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Purification and Initial Characterization of the ATP:Corrinoid Adenosyltransferase Encoded by the *cobA* Gene of *Salmonella typhimurium*

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The *cobA* gene of *Salmonella typhimurium* and its product were overexpressed to approximately 20% of the total cell protein. CobA was purified to 98% homogeneity; N-terminal sequence analysis (21 residues) of homogeneous protein confirmed the predicted amino acid sequence. ATP:corrinoid adenosyltransferase activity was demonstrated in vitro to be associated with CobA. This activity was optimal at pH 8 and 37°C. A quantitative preference was determined for Mn(II) cations and ATP. The apparent K_m of CobA for ATP was 2.8 μM, and that for cob(I)alamin was 5.2 μM. V_{max} was measured at 0.43 nmol/min. Cobinamide served as the substrate for CobA to yield adenosylcobinamide. Activity was stable at 4°C for several weeks but was lost rapidly at room temperature (50% overnight). Dithiothreitol was required to maintain the enzymatic activity of CobA.

The facultative anaerobe *Salmonella typhimurium* synthesizes and utilizes adenosylcobalamin (Ado-CBL). In this bacterium, de novo synthesis of Ado-CBL occurs under anaerobic growth conditions. However, assimilation of incomplete or complete corrinoids from the environment occurs under either aerobic or anaerobic growth conditions (12–14).

S. typhimurium cobA mutants display phenotypes consistent with a defect in the corrinoid adenosylation pathway (11). This pathway consists of three steps: (i) reduction of Co(III) to Co(II), (ii) reduction of Co(II) to Co(I), and (iii) transfer of the 5′-deoxyadenosine moiety from ATP to the reduced corrinoid (Fig. 1) (1, 29). The enzymes that catalyze these reactions have been partially purified and characterized in *Clostridium tetanomorphum* (27) and *Propionibacterium shermanii* (4). The genes encoding these enzymes, however, have not been identified in these organisms. Prior to this report, only the *Pseudomonas denitrificans cobO* gene and its gene product had been characterized (6).

In *S. typhimurium*, *cobA* is the only gene of the corrinoid adenosylation pathway that has been characterized (11, 24). Herein, we report the purification of the CobA protein and biochemical evidence that demonstrates CobA is the ATP:corrinoid adenosyltransferase in *S. typhimurium.*

MATERIALS AND METHODS

Strains, media, and growth conditions. The genotypes of bacterial strains and plasmids used in this study are listed in Table 1. The compositions of rich (nutrient broth) and Luria-Bertani broth and minimal media and the concentrations of auxotrophic requirements and antibiotics in the media have been described previously (5, 11, 28). Glucose (11 mM) was used as carbon and energy source. The cyano-CBL level was 15 nM, the dicyano-cobinamide [$(CN)_2$CBI] level was 15 nM, and the $CoCl_2$ level was 5 μM. *Escherichia coli* strains were grown in Luria-Bertani broth. All strains carrying plasmid pGP1-2 were grown at 30°C to prevent T7 RNA polymerase synthesis.

Genetic techniques. Transductions with mutant P22 phage HT*105/int-201* (20, 21) were performed at an approximate multiplicity of infection of 1 as described in reference 10. Crosses which selected for kanamycin or chloramphenicol resistance were preincubated for 2 h without shaking at either 30 or 37°C prior to plating on selective medium. All transductions performed in the presence of plasmid pGP1-2, either as a donor or as a plasmid present in the recipient cell, were incubated at 30°C.

Recombinant DNA techniques. (i) General. DNA manipulations were performed according to the method of Maniatis et al. unless otherwise noted (16). Plasmid DNA was prepared either by the alkaline lysis method (2) or by the Wizard Prep method (Promega, Madison, Wis.). All restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were purchased from New England Biolabs (Beverly, Mass.). Calf intestine phosphatase and T4 polynucleotide kinase were purchased from Promega. All enzymes were used according to the manufacturers' recommendations. DNA sequencing was performed by the double-strand method with Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio). Primers for sequencing were purchased from New England Biolabs (NEB 1201 reverse primer for M13mp19 and NEB 1227 for the T7 promoter). DNA fragments were isolated from agarose gels with the Gene Clean system (Bio 101, Inc., La Jolla, Calif.). Plasmids were introduced into bacterial strains by electroporation as described elsewhere (18).

