Purification and Characterization of CobT, the Nicotinate-mononucleotide:5,6-Dimethylbenzimidazole Phosphoribosyltransferase Enzyme from Salmonella typhimurium LT2

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We report the purification and biochemical characterization of the cobalamin biosynthetic enzyme nicotinate-mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase (CobT) from Salmonella typhimurium. CobT was overexpressed and the protein purified to approximately 97% homogeneity. NH₂-terminal sequence analysis confirmed that the protein encoded by cobT was purified. Homogeneous CobT catalyzed the synthesis of N¹-(5-phospho-o-D-ribosyl)-5,6-dimethylenzimidazol. The identity of high performance liquid chromatography-purified product was confirmed by fast atom bombardment mass spectrometry. CobT activity was optimal at 45 °C and pH 10.0. The apparent Kₘ for nicotinate mononucleotide was 680 μM; the apparent Kₘ for 5,6-dimethylenzimidazol was less than 10 μM. CobT used nicotinamide mononucleotide as a ribose phosphate donor. CobT phosphoribosylated alternative base substrates including benzimidazol, 4,5-dimethyl-1,2-phenylenediamine, imidazol, histidine, adenine, and guanine in vitro. The resulting ribotides were incorporated into cobamides that were differentially utilized by methionine synthase (EC 4.3.1.7), ethanolamine amionio-lyase (EC 4.3.1.13), and 1,2-propanediol dehydratase (EC 4.2.1.28) in vitro. The lack of base substrate specificity by CobT may explain the inability to isolate mutants blocked in the synthesis of 5,6-dimethylenzimidazol in this bacterium.

The primary cobamide synthesized by Salmonella typhimurium LT2 is Coo-5,6-dimethylenzimidazolyl-CoC adenosylcobamide (Ado-Cbl) (1) (Fig. 1). Cobamides containing compounds other than 5,6-dimethylenzimidazol (Me₂Bza) as lower (Coo) ligands have been isolated from other prokaryotes.

The lower ligands fall within two classes: (i) derivatives or analogs of benzimidazol (Bza) [e.g. 5-hydroxybenzimidazol, 5-methylenbenzimidazol, 5-methoxy-6-methylbenzimidazol, adenine] (3–5) and (ii) phenolic (e.g. p-cresol, phenol) (5, 6). It is unclear why some cobamide-producing prokaryotes have evolved to synthesize these alternative cobamides.

Cobamide-producing prokaryotes can be guided into synthesizing cobamides containing a lower ligand base different from the one usually found in the de novo synthesized cobamide by exogenously providing the alternative base to growing cells. Examples of this phenomenon termed “guided biosynthesis” have been documented (1, 7, 8).

Friedmann et al. (9) proposed that the cobalamin biosynthetic enzyme nicotinate-mononucleotide (NaMN):Me₂Bza phosphoribosyltransferase plays an important role in the incorporation of alternative lower ligand bases into cobamides in nature and during guided biosynthesis. This proposal was based on the observation that partially purified NaMN:Me₂Bza phosphoribosyltransferases from Propionibacterium shermanii and from Clostridium sticklandii were both shown to use a variety of base substrates in place of Me₂Bza in vitro (10, 11). It was proposed that this lack of base substrate specificity was likely responsible for the incorporation of alternative bases into cobamides in vivo. However, the incorporation of alternative NaMN:Me₂Bza phosphoribosyltransferase products generated in vitro into active cobamides in vivo was not demonstrated.

In this paper we report the purification and biochemical characterization of the NaMN:Me₂Bza phosphoribosyltransferase (CobT) from S. typhimurium. Previously we established a correlation between the presence of CobT and phosphoribosyltransferase activity using crude cell-free extracts containing increased levels of CobT protein (12). In this paper we demonstrate that homogeneous CobT is necessary and sufficient for the synthesis of N¹-(5-phospho-o-D-ribosyl)-5,6-dimethylenzimidazol (α-ribazole 5'-phosphate) (Fig. 2) and that the enzyme phosphoribosylates a wide range of alternative base substrates in vitro. We also present evidence that the resulting ribotides are incorporated into active cobamides in vivo. These results strongly support that CobT is responsible for the incorporation of alternative lower ligands into cobamides in S. typhimurium. Additionally, this result may help explain the inability to isolate mutants blocked in the synthesis of Me₂Bza in this bacterium.

