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Analysis of the Adenosylcobinamide Kinase/Adenosylcobinamide-phosphate Guanylyltransferase (CobU) Enzyme of *Salmonella typhimurium* LT2

IDENTIFICATION OF RESIDUE His-46 AS THE SITE OF GUANYLYLATION*

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CobU is a bifunctional enzyme involved in adenosylcobalamin (coenzyme B_{12}) biosynthesis in *Salmonella typhimurium* LT2. In this bacterium, CobU is the adenosylcobinamide kinase/adenosylcobinamide-phosphate guanylyltransferase needed to convert cobinamide to adenosylcobinamide-GDP during the late steps of adenosylcobalamin biosynthesis. The guanylyltransferase reaction has been proposed to proceed via a covalently modified CobU-GMP intermediate. Here we show that CobU requires a nucleoside upper ligand on cobinamide for substrate recognition, with the nucleoside base, but not the 2′-OH group of the ribose, being important for this recognition. During the kinase reaction, both the nucleotide base and the 2′-OH group of the ribose are important for γ-phosphate donor recognition, and GTP is the only nucleotide competent for the complete nucleotidyltransferase reaction. Analysis of the ATP:adenosylcobinamide kinase reaction shows CobU becomes less active during this reaction due to the formation of a covalent CobU-AMP complex that holds CobU in an altered conformation. Characterization of the GTP:adenosylcobinamide-phosphate guanylyltransferase reaction shows the covalent CobU-GMP intermediate is on the reaction pathway for the generation of adenosylcobinamide-GDP. Identification of a modified histidine and analysis of *cobU* mutants indicate that histidine 46 is the site of guanylylation.

A schematic of adenosylcobalamin (AdoCbl)[1] and the late steps of the biosynthesis of this coenzyme (referred to as nucleotide loop assembly) are shown in Fig. 1. In *Salmonella typhimurium* LT2, nucleotide loop assembly has been divided into two activation branches that involve four enzymes, CobU, CobT, CobC, and CobS (1, 2). CobT and CobC are involved in 5,6-dimethylbenzimidazole activation whereby 5,6-dimethylbenzimidazole is converted to its riboside, α-ribazole (3, 4) (Fig. 1). The second branch of the nucleotide loop assembly pathway is the cobinamide (Cbi) activation branch where adenosylcobinamide (AdoCbi) or adenosylcobinamide-phosphate (AdoCbi-P) is converted to the activated intermediate AdoCbi-GDP by the bifunctional enzyme CobU (5). The final step in AdoCbl biosynthesis is the condensation of AdoCbi-GDP with α-ribazole, which is catalyzed by cobalamin synthase (CobS) to yield AdoCbl (6).

Work reported in this paper, further analyzes the bifunctional enzyme CobU. This enzyme has kinase and guanylyltransferase activities. However, it appears that both activities are not required at all times. The kinase activity has been proposed to function only when *S. typhimurium* LT2 is assimilating Cbi from its environment (7).[2] In contrast, the guanylyltransferase activity is required for both the assimilation of exogenous Cbi and for *de novo* synthesis of AdoCbl (Fig. 1) (7).[2]

Biochemical and structural analysis on CobU have given insights into how this enzyme may work (5, 9, 10). Biochemical studies showed the reactions catalyzed by CobU are as follows: Reaction 1 (kinase): AdoCbi + NTP → AdoCbi-P + NDP; Reaction 2 (guanylyltransferase) (i) CobU + GTP → CobU-GMP + PP_i and (ii) CobU-GMP + AdoCbi-P → AdoCbi-GDP + CobU.

The kinase reaction (Reaction 1) can use either ATP or GTP as a γ-phosphate donor to generate the AdoCbi-P intermediate (5), as does its orthologue, CobP, in *Pseudomonas denitrificans* (11). The guanylyltransferase reaction is proposed to occur via two half-reactions (Reaction 2). First, the enzyme forms a CobU-GMP covalent phosphoramidate bond, followed by the transfer of the GMP moiety to AdoCbi-P to generate the final product AdoCbi-GDP (5).

Structural data showed CobU functions as a homotrimer, forming three prominent clefts with the conserved phosphate-binding loop of each monomer at the base of each cleft (9, 10). Co-crystallization of CobU with GTP determined there is a substantial substrate-induced conformational change associated with the formation of the CobU-GMP intermediate (10). This conformational change closes the clefts and, in the process, forms a GMP-specific binding pocket. The structural work

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[1] The abbreviations used are: AdoCbl, adenosylcobalamin; AdoCbi, adenosylcobinamide; AdoCbi-P, adenosylcobinamide-phosphate; AdoCbi-GDP, adenosylcobinamide guanosine diphosphate; Cbi, cobinamide; $(CN)_2$Cbi, dicyanocobinamide; $(CN)_2$Cbi-P, dicyanocobinamide phosphate; $(CN)_2$Cbi-GDP, dicyanocobinamide guanosine diphosphate; GuoCbi, guanosylcobinamide; InoCbi, inosylcobinamide; CyoCbi, cytosylcobinamide; dAdoCbi, 2′,5′-dideoxyadenosylcobinamide; AP, *R*-1-amino-*O*-2-propanol; AP-P, *R*-1-amino-propanol-*O*-2-phosphate; DTT, dithiothreitol; HPLC, high performance liquid chromatography; TCEP-HCl, Tris(2-carboxyethyl)phosphine hydrochloride; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

[2] Thomas, M. G., and Escalante-Semerena, J. C. (2000) *J. Bacteriol.* **182,** 4227–4233.

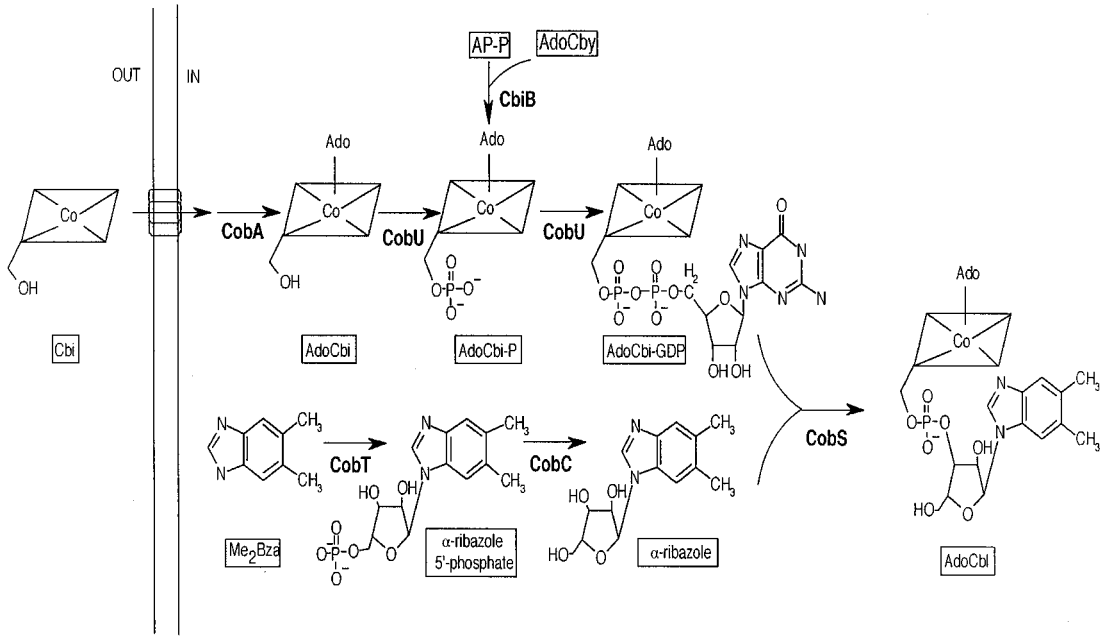

FIG. 1. **Schematic representation of the final steps of the *de novo* AdoCbl biosynthetic pathway and assimilation of exogenous Cbi by *S. typhimurium* LT2.** The *two parallel lines* at the *left* of the figure represent the cytoplasmic membrane with the location of the periplasm and cytoplasm indicated by *OUT* and *IN*, respectively. The *boxes* in the cytoplasmic membrane represent a Cbi transporter. The enzymes catalyzing each step in the pathway are in bold-face type and immediately below the reaction that they catalyze. The label for each intermediate of the biosynthetic pathway is *boxed below* each compound. *Me_2Bza*, 5,6-dimethylbenzimidazole.

also suggested there may be an overlap between the proposed GTP-binding site for guanylylation and the proposed nucleotide-binding site for phosphorylation. This raises the question of how this enzyme coordinates the binding of two different nucleotides for two separate enzymatic activities. Additionally, the finding that CobU functions as a guanylyltransferase or a kinase/guanylyltransferase raises questions as to how the enzyme binds two relatively large substrates, AdoCbi or AdoCbi-P, and discriminates between kinase or guanylyltransferase activities.

Interestingly, CobU was found structurally and topologically similar to the central domain of the RecA protein of *Escherichia coli* (9). CobU and RecA both contain a phosphate binding loop (Walker A box), a Walker B box, and a non-prolyl cis peptide bond in similar positions in the three-dimensional structures (9, 10, 12, 13). Additionally, both proteins bind nucleotides and three-dimensional structures have been solved for both proteins in the presence of nucleotide; GMP for CobU (10) and ADP for RecA (12). Based on these similarities, CobU and RecA most likely shared a common ancestor, and information obtained from the analysis of either protein may give insights into how both proteins function.

