CobD, a Novel Enzyme with L-Threonine-O-3-phosphate Decarboxylase Activity, Is Responsible for the Synthesis of (R)-1-Amino-2-propanol O-2-Phosphate, a Proposed New Intermediate in Cobalamin Biosynthesis in Salmonella typhimurium LT2∗

(Received for publication, September 17, 1997)

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The cobD gene of Salmonella typhimurium LT2 has been cloned, sequenced, and overexpressed. The overexpressed protein had a molecular mass of ~40 kDa, in agreement with the mass predicted by the deduced amino acid sequence (40.8 kDa). Computer analysis of the deduced amino acid sequence of CobD identified a consensus pyridoxal phosphate-binding motif. The role of CobD in cobalamin biosynthesis in this bacterium has been established. CobD was shown to decarboxylate L-threonine O-3-phosphate to yield (R)-1-amino-2-propanol O-2-phosphate. We propose that the latter is a substrate in the reaction catalyzed by the CbiB enzyme proposed to be responsible for the conversion of adenosylcobamic acid to adenosylcoaminamide and that the product of the reaction is adenosylcoaminamide phosphate, not adenosylcoaminamide as previously thought. The implications of these findings are discussed in light of the demonstrated kinase activity of the CobU enzyme (O'Toole, G. A., and Escalante-Semerena, J. C. (1995) J. Biol. Chem. 270, 23560–23569) responsible for the conversion of adenosylcoaminamide to adenosylcoaminamidine phosphate. These findings shed light on the strategy used by this bacterium for the assimilation of exogenous unphosphorylated coaminamide from its environment. To our knowledge, CobD is the first enzyme reported to have L-threonine O-3-phosphate decarboxylase activity, and computer analysis of its amino acid sequence suggests that it may be a member of a new class of pyridoxal phosphate-dependent decarboxylases.

The biochemistry of adenosylcobalamin biosynthesis has been studied for over 4 decades, with an accelerated pace of progress being accomplished in the last 15 years due to the application of genetic and recombinant DNA approaches (reviewed in Refs. 1–5). Several procaryotes have been used as model systems to study cobalamin biosynthesis. The best studied ones are the strictly respiration Pseudomonas denitrificans, the facultative anaerobe Salmonella typhimurium LT2, the aerotolerant anaerobe Propionibacterium freudenreichii (shermanii), and the strict anaerobe Eubacterium limosum. There are, however, key differences between the pathways leading to the synthesis of the corrin ring in these organisms. Most notable is the time of cobalt insertion into the macrocycle (6–11). While cobalt insertion in P. denitrificans occurs late in the pathway (12, 13), cobalt appears to be inserted very early in the synthesis of the corrin ring in S. typhimurium and P. freudenreichii (8, 10, 11). The majority of the reactions of the corrin ring biosynthetic pathway in P. denitrificans has been firmly established (2), whereas the identities of most of the intermediates of this pathway in S. typhimurium and P. freudenreichii have not been elucidated.

One important unanswered question about the synthesis of the corrin ring regards the metabolic origin of the (R)-1-amino-2-propanol moiety linking the macrocycle to the nucleotide (Fig. 1). Early studies performed on Streptomyces griseus demonstrated that in this bacterium, label from [15N]L-Thr was incorporated into the (R)-AP moiety of Cbl. However, evidence for direct decarboxylation of L-Thr was not obtained (14). Later, a series of studies by Ford and Friedmann (15–17) investigated the nonenzymatic decarboxylation of L-Thr. These authors showed that L-Thr decarboxylation occurred optimally at pH 8 when diaquocobic acid was present in a reaction mixture containing Tris-CI buffer and a reductant (e.g. glutathione, β-mercaptoethanol, or borohydride) (15, 16). This work led to a model for interactions between diaquocobic acid and L-Thr. Although the nature of the interaction between these two compounds was not established, it was demonstrated that for the interaction to occur, the cobalt ion in the ring had to be in its Co(II) oxidation state (17). While these studies presented a thought-provoking mechanism for the synthesis of (R)-AP and its incorporation into cobinamide, they failed to demonstrate that direct decarboxylation of L-Thr leads to (R)-AP synthesis.