**EXPERIMENTAL PROCEDURES**

**Strain Construction, Culture Media, and Growth Conditions**

The two bacterial strains used in this work were derivatives of S. typhimurium strain LT2 and contained metE205 ara-9 mutations in the background. Strain JE2461 (cobA367::Tn10id(Tc)/pGP1–2 T7 rpo " kan " pJO27 bla " + cobT" (cloning vector: pT7–5)) (12) was used to increase
Cbl. Strain JE3762 was unable to synthesize Cbl from its precursors cobinamide and Me₂Bza but synthesized Cbl when provided with α-ribazole 5'-phosphate. Two in vivo biological activity assays with different sensitivities were employed.

In Vivo Methionine Synthase Assay—Methionine synthase catalyzes the last step in methionine biosynthesis. The metE and metH genes of *S. typhimurium* LT2 encode different methionine synthase enzymes. The activity of MetH (EC 2.1.1.13) is Cbl-dependent, whereas that of MetE (EC 2.1.1.14) is Cbl-independent (16). Strain JE3762 carries a metE mutation that forces the cells to synthesize methionine using the MetH enzyme. Therefore, derivatives of metE mutants unable to synthesize Cbl are phenotypically methionine or Cbl auxotrophs (17). Approximately 25 Cbl molecules/cell satisfy the methionine requirement of a growing cell (18).

In Vivo Ethanalamine Ammonia-lyase and 1,2-Propanediol Dehydratase Assays—Ethanalamine ammonia-lyase (EC 4.3.1.7), the first enzyme in ethanalamine catabolism in *S. typhimurium* LT2, requires Ado-Cbl to function (19). 1,2-Propanediol dehydratase (EC 4.2.1.28), which catalyzes the first step in 1,2-propanediol catabolism in this bacterium, is also an Ado-Cbl-dependent enzyme (20, 21). Therefore, strains that cannot synthesize Ado-Cbl are unable to utilize 1,2-propanediol or ethanalamine as carbon and energy sources. Approximately 500 Cbl molecules/cell are needed for continued growth on ethanalamine (18).

**In Vivo Assessment of Cbl Biosynthesis**

Cbl biosynthesis was assessed in vivo by demanding strain JE3762 to grow under conditions that required the synthesis and utilization of α-ribazole 5'-phosphate. Strain JE3762 was unable to synthesize Cbl from its precursors cobinamide and Me₂Bza but synthesized Cbl when provided with α-ribazole 5'-phosphate. Two in vivo biological activity assays with different sensitivities were employed.

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the NaMN concentration was varied. For the kinetic analysis of
Me$_2$Bza, a saturating concentration of NaMN (100 nmol) was used
while the Me$_2$Bza concentration was varied. Data from four independ-
ent trials were analyzed with the nonlinear regression data analysis
program Enzfitter (Elsevier-Biosoft, Cambridge, United Kingdom).

**Alternative Substrate Analyses**

**Alternative Substrates for NaMN—Nicotinamide mononucleotide
(NMN) was substituted for NaMN in reaction mixtures containing
varying concentrations of NMN and a saturating concentration of $^{14}$C-
$^2$Me$_2$Bza (2 nmol). Data from three independent trials were analyzed
with the nonlinear regression data analysis program Enzfitter.

Ribose 5'-phosphate (0.96 µmol, 160 nmol, and 16 nmol) was substi-
tuted for NaMN in reaction mixtures.

**Alternative Substrates for Me$_2$Bza—Bza, Me$_2$Pda, imidazole, histi-
dine, adenine, or guanine (1 µmol each) was added to the reaction
mixture in lieu of Me$_2$Bza. Because these compounds were not radioac-
tive, formation of products was detected using a biological activity assay
(see above). If the reaction mixture restored the ability of strain JE3762
phosphorylase to make a physiologically active colasamide, growth was observed.

To increase the amount of product, assays were scaled up to 1 ml (50-fold)
and contained 15 µg of CobT and 3 µmol of NaMN. Assays were run at
pH 10.0, incubated for 1 h at 37 °C, heat inactivated at 90 °C for 15 min,
centrifuged for 10 min (14,800 × g) in a Marathon 13KMM microcentri-
fuge (Fisher Scientific), and the supernatant concentrated under vacu-
um using a SpeedVac concentrator (Savant Instruments, Inc., Farming-
dale, NY). Assays were resuspended in 50 µl of double-distilled
water, and a sample (10 µl) was tested for biological activity. Identical
assays were run without CobT to demonstrate the dependence of the
biological activity on CobT.

