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Regiospecific Formation of Cobamide Isomers Is Directed by CobT

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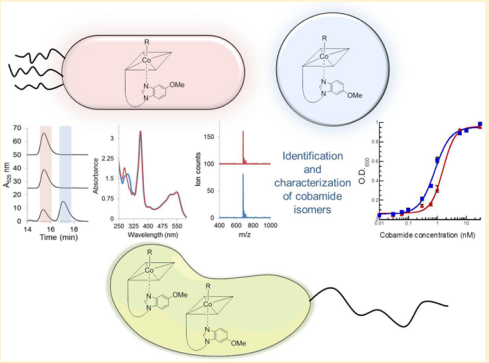
Supporting Information

ABSTRACT: Cobamides, which include vitamin B_{12} (cobalamin), are a class of modified tetrapyrroles synthesized exclusively by prokaryotes that function as cofactors for diverse biological processes. Cobamides contain a centrally bound cobalt ion that coordinates to upper and lower axial ligands. The lower ligand is covalently linked to a phosphoribosyl moiety through an alpha-glycosidic bond formed by the CobT enzyme. CobT can catalyze the phosphoribosylation of a variety of substrates. We investigated the ability of CobT to act on either of two nitrogen atoms within a single, asymmetric benzimidazole substrate to form two isomeric riboside phosphate products. Reactions containing asymmetric benzimidazoles as substrates for homologues of CobT from different bacteria resulted in the production of distinct ratios of two isomeric products, with some CobT homologues favoring the production of a single isomer and others forming a mixture of products. These preferences were reflected in the production of cobamide isomers with lower ligands attached in different orientations, some of which are novel cobamides that have not been characterized previously. Two isomers of methoxybenzimidazolylcobamide were found to be unequal in their ability to support ethanolamine ammonia-lyase dependent growth in *Salmonella enterica*, suggesting that CobT's regiospecificity could be biologically important. We also observed differences in pK_a, which can influence the reactivity of the cofactor and could contribute to these distinct biological activities. Relaxed regiospecificity was achieved by introducing a single point mutation in an active site residue of CobT. These new cobamide isomers could be used to probe the mechanisms of cobamide-dependent enzymes.



Nature has evolved a diverse array of enzyme specificities. On one end of the spectrum, many enzymes recognize one particular substrate and catalyze the synthesis of a single product. For example, substrate specificity in tRNA synthetases has evolved in order to avoid the production of potentially damaging mutant proteins.[1] At the other end of the spectrum, some enzymes such as alkaline phosphatases and some proteases recognize a type of bond or functional group and act on a broad range of molecules with that feature.[2,3] In between, some enzymes have dual substrate specificity such that they can accommodate two different substrates for the same reaction or perform two reactions on one substrate. For example, the enzyme PriA catalyzes Amadori rearrangement reactions in two similar substrates during the biosynthesis of both histidine and tryptophan in some bacteria.[4] Often, the precise specificity of an enzyme is critical for physiology.

An example of an enzyme that can accommodate multiple substrates is the bacterial CobT enzyme, which catalyzes the phosphoribosylation of various substituted benzimidazoles and purines as a step in the biosynthesis of vitamin B_{12} (cobalamin **17**) and other cobamides (Figure 1).[5−7] Homologues of CobT from diverse bacteria can catalyze the phosphoribosylation of several different substituted benzimidazoles as well as adenine *in vitro*. A distinct group of CobT homologues, called ArsAB, can additionally activate phenolic compounds.[8−14] Since the products of CobT are incorporated as the lower (α) axial ligand of cobamides (Figure 1), this promiscuous substrate specificity contributes to the diversity of cobamides produced by bacteria.[10,15−18] CobT's ability to accommodate multiple substrates can have positive impacts on physiology, as it enables cells to make use of multiple lower ligand bases available in the environment.[19−23] The ability to incorporate lower ligands to form cobamides that cannot be used as cofactors can also have detrimental effects.[13,24−26]

Here, we examine a new angle of CobT's catalytic promiscuity: its ability to catalyze the phosphoribosylation of asymmetric substituted benzimidazoles such as 5-hydroxybenzimidazole (5-OHBza **2**) or 5-methyoxybenzimidazole (5-OMeBza **3**) at either of two nitrogen atoms to form two isomeric products. We find that, despite CobT's promiscuity regarding substrate selectivity, some CobT homologues are specific for phosphoribosylation predominantly at only one of two nitrogen atoms. This regiospecificity is reflected in the orientation of the cobamide lower ligands produced *in vivo* as shown in Figure 1. The native specificity for the formation of a particular cobamide isomer by CobT can be relaxed by site-

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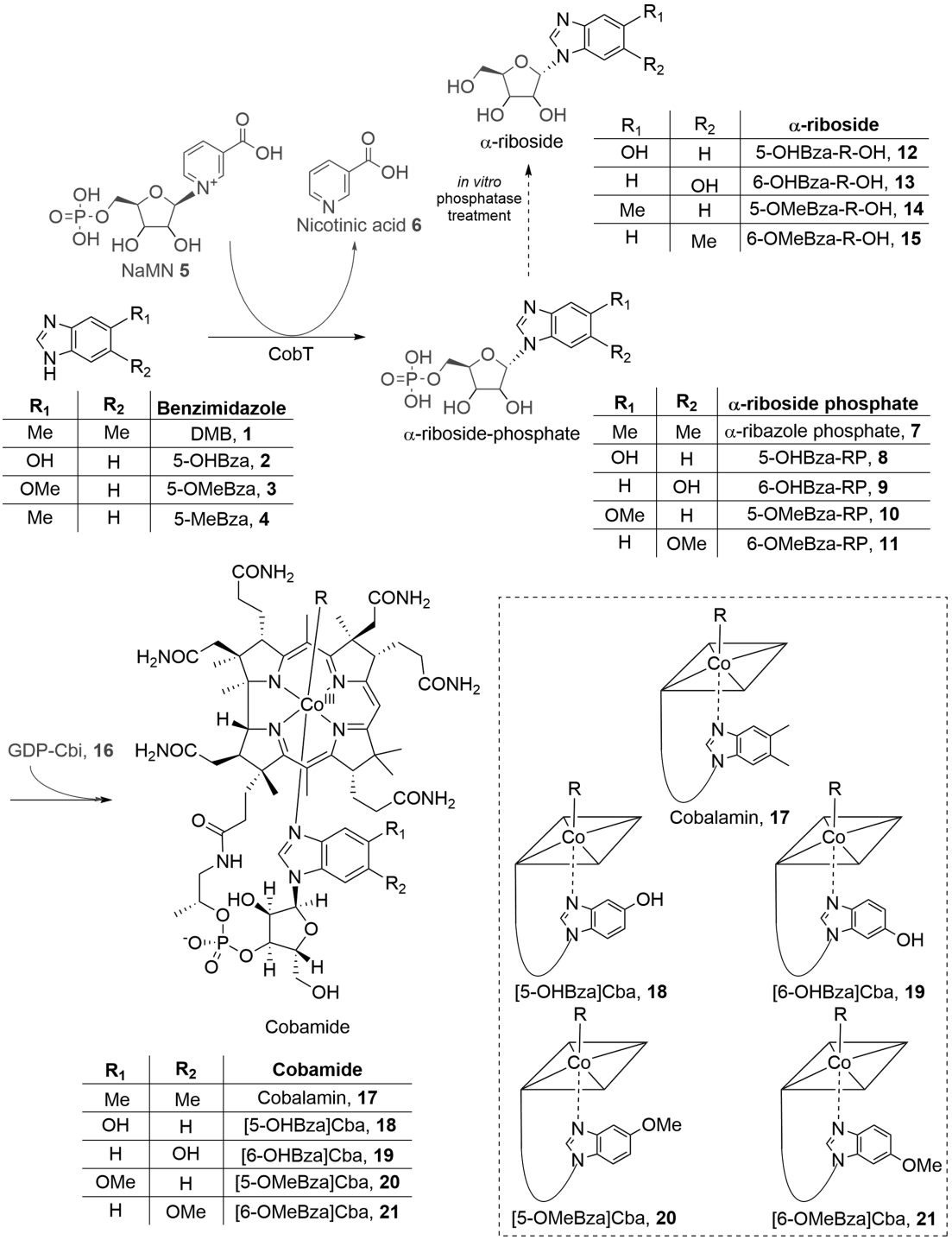