An alternative pathway for the synthesis of (R)-AP is via the enzymatic oxidation of L-Thr to α-amino-β-ketobutyrate by L-threonine 3-dehydrogenase (EC 1.1.1.103) (18, 19). This compound spontaneously decarboxylates to yield aminoacetone, which is reduced to (R)-AP by (R)-1-aminopropan-2-ol:NAD+ oxidation.

The abbreviations used are: (R)-AP, (R)-1-amino-2-propanol; Cbl, cobalamin; AdoCbi-P, 5′-deoxyadenosylcobinamide phosphate; AdoCbi, 5′-deoxyadenosylcobinamide; (CN)2Cby, dicyanocobyric acid; PCR, polymerase chain reaction; bp, base pair(s); IPTG, isopropyl-β-D-thiogalactopyranoside; PIPES, 1,4-piperazinediethanesulfonic acid; PLP, pyridoxal phosphate; HPLC, high performance liquid chromatography; oPA, o-phthalaldehyde; Thr-P, threonine O-phosphate.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 273, No. 5, Issue of January 30, pp. 2684–2691, 1998
Vol. 273, No. 5, Issue of January 30, pp. 2684–2691, 1998
Printed in U.S.A.

* This work was supported in part by United States Public Health Service Grant GM40313 from NIGMS (to J. C. E.-S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
+ The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) U90625.
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In Vivo Assessment of Cbl Biosynthesis

Cbl biosynthesis was assessed in vivo by demanding synthesis of methionine via the Cbl-dependent methionine synthetase, MetH (5-methyltetrahydrofolate-homocysteine methyltransferase, EC 2.1.1.13) (26, 27). cobD mutant JE2216 carried a mutation in metE encoding the Cbl-independent methionine synthetase, MetE (5-methyltetrahydropteroylglutamate-homocysteine methyltransferase, EC 2.1.1.14); hence, growth of the strain depended on the availability of either methionine or Cbl. As described elsewhere (21), Cbl biosynthesis in cobD mutants was restored when the medium was supplemented with (CN)2Cby and (R)-AP under aerobic growth conditions or by (R)-AP alone under anaerobic growth conditions. The need for (CN)2Cby under anaerobic conditions is bypassed because S. typhimurium synthesizes the corrin ring de novo when its environment is devoid of oxygen (27, 28).

Genetic Techniques

Transductions—All transductional crosses were performed using the high frequency transducing bacteriophage P22 mutant HT 105/1 int201 (29, 30). Transductants were purified and identified as phage-free as described (31).

Complementation Studies—Both plasmids pCOB62 and pCOBD6 and the corresponding vector-only controls pSU38 and pT7-7 were transformed into cobD mutant strain JE2216 and its recipient-deficient derivative JE4097. Transformants were tested for their ability to grow aerobically on minimal medium supplemented with glucose, (CN)2Cby, and ampicillin (pT7-7 derivatives) or kanamycin (pSU38 derivatives). Strains that grew under these conditions were assessed as positive for complementation of the cobD phenotype and therefore carried a plasmid with a functional cobD gene.

Recombinant DNA Techniques

Plasmid Isolation—Plasmid DNA was isolated from cultures using a QIAprep Spin Plasmid™ kit (QIAGEN Inc., Chatsworth, CA) and transformed into strains made competent using a standard calcium chloride method (32). Restriction endonucleases, T4 DNA ligase, and calf intestinal alkaline phosphatase were purchased from Promega (Madison, WI) and used in accordance with the manufacturer’s specifications. PCR products were isolated from Tris borate/EDTA-containing 1% agarose gels using the QIAquick™ gel extraction kit (QIAGEN Inc.).

DNA Sequencing—The complete DNA sequence of cobD was generated in part using the dye-deoxy method with the Sequenase® Version 2.0 kit (U. S. Biochemical Corp.) and by nonradioactive sequencing at the University of Wisconsin Biotechnology Center. The sequence presented is that of both strands being sequenced at least three times in their entirety. DNA sequence was analyzed using the software programs DNA Strider™, BLAST (33), MacTargsearch, and ProSite (34).