Phosphoribosyltransferase reactions were performed using $[^{14}$C]-adenine
in place of Me$_2$Bza. The 20-µl reactions used 100 nmol of
NaMN and 0.15 µg of CobT. The assay was incubated at 37 °C for 60
min.

**Chromatography and Spectroscopy**

HPLC purification of the CobT reaction product was performed as
described previously (12).

Native molecular mass analysis of CobT was performed using a
BioSep-SEC-S2000 column (300 × 7.8 mm; Phenomenex, Reno, NV)
equilibrated with 60 mM H$_2$PO$_4$, pH 7.0, at a rate of 1 ml/min. Protein
elution was monitored at 280 nm. Protein molecular weight standards
used included blue dextran, cytochrome c, carbonic anhydrase, alcohol
dehydrogenase, and β-amylase (Sigma). A 20-µl sample (4 mg/ml) was
injected. All samples were run twice and averaged. The elution time for
CobT was 7 min.

Thin layer chromatography analysis of the CobT reaction products
was performed as described previously (12) with the following modifi-
cations. Silica gel plates (20 × 10 cm) were used; 1-cm-wide lanes
separated by 0.5 cm were scored on the plates; plates were developed for
approximately 45 min using CHCl$_3$:MeOH (3:2).

UV-visible spectroscopy and fast atom bombardment mass spectrom-
eometry of the CobT reaction product were performed as described (12).

**Purification of CobT**

All purification steps were performed at 4 °C. The buffer used
throughout the purification was 50 mM Tris-HCl, pH 7.5 (at 4 °C).
Additions to this buffer are stated below. The buffers used throughout
the purification (except to resuspend the cells) contained ethylene glycol
or glycerol as indicated to stabilize the protein.

**Step 1. Cell Breakage and Crude Cell-free Extract Preparation**—A
2-liter culture of strain JE2461 was grown under conditions that over-
expressed cobT. The resulting 3.4 g of wet paste was resuspended in 45
ml of buffer. After the addition of protease inhibitors (EDTA, 1 mM;
phenylmethylsulfonyl fluoride, 0.5 mM), cells were broken by sonication
(18 min, 50% duty cycle; setting 5) using a model 550 Sonic Dismem-
brator (Fisher Scientific). To minimize heat denaturation of protein
during sonication, the extract was maintained at a temperature below
15 °C. Cell debris was removed by centrifuging at 44,000 × g for 1 h
(Sorvall SS-34 centrifuge; DuPont Instruments, Inc.).

**Step 2. Hydrophobic Interaction Chromatography**—Finely ground
Ultrapure™ ammonium sulfate (Schwarz/Mann, ICI Biomedicals Inc.,
Cleveland, OH) was added to the extract to 10% saturation, incubated
at 4 °C for 30 min, and centrifuged for 15 min at 10,000 × g to remove
precipitates. Ethylene glycol was added to the supernatant (10% (v/v),
i.e. 1.8 μl). The extract was loaded onto a phenyl-Sepharose CL-4B
(Sigma) column (2.5 × 60 cm, a 30-ml bed volume) equilibrated with
buffer containing 1.8 μl ethylene glycol and 10% saturation ammonium
sulfate. The column was equilibrated and developed at a flow rate of 30
ml/h. After loading, the column was washed with 30 ml of the equili-
trating buffer followed by a 150-ml linear gradient that simultaneously
decreased the amount of ammonium sulfate from 10% saturation to
zero and increased the ethylene glycol concentration from 1.8 to 8.9 μl.
The gradient was followed by a 60-ml wash with buffer containing 8.9 μl
ethylene glycol. CobT eluted from the column toward the end of
the gradient. Fractions containing CobT were identified by SDS-PAGE
and Coomassie Blue staining. CobT fractions were pooled and loaded
directly onto the next column.

**Step 3. Dye-Ligand Chromatography**—The sample was loaded onto
a 1.5 × 14-cm Cibracon Blue 3GA column (25-ml bed volume)
equilibrated with buffer containing 5.4 μl ethylene glycol at a flow rate
of 25 ml/h. After loading the sample, the column was washed with 50 ml
of buffer containing 1.4 μl glycerol and 200 mM NaCl; the column
was developed with a 125-ml linear gradient to buffer containing 1 mM
NaCl and 1.4 μl glycerol; the column was further washed with 50 ml of buffer
containing 2 mM NaCl and 1.4 μl glycerol. CobT eluted within the gradi-
ent, with the peak of protein detected at 450 mM NaCl. A cross-section
of the CobT-containing fractions revealed homogeneous CobT as judged
from Coomassie Blue-stained SDS-PAGE gels. These homogeneous
fractions were pooled, concentrated using a Centriprep-10 (Amicon,
Inc., Beverly, MA), and dialyzed overnight against buffer containing 2.7
μl glycerol. The enzyme was aliquoted and stored at −89 °C in a freezer.