The specificity of the CobU enzyme for the corrin ring substrate during the kinase reaction and the specificity of the enzyme for its nucleotide substrate for both the kinase and nucleotidyltransferase reactions was investigated. It is demonstrated that during the ATP:AdoCbi kinase reaction, in the absence of GTP, CobU is altered to a less active conformation due to the formation of a stable, covalent CobU-AMP complex. It is also shown that the CobU-GMP intermediate is a true intermediate of the guanylyltransferase reaction, and that histidine 46 is the site of guanylylation.

EXPERIMENTAL PROCEDURES

Bacteria, Culture Media, and Growth Conditions

Strain JE3207 ((*hisG-cob*)Δ299/pGP1–2 *rpo*$^+$ T7 RNA polymerase *kan*$^+$, pJO52 *cobU*$^+$) was used for the overexpression of wild-type CobU protein (5). Conditions for the overexpression of wild-type CobU protein were as follows. Strain JE3207 was grown at 30 °C in 2-liter batches of LB medium containing kanamycin (50 μg/ml) and ampicillin (100 μg/ml) in 4-liter Erlenmeyer flasks. Mid-log phase cultures (A_{650} = 0.6) were shifted to 42 °C for 1 h followed by expression at 37 °C for 2 h. After 2 h, cells were harvested and resuspended in 25 ml (per 2-liter batch) of 50 mM Tris-HCl, pH 8.0, at 4 °C containing 1.4 M glycerol. Cells were frozen at −20 °C until use.

Purification of CobU

Step 1: Preparation of Cell-free Extracts—Cell paste obtained from four 2-liter cultures of the overexpression strain JE3207 was used for each purification. Frozen cells were thawed and the following reagents were added: 10 mM (final concentration) dithiothreitol (DTT), 200 μM phenylmethanesulfonyl fluoride, 1 mM EDTA. Cells were broken by sonication (550 Sonic Dismembrator, Fisher Scientific, Itasca, IL) at setting 7, 50% duty. Sonication was performed four times for 5 min at 1-s intervals while keeping the sample below 15 °C. Cell debris was cleared from the extracts by centrifugation at 34,000 × *g* for 30 min in a Beckman J2–21 centrifuge using a KA21.50 rotor (Composite Rotor, Inc., Mountain View, CA).

Step 2: Ammonium Sulfate Precipitation—Finely ground Ultrapure™ ammonium sulfate (ICN Biomedicals Inc., Cleveland, OH) was added to the cell-free extracts to 30% saturation. The sample was incubated on ice with stirring for 30 min. Protein precipitate was pelleted by centrifugation at 14,000 × *g* (Beckman J2–21 centrifuge, KA21.50 rotor) for 20 min. Supernatant was recovered and ammonium sulfate was added to 55% saturation. Sample was incubated on ice with stirring for 30 min. Protein precipitate was pelleted by centrifugation as above. Supernatant was poured off, and the precipitated protein was resuspended in buffer A (50 mM Tris-HCl, pH 8.0, at 4 °C, 10 mM DTT, 200 mM phenylmethanesulfonyl fluoride, 1 mM EDTA). Resuspended protein was dialyzed against buffer A.

Step 3: Anion Exchange Chromatography—Dialyzed, ammonium sulfate-precipitated protein was loaded at 0.4 cm/min onto a 50-ml DEAE Sepharose fast-flow (Sigma) column (10 × 2.5 cm) pre-equilibrated with buffer A. After the sample was loaded, the column was washed with 250 ml of buffer A. CobU was eluted using a 500-ml linear gradient of 0–0.5 M NaCl in buffer A. Fractions containing CobU, detected by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie Blue staining, were pooled and dialyzed against buffer A.

Step 4: Dye-ligand Chromotography—Pooled fractions from the DEAE column were dialyzed against buffer B (50 mM Tris-HCl, pH 8.0, at 4 °C, 10 mM DTT, 200 μM phenylmethanesulfonyl fluoride, 5 mM $MgCl_2$). After dialysis, pooled fractions were loaded at 0.2 cm/min onto a 50-ml Cibacron Blue type 3000 (Sigma) column (10 × 2.5 cm) pre-

equilibrated with buffer B. After loading, the column was washed with 100 ml of buffer B. CobU was subsequently eluted with a 500-ml linear gradient of 0–2.0 M NaCl in buffer B. Fractions containing CobU, detected by 12% SDS-PAGE and Coomassie Blue staining, were pooled and dialyzed against buffer B.

Step 5: Size-exclusion Chromatography—Fractions from the Cibacron Blue column containing CobU were pooled, concentrated to a final volume of ~10 ml with Centriprep 10 concentrators (Amicon, Beverly, MA), and dialyzed against buffer C (1.8 M Tris-HCl, pH 8.0, at 4 °C, 5 mM DTT, 5 mM EDTA). After dialysis, sample was concentrated to ~5 ml and was loaded at 0.3 cm/min through a 130-ml Sephacryl S-300-HR (Sigma) column (75 × 1.5 cm) pre-equilibrated with buffer C. Fractions containing CobU, based on 12% SDS-PAGE and Coomassie Blue staining, were pooled and dialyzed extensively against buffer D (50 mM Tris-HCl, pH 8.0, at 4 °C, 5 mM DTT, 0.1 M NaCl, 680 mM glycerol). Protein was concentrated to 1.4 mg/ml and stored at −80 °C after drop-freezing in liquid nitrogen. Protein was stable under these conditions for months.

During size exclusion chromatography a high Tris-HCl concentration (1.8 M) was used as a mild denaturant, as described previously for actin purification (14). This step was included to enhance the release of any components that may be bound in the active site of CobU. The inclusion of the mild denaturant did not have a detectable effect on the specific activity of CobU when compared with the specific activity of CobU obtained through other protocols (data not shown). This denaturant was essential for consistent CobU crystal formation during structural analysis of the enzyme.[3]

Protein quantitation was determined by either the commercially available Bio-Rad Protein Assay kit or trichloroacetic acid-turbidimetric assay (15) both using bovine serum albumin as a standard. Assays gave comparable results and were consistent with protein concentration determined by amino acid analysis (data not shown).

Synthesis and Purification of Cbi substrates

Synthesis and purification of AdoCbi and AdoCbi-P were performed as described elsewhere.[2] For dAdoCbi, GuoCbi, InoCbi, and CyoCbi synthesis, the reactions mixtures were identical to those described[2] except 800 mM ATP was replaced by 800 mM dATP, GTP, ITP, or CTP. Purification and quantitation of Cbi substrates was performed as described elsewhere.[2]

CobU Activity Assays

Thin Layer Chromatography (TLC)—All reactions mixtures contained 50 mM Tris-HCl, pH 8.5, at 25 °C, 1.25 mM Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP-HCl), 10 mM $MgCl_2$, 680 mM glycerol, 0.1 M NaCl in addition to CobU and substrate at concentrations as indicated. Reactions were terminated by the addition of 5 μl of 0.1 M KCN, pH 10.0, and immediately incubated at 70 °C for 10 min. Samples were centrifuged for 30 s at 14,000 × *g* and 5 μl of each sample was spotted onto cellulose TLC plates (Macherey-Nagel Polygram™ CEL 400, Düren, Germany). Products and reactants were separated using ascending TLC with a solvent system of isobutyric acid:water:ammonium hydroxide (66:33:1) (5, 16). TLC resolution was allowed to run for 1.5 h, which corresponded to a 10-cm migration of the solvent front. All assays were performed in dim light to minimize the photolysis of the Co-C bond of the Cbi substrate.

To quantitate the amount of product synthesized, the TLC plate was dried, then cut in half to remove the unincorporated label. The portion of the TLC plate containing the $(CN)_2$Cbi-P or $(CN)_2$Cbi-GDP product was exposed to a PhosphorImager screen along with standards containing known amounts of radioactivity. A standard curve was generated, and determination of the product synthesized in each reaction mixture was based on this standard curve. The amount of product synthesized was calculated by adjusting for decay of radioactivity, the known ratio of radiolabeled to unlabeled nucleotide at time 0, and the ^{32}P specific activity of the sample determined by liquid scintillation counting. Calculations were periodically verified by cutting product spots from the TLC plate and determining the amount of radioactivity in each sample by liquid scintillation. At all times, the calculated values using the PhosphorImager standard curve were in agreement with the values obtained by scintillation counting.

High Performance Liquid Chromatography (HPLC) Assays—Reaction mixture components, assay conditions, and assay termination were the same as described above. The enzyme and substrate concentrations were as indicated. For the separation of the products and reactants a previously reported HPLC profile (11) was used with the following modifications. The chromatograph used was a computer-controlled Waters model 996 with a photodiode array detector and model 600 quaternary delivery system (Waters, Milford, MA). Separations were performed at room temperature on a LUNA 5 μ C_{18} (2) column (150 × 4.6 mm) (Phenomenex, Torrance, CA) at a flow rate of 1 ml/min. The solvents used were as follows: solvent A (0.1 M PO_4, pH 6.5, 10 mM KCN) and solvent B (0.1 M PO_4, pH 8.0, 100% acetonitrile (1:1), 10 mM KCN). The complete reaction mixture for each sample (20 μl) was loaded onto the column pre-equilibrated with 98% A, 2% B. The following protocol was used for separation. One-min isocratic development with 98% A, 2% B; 5-min linear gradient from 98% A, 2% B to 75% A, 25% B; 15-min linear gradient from 75% A, 25% B to 65% A, 35% B; 4-min linear gradient from 65% A, 35% B to 98% A, 2% B. Cbi products and substrate eluted at times indicated in Fig. 3*B*. Product generated was determined by analyzing the ratio of products ($(CN)_2$Cbi-P and $(CN)_2$Cbi-GDP) and substrate ($(CN)_2$Cbi). Quantitation of product generated using either the HPLC or TLC assay were in good agreement.