R_1	R_2	α-riboside
OH	H	5-OHBza-R-OH, **12**
H	OH	6-OHBza-R-OH, **13**
Me	H	5-OMeBza-R-OH, **14**
H	Me	6-OMeBza-R-OH, **15**

R_1	R_2	Benzimidazole
Me	Me	DMB, **1**
OH	H	5-OHBza, **2**
OMe	H	5-OMeBza, **3**
Me	H	5-MeBza, **4**

R_1	R_2	α-riboside phosphate
Me	Me	α-ribazole phosphate, **7**
OH	H	5-OHBza-RP, **8**
H	OH	6-OHBza-RP, **9**
OMe	H	5-OMeBza-RP, **10**
H	OMe	6-OMeBza-RP, **11**

R_1	R_2	Cobamide
Me	Me	Cobalamin, **17**
OH	H	[5-OHBza]Cba, **18**
H	OH	[6-OHBza]Cba, **19**
OMe	H	[5-OMeBza]Cba, **20**
H	OMe	[6-OMeBza]Cba, **21**

Figure 1. α-Phosphoribosylation of benzimidazole bases and incorporation into cobamides. CobT catalyzes the α-phosphoribosylation of a free benzimidazole base by using nicotinate mononucleotide (NaMN **5**) as the ribose-phosphate (RP) donor to form an α-riboside phosphate product. This product is subsequently incorporated into a cobamide. The phosphate group of the CobT product can be removed *in vitro* to produce α-ribosides. The numbers of the compounds discussed in the text are indicated.

directed mutagenesis of a single active site residue, suggesting that specificity in substrate binding orientation is part of the function of CobT and that this enzyme can be engineered to bind a single substrate in multiple orientations.

MATERIALS AND METHODS

***In Vitro* Reactions with Purified CobT Enzyme Homologues.** The overexpression and purification of His-tagged CobT enzyme homologues from *Salmonella enterica*, *Veillonella parvula*, and *Sinorhizobium meliloti* were performed as described.[14] Reactions contained 10 μM enzyme, 2 mM nicotinate mononucleotide (NaMN **5**), 250 μM lower ligand substrate, and 10 mM $MgCl_2$ in 50 mM Tris-HCl at pH 7.5 in a volume of 3 mL. The reactions were incubated at 37 °C for 48 h and subsequently quenched by addition of 4% formic acid and incubation at 100 °C for 1 min. Where indicated, the CobT product was dephosphorylated with calf intestinal phosphatase

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(New England Biolabs) in 125 mM Tris pH 7.9 and 10 mM $MgCl_2$ over 10 h at 25 °C to form the corresponding α-riboside (Figure 1). The reactions were incubated at room temperature for 14−16 h followed by heat inactivation at 100 °C for 1 min and filtration through a 10 000 MWCO filter (Pall). 5-OHBza **2** was synthesized from 5-methoxybenzimidazole (5-OMeBza **3**) as described.[13]

Purification of α-Riboside Phosphate and α-Riboside Products. Products of the CobT *in vitro* reactions were analyzed on an Agilent 1200 series high-performance liquid chromatography (HPLC) system equipped with an ultraviolet−visible (UV−vis) diode array detector. An Agilent Eclipse XDB C-18 column (5 μm, 4.6 × 150 mm) was used at a flow rate of 1 mL min^{-1} at 30 °C. Mobile phases used were 10 mM ammonium acetate pH 6.5 (solvent A) and methanol (solvent B). Samples were analyzed by the following method: 0% solvent B over 2 min, followed by a linear gradient of 0−15% solvent B over 1.5 min, 15 to 50% solvent B over 6.5 min, and 50 to 70% solvent B over 2 min, as previously described.[14] Individual products were isolated with an Agilent 1200 series fraction collector. The purified products were lyophilized to dryness, resuspended in deionized water, and lyophilized again to remove volatile salts. Purified samples were stored at −80 °C.

Characterization of the α-Riboside Phosphate Products by LC-MS. Purified α-riboside phosphates were analyzed by liquid chromatography−mass spectrometry (LC-MS) on an Agilent 6410 liquid chromatography-triple quadrupole mass spectrometer with the column and gradient elution method as described above.

^{1}H NMR Analysis of Purified α-Ribosides. Reactions containing CobT homologues from *S. enterica*, *V. parvula*, or *S. meliloti* were used to generate 0.5−1 mg of the α-riboside phosphate products. These products were then dephosphorylated as described above, purified by HPLC, and analyzed using a Bruker Biospin Avance II 900 MHz NMR spectrometer equipped with a TXI cryoprobe accessory at the California Institute for Quantitative Biosciences (QB3)-Berkeley core facility. Spectral assignments are as follows:

5-OMeBza-R-OH ***14***. The 1D ^{1}H spectrum shows the ribose ring protons (H_1' 6.41 ppm d, H_2' 4.60 ppm m, H_3' 4.46 ppm m, H_4' 4.41 ppm m, H_5' 3.93 ddd, H_5'' 3.78 ppm ddd), the benzimidazole ring protons (H_2 8.54 ppm s, H_7 7.59 ppm d, H_4 7.33 ppm d, H_6 7.10 ppm ddd), and the methyl group protons (CH_3 3.91 ppm), consistent with the expected structure of a 5-OMe substituted benzimidazole riboside.[27] A residual peak for the methyl protons of ammonium acetate buffer was observed at 1.91 ppm in these α-riboside NMR spectra.

6-OMeBza-R-OH ***15***. The 1D ^{1}H spectrum shows the ribose ring protons (H_1' 6.34 ppm d, H_2' 4.57 ppm m, H_3' 4.44 ppm m, H_4' 4.39 ppm m, H_5' 3.92, H_5'' 3.84 ppm ddd), the benzimidazole ring protons (H_2 8.37 ppm s, H_7 7.66 ppm d, H_4 7.20 ppm d, H_5 7.04 ppm dd), and the methyl group protons (CH_3 3.91 ppm), consistent with the expected structure of a 6-OMe substituted benzimidazole riboside.[27]

5-OHBza-R-OH ***12***. The 1D ^{1}H spectrum shows the ribose ring protons (H_1' 6.38 ppm d, H_2' 4.56 ppm m, H_3' 4.44 ppm m, H_4' 4.39 ppm m, H_5' 3.92, H_5'' 3.77 ppm ddd) and the benzimidazole ring protons (H_2 8.37 ppm s, H_7 7.51 ppm d, H_4 7.18 ppm d, H_6 6.97 ppm dd), consistent with the expected structure of a 5-OH substituted benzimidazole riboside.[27]

6-OHBza-R-OH ***13***. The 1D ^{1}H spectrum shows the ribose ring protons (H_1' 6.34 ppm d, H_2' 4.57 ppm m, H_3' 4.44 ppm m, H_4' 4.39 ppm m, H_5' 3.92, H_5'' 3.84 ppm ddd) and the benzimidazole ring protons (H_2 8.32 ppm s, H_7 7.60 ppm d, H_4 7.07 ppm d, H_5 6.94 ppm dd), consistent with the expected structure of a 6-OH substituted benzimidazole riboside.[27]

Further, 2D NMR analyses (^{1}H−^{13}C heteronuclear single quantum coherence [HSQC] and ^{1}H−^{13}C heteronuclear multiple-bond correlation [HMBC] spectra) of all the α-riboside isomers were used to verify their molecular structures (Supporting Information Figure 3A−H).