**NH$_2$-Terminal Sequencing**

The NH$_2$-terminal sequence of purified CobT was determined at the
Protein/Nucleic Acid Shared Facility at the Medical College of Wiscon-
sin (Milwaukee, WI) using conventional Edman sequence chemistry.
Sequence was conducted with a Beckman/Porton LF 3000 gas phase
sequenator. Data were reported using the Beckman system Gold
software.

**Other Procedures**

Protein concentrations were determined by a modification of the
turbidimetric method reported by Kunitz (22) using a standard curve
generated using known quantities of bovine serum albumin. These
values corresponded well to values obtained using the Bio-Rad Protein
Assay Kit, which is based on the Bradford dye-binding procedure (23).
SDS-PAGE was performed with 12% polyacrylamide gels (24) and
stained with Coomassie Blue (25). SDS-PAGE standards included phos-
phorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase,
and trypsin inhibitor (Bio-Rad). The native isoelectric point (pI) of CobT
was determined as reported (26). Conductivity measurements were
performed using a model 35 YSI conductivity meter (Yellow Springs,
OH). Densitometry scans were performed with a GS300 Transmittance/
Reflectance Scanning Densitometer (Hoefer Scientific Instruments,
San Francisco).

**RESULTS**

**cobT Overexpression and Visualization of CobT**

cobT was overexpressed using a construct where cobT was
transcribed from a bacteriophage T7 promoter (27). The cloning
of this construct was described previously (28). Based on den-
sitometry scans of Coomassie-stained SDS-PAGE gels, CobT
was judged to be approximately 12% of the total soluble protein
in crude cell-free extract of the overexpressing strain (data not
shown). The relative mobility of CobT in this system was
determined previously (28).

**Purification of CobT**

The purification of CobT from crude cell-free extract is sum-
mORIZED in Table I. CobT specific activity in the crude extract
(Table I) was determined 12 h after sonication and storage at
4 °C. This value (22 units/mg), was 1.5-fold lower than the
specific activity obtained immediately after sonication (33 units/mg)
suggesting inactivation or degradation of CobT. Inter-
Interestingly, after the initial loss of activity, the specific activity
did not decrease any further, even after 1 week of continued
storage at 4 °C (data not shown). One explanation for these
results may be that whatever was responsible for inactivating
or degrading CobT also became inactivated or degraded.
The purification of CobT is shown in Fig. 3. CobT was 97% pure as judged by densitometry scans of Coomassie-stained 12% SDS-PAGE (lane C, Fig. 3).

NH2-terminal Sequence

The NH2-terminal sequence (21 amino acids) of homogeneous CobT was determined by automated Edman degradation. The amino acid sequence of homogeneous CobT was identical to that predicted from the DNA sequence confirming that the cobT product had been purified. Additionally, the sequence established that as predicted elsewhere (29), the translational start site was 30 bases downstream of the start site originally proposed (30).

In Vitro Requirements for CobT Phosphoribosyltransferase Activity

Previously, using crude cell-free extract containing increased levels of CobT, we demonstrated that CobT was the NaMN:Me2Bza phosphoribosyltransferase responsible for the synthesis of α-ribazole 5'-phosphate, an intermediate in the assembly of the nucleotide loop of Cbl (Fig. 2) (12). Using homogeneous CobT, we repeated these experiments to investigate the exact requirements for this activity in vitro. After HPLC purification, the identity of the product was confirmed by UV-visible spectroscopy and mass spectral analysis to be α-ribazole 5'-phosphate (data not shown). In addition, the product was determined to be biologically active, i.e. it restored the ability of a cobT mutant strain (JE3762) to synthesize Cbl. Therefore, it was concluded that homogeneous CobT was determined by automated Edman degradation.

Characterization of the CobT Activity

pH and Temperature Optima—The optimal temperature for CobT activity was 45 °C (6.3 units/mg). CobT maintained 12% of its specific activity at 65 °C (0.8 unit/mg) and 45% at 30 °C (2.8 units/mg). Although maximum activity was obtained at 45 °C, activity assays were performed routinely at the more physiological temperature of 37 °C.

The optimal pH for CobT activity was pH 10.0 (6.5 units/mg). CobT maintained 28% of its specific activity at pH 8.0 (1.8 units/mg) and 2% at pH 11.0 (0.11 unit/mg).

