Structural Insights into the Mechanism of Four-Coordinate Cob(II)alamin Formation in the Active Site of the Salmonella enterica ATP:Co(I)rinoid Adenosyltransferase Enzyme: Critical Role of Residues Phe91 and Trp93

Theodore C. Moore,† Sean A. Newmister,‡ Ivan Rayment,*‡ and Jorge C. Escalante-Semerena*§

†Department of Bacteriology and ‡Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706, United States
§Department of Microbiology, University of Georgia, Athens, Georgia 30602-2605, United States

ABSTRACT: ATP:co(I)rinoid adenosyltransferases (ACATs) are enzymes that catalyze the formation of adenosylcobalamin (AdoCbl, coenzyme B12, or CoB12) from cobalamin and ATP. There are three families of ACATs, namely, CobA, EutT, and PduO. In Salmonella enterica, CobA is the housekeeping enzyme that is required for de novo AdoCbl synthesis and for salvaging incomplete precursors and cobalamin from the environment. Here, we report the crystal structure of CobA in complex with ATP, four-coordinate cobalamin, and five-coordinate cobalamin. This provides the first crystallographic evidence of the existence of cob(II)-alamin in the active site of CobA. The structure suggests a mechanism in which the enzyme adopts a closed conformation and two residues, Phe91 and Trp93, displace 5,6-dimethylbenzimidazole, the lower nucleotide ligand base of cobalamin, to generate a transient four-coordinate cobalamin, which is critical in the formation of the AdoCbl Co=C bond. In vivo and in vitro mutational analyses of Phe91 and Trp93 emphasize the important role of bulky hydrophobic side chains in the active site. The proposed manner in which CobA increases the redox potential of the cob(II)alamin/cob(I)alamin couple to facilitate formation of the Co=C bond appears to be analogous to that utilized by the PduO-type ACATs, where in both cases the polar coordination of the lower ligand to the cobalt ion is eliminated by placing that face of the corrin ring adjacent to a cluster of bulky hydrophobic side chains.

Cobalamin (Cbl, B12) is one of the largest cofactors in biology and is utilized by organisms across all domains of life.1,2 Cobalamin features a cobalt ion coordinated equatorially by the nitrogen atoms of a cyclic tetrapyrrole known as the corrin ring. The lower (Coα) axial ligand of Cbl is the purine analogue base 5,6-dimethylbenzimidazole (DMB), which is tethered to the corrin ring by a phosphodiester bond between an aminopropanol substituent of the ring and the phosphoryl moiety of the DMB-riboside monophosphate.3

In adenosylcobalamin (AdoCbl, coenzyme B12, or CoB12), the upper (Coβ) ligand is a 5′-deoxyadenosyl moiety covalently bound to the cobalt ion of the ring, forming a weak Co=C bond. Homolysis of the Co=C bond of AdoCbl results in five-coordinate cob(II)alamin and a 5′-deoxyadenosyl radical critical to the initiation of intramolecular rearrangements catalyzed by a variety of enzymes, such as ethanolamine ammonia-lyase,4 diol dehydratase,5 and methylmalonyl-CoA mutase,6 among others. AdoCbl is synthesized by a family of enzymes known as ATP:co(I)rinoid adenosyltransferases (ACATs). There are three nonhomologous types of ACATs, CobA, PduO, and EutT, which were named on the basis of their function in the enterobacterium Salmonella enterica.7−9 In this bacterium, CobA is the housekeeping ACAT involved in de novo AdoCbl synthesis and incomplete corrinoid salvaging.7 CobA has the broadest substrate specificity of the three ACAT types and recognizes both complete and incomplete corrinoids.10

Corrinoid adenosylation proceeds via a reactive nucleophilic Co′ species that is generated through a series of consecutive one-electron transfers to reduce the Co3+ ion.11 The bacterial cytoplasm has sufficient reducing power for reduction of Co3+ to Co2+, an event that removes the β-ligand.12 Further reduction to Co+ is thermodynamically difficult, because the Co2+/+ redox couple in solution (−610 mV) is beyond the reach of known biological reductants.13,14 ACATs raise the redox potential of the cob(II)alamin/cob(I)alamin couple to facilitate formation of the Co=C bond. In such species, the redox-active 3d orbital of cobalt is stabilized, resulting in an increase of ≥250 mV.18 In such an environment, cob(II)alamin can accept an electron from reduced flavodoxin A (FldA), to generate cob(I)alamin.12,19,20 Generation of cob(I)alamin is followed by a nucleophilic attack by Co′ on the 5′-carbon of the ATP cosubstrate, forming AdoCbl and releasing triplyphosphate (PPP).21 This is accomplished in Lactobacillus reuteri PduO ACAT (hereafter LrPduO) via
placement of the lower ligand coordination site of the cobalt ion in a hydrophobic environment. In LrPduO, Phe112 displaces DMB from its coordination bond with the cobalt ion to generate the four-coordinate intermediate. It was unknown whether this mechanism is shared by other nonhomologous ACATs or whether each ACAT has a distinct mechanism for achieving a four-coordinate cob(II)alamin.

