Reassessment of the Late Steps of Coenzyme B₁₂ Synthesis in *Salmonella enterica*: Evidence that Dephosphorylation of Adenosylcobalamin-5′-Phosphate by the CobC Phosphatase Is the Last Step of the Pathway

Carmen L. Zayas and Jorge C. Escalante-Semerena*

*Department of Bacteriology, University of Wisconsin—Madison, Madison, Wisconsin

Received 27 October 2006/Accepted 22 December 2006

We report that *cobC* strains of *Salmonella enterica* serovar Typhimurium are impaired in the ability to salvage cobyrinic acid (Cby), a de novo corrin ring biosynthetic intermediate, under aerobic growth conditions. In vivo and in vitro evidence support the conclusion that this new phenotype of *cobC* strains is due to the inability of serovar Typhimurium to dephosphorylate adenosylcobalamin-5′-phosphate (AdoCbl-5′-P), the product of the condensation of α-ribazole-5′-phosphate (α-RP) and adenosylcobinamide-GDP by the AdoCbl-5′-P synthase (CobS, EC 2.7.8.26) enzyme. Increased flux through the 5,6-dimethylbenzimidazole and cobinamide (Cbi) activation branches of the nucleotide loop assembly pathway in *cobC* strains restored AdoCbl-5′-P synthesis from Cby in a *cobC* strain. The rate of the CobS-catalyzed reaction was at least 2 orders of magnitude higher with α-RP than with α-ribazole as substrate. On the basis of the data reported herein, we conclude that removal of the phosphoryl group from AdoCbl-5′-P is the last step in AdoCbl biosynthesis in serovar Typhimurium and that the reaction is catalyzed by the AdoCbl-5′-P phosphatase (CobC) enzyme. Explanations for the correction of the Cby salvaging phenotype are discussed.

De novo synthesis of coenzyme B₁₂ (adenosylcobalamin [AdoCbl]) is a process at present in prokaryotes that requires at least 25 gene functions (4, 8, 9, 26, 43, 44, 47, 51, 57, 59–61). *Salmonella enterica* synthesizes coenzyme B₁₂ de novo under anaerobic conditions but salvages incomplete corrinoids (e.g., cobinamide [Cbi], cobyric acid [Cby]) from its environment under aerobic and anaerobic conditions (30, 61). Cbi and Cby are transported into the cell via the corrinoid-specific transport system comprised of the outer membrane protein BtuB (28), the periplasmic corrinoid Shuttle protein BtuF (11, 55), and the inner membrane proteins BtuCD (6, 20, 42). Once inside the cell, Cbi and Cby are adenosylated by the ATP:co(I)rinoid adenosyltransferase (CobA, EC 2.5.1.17) (10, 24, 48) enzyme, yielding AdoCbi or AdoCby, which enter the biosynthetic pathway at different points (Fig. 1).

Adenosylcobinamide (AdoCbi) is converted to AdoCbi-GDP in two steps catalyzed by the CobU enzyme (NTP: AdoCbi kinase, EC 2.7.7.62; GTP:AdoCbi-P guanylyltransferase, EC 2.7.1.156). AdoCby follows a different route. It is converted by the AdoCbi-P synthase (CbiB) enzyme to AdoCbi-P, which is converted by CobU to AdoCbi-GDP. A second set of reactions activates the lower ligand base, which in *S. enterica* serovar Typhimurium can be either 5,6-dimethylbenzimidazole (DMB) under aerobic conditions (31, 32) or adenine or 2-methyladenine under anaerobic conditions (33). In our current understanding of these reactions, the first step in lower ligand activation is catalyzed by a unique phosphoribosyltransferase enzyme (CobT in serovar Typhimurium; EC 2.7.8.26) (16–18, 52, 53), while the second step is thought to be catalyzed by a phosphatase enzyme (CobC in serovar Typhimurium; EC 3.1.3.73) (40) (Fig. 1). The CobT enzyme can use NaMN or NAD⁺ as substrate. When NAD⁺ and DMB are the substrates, the product of the CobT reaction is α-5,6-dimethylbenzimidazole adenine dinucleotide (α-DAD) (34), which is postulated to be cleaved by an uncharacterized hydrolase (Fig. 1). When DMB and NaMN are the substrates, the product of the CobT reaction is α-DMB-ribose-5′-phosphate (α-ribazole-P [α-RP]) (52), which is dephosphorylated to α-ribazole (α-R) by the phosphatase activity of CobC (40). The CobC enzyme was previously reported to also dephosphorylate AdoCbl-P in vitro, but the physiological meaning of this activity remained unclear (35).

According to our current understanding of the late steps of AdoCbl biosynthesis in serovar Typhimurium, the AdoCbl-5′-P synthase (CobS) enzyme is an integral membrane protein that catalyzes the condensation of AdoCbi-GDP and α-R to yield AdoCbl (Fig. 1) (35). However, the idea that α-R was the physiological substrate for CobS was first brought into question by our previous in vitro studies of the CobS enzyme, which showed that α-RP was also a substrate for the enzyme (35). In addition, earlier work with *Pseudomonas denitrificans* indicated that CobV (the CobS homolog in *P. denitrificans*) had higher affinity for α-RP than for α-R (K₉₀₀-RP, 2.7 μM; K₉₀₀-R, 7.8 μM) (13), suggesting that α-RP might be the preferred substrate for the enzyme in both bacteria. This idea was also consistent with results from earlier in vitro work with *Propionibacterium shermanii*, which suggested that Cbl-5′-phosphate was the last intermediate of the pathway (25). However, all of the above-mentioned studies were performed in vitro, and in vivo data supporting the conclusions have not been reported.