Bacterial Strains and Culture Conditions. *Sporomusa ovata* DSM 2662 was cultured at 30 °C anaerobically as previously described with 50 mM betaine as the carbon source.[24] *Veillonella parvula* DSM 2008 was cultured anaerobically with an atmosphere of 80% N_2 and 20% CO_2 at 37 °C in the *S. ovata* medium containing 10 g/L sodium DL-lactate instead of betaine and supplemented with 5 μg/mL putrescine. *S. meliloti* strains were cultured aerobically at 30 °C in M9 sucrose medium supplemented with cobalt and biotin as described.[13] *Salmonella enterica* serovar Typhimurium strain LT2 was cultured aerobically with the indicated benzimidazoles at 37 °C in NCE medium with 1,2-propanediol as the carbon source.[28] To analyze the biological activity of [OMeBza]Cba isomers, 5 mL of LB medium[29] was inoculated with an *S. enterica* colony and grown to saturation at 37 °C. Cells were harvested by centrifugation, washed with 0.85% NaCl, and diluted to an optical density at 600 nm ($O.D._{600}$) of 0.01 in a minimal medium containing glycerol as the carbon source and ethanolamine as the nitrogen source.[30] Varying concentrations of each [OMeBza]Cba isomer were added, and cultures were incubated at 37 °C for 36 h. The $O.D._{600}$ was monitored on a BioTek Synergy2 plate reader.

Corrinoid Extraction, HPLC Analysis, and Purification of Cobamide Isomers. For extraction and purification of cobamide isomers, *S. ovata* and *V. parvula* were cultured as described above in media containing either 500 μM 5-OMeBza **3** or 2 mM 5-OHBza **2**. *S. enterica* was cultured with 1 μM dicyanocobinamide and 1 μM 5-OMeBza **3**, and *S. meliloti* strains were cultured with 5 μM 5-OMeBza **3** or 5-OHBza **2**. Corrinoids were extracted with methanol twice from cell pellets and cyanated. An Agilent 1200 series HPLC system equipped with a UV-diode array detector was used to analyze the extracted corrinoids. Samples were injected onto an Agilent SB-Aq (5 μm, 4.6 × 150 mm column) at a flow rate of 1 mL/min with mobile phases of A, 0.1% formic acid in water and B, 0.1% formic acid in methanol. The column was maintained at 30 °C. Corrinoids were eluted with a linear gradient of 25% solvent B for 2 min, 25 to 34% solvent B over 11 min, and 34% to 70% solvent B over 3.5 min, as previously described.[13] Both isomers of [5(6)-OMeBza]Cba and [5(6)-OHBza]Cba were purified as follows. First, the cobamide isomers were purified and collected together on a 9.4 × 250 mm Eclipse Plus C18 column at 2 mL/min using a gradient of 18% solvent B over 2.5 min followed by 18−45% B over 18.5 min, with mobile phases A, 0.1% formic acid in water, and B, 0.1% formic acid in methanol, at 45 °C. Second, the mixed isomers were injected onto a 4.6 × 150 mm Zorbax SB-Aq reverse-phase column and separated at 1 mL/min using an isocratic method of 27% solvent B for the [OMeBza]Cba isomers and 25% B for the [OHBza]Cba isomers at 30 °C. Cobamides were quantified based on absorbance at 361 nm using an extinction coefficient of 28 060 mol^{-1} cm^{-1} using a BioTek Synergy2 plate reader.[24]

Characterization of Cobamides. UV−vis spectra were collected during HPLC purification using Agilent ChemStation

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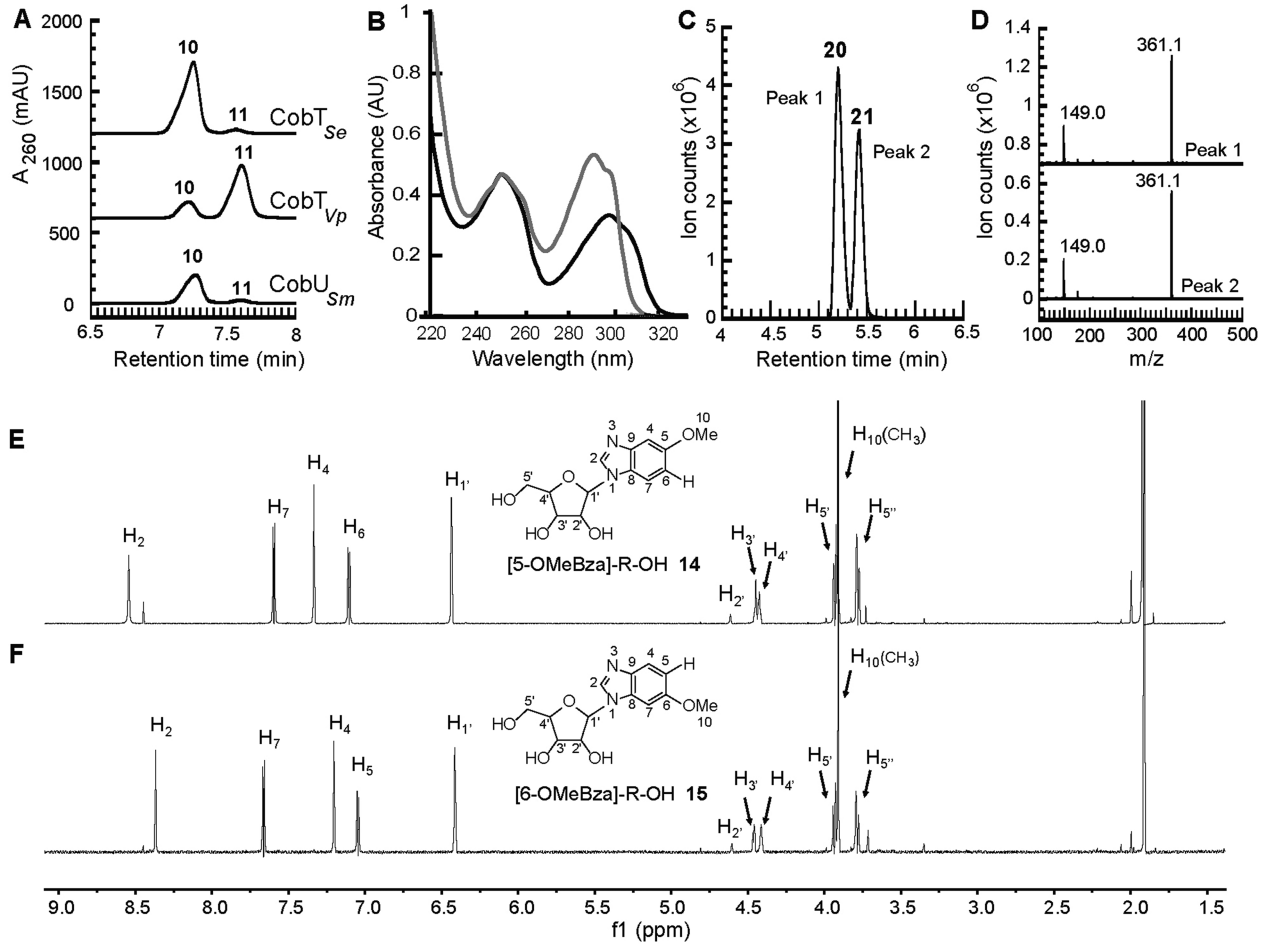