CobA is capable of generating the four-coordinate intermediate, however, such an intermediate has not been observed in the active site of CobA, so that the mechanism for the conversion of five- to four-coordinate cob(II)alamin in CobA was unknown. In earlier structural studies, prior to learning about the importance four-coordinate cob(II)alamin intermediates, CobA was crystallized in complex with HOCbl(III)alamin (HOCbl). In that structure, the cobalt ion of HOCbl is not in a suitable position for nucleophilic attack because it is located too far (>6 Å) from the 5'-carbon of ATP. Significantly, HOCbl is a Co³⁺ species that is not encountered by the enzyme in vivo.

To address the mechanism by which four-coordinate Cbl formation is supported in CobA, we have determined the structure of CobA in complex with cob(II)alamin and MgATP at 2.0 Å resolution. This revealed tetradentate coordination for the cobalt ion and provided insight into how this is accomplished in this class of ACATs. The structure was used to guide an investigation in vivo and in vitro of the components of the active site that appear to be critical for function.

### MATERIALS AND METHODS

**Strains, Culture Media, and Chemicals.** Strains used in this study are listed in Table S1 of the Supporting Information. Primers used for polymerase chain reaction-based site-directed mutagenesis are listed in Table S2 of the Supporting Information. Chemicals were purchased from Sigma and were used without further purification.

Minimal medium containing ethanolamine as a carbon, energy, and nitrogen source was used to assess AdoCbl biosynthesis via the adenosylcobalamin-dependent expression of the ethanolamine utilization operon of *S. enterica sv. Typhimurium* strain LT2 as described previously. The culture medium was supplemented with ethanolamine (90 mM), glycerol (0.5 mM), methionine (2 mM), MgSO₄ (1 mM), arabinose (0.5 mM), and trace minerals. AdoCbl precursors dicyanocobinamide [(CN)₂Cbi, 100 nM] and 5,6-dimethyl-arabinose (0.5 mM), and trace minerals were used as complex media. Lysogenic broth (LB) and Nutrient Broth (Difco Laboratories) were used as complex media.

**Protein Overproduction and Purification.** Overproduction and purification of tagless, wild-type CobA (CobAWT) protein were performed as described previously. CobAWT was concentrated [10000 molecular weight cutoff (MWCO) centrifugal filter, Millipore] to 20 mg/mL as determined by A₂₈₀ using the calculated molar extinction coefficient (23950 M⁻¹ cm⁻¹, ExPaSy). The protein was flash-frozen in liquid nitrogen and stored at −80 °C until it was used.

To facilitate overproduction and purification of CobA variants, the *S. enterica cobA* allele was cloned into a pTEV5 vector to direct synthesis of CobA proteins fused to an N-terminal, rTEV protease-cleavable H₆ tag. Mutant *cobA* alleles were constructed using the QuickChange II site-directed mutagenesis kit (Stratagene). The presence of the desired mutations was confirmed using BigDye Terminator DNA sequencing protocols (ABI PRISM); reaction mixtures were resolved at the University of Wisconsin Biotechnology Center. Plasmids directing the synthesis of the CobA variants were moved by electroporation into a strain of *Escherichia coli* BL21(DE3) carrying a null allele of *btuR*, the cobA homologue in this bacterium (Table S1 of the Supporting Information). Strains expressing different *cobA* alleles were inoculated into 2 L of LB containing ampicillin (100 µg/mL) and grown while being shaken at 37 °C to an OD₆₀₀ of ~0.6. At that point, the incubator temperature was decreased to 15 °C for 30 min before overnight induction with 1 mM IPTG. Cells were harvested (9000g for 15 min at 4 °C) and stored at −80 °C for at least 3 days. Cell pellets were resuspended in 100 mM tris(hydroxymethyl)aminomethane hydrochloride buffer (Tris-HCl) (pH 8 at 4 °C) with 500 mM NaCl, 70 mM imidazole, 1 mM tris(2-carboxyethyl)phosphine (TCEP), and protease inhibitor cocktail (Sigma) at a density of 3 mL of buffer per gram of cell pellet. Resuspended cells were lysed by two passages through a French pressure cell (10.3 MPa) at 4 °C. The insoluble fraction was removed by centrifugation (44000g for 45 min at 4 °C) followed by filtration through a 0.45 µm filter (Millipore). Clarified lysate was applied over a Ni-activated nitrilotriacetic acid (NTA) column (5 mL of HiPor resin, Thermo). After being loaded, the column was washed with 5 bed volumes of buffer A [100 mM Tris-HCl (pH 8 at 4 °C)] containing 500 mM NaCl, 70 mM imidazole, and 1 mM TCEP. CobA protein bound to the resin was eluted using 5 bed volumes of buffer B (buffer A containing 500 mM imidazole) and collected in 3 mL fractions. The location of CobA was established by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of fractions. Fractions containing high-purity CobA (12 mL) were combined and dialyzed (10000 MWCO, Pierce) with His-tagged recombinant tobacco etch virus (His7-rTEV) protease (1 mg/mL, 1:50, v/v) to cleave the His₆ tag. His₆-tagged rTEV was prepared as described elsewhere. The combined fractions were dialyzed against buffer C (buffer A containing 20 mM imidazole) with three buffer changes at 4 °C. The first and second dialyses lasted 3 h, and the third dialysis lasted 10 h. The dialyzed fractions were loaded onto a fresh Ni-NTA column and washed with 5 bed volumes of buffer A; the flow-through was collected in 3 mL fractions. The protein content of these fractions was analyzed by SDS–PAGE; those containing CobA of the highest purity were pooled (9 mL) and dialyzed against 1 L of 50 mM Tris-HCl (pH 8.0, 4 °C), 500 mM NaCl, 1 mM TCEP, and 10% (v/v) glycerol at 4 °C. Three changes of dialysis buffer were used at 4 °C. The first and second lasted 3 h, and the third lasted 10 h. The final dilution factor of the dialyzable material was 7.2 × 10⁻⁷. Proteins were concentrated (10000 MWCO centrifugal filter, Millipore); the concentration was measured by A₂₈₀ using the calculated molar extinction coefficients (Table S3 of the Supporting Information, ExPaSy), and the proteins were flash-frozen in liquid N₂ and stored at −80 °C. Flavodoxin A (FdaA) and ferredoxin (flavodoxin):NADP⁺ reductase (Fpr) were produced and purified as described previously.

