Purification and Characterization of the Bifunctional CobU Enzyme of Salmonella typhimurium LT2

EVIDENCE FOR A CobU-GMP INTERMEDIATE*

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The CobU protein of Salmonella typhimurium was overexpressed and purified to ~94% homogeneity. N-terminal sequencing of purified CobU confirmed the first 22 amino acids. In vitro assays showed that CobU has kinase and guanylyltransferase activities which catalyze the synthesis of adenosyl-cobinamide-GDP from adenosyl-cobinamide, via an adenosyl-cobinamide-phosphate intermediate. We present evidence that the transfer of the guanylyl moiety of GTP to adenosyl-cobinamide-phosphate proceeds via a phosphoramide-linked, enzyme-guanylyl intermediate.

In the presence of oxygen, kinase and guanylyltransferase activities of CobU were lost. Treatment of inactive CobU with dithiothreitol restored ~20% of the kinase and guanylyltransferase activities, indicating the involvement of sulfhydryl groups in enzyme activity. The sulfhydryl modifying agents 5,5'-dithiobis(2-nitrobenzoic acid) and N-ethylmaleimide abolished both CobU activities.

Native CobU protein was a dimer (~40 kDa) that functioned optimally at pH 8.8–9.0 and 37 °C. Substrates and kinetic parameters for both activities were determined. The preferred corrinoid substrate for this enzyme was adenosyl-cobinamide. In vitro experiments are consistent with previous genetic studies which had suggested that adenosyl-cobinamide was the preferred substrate of CobU, and that CobU functioned more efficiently in the absence of oxygen.

Assembly of the nucleotide loop of cobalamin in Salmonella typhimurium is thought to require the involvement of four enzymes, CobU, CobS, CobT, and CobC (Fig. 1). CobT and CobC act sequentially in the generation of dimethylbenzimidazole to yield 2,4-dimethylbenzimidazole (1), which is then dephosphorylated by CobC to generate α-ribazole. Homology to the CobP protein of Pseudomonas denitrificans (3, 4) and nutritional studies performed on S. typhimurium cob mutants (5) suggested that CobU was responsible for the synthesis of Ado1-CBI-GDP from Ado-CBI, via an Ado-CBI-P intermediate (Fig. 1). The CobS protein is thought to be the cobalamin synthase that catalyzes the synthesis of Ado-cobalamin from Ado-CBI-GDP and α-ribazole (2, 4, 5).

We report the overexpression and purification of the cobU gene product, document that the CobU protein has both kinase and guanylyltransferase activities required for the synthesis of Ado-CBI-GDP from Ado-CBI, and provide evidence that Ado-CBI-P is an intermediate in the synthesis of Ado-CBI-GDP. We also present evidence that the transfer of the guanylyl moiety of GTP to Ado-CBI-P proceeds via a phosphoramide-linked, CobU–GMP intermediate. The characterization of homogeneous CobU protein includes an analysis of substrate specificity, physical characteristics, and kinetic parameters.

Results from previous genetic studies suggested that oxygen negatively affected CobU activity and that the corrinoid substrate utilized by CobU was also affected by molecular oxygen in the environment (6). Specifically, it appeared that under aerobic conditions CobU could utilize Ado-CBI only as its corrinoid substrate. These results contrasted with the ability of this enzyme to utilize either Ado-CBI or H0-CBI (i.e. nonadenosylated CBI) under anaerobic conditions (6).

The in vitro data presented support the hypothesis that the preferred corrinoid substrate for the CobU enzyme is Ado-CBI. H0-CBI can also serve as substrate, albeit poorly. We documented oxygen lability of the kinase and guanylyltransferase activities of CobU, both of which can be restored by DTT. Chemical modification of sulfhydryl groups with DTNB or NEM strongly suggested that CobU activities require one or more of the 4 cysteinyl residues present in the protein.

EXPERIMENTAL PROCEDURES

Bacteria, Culture Media, and Growth Conditions

All bacterial strains used were derivatives of S. typhimurium strain LT2, and their genotypes are listed in Table I. Composition of culture media and growth conditions have been reported (6). Conditions for the overexpression of cobU were as follows. Strain J E3207 (DEL299 (hisG-cob)pGPI-2[p]p 052) was grown at 30 °C in 1-liter batches of LB medium containing kanamycin (20 μg per ml), and ampicillin (30 μg per ml) in 2-liter Erlenmeyer flasks. Mid-log phase cultures (~60 Klett units) were shifted to 42 °C for 40 min, followed by a shift to 37 °C for 12-16 h. Best expression was obtained when incubations (at 37 °C and 42 °C) were performed in water baths maintained at a 37 °C walk-in incubator.

1 The abbreviations used are: Ado, 5'-deoxyadenosyl; CBI, cobinamide; Ado-CBI-P, Ado-CBI-phosphate; HO, hydroxoc; (CN)2CBI, dicyano-CBI-phosphate; α-ribazole-S'-P, N'-5-phospho-α-riboyl-5,6-dimethylbenzimidazole; α-ribazole, α-riboyl-5,6-dimethylbenzimidazole; DTT, dithiothreitol; CobU–GMP, enzyme-guanylyl intermediate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid) NEM, N-ethylmaleimide; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

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mg of protein) made from a strain overexpressing CobA (JE2018) was added, and the vial was transferred to a 37 °C water bath. The reaction mixture was incubated for 2 h, then filtered to stop the reaction and remove particulates. The reaction mixture was then loaded onto a 3.5 × 2.5-cm column of the reverse phase resin LiChroprep RP-18 (EM Separations, Gibbstown, NJ) previously equilibrated with H₂O. The column was washed with 10 bed volumes of H₂O, then washed with 2 bed volumes of 100% methanol to elute the Ado-CBI. The sample was concentrated under vacuum (SpeedVac concentrator, Savant Instruments, Farmingdale, NY). All the manipulations were performed in the dark or in dim light to avoid photolysis of the C–Co bond.

The UV-visible spectrum of Ado-CBI showed no significant absorbance at 260 nm, indicating quantitative conversion of (CN)₂-CBI to Ado-CBI (data not shown). The extinction coefficient of Ado-CBI in H₂O at 457 nm was calculated to be 4,526 M⁻¹ cm⁻¹. Absorption maxima in H₂O were observed at 305 and 457 nm.