---

* Corresponding author. Mailing address: Department of Bacteriology, University of Wisconsin, 144A Enzyme Institute, 1710 University Avenue, Madison, WI 53726-4087. Phone: (608) 262-7379. Fax: (608) 265-7909. E-mail: escalante@bact.wisc.edu.

† Published ahead of print on 5 January 2007.
We took genetic and biochemical approaches to further investigating the timing of phosphate removal in serovar Typhimurium during the assembly of the nucleotide loop. This analysis was aided by the uncovering of a new phenotype of serovar Typhimurium cobC strains, which we argue provides the first in vivo support for the preference of the AdoCbl-5'-P synthase (CobS) enzyme for 5'-RP. We conclude that 5'-RP is the physiological substrate for CobS, that CobC catalyzes the last step of the AdoCbl biosynthetic pathway, and that removal of the 5'-O-P from AdoCbl-5'-P by CobC is the last step of the pathway.

**FIG. 1.** Late steps in corrin ring biosynthesis in serovar Typhimurium. When this work was initiated, this was the model of the enzymatic reactions needed for the last step in de novo corrin ring synthesis and nucleotide loop assembly in serovar Typhimurium. Shown are the enzymes, substrates, and intermediates of the pathway. Abbreviations: Cby, cobyric acid; AdoCby, adenosylcobyric acid; Cbi, cobinamide; AdoCbi, adenosylcobinamide; AdoCbi-P, adenosylcobinamide phosphate; AdoCbi-GDP, adenosylcobinamide GDP; AdoCbl, adenosylcobalamin; l-Thr, l-threonine; l-Thr-P, l-threonine phosphate; AP, aminopropanol; AP-P, aminopropanol phosphate; α-RP, α-ribazole-5'-phosphate; α-R, α-ribazole; DMB, 5,6-dimethylbenzimidazole; α-DAD, 5,6-dimethylbenzimidazole adenine dinucleotide; Nm, nicotinamide. CobC, α-ribazole-phosphate phosphatase; CobD, l-Thr O3-phosphate decarboxylase. The boxed reaction, proposed to be catalyzed by the CobC enzyme, came into question during the course of this work. The black box represents the corrinoid transport system (BtuBFCD) (20, 29, 55).

**MATERIALS AND METHODS**

**Bacterial strains and culture media.** Strains and plasmids used in this work are described in Table 1. All serovar Typhimurium strains used in these studies carry a null allele of the metE gene. The metE mutation inactivates the Cbl-independent methionine synthase enzyme (41), making growth of the cell dependent on the activity of the Cbl-dependent methionine synthase (MetH) enzyme (21, 27, 50). No-carbon E (NCE) medium (3, 56) was used to grow cells under chemically defined conditions. When added to the medium, the following supplements were present at the indicated concentrations: glucose, 11 mM; glycerol, 22 mM; MgSO₄, 1 mM; corrinoids (Cby, Cbi, and Cbl), 10 mM; l-threonine (l-Thr) and l-Thr-phosphate (l-Thr-P), 0.15 mM; 1-amino-2-propanol (AP), 10 mM; 5,6-dimethylbenzimidazole (DMB), 0.3 mM; Wolfe’s trace minerals (10 ml/liter) (1,
2. All corrinoids were added in their cyano form. Chemicals used in this work were commercially available, high-purity compounds. Dicyanocobamin [(CN)2Chl], cyanocobalamin (CNChl), tetracyanocobamide (CNcby), and DMB were purchased from Aldrich, and cyanocobalamin (CNCbl), L-Thr, L-Thr-P, and AP-P were purchased from Sigma. DMB was purchased from Aldrich, and cyanocobalamin (CNCby) was used as rich medium to culture serovar Typhimurium strains.

To full density (2 × 10^8 CFU/ml) of strain JE2718 (2), suspensions were passed through a 0.45-μm syringe filter (Nalgene). Corrinoids were purified from the sample with an Alltech 900-mg C8 Maxi-Clean cartridge (Alltech) equilibrated with double-distilled water (ddH2O). The cartridge was washed with 5 ml of ddH2O and eluted with 2 ml of methanol. Eluted samples were dried under vacuum as described above, suspended in 250 μl of ddH2O, and stored at 4°C until being used.

**RP-HPLC.** Corrinoids present in the samples were converted to their cyano form by the addition of KCN (1 mM; final reaction volume, 150 μl), followed by irradiation with a 60-W incandescent light at a distance of 6 cm on ice for 15 min. Samples were filtered with Spin-X centrifuge filters (Corning). An efficient reverse-phase high-performance liquid chromatography (RP-HPLC) method for the resolution of cyanocorrinoids has been reported (5). We used a Waters HPLC system equipped with an Alltima (Alltech) HP C8 5 μm column (150 by 4.6 mm) equilibrated and developed at a flow rate of 1 ml min^-1. The column was equilibrated with a 70% A-30% B buffer system (see below). A 25-min linear gradient was applied until the composition of the buffer system was 80% A-70% B. The solvents used were as follows: buffer A, 150 mM potassium phosphate buffer (pH 8.0) containing 10 mM KCN; buffer B, 100% methanol. Corrinoid elution from the column was detected with a Waters photodiode array detector. Authentic CNCby and CNChl were used as standards.

**Mass spectrometry.** HPLC-purified compounds were dried under vacuum, suspended in 1 ml of ddH2O, loaded onto an Alltech C8 Maxi-Clean cartridge, and processed as described above; corrinoids were eluted with 1 ml of 100% methanol. Samples were dried under vacuum. Dried samples were suspended in 50 μl of ddH2O and filtered on 0.22-μm Spin-X columns (Corning). Mass spectrometry analysis of the material was performed at the Mass Spectrometry Facility of the University of Wisconsin—Madison Biotechnology Center. Mass spectra were obtained with an MDS Sciex 4000 matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometer.