Detection of CobU-[^{32}P]NMP Complex

Formation of the CobU-[^{32}P]AMP Complex during the ATP:AdoCbi Kinase Reaction—CobU (60 nM) was incubated in the following 20-μl reaction mixtures. Tris-HCl (100 mM), pH 8.5, at 25 °C, 5 mM $MgCl_2$, 30 μM AdoCbi, 100 μM ATP, 0.3 μM [α-^{32}P]ATP (800 Ci/mmol) (NEN Life Science Products) in the presence or absence of 100 μM GTP. Reactions were incubated for 20 min at 25 °C. Reactions were terminated by the addition of 20 μl of 2 × loading buffer (17) and incubated at 70 °C for 10 min. Separation of the unincorporated label and CobU-[α-^{32}P]AMP was achieved by 12% SDS-PAGE. The gel was dried and exposed to a PhosphorImaging plate for visualization.

Stability of the CobU-[^{32}P]AMP Complex to Acid and Alkali Treatment—Analysis of the chemical stability of the CobU-[^{32}P]AMP complex was performed as described previously (18) with the following modifications. A 120-μl reaction mixture containing 50 mM Tris-HCl, pH 8.5, at 25 °C, 10 mM $MgCl_2$, 1.25 mM TCEP-HCl, 680 mM glycerol, 0.1 M NaCl, 0.1 μM [α-^{32}P]ATP (800 Ci/mmol), and 0.86 μM CobU was incubated at 25 °C. After 10 min, the reaction was stopped by the addition of 18 μl of stop buffer (10% SDS, 1.5 M DTT, 1.36 M glycerol) and the sample was heated to 70 °C for 10 min. Twenty-three-μl samples of the reaction mixture were then transferred to fresh tubes containing 5 μl of 1.0 M HCl, 0.16 M HCl, ddH_20, 1.8 M NaOH, or 3.3 M NaOH to alter the final pH to ~1, 3, 8.5, 11, and 13, respectively. Twenty-eight μl of 2 × loading buffer (17) was then added to each sample and heated to 70 °C for 10 min. Samples were resolved on 12% SDS-PAGE and visualized by PhosphorImaging. For the analysis of the CobU-[^{32}P]GMP chemical stability, the same procedure was followed, however, [α-^{32}P]GTP replaced [α-^{32}P]ATP.

Nucleotide Exchange Reactions

Starting reaction mixtures for CobU-[^{32}P]GMP exchange reactions contained the following: Tris-HCl (50 mM), pH 8.5, at 25 °C, 10 mM $MgCl_2$, 1.25 mM TCEP-HCl, 680 mM glycerol, 0.1 M NaCl, 52 nM [α-^{32}P]GTP (800 Ci/mmol), and 0.25 μM CobU (trimer). This concentration of label to enzyme was chosen to minimize the formation of more than one molecule of CobU-[^{32}P]GMP per trimer. Reaction mixtures (180 μl) were incubated for 5 min at 25 °C. At time 0, a 15-μl sample was removed and added to 15 μl of 2 × loading buffer (17). After removal of the zero time point sample, nonradioactive GTP was added to a final concentration of 2 mM to bring the final volume to 220 μl. Samples were mixed by pipetting, and 20-μl samples were removed at specified time points and added to 20 μl of 2 × loading buffer (17). After the addition of loading buffer, the reactions were incubated at 70 °C for 10 min. Equal protein concentrations were separated by 15% SDS-PAGE to differentiate between covalently bound and unincorporated label. Identical reactions were run for the ATP nucleotide exchange reactions where [α-^{32}P]ATP replaced [α-^{32}P]GTP and nonradioactive ATP was added to a final concentration of 2 mM instead of GTP.

Characterization of the Guanylylated Amino Acid Residue

A previously described protocol (19, 20), with minor modifications, was used to characterize the guanylylated amino acid residue with minor modifications. CobU (1.4 μg) was added to a reaction mixture (100 μl final volume) containing 50 mM Tris-HCl, pH 8.5, at 25 °C, 10 mM $MgCl_2$, 1.25 mM TCEP-HCl, 680 mM glycerol, 0.1 M NaCl, 10 μCi of [α-^{32}P]GTP (800 Ci/mmol) and incubated for 10 min at 25 °C to allow formation of the CobU- [^{32}P]GMP intermediate. The reaction was terminated by the addition of 200 μl of 15% trichloroacetic acid and

[3] T. B. Thompson and I. Rayment, unpublished data.

incubated on ice 30 min. Precipitated protein was recovered by centrifugation (14,000 × *g* for 5 min). The pellet was washed once with 200 μl of 10% trichloroacetic acid followed by 3 washes with 0.5 ml of cold acetone. After the final acetone wash, the pellet was allowed to dry. The precipitated protein was resuspended in 100 μl of 50 mM sodium carbonate, pH 10.5, 1% SDS. Sodium periodate ($NaIO_4$) was added to a final concentration of 10 mM and incubated at room temperature in the dark for 30 min. Ethylene glycol was added to a final concentration of 50 mM to quench periodate. pH was adjusted to 11 with 1 M NaOH and the sample was incubated at 50 °C for 1 h. NaOH was then added to a final concentration of 3 M and the sample was incubated at 110 °C for 3 h to hydrolyze the protein. The hydrolysate was neutralized with 10% (v/v) perchloric acid ($HClO_4$) and the precipitate was removed by centrifugation at 13,000 × *g* (Marathon 13 K/M microcentrifuge, Fisher Scientific) for 5 min. A 5-μl sample of the supernatant was mixed with carrier standard amino acids (phospholysine, phosphohistidine, and phosphoarginine) and analyzed by silica gel TLC (20, 21) along with samples of the individual phosphorylated amino acids. Phosphohistidine and phospholysine standards were synthesized as described previously (22), while phosphoarginine was commercially available (Sigma).

Generation of Site-directed Mutations in cobU

To generate the *cobU*H46A mutation a polymerase chain reaction (PCR) that incorporated the phosphorylated mutagenic oligonucleotide (5′-GAGAATTCAGCAT**GCT**AAAGATGGCA-3′) was used (23). The bases in bold-face type identify the altered codon. The external primers used in the mutagenesis were the −40 5′-GTTTTCCCAGTCACGAC-3′) and reverse (5′-AGCGGATAACAATTTCACACAGGA-3′) universal primers and the DNA template used was plasmid pJO51 (5). The PCR-amplified product was cloned into pUC119 using *Bam*HI/*Hin*dIII sites and then subcloned into the overexpression vector pT7-7 using *Nde*I/*Hin*dIII restriction sites. The mutated, overexpression plasmid is pCOBU9 (*cobU*H46A).

To generate the *cobU*H46N, *cobU*H45A/H46A, and *cobU*H45A mutations the *Eco*RI site immediately upstream of the histidine codons was used as a convenient restriction site in each mutagenic primer. Each mutagenic primer was used in a PCR reaction with the −40 primer shown above. The DNA template was pJO51 (5), which allowed amplification from the internal *Eco*RI site of *cobU* to the external −40 primer site. The PCR product was then digested with *Eco*RI/*Hin*dIII and the digested fragment was cloned into *Eco*RI/*Hin*dIII-digested pJO52. This resulted in the exchange of the wild-type 3′-half of *cobU* with the mutagenized PCR product. The mutagenic primers used were as follows: H46N (5′-GCGAGAATTCAGCAT**AAT**AAAGATGGCAGG-3′), H45A/H46A (5′-GCGAGAATTCAG**GCTGCT**AAAGATGGCAGG-3′), and H45A (5′-GCGAGAATTCAG**GCG**CATAAAGATGGCAGG-3′). The bases in bold-face type indicate the altered codon(s). The resulting plasmids carrying the *cobU*H46N, *cobU*H45A/H46A, and *cobU*H45A mutations were pCOBU10, pCOBU11, and pCOBU12, respectively. All mutant clones were sequenced to ensure correct incorporation of the mutated codon, and that no additional PCR-induced mutations were present. Nonradioactive sequencing was performed at the University of Wisconsin, Madison Biotechnology Center. Plasmids pCOBU9-12 were individually transformed into JE2587 ((*hisG-cob*)Δ299/pGP1-2 *rpo*$^+$ T7 RNA polymerase *kan*$^+$) and used for overexpression and purification of the respective proteins as described above for wild-type CobU.

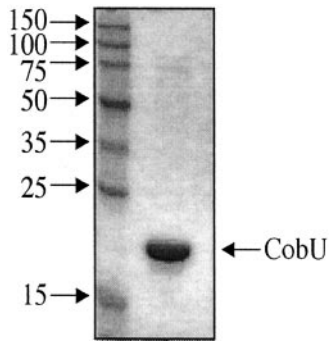

FIG. 2. **SDS-PAGE analysis of purified CobU protein.** Purified CobU (6.25 μg) was resolved by 12% SDS-PAGE and stained with Coomassie Blue. Size markers are indicated by *arrows* on the *left* with molecular mass shown in kilodaltons.