Synthesis of Radiolabeled Ado-CBI-P

This compound was synthesized in vitro, utilizing the ATP:CBI kinase assay described below. CobU enzyme was added in excess (22.5 μg) to a scaled-up reaction mixture (1 ml) containing [γ⁻³²P]ATP, specific radioactivity = 10 Ci mmol⁻¹ (DuPont NEN), and the reaction was allowed to proceed for 120 min. Radiolabeled Ado-CBI⁻³²P was separated from unreacted [γ⁻³²P]ATP by reverse phase chromatography on LiChroprep RP-18 as described above. Specific radioactivity was determined to be 0.0039 Ci mmol⁻¹. These reaction conditions resulted in quantitative conversion of Ado-CBI to Ado-CBI⁻³²P. Unlabeled Ado-CBI⁻³²P was generated as described above, except the radiolabeled ATP was replaced with unlabeled ATP.

In Vitro Assays for CobU Activities

ATP:Ado-CBI Kinase Assay

The assay for kinase activity was a modification of a reported protocol (3). The reaction mixture contained Tris-HCl, 0.1 M, pH 8.8, at 37 °C; MgCl₂, 2.5 mM; GTP, 2 mM; [γ⁻³²P]GTP, 3.34 μCi, specific radioactivity = 30 Ci mmol⁻¹ (DuPont NEN); CobU, 180 μg; protein, 0.1–0.4 μg; final volume, 20 μl. The reaction mix lacking ATP was prepared on ice and preincubated for 5 min at 37 °C; the reaction was initiated by the addition of ATP and terminated by the addition of 20 μl of 20 mM KCl followed by a 10-min incubation at 80 °C. Samples were centrifuged at 15,850 × g for 10 min in a Marathon 13K/M microcentrifuge (Fisher), and the supernatant was transferred to a fresh tube. One unit of activity was defined as the amount of enzyme required to synthesize 1 nmol of Ado-CBI-P/min. Specific activity was defined as units/μg of protein.

As shown below, ATP did not serve as an efficient substrate for the transferase activity of CobU. Therefore, in the absence of GTP, the conditions for the Ado-CBI kinase assay allowed us to document the synthesis of the intermediate Ado-CBI⁻³²P.

GTP:Ado-CBI Kinase, Ado-CBI⁻³²P Guanylyltransferase Activity

This assay was used to monitor the conversion of Ado-CBI to Ado-CBI-GDP. This assay was used to monitor the activity of fractions during the purification of CobU.

GTP:Ado-CBI⁻³²P Guanylyltransferase Assay (Protocol A)

This assay was a modification of a reported protocol (3). The reaction mix contained Tris-HCl, 0.1 M, pH 8.8, at 37 °C; MgCl₂, 2.5 mM; GTP, 2 mM; [γ⁻³²P]GTP, 3.34 μCi, specific radioactivity = 30 Ci mmol⁻¹ (DuPont NEN); Ado-CBI⁻³²P, 300 μg; protein, 0.1–0.4 μg; final volume, 20 μl. The reaction mix was handled as described above. One unit of activity was defined as the amount of enzyme required to synthesize 1 nmol of Ado-CBI-GDP/min. GTP served as a substrate for both the kinase and transferase reactions (see below). Therefore, this assay allowed a convenient means to monitor the conversion of Ado-CBI to Ado-CBI-GDP.

GTP:Ado-CBI⁻³²P Guanylyltransferase Assay (Protocol B)

The reaction mix contained Tris-HCl, 0.1 M, pH 8.8, at 37 °C; MgCl₂, 2.5 mM; GTP (or indicated nucleotide), 150 μM; Ado-CBI⁻³²P, 300 μg; specific radioactivity = 0.0039 Ci mmol⁻¹; enzyme, 0.1–0.4 μg in a final volume of 20 μl. The reaction mix was handled as described above. One unit of activity was defined as the amount of enzyme required to synthesize 1 nmol of Ado-CBI-GDP/min.

Quantitation of Ado-CBI⁻³²P and Ado-CBI-GDP

In all assays, radiolabeled substrates were diluted with a known amount of unlabeled substrates. To quantitate the amount of product synthesized, the radiolabeled substrate incorporated into the product was determined (see below) and adjusted for decay of the radioactivity and counting efficiency of the scintillation counter. The total product syn-
was converted to its dicyano derivative (CN)\textsubscript{2}CBI by addition of an equal volume of 20 mM KCN and heating at 80°C for 10 min, and purified in two steps. In System 1, the sample was loaded on a BIOSEP-DEAE-P (75 mm x 7.8 mm, Phenomenex, Torrance, CA) column equilibrated with potassium phosphate buffer (0.1 M, pH 6.5) containing 10 mM KCN. The column was washed with 3 min with equilibration buffer, then developed with a 30 min concave gradient (curve 8) of NaCl from 0 to 600 mM in the potassium phosphate buffer (0.1 M, pH 6.5) containing 10 mM KCN. The column was washed with 800 mM NaCl in potassium phosphate buffer (0.1 M, pH 6.5) containing 10 mM KCN for 5 min after the completion of the gradient. Elution of (CN)\textsubscript{2}CBI-P was monitored at 365 nm and the flow rate was 0.3 ml min\textsuperscript{-1}. In System 2, the column described above was equilibrated with potassium phosphate buffer (0.05 M, pH 6.5) containing 5 mM KCN, and developed with an isocratic system of potassium phosphate buffer (0.05 M, pH 6.5) containing 5 mM KCN at a flow rate of 1 ml min\textsuperscript{-1}.

Purification of (CN)\textsubscript{2}CBI-GDP—The purification of (CN)\textsubscript{2}CBI-GDP was performed as reported without modifications (5).

**Paper Chromatography**

The substrates and products of assays were routinely resolved by ascending paper chromatography on Whatman No. 1 paper (Whatman) in a solvent system of isobutyric acid-water-ammonia (66:33:1) (15). The solvent front was allowed to migrate between 10 and 15 cm, the paper was dried, and an autoradiograph was prepared. Using the autoradiograph as a guide, each radioactive spot was cut from the chromatograph mixture. In some experiments, the relative radioactivity of each radioactive spot was determined by analysis with a PhosphorImager (445I, Molecular Dynamics, Sunnyvale, CA).

**Spectroscopic Analysis**

UV-visible spectroscopy and fast atom bombardment mass spectrometry were performed as reported elsewhere (1).

**Overexpression and Visualization of CobU**

Overexpression of CobU was performed as previously described (16–19) and visualized by 12% SDS-PAGE (20), followed by Coomassie Blue staining (19). The native pl of CobU was determined as reported elsewhere (21).

**Purification of CobU**

All purification steps were performed at 4°C.