**Biochemical procedures. (i) In vitro i-Thr kinase activity assay.** We used radioiodinated i-Thr to increase the sensitivity of the assay used to investigate whether CobC had i-Thr kinase activity. We resolved reagents from products by thin-layer chromatography (TLC) on polyethyleneimine (PEI) plates developed with a 95% (vol/vol) ethanol:2.5 M ammonium acetate (1:1) mobile phase. Visualization of the results was achieved with a Packard Cyclone storage phosphor imaging system (Packard). Reaction mixtures contained [35S]C-1-Thr (6.55 mCi/mmol; 50, 150, 250, and 750 nmol) and a number of potential phosphate donors, including ATP and GTP (10, 20, and 50 nmol); α-PR (20 nmol); α-DAD (5 and 20 nmol); and sodium pyrophosphate (10 mM). We also tested the effects of trace

---

**TABLE 1. Strains and plasmids**

| Strain or plasmid | Relevant genotype | Reference or source |
|-------------------|------------------|---------------------|
| **S. enterica strains** | metE205 ara-9 | K. Sanderson via J. Roth |
| **TR6583 (formerly SA2929)** | Laboratory collection |
| **Derivatives of TR6583** | Laboratory collection |
| JE2216 | cobD1302 :: Tn10d (cat^R)^d | Laboratory collection |
| JE2718 | ΔcobC1151 | Laboratory collection |
| JE2719 | cobD1302 :: Tn10d (cat^R) | Laboratory collection |
| JE7489 | ΔcobC1151/pJO46 | Laboratory collection |
| JE7488 | ΔcobC1151/pT7-7 | Laboratory collection |
| JE7490 | ΔcobC1151/pT7-5 | Laboratory collection |
| JE7777 | ΔcobC1151/pCBIB4 | Laboratory collection |
| JE8248 | ΔcobC1151 | Laboratory collection |
| JE9469 | ΔcobC1151/pCOBS8 | Laboratory collection |

**E. coli C41(DE3)**

| Plasmids | Relevant genotype | Reference or source |
|----------|------------------|---------------------|
| pJO46 | cobC^+ in pT7-5; bla^+ | Novagen |
| pT7-5, pT7-7 | Cloning vectors; bla^+ | Novagen |
| pCBIB4 | cbiB^+ in pT7-7; bla^+ | Novagen |
| pCOBS5 | cobS^+ in pET15b; bla^+ | Novagen |
| pCOBS8 | cobS^+ in pT7-7; bla^+ | Novagen |
| pET-15b | N-terminal H^6 tag vector; bla^+ | Novagen |

---

a. *S. enterica* strains are derivatives of serovar Typhimurium strain LT2. Strains and plasmids were constructed during the course of this work unless stated otherwise.

b. Described in reference 22.

c. Referred to in the text as Tn10d(ter^+) (58).

d. Described in reference 36.
minerals, deproteinized extract, and pure CobD enzyme (2.5 μg), purified as described previously (15).

(ii) CobS-enriched cell extracts. Ten milliliters of lysogenic broth containing ampicillin (100 μg/ml) was inoculated with a fresh transformant of Escherichia coli strain C41(DE3) carrying pCD8S5 (cobS5) and incubated overnight at 37°C. Two liters of lysogenic broth supplemented with ampicillin (100 μg/ml) in a 4-liter flask were inoculated with 10 ml of the overnight culture and incubated at 37°C with continuous shaking (200 rpm). Isopropyl-β-D-thiogalactopyranoside was added to the cell culture at a final concentration of 0.4 mM when it reached a cell density at 650 nm of ~0.6. Cultures were incubated at 37°C for 3 h after induction and harvested by centrifugation as described for corrinoid extractions, and the cell paste (4.5 g, wet weight) was stored at −20°C until being used. The same procedure was performed with E. coli strain C41(DE3) carrying the empty pET3b plasmid as negative control.

Cell paste was resuspended in 30 ml of 0.1 M Tris-Cl buffer (pH 7.9, 24°C), containing 500 μl of protease inhibitor cocktail for His-tagged proteins (Sigma). Cells were broken with a French pressure cell operating at 8 × 106 kPa. Cell extracts were obtained after centrifugation at 4°C at 5,000 × g for 15 min. Cell membranes were obtained from a high speed spin at 75,000 × g for 90 min with a JA-25.50 rotor in an Avanti J-25 Beckman/Coulter refrigerated centrifuge. Membranes were resuspended in 10 ml of 0.1 M Tris-Cl buffer (pH 7.9, 24°C) with a glass homogenizer and were solubilized by the addition of DHPC (1:2, diheptanoyl-sn-glycero-3-phospholcholine) to a final concentration of 15 mM. DHPC was added slowly to avoid denaturation of proteins. The detergent-containing CobS-enriched extract was incubated on ice for 30 min and centrifuged at 4°C at 75,000 × g for 30 min. Glycerol (10% [vol/vol], final concentration) was added to the soluble membrane extract, which was flash frozen in liquid nitrogen prior to storage at −80°C. Protein concentration was determined with a Bradford Bio-Rad kit (7).

(iii) Synthesis of CobS substrates. Protocols for the synthesis and purification of AdoCbi-GDP and α-RP have been described (35, 37). In this work, we used an Altima HP C18 AQ 5 μ column (150 by 4.6 mm) (Alltech) and a Waters 600 HPLC system to resolve products from reagents. α-R was derived from α-RP by treatment with shrimp alkaline phosphatase (1 U/ml; Promega) followed by purification with a C8 SepPak cartridge (Waters). Prior to use, the C8 cartridge was equilibrated with ddH2O, washed with 10 ml of ddH2O after sample application, and eluted with 2 ml of 100% methanol. α-R was dried under vacuum as described above, suspended in 150 μl of ddH2O, and stored at −20°C until being used.