(ii) Site-directed mutagenesis. Site-directed mutagenesis was performed according to the method of Deng and Nickoloff (8). Oligonucleotides used for the mutagenesis were purchased from National Biosciences (Plymouth, Minn.). Mutagenesis was performed with plasmid pCOBA6 (*cobA* gene in vector pSU19 [17]). Two oligonucleotides were designed for the mutagenesis: a selective oligonucleotide and a nonselective oligonucleotide.

The selective oligonucleotide, 5′-GTTTTCAC**T**ATGGGCAAATAT-3′, was 21 nucleotides in length and had a single nucleotide change (shown by the boldface letter) which eliminated the unique *Nco*I site within the chloramphenicol resistance *cat* gene of the pSU vector without affecting the function of the chloramphenicol acetyltransferase enzyme. Elimination of this site provided a way to counterselect against nonmutagenized plasmid. Mutagenized, *Nco*I-resistant plasmids are enriched for after a few cycles of cutting with *Nco*I enzyme followed by electroporation. The poor frequency of electroporation for linear DNA increases the probability of isolating mutagenized plasmids.

The nonselective oligonucleotide, 5′-CAGGAATCGCC**AT**ATGAGTGAT GA-3′, was 24 nucleotides in length and had two nucleotide changes (shown by boldface letters) that created an *Nde*I site directly upstream of the *cobA* coding sequence without affecting the open reading frame; the resulting plasmid was pCOBA14. Mutagenesis was confirmed by DNA sequencing of the region. The *Nde*I-*Bam*HI fragment of pCOBA14 carrying *cobA* was cloned into the T7 transcription/translation vector pET-3a (23) to yield the *cobA*-overexpression plasmid pCOBA15 (Fig. 2). The junction between the T7 promoter and the *cobA* gene in pCOBA15 was sequenced to confirm that no other mutations were introduced during cloning.

ATP:corrinoid adenosylation activity assay. A modification of the in vitro ATP:CBL adenosyltransferase assay described by Vitols et al. (27) was employed to determine if this enzymatic activity was associated with CobA.

(i) Assay conditions and protocol. The assay was performed under strictly anoxic conditions to prevent oxidation of hydroxo-cob(I)alamin. Anoxic conditions were maintained as described elsewhere (10). The reaction mixture contained the following: Tris-HCl (pH 8.0) at 37°C, 200 nmol; hydroxo-cob(III) alamin, 50 nmol; ATP, 400 nmol; $MnCl_2$, 800 nmol; and $CoCl_2$, 100 μg (final volume of 0.9 ml). The reaction mixture was degassed with oxygen-free nitrogen for 30 min by bubbling the oxygen-free N_2 through the solution. A cuvette

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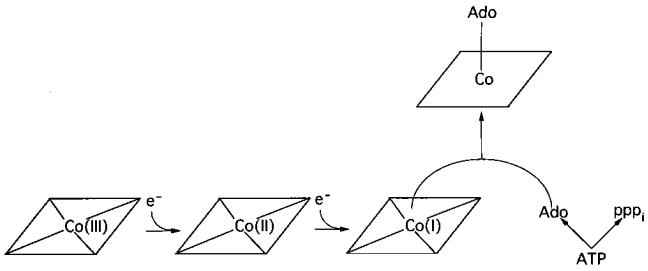

FIG. 1. Schematic representation of the sequence of reactions leading to corrinoid adenosylation. Abbreviations: Ado, 5′-deoxyadenosine; e^-, electron.