RESULTS

Purification of CobU—Fig. 2 shows a sample of purified CobU following the protocol outlined under "Experimental Procedures." This protocol resulted in a 10-fold purification of CobU to >95% homogeneity based on densitometry (data not shown). This protocol allowed for a more rapid and reproducible purification of CobU than previously described (5, 9).

Cbi Kinase and Cbi-P Guanylyltransferase Assays—Two different assays were used to detect product formation during the Cbi kinase or Cbi-P nucleotidyltransferase reactions. The first assay relied on ^{32}P-labeled nucleotide and separation of the incorporated and unincorporated label using ascending TLC (Fig. 3*A*). This assay system could be used to detect both the Cbi kinase and subsequent Cbi-P guanylyltransferase reactions when γ-^{32}P-labeled nucleotide was used (Fig. 3*A*). The nucleotidyltransferase reaction was followed using the same TLC system by substituting α-^{32}P-labeled nucleotide for the γ-^{32}P-labeled nucleotide.

The second assay was an HPLC assay that separated $(CN)_2$Cbi, $(CN)_2$Cbi-P, and $(CN)_2$Cbi-GDP (Fig. 3*B*). The HPLC

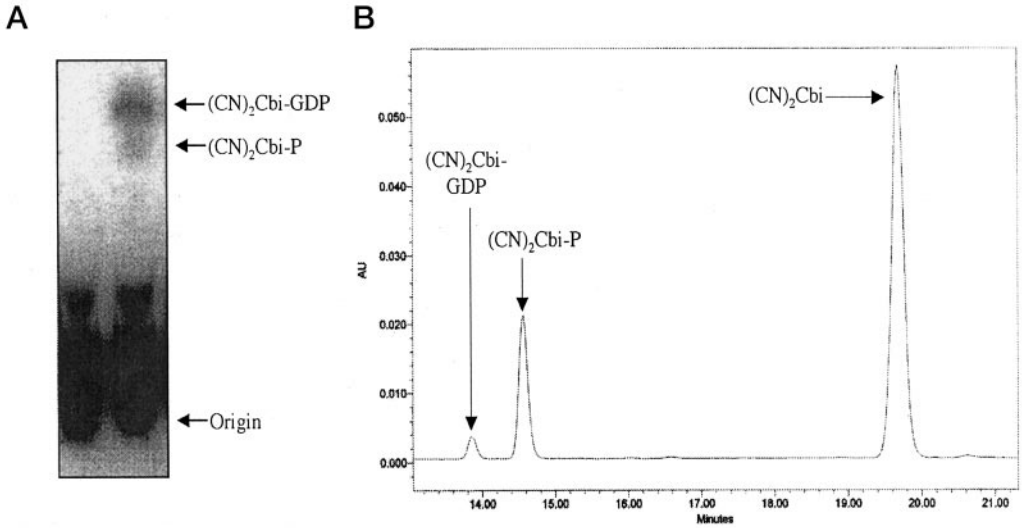

FIG. 3. **Representative end points of TLC and HPLC assays of the GTP:AdoCbi kinase reaction.** *Panel A,* representative TLC assay separating unincorporated [γ-^{32}P]GTP from label incorporated into $(CN)_2$Cbi-P or $(CN)_2$-GDP. *Origin*, $(CN)_2$Cbi-P and $(CN)_2$Cbi-GDP are indicated by *arrows*. *Panel B*, representative HPLC assay separating $(CN)_2$Cbi, $(CN)_2$Cbi-P, and $(CN)_2$Cbi-GDP. Each product is identified by an *arrow* and label.

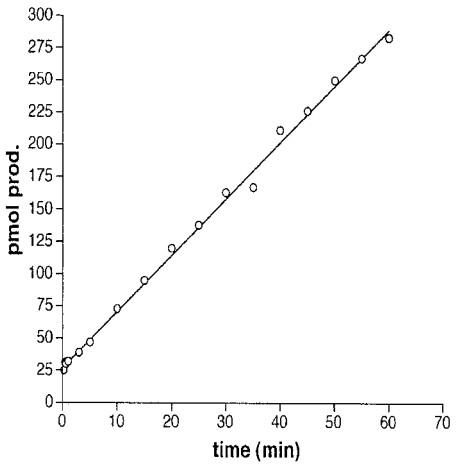

FIG. 4. **Representative progress curve of the GTP:AdoCbi kinase reaction showing an initial burst of product formation.** Product formation was followed using the TLC assay system. Buffer conditions were as described under "Experimental Procedures," enzyme and substrate concentrations were as follows: 60 nM CobU, 50 μM AdoCbi, 100 μM GTP; 0.43 μM [γ-^{32}P]GTP (30 Ci/mmol). Total volume of the reaction was 380 ml after the addition of enzyme to start the reaction. Twenty-μl samples were removed at the indicated times, added to fresh tubes containing 5 μl of 0.1 mM KCN, pH 10.0, and heated to 70 °C for 10 min. Earliest time points were at 15, 30, 45, and 60 s, followed by 3 min, 5 min, and then every 5 min until 60 min. Product generated included both AdoCbi-P and AdoCbi-GDP and is calculated for a 20-μl reaction volume.

profile for separating Cbi substrate and products was a minor modification of a previously described procedure (11). Quantitation of the product generated using either the TLC or HPLC system was in good agreement.

Progress Curves of the GTP:AdoCbi Kinase Reaction—To determine relevant kinetic values for the GTP:AdoCbi kinase reaction, a linear range of the reaction was determined using the TLC assay system, when [γ-^{32}P]GTP was used as the γ-phosphate donor. It should be noted that analysis of the GTP:AdoCbi kinase reaction alone was not possible. A low level (<10% of the total product) of $(CN)_2$Cbi-GDP was always detected (Fig. 3, *A* and *B*) and was included in all product formation calculations. While the progress curve of the reaction did show linearity, there was an unexplained initial burst of activity for the GTP:AdoCbi kinase reaction within the first few seconds of the assay followed by linearity for longer than 1 h (Fig. 4). Both the TLC and HPLC assay systems detected this burst suggesting it was not due to a quantitation artifact of the TLC system (data not shown). Extrapolation of the linear portion of the curve through the *y* axis determined that the burst was above the number of moles of enzyme used in the assay thereby eliminating a single turnover event followed by a new steady state. The size of this burst varied with both enzyme concentration and substrate concentration (data not shown). Lowering the enzyme concentration, in an attempt to eliminate possible product inhibition, did not eliminate the burst, at least to the detection limit of the TLC assays. Analysis of the linear portion of the progress curve while varying substrate concentrations suggested that the linear portion of the progress curve did not reflect steady-state conditions that would allow kinetic parameters to be determined (data not shown). The same burst and subsequent linear product formation was also seen for the GTP:AdoCbi-P guanylyltransferase reaction (data not shown). With this limitation, the enzyme was characterized using end point assays whereby product formation could be determined but not kinetic parameters.

Cbi Substrate Specificity for the GTP:Cbi Kinase Reaction—To understand substrate recognition elements on Cbi, the upper ligand of Cbi was altered and assayed for product formation. The TLC assay system, with [γ-^{32}P]GTP as the γ-phosphate donor, was used to quantitate product formation. No kinase activity was detected with $(CN)_2$Cbi even at millimolar concentrations (Table I). Furthermore, no competition between $(CN)_2$Cbi and AdoCbi was observed even when $(CN)_2$Cbi was in a 100-fold molar excess. This result suggested that $(CN)_2$Cbi did not bind to the enzyme, at least in a position that would allow catalysis or competition with the natural substrate. Alternatively, the enzyme has a much higher affinity for AdoCbi than $(CN)_2$Cbi. One possible reason for the absence of any detectable activity with $(CN)_2$Cbi was that CobU might require a nucleoside upper ligand. To investigate what moieties of the nucleoside upper ligand were important for recognition, the nucleoside base or ribose was altered, and product formation was compared (Table I). Removing the 2′-OH group of the ribose (dAdoCbi) did not have any detectable effect on product formation relative to AdoCbi. However, changing the nucleoside base did reduce product formation by approximately 40% (Table I). Approximately the same reduction in product formation was seen with both alternative purine upper ligands (GuoCbi and InoCbi) and the one pyrimidine upper ligand (CyoCbi). These data suggested a nucleoside upper ligand was required for substrate recognition, with an adenosine base being favored. It was also noted that all Cbi derivatives were substrates for the guanylyltransferase reaction based on the detection of Cbi-GDP in all the above mentioned reactions.