(iv) In vitro synthesis of AdoChl and AdoChl-P. Reaction mixtures contained AdoChl-GDP (0.0035 μmol), α-RP, or α-R (0.003 μmol; 2′-(α-cyclohexylamino)-ethanesulfonic acid buffer (pH 9, 5 μmol); MgCl2 (0.4 μmol); and H2-CobS5-enriched membrane extract (5 μg) in a final volume of 100 μl. Reaction mixtures were incubated at 37°C for 1 h and stopped by the addition of 50 μl of 24 mM KCN (1.2 μmol) and incubation at 80°C for 10 min. All steps were performed in dim light to minimize cleavage of the C-C bond of adenosylated corrinoids.

(v) Bioassays for detection of CobS activity. Strain JE3248 (ΔcobS) was used as indicator strain. Cells of an overnight NB culture were washed twice with sterile saline. Two hundred microliters of culture was added to 4 ml of molten 0.7% (wt/vol) agar and overlaid on E Medium (19) supplemented with glucose. After overnight incubation at 37°C, the diameter of the zone of growth is reported in centimeters.

(vi) RP-HPLC analysis of cobalamin synthase (CobS) reaction products. Reaction products were separated with a System Gold HPLC system (Beckman/ Coulter) equipped with an Alltech HP C18 AQ 5 μ column (150 by 4.6 mm) (Alltech). System II for corrinoid separation method was employed as described previously (3). For quantitative purposes, a standard curve for CNChl was constructed. The lower limit of detection of CNChl was 5 pmol, with an upper limit of 2,000 pmol (r2 = 0.9999). A third of the reaction mixture volume (50 μl) was injected, and the total amount of product was calculated by multiplying the amount of CNChl detected by a factor of 3.

RESULTS

Serovar Typhimurium cobC strains cannot salvage cohyric acid (Cby). During the course of studies aimed at identifying the gene encoding the enzyme responsible for the phosphorylation of l-Thr and AP, we uncovered a new phenotype for cobC strains of serovar Typhimurium. We used Tn10Δ(ltet’)

transposon mutagenesis to isolate derivatives of strain JE2216 [cobD1302::Tn10Δ(lcat’)] that failed to salvage Cby when provided with AP but grew well when provided with Cbi (Fig. 1).

To demonstrate that a single Tn10Δ(ltet’)

element caused the observed inability to make AdoCbl from Cby and AP, P22 phage was grown on the original mutant strain and the phage lysate was used as donor to transduce strain JE2216 to tetra-cycline resistance; the reconstructed strain failed to salvage Cby in the presence of AP. Sequencing of the DNA flanking the Tn10Δ(ltet’)

element showed that the transposon was inserted in the cobC gene (data not shown). The inability of the reconstructed strains to convert Cby and AP to AdoCbl was 100% cotransducible with a Tn10Δ(ltet’)

element located within the cobC gene (40). This growth defect was also observed with a ΔcobC strain (JE2217). Although the extent of the deletion in strain JE2217 has not been established, it does not affect the expression of the adjacent cobD gene (data not shown).

To confirm this new phenotype of cobC strains, we tested a previously isolated cobD cobC strain (JE4724) from our laboratory strain collection. Indeed, strain JE4724 failed to convert Cby and AP to Cbl but efficiently converted cobinamide (Cbi) to Cbl. This new phenotype of a cobC strain suggested a block in de novo corrin ring biosynthesis caused by the apparent inactivation of the kinase responsible for the phosphorylation of l-Thr or AP. As predicted by the pathway shown in Fig. 1, the absence of AP-P would prevent conversion of Cby to Cbi-P by the Cbi-P synthase (CbiB) enzyme.

Supplements that bypass the need for CobC function during Cbl synthesis from Cby and AP in a cobC strain. (i) Addition of l-Thr-P to the medium. Given the structural similarity between l-Thr and AP (Fig. 1), we investigated whether the lack of CobC would affect the synthesis of l-Thr-P or AP-P from l-Thr or AP, respectively. Because AP-P was not commercially available but l-Thr-P was, all subsequent studies were performed with l-Thr-P. We assessed the responsiveness of serovar Typhimurium strain JE2718 (ΔcobC1151) to l-Thr-P during growth in minimal medium containing glucose and CNChl, with or without DMB; a cobC’ strain (TR6583) was used as positive control. l-Thr-P restored the conversion of Cby to AdoCbl by a cobC strain (Fig. 2A). This result was consistent with a block in the phosphorylation of l-Thr in a cobC strain. Whether the effect was direct or indirect remained unclear. Surprisingly, addition of DMB in lieu of L-Thr-P to the medium.

whether the effect was direct or indirect remained unclear. Surprisingly, addition of DMB in lieu of L-Thr-P to the medium.

whether the effect was direct or indirect remained unclear. Surprisingly, addition of DMB in lieu of L-Thr-P to the medium.
down the pathway, perhaps related to the activity of the AdoCbl-5'-P synthase (CobS) enzyme. To test this possibility, we increased the level of AdoCbi-P synthase (CbiB) enzyme in the cell by placing the cbiB gene on a plasmid with a promoter that was no longer controlled by oxygen (23). We assessed growth of the cobC strain carrying plasmid pCBIB4 (cbiB×) in minimal medium with glycerol and Cby, with or without DMB (Fig. 3A). We note that residual expression of cbiB× from the plasmid used in this experiment was previously reported to restore AdoCbl synthesis in a cbiB strain (61).

Growth of strain JE7777 (cobC/pCBIB4) on medium containing Cby (without DMB supplementation) was greatly improved compared to the growth of control strains (Fig. 3A). Increasing the level of the CbiB enzyme had the same effect on Cby salvaging as the addition of L-Thr-P or DMB to the culture medium (Fig. 2). When DMB was added to the medium, growth of the control strains (Fig. 3B) was strongly stimulated, and the combination of DMB and higher levels of CbiB enzyme resulted in growth of the cobC strain that was indistinguishable from that of the cobC+ strain (Fig. 3B).