**Step 1: Preparation of Cell-free Extracts**

Cells obtained from 18 liters of the CobU overexpressing strain JE3207 were harvested and resuspended in Tris-HCl, 0.1 M, pH 8.0 at 4°C containing DTT, 10 mM. Cells were broken by sonication (Branson Ultrasonics Corp., Danbury, CT) at setting 7, 50% duty cycle. Sonication was performed six times at 5-min intervals, and the sample was allowed to cool to less than 15°C before resuming sonication. Cell debris was cleared from the extracts by centrifugation at 13,400 x g for 1.5 h (Servall RC-5B refrigerated centrifuge, DuPont Instruments, Wilmington, DE). Serine protease inhibitor phenylmethylsulfonyl fluoride was then added to a final concentration of 1 mM.

**Protein Concentration Assay**

Preliminary protein concentrations were determined by a modification of the turbidimetric method reported by Kunitz (13). More accurate determinations of protein concentrations were achieved by the BCA method (14) using a commercially available kit from Fierce Chemical Co. (Rockford, IL).

**Identification of Products of the CobU-catalyzed Reactions**

To identify the products of the CobU-catalyzed kinase and guanylyltransferase reactions, the CobU assay reaction mixtures described above were increased to 100 μl, Ado-CBI was increased to 0.4 mM, in a reaction mixture which contained only unlabeled nucleotide triphosphates. Following a 4-h incubation at 37°C, products were derivatized under control of the phage T7 promoter and ribosome binding site.

**Chromatographic Methods**

**HPLC**

Products of the large scale CobU-catalyzed reactions were purified by HPLC. The chromatograph used was a computer-controlled Waters model 961 Plus equipped with a photodiode array detector and a model 600 multisolvant delivery system (Waters, Milford, MA).

Purification of (CN)\textsubscript{2}CBI-P—Enzymically synthesized Ado-CBI-P...
Step 2: Hydrophobic Interaction Chromatography

Finely ground Ultrapure ammonium sulfate (Schwarz/Mann, ICN Biomedicals Inc., Cleveland, OH) was added to crude cell-free extract to 1% saturation. The extract was centrifuged at 10,000 x g for 15 min to remove precipitates. The resulting supernatant was loaded onto a 35-ml bed volume phenyl-Sepharose CL-4B column (Sigma) equilibrated at 4°C with 1% ammonium sulfate in Tris-HCl, 0.1M, pH 8.0 at 4°C, containing 10 mM DTT. The column was washed with 3 bed volumes of the elution buffer. CobU was eluted with 50% ethylene glycol in Tris-HCl, 0.1 mM, pH 8.0 at 4°C, containing 10 mM DTT. The flow rate of the column was 30 ml h⁻¹.

Step 3: Dye Ligand Chromatography

Dye ligand chromatography was used as a negative purification step. Pooled fractions from the phenyl-Sepharose column were dialyzed against Tris-HCl, 0.1 mM, pH 8.0 at 4°C, with 10 mM DTT to remove ethylene glycol. A 15-ml column of Cibacron blue type 3000 (Sigma) was equilibrated with Tris-HCl, 0.1 mM, pH 8.0 at 4°C containing 10 mM DTT plus 5 mM MgCl₂. The dialyzed CobU-containing sample was loaded onto the column at a flow rate of 15 ml h⁻¹. CobU was recovered from the flow-through and the first bed volume of the wash buffer.

Step 5: Anion Exchange Chromatography

The pooled CobU-containing samples from the previous step was loaded onto a 20-ml column of DEAE Sephadex A-25 equilibrated with Tris-HCl, 0.1 mM, pH 8.0 at 4°C containing 10 mM DTT, at a flow rate of 15 ml h⁻¹. The column was washed with 2 bed volumes of the equilibration buffer and eluted with a 5 x bed volume gradient of 0–0.5 M NaCl in the same buffer. CobU activity eluted when the salt concentration was between 300 and 500 mM NaCl and in the 500 mM NaCl wash (data not shown).

RESULTS

Overexpression and Visualization of CobU

Expression of the cobU gene and its gene product was cloned under the control of a strong phage T7 promoter and ribosome binding site. Using two plasmid phage T7-derived overexpression systems (16–19), CobU was produced to a level >100-fold higher than chromosomal level produced by the wild-type strain (as judged by densitometry, data not shown). An extract made from a strain carrying the cobU overexpression plasmid (J E3207) showed a prominent band at approximately 22 kDa when analyzed by SDS-PAGE (Fig. 3, Panel A, lane 2). This prominent band was absent from extracts of a control strain (J E3420), which carried only the overexpression vector pT7-7 (Fig. 3, Panel A, lane 1).

Activity Assays for the CobU Activities

ATP:Ado-CBI Kinase Activity—Fig. 4, Panel A, shows an autoradiograph prepared from a representative ATP:Ado-CBI kinase assay analyzed by ascending paper chromatography. The inclusion of radiolabeled [γ-³²P]ATP in the reaction mixture results in the synthesis of radiolabeled Ado-CBI-P (Fig. 4, Panel A, lane 1). In this representative experiment, extracts derived from CobU overexpressing strain (J E3207) had a specific activity of Ado-CBI-P synthesis of 41.5 nmol min⁻¹ mg⁻¹ of protein. This level of activity was approximately 20-fold higher than that measured in extracts made from a strain carrying only the overexpression vector (J E3420, specific activity = 1.9, Fig. 4, Panel A, lane 2).

The relative mobility of radiolabeled (CN)₂CBI-P on the chromatogram was 0.77, a value consistent with that reported in the literature (15). This radioactive product comigrated with authentic (CN)₂CBI-P; unlabeled (CN)₂CBI-P was identified on the chromatogram by its characteristic red-purple color.

In this paper chromatography system, (CN)₂CBI migrated with an Rₜ = 0.92, ATP migrated with an Rₜ = 0.38, and the hydrolysis of ATP resulted in labeled inorganic phosphate which remained near the origin. The synthesis of Ado-CBI-P was dependent on overexpression of CobU and the presence of Ado-CBI and ATP in the reaction mixture. Heat treatment of cell-free extracts resulted in loss of ATP:Ado-CBI kinase activity.

Guanylyltransferase Activity—Fig. 4, Panel B, shows the results of a representative GTP:Ado-CBI kinase, Ado-CBI-P guanylyltransferase assay analyzed by ascending paper chromatography. The inclusion of radiolabeled [γ-³²P]GTP in the reaction mixture resulted in the synthesis of radiolabeled Ado-CBI-P (Fig. 4, Panel B, lane 1). In this representative experiment, extracts derived from the CobU overexpressing strain (J E3207) had a specific activity of Ado-CBI-P synthesis of 218 nmol/min/mg of protein. This level of activity was approximately 400-fold higher than that measured in extracts made from a strain carrying only the overexpression vector (J E3420, specific activity = 0.53, Fig. 4, Panel A, lane 2).