TABLE I
CobU specificity for the Cbi upper ligand

Cobinamide substrate	Product generated[a]	Product generated relative to AdoCbi
	pmol	*%*
AdoCbi	29.2 ± 0.6	100
dAdoCbi	29.7 ± 1.2	102
GuoCbi	18.1 ± 0.8	62
InoCbi	16.0 ± 0.1	55
CyoCbi	19.1 ± 2.6	65
$(CN)_2$Cbi	ND[b]	NA[c]

[a] Product detected using the GTP:Cbi kinase TLC assays. Each reaction (20 μl) contained 50 mM Tris-HCl, pH 8.5, at 25 °C, 10 mM $MgCl_2$, 1.25 mM TCEP-HCl, 680 mM glycerol, 0.1 M NaCl, 100 μM GTP; 0.5 μCi of [γ-^{32}P]GTP (30 Ci/mmol); 6 nM CobU (trimer), and 50 μM of the indicated Cbi substrate. Reactions were run for 20 min and terminated by the addition of 5 μl of 0.1 M KCN pH 10.0, and heating the sample at 70 °C for 10 min. 5 μl of each terminated reaction was spotted onto cellulose TCL plates and products were resolved and quantitated as described under "Experimental Procedures." Product generated included both Cbi-P and Cbi-GDP.

[b] ND, none detected.

[c] NA, not applicable.

Nucleotide Specificity of the Kinase and Nucleotidyltransferase Reactions—It was previously determined that both CobU (5) and its orthologue in *P. denitrificans*, CobP (11), can use either GTP or ATP as the γ-phosphate donor in the kinase reaction. Furthermore, it was determined that the addition of GTP to the ATP:AdoCbi kinase reaction stimulated the ATP-dependent kinase reaction (5, 11). Because of this GTP effect, γ-phosphate donors were tested in the presence or absence of GTP (Table II). These assays were performed using a combination of the HPLC and TLC assay systems. First, product formation was monitored with the HPLC assay system when 100 μM of the various γ-phosphate donors was tested for product formation in the presence or absence of 15 μM GTP. This assay system was used because of the limitation of commercially available γ-^{32}P-labeled nucleotides. For the reactions containing 15 μM GTP, a parallel reaction was run that included γ-labeled GTP to allow quantitation, via TLC assays, of the

TABLE II
Nucleotide specificity for the AdoCbi kinase reaction

Substrate	Picomole of Product generated[a]	Picomole of Product generated in presence of 15 μM GTP[b]
GTP	231	114[c] (100%)
ATP	50	218 (10%)
CTP	0	32 (100%)
UTP	0	66 (100%)
dGTP	43	60 (37%)
dATP	7	109 (21%)
dCTP	0	33 (100%)

[a] Product detected using HPLC assays. Each reaction (20 μl) contained 50 mM Tris-HCl, pH 8.5, at 25 °C, 10 mM $MgCl_2$, 1.25 mM TCEP-HCl, 680 mM glycerol, 0.1 M NaCl, 60 nM CobU, and 100 μM of the indicated nucleotide substrate. Reactions were run for 20 min and terminated by the addition of 5 μl of 0.1 M KCN, pH 10.0, and heating the sample at 70 °C for 10 min. Each terminated reaction was run through HPLC and products were resolved and quantitated as described under "Experimental Procedures." Product generated included both Cbi-P (and Cbi-GDP in the case of GTP). Each value is the average of two independent reactions.

[b] Product detected using the HPLC assays indicated by number on the left. The percentages in parentheses indicate the amount of product derived from added GTP based on parallel TLC assays employing [γ-^{32}P]GTP as a tracer. Reactions were performed as described above, however, with the addition of 15 μM GTP in each reaction.

[c] Reaction was run as described above, except the 15 μM GTP was the only nucleotide added to the reaction mixture. This value, therefore, represents the maximum product generated from the 15 μM GTP added to each reaction.

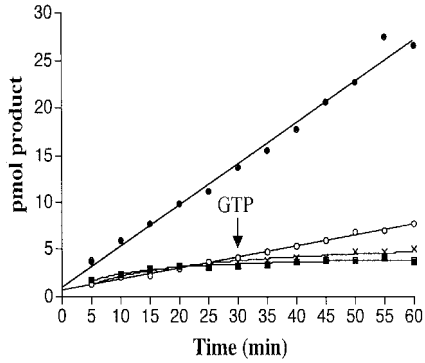

FIG. 5. **Representative progress curve of the ATP:AdoCbi kinase reaction in the presence or absence of GTP.** Product formation was followed using the TLC assay system. Buffer conditions were as described under "Experimental Procedures," enzyme and substrate concentrations were as follows: 6 nM CobU (trimer), 15 μM AdoCbi, 100 μM ATP, 0.5 μM [γ-^{32}P]ATP (30 Ci/mmol), and the presence or absence of 100 μM GTP as indicated. Total volume of the reaction was 280 μl. 20-μl samples were removed at 5-min intervals for 60 min and product was calculated for a 20-μl reaction volume. Curves are as follows: *solid squares*, 100 mM ATP alone; *solid circles*, 100 μM GTP addition at the start of the reaction; ×, 100 μM GTP addition after 30 of the ATP:AdoCbi kinase reaction; *open circles*, preincubation of CobU with 100 μM ATP prior to the addition of 100 μM GTP and 15 μM AdoCbi. The *arrow* indicates the time of GTP addition for reaction B.

amount of product derived from the low level of added GTP. From this analysis, the kinase reaction appears to be specific for a purine nucleotide (Table II). When each nucleotide was tested individually, only GTP, ATP, dGTP, and dATP generated detectable product. For the purine nucleotides, removal of the 2′-OH group of the ribosyl moiety had negative affects on product generation suggesting the nucleotide-binding site for the kinase reaction recognizes the ribosyl moiety in addition to the nucleotide base. The addition of a low level of GTP to each reaction stimulated the use of ATP and dATP as substrates for the kinase reaction (Table II). Interestingly, dGTP had a negative effect on product formation since the amount of product formed in the presence of both dGTP and GTP was not additive, but rather it was comparable to the amount seen with dGTP alone. Finally, the pyrimidine nucleotides were not substrates for the kinase reaction when tested individually or in the presence of a low level of GTP (Table II). The purine nucleotides did bind to CobU since they reduced GTP-dependent product formation, however, once bound, they were not competent for catalysis.

All nucleotides listed in Table II were tested for nucleotidyltransferase activity using the HPLC assay system. Only GTP showed detectable transferase activity (data not shown). This result was consistent the GTP-specific binding pocket observed in the structure of CobU co-crystallized with GMP (10). The structure also suggested that dGTP would not be an efficient substrate due to specific interactions of enzyme side chains with the 2′-OH group of the ribose (10) and, as expected, nucleotidyltransferase activity with dGTP was not detected (data not shown). It was previously reported that ATP was a substrate for the transferase reaction but at only 25% of the activity seen with GTP (5). In the present study no $(CN)_2$Cbi-ADP product could be detected (see below).

Characterization of the ATP:AdoCbi Kinase Reaction—As discussed above, ATP can serve as the γ-phosphate donor during the kinase reaction. It was previously thought that the ATP:AdoCbi kinase reaction could be studied independently from the transferase reaction because the latter reaction favors GTP (5). Initial attempts to determine a linear range of the ATP:AdoCbi kinase reaction were unsuccessful; only a hyperbolic curve was seen regardless of enzyme or substrate concentration tested (Fig. 5).

One interpretation of these results was that, during the ATP:AdoCbi kinase reaction, CobU was becoming altered in a way that reduced its activity. This reduced CobU activity was not seen when ATP was replaced by GTP (Fig. 5). Furthermore, as shown in Table II, GTP appeared to have a stimulatory effect on the ATP:AdoCbi kinase reaction. One explanation for this stimulatory effect was that GTP protected the enzyme from the ATP-dependent reduction in enzyme activity. This idea was tested in three different ways: 1) GTP was added at the start of the reaction; 2) GTP was added after the reaction had proceeded for 30 min; and 3) CobU was preincubated with ATP alone for 5 min, then the reaction was started by the addition of GTP and AdoCbi. The addition of GTP at the start of the reaction allowed the enzyme to produce product at a linear rate for greater than 1 h (Fig. 5). The addition of GTP to the ATP:AdoCbi kinase reaction after 30 min did not restore the reaction rate to the level seen when GTP was added at the start of the reaction (Fig. 5), suggesting that GTP could no longer increase the activity of CobU. Finally, preincubation of CobU with ATP prior to the start of the reaction resulted in an enzyme with a reduced rate of product formation. Therefore, it appears that a preincubation of CobU with ATP resulted in a less active enzyme. These data suggested that during the ATP:AdoCbi kinase reaction, in the absence of GTP, CobU became altered in a way that reduced its catalytic activity.

