

Role of Feedback Regulation of Pantothenate Kinase (CoaA) in Control of Coenzyme A Levels in *Escherichia coli*

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Pantothenate kinase (CoaA) is a key regulator of coenzyme A (CoA) biosynthesis in *Escherichia coli*, and its activity is controlled by feedback inhibition by CoA and its thioesters. The importance of feedback inhibition in the control of the intracellular CoA levels was tested by constructing three site-directed mutants of CoaA that were predicted to be feedback resistant based on the crystal structure of the CoaA-CoA binary complex. CoaA[R106A], CoaA[H177Q], and CoaA[F247V] were purified and shown to retain significant catalytic activity and be refractory to inhibition by CoA. CoaA[R106A] retained 50% of the catalytic activity of CoaA, whereas the CoaA[H177Q] and CoaA[F247V] mutants were less active. The importance of feedback control of CoaA to the intracellular CoA levels was assessed by expressing either CoaA or CoaA[R106A] in strain ANS3 [*coaA15*(Ts) *panD2*]. Cells expressing CoaA[R106A] had significantly higher levels of phosphorylated pantothenate-derived metabolites and CoA in vivo and excreted significantly more 4'-phosphopantetheine into the medium compared to cells expressing the wild-type protein. These data illustrate the key role of feedback regulation of pantothenate kinase in the control of intracellular CoA levels.

Coenzyme A (CoA) is the major acyl group carrier in living systems and is synthesized by a series of enzymatic steps beginning with the vitamin pantothenate (6). All of the genes and enzymes involved in the biosynthetic pathway have been identified for *Escherichia coli* (Fig. 1). The pathway is initiated by pantothenate kinase (CoaA) (ATP:D-pantothenate 4'-phosphotransferase [EC 2.7.1.33]). Cysteine is next added to the phosphopantothenate by 4'-phosphopantothenoylcysteine synthase and rapidly decarboxylated to 4'-phosphopantetheine (P-PanSH). These two steps are carried out by a single bifunctional polypeptide (Dfp, renamed CoaBC) (15). The last two steps are carried out by P-PanSH adenyltransferase (CoaD) (4, 5) followed by the addition of the 3'-ribose phosphate by dephospho-CoA kinase (11). *E. coli* is capable of de novo pantothenate biosynthesis (6), or it can import pantothenate from the medium via a sodium-dependent active transport process (7, 18, 19).

CoaA is proposed as the master regulator of CoA biosynthesis, with CoaD as a secondary site for pathway regulation (6). Metabolic labeling experiments established that the utilization, rather than the production, of pantothenate governs the intracellular CoA level (8, 9). This work was followed by the demonstration that the CoaA gene is essential (20) and that the enzyme was regulated most potently by CoA and to a lesser extent by its thioesters (17). CoaA exists as a homodimer that utilizes a compulsory ordered mechanism with ATP as the first substrate and exhibits highly cooperative ATP binding (14). Kinetic analysis revealed that CoA inhibition is competitive with ATP, and Lys101 is a key residue involved in the binding of both the nucleotide substrate and inhibitor (14). Cellular CoA levels fluctuate depending on growth conditions,

and carbon source shift experiments support the importance of CoA, as opposed to acetyl-CoA, as the primary regulator of CoaA in vivo (16, 17). A second-site revertant of the temperature-sensitive *coaA* allele was isolated that exhibited feedback-resistant properties in crude cell extracts coupled with higher intracellular CoA levels than those for the strain carrying *coaA*(Ts) (16). These data point to feedback regulation of CoaA as a key determinant of the intracellular CoA level in *E. coli*, and the similarities in the regulatory mechanism for bacterial pantothenate kinase compared to the structurally diverse eukaryotic proteins (2, 12) support the hypothesis that feedback regulation is fundamental to the control of CoA content in all organisms.

Recently the crystal structure of CoaA was determined in complex with either ATP or CoA (21). These structures reveal that ATP and CoA bind to the enzyme in distinctly different ways but that their phosphodiester binding sites overlap at Lys101, explaining the kinetic competition between the CoA regulator and the ATP substrate. The more potent inhibition of CoaA by CoA than by acetyl-CoA is explained by the location of the CoA thiol group within a box of aromatic amino acid side chains that does not favor the binding of the bulky acetyl group attached to the thiol of acetyl-CoA. Three residues are identified in the CoaA-CoA binary complex that make important contacts with CoA but do not interact with ATP (Fig. 2). His177 and Phe247 form a hydrophobic pocket with the adenine moiety of CoA sandwiched between. However, His177 may also contribute to catalysis. Modeling of the postulated pentacoordinate transition state onto the CoaA-AMPPNP [adenosine 5'-(β,γ -imido)triphosphate] binary structure places His177 within 5 Å of the apical oxygen, and the high B factor of His177 in the PanK-AMPPNP structure opens the possibility that it may move closer to the active site pocket during catalysis and stabilize the transition state (21). Arg106 is postulated to be an important and specific requirement for