Growth of the ΔcobC strain did not improve when the level of AdoCbl-5'-P synthase (CobS) enzyme was increased. On the contrary, increased levels of CobS resulted in a drastic Cby salvaging deficiency in the cobC strain (Fig. 4). Growth of a cobC strain carrying plasmid pCOBS8 (cobS×) was assessed in minimal medium containing glycerol and Cby, with or without DMB supplementation. Growth of strain JE9469 (ΔcobC/pCOBS8 cobS×) in medium lacking DMB was significantly reduced compared to control strains (Fig. 4A). Addition of DMB to the medium stimulated growth of strain JE9469 (Fig. 4B), but growth was not as robust as that of the control strain (Fig. 4B). We note that increased levels of CobS enzyme did not have a negative effect on the growth of a cobC strain when Cbl was present in the medium, suggesting that high levels of

FIG. 2. Effects of L-Thr-P and DMB supplementation on Cby salvaging. In both panels, Cbl-dependent growth of serovar Typhimurium strains was assessed in minimal medium supplemented with glucose and Cby. (A) Serovar Typhimurium strain JE2718 (ΔcobC). (B) Serovar Typhimurium strain TR6S83 (cobC+). Conditions for the experiments were as described in Materials and Methods.

FIG. 3. Cby salvaging by a cobC strain is restored when expression of CbiB is increased and exogenous DMB is added to the medium. Cbl-dependent growth of serovar Typhimurium strains was assessed in minimal medium supplemented with glycerol and Cby (A) and Cby plus DMB (B). Plasmids introduced into strain JE2718 (ΔcobC): empty plasmids pT7-7 and pT7-5 (used as VOCs [vector-only controls]), plasmid pJO46 (cobC+), and plasmid pCBIB4 (cbiB×).
CobS somehow prevent synthesis of AdoCbl from Cby in this strain (data not shown).

A cobC strain accumulates AdoCbl-P. We used RP-HPLC and MS analysis (Fig. 5 and 6) to analyze the corrinoid content in a serovar Typhimurium ΔcobC strain (JE2718) grown in minimal NCE-glycerol medium supplemented with Cby and DMB. Under these conditions, we detected the accumulation of substantial amounts of Cbl-P and minor amounts of AdoCbl (Fig. 5). The identities of Cbl-P (12.5 min) and Cbl (16.3 min) were confirmed by their UV-visible spectra (not shown), by their masses (Fig. 6), and by the shift in retention time after treatment with alkaline phosphatase (Fig. 5). Upon dephosphorylation, the Cbl-P retention time shifted from 12.5 min to 16.3 min.

α-RP is the preferred substrate of the AdoCbl-5′-P synthase (CobS) enzyme. A physiological explanation for the results presented in Fig. 2 and 3 can be gleaned from Fig. 1. One possible explanation was that α-RP, not α-R (as shown in Fig. 1), was the preferred substrate of CobS. Results shown in Fig. 7 support this idea. To investigate this possibility, we used bioassays to analyze the activity of CobS as a function of α-RP or α-R. Data shown in Fig. 7 (inset) reflect the amount of complete corrinoids synthesized by CobS when the enzyme was incubated with saturating levels of AdoCbi-GDP and equimolar amounts of α-RP or α-R under the same conditions. The presence of Cbl in the reaction mixture was detected by a bioassay that employed a Cbl auxotroph as the indicator strain (JE8248 ΔcobS). We interpreted the results from these experiments to mean that α-RP was a better substrate for CobS than α-R.
We performed RP-HPLC to get a quantitative assessment of CobS activity as a function of \(\text{H}9251\)-RP or \(\text{H}9251\)-R. Under the same assay conditions, CobS converted 2,500 pmol of \(\text{H}9251\)-RP into AdoCbl-P in 30 s (Fig. 7), while CobS converted only 20 pmol of \(\text{H}9251\)-R into Cbl in 30 s (Fig. 7). Together, these results indicate that \(\text{H}9251\)-RP was the preferred substrate of CobS.

DISCUSSION

We report here the first in vivo evidence that the last step of the AdoCbl biosynthetic pathway in serovar Typhimurium is the dephosphorylation of AdoCbl-5'-P and that the CobC enzyme catalyzes this reaction. Our in vivo evidence is strongly supported by results from in vitro experiments. This new knowledge changes our current view of the late steps of AdoCbl biosynthesis in several ways. First, it does not support the idea that the condensation of AdoCbi-GDP and \(\text{H}9251\)-R by the cobalamin synthase (CobS) enzyme is the terminal step of the pathway. Second, it brings into question whether \(\text{H}9251\)-R is an intermediate of the pathway, as previously suggested (40, 44, 57). Although these conclusions directly apply to coenzyme B12-producing prokaryotes.

Why are cobC strains unable to salvage Cby? The block of Cby salvaging in cobC strains is a complex phenotype that we explain by modifying the sequence of reactions presented in Fig. 1 to that presented in Fig. 8. Two key modifications are noted. First, \(\alpha\)-RP, not \(\alpha\)-R, is the cosubstrate for the AdoCbl-5'-P synthase (CobS) enzyme, and second, CobC is the AdoCbl-P phosphatase that converts AdoCbl-P to AdoCbl, the end product of the pathway. Dephosphorylation of AdoCbl-P by CobC is not an unprecedented idea. In fact, evidence that CobC dephosphorylates AdoCbl-P in vitro was previously reported, but the physiological significance of this activity was not explored (35).