Formation of a CobU-[^{32}P]AMP Complex—It was previously shown that CobU forms a covalent CobU-GMP intermediate (5) and the formation of this intermediate, and its subsequent hydrolysis, results in a significant conformational change in CobU (9, 10). This covalent intermediate and the conformational change associated with it may be the reason for the observed protection of CobU by GTP during the ATP:AdoCbi kinase reaction. In the absence of GTP, it was possible that the enzyme formed a CobU-AMP covalent complex that reduced CobU activity. This reduction in activity would occur because the AMP moiety could not be transferred to AdoCbi-P and would therefore be trapped as a CobU-AMP covalent complex. To address this question, the formation of a CobU-[^{32}P]AMP complex was assayed for during the ATP:AdoCbi kinase reac-

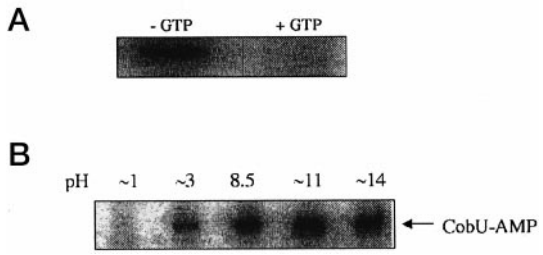

FIG. 6. **Evidence for the formation of a CobU-[^{32}P]AMP complex during the ATP:AdoCbi kinase reaction and support for a phosphoramidate CobU-[^{32}P]AMP covalent bond.** *Panel A,* 12% SDS-PAGE and PhosphorImaging analysis of the CobU-[^{32}P]AMP formation during the ATP:AdoCbi kinase reaction in the presence or absence of GTP. Reaction conditions were as follows: 60 nM CobU, 30 μM AdoCbi, 100 μM ATP, 0.3 μM [α-^{32}P]ATP (800 Ci/mmol), in the presence or absence of 100 μM GTP (20 μl final volume). Reactions were terminated by the addition of 20 μl of 2 $\times$ loading buffer and incubation at 70 °C for 10 min. A 0.71-μg sample of CobU was loaded per lane. *Panel B,* 12% SDS-PAGE and PhosphorImaging analysis of the chemical stability of the CobU-[^{32}P]AMP covalent complex. pH noted *above* each lane indicates the final pH of the sample, based on pH paper analysis, after denaturation of the enzyme and addition of HCl or NaOH. A 0.26-μg sample of CobU was loaded per lane.

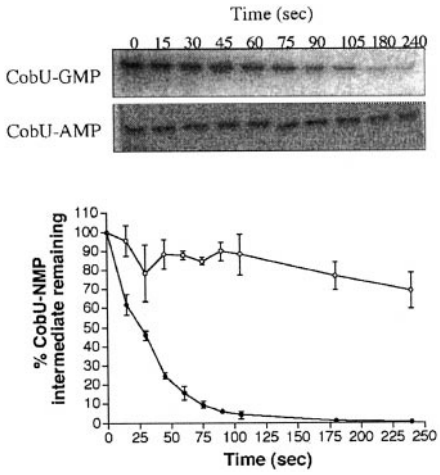

FIG. 7. **Nucleotide exchange between CobU-[^{32}P]NMP and nonradioactive nucleotide.** The *two panels above* the graph are representative exchange assays showing CobU-[^{32}P]NMP separated by 15% SDS-PAGE and visualized by a PhosphorImager. Time points (s) after the addition of excess nonradioactive nucleotide is indicated *above* each lane. A total of 0.05 μg CobU was loaded per lane. The *graph below* shows the results of three independent assays in which the amount of remaining CobU-[^{32}P]NMP was quantitated relative to the zero time point. *Open circles*, CobU-[^{32}P]AMP; *solid circles*, CobU-[^{32}P]GMP. *Bars* indicate standard deviation.

tion in the presence or absence of GTP. A CobU-[^{32}P]AMP intermediate did form during the reaction, but only in the absence of GTP (Fig. 6*A*). The failure of this intermediate to form when equimolar concentrations of GTP and ATP were used suggested the enzyme had a higher affinity for GTP than ATP in the formation of the CobU-NMP intermediate. Consistent with this interpretation, a 10-fold molar excess of ATP also did not have an effect on CobU-GMP formation (data not shown). These data support the conclusions of the structural work, which identified a binding site that favors GMP (9, 10).

Further analysis of the CobU-AMP complex suggested that the covalent bond was a phosphoramidate bond, similar to that seen with the CobU-GMP intermediate (5), since it was sensitive to acidic but resistant to alkali conditions (Fig. 6*B*). This would support the conclusion that the CobU-AMP covalent complex forms at the same residue as the CobU-GMP intermediate. The reason for GTP not restoring the ATP:AdoCbi kinase reaction to the rate seen when GTP was added at the start of the reaction would be due to the site being blocked by the CobU-AMP complex. In support of this conclusion, the addition of [α-^{32}P]GTP after 40 min of the ATP:AdoCbi kinase reaction failed to form a detectable CobU-[^{32}P]GMP intermediate 15 min after [α-^{32}P]GTP addition (data not shown).

Nucleotide Exchange Rates of the CobU-[^{32}P]GMP and CobU-[^{32}P]AMP Complexes—To address the question of whether the two forms of the enzyme, CobU-GMP and CobU-AMP, were conformationally distinct, nucleotide exchange rates were determined. CobU was incubated with a low concentration of either [α-^{32}P]GTP or [α-^{32}P]ATP to allow formation of the covalent complex. Following the formation of the complex, a >30,000-fold molar excess of unlabeled nucleotide as added to follow exchange with CobU-[^{32}P]NMP. The exchange rates of CobU-GMP *versus* CobU-AMP were significantly different (Fig. 7). The CobU-GMP complex showed a half-life of ~23 s while the CobU-AMP complex had a half-life of >4 min. This result supported the model that ATP reduces CobU activity by forming a covalent complex that traps CobU in an altered, less active conformation.

Pulse-chase Experiments of the CobU-[^{32}P]GMP and CobU-[^{32}P]AMP Complexes—While an earlier study (5), and the experiments discussed above, clearly showed the existence of a CobU-GMP covalent complex, they did not directly show that this intermediate is on the reaction pathway for the guanylyltransferase reaction. Furthermore, work on CobP, the orthologue of CobU from *P. denitrificans*, lead to the proposal that there are three different GTP-binding sites on the enzyme: one for the kinase reaction, one for the guanylyltransferase reaction, and a final site that was proposed to be a kinase activation site (11). Therefore, it was essential to determine whether the observed CobU-GMP complex was on the reaction pathway to AdoCbi-GDP.

To address this question directly, a pulse-chase experiment was performed wherein CobU was first incubated with a limiting amount of [α-^{32}P]GTP to allow a small percentage of the enzyme to form a labeled CobU-[^{32}P]GMP complex. The [^{32}P]GMP moiety of this complex was subsequently chased to the product AdoCbi-[^{32}P]GDP by the addition of a >30,000-fold molar excess of GTP and corrin substrate, either AdoCbi (Fig. 8*A*) or AdoCbi-P (Fig. 8*B*). AdoCbi-[^{32}P]GDP had a slightly slower mobility through a 12% SDS-PAGE system allowing us to distinguish between products (Fig. 8, *A* and *B*). To ensure this new species was AdoCbi-[^{32}P]GDP, the material was cut and eluted from the gel and resolved using the TLC assay system. The eluted band had an R_F value equivalent to a AdoCbi-[^{32}P]GDP standard. Additionally, the band was orange, consistent with it being AdoCbi-GDP. These data support the conclusion that the CobU-GMP intermediate is on the reaction pathway to AdoCbi-GDP synthesis.

The pulse-chase system was used to give further evidence that the CobU-[^{32}P]AMP complex was not competent for the transferase reaction (Fig. 8*C*). Additionally, adenylyltransferase activity could not be detected using the TLC or HPLC assays even when an excess of enzyme or substrate was used. These data were consistent with prior results with CobP, the orthologue of CobU in *P. denitrificans* where ATP was not a substrate for the transferase reaction (11). Therefore, ATP appears to proceed to the first step of the transferase reaction, but is not competent for the subsequent transfer to AdoCbi-P thereby trapping the enzyme in the less active CobU-AMP complex. This complex does not form in the presence of GTP because the enzyme has a higher affinity for GTP.

Evidence for a Histidine-linked Phosphoramidate CobU-GMP Bond—Prior work determined that the covalent CobU-GMP intermediate likely involves a phosphoramidate bond (5).

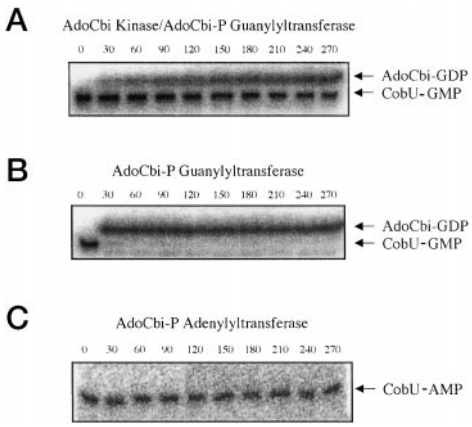

FIG. 8. **Pulse-chase experiments following CobU-[^{32}P]NMP conversion to AdoCbi-[^{32}P]NDP.** Assays were performed as described for the nucleotide exchange assays, however, in addition to nonradioactive nucleotide, AdoCbi or AdoCbi-P was added as indicated. *Panel A,* representative pulse-chase experiment where CobU-[^{32}P]GMP is formed prior to the addition of excess of nonradioactive GTP and 50 μM AdoCbi (final concentration). *Panel B,* representative pulse-chase experiment where CobU-[^{32}P]GMP is formed prior to the addition of excess nonradioactive GTP and 20 μM AdoCbi-P (final concentration). *Panel C,* representative pulse-chase experiment where CobU-[^{32}P]AMP is formed prior to the addition of excess nonradioactive ATP and 20 μM AdoCbi-P (final concentration). For all reactions, an initial sample of the reaction mixture was removed prior to the addition of nonradioactive NTP and corrin for the zero time point. Equal amounts of CobU were loaded per lane. *Numbers above* each lane indicate seconds after chase was begun. Separation of CobU-[^{32}P]NMP and AdoCbi-[^{32}P]NDP was performed by 12% SDS-PAGE and labeled products were visualized by PhosphorImager. The title for each panel indicates reaction(s) that must occur to allow detection of AdoCbi-[^{32}P]NDP product. *Arrows* identify the labeled products.