The relative mobility of radiolabeled (CN)₂CBI-GDP was 0.56, a value consistent with the literature (15). This radioactive product comigrated with authentic (CN)₂CBI-GDP and was identified on the paper chromatogram by its characteristic red-purple color (data not shown).

The synthesis of Ado-CBI-GDP was dependent on overexpression of CobU and the presence of Ado-CBI and GTP in the reaction mixture. Heat treatment of cell-free extracts resulted in loss of GTP:Ado-CBI kinase, Ado-CBI-P guanylyltransferase activities.

Ado-CBI-P Is an Intermediate in the Synthesis of Ado-CBI-GDP —As described above, when Ado-CBI was incubated in the presence of CobU and [γ-³²P]ATP, radiolabeled Ado-CBI-P was synthesized. The addition of unlabeled GTP to this reaction mixture resulted in the synthesis of radiolabeled Ado-CBI-GDP.
There were two important implications of these results. First, CobU catalyzed the transfer of the γ-phosphate of ATP to Ado-CBI to generate Ado-CBI-P. Second, the retention of the label in the product of the reaction (i.e. Ado-CBI-GDP) after addition of unlabeled GTP to the reaction mixture, indicated that the phosphate group donated by ATP was retained in Ado-CBI-GDP. Hence, we concluded that the GMP moiety of GTP was donated to Ado-CBI-P with the release of pyrophosphate. The UV-visible spectrum, and the molecular mass of the cyano derivative of the end product of the guanylyltransferase reaction was consistent with this compound being (CN)$_2$CBI-GDP (see below).

**Purification and Identification of the Products of the CobU-catalyzed Reactions**

Product of the Kinase Reaction—The cyano derivative of the product of the kinase reaction was shown to be (CN)$_2$CBI-P. The products of the scaled-up ATP:Ado-CBI kinase reaction using highly purified CobU (94% purity) were analyzed by reverse-phase HPLC in two steps. First, the cyano derivative of the prominent product of the reaction had a (CN)$_2$CBI-like spectrum and eluted with a retention time between 7 and 10 min (using System 1, see “Experimental Procedures”). (CN)$_2$CBI-P eluted in a poorly resolved peak adjacent to (CN)$_2$CBI. Fractions collected between 7 to 10 min were pooled and analyzed using System 2 (see “Experimental Procedures”). (CN)$_2$CBI-P eluted at 3 min as a single well defined peak. The identity of the prominent peak eluted from System 2 was established as (CN)$_2$CBI-P by UV-visible spectroscopy and fast atom bombardment mass spectrometry (data not shown).

Product of the Guanylyltransferase Reaction—The product of the CobU-catalyzed GTP:Ado-CBI kinase, Ado-CBI-P guanylyltransferase activities was shown to be Ado-CBI-GDP. The product of these reactions was analyzed by reverse-phase HPLC after derivatization with KCN; the prominent peak comigrated with authentic (CN)$_2$GDP on ascending paper chromatography. Verification of the identity of the product of the guanylyl transfer reaction was established by UV-visible spectroscopy and fast atom bombardment mass spectrometry (data not shown).

**Purification of CobU**

Fig. 3, Panel B, lane 2, shows a sample of purified CobU as analyzed by SDS-PAGE (12%). Molecular weight size markers are shown in lane 1. The CobU protein was ~3-4 fold enriched after these purification steps (Table II) and >94% pure as judged by densitometry (data not shown).

**Some Properties of the CobU Enzyme**

Under the conditions described above, both kinase and guanylyltransferase activity assays were shown to be linear with time (for at least 30 min) and with protein (0.1-0.4 μg of protein). The optimal pH for both the kinase and guanylyltransferase activities was between pH 8.8 and 9.0, but over 50% of both CobU activities were retained between pH 8.5 and 9.5 (data not shown). The optimal reaction temperature was 37°C, but CobU retained at least 50% of its activities when assayed between 25 and 60°C. Preincubation of CobU at temperatures up to 60°C (for 10 min), resulted in little effect on either activity (subsequently determined at 37°C). Preincubation at temperatures above 60°C resulted in drastic decreases in both CobU activities (data not shown).

The molecular mass of homogeneous CobU, as judged by SDS-PAGE, was estimated at 22 kDa, a value in agreement with a previous report (5). The predicted mass of CobU, based on the DNA sequence of the cobU gene, is 19.7 kDa (4). As determined by nondenaturing gel electrophoresis (22), the native molecular mass was estimated to be 49 kDa. These data were consistent with CobU functioning as a dimer. The experimentally determined isoelectric point of the native CobU enzyme identified two species at pI of 7.2 and 7.5, a sharp departure from the pI predicted for the denatured protein (pI = 5.3).

**Kinetic Parameters**

Kinetic parameters for the kinase and guanylyltransferase activities were determined in order to optimize assay conditions (Table III). The V$_{max}$ values for both the kinase and guanylyltransferase reactions were consistently ~75% lower when the concentration of the NTP varied than when the concentration of the NTP was fixed. Because most or all this difference in these values could be accounted for by the standard deviation values, it was inferred that the data reflect some variability in the assay. However, additional kinetic studies may be required to fully evaluate these results.

**CobU Substrates**

Corrinoids—Only Ado-CBI served as an efficient substrate for the synthesis of Ado-CBI-P or Ado-CBI-GDP (Table IV), a result consistent with genetic studies (6). HO-CBI also served as substrate for CobU, albeit poorly. An approximately 14-fold decrease in the specific activity was measured for the synthesis of HO-CBI-P or HO-CBI-GDP from HO-CBI as compared to Ado-CBI. (CN)$_2$CBI was also an extremely poor substrate for both CobU activities. These results were consistent with the critical role of the upper ligand for CobU activity.

**Triphosphate Nucleotides**—Table IV summarizes the results of assays using various nucleotide triphosphates as substrates for the kinase and nucleotidytransferase activities.

An examination of the CobU amino acid sequence revealed two partial nucleotide triphosphate binding domains (5). Near the N terminus of CobU there is a portion of the ATP binding domain which specifies phosphate binding, suggesting some flexibility in substrate binding. Consistent with this observa-
tion, either ATP or GTP served as phosphate donors for the synthesis of Ado-CBI-P. In fact, the specific activity for the kinase reaction was approximately 10-fold higher when GTP was used as substrate than when ATP was provided at the same concentration. These results did not necessarily imply that GTP was the phosphate donor in vivo, because these increased reaction rates could be the result of an active guanylyltransferase function. For example, the presence of 100 μM unlabeled GTP stimulated the formation of GTP was lowered to 150 μM. When the concentration of GTP was varied, the GTP concentration was held at 3 mM in preliminary experiments. In a subsequent experiment, the concentration of GTP was lowered to 150 μM. When the concentration of GTP was varied, the Ado-CBI-P concentration was held at 300 μM. In all experiments, CobU enzyme was used at 6.1 pmol of dimer per reaction.