Figure 2. *In vitro* characterization of isomeric products of CobT. (A) HPLC chromatogram of reactions with CobT$_{Se}$, CobT$_{Vp}$, and CobU$_{Sm}$ and the substrate 5-OMeBza **3**, with absorbance monitored at 260 nm (A_{260}). (B) UV−vis spectra of α-5-OMeBza-RP **10** (black) and α-6-OMeBza-RP **11** (gray) normalized to the same molar equivalents. (C) LC-MS showing the EIC of the α-riboside phosphate products purified by HPLC from reactions containing CobU$_{Sm}$ and 5-OMeBza **3**. (D) Extracted MS spectra of peak 1 (top) and peak 2 (bottom) from panel C. (E) 1D ^{1}H NMR of 5-OMeBza-R-OH **14**. (F) 1D ^{1}H NMR of 6-OMeBza-R-OH **15**.

software. Following HPLC purification, cobamides were further characterized on an Agilent 6410 liquid chromatograph-triple quadrupole mass spectrometer using the 4.6 × 150 Zorbax SB-Aq column as previously described,[13] to identify cobamides based on their characteristic transitions and retention times.[23] Samples were also prepared in 50% acetonitrile in water and analyzed using an Agilent 1200 series liquid chromatograph in line with an Agilent 6520 Q-TOF at the Energy Biosciences Institute Mass Spectrometry Facility. The error of the instrument was less than 5 ppm, providing high mass-accuracy MS spectra of the compounds. For NMR analyses of cobamides, samples were prepared and analyzed as described above for the α-ribosides. Spectral assignments are as follows:

*[5-OMeBza]Cba **20**.* The 1D ^{1}H spectrum shows the ribose ring protons (H_1' 6.38 ppm d, H_2' [overlapping with HDO signal], H_3' 4.26 ppm m, H_4' 4.05 ppm m, H_5' 3.91, H_5'' 3.73 ppm ddd), the benzimidazole ring protons (H_2 7.16 ppm s, H_7 7.45 ppm d, H_4 6.28 ppm d, H_6 7.07 ppm dd), and the methyl protons at 3.82 ppm s, consistent with the expected structure of 5-OMeBza as the lower ligand of the cobamide.

*[6-OMeBza]Cba **21**.* The 1D ^{1}H spectrum shows the ribose ring protons (H_1' 6.37 ppm d, H_2' [overlapping with HDO signal], H_3' 4.28 ppm m, H_4' 4.05 ppm m, H_5' 3.91, H_5'' 3.73 ppm ddd), the benzimidazole ring protons (H_2 7.12 ppm s, H_7 7.02 ppm d, H_4 6.94 ppm d, H_5 6.66 ppm dd), and the methyl protons at 3.79 ppm s, consistent with the expected structure of 6-OMeBza as the lower ligand of the cobamide.

*[5-OHBza]Cba **18**.* The 1D ^{1}H spectrum shows the ribose ring protons (H_1' 6.33 ppm d, H_2' [overlapping with HDO signal], H_3' 4.28 ppm m, H_4' 4.05 ppm m, H_5' 3.91, H_5'' 3.73 ppm ddd) and the benzimidazole ring protons (H_2 7.12 ppm s, H_7 7.37 ppm d, H_4 6.19 ppm d, H_6 6.92 ppm dd), consistent with the expected structure of 5-OHBza as the lower ligand of the cobamide.[31]

*[6-OHBza]Cba **19**.* The 1D ^{1}H spectrum shows the ribose ring protons (H_1' 6.59 ppm d, H_2' [overlapping with HDO signal], H_3' 4.28 ppm m, H_4' 4.05 ppm m, H_5' 3.91, H_5'' 3.73 ppm ddd), the benzimidazole ring protons (H_2 7.09 ppm s, H_7 6.89 ppm d, H_4 6.82 ppm d, H_5 6.60 ppm dd), consistent with the expected structure of 6-OHBza as the lower ligand of the cobamide.

The structure of each cobamide was further verified by 2D HSQC or Heteronuclear Multiple Quantum Coherence (HMQC) NMR spectroscopy.

Cobamide Adenosylation and pK_a Determination. Purified, cyanated [OMeBza]Cba isomers and cyanocobalamin were converted to their adenosylated forms (where the upper ligand [CN] was replaced with 5′-deoxyadenosine) using a

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method modified from that described by Brown et al.[32,33] Briefly, cobamides in concentrations ranging from 130 to 350 μM were prepared in 5% ammonium chloride and transferred to an anaerobic chamber (Coy laboratories) for 30 min. Zinc metal was prepared in the anaerobic chamber by soaking in 1 M HCl for 30 min and subsequently added to the cobamide solutions. Approximately 10-fold molar excess of 5′-chloro-5′-deoxyadenosine was added to each solution, and solutions were stirred in the dark for 2 h. Unreacted 5′-chloro-5′-deoxyadenosine and salts were removed by applying the reactions to Sep-Pak C_{18} cartridges (Waters), washing with water, and eluting with 100% methanol. The samples were dried in the dark at 45 °C under reduced pressure. Purity was established by UV−vis spectroscopy.

For pK_a measurements, combinations of citric acid and dibasic sodium phosphate buffers were prepared to provide buffering over a pH range from 2.6 to 7.0. Cobamide samples and buffers were combined in a 1:1 ratio (v/v), and the base-off/base-on conformations at each pH were determined based on their UV−vis spectra at wavelengths between 400 and 600 nm.[34,35] pK_a values were calculated from these data using the KaleidaGraph program by fitting to the equation $y = m_1 + (m_2 - m_1)/(1 + (x/m_3)^{m_4})$, where y, m_1, m_2, m_3, m_4, and x represent the ratio of absorbance at two wavelengths corresponding to the base-off or base-on conformation, the maximum and minimum of the ratio of absorbance, the pK_a of the compound, the slope of the line at the pK_a, and the pH of the sample, respectively. The error in the pK_a measurement was calculated by the program.

RESULTS AND DISCUSSION

CobT Enzyme Homologues React with Asymmetric Substrates to Form Two Isomeric Products. Three bacterial CobT homologues, CobT$_{Se}$ from *Salmonella enterica*, CobT$_{Vp}$ from *Veillonella parvula*, and CobU$_{Sm}$ from *Sinorhizobium meliloti*, were purified, and their reactions with 5-OMeBza **3** were monitored by HPLC. Two products with distinct retention times were observed in different ratios for each of the CobT homologues. This result indicates that unique molecular features encoded in each CobT homologue control the ratio of the two products (Figure 2A). The UV−vis spectra of the two products were distinct, suggesting a difference in structure (Figure 2B).