containing 4 mg of solid KBH_4 and 2 mg of Zn dust was fitted with a red butyl rubber stopper and degassed on ice for 10 min with oxygen-free N_2; 0.9 ml of degassed reaction mixture was added to the cuvette with a syringe previously flushed with oxygen-free N_2. Reduction of cob(III)alamin to cob(I)alamin occurred within 10 min, and cob(I)alamin was identified by a change in the color of the solution from red to dark green-black. During reduction, the cuvette was constantly flushed with oxygen-free N_2 to prevent oxidation of cob(I)alamin. Enzyme was anoxically added to the cuvette with a Hamilton syringe (Hamilton Co., Reno, Nev.) previously flushed with anoxic water. The reaction was initiated by incubating the cuvette at 37°C in the dark. The reaction was allowed to proceed for 30 min, and the UV-visible spectrum of the product was measured on a Perkin-Elmer Lambda 6 spectrophotometer furnished with UV Data Manager software (Perkin-Elmer Corp., Norwalk, Conn.). The reproducibility of the assay was achieved when cuvettes were stored in the anoxic chamber (Coy Laboratory Products, Inc., Grass Lake, Mich.) for at least 24 h prior to use.

In the latter stages of this study, it was found that Zn dust was not required for the in vitro adenosylation assay. Thus, all assays performed with homogeneous CobA were performed in reaction mixtures lacking Zn.

(ii) Quantitation of the product. The amount of Ado-CBL produced in the reaction was quantitated by measuring the A_{525} of the reaction mixture before and after 5 min of irradiation under a 150-W tungsten light at a distance of about 15 to 20 cm. Under these conditions, Ado-CBL is converted by homolytic cleavage of the Co-C bond to cob(II)alamin (3). The concentration of Ado-CBL in the mixture was calculated by the formula $\Delta A_{525} = \Delta\varepsilon CL$, where ΔA_{525} is the difference in A_{525} between Ado-CBL and cob(II)alamin, $\Delta\varepsilon$ (0.0048) (27), is the millimolar extinction coefficient at 525 nm between Ado-CBL and cob(II)alamin, and L is the cuvette light path (in our case, 1 cm). The concentration of Ado-CBL was determined by solving for C.

One unit of CobA activity was defined as the amount of enzyme required to generate 1 nmol of Ado-CBL per min. All in vitro assay results reported are the average of a minimum of two determinations.

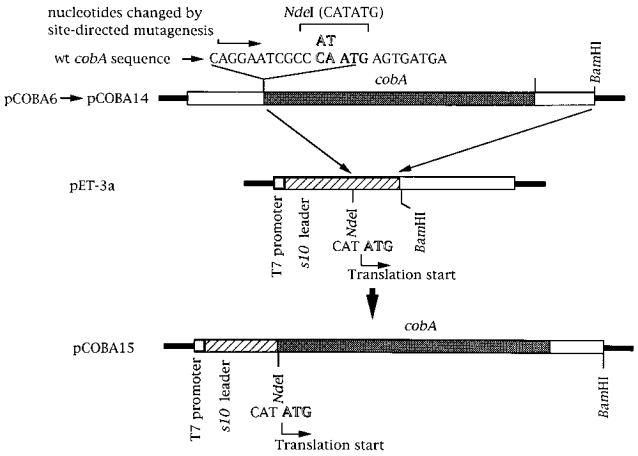

FIG. 2. Construction of a *cobA* overexpression vector. A unique *Nde*I restriction site was generated on plasmid pCOBA6 by site-directed mutagenesis. Introduction of the *Nde*I site allowed the subcloning of the *cobA* open reading frame (encoded on an *Nde*I-*Bam*HI fragment) into a T7 overexpression vector, pET-3a, in the correct reading frame. The resulting plasmid, pCOBA15, directed the synthesis of CobA protein to approximately 20% of the total cellular protein upon induction in *S. typhimurium*.