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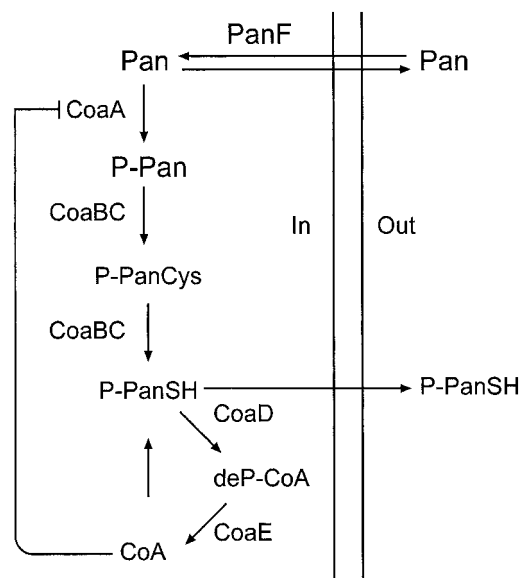


FIG. 1. The CoA biosynthetic pathway for *E. coli*. The pathway for the biosynthesis of CoA is defined for *E. coli* and consists of a series of five enzymes (6). Pantothenate (Pan) is transported into the cell by a sodium-dependent transporter (PanF), and efflux occurs by a separate mechanism using a yet unidentified protein(s) (7, 18, 19). Pantothenate kinase, the product of the *coaA* gene (13, 20), catalyzes the first step in the pathway, and this enzyme is potently feedback inhibited by CoA (14, 17). The next two steps are catalyzed by the bifunctional phosphopantothenoylcysteine (P-PanCys) synthase/P-PanCys decarboxylase encoded by the *coaBC* gene (formerly *dfp*) (15). The *coaD* gene produces the P-PanSH adenylyltransferase (4, 5), and the *coaE* gene encodes the last enzyme in the pathway, dephospho-CoA (deP-CoA) kinase (11). CoA is degraded by a phosphodiesterase to 3',5'-ADP and P-PanSH (16), but the gene encoding this activity is unknown. P-PanSH exits the cell by an unknown mechanism, but *E. coli* cannot incorporate extracellular P-PanSH into CoA (8, 9).

CoA binding since it forms a salt bridge with the phosphate attached to the 3'-hydroxyl of the CoA ribose to stabilize the inhibited complex. This surface residue does not have a role in catalysis. The goal of this study was to evaluate the importance of these three residues to feedback inhibition of CoA by CoA and to assess the contribution of feedback regulation to the control of cellular pantothenate metabolism and CoA content *in vivo*.

MATERIALS AND METHODS

Materials. Sources of supplies were the following: American Radiolabeled Chemicals, D-[1-¹⁴C]pantothenate (specific activity, 53.5 mCi/mmol); Invitrogen, pBAD vector; Bio-Rad, Bradford dye-binding protein assay solution; Analtech Inc., 250- μ m Silica Gel H plates; Fisher Scientific, Scintisafe 30%; Novagen, pET15b vector; Promega, restriction endonucleases and T4 DNA ligase; Qiagen, Quiaquick kit and P100 columns; Sigma Chemical Co., CoA and CoA thioesters, ATP, and pantothenate; Whatman, DE81 filter circles. All other materials were reagent grade or better.

Bacterial strains. Strain DV70 [*coaA15*(Ts) *panD2*] is a tetracycline-sensitive derivative of strain DV62 (20). This strain is unable to synthesize CoA or grow at 42°C, and extracts from cells grown at 30°C have less than 20% of the wild-type pantothenate kinase-specific activity (20). Strain ANS3 is an Δ *ara714* *leu::Tn10* derivative of strain DV70 that has a deletion in arabinose metabolism in its genetic background.

Mutant construction and protein purification. The *coaA* DNA in plasmid pWS7-13-2 (13) (GenBank/EMBL data bank accession no. M90071) encoding the wild-type *E. coli* K-12 CoaA protein was mutagenized using overlap exten-

sion PCR (3) to introduce mutations at R106A, F247V, and H177Q in the protein sequence, and the PCR products were subcloned into plasmid pCR2.1 (Invitrogen). The resulting plasmids from each mutated gene were sequenced to verify the introduction of the desired mutation and the absence of PCR artifacts. The sequences were determined on both strands by automated DNA sequencing at the Hartwell Center of St. Jude Children's Research Hospital. In the CoaA [F247V] mutant, the sequence 5'-AAATTCC-3' was changed to 5'-AAAGTCC; in the CoaA[R106A] mutant, the sequence 5'-CCCGTGT was changed to 5'-CCGCTGT; in the CoaA[H177Q] mutant, the sequence 5'-CATCTT was changed to 5'-CAGCTT (underlining shows sequence alteration). DNA fragments containing the mutated regions were ligated into the pET15b-CoaA expression vector constructed previously (2).

Strain BL21(DE3) (Novagen) was transformed with each plasmid, and ampicillin-resistant single isolate transformants were cultured to mid-log phase, frozen at -70°C in Luria broth containing 7% dimethyl sulfoxide, and screened for overexpression of the bPanK protein by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis after isopropyl- β -D-thiogalactopyranoside induction. Frozen stocks were inoculated into overnight cultures as a series of 1:100 dilutions, and the next morning those cultures, in early-mid-log phase, were added to