Subcloning of cobD—Two oligonucleotide primers were used to amplify the ~125 to +1148 region of cobD of pCOBC4 using PCR methodology. Each primer contained a 3′-region complementary to pCOBC4 followed by a 5′-noncomplementary end that generated either an EcoRI (primer 1) or a BamHI (primer 2) restriction endonuclease site. Amplification between the two primers was performed using Vent™ polymerase (New England Biolabs Inc., Beverly, MA) in a Temp-Tronic Thermocycler (Barnstead Thermolyne, Dubuque, IA). Reaction conditions were as follows: denaturation at 94 °C for 2 min, annealing at 50 °C for 1.5 min, and extension at 72 °C for 1 min. The primers used were as follows: primer 1 (5′-CCCAGATTCGCCGAGCCACGCAGCCAACA-TCGC-3′), which hybridized to the −125 to −100 region upstream of cobD and generated an EcoRI restriction endonuclease site; and primer 2 (5′-CCCCGATTCGAGAAAAGCCGATCTCCTGCAGCAG3′), which hybridized to the +1123 to +1148 region downstream of cobD and generated a BamHI restriction endonuclease site. These primers were used to generate a 1291-bp fragment, which was gel-purified, digested with EcoRI and BamHI, and ligated into the intermediate copy number vector pSU36 (35) to generate plasmid pCOBD2.

Overexpression and Visualization of CobD—Two oligonucleotide primers were used to amplify the ~16 to +1148 region of cobD of plasmid pCOBC4 by PCR. One primer (primer 1) contained a 3′-region complementary to plasmid pCOBC4 followed by a 5′-noncomplementary end that generated a BamHI restriction endonuclease site. The second primer (primer 2) was designed to introduce an Xdel restriction

** EXPERIMENTAL PROCEDURES **

** Bacterial Strains **

All strains used in this study were derivatives of *S. typhimurium* LT2, unless noted, and their genotypes are listed in Table I. *Tn106d(Cm)* refers to the transposition-defective element described by Elliott and Roth (22).

** Culture Media, Chemicals, and Growth Conditions **

The E minimal medium of Vogel and Bonner (23) was routinely supplemented with 11 mm glucose as the carbon/energy source. Difco Nutrient Broth™ (8 g/liter) with NaCl (5 g/liter) added was used as the rich medium. LB broth was used for experiments involving plasmid manipulation and for protein overexpression. Difco Granulated Agar™ (15 g/liter) was added for solid support. When present in the medium, (CN)2Cby and dicyanocobinamide were at 45 mM, and (R)-AP was at 1.3 mM. Concentrations of antibiotics were as described elsewhere (24). All chemicals were purchased from Sigma, except (CN)2Cby, which was a gift from F. Blanche (Rhône-Poulenc Rorer S. A., Vitry-sur-Seine Cedex, France); l- and dl-lactaldehydes were synthesized as described (25). Cell density of cultures was monitored with a Klett photoelectric colorimeter (Manostat Corp., New York, NY) or with a Spectronic 20D spectrophotometer (Milton Roy Co., Rochester, NY).
endonuclease site immediately 5' to the translation initiation site of cobD. Amplification between the two primers was performed using Vent®(exo−) polymerase (New England Biolabs Inc.). Reaction conditions were: denaturation at 94 °C for 2 min, annealing at 50 °C for 1.5 min, and extension at 72 °C for 1 min. The primers were as follows: primer 2 and primer 3 (5'-TTTTGGCTGGAGGCATAT-3'). These primers generated a 1173-bp fragment, which was purified, digested with BglII and BstXI, and ligated into the T7 overexpression vector pT7-7 (36). The resulting plasmid (pCLOBD6) and pT7-7 were transformed into Escherichia coli strain BL21/DE3, generating strains JE4094 and JE4096, respectively. These strains contained the T7 RNA polymerase in a λ-lysogen under control of an IPTG-inducible promoter.

CobD overexpression was performed as follows. A 0.1% (v/v) inoculum was added to LB broth containing ampicillin (100 μg/ml) and grown at 30 °C with shaking to ~70 Klett units. IPTG was added to 400 μM, and incubation was continued for ~12 h. A 100-μl sample of culture was pelleted, and 100 μl of 2 × sample buffer (37) was added and heated at 100 °C for 5 min. Cell-free extracts were cleared after spinning at 12,000 × g for 5 min in a Sorvall SS34 rotor RC5B low speed ultracentrifuge (DuPont).