Temperature Stability—CobT was preincubated for 10 min at several temperatures by itself, or with NaMN (20 nmol), or with Me2Bza (20 nmol). CobT was then assayed for phosphoribosyltransferase activity at 37 °C. The specific activities were compared with the one observed when CobT was preincubated on ice. Preincubation at 37 °C with no additions resulted in a drop in specific activity (2.5 units/mg versus 5.4 units/mg, i.e. 46% of the specific activity of CobT kept on ice). However, CobT activity was stable to temperatures as high as 50 °C when preincubated with NaMN (4.5 units/mg). We concluded that NaMN has a stabilizing effect on CobT at increased temperatures. Me2Bza did not increase CobT stability at any temperature (data not shown).

Substrate Inhibition—CobT activity was inhibited about 2.6 fold at increased Me2Bza concentrations. CobT activity at 1.0 mM Me2Bza was 5.9 units/mg compared with 15.1 units/mg at 0.1 mM Me2Bza.

Other Properties

Linearity of the Reaction—CobT activity was linear with respect to protein concentration up to 0.02 μg. CobT activity was linear with respect to time up to 12 min.

Native Oligomeric State—Based on the cobT nucleotide sequence, CobT was predicted to be 356 amino acids in length with a molecular mass of 36,560 Da. This value corresponded well to the position of homogeneous CobT on 12% SDS-PAGE gels (Fig. 3, lane C). Native molecular mass was determined by gel filtration chromatography to be about 68,000 Da, suggesting that native CobT was a dimer.

Isoelectric Point and Extinction Coefficient—The experimentally determined isoelectric point revealed two bands on isoelectric focusing gels corresponding to a pI of 5.8 and 5.6. Both points differ from the predicted isoelectric point for denatured protein, 6.1. The molar extinction coefficient of CobT at 280 nm was predicted to be 9,970 using the Edelhoch method as described (31).

Kinetic Parameters

Based on data from four independent trials, the apparent Km value of CobT for NaMN was 680 μM, the Vmax was 134 pmol of product/min, and the kcat was 817 pmol of product/min/pmol of CobT dimer. The apparent Km value (680 μM) was similar to the CobT homolog from C. sticklandii (700 μM) (11) and 8-fold higher than the homolog from Pseudomonas denitrificans (83 μM) (32).

The apparent Km value of CobT for Me2Bza could not be determined because of the low specific activity of [2-14C]Me2Bza. However, we were able to conclude that the apparent Km value was less than 10 μM (data not shown). The P. denitrificans homolog had a Km for Me2Bza of 16 μM (32). The Km for Me2Bza of the C. sticklandii homolog was not determined.

Substrate Specificity

Ribose Phosphate Donor—NMN and ribose 5'-phosphate were both tried in lieu of NaMN in phosphoribosyltransferase reactions. CobT used NMN as substrate in vitro; however, CobT phosphoribosyltransferase activity was inhibited at [NMN] > 40 mM. Because of this inhibition, kinetic parameters...
were derived from a rate curve that did not reach saturation. Based on data from three independent trials, the apparent $K_m$ of CobT for NMN was 30 mM, 44-fold higher than the one measured for NaMN. This supports the idea that NaMN is the in vivo substrate for CobT. The $V_{max}$ was 114 pmol of product/min, and the $k_{cat}$ was 695 pmol of product/min/pmol of CobT dimer. The CobT utilization of NMN was in contrast to the C. sticklandii homolog, which did not utilize NMN as a substrate (11). Ribose 5'-phosphate was not utilized by CobT.

(FIG. 4) Evidence of CobT utilization of Me₂Bza, Bza, and Me₂Pda using the methionine synthase biological activity assay. CobT phosphoribosyltransferase reactions were performed with Me₂Pda (plate B) and Bza (plate C) in place of Me₂Bza (plate A). Growth of strain JE3762 in response to the CobT reaction mixtures indicated that CobT utilized Me₂Bza, Bza, and Me₂Pda in vitro and that the products were transformed into active cobamides utilized by methionine synthase in vivo. Biological activity assays were performed as described under “Experimental Procedures.” No CobT refers to reaction mixtures containing no enzyme.