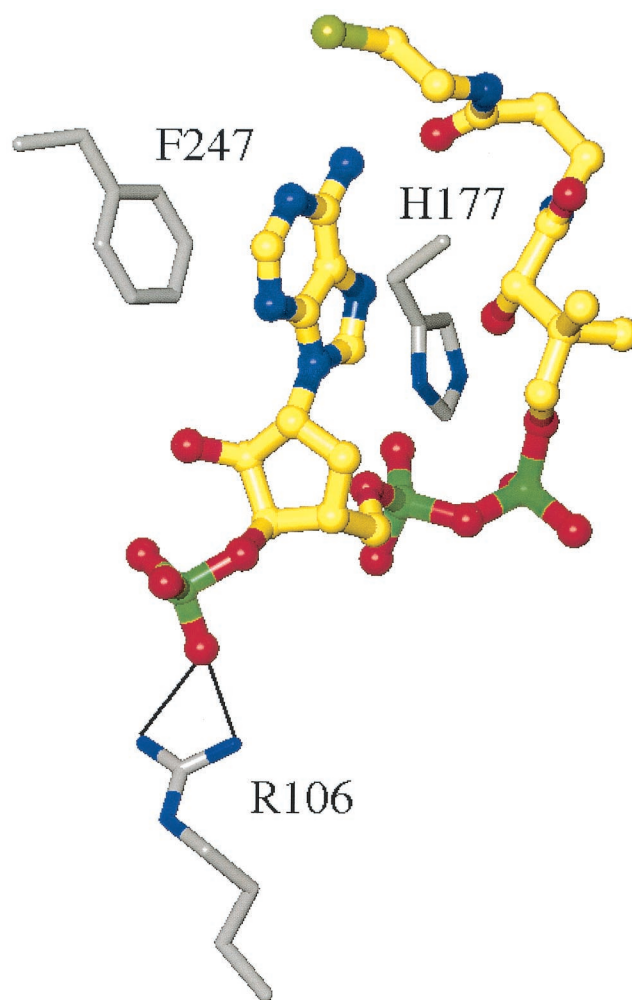


FIG. 2. View of the CoA inhibitor binding site illustrating the location of the three key amino acid residues critical to the interaction of CoA with the enzyme. CoA is potently inhibited by nonesterified CoA that fits into a pocket adjacent to, and overlapping with, the ATP binding site. His177 and Phe247 form a hydrophobic sandwich that interacts with the adenine group of CoA, and Arg106 forms a salt bridge with the phosphorylated 3'-hydroxyl group of CoA. See Yun et al. (21) and Protein Data Bank accession numbers 1esm and 1esn for complete details of CoA-CoA and CoA-AMPPNP interactions.

500 ml of Luria broth and grown to a density of about 5×10^8 cells/ml. Isopropyl- β -D-thiogalactopyranoside was added to a final concentration of 1 mM, and incubation continued for 3 h at 37°C. Cells were harvested, and the proteins were purified on Ni-nitrilotriacetic acid (NTA) agarose resin (Qiagen) as described previously (2). Following purification and concentration, the enzyme preparations were stored at -20°C in 50% glycerol and retained full activity for several months. Purity was assessed on 12% SDS-polyacrylamide gels, and protein determinations were made using the Bradford method (1) with γ -globulin as a standard.

Construction of expression vectors. The wild-type and R106A *coaA* DNAs in pET-15b were subcloned into the pBluescript II KS(+) vector along with a His tag fused in-frame to the 5' end of the coding sequences following digestion with *Xba*I and *Bam*HI to yield plasmids pPJ161 and pPJ163. Subsequently, the wild-type and R106A *coaA* DNAs were subcloned into the pBAD/myc-HisA vector (Invitrogen) following digestion with *Eco*RI and *Nco*I to yield plasmids pAN26 (wild-type) and pAN28 (R106A). Plasmids were transformed into strain ANS3.

Aliquots containing approximately 10^9 cells from overnight cultures were grown at 42°C in minimal medium E (10) containing 0.4% glucose, 0.01% methionine, 0.01% leucine, and 40 μM pantothenate. Cells were lysed in 2 $\times$ SDS sample buffer and boiled before loading onto SDS-10% polyacrylamide gels. Following electrophoresis, the separated proteins plus prestained protein standards were transferred to nitrocellulose by electroporation. Expression of the CoaA proteins was verified by Western blotting using a rabbit polyclonal immunoglobulin G anti-His-tag primary antibody (His-probe; Santa Cruz Biotechnology) and anti-rabbit immunoglobulin horseradish peroxidase-linked whole antibody from donkey (Amersham) as a secondary antibody. Western blotting was performed according to the manufacturer of the ECL detection kit.

Pantothenate kinase and CoA binding assays. Enzyme assays were performed as described previously (17) and were linear with time and protein input. Standard assays contained 90 μM D-[1- ^{14}C]pantothenate ($\sim 100,000$ dpm), 1 mM ATP, 10 mM MgCl_2 , 0.1 M Tris-HCl (pH 7.5), and CoaA protein in a final volume of 40 μl . The mixture was incubated for 10 min at 37°C, and the reaction was stopped by depositing a 30- μl aliquot onto a Whatman DE81 ion exchange filter disk that was washed in three changes of 1% acetic acid in 95% ethanol (25 ml/disk) to remove unreacted pantothenate. P-Pan was quantitated by scintillation counting of the dried disk in 3 ml of scintillation solution and comparison with an unwashed disk representing the total input pantothenate.