This new model explains all the data reported in this paper. First, a cobC strain cannot salvage Cby because it makes AdoCbl-P, which cannot be dephosphorylated in the absence of CobC; hence, AdoCbl-P cannot be used by Cbl-dependent methionine synthase. Cby salvaging is restored in a cobC strain when either L-Thr-P or DMB is added to the medium or the level of cobinamide-P synthase (CbiB) enzyme is increased. We propose that Cby salvaging is restored under these conditions because the amount of AdoCbl-5'-P made by CobS is higher and the putative nonspecific phosphatase that dephosphorylates AdoCbl-5'-P requires higher levels of it before it can be used as substrate. There is precedence for such a scenario in AdoCbl biosynthesis in serovar Typhimurium (54). CobS can synthesize more AdoCbl-5'-P when it is saturated with either AdoCbl-GDP (CbiB, L-Thr-P effect) or \(\alpha\)-RP (DMB effect).

FIG. 7. HPLC and bioassay analyses of CobS products. Shown are the RP-HPLC analysis of the product of the CobS reaction and the results of bioassays (inset) used to detect AdoCbl-P synthesized from AdoCbi-GDP and \(\alpha\)-RP (black trace) or Cbl from \(\alpha\)-R (gray trace). AdoCbi-GDP, Cbl-P, and Cbl eluted at 3.6 min, 9.9 min, and 13.6 min, respectively. A 1-μl sample of a 1:10 dilution of in vitro reactions was used in the bioassay. (Inset) A, Cbl generated by CobS from \(\alpha\)-RP and AdoCbi-GDP (2.5 cm in diameter); B, Cbl generated by CobS from \(\alpha\)-R and AdoCbi-GDP (1.9 cm in diameter); C, CNCbl control (1.8 cm in diameter).

FIG. 8. Last two steps of the nucleotide loop assembly pathway in serovar Typhimurium. Steps for the synthesis of AdoCbl-GDP and \(\alpha\)-RP remain as in Fig. 1.
Other important conclusions. (i) CobC does not phosphorylate \(\text{L-Thr} \) or AP. Even though the inability of a cobC strain to salvage Cby is corrected when \(\text{L-Thr-P} \) is present in the medium, one should not conclude that the CobC protein has \(\text{L-Thr} \) kinase activity. If a cobC strain were truly blocked in \(\text{L-Thr} \) phosphorylation, AdoCbl synthesis would be correctible only by the addition of \(\text{L-Thr-P} \). The suggestion that CobC phosphorylates \(\text{L-Thr} \) is further weakened by the correction of the Cby phenotype by the addition of DMB or the increase in the level of AdoCbi-P synthase (CbiB) enzyme. Instead, we propose that all the above conditions that restore Cby salvaging in cobC strains do so by increasing the level of \(\text{AdoCbi-GDP} \) or the level of \(\text{Cbi} \)-AP, with the concomitant increase in AdoCbl-5'-P synthesized by CobB. The identity of the \(\text{L-Thr} \) kinase enzyme involved in AdoCbl synthesis remains unknown.

(ii) On the previously reported growth behavior of cobC strains. Previous studies of CobC by our laboratory showed that under highly aerobic growth conditions, a serovar Typhimurium cobC strain required DMB to grow, but under anaerobic conditions the cobC strain grew well without the addition of DMB (40). While work presented here provides plausible explanations for the effect of DMB on the aerobic growth of cobC strains, it does not provide insights into why DMB supplementation is not needed during anaerobic growth. We hypothesize that since DMB is not synthesized by serovar Typhimurium under anaerobic conditions (33), the incorporation of adenine or methyl-adenine into \(\text{B}_{12} \) may be more efficient, hence eliminating the need for exogenous DMB.

(iii) In wild-type serovar Typhimurium, the conversion of AdoCby to AdoCbi-P by the Cbi enzyme is limited under aerobic conditions. This is not unexpected, given that under aerobic growth conditions, expression of the \(\text{chi} \) genes is low but not off (23). The low level of CbiB made by serovar Typhimurium under aerobic conditions is clearly sufficient to salvage Cby as long as the CobC enzyme is functional, probably reflecting on the differences in affinity of CobC and the putative nonspecific phosphatase acting on AdoCby-P. Efforts to identify the putative nonspecific AdoCbl-P phosphatase are ongoing.

ACKNOWLEDGMENTS

This work was supported by NIH grant GM40313 to J.C.E.-S. C.L.Z. was supported in part by the Ruth L. Kirstein Research Service award (NRSA) fellowship F31-GM64099.

K. R. Brushaber and J. Zawacki, from our research group, first noted the inability of cobC mutants of serovar Typhimurium to use aminopropanol when cells were fed with cobyric acid. We thank P. Atlas, R. K. R. Brushaber and J. Zawacki, from our research group, first identified growth of \(\text{Methanobacterium ruminantium} \) in a pressurized atmosphere. J. Bacteriol. 173:789–808.

REFERENCES

1. Atlas, R. K. 1995. Handbook of media for environmental microbiology. CRC Press, Boca Raton, FL.

2. Balch, W. E., and R. S. Wolfe. 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of \(\text{Methanobacterium rumi} \) in a pressurized atmosphere. Appl. Environ. Microbiol. 32:781–787.

3. Berkowitz, D., J. M. Hushon, H. J. Whitfield, J. Roth, and B. N. Ames. 1968. Procedure for identifying nonsense mutations. J. Bacteriol. 96:215–220.

4. Cameron, B., F. Blanché, M. C. Rouyez, D. Bisch, A. Famechon, M. Couder, L. Cauchois, D. Thibaut, L. Debussche, and J. Crouzet. 1991. Genetic analysis, nucleotide sequence, and products of two \(\text{Pseudomonas denitrificans} \) genes encoding nicotine-nucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase and cobalamin (5'-phosphate) synthase. J. Bacteriol. 173:6066–6073.