### Table III

| Activity            | Substrate | Apparent $K_m$ | $V_{max}$ | $K_{cat}$ |
|---------------------|-----------|----------------|-----------|-----------|
| Kinase              | Ado-CBI   | 66 ± 14        | 0.0916 ± 0.016 | 15.1      |
|                     | ATP       | 206 ± 65       | 0.0695 ± 0.011 | 11.5      |
| Guanylyltransferase | Ado-CBI-P | 102 ± 10       | 0.0339 ± 0.0085 | 5.6       |
|                     | GTP       | 21.3 ± 4.3     | 0.0214 ± 0.0013 | 3.5       |

### Table IV

| Substrate | Specific activity |
|-----------|------------------|
| Cobinamide |                 |
| Ado-CBI   | 153*            |
| HO-(CN)$_2$ | 5               |
| Nucleotides |               |
| GTP       | 1298            |
| ATP       | 152             |
| UTP       | NT*             |
| CTP       | NT*             |

* Nanomoles of product/min. The data presented are the average of three determinations.

**Evidence for the Formation of a CobU–GMP Intermediate**

The evidence presented above demonstrated that CobU can catalyze the transfer of the GMP moiety of GTP to Ado-CBI-P. The enzyme galactose-1-phosphate uridylyltransferase catalyzes a reaction that is analogous to the reaction catalyzed by CobU, that is, the transfer of the the UMP moiety of UDP-galactose to the phosphorylated substrate galactose-1-P. Frey and co-workers have reported evidence that galactose-1-phosphate uridylyltransferase performs this reaction via an enzyme-bound uridylyl intermediate (23–25).

To test whether CobU forms an CobU–GMP intermediate, we performed the experiment shown in Fig. 5. As shown in lane 2, Panels A and B, the incubation of CobU with [α-32P]GTP resulted in substantial radioactivity associated with the CobU protein band. Incubation of CobU with [γ-32P]GTP labeled in the α-position instead of the γ-position resulted in no radiolabel associated with CobU (lane 1, Panels A and B). Lanes 3–5, Panels A and B, demonstrated that both CobU and [α-32P]GTP were required for the formation of the radiolabeled protein band.

Inclusion of a 100-fold molar excess of unlabeled GTP (Fig. 5, Panel A, lane 6) resulted in a 30-fold decrease in label incorporated into the CobU protein when compared to lane 2. The inclusion of a 100-fold excess of unlabeled GTP resulted in only a 0.4-fold decrease in label incorporated into the CobU protein. As described above, both ATP and GTP can serve as substrates for the kinase activity, but only GTP is an efficient substrate for the guanylyltransferase activity. The ability of GTP (but not ATP) to compete with the radiolabeled GTP suggested that the association of radiolabel with CobU occurred at a site required specifically for GTP binding and presumably for the guanylyl transfer reaction.

Based on the data presented in this section, it is formally possible that CobU forms an enzyme-GDP intermediate. Two lines of evidence argue against such a conclusion. The cyanodependent inhibition of the CobU-catalyzed reaction has a molecular mass identical to (CN)$_2$CBI-GDP. Further, the phosphate moiety of Ado-CBI-P was retained in Ado-CBI-GDP, suggesting that a GMP moiety (not a GDP moiety) was trans-
ferred to Ado-CBI-P to generate Ado-CBI-GDP. Taken together, these data indicated that the GMP moiety of GTP, not the GDP moiety, was transferred to the CobU enzyme.

The data presented above indicated that GMP was associated with the CobU protein. Further, the label associated with CobU was retained after treatment of the protein with SDS, heating to 80 °C, and analysis by SDS-PAGE. These observations indicated that GMP had formed a stable covalent bond with the CobU protein. This point is addressed in more detail in the next section.

The Sensitivity of the CobU–GMP Intermediate to Acid and Stability to Base Is Consistent with a Phosphoramidate Linkage

We suspected that GMP had formed a covalent bond to the CobU enzyme. This linkage could be to the carboxyl group of Glu or Asp, the ε-amino group of Lys, or the imidazole side chain of His. To distinguish among these possibilities we assessed the stability of the CobU–GMP to acid and alkali conditions, using reaction conditions similar to those reported (26).

Fig. 6, lanes 1 and 2, shows that a 10-min incubation at 55 °C (with no addition of acid or base) resulted in CobU protein which retained the radiolabeled GMP. Treatment with acid (pH of 1.5) at 55 °C for 10 min resulted in a 38-fold decrease in radiolabel associated with the CobU protein (lanes 3 and 4). The CobU protein, however, was still visible on Coomassie Blue-stained polyacrylamide gels at levels comparable to the control lanes (data not shown). Treatment with base (pH of 13.0) resulted in no loss of radiolabel (lanes 5 and 6).

These data showed that the CobU–GMP intermediate was sensitive to treatment with acid, but not to treatment with base. These data argue against a phosphoester bond, which should be sensitive to both acidic and alkali conditions. This behavior was consistent with a phosphoramidate linkage.

The CobU–GMP Intermediate Drives the Synthesis of Ado-CBI-GDP

The data above indicated that incubation of CobU with GTP resulted in the formation of an enzyme-bound guanylyl intermediate. However, we had not yet established whether this intermediate could drive the synthesis of Ado-CBI-GDP if provided with its other substrate, Ado-CBI-P.

To address this point, we performed the experiment described in Table V. Briefly, the CobU–GMP intermediate was generated and dialyzed against buffer to remove unreacted [α-32P]GTP. As shown in Table V, the purified CobU–GMP intermediate was capable of driving Ado-CBI-GDP synthesis only if provided with Ado-CBI-P, or Ado-CBI and ATP. These results allowed two conclusions. First, the CobU–GMP inter-

### Table V

| Addition to the reaction mixture | Concentration | Ado-CBI-GDP synthesized |
|---------------------------------|---------------|-------------------------|
| None                            |               | ND<sup>a</sup>          |
| Ado-CBI-P                       | 180 μM        | 96.0                    |
| Ado-CBI                         | 180 μM        | ND<sup>b</sup>          |
| ATP                             | 2 mM          | ND<sup>b</sup>          |
| Ado-CBI & ATP                   | 180 μM + 2 mM | 56.0                    |

<sup>a</sup> The reaction mixture contained the CobU–GMP intermediate (prepared as described above), 12 nmol; Tris-HCl, 0.1 M, pH 8.8, at 37 °C; MgCl<sub>2</sub>, 2.5 mM; and the indicated additions. The representative values reported here are the average of two determinations.