The ability of the wild-type protein and the three mutants to bind CoA was directly measured using equilibrium dialysis. Disposal equilibrium biodialyzers (Nest Group Inc.) were loaded with 90 μl of buffer (100 mM sodium phosphate [pH 7.5], 100 mM NaCl, 2.5 mM MgCl_2 , 1-mg/ml bovine serum albumin) containing 20 μM CoA on one side and 5.5 μM pantothenate kinase protein in the same buffer on the other side. The dialyzers were shaken for 48 h at 4°C, and a 20- μl aliquot was removed to determine CoA using the thiol quantitation kit (Molecular Probes) following the directions provided by the manufacturer, except that the volume was reduced to 300 μl . The [bound + free] CoA on the protein side was subtracted from the free CoA concentration on the buffer side to determine the amount bound. Controls without pantothenate kinase protein were used to verify that CoA equilibrium between the chambers was achieved.

Metabolic labeling. Overnight cultures of strains ANS3/pAN26 and ANS3/pAN28 were grown in minimal E medium (10) containing 0.4% glucose, 0.01% methionine, and 0.01% leucine, without pantothenate, to deplete the intracellular pools of pantothenate metabolites (8). Cells from the overnight cultures were inoculated into fresh medium of the same composition that included the indicated concentration of D-[1- ^{14}C]pantothenate. Cells were harvested at a density of 7×10^8 cells/ml and washed twice with medium E. Cells were lysed as previously described (8), and CoA and intermediates in the CoA biosynthetic pathway were separated and identified by thin-layer chromatography on Silica Gel H layers developed with butanol-acetic acid-water (5:2:4 [vol/vol]). Total conversion of pantothenate to phosphorylated metabolites was determined using thin-layer chromatography on Silica Gel H layers developed with ethanol-ammonium hydroxide (4:1 [vol/vol]). Migration of standards was the same as the Rf values reported previously (8).

RESULTS

Critical residues involved in CoA binding to CoaA. Inspection of the X-ray crystal structures of the CoaA-CoA and CoaA-AMPPNP binary complexes revealed that ATP and CoA bind to CoaA in different orientations, with their respective adenine moieties binding to nonoverlapping subsets of

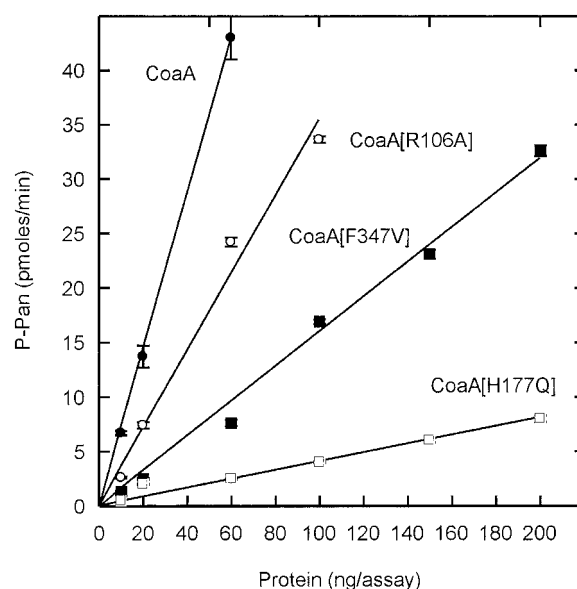


FIG. 3. Catalytic activity of the CoaA mutants compared to wild-type CoaA. CoaA and the three mutant forms were expressed and purified by affinity chromatography as described in Materials and Methods. The enzyme-specific activities were calculated from the slope of the lines determined by linear regression analysis of the triplicate assays and were as follows: CoaA (●), 650 nmol/min/ μg ; CoaA [R106A] (○), 350 nmol/min/ μg ; CoaA[F347V] (■), 160 nmol/min/ μg ; and CoaA[H177Q] (□), 40 nmol/min/ μg .

amino acid residues (21). The two nucleotides occupy the same area of the active site at Lys101, which binds the phosphodiester of either molecule, and accordingly, CoaA[K101M] is catalytically inactive and fails to bind ATP or CoA (14). We focused on modifications of the amino acids that interact with the adenine of CoA to generate mutant proteins that were catalytically competent but refractory to feedback inhibition by CoA. This analysis identified three residues, Arg106, His177, and Phe247, as being involved in CoA recognition but not ATP binding (Fig. 2). His177 and Phe247 interact with the adenine group via stacking interactions, and Arg106 forms a salt bridge with the 3' phosphate of CoA.

Activity and feedback regulation of CoaA mutants. We investigated the role of these three residues in the feedback regulation of CoaA by constructing the CoaA[R106A], CoaA [H177Q], and CoaA[F247V] mutants. CoaA and its three mutant forms were expressed and purified to homogeneity by affinity chromatography. The mutant proteins retained significant levels of pantothenate kinase activity (Fig. 3). CoaA [H177Q] was the least active of the mutants, exhibiting 6% of the wild-type activity, and CoaA[F247V] retained 25% (Fig. 3). CoaA[R106A] was the least perturbed of all the mutants, with a specific activity that was 54% of the wild-type protein. Since the CoaA[K101M] mutant was essentially inactive (less than 0.001% of CoaA) (14), these data indicated that the three residues do not play a major role in catalysis, nor do they significantly participate in ATP or pantothenate binding to the enzyme. The enzymatic differences between the proteins arose from effects on the V_{max} values, since graphical determination of the K_m s for ATP (CoaA, 280 μM ; CoaA[R106A], 210 μM ; CoaA[H177Q], 210 μM ; CoaA[F247V], 220 μM) and panto-