Biochemical Techniques

In Vitro Assays for CobD Activity—Cell-free extracts used in the in vitro activity assays were obtained from 1-liter cultures grown in LB broth containing ampicillin (100 μg/ml). Cultures were shaken at 30 °C in 2-liter Erlenmeyer flasks. After induction, cells were pelleted, resuspended in 20 ml of 50 mM PIPES (pH 6.8), and broken by passing twice through a French pressure cell at 8058 kips/cals. Membrane and soluble fractions were separated by centrifugation at 40,000 × g for 1.5 h. The supernatant was dialyzed at 4 °C against 50 mM PIPES and 10 mM dithiothreitol (pH 6.8) and kept under N2 with 10 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride added. All reactions were performed in 1.5-ml Eppendorf tubes (Fisher).

Aminotransferase Activity Assays—Aminotransferase assay mixtures (100-μl final volume) contained 50 nmol of L- or D-lactaldehyde, 50 nmol of one of the 20 L-amino acids, 50 nmol of PLP, and 5 μmol of PIPES (pH 6.8).

Decarboxylase Activity Assays—Decarboxylase assay mixtures (100-μl final volume) contained 50 nmol of substrate (L- or D-threonine or L- or D-threonine O-3-phosphate), 50 nmol of PLP, and 5 μmol of PIPES (pH 6.8).

General Assay and Derivatization Conditions—Both aminotransferase and decarboxylase assays were started by the addition of ~100 μg of protein. The reaction mixtures were incubated at 37 °C for either 30 or 90 min as indicated. The reactions were terminated, and proteins were precipitated by incubation at 100 °C for 10 min. Precipitated proteins were removed by a 1-min centrifugation using a Marathon® 13KCM microcentrifuge (Fisher). Samples were filtered with a Spin-X® filter to remove debris. Primary amines in the mixture (5-μl sample) were derivatized, and 15 μl of this mixture (~1 nmol of substrate) was analyzed by HPLC as described above.

High Performance Liquid Chromatography—Thio-substituted isoin- doles were separated utilizing reverse-phase HPLC with a Prodigy™ 5 ODS-2 column (250 × 4.60 mm, 5 μm; Phenomenex Inc., Torrance, CA). pαA derivatives were resolved with a gradient from solvent A (1:19:80 tetrahydrofuran, methanol, and 50 mM sodium acetate (pH 5.9)) to solvent B (8:2 methanol and 50 mM sodium acetate (pH 5.9)) at a flow rate of 0.7 ml/min as follows: 100% solvent A for 5 min, a 5-min linear gradient to 50% solvent A and 50% solvent B, and a 25-min linear gradient to 100% solvent B, followed by a 5-min linear gradient to 100% solvent A. Under these conditions, derivatized standards of L-Thr-P, L-Thr, and (R)-Thr-AP eluted with retention times of 23, 28, and 35 min, respectively. Elution was monitored with a Waters 470 scanning fluo- rescence detector set at 330 nm (excitation), 418 nm (emission), 1.5 s (filter), 16 (attenuation), and ×100 (gain).

Alkaline Phosphatase Assays—Alkaline phosphatase treatments of cobD reaction mixtures were performed as follows. A 20-μl sample of the reaction mixture was diluted to 28 μl with alkaline phosphatase buffer (50 mM Tris-HCl (pH 9.5), 1 mM MgCl2, 100 μM ZnCl2, and 1 mM spermidine) containing 2 units of calf intestinal alkaline phosphatase. The mixture was incubated at 37 °C for 30 min. The reaction was terminated, and proteins were precipitated by incubation at 100 °C for 10 min and removed by centrifugation. The sample was then filtered with a Spin-X® filter to remove debris. Primary amines in the mixture (5-μl sample) were derivatized, and 15 μl of this mixture (~1 nmol of substrate) was analyzed by HPLC as described above. Alkaline phosphatase treatment was used as a standard for this procedure, which, after treatment and derivatization, eluted with a retention time of 28 min (same as L-Thr).

Protein Analysis—Proteins present in soluble cell-free extracts were resolved on 12% SDS-polyacrylamide gels using the Laemmli system (35 hexamer (TTGCGC) had only a 23 bp 5′ to the putative translation start site. The 23 nucleotide sequence of the cobD promoter was identified 23 bp 5′ to the putative translation start site. The 10 hexamer (TAAAAT) had a five out of six match with the consensus sequence, whereas the 35 hexamer (TTGGCC) had only a three out of six match. A putative ribosome-binding site sequence (Shine-Dalgarno sequence; GGAGG) was located 4 bp upstream of the initiation codon. This sequence analysis suggested that cobD and cobC were divergently transcribed and that only 1 bp separated the translation initiation codons of the two genes (Fig. 2). If the putative promoter and Shine-Dalgarno sequences of cobD were correct, they reside within the open