5. Chan, R. K., D. Botstein, T. Watanabe, and Y. Ogata. 1972. Specialized transduction of tetracycline resistance by phage P22 in \(\text{Salmonella typhi} \). II. Properties of a transducing lysate. Virology 50:831–837.

6. Cheong, C. G., C. B. Bauer, K. R. Brushaber, J. C. Escalante-Semerena, and I. Rayment. 2002. Three-dimensional structure of the \(\text{l-threonine-O-3-phosphate} \) decarboxylase (CobF) enzyme from \(\text{Salmonella enterica} \). Biochemistry 41:7986-7998.

7. Cheong, C. G., J. C. Escalante-Semerena, and I. Rayment. 2002. Capture of a labile substrate by expulsion of water molecules from the active site of nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase (CobF) from \(\text{Salmonella enterica} \). J. Biol. Chem. 277:11203–11207.

8. Cheong, C. G., J. C. Escalante-Semerena, and I. Rayment. 1999. The three-dimensional structures of nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase (CobF) from \(\text{Salmonella typhimurium} \) complexed with 5,6-dimethylbenzimidazole and its reaction products determined to 1.9Å resolution. Biochemistry 38:16125–16135.

9. Davis, R. W., D. Botstein, and J. R. Roth. 1980. A manual for genetic engineering: advanced bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

10. DeVeaux, L. C., D. S. Clevenson, C. Bradbeer, and R. J. Kadner. 1986. Identification of the \(\text{B}_{12} \) receptor protein in the outer membrane of \(\text{Salmonella typhimurium} \). J. Bacteriol. 167:929–937.

11. Drennan, C. L., S. Huang, J. T. Drummond, R. G. Matthews, and M. L. Ludヴィ. 1994. How a protein binds B12: a 3.3Å X-ray structure of B12-binding domains of methionine synthase. Science 266:1660–1674.

12. Elliott, T., and J. R. Roth. 1988. Characterization of Tn10-cam: a transposon-defective Tn10 specifying chloramphenicol resistance. Mol. Gen. Genet. 213:332–338.

13. Escalante-Semerena, J. C., and J. R. Roth. 1987. Regulation of cobalamin biosynthetic operons in \(\text{Salmonella typhimurium} \). J. Bacteriol. 169:2251–2258.

14. Escalante-Semerena, J. C., S. J. Suh, and J. R. Roth. 1990. cobF function is required for both de novo cobalamin biosynthesis and assimilation of exogenous corrinoids in \(\text{Salmonella typhimurium} \). J. Bacteriol. 172:273–280.

15. Friedmann, H. C. 1968. Vitamin B12 biosynthesis. Evidence for a new precursor vitamin B12 5'-phosphate. J. Biol. Chem. 243:2065–2075.

16. Gray, M. J., and J. C. Escalante-Semerena. 2002. Single enzyme conversion of FMNH2 to 5,6-dimethylbenzimidazole, the lower ligand of \(\text{B}_{12} \) in \(\text{Escherichia coli} \). J. Bacteriol. 184:904–908.

17. Heller, K., and R. J. Kadner. 1985. Nucleotide sequence of the gene for the \(\text{B}_{12} \) receptor protein in the outer membrane of \(\text{Escherichia coli} \). J. Bacteriol. 161:904–908.

18. Keller, K., B. J. Mann, and R. J. Kadner. 1985. Cloning and expression of the gene for the \(\text{B}_{12} \) receptor protein in the outer membrane of \(\text{Salmonella enterica} \). J. Bacteriol. 167:906–909.

19. Jeter, R. M., B. M. Olivera, and J. R. Roth. 1984. \(\text{Salmonella typhimurium} \) synthesizes cobalamin (vitamin \(\text{B}_{12} \)) de novo under anaerobic growth conditions. J. Bacteriol. 159:206–215.

20. Borths, E. L., R. Poolman, R. N. Hvorup, K. P. Locher, and D. C. Rees. 2005. In vitro functional characterization of \(\text{BtuCD-F} \)-dependent vitamin B12 uptake. Biochemistry 44:16301–16309.