LC-MS analysis of the two products formed in reactions containing CobT$_{Vp}$ and 5-OMeBza **3** revealed that the two chromatographically distinct species are indistinguishable by mass spectrometry (Figure 2C,D). In both cases, the extracted MS from the extracted ion chromatograms (EIC) at 361 m/z (corresponding to the mass of OMeBza-RP) showed an additional peak at 149 m/z, which matches the expected m/z of the singly charged 5-OMeBza **3** fragmentation product (Figure 2D). Similarly, the two products formed by CobU$_{Sm}$ with another asymmetric substrate, 5-OHBza **2** were found to have distinct UV−vis spectra and identical mass spectra (Figure S1A−D, Supporting Information). In contrast, a single α-riboside phosphate product was formed in reactions containing the symmetric substrates 5,6-dimethylbenzimidazole (DMB) **1** or benzimidazole ([13] and data not shown). Together, these results suggest that in reactions containing asymmetric substrates, the two products formed are isomers.

To characterize the structures of these compounds, we purified the two products from large-scale reactions of CobT$_{Se}$ and CobT$_{Vp}$ with 5-OMeBza **3** for analysis by NMR spectroscopy. Because the phosphate groups were partially hydrolyzed during the purification process, the products were enzymatically dephosphorylated prior to analysis. 1D ^{1}H NMR analysis of the two products revealed small differences in the chemical shifts of peaks in the downfield region (Figure 2E,F), particularly in the benzimidazole ring protons (H_2 8.54 versus 8.37 ppm s, H_7 7.59 versus 7.66 ppm d, H_4 7.33 versus 7.20 ppm d, H_6 7.10 versus H_5 7.04 ppm ddd) and the H1′ ribose proton (6.41 versus 6.34 ppm), consistent with the two compounds having differences in the orientation of the lower ligand.

The orientation of the base with respect to the ribose ring could differ in either of two ways. First, the linkage between the base and the ribose ring can be in either the α or β orientation, and second, the base can be attached via either of the benzimidazole nitrogen atoms. We observed that the coupling constants for H1′ with H2′ were nearly identical in the two isomers of OMeBza-R-OH and OHBza-R-OH (4.2−4.3 Hz), which effectively rules out the possibility that the two products are stereoisomers with one containing an α linkage between the base and the ribose ring and the other with a β linkage (Figure S2, Supporting Information). In support of this interpretation, a comparison to a standard of commercially available β-adenosine, in which the coupling constant of the equivalent protons was measured as 6.2 Hz (data not shown), suggests that the CobT products are not in the β orientation.[27] The assignment of these compounds as α-ribosides is in agreement with several previous studies that show that CobT enzymes specifically catalyze the formation of α-riboside phosphates.[16−18] Next, to examine the possibility that the two isomers differ in the attachment of the benzimidazole to the ribose ring via nitrogen atoms N1 or N3, we analyzed the two OMeBza-R-OH isomers by 2D HSQC (Figure S3A,B, Supporting Information) and HMBC NMR (Figure S3C,D) to confirm the absolute identity of these compounds. The HMBC spectrum of the first isomer (peak 1 in Figure 2C, treated with phosphatase, numbered as shown in Figure S3C) showed an H−C coupling between H1′ and benzimidazole ring C8 and an absence of coupling between H1′ and the more distant benzimidazole ring C9, confirming that the riboside is 5-OMeBza-OH **14** (Figure S3C). Similarly, the HMBC spectrum of the second isomer (peak 2 in Figure 2C, treated with phosphatase, numbered as shown in Figure S3D) showed an H−C coupling between H1′ and benzimidazole ring C8 and an absence of coupling between H1′ and benzimidazole ring C9, confirming that the riboside form of peak 2 is 6-OMeBza-OH **15** (Figure S3D). Similar results were observed when the two products purified from reactions containing 5-OHBza **2** were analyzed by 2D NMR (Figure S3E−H). Consistent with these assignments, it was previously established by X-ray crystallography that CobT$_{Se}$ binds asymmetric benzimidazoles in the orientation that would produce 5-substituted α-riboside phosphates.[10] Taken together, these results demonstrate that CobT can catalyze the α-phosphoribosylation of either of the two nitrogen atoms of asymmetric benzimidazoles and that CobT homologues from different organisms have distinct preferences for substrate orientation.

Characterization of Cobamide Structural Isomers Produced in Bacteria by Guided Biosynthesis. We next tested whether the observed preferences of CobT homologues for the biosynthesis of two isomeric phosphoribosylated products could result in the production of cobamide isomers with lower ligands in different orientations. The ratios of

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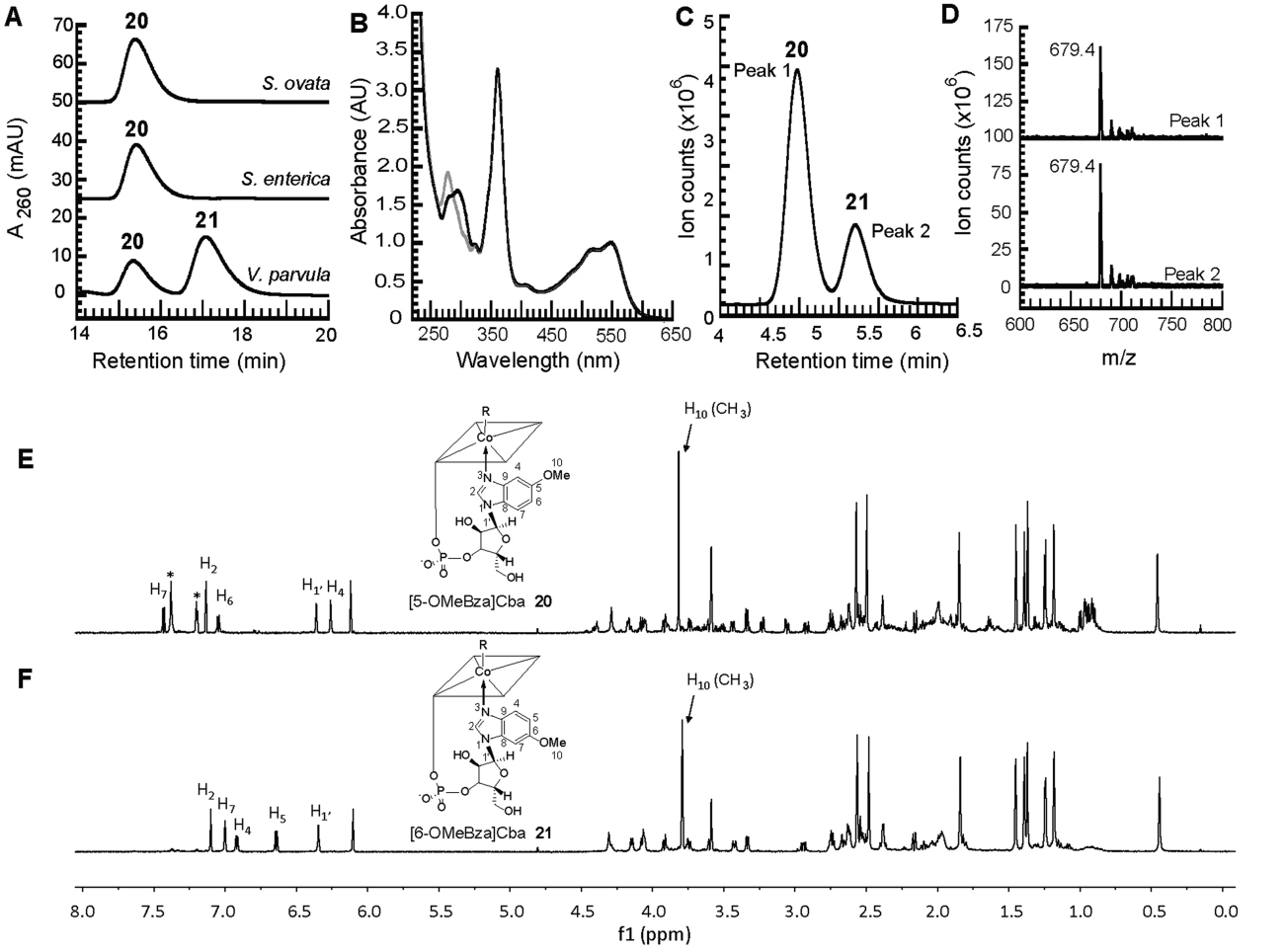