Protein techniques. Protein concentration was measured with the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.) after precipitating the proteins with trichloroacetic acid and washing the pellet once with 70% ethanol to eliminate dithiothreitol (DTT) interference. All electrophoretic protein analyses were performed with the Bio-Rad Mini Protean II system (Bio-Rad Laboratories, Richmond, Calif.). Nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed on 7, 8, 9, and 10% polyacrylamide gels; molecular weight standards were purchased from Sigma (Sigma Chemical Co., St. Louis, Mo.). Sodium dodecyl sulfate (SDS)-PAGE (15) was performed in 12% polyacrylamide gels. Molecular weight standards were purchased from Bio-Rad.

CobA overexpression. CobA was overexpressed in *S. typhimurium* JE2884 according to Tabor's two-plasmid method (25). Briefly, Luria-Bertani broth (100

TABLE 1. Strains and plasmids used in this study[a]

Strain or plasmid	Genotype	Source or reference
Strains		
E. coli		
BMH71-18 (*mutS*)	F′ *lacI*^{q} Δ(*lacZ*)M15 *proA*$^+$*B*$^+$/Δ(*lac-proAB*) *supE thi mutS*::Tn*10*	31
DH5αF′	F′/*endA! hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*) *U169 deoR* [ϕ80d*lac*Δ (*lacZ*)M15]	19, 30
S. typhimurium		
JR501	*hsdSA29 hsdSB121 hsdL6 metA22 metE551 trpC2 ilv-452 rpsL120 xyl-404 galE719 H1-b H2-e,n,x* [cured of *Fels2* (−)] *fla-66 nml*	26
TR6583[b]	*metE205 ara-9*	K. Sanderson via J. Roth
Derivatives of TR6583		
JE1445	DE *902* (*trp-cobA*)	Laboratory collection
JE2884	DE *902* (*trp-cobA*)/pCOBA15 pGP1-2	This study
JE2888	DE *902* (*trp-cobA*)/pET-3a pGP1-2	This study
Plasmids		
pET-3a	T7 transcription/translation vector (Km^r)	pGP1-2 T7 (23)
RNA polymerase		S. Tabor
pSU19	Cloning vector (Cm^r)	17
pCOBA6	*cobA* in pSU19	This study
pCOBA14 *Nco*I restriction site	pCOBA6 with *Nde*I restriction generated and destroyed	This study
pCOBA15 (*cobA* overexpression plasmid)	*cobA* cloned in pET-3a	This study

[a] All *S. typhimurium* strains are derivatives of the LT2 strain. For transfer of plasmids between *E. coli* and *S. typhimurium*, the plasmids were first passed through their respective modification-proficient, restriction-deficient strains: DH5αF′ for *E. coli* and JR501 for *S. typhimurium.*

[b] Formerly SA2779.

TABLE 2. CobA purification profile

Procedure	Vol (ml)	Total protein (mg)	Protein (mg/ml)	Sp act (U/mg of protein)	Total U	Yield (%)	Purification (fold)
Soluble cell extract	10.0	51.0	5.1	13.2	673.2	100	0
$(NH_4)_2SO_4$ precipitation	8.0	30.7	3.84	18.8	577.5	60.2	1.4
Phenyl-Sepharose	4.0	9.2	2.31	46.8	432.4	18.1	3.5
Cibacron Blue	6.5	5.7	0.87	51.9	293.5	11.2	3.9
Hydroxyapatite	4.0	5.1	1.27	53.1	269.7	10.0	4.0

ml) containing 22 mM glucose, ampicillin, and kanamycin was inoculated with 1 ml of an overnight culture of strain JE2884 and grown at 30°C to 80 to 90 Klett units. The temperature was shifted to 42°C for 30 min to induce expression of T7 RNA polymerase, followed by further growth at 37°C for 90 min to express *cobA*. Approximately 50 to 60% of the CobA protein formed inclusion bodies under these conditions; attempts to increase the amount of soluble CobA protein failed.