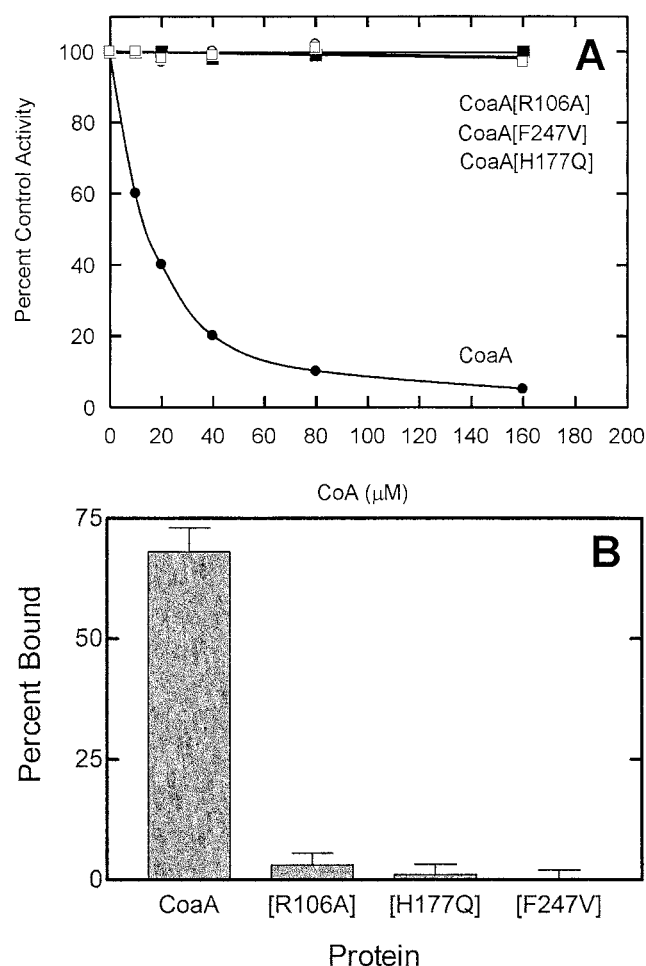


FIG. 4. (A) Feedback inhibition of CoaA mutants compared to that of wild-type CoaA. The ability of CoA to function as an inhibitor of pantothenate kinase activity was addressed by comparing the effects of various concentrations of CoA on the biochemical activity of CoaA (●), CoaA[R106A] (○), CoaA[F247V] (■), and CoaA[H177Q] (□) using the *in vitro* assay described in Materials and Methods. (B) CoA binding of CoaA protein mutants compared to that of wild-type CoaA. The equilibrium binding of CoA to the wild-type and mutant proteins was determined following dialysis with the ligand at 4°C as described in Materials and Methods.

thenate (CoaA, 28 μM ; CoaA[R106A], 20 μM ; CoaA[H177Q], 42 μM ; CoaA[F247V], 23 μM) did not reflect any significant differences among the four proteins.

If all three residues were required for the binding of CoA to its regulatory site, each of the mutant proteins in this study should be refractory to feedback inhibition by CoA. An *in vitro* biochemical assay was used to compare the sensitivity to CoA inhibition of the three mutants (Fig. 4A). All of the mutants were refractory to CoA inhibition within the concentration ranges tested, which potentially inactivated wild-type CoaA protein. The mutant proteins also were not inhibited by acetyl-CoA (data not shown). Neither CoaA nor any of the three mutants was inhibited by dephospho-CoA. To confirm that the mutant proteins did not bind CoA, an equilibrium dialysis experiment was performed as described in Materials and Methods. The wild-type CoaA protein bound CoA with high

affinity (see reference 14 for a detailed analysis); however, none of the mutant proteins exhibited significant CoA binding (Fig. 4B). In this experiment, the protein concentrations were high (5.5 μM) compared to the 10 μM concentration of CoA at equilibrium in order to detect low-affinity binding by the mutant proteins. Nonetheless, a signal was not observed with the mutant proteins. These data clearly illustrate that residues Arg106, His177, and Phe247 in CoaA are all required for feedback regulation of the enzyme by CoA.

Physiological consequences of eliminating feedback regulation of CoaA. To test the role of feedback regulation on the control of intracellular CoA levels, we selected CoaA[R106A] for the *in vivo* experiments because this enzyme's catalytic activity was closest to that of wild-type CoaA (Fig. 3) and it was refractory to feedback regulation by CoA (Fig. 4). We used strain ANS3 [*coaA15*(Ts) *panD2*] as the host because this strain is conditionally defective in pantothenate kinase due to the temperature-sensitive *coaA* allele, and the *panD* mutation prevented the synthesis of endogenous pantothenate. The next experiments determined the vector most appropriate for expression of the wild-type and mutant genes at a level that approximated the capacity for untransformed wild-type cells to utilize pantothenate. CoaA is not an abundant protein (14), and its expression from a multicopy plasmid, such as pBlue-script or pBR322, led to a large increase in cellular CoA concentrations due to the high levels of intracellular pantothenate kinase (13). We selected the arabinose-regulated pBAD vector, and in a series of control experiments with strain ANS3 harboring the wild-type *coaA* gene cloned into this vector (pAN26) at different arabinose concentrations, we found that the small amount of *coaA* expression from the vector in the absence of exogenous arabinose was sufficient to complement the temperature-sensitive growth phenotype of ANS3. Also, the amount of pantothenate utilization was similar to the utilization of pantothenate by strain SJ16 (*panD2*), the parent of strain DV70 containing a single copy of the wild-type *coaA* gene.