### Table I

| Strain | Genotype | Source |
|--------|----------|--------|
| *Salmonella* | | |
| LT2 | Wild type | D. Downs |
| TR6583 | metE205 ara-9 derivative of strain LT2 | K. Sanderson via J. Roth |
| JE2216 | metE205 ara-9 cobD::Tn10d(Cm) | |
| JE4097 | srl202::Tn10d(Tc)creA derivative of JE2216 | |
| **E. coli** | | |
| BL21/DE3 | hadS gal (Acta857 ind1 Sam7 nin5 lacUV5-T; gene 1) | Novagen |
| JE4094 | BL21/DE3/pCLOB6 | |
| JE4096 | BL21/DE3/pT7-7 | |
| **Plasmids** | | |
| pCLOBD4 | cobD′ · cobD′ · Ap′ · Cm′ · vector pBR328 | E. Martínez |
| pCDOB2 | cobD′ · Km′; vector pSU38 | S. Tabor |
| pCODB6 | cobD′ · Ap′; pT7-7 overexpression clone | |
| pSU38 | cloning vector, Km' | |
| pT7-7 | overexpression vector, Ap' | |

Novel Decarboxylase Encoded by the cobD gene of Salmonella

Unless otherwise stated, strains or plasmids were generated during the course of this work.

### RESULTS

Nucleotide Sequence of cobD and Predicted Amino Acid Sequence of CobD—Previously reported work from our laboratory identified 211 bp of the 5′-end of the cobD gene (41). We determined the remaining sequence of cobD using plasmid pCLOBD (cobC′ · cobD′). Analysis of the sequence revealed an open reading frame of 1095 bp with an OPAL (TGA) stop codon (Fig. 2). The sequence predicted a polypeptide of 364 amino acids with a molecular mass of 40,810 Da.

A putative promoter was identified using the MacTargsearch program. The −10 region of the cobD promoter was identified 23 bp 5′ to the putative translation start site. The −10 hexamer (TAAAAT) had a five out of six match with the consensus sequence, whereas the −35 hexamer (TTGGCC) had only a three out of six match. A putative ribosome-binding site sequence (Shine-Dalgarno sequence; GGAGG) was located 4 bp upstream of the initiation codon. This sequence analysis suggested that cobD and cobC were divergently transcribed and that only 1 bp separated the translation initiation codons of the two genes (Fig. 2). If the putative promoter and Shine-Dalgarno sequences of cobD were correct, they reside within the open

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The predicted CobD polypeptide contained a consensus PLP-binding motif (SLTKFYAIPGLRLG) and showed striking homology to the histidinol-phosphate transaminases (EC 2.6.1.9) and, to a lesser extent, to the aspartate transaminases (EC 2.6.1.1) and tyrosine transaminases (EC 2.6.1.5) of many organisms. CobD was most similar to HisH, the histidinol-phosphate transaminase from *Bacillus subtilis* (GenBank™ accession number M15409), sharing 28% identity and 68% similarity at the amino acid level. Additionally, CobD is 28% identical and 67% similar to the CobC protein of *P. denitrificans* (GenBank™ accession number M32223), thought to be involved in (R)-AP synthesis (11).

**Subcloning of cobD**—Two oligonucleotide primers were used to amplify the cobD region of pCOBC4 using PCR methodology. Primers 1 and 2 contained a 5'-region complementary to pCOBC4 and a 3'-noncomplementary end that generated either an *Eco*RI or a *Bam*HI restriction enzyme site. These primers were used to amplify the 2125 to 11148 region of pCOBC4. This fragment contained the entire cobD gene plus some flanking sequences. The 1291-bp fragment was digested with *Eco*RI and *Bam*HI and ligated into the intermediate copy number vector pSU38 to generate pCOBD2. This plasmid complemented CobD function, i.e., restored cobalamin biosynthesis from (CN)2Cby without the addition of (R)-AP in cobD mutants. CobD was resequenced from pCOBD2 to ensure that no mutations were introduced during amplification.