Figure 3. Analysis of [5-OMeBza]Cba **20** and [6-OMeBza]Cba **21** produced by guided biosynthesis. (A) HPLC chromatograms of corrinoid extracts of *S. enterica*, *V. parvula*, and *S. ovata* cultures grown with 5-OMeBza **3**, with absorbance monitored at 525 nm (A_{525}). (B) UV−vis spectra of [5-OMeBza]Cba **20** (black) and [6-OMeBza]Cba **21** (gray), normalized to the absorbance at 550 nm (A_{550}). (C) LC-MS chromatogram of a representative corrinoid extract from *S. meliloti bluB* grown with 5-OMeBza **3**. LC-MS spectra showing the extracted ion chromatogram at 679.3 *m*/*z*, corresponding to the doubly charged [5-OMeBza]Cba **20** and [6-OMeBza]Cba **21**. (D) Extracted MS spectra of peak 1 (top) and peak 2 (bottom) from panel C. (E) 1D ^{1}H NMR spectrum of [5-OMeBza]Cba **20**. Peaks marked with an asterisk (*) indicate impurities in the sample. (F) 1D ^{1}H NMR spectrum of [6-OMeBza]Cba **21**.

cobamides produced by guided biosynthesis (the addition of an exogenous lower ligand base, which is incorporated into a cobamide) in cultures of *S. enterica*, *V. parvula*, and *Sporomusa ovata* grown with 5-OMeBza **3** were examined by HPLC. Only one cobamide peak was detected in *S. enterica* and *S. ovata*, while two peaks were observed in extracts from *V. parvula* (Figure 3A). We also previously observed two peaks in corrinoid extracts of *S. meliloti* cultured with 5-OMeBza **3**.[13] The relative levels of the two cobamide isomers formed by guided biosynthesis in *S. enterica* and *V. parvula* resemble those of the corresponding *in vitro* α-riboside phosphate isomers by $CobT_{Se}$ and $CobT_{Vp}$ (compare Figures 2A and 3A). The UV−vis spectra of the two cobamides are indistinguishable at wavelengths higher than 350 nm, a region dominated by the absorbance properties of the corrin ring, but are distinct between 250 and 350 nm, indicative of a difference in the lower ligand[36] (Figure 3B). The differences in the UV−vis spectra of these cobamides mirror those of the corresponding α-riboside phosphate isomers (Figure 2B). Parallel experiments with 5-OHBza **2** showed that two cobamides with distinct UV−vis spectra were formed in *V. parvula*, while *S. ovata* appeared to produce only one cobamide (Figure S4A,B).

The [OMeBza]Cba isomers were found to be indistinguishable by mass spectrometry, further confirming their identities as isomers. LC-MS analysis showed that both spectra contain peaks with *m*/*z* values that match those of the doubly charged cobamide (679.4 *m*/*z*) (Figure 3C,D). Furthermore, analysis of these products by ESI-TOF-MS showed *m*/*z* values of 679.2808 and 679.2806, which correspond to the predicted exact mass of the doubly charged [OMeBza]Cba (data not shown). Similar results were obtained for the [OHBza]Cba isomers (Figure S4C,D).

Each purified cobamide was also analyzed by 1D ^{1}H NMR. We assigned the first purified peak of the [OHBza]Cba isomers (Figure S4E) as [5-OHBza]Cba **18** because its 1D ^{1}H NMR spectrum is identical to that of the previously reported [5-OHBza]Cba **18**, which was additionally characterized by UV−vis spectroscopy, circular dichroism, and fast atom bombardment mass spectrometry.[31] The ^{1}H NMR spectrum of [5-OHBza]Cba **18** differs from that of the second purified peak (H_2 7.12 vs 7.09 ppm, s; H_7 7.37 vs 6.89 ppm, d; H_6 6.92 vs H_5 6.60 ppm, d; and H_4 6.19 vs 6.82 ppm, d) (Figure S4F), and therefore we assigned the second peak to be [6-OHBza]Cba **19**.

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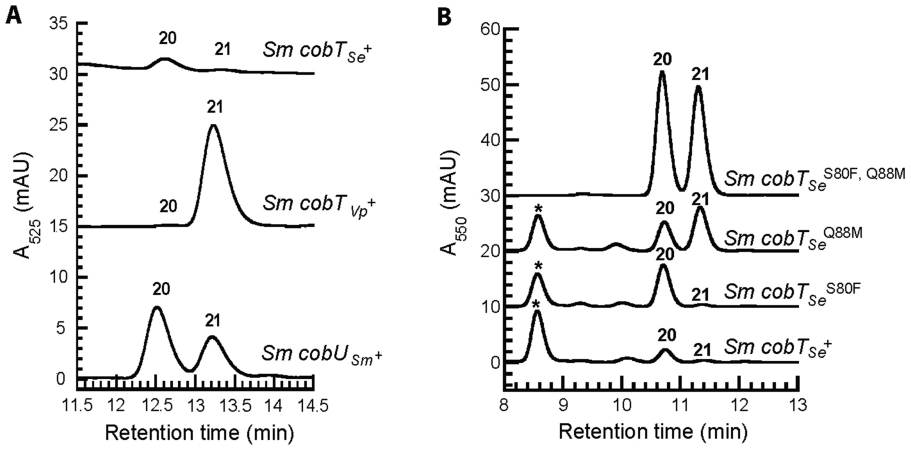

Figure 4. Heterologous expression of *cobT* homologues in *S. meliloti*. (A) HPLC analysis of corrinoids extracted from *Sm* $cobT_{Se}^{+}$, *Sm* $cobT_{Vp}^{+}$, and *Sm* $cobU_{Sm}^{+}$ grown with 5-OMeBza **3**. (B) HPLC analysis of corrinoids extracted from *Sm* $cobT_{Se}^{+}$, *Sm* $cobT_{Se}^{S80F}$, *Sm* $cobT_{Se}^{Q88M}$, and *Sm* $cobT_{Se}^{S80F, Q88M}$ grown with 5-OMeBza **3**. Asterisks (*) indicate the presence of adeninylcobamide, the native corrinoid of *S. enterica*.

The ^{1}H NMR spectrum of the first purified peak of the [OMeBza]Cba isomers is very similar to that of [5-OHBza]Cba **18** (Figure S5A). Except for the distinct CH_3-protons of the methoxy substituent at 3.58 ppm, the peak pattern is identical for the benzimidazole H_2, H_4, $H_{5/6}$ and H_7 and the H_1' ribose protons for these two cobamides. Similarly, the ^{1}H NMR spectrum of the second purified peak of the [OMeBza]Cba isomers is very similar to that of [6-OHBza]Cba **19** (Figure S5B). On the basis of these similarities, we assigned the first [OMeBza]Cba peak as [5-OMeBza]Cba **20** and the second as [6-OMeBza]Cba **21**. These results were further confirmed by 2D ^{1}H−^{13}C heteronuclear multiple quantum coherence (HMQC) or HSQC NMR (Figure S6).