**Crystallography.** Crystallization conditions were analyzed using a 144-condition sparse matrix screen developed in the Raymont laboratory. All crystals of tagless CobA were grown by hanging-drop vapor diffusion in an anoxic chamber (Coy) at 20–25 °C. CobA was thawed and dialyzed three times against 1 L of 20 mM Tris-HCl (pH 8.0, 25 °C) for 30 min each at 25 °C to remove glycerol. A reaction mixture containing 20 µg/mL Fpr, 20 mM NADH, 3 mM ATP, 4.5 mM MgCl₂, and 2 mM HOCbl was constituted at room temperature inside an
anoxic chamber (90% N2/10% H2) to reduce HO-cob(III)-alamin to cob(II)alamin. The reduction was performed inside the anoxic chamber to prevent the rapid oxidation of cob(II)alamin to cob(III)alamin. Anoxic CobA was added to the reaction mixture after preincubation for 20 min at 25 °C. The final concentration of CobA in the mixture was 10 mg/mL. CobA was cocryocrystallized with MgATP and cob(II)alamin by mixing 2 μL of the reaction mixture with 2 μL of well solution composed of 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6.0), 320 mM NaCl, and 19.6% (w/v) polyethylene glycol 4000 (PEG4000).

Brown, orthorhombic crystals (0.1 mm × 0.5 mm) were observed after 48 h. The crystals were incrementally transferred in two steps to a cryoprotectant solution that contained 22.5% (w/v) PEG4000, 13.8% (v/v) ethylene glycol, 100 mM MES (pH 6.0), 240 mM NaCl, 0.5 mM HOCbl, 20 μg/mL Fpr, 10 mM NADH, 1 mM ATP, and 1.5 mM MgCl2 in acrylic batch plates inside the anaerobic chamber. The plates containing the crystals were moved into an O2-free argon bath for ease of manipulation before being frozen. The crystals were briefly exposed to oxygen (≤1 s) while being flash-frozen in liquid nitrogen. Tagless CobA in complex with MgATP and cob(II)alamin crystallized in space group P212121 with one homodimer of CobA per asymmetric unit and the following unit cell dimensions: a = 59.7 Å, b = 74.2 Å, c = 92.4 Å. Each chain contained ATP; one chain contained four-coordinate cob(II)alamin, and the other contained five-coordinate cob(II)alamin.

X-ray Data Collection and Structure Refinement. X-ray data for the CobA-cob(II)alamin-MgATP complex were collected at 100 K on Structural Biology Center beamline 19BM at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL). Diffraction data were integrated and scaled with HKL3000. Data collection statistics are listed in Table 1. The structure of the CobA-cob(II)alamin-MgATP complex was determined using the apo form of CobA (Protein Data Bank entry 1G5R) as a molecular replacement search model in Molrep. The final model was generated with alternate cycles of manual model building and least-squares refinement using Coop and Refmac. Refinement statistics are listed in Table 1.

In Vivo Assessment of CobA Variant Function. Mutant cobα alleles encoding specific CobA variants were constructed on plasmid pCOBA70 (Table S2 of the Supporting Information) using the QuickChangeII site-directed mutagenesis kit (Stratagene). Plasmids carrying mutant cobα alleles were moved by electroporation into a strain harboring a null allele of cobA [JE15023 (Table S1 of the Supporting Information)]. The functionality of CobA variants was assessed in vivo for their ability to restore AdoCbl synthesis in a ΔcobA strain during growth on ethanolamine as the sole source of carbon, energy, and nitrogen. Strains were grown to full density overnight in Nutrient Broth with ampicillin (100 μg/mL). A 20 μL sample was used to inoculate fresh minimal medium containing ethanolamine, (CN)2Cbi, and DMB (1:40, v/v) in 96-well plates; each culture was analyzed in triplicate. Growth behavior was monitored for 48 h using a BioTek ELx808 Ultra microplate reader. Data were collected at 630 nm every 1800 s at 30 or 37 °C. Plates were shaken for 1795 s between readings.

In Vitro Assessment of CobA Variant Function. Continuous spectrophotometric assays of CobA activity were performed using either cob(II)alamin or cob(I)alamin substrate as described previously, with the following modifications.