(FIG. 5) Evidence of CobT utilization of histidine, adenine, guanine, and imidazole using the ethanolamine ammonia-lyase biological activity assay. CobT phosphoribosyltransferase reactions were performed with adenine (plate A), guanine (plate B), histidine (plate C), and imidazole (plate D) in place of Me₂Bza. Growth of strain JE3762 in response to the CobT reaction mixtures indicated that CobT utilized histidine, adenine, guanine, and imidazole in vitro and that the products were transformed into active cobamides utilized by ethanolamine ammonia-lyase in vivo. Biological activity assays were performed as described under “Experimental Procedures.” No CobT refers to reaction mixtures containing no enzyme. In plate B precipitate (not growth) is observed where the No CobT reaction mixture was spotted.

**DISCUSSION**

We have demonstrated CobT recognition of a variety of bases as substrates in vitro and the incorporation of these alternative products into active cobamides in vitro. We know that the resulting cobamides in the case of the histidine, adenine, guanine, and imidazole reactions are not transformed into Cbl because no growth response was observed when methionine synthase activity was demanded. This supports that S. typhimurium incorporated the CobT in vitro reaction products directly into a cobamide in vivo. These results provide strong support for the role of the NaMN:Me₂Bza phosphoribosyltransferase in the incorporation of alternative lower ligands into cobamides in nature and during guided biosynthesis. Additionally, these results demonstrate that methionine synthase, ethanolamine ammonia-lyase, and 1,2-propanediol dehydratase have different requirements for the identity of the lower ligand.

Methionine synthase was more stringent than ethanolamine ammonia-lyase or 1,2-propanediol dehydratase in its utilization of different cobamides in vitro. One explanation is that the alternative lower ligands may prevent the enzyme from binding the cobamide. The structure of the methionine synthase domain that binds Cbl has been solved (33). The structure indicates that Me₂Bza and the nucleotide loop swing away from the corrin ring and are bound by the enzyme in a pocket. To achieve this conformation, the coordination bond between...
Me₄Bza and cobalt is replaced by a coordination bond between a histidine residue (His¹⁷⁵) of the enzyme and cobalt. Perhaps the lower ligands of the alternative cobamides generated in our experiments did not fit into the binding pocket or were locked into a base-on conformation. If the inability to bind was the reason for the lack of activity, we would predict that ethanolamine ammonia-lyase and 1,2-propanediol dehydratase bind cobamides differently than methionine synthase. The structure of the Cbl binding domains of ethanolamine ammonia-lyase and 1,2-propanediol dehydratase have not been determined to date.

However, our results suggest that methionine synthase can use alternative cobamides that contain lower ligands structurally similar to Me₄Bza, since the products of the Bza and Me₂Pda reactions resulted in the in vivo synthesis of cobamides utilized by this enzyme. We cannot rule out that Me₂Pda may have been converted to Me₄Bza in vivo. However, in the case of Bza, we know that S. typhimurium synthesizes a physiologically active cobamide with Bza as the lower ligand when supplied with exogenous Bza (1), supporting the argument that benzimidazolyl-cobamide was generated in this assay. This suggests that methionine synthase is able to utilize cobamide containing Bza as the lower ligand.

The ability of methionine synthase to utilize alternative cobamides is further supported by Eberhard et al. (34) who showed that a variety of cobamides containing different lower ligands supported growth of an Escherichia coli strain that required active cobamide for methionine synthase activity. However, in contrast to our results, they also saw growth with imidazolyl-cobamide. It has been reported previously that some prokaryotes when supplied with exogenous complete cobamides can replace the alternative lower ligands with their natural base (8, 9). Perhaps this explains the in vivo activity observed for imidazolyl-cobamide.

Identification of Me₄Bza biosynthetic genes and isolation of their products has been elusive. The only Me₄Bza auxotrophs isolated in S. typhimurium carry mutations in cobT. We have proposed previously that cobT mutants are Me₄Bza auxotrophs because a protein referred to as CobB substitutes for CobT activity when endogenous Me₂Bza levels are increased (12). Although we have not ruled out that CobT may in addition to its phosphoribosyltransferase activity be involved in Me₂Bza biosynthesis, we predict that CobT is not solely responsible as suggested elsewhere (35). There are a number of reasons that may explain the difficulty with isolating mutations in Me₂Bza biosynthetic genes: (i) null alleles of Me₂Bza biosynthetic genes may be lethal or (ii) more than one Me₂Bza biosynthetic pathway could exist in S. typhimurium. Alternatively, based on the results presented herein, it is possible that in vivo more than one base can substitute for Me₄Bza. Therefore Me₄Bza auxotrophy would not be observed unless the synthesis of all potential lower ligands was eliminated.

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2 A. Tsang, unpublished results.