Metabolic labeling with [1- ^{14}C]pantothenate was used to uniformly label the intracellular pool, and the radiolabeled cells were analyzed by extraction and thin-layer chromatography as described in Materials and Methods. Since the *panD* mutant strain used in this work was unable to synthesize endogenous pantothenate, most experiments challenged the cell with increasing quantities of extracellular pantothenate to investigate the ability of the cells to regulate their CoA levels in the presence of excess precursor. We first compared the total phosphorylated pantothenate-derived metabolites in the cells plus the culture medium (Fig. 5). Cultures of strain ANS3/pAN28 that expressed the feedback-resistant CoaA[R106A] mutant had significantly higher levels of phosphorylated pantothenate-derived metabolites, clearly illustrating that the flux of pantothenate through CoaA[R106A] was higher than that of its wild-type counterpart. Next, the intracellular levels of labeled CoA and P-PanSH were determined (Fig. 6). Again, strain ANS3/pAN28 expressing the CoaA[R106A] mutant protein contained significantly higher concentrations of CoA and P-PanSH than the same strain (ANS3/pAN26) expressing the wild-type gene. Importantly, the levels of intracellular P-PanSH were more elevated than CoA, illustrating that CoaD was a second site for CoA regulation that became more

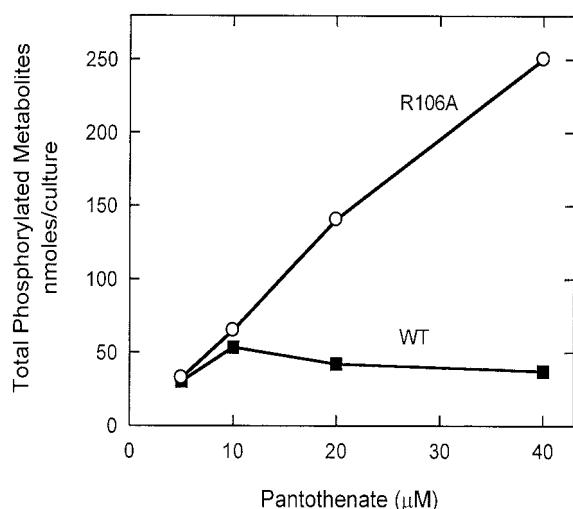


FIG. 5. A comparison of the accumulation of intracellular pantothenate metabolites in cells expressing either wild-type CoaA (WT) or mutant CoaA[R106A]. Strain ANS3 expressing either *coaA* or *coaA* [R106A] was grown in the presence of the indicated concentrations of [$1\text{-}^{14}\text{C}$]pantothenate as described in Materials and Methods. The cells were harvested and extracted, and the soluble fractions separated by chromatography on Silica Gel H thin layers developed with ethanol-ammonium hydroxide (4:1 [vol/vol]) to separate phosphorylated metabolites from pantothenate.

readily apparent when regulation at the CoaA step was circumvented by the feedback-resistant mutation.

Since *E. coli* excretes quantities of P-PanSH into the medium (8, 9, 16), we next analyzed the extracellular metabolites to determine if the strain expressing the feedback-resistant CoaA exported larger amounts of this intermediate (Fig. 7). When the cells were grown in the presence of 40 μM pantothenate, strain ANS3/pAN26 expressing the wild-type allele produced extracellular P-PanSH, but most of the extracellular pantothenate remained unmodified. In contrast, strain ANS3/pAN28 expressing the *coaA*[R106A] mutant allele consumed much higher levels of pantothenate and excreted high levels of P-PanSH. In contrast, both strains had comparable levels of extracellular P-PanSH at 5 μM pantothenate, a limiting concentration of precursor that is mostly converted to intracellular CoA.

DISCUSSION

Our work identifies three of the key residues involved in specifying the binding site for the feedback inhibitor CoA on *E. coli* CoaA. The X-ray structure of the CoaA-CoA binary complex suggested that four residues make significant contributions to the binding of CoA to the enzyme. Lys101 interacts with the phosphodiester of CoA, and the CoaA[K101M] mutant is unable to bind CoA (14). However, Lys101 is also important to the binding of the ATP phosphodiester, and thus, CoaA[K101M] does not bind ATP and is catalytically inactive (14). In addition, there are three residues, Arg106, His177, and Phe247, that make specific contacts with CoA but not with ATP (21). Arg106 forms a salt bridge with the 3' phosphate of CoA, and Phe247 and His177 interact with the adenine base (Fig. 2). These four residues are completely conserved in 19 bacterial pantothenate kinase sequences. The predictions from

the structure were borne out by the analysis of mutant CoaA proteins. CoA did not inhibit the activity of CoaA[R106A], CoaA[H177Q], and CoaA[F247V] (Fig. 4A), and CoA binding was not observed in equilibrium dialysis (Fig. 4B), suggesting that the allosteric regulator failed to interact with these mutant proteins. In all cases, the three mutant CoaA proteins retain significant catalytic activity, illustrating that these residues are not essential for catalysis. On the other hand, the CoaA [H177Q] mutant had the lowest level of catalytic activity of the mutant proteins, suggesting that His177 may possibly have a role in accelerating catalysis even though it is not essential. However, overlaying the ADP-vanadate structure, a model for the pentacoordinate transition state, into the CoaA-AMPPNP structure shows that His177 is located 5.0 Å from the apical oxygen of the vanadate. This is too far away to donate a hydrogen bond to the putative transition state complex, arguing against a role for His177 in catalysis, but additional structures need to be determined to verify this point, since the loop containing His177 may move closer to the active site in the CoaA-ATP-pantothenate ternary complex.