Table 1. Data Collection and Refinement Statistics for the CobA-ATP-B12 Complex

| Space group | P212121 |
| Resolution (Å) | 0.979 |
| No. of measured reflections | 647369 |
| No. of unique reflections | 30580 |
| Redundancy | 4.7 (4.8) |
| Completeness (%) | 98.4 (99.8) |
| Average 1/σ | 29.4 (3.6) |
| Ramachandran plot (%) | |
| Most favored | 97.8 |
| Allowed | 2.2 |
| Disallowed | 0.0 |
| Root-mean-square deviation |
| Bond lengths (Å) | 0.020 |
| Bond angles (deg) | 2.657 |

All reaction mixtures contained 0.2 M Tris-HCl (pH 8, 37 °C), 1.5 mM MgCl2, 0.5–50 μM HOCbl, and 1–100 μM ATP. Two assays were used to quantify CobA activity: (i) the Co+ assay, in which 0.5 mM Ti(III)citrate was used to reduce cob(III)alamin to cob(I)alamin, and (ii) the Co2+ assay. The reaction mixture of this assay included 44 μM FldA, and 1 mM NADH to reduce cob(III)alamin to cob(II)alamin.

RESULTS AND DISCUSSION

Evidence of the Existence of Four-Coordinate Cob(II)alamin in the Active Site of CobA. CobA was crystallized under anaerobic conditions in the presence of cob(II)alamin in an effort to generate a structure of the enzyme with the physiologically relevant substrate in its active site. The structure of the CobA-cob(II)alamin-MgATP complex was determined at 2.0 Å resolution; its overall fold is shown in Figure 1. The protein crystallized with a dimer in the asymmetric unit. The electron density is well-defined for both subunits in the dimer. The final model extends continuously from amino acid Tyr6 to Cys270. Both active sites contain an unequivocal electron density for MgATP and a corrinoid.

In the active site of CobA, MgATP is oriented in a unique manner in the opposite orientation compared to MgATP in the other active site.

In the active site of CobA, MgATP is oriented in a unique manner in the opposite orientation compared to MgATP in the other active site.
enzymes with this fold. In this way, the \( \gamma \)-phosphate resides at the location normally occupied by the \( \alpha \)-phosphate in other nucleotide hydrolases. This facilitates the transfer of the 5′-carbon of the ribose to the cobalt ion.

The earlier structure of the CobA-hydroxycob(III)-alamin-MgATP complex also crystallized with a molecular dimer in the crystallographic asymmetric unit, where both active sites contained MgATP but only one bound cob(III)-alamin. In the latter case, the N-terminal section of the polypeptide chain from the symmetry-related subunit folded over the cobalamin and DMB remained bound to the central cobalt ion. In this way, sections of both subunits of the dimer contributed to the active site. Conversely, the corresponding section of the opposing polypeptide chain was disordered in the active site that lacked hydroxycob(III)alamin. In the crystal structure presented here, there is also a dimer in the asymmetric unit but both active sites include cob(II)alamin and MgATP, but even here there is asymmetry in the molecular dimer, as described below.

In the structure of the CobA-cob(II)alamin-MgATP complex, one site is occupied by four-coordinate cob(II)alamin while the other site is occupied by five-coordinate cob(II)alamin. In the active site that contains the four-coordinate cob(II)alamin (subunit B), the N-terminal helix of subunit A extends over the active site of subunit B, while the N-terminal helix of subunit B is disordered. Figures 1–5 were prepared with Pymol.44

Figure 1. Cartoon representation of the S. enterica CobA homodimer. Each subunit binds one molecule of MgATP and one molecule of cob(II)alamin. Subunit B (colored wheat) binds a four-coordinate cob(II)alamin, whereas subunit A (colored white) binds a five-coordinate cob(II)alamin. The N-terminal helix of subunit A extends over the active site of subunit B, while the N-terminal helix of subunit B is disordered. Figures 1–5 were prepared with Pymol.44

Figure 2. Stereoview of the electron density for four-coordinate cob(II)alamin (A), five-coordinate cob(II)alamin (B), and MgATP. Electron density [2.0\( \sigma \) for four-coordinate cob(II)alamin and MgATP, 1.5\( \sigma \) for five-coordinate cob(II)alamin] was calculated from coefficients of the form \( F_o - F_c \), where cob(II)alamin and MgATP were omitted from phase calculation and refinement. The electron density was not as well-defined in the five-coordinate active site.
segments of cob(II)alamin are solvent-exposed and do not contribute to substrate binding. This observation is consistent with the broad specificity of CobA and its role as a corrinoid salvaging enzyme. The square-planar structure of the Co(II) ion and its four nitrogen ligands from the corrin ring together with the lack of axial ligands reveal the presence of four-coordinate cob(II)alamin. A similar structure was observed in the active site of the *L. reuteri* PduO (*Lr* PduO) ACAT.23

In contrast, the other active site (subunit A) contains a five-coordinate cob(II)alamin. Here, the DMB ligand remains coordinated via N3 to the central Co(II) ion. Additionally, the N-terminal helix from opposing subunit B is disordered and could not be modeled because of the lack of electron density. The electron density level for the ligands in this active site is somewhat lower than that observed in the four-coordinate site, suggesting a lower occupancy.

**Figure 3.** Comparison of the polypeptide chain and cob(II)alamin for the four- and five-coordinate states. This shows a stereoribbon representation of the superposition of the four- and five-coordinate states. The four-coordinate state is colored wheat and brown and is denoted as the "closed" conformation of the protein. The five-coordinate species is colored white and light gray and is denoted as the “open” conformation of the protein. The protein fold is essentially identical for both subunits except for the N-terminal helix that is ordered in subunit A and a loop between Met87 and Cys105 (colored brown and gray in the closed and open states, respectively). This loop is well-ordered in both active sites but rotates to exclude DMB in the four-coordinate state. MgATP was excluded for the sake of clarity.