CobA purification. (i) Growth of CobA-overproducing cells and preparation of soluble protein extract. Cells obtained from a 200-ml culture of the *cobA*-overexpressing strain JE2884 were resuspended in 12 ml of ice-cold buffer A (50 mM Tris-HCl [pH 8.0] at 4°C, 5 mM DTT) containing 16 μg of the serine protease inhibitor phenylmethylsulfonyl fluoride per ml. Resuspended cells were lysed by sonication (Branson Ultrasonics Corp., Danbury, Conn.) with a microtip at a 50% duty cycle on the setting of 7. Sonication was performed for 7.5 min with intermittent cooling after every 2.5 min. Cell breakage was monitored under the microscope until at least 90% was observed. Cytosolic proteins (59.1 mg) were separated by high-speed centrifugation at 40,000 × *g* for 2 h at 4°C. Subsequent steps were performed at 4°C. Enrichment of the CobA protein extracts was monitored at each step by SDS-PAGE and in vitro with the CBL adenosylation assay.

(ii) Step 1: ammonium sulfate precipitation. CobA precipitated out of solution between 30 and 50% saturation of ammonium sulfate. Protein was pelleted by centrifugation at 10,000 × *g* for 10 min, resuspended in buffer A containing 20% ammonium sulfate, and pooled.

(iii) Step 2: hydrophobic interaction chromatography on phenyl-Sepharose. A phenyl-Sepharose (Sigma) column (1.5 by 5.7 cm, 10-ml bed volume) equilibrated with 20% ammonium sulfate in buffer A was used to further purify CobA. Proteins were eluted from the column with a 50-ml reverse linear gradient (20% to 0%) of ammonium sulfate in buffer A at a flow rate of 11 cm h^{-1}. CobA-containing fractions were pooled, and proteins were precipitated by bringing the ammonium sulfate concentration in the buffer to 65% saturation. The pellet was resuspended in 4 ml of buffer A. A total of 9.3 mg of protein was recovered.

(iv) Step 3: dye-ligand chromatography on Cibacron Blue. Two milliliters of Cibacron Blue 3GA type 3000 resin (Sigma) was equilibrated with 0.2 M KCl in buffer A. After loading the CobA-containing sample, the column was washed with 10 ml of starting buffer. Proteins were eluted with a linear gradient of 0.2 to 2 M KCl in buffer A in a volume of 20 ml. Protein (5.6 mg) was recovered in 6.5 ml. CobA-containing fractions were desalted and concentrated with a Centri-Prep 10 (Amicon, Inc., Beverly, Mass.).

(v) Step 4: hydroxyapatite chromatography. Purified CobA was loaded onto a hydroxyapatite column (Bio-Rad Laboratories) (1 by 6.4 cm, 5-ml bed volume) equilibrated with 20 mM potassium phosphate buffer (pH 8.0) containing 5 mM DTT. After loading, the column was washed with 15 ml of the starting buffer. CobA was eluted from the column with a 25-ml linear gradient of 20 to 250 mM phosphate buffer (pH 8.0) containing 5 mM DTT at a flow rate of 4.6 cm h^{-1}. Fractions containing CobA were pooled, and the buffer was exchanged with buffer A with a Centri-Prep 10. The final yield of CobA was 5.1 mg. Densitometry (Hoefer GS300 densitometer; Hoefer Scientific Instruments, San Francisco, Calif.) of a Coomassie blue-stained denaturing gel indicated that CobA was 98% homogeneous.

N-terminus sequencing. The N-terminus sequence of purified CobA was determined by the Protein Sequence and Characterization Service of Nucleic Acid and Protein Facility in the University of Wisconsin Biotechnology Center, Madison. Protein sequencing was conducted with an automated model HP G1005A protein sequencer with an HP G1001A Sample Prep station, and an on-line PTH-amino acid analyzer based on the HP 1090M high-performance liquid chromatograph (Hewlett-Packard Co., Palo Alto, Calif.).

RESULTS AND DISCUSSION

Purification of CobA. (i) Starting levels of CobA in the cell. The CobA levels produced directly by our previously reported overexpression vector were relatively low (24). We dramatically improved these levels by replacing the *cobA* ribosome binding site with the one for the phage T7 *s10* gene. Overexpression of *cobA* directed by the new overexpression plasmid pCOBA15 resulted in accumulation of CobA to approximately 20% of the total cell protein (data not shown). As reported, CobA ran as a 25-kDa protein by SDS-PAGE (12% polyacrylamide) (24).