**Overexpression of CobD**—Two oligonucleotide primers (primers 2 and 3) were used to amplify the cobD region of pCOBC4 using PCR. Primer 2 contained a 3'-region complementary to pCOBC4 and a 5'-noncomplementary end that generated a *Nde*I restriction site immediately 5' to the translation start site of cobD. The *Nde*I restriction site was required to align cobD with the efficient ribosome-binding site of the T7 overexpression vector pT7-7. Primers 2 and 3 were used to amplify the 216 to 11148 region of cobD. The 1173-bp fragment generated was digested with *Nde*I and *Bam*HI and ligated into pT7-7 digested with the same enzymes to generate plasmid pCOBD6. CobD was resequenced from pCOBD6 to ensure that no mutations were introduced during amplification. Plasmid pCOBD6 and the control overexpression vector (pT7-7) were transformed into *E. coli* strain BL21/DE3 to generate strains JE4094 (pCOBD6) and JE4096 (pT7-7), which were used in overexpression experiments. Following induction, proteins in the crude cell-free extracts were resolved by SDS-polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining. As shown in Fig. 3, cell-free extracts of strain JE4094 grown in the presence of IPTG (lane B) contained an extra protein band of 40 kDa compared with the strain containing the expression vector (pT7-7) lacking cobD (lane D). Cell-free extracts of strain JE4094 grown without IPTG (lane A) contained drastically reduced amounts of the 40-kDa protein. The molecular mass of the overexpressed protein correlated well with the predicted molecular mass of 40.8 kDa for CobD.

CobD Does Not Have Lactaldehyde Aminotransferase Activity—Computer analysis of cobD sequence data suggested CobD using PCR. Primer 2 contained a 3'-region complementary to pCOBC4 and a 5'-noncomplementary end that generated a *Bam*HI restriction enzyme site. Primer 3 was designed to introduce an *Nde*I restriction site immediately 5' to the translation start site of cobD. The *Nde*I restriction site was required to align cobD with the efficient ribosome-binding site of the T7 overexpression vector pT7-7. Primers 2 and 3 were used to amplify the 216 to 11148 region of cobD. The 1173-bp fragment generated was digested with *Nde*I and *Bam*HI and ligated into pT7-7 digested with the same enzymes to generate plasmid pCOBD6. CobD was resequenced from pCOBD6 to ensure that no mutations were introduced during amplification. Plasmid pCOBD6 and the control overexpression vector (pT7-7) were transformed into *E. coli* strain BL21/DE3 to generate strains JE4094 (pCOBD6) and JE4096 (pT7-7), which were used in overexpression experiments. Following induction, proteins in the crude cell-free extracts were resolved by SDS-polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining. As shown in Fig. 3, cell-free extracts of strain JE4094 grown in the presence of IPTG (lane B) contained an extra protein band of 40 kDa compared with the strain containing the expression vector (pT7-7) lacking cobD (lane D). Cell-free extracts of strain JE4094 grown without IPTG (lane A) contained drastically reduced amounts of the 40-kDa protein. The molecular mass of the overexpressed protein correlated well with the predicted molecular mass of 40.8 kDa for CobD.

Novel Decarboxylase Encoded by the cobD gene of Salmonella
Novel Decarboxylase Encoded by the cobD gene of Salmonella

Role of CobD in Cobalamin Biosynthesis in S. typhimurium—CobD is the first enzyme reported to be able to decarboxylate l-threonine O-3-phosphate to yield (R)-1-amino-2-propanol O-2-phosphate. In vitro data indicate that the CobD enzyme has stereospecificity for L-Thr-P and is unable to decarboxylate the d-isomer of this compound. Although it was not investigated in this work, the existence of a consensus PLP-binding motif within cobD makes it likely that this is a member of the PLP-dependent decarboxylases. The fact that the phosphate group in the substrate is retained in the product raises important mechanistic questions that will be best addressed when homogeneous protein becomes available.

The meaning of the homology of CobD to class I PLP-dependent aminotransferases and the lack of homology of this enzyme to other PLP-dependent decarboxylases suggests that CobD may be a member of a new family of decarboxylases that evolved from a common ancestor of class I PLP-dependent aminotransferase enzymes.

