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 CblB enzyme proposed to be responsible for the conversion of adenosylcobyric acid to adenosylcobinamide and that the product of the reaction is adenosylcobinamide phosphate, not adenosylcobinamide 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 adenosylcobinamide to adenosylcobinamide phosphate. These findings shed light on the strategy used by this bacterium for the assimilation of exogenous unphosphorylated cobinamide 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 respiring *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)-AP3 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 diaquocobyric acid was present in a reaction mixture containing Tris-Cl buffer and a reductant (e.g. glutathione, β-mercaptoethanol, or borohydride) (15, 16). This work led to a model for interactions between diaquocobyric 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 aminoacetonitrile, which is reduced to (R)-AP by (R)-1-aminopropan-2-ol:NAD+1.

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* The abbreviations used are: (R)-AP, (R)-1-amino-2-propanol; Cbl, cobalamin; AdoCbl-P, 5′-deoxyadenosylcobinamide phosphate; AdoCbl, 5′-deoxyadenosylcobinamide; (CN)2Cby, dicyanocobyric acid; PCR, polynucleotide chain reaction; bp, base pair(s); IPTG, isopropyl-β-D-thiogalactopyranoside; PIPES, 1,4-piperazineethanesulfonic acid; PLP, pyridoxal phosphate; HPLC, high performance liquid chromatography; oPA, o-phthalaldehyde; Thr-P, threonine 0-phosphate.

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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 synthase, 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 synthase, 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 crossovers were performed using the high frequency transducing bacteriophage P22 mutant HT 105/1 int-201 (29, 30). Transductants were purified and identified as phage-free as described (31).

Complementation Studies—Both plasmids pCOBD2 and pCOBD6 and the corresponding vector-only controls pSU38 and pT7-7 were transformed into cobD mutant strain JE2216 and its recombination-deficient derivative JE4097. Transformants were tested for their ability to grow aerobicly 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 treatment (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. Plasmid 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 dideoxy 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 the result 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).

Subeloning 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’-CCGGATTCCTGGCCATGCCAAGCAGCACAACCTCGC-3’), which hybridized to the +125 to +110 region upstream of cobD and generated an EcoRI restriction endonuclease site; and primer 2 (5’-CCGGATCCGGAGAAAGCGGAATCTCAGGGGAG-3’), 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 pSU38 (35) to generate plasmid pCOBD2.

Overexpression and Visualization of CobD—Two oligonucleotide primers were used to amplify the 5’ to +1148 region of cobD of plasmid pCOBC4 by PCR. One primer (primer 2) 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 3) was designed to introduce an XhoI restriction site.
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 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. For the primers used as controls: primer 2 and primer 3 (5′-TGTGGCGTGAGGATAT- GCCCTTTATACACCG-3′), which hybridized to the −16 to +19 region of cobD and generated an Ndel restriction endonuclease site. These primers generated a 1173-bp fragment, which was purified, digested with NdeI and BamHI, and ligated into the T7 overexpression vector pT7-7 (36). The resulting plasmid (pCOBD6) and pT7-7 were transformed into Escherichia coli strain BL21/ADE3, 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) 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).

High Performance Liquid Chromatography—Thio-substituted isoindoles were separated utilizing reverse-phase HPLC with a Prodigy™ 5 ODS-2 column (250 × 4.60 mm, 5 μm; Phenomenex Inc., Torrance, CA). oPA 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 t-Thr-P, l-Thr, and (R)-3-Acetylthreonine were eluted at 23, 28, and 35 min, respectively. Elution was monitored with a Waters 470 scanning fluorescence 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 9.3), 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.

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 pCOBC4 (cobC′-cobD′). Analysis of the sequence revealed an open reading frame of 1095 bp with a TGG/CTG 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 (TATAAA) had a five out of six match with the consensus sequence, whereas the −35 hexamer (TTGCGG) had only a three out of six match. A putative ribosome-binding site sequence (Shine-Dalgorno 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 reading frame.
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 \textit{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 \textit{P. denitrificans} (GenBank™ accession number M32223), thought to be involved in (R)-AP synthesis (11).

Subcloning of \textit{cobD}—Two oligonucleotide primers were used to amplify the \textit{cobD} region of pCOBC4 using PCR methodology. Primers 1 and 2 contained a 3'-region complementary to pCOBC4 and a 5'-noncomplementary end that generated either an \textit{EcoRI} or a \textit{BamHI} restriction enzyme site. These primers were used to amplify the 2125 to 11148 region of pCOBC4. This fragment contained the entire \textit{cobD} gene plus some flanking sequences. The 1291-bp fragment was digested with \textit{EcoRI} and \textit{BamHI} and ligated into the intermediate copy number vector pSU38 to generate pCOBD2. This plasmid complemented \textit{CobD} function, i.e. restored cobalamin biosynthesis from (CN)$_2$Cby without the addition of (R)-AP in \textit{cobD} mutants. \textit{cobD} was resequenced from pCOBD2 to ensure that no mutations were introduced during amplification.