Heterologous Expression and Mutagenesis Demonstrate the Role of CobT in Controlling Cobamide Lower Ligand Orientation. To determine the influence of CobT on cobamide isomer formation *in vivo*, we investigated CobT activity in a heterologous bacterial system. *cobT* homologues from *S. enterica*, *V. parvula*, and *S. meliloti* were expressed in the *S. meliloti bluB cobU* mutant (which cannot synthesize DMB or phosphoribosylate a lower ligand base).[13] These strains are abbreviated as *Sm* $cobT_{Se}^{+}$, *Sm* $cobT_{Vp}^{+}$, and *Sm* $cobU_{Sm}^{+}$, respectively. The levels of each cobamide isomer in these strains were similar to the trend seen in the products of the corresponding CobT enzymes *in vitro*, as well as in the corresponding organisms from which the *cobT* genes were derived (compare Figure 4A with Figures 2A and 3A and see ref 13). This suggests that the ratio of the two isomers *in vivo* is largely determined by substrate binding preferences in CobT.

We next explored the features of CobT's active site that may contribute to the observed preferences for substrate orientation by examining strains containing point mutations in the active site of $CobT_{Se}$ that were previously shown to alter substrate specificity and selectivity.[13,37] The levels of [5-OMeBza]Cba **20** and [6-OMeBza]Cba **21** were examined in *S. meliloti bluB cobU* strains expressing each of two point mutations, S80F and Q88M, alone and in combination, when cultured with 5-OMeBza **3**. We previously found that these mutations alter the relative specificity for DMB versus adenine *in vivo*.[13] The *S. meliloti* strain expressing $CobT_{Se}$ Q88M (*Sm* $cobT_{Se}^{Q88M}$) showed a 9-fold increase in the production of [6-OMeBza]Cba **21** relative to [5-OMeBza]Cba **20** in comparison to the strain expressing wild type $CobT_{Se}$ (*Sm* $cobT_{Se}^{+}$; Figure 4B). In contrast, the S80F mutation caused an increase in selectivity for 5-OMeBza **3** over adenine, as observed previously for DMB **1**,[13] ,but did not affect the ratios of the [OMeBza]Cba isomers. The strain expressing the CobT S80F, Q88M double mutant showed the highest selectivity for 5-OMeBza **3** over adenine, but a slightly lower ratio of [6-OMeBza]Cba **20** to [5-OMeBza]Cba **21** than the Q88M single mutant (Figure 4B). These results suggest that a single point mutation in the active site can significantly alter the substrate binding orientation. Previous structural studies indicated that $CobT_{Se}$ preferentially binds asymmetric benzimidazoles such as 5-OMeBza **3** and 5-MeBza **4** and purines such as adenine in a single orientation that would lead to the transfer of the ribose-phosphate group of NaMN to one of the two imidazole nitrogen atoms.[10,11] Binding of 5-OMeBza **3** in the orientation poised for formation of [5-OMeBza]-RP **10** may be favored in the wild type $CobT_{Se}$ active site because residue Q88 is positioned in the vicinity (4 Å) of the methoxy group.[10] Substitution of a methionine residue at this position could result in a loss of specificity in binding orientation and lead to the observed production of both [OMeBza]Cba isomers. $CobU_{Sm}$, which forms both isomers (Figures 2A, 4A, and ref 13), has a methionine residue at this position, providing additional validation for the observed effect of the Q88M mutation in $CobT_{Se}$. Nevertheless, additional active site residues likely influence substrate binding orientation, based on the fact that in $CobT_{Vp}$, an enzyme that favors the formation of [6-OMeBza]Cba **21** (Figure 4A), a glutamine residue is present at this position.

[OMeBza]Cba Isomers Have Different Chemical and Biological Properties. Given that some bacteria (including *S. enterica*) are thought to take up free benzimidazole bases in the environment for incorporation into cobamides by guided biosynthesis,[19−23] we speculate that it may be beneficial for these organisms to selectively produce one cobamide isomer that may function better as a cofactor *in vivo*. To test this hypothesis, growth of wild type *S. enterica* was assayed under cobamide-dependent conditions in the presence of either [5-OMeBza]Cba **20** or [6-OMeBza]Cba **21**. Growth with ethanolamine as the sole nitrogen source was chosen because the cobamide-dependent ethanolamine ammonia-lyase enzyme required for ethanolamine metabolism binds the cobamide in the base-on form, and thus we reasoned that a small change in the lower ligand could significantly influence catalysis.[38,39] The

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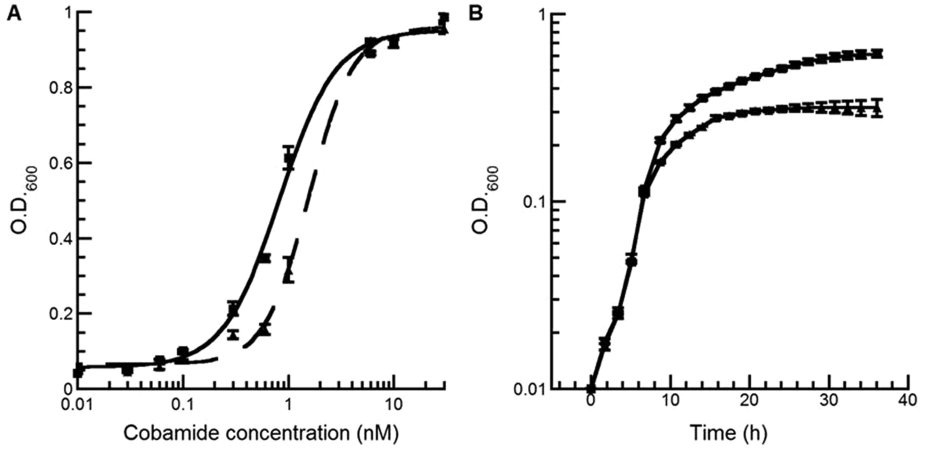

Figure 5. Growth of *S. enterica* with [5-OMeBza]Cba **20** and [6-OMeBza]Cba **21**. (A) Wild type *S. enterica* was grown with ethanolamine as the sole nitrogen source and varying concentrations of purified [5-OMeBza]Cba **20** (triangles, dashed line) or [6-OMeBza]Cba **21** (squares, solid line). (B) Growth curve of *S. enterica* in the same medium supplemented with 1 nM of either [5-OMeBza]Cba **20** (triangles) or [6-OMeBza]Cba **21** (squares). Values represent the average of three independent cultures, and error bars represent the standard error.