Understanding the Role of CobD Changes Our View of the de Novo Corrin Ring Biosynthetic Pathway—On the basis of our data, we propose the model shown in Fig. 6 for the last step of de novo corrin ring biosynthesis in S. typhimurium, a reaction currently thought to be catalyzed by the ChBI protein. In this model, l-Thr is phosphorylated by an uncharacterized kinase to yield l-Thr-P, which is decarboxylated by CobD to yield (R)-1-
amino-2-propanol-O-2-phosphate. The latter is proposed to be the true cosubstrate for the CbiB enzyme. Thus, we propose that the end product of the de novo pathway for corrin ring biosynthesis is AdoCbi-P, not AdoCbi as previously thought.

There is evidence that several proteins, including one homologous to CobD, may be involved in the attachment of (R)-AP in _P. denitrificans_. It is interesting that Rémy et al. (42) found an unexpectedly high $K_m$ ($\approx 20$ m\text{M}) for (R)-AP in an in vitro system that generated AdoCbi from (R)-AP and 5'-deoxyadenosylcobyric acid. This high $K_m$ for (R)-AP suggests that the correct substrate for this reaction may be (R)-1-amino-2-propanol-O-2-phosphate in this organism as well.

Timing of Decarboxylation—It is not clear what the timing of the decarboxylation is. It is possible that L-Thr-P is the substrate for CbiB and that carboxylated AdoCbi-P is the substrate for CobD. At this point, no evidence is available to rule out this possibility, although we feel that it is an unlikely scenario given the putative involvement of PLP in the reaction. If the mechanism of catalysis involves the formation of an imine (Schiff base) between PLP and the $\alpha$-amino group of L-Thr-P prior to decarboxylation, such a bond would not be possible if the $\alpha$-amino group were derivatizing the propionyl substituent of ring D of the macrocycle (Fig. 1). If, however, decarboxylation occurs after amidation of the propionyl substituent, then the interaction of PLP with the secondary amine at C-$\alpha$ would yield an enamine. Further insights into this problem require the biochemical analysis of the CbiB protein.

**Explanations for the Observed Phenotypes of cobD Mutants**—Since adenosylcobalamin biosynthesis in cobD mutants is restored by exogenously supplied (R)-AP (21), it is assumed that this compound must be phosphorylated before it can be used as substrate by the CbiB enzyme (Fig. 6). Alternatively, CbiB may be able to catalyze the synthesis of AdoCbi from 5'-deoxyadenosylcobyric acid if an excess of unphosphorylated (R)-AP is available. If CbiB cannot use (R)-AP as substrate, we predict the existence of a kinase enzyme to convert (R)-AP into (R)-1-amino-2-propanol-O-2-phosphate (Fig. 6). Again, insights into this problem must await the biochemical analysis of the CbiB protein.
The role of the CbiB enzyme has not been documented in *S. typhimurium*. Phosphorylation of t-Thr and (R)-AP could be performed by the same kinase. Kinase activities are hypothetical.

**FIG. 6. Role of CobD in the conversion of 5′-deoxyadenosylcobyric acid to AdoCbi-P.** The role of the CbiB enzyme has not been documented in *S. typhimurium*. Phosphorylation of t-Thr and (R)-AP could be performed by the same kinase. Kinase activities are hypothetical.

**FIG. 7. E. coli sequence homology to the S. typhimurium cobC/cobD region.** Shown is a representation of the cobC region of *S. typhimurium* (*S.t.*) compared with the *E. coli* (*E.c.*) cobC homolog phpB. *phpB* was 68% identical at the nucleotide level to the final 612 bp of *cobC*. The remaining 93 bp of the 5′-end of *cobC* showed no identity to the region upstream of *phpB*. There was no identity to all but the extreme 3′-end of *cobD*. A, the 155-bp region immediately 3′ to *cobC* shared 79% identity with the region 3′ to *phpB*. B, *phpB* was 68% identical to the 3′-end of *cobC*. *phpB* was 68% identical and 85% similar to this region of *CobC* at the protein level. C, the first 93 bp of *cobC* did not share homology with the *phpB* region of *E. coli*. D, the last 25 bp of *cobD* and 103 bp 3′ to *cobD* shared 74% identity with the 3′-end of *orfUU*, † this 22-bp region between *phpB* and *orfUU* was not homologous to any portion of the cobC/cobD region; ‡ these two regions are 53-bp inverted repeats. The repeat 3′ to *cobC* was not homologous to *E. coli* sequence 3′ to *phpB*.