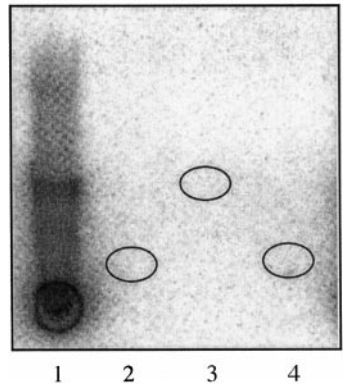

FIG. 9. **TLC separation of CobU-[^{32}P]P hydrolysate amino acids and phosphoamino acid standards.** Representative TLC separation of CobU-[^{32}P]P hydrolysate (*lane 1*) and phoshoamino acid standards: phospholysine (*lane 2*); phosphohistidine (*lane 3*); and phosphoarginine (*lane 4*). *Circles* in *lanes 2–4* identify site of migration of nonradioactive standards after staining TLC with ninhydrin. Radiation above His ~ [^{32}P]P in lane one is likely peptides that have not been fully hydrolyzed (20) and radiation remaining at origin is [^{32}P]P_i released during sample treatment (20).

This suggested that the residue involved was an arginine, lysine, or histidine. Analysis of sequence alignments between CobU and its orthologues determined that there is only one conserved histidine (His-46), one conserved lysine (Lys-12), and four conserved arginines (Arg-9, Arg-42, Arg-148, and Arg-151) (9). To determine which residue was modified, we used a technique developed by Yang *et al.* (19) that cleaves off the nucleoside from the modified protein and leaves the phosphorylated amino acid. The protein is subsequently hydrolyzed to its single amino acids and the amino acids are separated by TLC (Fig. 9) (20, 21). These data show the presence of a phosphohistidine, not a phosphoarginine or phospholysine.

Identification of His-46 as the Residue Involved in CobU-GMP Formation—The strongest candidate for the site of covalent GMP modification was the histidinyl residue at position 46 (His-46). This conclusion was based on the phosphohistidine results discussed above, the presence of only one conserved histidinyl residue (His-46) in CobU and its orthologues (9), and structural data showing that His-46 is brought within close proximity to the α-phosphate of GMP following the substrate-induced conformational change in CobU (9, 10).

To address whether His-46 is the site of the CobU-GMP intermediate, the His-46 residue was changed to an alanine (CobUH46A) or asparagine (CobUH46N). A histidinyl residue was noted at position 45 in the CobU sequence and was targeted for mutagenesis to address whether this residue would replace a mutated His-46 residue. Therefore, the double mutant H45A/H46A and the single H45A mutant proteins were also characterized. All four mutant proteins were purified to homogeneity using the same protocol as described for wild-type CobU (data not shown). The proteins were analyzed by size exclusion HPLC and were found to have the same oligomeric state as wild-type CobU (data not shown) suggesting no drastic structural changes occurred.

The mutant proteins were analyzed for their ability to form a CobU-[^{32}P]GMP intermediate compared with wild-type CobU. As expected, altering the His-46 residue resulted in a significant reduction of CobU-[^{32}P]GMP formation (Fig. 10, Table III). The remaining label was attributed to guanylylation of His-45. Consistent with this interpretation, the double mutant, H45A/H46A, did not form a CobU-[^{32}P]GMP intermediate (Fig. 10). Further support for the formation of a CobUH45-[^{32}P]GMP intermediate was the finding that the bond formed on both the H46A and H46N mutant proteins was acid-sensitive but alkali-resistant, consistent with the formation of a phosphoramidate bond (data not shown). It should be noted that under the conditions described in Fig. 10, the concentration of [^{32}P]GTP is not in excess relative to the concentration of enzyme. The addition of nonradioactive GTP to the reaction mixture, in an effort to saturate the enzyme with GTP, abolished all detectable E-[^{32}P]GMP intermediates on CobUH46A and CobUH46N. Therefore, under the conditions shown in Fig. 10, relative levels of *E*-[^{32}P]GMP intermediates cannot be directly compared.

Functional Characterization of Mutant Proteins—The specific activities of the mutant proteins for both the GTP:AdoCbi kinase and GTP:AdoCbi-P guanylyltransferase reactions were determined (Table III). Altering the His-46 residue to either H46A or H46N had drastic effects on the specific activities of both the kinase and guanylyltransferase activities (Table III). Both the kinase and guanylyltransferase activities were reduced by 4 orders of magnitude relative to wild-type CobU. As expected, neither kinase or guanylyltransferase activities were detected when characterizing the H45A/H46A double mutant. The H45A mutant protein did have a 5- or 3-fold effect on the kinase or guanylyltransferase activities, respectively, and may suggest a role for His-45 in positioning the His-46 residue for optimal catalysis. The fold effects seen for the kinase and guanylyltransferase activities was also reflected in the level of the initial product burst (data not shown). The negative affect the mutations have on both the kinase and guanylyltransferase activities suggests the formation of the CobU-GMP intermediate may be required for both the kinase and guanylyltransferase activities. This is supported by structural work where a significant conformation change in all three large active site clefts occurs in the presence of a CobU-GMP complex (9, 10). This conformational change may be essential for optimal kinase activity.

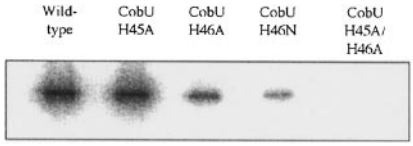

FIG. 10. **Formation of the CobU-[^{32}P]GMP intermediate on wild-type CobU and mutant CobU proteins.** A total of 0.2 μg of each protein was incubated for 5 min with 2 μCi of [α-^{32}P]GTP (800 Ci/mmol) in a 20-μl reaction. Reactions were stopped by the addition of an equal volume of 2 $\times$ loading buffer and heated to 70 °C for 10 min. Proteins were separated by 15% SDS-PAGE and the CobU-[^{32}P]GMP intermediate visualized by PhosphorImaging. Each lane contained 0.05 μg of protein. Wild-type or mutant CobU protein is identified *above* each lane.

It is intriguing that the kinase and guanylyltransferase activities have similar specific activities. This might be expected for an enzyme that must coordinate two catalytic activities. However, the turnover of the enzyme is more complicated. As stated above, we were unable to analyze the kinase reaction independent of the guanylyltransferase reaction. Thus, the rate of the kinase reaction is made up of two reactions, the kinase and guanylyltransferase. Additionally, the rate of the guanylyltransferase reaction while the kinase reaction is proceeding is ~5-fold reduced relative to the kinase reaction (data not shown). This observation suggests that the kinase reaction has negative effects on the guanylyltransferase reaction. It is not obvious how the enzyme coordinates these activities *in vivo*.

DISCUSSION

CobU AdoCbi Kinase and AdoCbi-P Guanylyltransferase Reactions Result in an Initial Burst in Product Formation—There is an initial burst in product formation during both the kinase and guanylyltransferase reactions followed by a new rate of product formation. The currently defined assay conditions could not be manipulated to analyze this burst and suggests the definition of true kinetic parameters will require the development of pre-steady state kinetic techniques. Since CobU turns over multiple times prior to the new linear rate of product formation, one possible reason for the reduced rate in turnover is the reaction is affected by product inhibition. For the AdoCbi kinase reaction, the products are: AdoCbi-P, AdoCbi-GDP, GMP, GDP, and PP_i. Preliminary results suggest both PP_i and AdoCbi-P inhibit the kinase reaction, in addition to PP_i inhibiting the guanylyltransferase reaction when substrates and products are in equimolar concentrations (data not shown). This suggests the enzyme has comparable affinities for both the substrates and products. There is also the possibility that GDP may be inhibitory based on the finding that RecA protein is inhibited by ADP (24–26), one of the products of ATP hydrolysis during RecA protein function, however, this has yet to be investigated for CobU. Product inhibition may not be the only complication since preliminary results also suggest AdoCbi-P shows substrate inhibition of the guanylyltransferase reaction (data not shown). In light of these complications and the need for pre-steady state kinetic analysis of CobU the previously reported kinetic values for CobU (5) must be re-evaluated.

A Nucleoside Upper Ligand on Cbi Is Required for Recognition and Catalysis by CobU—The finding that $(CN)_2$Cbi is not competent for catalysis or for competition with AdoCbi is surprising since the corrin ring is such a substantial part of the AdoCbi structure. These data have important implications for Cbi recognition by CobU. The upper ligand nucleoside and the aminopropanol group of AdoCbi, the target of modification, reside on opposite faces of the corrin ring. Hence, AdoCbi must bind to CobU in a way that allows both faces of the corrin ring to be recognized and would eliminate a model where only the aminopropanol side group is presented into the catalytic clefts of CobU.

While CobU requires a nucleoside upper ligand, it appears to have some flexibility in the nucleosides that can substitute for the natural 5′-deoxyadenosyl upper ligand and remain competent for catalysis. So why will the enzyme not use $(CN)_2$Cbi? Analysis of the structure of $(CN)_2$Cbl showed when the upper ligand is a cyano group, the upper face of the corrin ring is hydrophobic (27). The addition of a nucleoside to the upper face of the corrin ring disrupts this hydrophobicity (27). By analogy, it is assumed the upper face of $(CN)_2$Cbi will also be hydrophobic and the addition of a nucleoside will disrupt this hydrophobicity. Therefore, the upper ligand may be important for generating a hydrophilic upper face of the corrin ring in addition to positioning the aminopropanol side group of AdoCbi for catalysis.