**Figure 4.** Stereoview of the corrin binding site for four-coordinate cob(II)alamin. The corrin ring sits across the MgATP and interacts with a constellation of polar and hydrophobic side chains around the periphery of the corrin ring. The large hydrophobic side chains are colored gray.22 The loop that extends over the corrin ring and displaces DMB in the five-coordinate state (Ala88–Asn97) was omitted for the sake of clarity.

**Interactions of the Corrin Ring with Side Chains in the Active Site.** The protein cores of the two subunits in the asymmetric unit are highly similar and show a root-mean-square difference of only 0.19 Å between 129 structurally equivalent α-carbon atoms. The only significant differences occur in the regions that interact with the corrin ring and relate to differences between the four and five coordination of cob(II)alamin. These differences are discussed later. Overall, the corrin ring binds in a similar location in both active sites but is shifted ∼0.7 Å further into the binding pocket in the case of the four-coordinate cob(II)alamin (Figure 3). In both active sites, the corrin ring lies on top of the MgATP so that most of the interactions occur around the periphery of the corrinoid. The four-coordinate cob(II)alamin experiences more interactions than the five-coordinate cob(II)alamin as a consequence of the displacement of DMB and the small movement further into the active site. However, the interactions in the...
A constellation of hydrophobic residues and hydrophobic components of polar side chains surrounded the corrin ring (Figure 4). The hydrophobic nature of the binding site was noted in the earlier structure of CobA complexed with hydroxycob(III)alamin, though most of those residues were not in contact with the corrinoid because the cobalt ion was positioned ~6.1 Å from the S'-carbon of ATP. This distance was presumed to be due to the hydroxyl β-ligand, which was not visualized in the crystal structure but helped explain why the substrate did not bind in the hydrophobic active site. In this structure, the corrin ring is nestled more deeply in the binding pocket and is in the proximity of Ile65, Trp69, Arg161, Phe184, and Tyr196. This serves to bring the Co(II) ion significantly closer to the MgATP. In the current structure, the Co(II) ion is 3.1 Å from its target and is well-positioned to initiate nucleophilic attack once it is reduced to cob(I)alamin.37 This closer positioning of the corrin ring to MgATP seen in the figure 5. Stereo view of the conformational change between the open and closed states of CobA for the loop between Met87 and Cys105. This shows a superposition of the four-coordinate cob(II)alamin closed state and the open state observed in the five-coordinate complex. The closed state is colored wheat and brown, whereas the open state of the cob(II)alamin complex is colored white. The conformation of Met87–Cys105 is also seen in the MgATP and substrate free CobA determined previously. The loops for these structures are colored light cyan and blue, respectively. In the closed state, Phe91 and Trp93 rotate and translate from a partially buried location within the loop to a stacking position ~4 Å above the corrin ring. The side chains for Phe91 and Trp93 move by ~12.1 and 7.5 Å, respectively. The ordered N-terminal helix from the opposing helix is colored wheat for the closed complex. This contributes three hydrophobic side chains to the corrin binding pocket. The coordinates for the structures of MgATP and substrate free CobA were taken from Protein Data Bank entries 1G5T and 1G5R, respectively.

Figure 4. Structural Basis for the Formation of Four-Coordinate Cob(II)alamin. In the four-coordinate state, the lower ligand (DMB) and entire nucleotide arm are displaced by Phe91 and Trp93 and the N-terminal helix from the opposing subunit (Figure 5). This yields a closed active site or conformation for the enzyme. Relative to the five-coordinate cob(II)alamin state, this displacement involves a conformational change in the loop that extends from Met87 to Cys105 and includes a change in the orientation of Phe91 and Trp93. The structure of this loop in the five-coordinate state is essentially identical to that seen in the substrate free and MgATP-bound forms reported earlier (Figure 5).22 In this case, the active site adopts an open conformation. This suggests that the active site can adopt two stable conformational states, the first of which (open) arises in the absence of substrate, in the presence of MgATP, and in the five-coordinate state. The second conformation (closed) occurs only with four-coordinate cob(II)alamin. Examination of the crystal lattice for the current structure indicates that the active site that carries the five-coordinate cob(II)alamin complex is maintained in the open state by crystal packing forces and thus neither implies nor excludes negative cooperativity between the active sites.
As shown in Figure 5, Phe91 and Trp93 move a considerable distance during the transition from the open to the closed conformation. The α-carbons of these residues move 7.1 and 5.1 Å, respectively, whereas the side chains themselves move ~12.1 and 7.5 Å, respectively (Figure 5). Interestingly, these side chains are mostly buried in both the open and closed conformations, suggesting that there is a small difference in hydrophobic stabilization between states. Thus, in the closed state, the Co(II) is placed in a hydrophobic environment that serves to eliminate water from the α-face of the corrin ring that would quench the cob(I)alamin nucleophile.38 The aromatic side chains of residues 91 and 93 are approximately orthogonal to the corrin ring in the closed conformation. This orientation makes π–π stacking with the corrin ring unlikely, although these residues might be stabilizing cobalamin via π–σ interactions.40

The N-terminal helix of the adjacent subunit provides additional hydrophobic cover to the α-face of the corrin ring. The side chains of Val13 and Val17 are orthogonal to the face of Trp93, whereas Val21 contacts the hydrophobic component of the α-propionamide on pyrrole ring A (Figure 5).