Overexpression of \textit{CobD}—Two oligonucleotide primers (primers 2 and 3) were used to amplify the \textit{cobD} region of pCOBC4 using PCR. Primer 2 contained a 3'-region complementary to pCOBC4 and a 5'-noncomplementary end that generated a \textit{BamHI} restriction enzyme site. Primer 3 was designed to introduce an \textit{NdeI} restriction site immediately 5'- to the translation start site of \textit{cobD}. The \textit{NdeI} restriction site was required to align \textit{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 \textit{cobD}. The 1173-bp fragment generated was digested with \textit{NdeI} and \textit{BamHI} and ligated into pT7-7 digested with the same enzymes to generate plasmid pCOBD6. \textit{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 \textit{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 \textit{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 \textit{cobD} sequence data suggested CobD using PCR. Primer 2 contained a 3'-region complementary to pCOBC4 and a 5'-noncomplementary end that generated a \textit{BamHI} restriction enzyme site. Primer 3 was designed to introduce an \textit{NdeI} restriction site immediately 5'- to the translation start site of \textit{cobD}. The \textit{NdeI} restriction site was required to align \textit{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 pCOBC4. This fragment contained the entire \textit{cobD} gene plus some flanking sequences. The 1291-bp fragment was digested with \textit{EcoRI} and \textit{BamHI} and ligated into the intermediate copy number vector pSU38 to generate pCOBD2. This plasmid complemented \textit{CobD} function, i.e. restored cobalamin biosynthesis from (CN)$_2$Cby without the addition of (R)-AP in \textit{cobD} mutants. \textit{cobD} was resequenced from pCOBD2 to ensure that no mutations were introduced during amplification. Plasmid pCOBD6 and the control overexpression vector (pT7-7) were transformed into \textit{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 \textit{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

CobD Has L-Threonine-O-3-phosphate Decarboxylase Activity—To investigate whether cobD encoded ω-threonine-O-3-phosphate decarboxylase, activity assays were performed on crude extracts of JE4094 (pCOBD6) and JE4096 (pT7-7) after induction of T7 RNA polymerase. The assays were performed by providing ω-Thr-P as substrate. After incubation with CobD-enriched cell-free extract, primary amine-containing compounds were analyzed by reverse-phase HPLC after derivatezation with oPA as described above.

Assay mixtures containing cell-free extract of strain JE4094 were found to contain DL-Thr-P (elution time, 23 min) and one additional compound (elution time, 30 min) that was absent from mixtures containing cell-free extract of the control strain JE4096 (data not shown). Extended incubation (1.5 h) resulted in roughly equivalent amounts of substrate and product, suggesting that only one stereoisomer of DL-Thr-P was converted to product.

To determine which stereoisomer was the substrate for CobD, assays were performed with L-threonine O-3-phosphate, whose stereochemistry at the β-carbon is the same as that of (R)-AP. Assay mixtures containing cell-free extract of strain JE4094 (pCOBD6) converted more than half of L-Thr-P to product (elution time, 30 min) after a 30-min incubation (Fig. 4B). Extended incubation (1.5 h) resulted in complete conversion to product (Fig. 4C), indicating that L-Thr-P was the substrate for CobD. Control assay mixtures containing cell-free extract of strain JE4096 (pT7-7) did not contain detectable levels of product (Fig. 4A).

The Product of the CobD-catalyzed Reaction Is (R)-1-Amino-2-propanol O-2-phosphate—The PLP-dependent decarboxylation of L-threonine O-3-phosphate predicted the formation of either (R)-1-amino-2-propanol or (R)-1-amino-2-propanol O-2-phosphate depending on the reaction mechanism. The product of the reaction had a different elution time (30 min) compared with authentic (R)-AP (35 min), suggesting that the phosphate group was retained in the product. To test for the presence of the phosphate in the product, reaction mixtures were subjected to treatment with alkaline phosphatase. As shown in Fig. 5, this treatment changed the elution time of the product from 30 to 35 min, which corresponded with that of authentic (R)-AP. Alkaline phosphatase treatment of reaction mixtures incubated for only 30 min (i.e. incomplete) contained an additional compound whose elution time (28 min) was in good agreement with that of authentic L-Thr (data not shown).

DISCUSSION

We discuss below several important contributions to the field of cobalamin biosynthesis made by the work presented in this paper.

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 CbiB 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-

Fig. 3. Overexpression of cobD. Shown is a Coomassie Blue-stained 12% SDS-polyacrylamide gel used to analyze proteins in cell-free extracts of strains carrying phage T7 expression vectors. Phage T7 RNA polymerase was provided by an IPTG-inducible promoter. Lane A, strain JE4094 (pCOBD6 cobD<sup⁺)</sup>) not induced with IPTG; lane B, strain JE4094 induced with IPTG (a protein band with an apparent molecular mass of ∼40 kDa is labeled as CobD); lane C, strain JE4096 (pT7-7 lacking cobD) not induced with IPTG; lane D, strain JE4096 induced with IPTG. Numbers on the right represent the molecular masses of the following proteins (top to bottom): β-phosphorylase (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), and lysozyme (14 kDa).
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 $K_m$ (20 mM) 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-5 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 CobU enzyme in the synthesis of AdoCbi from CobU, we hypothesize that under anaerobic growth conditions, the kinase activity of CobU is not required for the assembly of the nucleotide loop because the product of the CobU enzyme in the synthesis of AdoCbi-GDP from AdoCbi via an AdoCbi-P intermediate (43). To reconcile the 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 adjacent in S. typhimurium.

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 cobD 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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