<sup>b</sup> ND, none detected.

mediate was functional for the synthesis of Ado-CBI-GDP. Second, the CobU–GMP intermediate was competent for the synthesis of Ado-CBI-P from Ado-CBI and ATP. From this second observation we inferred that the two activities of the protein function independently, a result consistent with genetic experiments.<sup>2</sup>

Each CobU Monomer Binds a GMP Moiety

As described above, CobU probably functions as a dimer. We were interested in determining whether both monomers of the CobU dimer were capable of catalyzing a guanylyl transfer. To test this idea, 1.25 pmol of CobU dimer were incubated in the presence of a 10-fold molar excess of [α-32P]GTP (specific radioactivity = 800 Ci mmol<sup>-1</sup>) in a 10-μl reaction mixture containing 2.5 mM MgCl<sub>2</sub> in Tris-HCl, 0.1 M, pH 8.8, at 37 °C. The reaction mixture was incubated at 37 °C for 10 min, then 2 μl of 6 × SDS loading buffer were added, and the samples were incubated at 80 °C for 10 min. Each sample was analyzed by SDS-PAGE (12%), and the radiolabeled CobU band was excised, and the radioactivity of the band was determined by scintillation counting. Using the known specific activity of the [α-32P]GTP, the moles of GMP in each sample were determined.

A ratio of 1.56 ± 0.27 mol of GMP per mol of CobU dimer was calculated based on results from four trials. These data suggested that each CobU monomer bound a GMP moiety and, based on the results in the preceding section, can subsequently catalyze a guanylyl transfer. Based on this result, we propose that both monomers of the CobU dimer are functionally equivalent and can likewise catalyze the kinase reaction.

Reducing Agents Are Required to Maintain CobU Activities

Crude cell-free extracts of strains overexpressing CobU did not require the addition of reducing agents in order to detect either the kinase or guanylyltransferase activities. However, during the initial stages of purification, CobU rapidly lost both of its activities. The addition of 10 mM DTT to the reaction mixture stimulated both kinase and guanylyltransferase activities (data not shown). Therefore, DTT was included in all steps of the CobU purification, and the enzyme was routinely stored under oxygen-free N<sub>2</sub>.

Thiol-reducing agents have been reported to react and form

<sup>2</sup> G. A. O'Toole and J. C. Escalante-Semerena, unpublished results.
complexes with corrinoids (27). Therefore, it was not clear whether DTT affected the protein, the corrinoid substrate, or both. To address this concern we performed the following experiment. A sample of purified CobU was stored at 4°C in air for approximately 2 weeks. The kinase and guanylyltransferase activities of this sample decreased to <1% of their original activity (the specific activity for the kinase reaction decreased from 83 to 0.7, and for the guanylyltransferase reaction the specific activity decreased from 76 to 0.4). This form of CobU enzyme was referred to as “oxidized CobU.” Enzyme stored at 4°C under a N₂ headspace retained >98% of its activity for at least 3 weeks (data not shown).

DTT (10 mM) was added to one half of the oxidized CobU sample and incubated on ice for 2 h; an equal volume of H₂O was added to the other half of the sample. The DTT-treated sample (0.5 ml) was dialyzed against 2 × 10⁻³ liter of anoxic Tris-HCl, 0.1 M, pH 8.0 at 4°C, to remove the DTT (this represents a 4 × 10⁻³-fold dilution). The untreated sample was dialyzed against the same type and volume of oxic buffer. DTT treatment of oxidized CobU resulted in an 18-fold increase in kinase activity (specific activity increased from 0.7 to 12.6), and a 42-fold increase in guanylyltransferase activity (specific activity increased from 0.4 to 17.0). The activities of the DTT-treated CobU represent 15–20% of the original specific activities. Untreated CobU showed no change in either activity. Addition of DTT to a reaction mixture containing reduced CobU enzyme did not further stimulate activity, suggesting that the effect of DTT was due to the reduction of disulfide bonds in the enzyme (data not shown).

We quantitated the total moles of sulfhydryl groups in reduced and oxidized CobU by the method of Habeeb (28). DTT-treated CobU had 4 mol of reduced sulfhydryl groups per monomer of CobU, a result consistent with the 4 cysteinyl residues per CobU polypeptide. Untreated CobU showed <1 mol of reduced sulfhydryl group per monomer of CobU. Therefore, the presence of reduced sulfhydryl groups correlated with active kinase and guanylyltransferase functions of CobU.

Evidence for the Involvement of Sulfhydryl Groups in the Kinase and Guanylyltransferase Activities

To investigate the involvement of sulfhydryl groups in CobU activities we tested the effect of sulfhydryl-reactive compounds on CobU kinase and guanylyltransferase activities. An analysis of available sulfhydryl groups in the native CobU protein with DTNB was performed as reported elsewhere (28). We observed a single class of sulfhydryl residues, which were fully modified with DTNB (at 0.33 and 1 mM) in less than 2 min (assays were monitored for a total of 30 min). We determined a value of 4 available sulfhydryl groups per dimer of CobU; each CobU dimer has 8 cysteinyl residues. The rapid modification of DTNB by CobU suggests that the sulfhydryl residues are readily accessible to DTNB. In fact, the rate of modification of CobU by DTNB is similar to the rate reported for small molecules such as cysteine (28).

We tested whether the sulfhydryl modifying agents DTNB and NEM inhibited CobU kinase and guanylyltransferase activities. CobU was incubated at 37°C in the presence of 1 mM DTNB and NEM for 10 min, then assayed immediately for its kinase and guanylyltransferase activities. The kinase activity of CobU (original specific activity = 231) was inhibited 95% by treatment with 1 mM DTNB (specific activity = 11) and inhibited 94% by treatment with 1 mM NEM (specific activity = 14). The guanylyltransferase activity (original specific activity = 93) was inhibited 92% (specific activity = 8) and 87% (specific activity = 13) by treatment with DTNB and NEM, respectively. The buffer used to prepare the DTNB and NEM solutions (0.1 M NaPi, pH 8.0) had no effect on either CobU activity (data not shown). Taken together with the study above, these data suggested that one or more of the rapidly modified sulfhydryl groups is required for CobU kinase and guanylyltransferase activities.

The Effect of Substrates on Inhibition by DTNB

In general, substrates provided little protection from inhibition by DTNB. CobU enzyme was preincubated with individual substrates under typical assay conditions (see “Experimental Procedures”) at 37°C for 10 min, then DTNB was added to 1 mM, and the reaction mixtures were incubated for an additional 10 min at 37°C.