The structure of the four-coordinate substrate suggests that Phe91 and Trp93 play a critical role in CobA function. This role was tested by site-directed mutagenesis. Both in vitro and in vivo analyses were performed, as each provided unique information about the contribution of these residues to adenosyltransferase activity. The in vivo analysis is a sensitive test of the ability of a variant to produce adenosylated corrinoid, as only small amounts are needed for growth. However, the in vivo analysis cannot distinguish between problems of protein expression and folding versus a lack of enzymatic activity. The biochemical analysis provides insight into the molecular consequences of a mutation but does not necessarily indicate how it might influence the biological fitness of its host. The effects of mutations on in vivo function are described first.

Residues Phe91 and Trp93 Are Critical for the Function of CobA in Vivo. A series of mutant cobA alleles were constructed and placed under the control of an inducible promoter for expression, and the resulting variant proteins were tested for their ability to restore AdoCbl synthesis in a ΔcobA strain in vivo. S. enterica cannot synthesize AdoCbl de novo under aerobic conditions4 but can scavenge incomplete corrinoids. AdoCbl is required for induction of the eut operon in S. enterica, which allows the strain to catabolize ethanolamine as a carbon, nitrogen, and energy source.39 The precursor Cbi was added to the medium instead of Cbl to prevent false positives via the Cbl-specific ACAT, EutT. CobA is capable of adenosylating Cbi and Cbl.7 AdoCbi proceeds via S. enterica’s corrinoid salvaging pathway to become AdoCbl.1

Variant CobA proteins with conservative substitutions at these positions (i.e., F91Y, W93F, and W93Y) retained sufficient activity to support AdoCbl synthesis at 37 °C, resulting in wild-type growth of the ΔcobA strain (Figure 6A, group 1). Interestingly, a variant in which residues Phe91 and Trp93 were switched (i.e., CobAW93F, W93F) also retained activity similar to that of the wild-type enzyme (Figure 7B). Figures 6 and 7 were made using Prism 4 (GraphPad, 2003).

Residues Phe91 and Trp93 Are Needed To Displace the Lower Ligand of Five-Coordinate Cob(II)alamin. In general, CobA variants that synthesized enough AdoCbl to support growth of a ΔcobA strain on ethanolamine had specific activities similar to that of the wild-type enzyme (Figure 7B) with two exceptions that are discussed below. All CobA variants that supported growth of the ΔcobA strain on ethanolamine had substitutions that retained aromatic side chains. On the basis of the structure reported here, and extensive analysis of the mechanism of the LrPduO ACAT, we suggest that hydrophobic side chains at positions 91 and 93 play a role in the conversion of five- to four-coordinate cob(II)alamin to allow the formation of the cob(I)alamin nucleophile. In support of this hypothesis, alanine substitutions at either Phe91 or Trp93 resulted in variant enzymes that are unable to produce sufficient AdoCbl for growth on ethanolamine.
variant has a detrimental effect in vivo that is not evident in the in vitro assay. Further work is required to elucidate the effects of this variant on corrinoid adenosylation.

The effects of substitutions at position Phe91 were more severe than those at Trp93, which might be expected on the basis of the closer proximity of its side chain to the corrin ring. CobA<sup>F91A</sup>, CobA<sup>F91H</sup>, and CobA<sup>F91P</sup> variants lost >95% of their activity relative to that of CobAWT in the Co<sup>2+</sup> assay. Surprisingly, a CobA<sup>F91Y</sup> variant retained sufficient enzymatic activity (85% of that of CobAWT) to support growth of the ΔcobA strain on ethanolamine.

The effect of histidine in variant CobA<sup>P91H</sup> was very similar to that reported for the LpPduO ACAT, where replacement of the critical Phe112 residue with histidine inactivated the enzyme by mimicking the effect of the coordination of the imidazole ring of DMB with the cobalt ion. It is unknown whether the imidazole side chain of the CobA<sup>P91H</sup> variant interacts with the cobalt ion, but the predicted proximity of the side chain makes this likely.

**Kinetic Analyses of CobA Variants.** As described above, two assays were used to evaluate CobA activity in vitro. The first assay (termed the Co<sup>2+</sup> assay) used a flavoprotein reductase (Fpr) and NADH to reduce cob(III)alamin to cob(I)alamin via reduced flavodoxin A (FldA). The latter is an electron transfer protein specifically recognized by CobA<sup>12,19</sup>. Reduced FldA supplies the electron needed to reduce cob(I)alamin to cob(II)alamin in the active site of CobA. Because the Co<sup>2+</sup> assay measures the rates of reduction and adenosylation, we consider this assay to be more reflective of what happens in vivo.

The second assay (termed the Co<sup>+</sup> assay) bypasses the need for the Fpr/FldA system by chemically reducing cob(III)alamin to cob(I)alamin using Ti(III)citrate. Cob(I)alamin is a four-coordinate species in solution<sup>41</sup> and needs only to be positioned adjacent to ATP to perform a nucleophilic attack on the S'-carbon of ATP to yield AdoCbl and PPP<sup>41</sup>. With the exception of CobA<sup>F91D</sup>, all CobA variants had detectable activity with the Co<sup>+</sup> assay (Figure 7A and Table 2). Furthermore, the <i>K<sub>m</sub></i> values of all other variant proteins were <5-fold different than those of CobAWT with respect to cob(I)alamin and ATP. The reason for the lack of activity in the CobA<sup>F91D</sup> variant is unknown, although placement of a polar, charged side chain in the proximity of several hydrophobic groups may be detrimental to protein function. Interestingly, the CobA<sup>W93D</sup> retains ~40% specific activity relative to that of the wild type in the Co<sup>+</sup> assay, further indicating a greater tolerance for amino acid variation at this position.