21. Bradford, M. R. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
31. Johnson, M. G., and J. C. Escalante-Semerena. 1992. Identification of 5,6-dimethylbenzimidazole as the CoE ligand of the cobamide synthesized by Salmonella typhimurium. Nutritional characterization of mutants defective in biosynthesis of the imidazole ring. J. Biol. Chem. 267:13302–13305.
32. Keck, B., M. Munder, and P. Renz. 1998. Biosynthesis of cobalamin in Salmonella typhimurium: transformation of riboflavin into the 5,6-dimethylbenzimidazole moiety. Arch. Microbiol. 171:66–68.
33. Keck, B., and P. Renz. 2000. Salmonella typhimurium forms adenylcobamide and 2-methyladenylcobamide, but no detectable cobalamin during strictly anaerobic growth. Arch. Microbiol. 173:76–77.
34. Maggio-Hall, L. A., and J. C. Escalante-Semerena. 2003. Alpha-5,6-dimethylbenzimidazole adenosine dinucleotide (alpha-DAD), a putative new intermediate of coenzyme B12 biosynthesis in Salmonella typhimurium. Microbiology 149:983–990.
35. Maggio-Hall, L. A., and J. C. Escalante-Semerena. 1999. In vitro synthesis of the nucleotide loop of cobalamin by Salmonella typhimurium enzymes. Proc. Natl. Acad. Sci. USA 96:11798–11803.
36. Miroux, B., and J. E. Walker. 1996. Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. J. Mol. Biol. 260:289–298.
37. O'Toole, G. A., and J. C. Escalante-Semerena. 1995. Purification and characterization of the bifunctional CobU enzyme of Salmonella typhimurium LT2. Evidence for a CobU-GMP intermediate. J. Biol. Chem. 270:23560–23569.
38. O'Toole, G. A., and R. Kolter. 1998. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. Mol. Microbiol. 28:449–461.
39. O’Toole, G. A., M. R. Rondon, and J. C. Escalante-Semerena. 1993. Analysis of mutants of Salmonella typhimurium defective in the synthesis of the nucleotide loop of cobalamin. J. Bacteriol. 175:3317–3326.
40. O’Toole, G. A., J. R. Trzebiatowski, and J. C. Escalante-Semerena. 1994. The cobC gene of Salmonella typhimurium codes for a novel phosphatase involved in the assembly of the nucleotide loop of cobalamin. J. Biol. Chem. 269:26503–26511.
41. Pearson, K. Z. S., Zhou, A. E. Smith, R. G. Matthews, and J. E. Penner-Hahn. 2001. Characterization of the zinc sites in cobalamin-independent and cobalamin-dependent methionine synthase using zinc and selenium X-ray absorption spectroscopy. Biochemistry 40:987–993.
42. Rioz, C. R., and R. J. Kudner. 1989. Vitamin B12 transport in Escherichia coli K12 does not require the btuE gene of the btuCED operon. Mol. Gen. Genet. 217:301–308.
43. Rodionov, D. A., A. G. Vitreschak, A. A. Mironov, and M. S. Gelfand. 2003. Comparative genomics of the vitamin B12 metabolism and regulation in prokaryotes. J. Biol. Chem. 278:41148–41159.
44. Roth, J. R., J. G. Lawrence, and T. A. Bobik. 1996. Cobalamin (coenzyme B12) biosynthesis and biological significance. Annu. Rev. Microbiol. 50:137–181.
45. Schmieder, H. 1971. A method for detection of phage mutants with altered transduction ability. Mol. Gen. Genet. 106:378–381.
46. Schmieder, H., and H. Bakharski. 1973. The origin of DNA in transducing particles of P22 mutants with increased transduction frequencies (HT-mutants). Mol. Gen. Genet. 120:181–190.
47. Scott, A. I. 2003. Discovering nature’s diverse pathways to vitamin B12: a 35-year odyssey. J. Org. Chem. 68:2529–2539.
48. Suh, S.-J., and J. C. Escalante-Semerena. 1995. Purification and initial characterization of the ATP-riboseadenosyltransferase encoded by the cob4 gene of Salmonella typhimurium. J. Bacteriol. 177:921–925.
49. Tabor, S. 1990. Expression using the T7 RNA polymerase/promoter system, p. 16.21–16.22. In F. M. Ausubel, R. Brent, R. E. Kingston, D. M. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 2. Wiley Interscience, New York, NY.
50. Taylor, R. T., and H. Weissbach. 1973. N5-methyleneetrahydrofolate-homocysteine methyltransferases, p. 121–165. In P. D. Boyer (ed.), The enzymes, vol. 9. Academic Press, Inc., New York, NY.
51. Thomas, M. G., and J. C. Escalante-Semerena. 2000. Identification of an alternative nucleoside triphosphate:5'-deoxyadenosylcobinamide phosphate nucleotidyltransferase in Methanobacterium thermoautotrophicum ΔH. J. Bacteriol. 182:4227–4233.
52. Trzebiatowski, J. R., and J. C. Escalante-Semerena. 1997. Purification and characterization of CobE, the nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase enzyme from Salmonella typhimurium LT2. J. Biol. Chem. 272:17662–17667.
53. Trzebiatowski, J. R., G. A. O’Toole, and J. C. Escalante-Semerena. 1994. The cobE gene of Salmonella typhimurium encodes the NaMN:5,6-dimethylbenzimidazole phosphoribosyltransferase responsible for the synthesis of N3-(5-phospho-u-d-ribo-fosyl)-5,6-dimethylbenzimidazole, an intermediate in the synthesis of the nucleotide loop of cobalamin. J. Bacteriol. 176:3568–3575.
54. Tsang, A. W., and J. C. Escalante-Semerena. 1998. CobB, a new member of the SIR2 family of eucaryotic regulatory proteins, is required to compensate for the lack of nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase activity in cobE mutants during cobalamin biosynthesis in Salmonella typhimurium LT2. J. Biol. Chem. 273:31788–31794.
55. Van Biber, M., C. Bradbeer, N. Clark, and J. R. Roth. 1999. A new class of cobalamin transport mutants (btuF) provides genetic evidence for a periplasmic binding protein in Salmonella typhimurium. J. Bacteriol. 181:5539–5541.
56. Vogel, H. J., and D. M. Bonner. 1956. Acetylcornitrate of Escherichia coli: partial purification, and some properties. J. Biol. Chem. 218:97–106.
57. Warren, M. J. E., R. E. Raus, H. L. Schubert, and J. C. Escalante-Semerena. 2002. The biosynthesis of adenosylcobalamin (vitamin B12). Nat. Prod. Rep. 19:390–412.
58. Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner. 1984. New Tn10 derivatives for transposon mutagenesis and for construction of lacZ operon fusions by transposition. Gene 32:369–379.
59. Woodson, J. D., and J. C. Escalante-Semerena. 2004. CbiZ, an amidohydro-lase enzyme required for salvaging the coenzyme B12 precursor cobinamide in archaea. Proc. Natl. Acad. Sci. USA 101:1591–1596.
60. Woodson, J. D., R. F. Peck, M. P. Krebs, and J. C. Escalante-Semerena. 2003. The cobY gene of the archaean Halobacterium sp. strain NRC-1 is required for de novo cobamide synthesis. J. Bacteriol. 185:311–316.
61. Woodson, J. D., C. L. Zayas, and J. C. Escalante-Semerena. 2003. A new pathway for salvaging the coenzyme B12 precursor cobinamide in archaea requires cobinamide-phosphate synthase (Chib) enzyme activity. J. Bacteriol. 185:7193–7201.