(ii) ATP:corrinoid adenosyltransferase activity of CobA. ATP:CBL adenosyltransferase specific activity in cell extract of the CobA-overproducing strain (JE2884) was 132-fold higher than that measured in the isogenic strain lacking pCOBA15 (JE2888). CobA-dependent synthesis of Ado-CBI from cob(I)inamide and ATP was also demonstrated but not quantitated. No activity was detected in cell extract of the nonoverproducing strain TR6583, despite the fact that this strain is genotypically *cobA*$^+$, suggesting that wild-type levels of CobA may be very low.

The enzymatic activity of CobA was lost in the absence of DTT, suggesting the involvement of cysteinyl residues in the enzymatic activity of the protein.

(iii) Correlation between CobA enzymatic activities and the phenotypes of *cobA* mutants. The in vitro data presented above explain the observed phenotypes of the *cobA* mutants. *cobA* mutants fail to synthesize Ado-CBL from exogenous, nonadenosylated CBI or CBL. Ado-CBI corrects this phenotype (11).

Purification of CobA and some basic properties of the protein. Table 2 shows the purification profile of CobA. A fourfold increase in the specific activity of the activity in crude cell extract was sufficient to generate a homogeneous preparation of CobA.

CobA interacted strongly with phenyl-Sepharose, requiring a concentration of ammonium sulfate in the buffer close to zero (data not shown). CobA also bound to Cibacron Blue resin very tightly in the absence of magnesium in the buffer; a high concentration of KCl (1.5 to 2 M) was needed to dissociate CobA from this resin. Hydroxyapatite, the final step in the purification, removed minor impurities and resulted in 98% homogeneous CobA. The protein yield was 10%: i.e., 5 mg of homogeneous CobA from 59 mg of starting protein. The same yield was obtained from a scaled-up purification of CobA (0.16 g of homogeneous CobA from 1.5 g of protein). Figure 3 illustrates the purification of CobA from crude cell extract to homogeneity. Nondenaturing PAGE showed biologically active CobA to be dimeric (ca. 42 kDa [data not shown]).

Temperature stability. The enzymatic activity of homogeneous CobA was stable for several weeks at 4°C when 5 mM DTT was included in the buffer. At room temperature, the protein retained approximately 50% of its activity after overnight incubation (data not shown). Enzymatic activity was lost upon repeated freezing and thawing. In the absence of CBL and ATP, CobA precipitated out of solution when concentrated to greater than 6 to 7 mg ml^{-1}. In the presence of CBL and ATP, CobA was concentrated to more than 18 mg ml^{-1} (17a).

The stability of CobA activity at temperatures ≥37°C was measured after preincubation of the enzyme at any given temperature for 10 min before adding it to the reaction mixture.

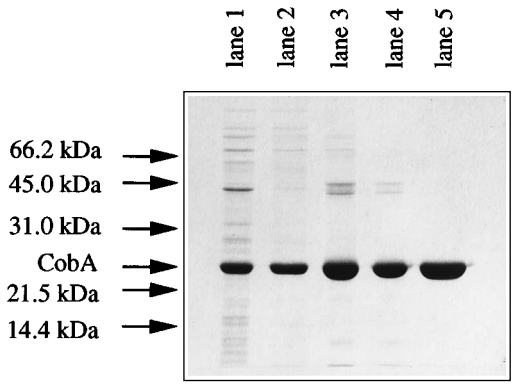

FIG. 3. Purification of CobA as shown by SDS-PAGE. Approximately 8 μg of total protein was loaded in each lane. Lanes: 1, soluble fraction of crude cell extract; 2, purification after ammonium sulfate precipitation; 3, purification after phenyl-Sepharose chromatography; 4, purification after Cibacron Blue chromatography; 5, purification after hydroxyapatite chromatography.