Ado-CBI and ATP (for the kinase activity) and Ado-CBI-P (for the guanylyltransferase activity) afforded no protection from inhibition by DTNB. After incubation with substrate and inhibitor, unreacted compounds were separated from CobU on a desalting column of Sephadex G-25. CobU was assayed immediately for the appropriate activity. In all cases, the activity of CobU treated with DTNB in the presence of substrate was no higher than CobU treated with DTNB in the absence of substrate (data not shown). Untreated CobU served as a positive control and retained both activities after the 20 min of incubation at 37°C and passage over the sizing column (data not shown).

GTP provided slight protection from inhibition of the guanylyltransferase activity. CobU was incubated with 150 μM GTP for 10 min to allow formation of the CobU—GMP intermediate, then treated with DTNB as above. Dialysis against 2 × 10⁻³ ml of 0.1 M Tris-HCl, pH 8.0 at 4°C, in a Centricon-10 concentrator removed the unreacted GTP and DTNB, but the CobU-GMP intermediate is stable under these conditions (see section on CobU-GMP intermediate formation above). GTP-treated CobU (specific activity = 1.25 nmol of Ado-CBI-GDP/min/mg) had 3-fold more activity than the enzyme treated with DTNB in the absence of GTP (0.34 nmol/min/mg). However, control enzyme not treated with DTNB had a specific activity of 50.4 nmol/min/mg. Therefore, even in the presence of GTP, DTNB inhibited the guanylyltransferase activity ~95%. This GTP-protected CobU had no detectable kinase activity (data not shown).

The activity of DTNB-modified CobU could be partially restored by treatment with DTT. CobU was treated with DTNB and dialyzed as described above. The enzyme was subsequently incubated with 10 mM DTT at 37°C for 15 min, then immediately assayed for both kinase and guanylyltransferase activity. The kinase activity was stimulated ~4-fold by treatment with DTT (specific activity increased from 1.0 to 3.8 nmol of Ado-CBI-P/min/mg). The guanylyltransferase activity was stimulated 6-fold by treatment with DTT (specific activity increased from 1.1 to 6.4 nmol of Ado-CBI-GDP/min/mg). However, this DTT-mediated increase occurs in a specific activity ~10-fold below that of untreated control enzyme (specific activity = 50.4). There was no increase in either activity when the time of DTT treatment was increased to 60 min (data not shown).

At present, it is not clear whether cysteinyl residues are required for one or both CobU activities or, if so, how many cysteinyl residues are involved. It is possible that a modified cysteinyl residue is required for only one activity, and the loss of the second activity is simply the consequence of an indirect effect(s) of the modifying agents. Site-directed mutagenesis of each cysteinyl residue is in progress to evaluate the involvement of each of these residues in CobU activities.
CobU Does Not Have Detectable ATP:Adenosyltransferase Activity

We had previously shown that CobU plays a key role in the assimilation of nonadenosylated CBI into cobalamin under anaerobic conditions in cobA mutants. cobA mutants lack ATP:corrinoid adenosyltransferase activity (6, 29). One interpretation of these genetic studies was that CobU had an ATP:CBI adenosyltransferase activity which catalyzed the synthesis of Ado-CBI, the preferred substrate for the CobU enzyme (6). To test this hypothesis we used homogeneous CobU in ATP:corrinoid adenosyltransferase assays, performed as reported (12).

Control assays with the ATP:corrinoid adenosyltransferase enzyme (CobA), resulted in the synthesis of Ado-CBI from CO(I)binamide and ATP (12). The synthesis of Ado-CBI was monitored by two methods. In a spectrophotometric assay, the synthesis of Ado-CBI was monitored at 457 nm (12). In a bioassay, Ado-CBI (but not nonadenosylated CBI) restored growth of a cobA mutant on minimal medium (29). This bioassay is capable of detecting picomolar quantities of corrinoids.

When homogeneous CobU (20 μg per assay) was substituted for CobA in the assay, we were unable to detect Ado-CBI synthesis. We concluded that CobU did not have ATP:CBI adenosyltransferase activity in vitro. Alternative models to explain the role of CobU in the assimilation of nonadenosylated corrinoids under anaerobic conditions should focus on the specificity of the enzyme for its corrinoid substrate as a function of oxygen in the environment.

DISCUSSION

CobU Is a Bifunctional Enzyme That Catalyzes the Synthesis of Ado-CBI-GDP from Ado-CBI via an Ado-CBI-P Intermediate—The CobU protein of S. typhimurium was purified to >94% purity. The experimentally determined N-terminal sequence of the purified protein showed a 22 of 22 match with the amino acid sequence predicted from the DNA sequence of CobU (4, 6). These data confirmed the purification of CobU and suggested that the N terminus of CobU is not modified.

The kinase activity of CobU catalyzes the phosphorylation of Ado-CBI at the expense of ATP to yield Ado-CBI-P. The γ-phosphate of ATP is donated to Ado-CBI in this reaction, and remains a part of the product of the CobU-catalyzed reaction, Ado-CBI-GDP. In S. typhimurium, the γ-phosphate of ATP probably remains as part of the end product of the pathway, Ado-cobalamin (1, 2).

The second CobU activity catalyzes the transfer of the guanylyl moiety of GTP to Ado-CBI-P. We presented evidence that the transfer of the guanylyl moiety occurs via an enzyme-guanylyl intermediate (Fig. 5). This intermediate is acid-labile and alkali-resistant (Fig. 6). The properties of the enzyme-guanylyl intermediate are reminiscent of the phosphoramidate linkages observed in galactose-1-phosphate uridylyltransferase (23–25) and histidine kinases such as FixL (26).

Release of the GMP moiety from the CobU-GMP intermediate is driven by the presence of Ado-CBI-P (provided exogenously or generated in situ), but not by Ado-CBI. This finding is consistent with the identification of Ado-CBI-P as an intermediate in the synthesis of Ado-CBI-GDP and supports the sequence of reactions as phosphorylation followed by guanylyl transfer.

Two additional lines of evidence support the bifunctionality of CobU. Overexpression of the CobU polypeptide (Fig. 3, Panel A) results in the overexpression of both kinase and guanylyltransferase activities (Fig. 4) and both enzymatic activities copurified through three chromatography steps to >94% homogeneity (Table II).

What Residues Are Required for the Formation of the CobU-GMP Intermediate?—We have identified an allele of cobU which results in an amino acid substitution at His-46. The H46Y amino acid change results in a CobU protein unable to support cobalamin synthesis in vivo.3 His-46 is adjacent to a second histidyl residue at position 45. Both residues are conserved between the CobU protein and the CobP protein of P. denitrificans (data not shown). Interestingly, Frey and coworkers identified two nearby histidyl residues in galactose-1-phosphate uridylyltransferase (His-164 and His-166) which were required for the formation of the uridylyl-enzyme intermediate (25). We are currently using site-directed mutagenesis to determine if His-45 and/or His-46 are required for the formation of the CobU-GMP intermediate.