**Implications of the Synthesis of AdoCbi-P on Our View of the Late Steps of the Cobalamin Biosynthetic Pathway (i.e. the Nucleotide Loop Assembly Pathway)**—Our proposal that *AdoCbi-P* is the end product of corrin ring biosynthesis raises an important question regarding the role of the kinase activity of the *CobU* enzyme in the synthesis of *AdoCbi-GDP* from *AdoCbi* via an *AdoCbi-P* intermediate (43). To reconcile the results presented herein with the documented kinase activity of *CobU*, we hypothesize that under anaerobic growth conditions (e.g. in the gut) when *S. typhimurium* can synthesize the corrin ring *de novo*, the kinase activity of *CobU* is not required for the assembly of the nucleotide loop because the product of the *CbiB* reaction is *AdoCbi-P*. However, the kinase activity of *CobU* would become relevant under aerobic growth conditions (e.g. free-living) when unphosphorylated *cobinamide* may be present in the environment. A prediction from this hypothesis is that mutant *CobU* enzymes lacking only kinase activity should display a nucleotide loop assembly phenotype under aerobic growth conditions, but should be unable to synthesize adenosylcobalamin *de novo* under anaerobic growth conditions.

It is possible, however, that (R)-1-amino-2-propanol O-2-phosphate may be dephosphorylated before, during, or after attachment to 5′-deoxyadenosylcobyric acid. In this scenario, the end product would be *AdoCbi*, and the kinase activity of *CobU* would be required for synthesis of the nucleotide loop regardless of growth conditions. These ideas are currently under investigation.

**Implications of the Organization of the cobD and cobC Genes—**Analysis of nucleotide sequence data raises important questions regarding regulation of expression of *cobD* and its neighbor, *cobC*. The fact that *cobD* is separated by only 1 base pair from the neighboring, divergently transcribed *cobC* gene places the putative regulatory region for *cobD* within the *cobC* coding sequence. Similarly, the putative *cobC* promoter appears to be located within the *cobD* coding sequence. The effect of this organization on the transcription of both genes is unclear and is currently under investigation. If this organization were correct, we predict that insertions of transposable elements proximal to the 5′-end of either *cobD* or *cobC* should affect both gene functions, resulting in strains displaying additional phenotypes to those expected for *cobC* or *cobD* mutants with lesions outside the overlapping regions.

**Comparison of the cobC/cobD Region of *S. typhimurium* with *E. coli*—**The *E. coli* *phpB* gene (GenBank™ accession number U23163) showed homology to *cobC*. When comparing the two genes, we found that *cobC* contained an additional 93 bp at the 5′-end. Over the region that *phpB* shares homology with *cobC*,
they were 68% identical (Fig. 7). At the protein level, they were 68% identical and 85% similar. Although E. coli has homologs to many S. typhimurium genes involved in the later steps of Cbl biosynthesis, we did not identify a homolog to cobD. Since cobC and cobD are adjacent in S. typhimurium, we analyzed the phpB region of E. coli further (GenBank™ accession number AE000168 U00096) (44). We found that E. coli contained regions homologous to sequences 3’ to both cobC and cobD. Interestingly, the last 25 bp of cobD and 103 bp immediately 3’ to cobD shared 74% sequence identity with the 3’-end of the hypothetical orfUU gene of E. coli. The function of OrfUU is not known, but appears to be essential for growth. We do not have additional sequence 3’ to cobD to determine whether S. typhimurium possesses a homolog to orfUU.

It appears as though the 93 bp of the 5’-end of cobC and the 1070 bp 5’ of cobD (1164 bp total) were either gained by S. typhimurium or deleted from E. coli. In E. coli, phpB and orfUU are separated by an intergenic region of only 22 bp that are repeated. Because this repeat spans all the sequence 3’, these regions spanned from bp 983 to 1

Further analysis of the S. typhimurium cobC/cobD region identified two 53-bp regions of identical inverted repeats. One of these regions spanned from bp +883 to +935 of the cobD sequence. The second region was located 158–210 bp 3’ to cobC. This second region is 3’ to a region with 79% identity to E. coli; however, the homology stopped abruptly at the beginning of the repeat. Because this repeat spans all the sequence 3’ to cobC that we have so far obtained, the size of the repeat may be larger than 53 bp. The evolutionary significance of these inverted repeats is unclear and is currently under investigation.

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*J. Biol. Chem.* 1998, 273:2684-2691.
doi: 10.1074/jbc.273.5.2684

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