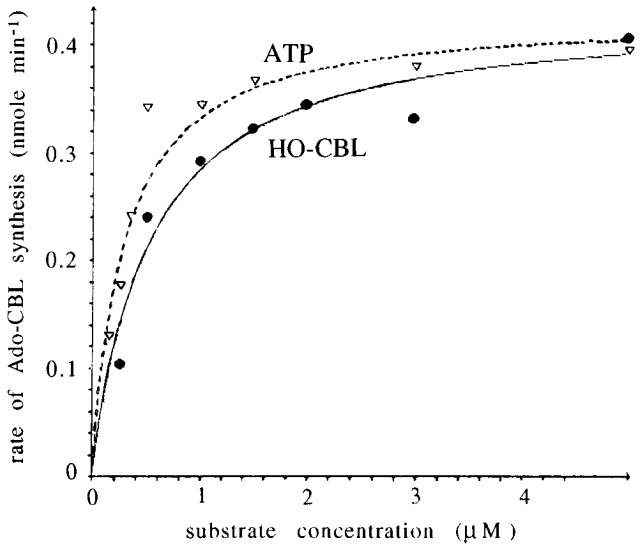

FIG. 4. Determination of kinetic constants for CBL and ATP. When the concentration of CBL in the reaction mixture was varied, the ATP concentration in the assay was maintained at 0.4 mM. When the concentration of ATP in the reaction mixture was varied, CBL was maintained at 20 μM. Data were analyzed with the nonlinear regression data analysis program Enzfitter (Elsevier-Biosoft, Cambridge, United Kingdom). The assay was performed as described in Materials and Methods; in all cases, the reaction mixture contained 4 μg of CobA. HO-CBL, hydroxo-cob(III)alamin.

The following data are expressed as preincubation temperature, specific activity remaining, and relative activity; note that the activity remaining was measured at 37°C after treatment and expressed as a percentage of the activity remaining after preincubation at 37°C: 37°C, 141.4 U mg of protein^{-1}, 100%; 45°C, 141 U mg of protein^{-1}, 100%; 50°C, 120.7 U mg of protein^{-1}, 85%; 55°C, 89.7 U mg of protein^{-1}, 63%; 60°C, 29.7 U mg of protein^{-1}, 21%; 65°C, 0.2 U mg of protein^{-1}, 1%; and 70°C, remaining specific activity not detectible and relative activity not measurable. A sharp decrease in activity was observed after preincubation at temperatures higher than 55°C.

N-terminal sequence. The N-terminus of homogeneous CobA protein was determined by automated Edman degradation. The N-terminal sequence of the first 21 amino acid residues was determined to be MSDERYQQRQQKVKDRVD ARV, a perfect match to the predicted amino acid sequence of CobA (24).

Linearity of the reaction. Enzymatic activity was proportional to the enzyme concentration up to 30 μg of protein. The assay was performed at 37°C for 15 min with increasing amounts of CobA. The reaction rate, measured in nanomoles of Ado-CBL generated per minute, was linear from 5 μg of CobA (0.42 nmol of Ado-CBL min^{-1}) to 30 μg of CobA (2.73 nmol of Ado-CBL min^{-1}).

Specificity for the donor nucleotide. The ability of CobA to use other nucleotides as the donor molecule for the upper ligand of CBL was measured. The assay was performed with 29 μg of enzyme at 37°C for 10 min. We found that ATP was the best substrate (specific activity of 120 U mg of protein^{-1}), and this value was arbitrarily set as 100% activity. With CTP, we observed 98% activity, with UTP we observed 88% activity, and with GTP we observed 37% activity. It is interesting that GTP, which is structurally the most similar to ATP, is the poorest substrate for CobA. This may suggest the way CobA recognizes and binds ATP. Having an extra amino group must somehow affect the binding of GTP to CobA, either by steric hindrance or a charge effect. Conversely, a CBL with a guanosyl moiety as the upper ligand may be less stable under the assay conditions.