Purine Nucleoside Triphosphates Are the Preferred γ-Phosphate Donors—CobU will only use purine nucleotides for the kinase reactions. Additionally, the 2′-OH group of the ribosyl moiety of the purine nucleotide is important (Table II). Comparison of the location of the nucleotides bound in the structures of CobU-GMP (10) and RecA-ADP (12) may give some insight into why CobU requires the 2′-OH group of the nucleoside for both kinase reactions. The GMP bound to CobU identified the guanylyltransferase nucleotide-binding site while the ADP bound to RecA identified the nucleotide-binding site for ATP hydrolysis. Assuming the ADP-binding site of RecA was equivalent to the kinase nucleotide-binding site of CobU, modeling of the ADP molecule of the RecA-ADP structure onto the CobU-GMP structure would suggest the phosphates of the kinase nucleotide would be bound to the P-loop of one CobU monomer, while the hydroxyls of the ribosyl moiety would point in the direction of conserved residues on a neighboring CobU monomer (data not shown). Further analysis of CobU will be required to determine if this model is correct.

Interestingly, CobU binds CTP, UTP, or dCTP, because they all compete against GTP for product formation (Table II). However, binding of these nucleotides does not result in a conformation that is competent for catalysis, suggesting the kinase nucleotide binding has flexibility for nucleotide binding but only purines bind in a catalytically competent conformation. Comparing what is known about the nucleotide-binding site of the RecA protein, this protein will bind a number of nucleotides, only two of which, ATP and dATP, are competent for stimulating RecA-dependent catalysis (28).

Formation of a CobU-AMP Complex during the ATP:AdoCbi Kinase Reaction—The data presented in Figs. 6 and 7 support the conclusion that during the ATP:AdoCbi kinase reaction, in the absence of GTP, CobU forms a phosphoramidate-linked CobU-AMP complex that alters the conformation of CobU resulting in a less active enzyme. Structural data showed that the 6-oxo and 2-amino groups of guanine form specific hydrogen bonds with conserved amino acid side chains of Lys-47, Arg-50, and Glu-58 (Fig. 11) (10). This 6-oxo group of GMP would be replaced by an amine in AMP and would likely result in unfavorable protein-AMP interactions. Furthermore, the absence of a 2-amino group on AMP may introduce another unfavorable interaction in the binding pocket further disrupting the conformation of CobU. The inability of the enzyme to subsequently transfer the covalently bound AMP to AdoCbi-P would then trap the enzyme in this altered conformation.

It has been previously argued that ATP is the favored substrate for the AdoCbi kinase reaction for both CobU (5) and its orthologue, CobP, from *P. denitrificans* (11). In both studies, this was argued based on the finding that the addition of GTP to the ATP:AdoCbi kinase reaction had a stimulatory affect on the ATP-dependent kinase reaction. This stimulatory effect

TABLE III
Analysis of GTP:AdoCbi kinase and GTP:AdoCbi-P guanylyltransferase activities of wild-type and mutant CobU enzymes

Enzyme	GTP:kinase specific activity[a]	Reduction in kinase activity	GTP:guanylyltransferase specific activity[b]	Reduction in guanylyltransferase activity
		-fold		*-fold*
CobU	1166 ± 100		670 ± 92	
CobUH46A	0.03 ± 0.01	38,867	0.03 ± 0.01	22,367
CobUH46N	0.08 ± 0.01	14,575	0.02 ± 0.01	33,500
CobUH45A/H46A	ND[c]	NA[d]	ND	NA
CobUH45A	251 ± 90	4.7	205 ± 88	3.3

[a] Reactions (112 μl) mixtures included 50 mM Tris-HCl, pH 8.5, at 25 °C, 10 mM $MgCl_2$, 1.25 mM TCEP-HCl, 680 mM glycerol, 0.1 M NaCl, 100 μM GTP, 2 μCi of [γ-^{32}P] GTP (30 Ci/mmol), 50 μM AdoCbi and the following concentrations of protein: CobU (6 nM), CobUH46A (3.2 μM), CobUH46N (4.7 μM), CobUH45A/H46A (4.9 μM), and CobUH45A (6 nM). 20-μl samples were removed at 5, 10, 15, and 20 min and added to fresh tubes containing 5 μl of 0.1 M KCN, pH 10.0, and then incubated at 70 °C for 10 min. The linear portion of the curve was used to define the rate, which was divided by the nanomole of CobU active site (assuming 3 active sites per trimer). Specific activity is reported as nanomole of product min^{-1} nmol of $CobU^{-1}$.

[b] Reactions were performed as described above except that [γ-^{32}P] GTP was replaced with 5 μCi of [α-^{32}P] GTP (800 Ci/mmol) and AdoCbi was replaced by 20 μM AdoCbi-P. Specific activities were calculated as described above.

[c] ND, none detected.

[d] NA, not applicable.

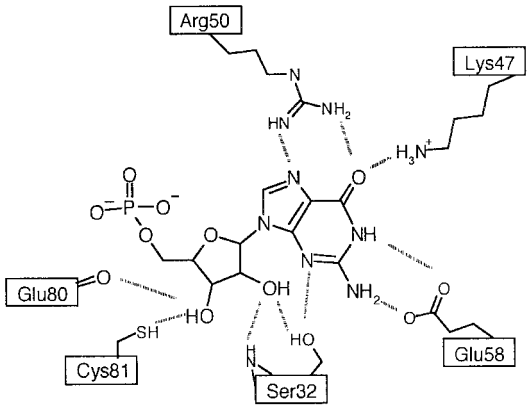

FIG. 11. **Amino acids of CobU that interact with bound GMP.** The amino acids of CobU that interact with bound GMP based on the structure of CobU-GMP (10). The *dashed lines* between amino acids and GMP highlight regions of interaction.

was used to argue for metabolic regulation of Cbi and Cbi-P levels in the cell (11). Based on the findings reported in this current study, this can be reinterpreted. As shown in Figs. 6 and 7, CobU, and presumably CobP, will form a CobU-AMP complex that alters the conformation of the enzyme compared with the CobU-GMP complex. Therefore, the stimulatory effect of GTP may actually be due to an altered, and more catalytically relevant, form of the enzyme. Thus, the enzyme in the presence of ATP or GTP alone cannot be directly compared.

CobU Is a Unique Guanylyltransferase—Data were presented to show that CobU catalyzes the guanylyltransferase reaction via a CobU-GMP intermediate that forms on the His-46 residue. Currently, there are only four enzymes, including CobU, known to function as guanylyltransferases. The other three enzymes are: 1) mRNA capping enzyme (29–32); 2) mannose-1-phophate guanylyltransferase (33); and 3) the recently identified GTP:GTP guanylyltransferase from *Artemia* (34). Of the four enzymes, three are known to proceed via a covalent phosphoramidate-linked *E*-GMP intermediate. A lysyl residue functions in mRNA capping enzyme (35, 36), while CobU and GTP:GTP guanylyltransferase (20) appear to use a histidinyl residue. Preliminary analysis of the GTP:GTP guanylyltransferase suggests it will not be homologous to CobU (20, 34) and support the conclusion that CobU is a unique guanylyltransferase (10).

Conclusions—The catalytic mechanism of CobU is complex. Here we have presented the biochemical analysis of the AdoCbi kinase/AdoCbi-P guanylyltransferase enzyme (CobU) of *S. typhimurium*. The *cobU* gene encodes for relatively a small (180 amino acid) polypeptide that binds four different substrates, generates and releases five products, and discriminates between functioning as a kinase/guanylyltransferase or just as a guanylyltransferase. The apparent overlap of the nucleotide-binding sites of the enzyme (10) would demand a precise order of substrate binding and product release, which may be impossible to achieve with the enzyme in isolation. Support for this idea comes from the finding that the cobalamin synthase of *P. denitrificans* cannot be purified to homogeneity due to the enzyme being present in a large, non-dissociable enzyme complex (37). Since CobU function is required immediately upstream of cobalamin synthase activity, CobU may be a member of a similar complex in *S. typhimurium*.

CobU Is an Inefficient Enzyme—A second possibility for the complications seen in this study is that CobU has yet to evolve into an efficient enzyme. One hypothesis is CobU and RecA shared a common ancestor that was a kinase. Both of these enzymes have a Rossman fold and show structural similarity to a number of kinases (9, 38), while having topologically similarity only to each other. From an evolutionary standpoint, RecA has retained the ATP binding and nucleotide hydrolysis functions while CobU gained the extra guanylyltransferase activity of this common ancestor. This additional activity for CobU may cause problems because of the limited space for binding two nucleotides for these two different functions. Alternatively, CobU may have evolved from RecA. RecA homologues have been found in all three domains of life (8) while to date, CobU orthologues have been found only in bacteria (9).[2] Furthermore, it has been recently shown that archaea encode an non-orthologous AdoCbi-P nucleotidyltransferase that replaces CobU function in these organisms.[2] Therefore, CobU may have gained guanylyltransferase and kinase activities but has yet to evolve into an efficient form. However, because AdoCbl is required at low levels in bacteria, there may not be sufficient selective pressure for this enzyme to become more efficient.

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