Oxygen Lability of CobU Enzymatic Activities and a Role for Cysteinyl Residues—The loss of CobU activity in vitro in the absence of a reducing agent is consistent with findings from genetic experiments which suggested that CobU functioned more efficiently when cells were grown anaerobically than aerobically (6). The requirement for DTT to maintain both kinase and guanylyltransferase activities, and the results of DTNB and NEM inhibition studies, is diagnostic of a role for one or more of the 4 cysteinyl residues present in the CobU enzyme. However, it is still not clear if a cysteinyl residue participates at the active site(s) of this enzyme.

Comparison of the CobU amino acid sequence to its homolog CobP in P. denitrificans showed that cysteine at position 81 in CobU is conserved between these two proteins, suggesting an important role of this residue in enzyme activity(ies). This idea has been verified by genetic means. Site-directed mutagenesis of CobU at this position was performed to change the cysteine to alanine (C81A). The C81A allele of cobU resulted in loss of CobU function in vivo and in vitro (data to be presented elsewhere).

Although CobU has 4 cysteinyl residues, CobP has only the single conserved cysteinyl residue and the CobP enzyme does not appear to require reducing agents to maintain its activities (3). Interestingly, Wong et al. (24) reported that galactose-1-phosphate uridylyltransferase also had an essential sulfhydryl group, probably located outside of the active site of the enzyme. Studies on the involvement of the remaining cysteinyl residues in CobU enzyme activity(ies) is in progress.

Specificity of CobU for Its Corrinoid Substrate—Previously reported genetic studies suggested that CobU could assimilate non-adenosylated corrinoids into cobalamin anaerobically, but not aerobically. We proposed two models to address these findings (6). One model proposed that CobU was capable of catalyzing the synthesis of Ado-CBI, then utilizing this substrate for the synthesis of Ado-CBI-GDP. The data presented here (i.e. the inability of CobU to catalyze the synthesis of detectable quantities of Ado-CBI in vitro) and the lack of amino acid similarity between CobU and adenosyltransferase enzymes (data not shown) argue against such a model.

An alternative model proposed that CobU could utilize non-adenosylated corrinoids as substrates in the absence of oxygen. Our in vitro data show that CobU can utilize HO-CBI, although Ado-CBI is clearly the preferred substrate. Therefore, it appears that CobU can catalyze the synthesis of CBI-GDP from non-adenosylated CBI, albeit inefficiently. This low level of CBI-GDP synthesis, however, appears to be sufficient to satisfy the cell’s requirement for cobalamin under anaerobic growth conditions (6). Structural work on CobU (currently in progress) will prove valuable in understanding how corrinoid substrate specificity is established by the primary structure of this protein.

3 J. C. Escalante-Semerena, unpublished results.
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REFERENCES

1. Trzebiatowski, J. R., O'Toole, G. A., and Escalante-Semerena, J. C. (1994) J. Bacteriol. 176, 3568–3575
2. O'Toole, G. A., Trzebiatowski, J. R., and Escalante-Semerena, J. C. (1994) J. Biol. Chem. 269, 26503–26511
3. Blanche, F., Debussche, L., Famechon, A., Thibaut, D., Cameron, B., and Crouzet, J. (1991) J. Bacteriol. 173, 6052–6057
4. Roth, J. R., Lawrence, J. G., Rubenfield, M., Kiessig-Higgens, S., and Church, G. M. (1993) J. Bacteriol. 175, 3303–3316
5. O'Toole, G. A., Rondon, M. R., and Escalante-Semerena, J. C. (1993) J. Bacteriol. 175, 3317–3326
6. O'Toole, G. A., and Escalante-Semerena, J. C. (1993) J. Bacteriol. 175, 6328–6336
7. Vieira, J., and Messing, J. (1987) Methods Enzymol. 153, 3
8. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1983) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
9. Kunkel, T. A. (1987) in Current Protocols in Molecular Biology (Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Vol. 2, pp. 16.2.1–16.2.11, Wiley Interscience, New York
10. Sasse, J. (1991) in Current Protocols in Molecular Biology (Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Vol. 1, pp. 10.6.1–10.6.6, Wiley Interscience, New York
11. Debussche, L., Couder, M., Thibaut, D., Cameron, B., Crouzet, J., and Blanche, F. (1991) J. Bacteriol. 173, 6300–6302
12. Suh, S.-J., and Escalante-Semerena, J. C. (1995) J. Bacteriol. 177, 921–925
13. Kunitz, M. J. (1952) J. Gen. Physiol. 35, 423–450
14. Smith, P., Krohn, R., Hermanson, A., Maia, A., Gartner, F., Provenzano, M., Fujimoto, E., Goeke, N., Olson, B., and Kleny, D. (1985) Anal. Biochem. 150, 76–85
15. Ronzio, R. A., and Barker, H. A. (1967) Biochemistry 6, 2344–2354
16. Tabor, S., and Richardson, C. C. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1074–1078
17. Tabor, S., and Richardson, C. C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4767–4771
18. Tabor, S. (1990) in Current Protocols in Molecular Biology (Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Vol. 2, pp. 16.2.1–16.2.11, Wiley Interscience, New York
19. Sasse, J. (1991) in Current Protocols in Molecular Biology (Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Vol. 1, pp. 10.6.1–10.6.6, Wiley Interscience, New York
20. Laemmli, U. K. (1970) Nature 227, 680–685
21. Bollag, D. M., and Edelstein, S. J. (1991) Protein Methods, pp. 162–180, John Wiley & Sons, New York
22. Ferguson, K. A. (1964) Metabolism 13, 985–1002
23. Wong, L.-j., and Frey, P. A. (1974) J. Biol. Chem. 249, 2322–2324
24. Wong, L.-j., Rex Sheu, K.-F., Lee, S.-L., and Frey, P. A. (1977) Biochemistry 16, 1010–1014
25. Field, T. L., Reznikoff, W. S., and Frey, P. A. (1989) Biochemistry 28, 2094–2099
26. Giles-Gonzalez, M. A., Ditta, G. S., and Helinski, D. R. (1991) Nature 350, 170–172
27. Aronovitch, J., and Grossowicz, N. (1962) Biochem. Biophys. Res. Commun. 8, 416–420
28. Haberb, A. F. S. A. (1972) Methods Enzymol. 25, 457–464
29. Escalante-Semerena, J. C., Suh, S.-J., and Roth, J. R. (1990) J. Bacteriol. 172, 273–280

Synthesis of Ado-CBI-GDP in S. typhimurium LT2

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