Determination of pH and temperature optima. (i) pH. Adenosyltransferase activity was measured at pH 7.0, 7.5, 8.0, and 8.5. The assay was performed with 29 μg of enzyme at 37°C for 30 min. Under the conditions tested, enzyme was optimally active at both pH 8.0 and pH 8.5. A sharp decrease in CobA activity (36% drop) was measured at pH 7.5, and a further decrease in activity (67% drop) was measured at pH 7.0. We were unable to assess CobA activity at a pH greater than 8.5 because cob(I)alamin could not be generated under these conditions.

(ii) Temperature. Adenosyltransferase activity was measured at 25, 30, 37, 42, and 50°C. The assay was performed with 29 μg of enzyme for 10 min at the temperatures given above. The reaction mixtures containing cob(I)alamin were preincubated at the temperatures given above to equilibrate the temperature of the reaction mixtures prior to adding the enzyme. The relative specific activities of the enzyme were as follows: 34% at 25°C, 73% at 30°C, 100% at 37°C, 93% at 42°C, and 39% at 50°C. On the basis of these results, we concluded that the optimal temperature for CobA activity was 37°C. We note, however, that because Tris-HCl (pH 8.0 at 37°C) was used as the buffer in the assay, the decrease in CobA activity observed at 50°C may partially be due to a decrease in the pH of the buffer. Specifically, at 50°C, the pH of the buffer used was 7.7.

Divalent cation requirement. We tested for a divalent cation requirement for the CobA enzymatic activity. At 800 μM divalent cation in the reaction mixture, the best CobA activity was observed when Mn(II) was used. This was arbitrarily set as the 100% activity value. With Mg(II), we observed 77% of the optimal activity; with Co(II) (100 μg ml^{-1} = 420 μM), we observed only 15% of the activity. The last value for Co(II) was measured only to assess the background level of CobA activity due to the Co(II) in the reaction mixture. The Co(II) was included in the reaction mixture for the sole purpose of aiding in the generation of cob(I)alamin (9).

***K_m* and *V_{max}* measurements.** The in vitro assays for determining the kinetic constants were performed at pH 8.0 and 37°C for CBL and ATP. The dependence of CobA activity on the CBL concentration is illustrated in Fig. 4. Under the stated reaction conditions, the apparent K_m of CobA for CBL was determined to be 5.2 μM, with a V_{max} of 0.43 nmol/min. The

dependence on the ATP concentration is shown in Fig. 4. The apparent K_m of CobA for ATP was determined to be 2.8 μM, with a V_{max} of 0.43 nmol/min. Inconsistent results were obtained during the determination of the K_m for ATP when the enzyme was not mixed with ATP prior to being added to the reaction mixture. For this reason, CobA was preincubated with ATP on ice for 5 min prior to its addition to the reaction mixture. This observation suggests that CobA may bind its substrates in a specific order; i.e., ATP may bind first, followed by binding of the corrinoid.

In summary, we have purified CobA from an overproducing strain and performed initial characterization of the protein that demonstrated its function as the ATP:corrinoid adenosyltransferase of *S. typhimurium*. On the basis of the available genetic data, CobA seems capable of adenosylating at least three different corrinoid compounds: an unidentified biosynthetic intermediate, CBI, and CBL (11). We have demonstrated that CobA can utilize CBI and CBL as substrates for its enzymatic activity. Identification of the de novo corrin ring biosynthetic intermediate that is the substrate for CobA is in progress.

Recent evidence suggests that cobalt insertion may occur at a relatively early stage of the corrin ring biosynthesis in *S. typhimurium* (22). This suggests a potentially different path for the corrin ring biosynthesis from that of *P. denitrificans*, in which the cobalt insertion occurs at a relatively late stage of the corrin ring biosynthesis at hydrogenobyrinic acid *a*,*c*-diamide (7). If the cobalt insertion occurs early in *S. typhimurium*, we would also expect corrinoid adenosylation to occur earlier than in *P. denitrificans.*

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