Our results provide support for the pivotal role for feedback

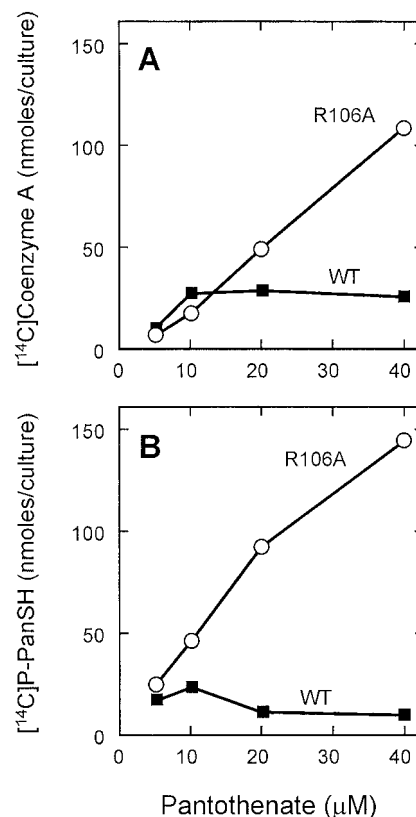


FIG. 6. A comparison of the intracellular levels of CoA (A) and P-PanSH (B) in cells expressing either wild-type (WT) CoaA or mutant CoaA[R106A]. Strain ANS3 expressing either *coaA* or *coaA* [R106A] was grown in the presence of the indicated concentrations of [$1\text{-}^{14}\text{C}$]pantothenate as described in Materials and Methods. The cells were harvested and extracted, and the soluble fractions separated by chromatography on Silica Gel thin layers developed with butanol-acetic acid-water (5/2/4). Radioactive zones corresponding to CoA and P-PanSH were scraped from the plate and quantitated by scintillation counting.

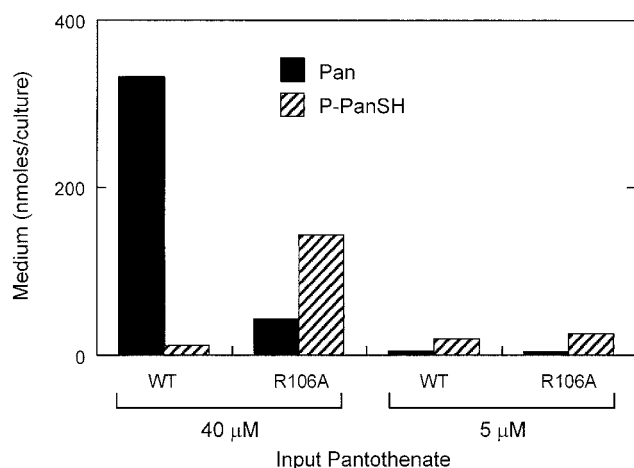


FIG. 7. A comparison of the extracellular pantothenate metabolites derived from cells expressing either wild-type CoaA or mutant CoaA[R106A]. Strain ANS3 harboring a plasmid expressing either *coaA* (pAN26) or *coaA*[R106A] (pAN28) was labeled with [14 C]pantothenate at 40 or 5 μ M and harvested at a concentration of 8×10^8 cells/ml. The cells were removed from the medium by filtration, and the medium was fractionated by chromatography on Silica Gel H thin layers developed either with butanol-acetic acid-water (5:2:4 [vol/vol]) or ethanol-ammonium hydroxide (4:1 [vol/vol]) to quantitate P-PanSH and pantothenate. Radioactive zones corresponding to either pantothenate or P-PanSH were scraped from the plate and quantitated by liquid scintillation counting.

regulation in the control of intracellular CoA concentrations. *E. coli* produces about 15 times more pantothenate than it uses for CoA biosynthesis, and the excess vitamin is excreted (8); thus, these bacteria possess an efficient mechanism to prevent uncontrolled metabolism of the vitamin. Cells expressing the feedback-resistant CoaA[R106A] mutant protein have significantly elevated levels of intracellular phosphorylated pantothenate-derived metabolites (Fig. 4) that translates into a higher CoA content (Fig. 5). Previous work is consistent with a major role for feedback regulation in limiting the conversion of pantothenate to CoA. Results of carbon source shift experiments are consistent with regulation of CoA levels occurring within minutes, a time frame that is inconsistent with gene expression being a major contributor to regulation by a fuel source (16, 17). The concentrations of CoA that potently inhibit pantothenate kinase correlate well with the concentrations of CoA found in vivo, strongly supporting a role for CoA, as opposed to acetyl-CoA, as the most potent inhibitory ligand (8, 17).