In contrast to the Co<sup>+</sup> assay, the Co<sup>2+</sup> assay showed a much greater variation in kinetic parameters among variants with <30% CobAWT activity (Table 3). The apparent Michaelis constant (<i>K<sub>m</sub></i>) values were higher for CobA<sup>P91F</sup>, CobA<sup>P91W</sup>, and CobA<sup>P91H</sup> variants than for CobAWT, indicating that such substitutions affect the ability of the enzyme to bind the substrate under saturating conditions. Surprisingly, the cob(I)alamin <i>K<sub>m</sub></i> values of CobA<sup>F91L</sup>, CobA<sup>F91W</sup>, and CobA<sup>F91H</sup> were 3-, 4-, and 8-fold lower, respectively, than that of CobAWT.

Turnover (<i>k<sub>cat</sub></i>) values were relatively unchanged across all variants, with the exception of 2- and 10-fold enhancements for CobA<sup>P91W</sup> with respect to cobalamin and ATP, respectively, and a >10-fold decrease with respect to cob(I)alamin for CobA<sup>P91F</sup>. Notable exceptions are discussed below.
The cob(II)alamin was generated chemically using Ti(III)citrate as the reductant.

While the specific activity of CobAWT increased >30-fold when cob(I)alamin was used as a substrate, other variants increased only a few-fold over their specific activity with cob(II)alamin (Figure 7). Kinetic parameters indicated that many of the variants with <10% of the CobAWT activity also had 10–15-fold lower catalytic efficiencies with respect to ATP (Table 3). Explanations for these observations are not obvious from available data and need to be investigated further.

**Effect of an F91W Substitution on CobA Activity.** The formation of four-coordinate cob(II)alamin requires a hydrophobic residue of sufficient bulk to displace the α-ligand base. Given that the activity of the CobA_F91W, W93F variant was similar to that of CobAWT in the Co2+ assay (Figures 5 and 6), we hypothesized that CobA_F91W might also exhibit high levels of activity. Surprisingly, the CobA_F91V variant had a >10-fold decrease in \( k_{cat} \) values and >10-fold increase in \( K_M \) for cob(II)alamin relative to those of CobAWT. The CobA_F91H variant also failed to support growth of A.cholela strain (Figure S5A). In contrast, \( K_M \) values for ATP were lower than those of CobA_F91W, and the turnover number was only a few-fold lower than that of CobAWT in the Co2+ assay. Collectively, these data suggest that the F91W replacement affects cob(II)alamin binding or the formation of four-coordinate cob(II)alamin. Kinetic data obtained using the Co2+ assay (Table 2) support the possibility that smaller ligand base displacement is the process affected by this replacement, because the kinetic values for CobA_F91W did not differ significantly from those of CobAWT. This information suggests that binding of cob(II)alamin to CobA_F91W is not impaired, but because cob(II)alamin is a four-coordinate species, the reaction can occur as efficiently as in CobAWT.

**The F91Y Substitution Makes the S. enterica CobA a Better Enzyme in Vitro.** Strikingly, one CobA variant was a better enzyme than CobAWT in vitro in both the Co2+ and Co2+ assays. In the Co2+ assay, the CobA_F91Y variant had a ∼4-fold lower \( K_M \) for cob(II)alamin and an ∼20-fold lower \( K_M \) for ATP. In the more physiologically relevant Co2+ assay, the CobA_F91Y variant displayed an ∼3-fold higher \( k_{cat} \) and an ∼10-fold higher catalytic efficiency (\( k_{cat}/K_M \)) than CobAWT. These results were surprising because, on the basis of the structure, it would be predicted that the additional polar hydroxyl group might interfere with ligand binding, coordinate with the cobalt ion, or slow product formation or release. Bioinformatic analysis shows that residue Phe91 is conserved among members of the CobA family (Figure S1 of the Supporting Information). Several species of the genus *Ralstonia* have a tyrosine in place of phenylalanine in the equivalent residue of their putative CobA proteins. Several species, including the plant pathogen *Ralstonia*
solanacearum, contain the complete suite of AdoCbl bio-
synthetic genes, several B_{12} dependent enzymes, and additional
ACATs.\textsuperscript{45} To the best of our knowledge, this is the only
variation of this residue tolerated in nature, but this does not
explain why this variant has improved kinetic parameters or
why it is not seen more frequently.

## CONCLUSIONS

The structure and mutational and kinetic analyses of the
housekeeping CobA ACAT of \textit{S. enterica} in complex with
cob(II)alamin and MgATP offer new insights into its
mechanism of catalysis. The structure also revealed how the
catalytically important four-coordinate cob(II)alamin inter-
mediate binds to the active site of the enzyme. Although
CobA- and PduO-type ACATs are structurally dissimilar, both
types of enzymes use similar mechanisms to accomplish
corrinoid adenosylation. In CobA, smaller ligand base displace-
ment appears to be the result of a coordinated effect of residues
Phe91 and Trp93, neither of which can coordinate to the cobalt
ion of the ring, bringing the redox potential of the Co^{3+}/Co^{+}
pair within reach of the FMNH\textsubscript{2} cofactor of flavodoxin A
(FlD). The importance of residues Phe91 and Trp93 was
confirmed in vitro and in vivo.

## ASSOCIATED CONTENT

- Supporting Information
  - Strain tables, plasmid tables, extinction coefficients, and a
    multiple-sequence alignment plot. This material is available free
    of charge via the Internet at http://pubs.acs.org.
  - Accession Codes

X-ray coordinates for the cob(II)alamin-CobA complex have
been deposited in the Research Collaboratory for Structural
Bioinformatics as Protein Data Bank entry 4HUT.

## AUTHOR INFORMATION

Corresponding Author
J.C.E.-S.: Department of Microbiology, University of Georgia,
527 Biological Sciences Building, 120 Cedar St., Athens, GA
30602; phone, (706) 542-2651; Fax, (706) 542-2815; e-mail,
jescala@uga.edu. I.R.: Department of Biochemistry, University
of Wisconsin, 433 Babcock Dr., Madison, WI 53706; phone,
(608) 262-0437; fax, (608) 262-1319; e-mail, ivan.raymont@
biochem.wisc.edu.

Funding
This work was supported by National Institutes of Health
Grants R37 GM40313 to J.C.E.-S. and R01 GM083987 to I.R.
T.C.M. was supported in part by Chemistry and Biology
Interface Training (CBIT) Grant T32 GM008505. Use of the
SBC BM19 beamline at the Argonne National Laboratory
Advanced Photon Source was supported by the U.S. Depart-
ment of Energy, Office of Energy Research, under Contract
W-31-109-ENG-38.

Notes
The authors declare no competing financial interest.

## ABBREVIATIONS
ACAT, ATP:co(I)rinoid adenosyltransferase; Cbl, cobalamin;
Cot, cobalamin α-ligand; Coβ, cobalamin β-ligand; DMB, 5,6-
dimethylbenzimidazole; Cbl, cobalamin; Cbi, cobinamide;
AdoCbl, adenosylcobalamin; CoB\textsubscript{12}, coenzyme B_{12};
HOCbl, hydroxycobalamin; PPP, triplyphosphate; IrPduO, \textit{L. reuteri}
PduO; Tris-HCl, tris(hydroxymethyl)aminomethane hydro-
chloride; TCEP, tris(2-carboxyethyl)phosphine; rTEV, re-
combinant tobacco etch virus; NTA, nitritoltriacetic acid;
FAD, flavin adenine dinucleotide; FlD, flavodoxin A; Fpr,
ferredoxin (flavodoxin):NADPH reductase; NADH, reduced
nicotinamide adenine dinucleotide; MES, 2-(N-morpholino)
ethanesulfonic acid.

## REFERENCES

1. Warren, M. J., Raux, E., Schubert, H. L., and Escalante-Semerena,
Vitamin B12: Unique metalorganic compounds and the most complex vitamins. Molecules 15, 3228–3259.
3. Lenhert, P. G., and Hodgkin, D. C. (1961) Structure of the 5,6-
ammonia-lyase, a B12-dependent enzyme. VII. The mechanism of hydrogen transfer. J. Biol. Chem. 245, 6125–6133.
radical intermediates by coenzyme B\textsubscript{12}-dependent methylmalonyl-CoA
function is required for both de novo cobalamin biosynthesis and
assimilation of exogenous corrinoids in \textit{Salmonella typhimurium}. J.
Identification of the 5,6-dimethylbenzimidazole as the CO ligand of the
coenzyme B-subunit of the corrinoid synthesized by \textit{Salmonella typhimurium}. Nutritional
characterization of mutants defective in biosynthesis of the imidazole
ring. J. Biol. Chem. 267, 13300–13305.
eutT gene of \textit{Salmonella enterica} encodes an oxygen-labile, metal-
186, 5708–5714.
10. IUPAC-IUB Commission on Biochemical Nomenclature
Biochemistry 13, 1555–1560.
of ATP:cob(I)alamin adenosyltransferases in the conversion of B_{12} to
vitro reducing system for the enzymic conversion of cobalamin to
13. Olteanu, H., Wolters, K. R., Munro, A. W., Scrutton, N. S., and
the common polymorphic variants of human methionine synthase
(2003) Spectroscopic and computational studies of Co\textsuperscript{3+}-corrinoids:
Spectral and electronic properties of the B\textsubscript{12} cofactors and biologically
Spectroscopic and computational studies of Co\textsubscript{2+}-corrinoids: Spectral
and electronic properties of the biologically relevant base-on and base-
off forms of Co\textsubscript{2+}corbamin. J. Am. Chem. Soc. 126, 9735–9749.
17. Park, K., Mera, P. E., Escalante-Semerena, J. C., and Brunold, T.
adenosyltransferase PduO from \textit{Lactobacillus reuteri}: Substrate
specifcity and insights into the mechanism of Co(II)-corrinoid
18. Stich, T. A., Buan, N. R., Escalante-Semerena, J. C., and
Brunold, T. C. (2005) Spectroscopic and computational studies of the
ATP:corrinoid adenosyltransferase (CobA) from \textit{Salmonella enterica}.