Our results also support the proposed role of CoaD, P-PanSH adenylyltransferase, as a secondary point for control of cellular CoA content. The extracellular P-PanSH that accumulates in cells expressing the CoaA[R106A] mutant may arise from two sources. Regulation at the adenylyltransferase step would lead to the accumulation of P-PanSH followed by its expulsion from the cell. Purified CoaD contains bound CoA, suggesting that this enzyme may also be subject to feedback regulation by CoA (4). However, more detailed studies will be required to determine the mechanism for CoA regulation and its importance to the overall control of CoaD activity and cellular CoA levels. Alternatively, P-PanSH may arise from CoA degradation. This process is carried out by an unidentified enzyme, but CoA hydrolysis to P-PanSH makes a signifi-

cant contribution to lowering CoA levels during metabolic readjustments (16). Once P-PanSH exits the cell, it cannot be taken up by *E. coli* and used for CoA biosynthesis (9); therefore, excretion of P-PanSH is an irreversible response to the overproduction of phosphopantothenate arising from the dysregulation of pantothenate kinase.

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REFERENCES

- Bradford, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Calder, R. B., R. S. B. Williams, G. Ramaswamy, C. O. Rock, E. Campbell, S. E. Unkles, J. R. Kinghorn, and S. Jackowski. 1999. Cloning and characterization of a eukaryotic pantothenate kinase gene (*panK*) from *Aspergillus nidulans*. *J. Biol. Chem.* **274**:2014–2020.
- Cormack, B. 1997. Directed mutagenesis using the polymerase chain reaction, p. 8.5.1–8.5.10. In F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), *Current protocols in molecular biology*. John Wiley & Sons, Inc., New York, N.Y.
- Geerlof, A., A. Lewendon, and W. V. Shaw. 1999. Purification and characterization of phosphopantetheine adenylyltransferase from *Escherichia coli*. *J. Biol. Chem.* **274**:27105–27111.
- Izard, T., and A. Geerlof. 1999. The crystal structure of a novel bacterial adenylyltransferase reveals half of sites reactivity. *EMBO J.* **18**:2021–2030.
- Jackowski, S. 1996. Biosynthesis of pantothenic acid and coenzyme A, p. 687–694. In F. C. Neidhardt, R. Curtiss, C. A. Gross, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. Reznikoff, M. Riley, M. Schaechter, and H. E. Umberger (ed.), *Escherichia coli and Salmonella typhimurium: cellular and molecular biology*. American Society for Microbiology, Washington, D.C.
- Jackowski, S., and J.-H. Alix. 1990. Cloning, sequence, and expression of the pantothenate permease (*panF*) gene of *Escherichia coli*. *J. Bacteriol.* **172**:3842–3848.
- Jackowski, S., and C. O. Rock. 1981. Regulation of coenzyme A biosynthesis. *J. Bacteriol.* **148**:926–932.
- Jackowski, S., and C. O. Rock. 1984. Metabolism of 4'-phosphopantetheine in *Escherichia coli*. *J. Bacteriol.* **158**:115–120.
- Miller, J. H. 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Mishra, P., P. K. Park, and D. G. Drueckhammer. 2001. Identification of *yacE* (*coaE*) as the structural gene for dephosphocoenzyme A kinase in *Escherichia coli* K-12. *J. Bacteriol.* **183**:2774–2778.
- Rock, C. O., R. B. Calder, M. A. Karim, and S. Jackowski. 2000. Pantothenate kinase regulation of the intracellular concentration of coenzyme A. *J. Biol. Chem.* **275**:1377–1383.
- Song, W.-J., and S. Jackowski. 1992. Cloning, sequencing, and expression of the pantothenate kinase (*coaA*) gene of *Escherichia coli*. *J. Bacteriol.* **174**:6411–6417.
- Song, W.-J., and S. Jackowski. 1994. Kinetics and regulation of pantothenate kinase from *Escherichia coli*. *J. Biol. Chem.* **269**:27051–27058.
- Strauss, E., C. Kinsland, Y. Ge, F. W. McLafferty, and T. P. Begley. 2001. Phosphopantothentoylcysteine synthetase from *Escherichia coli*. Identification and characterization of the last unidentified coenzyme A biosynthetic enzyme in bacteria. *J. Biol. Chem.* **276**:13513–13516.
- Vallari, D. S., and S. Jackowski. 1988. Biosynthesis and degradation both contribute to the regulation of coenzyme A content in *Escherichia coli*. *J. Bacteriol.* **170**:3961–3966.
- Vallari, D. S., S. Jackowski, and C. O. Rock. 1987. Regulation of pantothenate kinase by coenzyme A and its thioesters. *J. Biol. Chem.* **262**:2468–2471.
- Vallari, D. S., and C. O. Rock. 1985. Isolation and characterization of *Escherichia coli* pantothenate permease (*panF*) mutants. *J. Bacteriol.* **164**:136–142.
- Vallari, D. S., and C. O. Rock. 1985. Pantothenate transport in *Escherichia coli*. *J. Bacteriol.* **162**:1156–1161.
- Vallari, D. S., and C. O. Rock. 1987. Isolation and characterization of temperature-sensitive pantothenate kinase (*coaA*) mutants of *Escherichia coli*. *J. Bacteriol.* **169**:5795–5800.
- Yun, M., C.-G. Park, J.-Y. Kim, C. O. Rock, S. Jackowski, and H.-W. Park. 2000. Structural basis for the feedback regulation of *Escherichia coli* pantothenate kinase by coenzyme A. *J. Biol. Chem.* **275**:28093–28099.