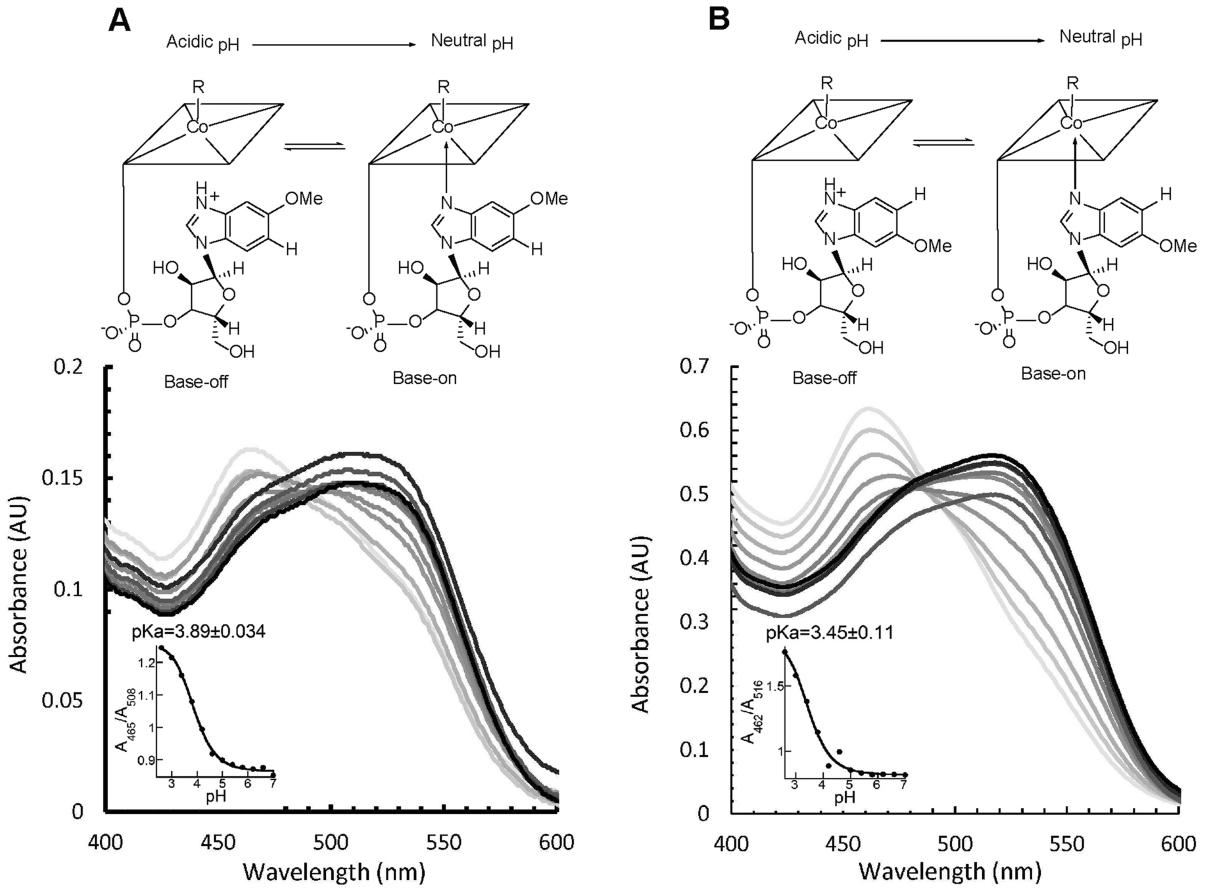

Figure 6. Spectrophotometric determination of pK_a values for β-adenosylated [5-OMeBza]Cba and [6-OMeBza]Cba. UV−vis spectra are shown for (A) β-adenosyl-[5-OMeBza]Cba and (B) β-adenosyl-[6-OMeBza]Cba in buffers ranging from pH 2.5 (gray) to 7.0 (black). Inset: ratios of absorbance at the local maxima and minima were plotted to determine pK_a values of each compound.

cobamide biosynthetic genes are expressed only under anaerobic conditions in *S. enterica*,[40] and therefore under aerobic conditions with ethanolamine as the sole nitrogen source *S. enterica* is phenotypically a cobamide auxotroph. An analysis of the maximum growth achieved with different concentrations of each cobamide isomer under these conditions revealed a distinct response to each isomer, as the concentration required for half-maximal growth yield (EC_{50}) was found to be 1.6 ± 0.2 nM for [5-OMeBza]Cba **20** and 0.82 ± 0.06 nM for [6-OMeBza]Cba **21** (Figure 5A). Examination of growth over time with a single concentration of each cobamide isomer (1 nM) showed the same growth rates in both conditions but slightly different final growth yields (Figure 5B). The observed differences in growth could not be

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attributed to preferential uptake of [6-OMeBza]Cba **21** because identical levels of each cobamide were detected in cultures grown with either isomer at 0.3 and 3 nM (data not shown). These results demonstrate that [6-OMeBza]Cba **21** slightly enhances growth of *S. enterica* in comparison to [5-OMeBza]-Cba **20**. Interestingly, this result is the opposite of that predicted by the preference of $CobT_{Se}$, since [5-OMeBza]Cba **20** formation is favored by $CobT_{Se}$ both *in vitro* (Figure 2A) and *in vivo* (Figures 3A and 4A).

We next investigated the chemical basis of the observed biological distinction between [5-OMeBza]Cba **20** and [6-OMeBza]Cba **21**. In ethanolamine ammonia-lyase and other base-on cobamide-dependent enzymes, the bond between the Co ion and the upper ligand, 5′-deoxyadenosine, is transiently broken.[41] The strength of this bond is influenced by the strength of the interaction between the Co ion and the lower ligand.[42] To measure the impact of lower ligand orientation on the strength of this bond, we determined the pK_a values of the adenosylated forms of [5-OMeBza]Cba **20** and [6-OMeBza]-Cba **21** based on the spectrophotometrically discernible transition between the unprotonated base-on and protonated base-off forms (Figure 6).[35] As a control, we measured the pK_a of 5′-deoxyadenosylcobalamin using this method and found it to be identical to the reported value (3.50 ± 0.054) (data not shown).[35] The pK_a of β-adenosyl-[5-OMeBza]Cba was found to be 3.9, while β-adenosyl-[6-OMeBza]Cba was found to have a pK_a of 3.5 (Figure 6), indicating a relatively stronger Co-lower ligand bond and consequently a more labile upper ligand in [6-OMeBza]Cba **21**. It is perhaps for this reason that [6-OMeBza]Cba **21** functions slightly better during cobamide-dependent growth in *S. enterica*.

CONCLUSION

The bacterial CobT enzyme is known for its ability to catalyze the phosphoribosylation of a wide range of substrates. Here we have found that, in addition to its promiscuity in accommodating multiple substrates, some homologues of CobT can phosphoribosylate either of two sites on a single substrate, while others act in a highly regiospecific manner. We have further shown that homologues of CobT form different ratios of isomers *in vitro*, and that this regiospecificity informs the *in vivo* production of cobamide isomers with lower ligands attached in different orientations. Although both isomers of [OMeBza]Cba function as cofactors for *S. enterica* ethanolamine ammonia-lyase, they have modestly different biological activities, as reflected by the ability of each to support corrinoid-dependent growth of *S. enterica*. Remarkably, a single, conservative point mutation in the active site of $CobT_{Se}$ resulted in a loss of regiospecificity.

Interestingly, we found that the previously uncharacterized [6-OMeBza]Cba **21** isomer, which has not been observed as a native cobamide of any organism, has slightly higher biological activity in *S. enterica* than [5-OMeBza]Cba **20**. We hypothesize that differences in isomer reactivity, as indicated by the observed variation in pK_a, could explain the observed differences in the biological activities of the two isomers. It is also possible that the higher activity of [6-OMeBza]Cba **21** is due to a greater similarity between this cobamide and one of the native cobamides of *S. enterica*, Factor A (2-methyladeninylcobamide).[43] Specifically, the orientation of the methoxy substituent in [6-OMeBza]Cba **21** is more similar than that of [5-OMeBza]Cba **20** to the orientation of the methyl group in Factor A. The *S. enterica* ethanolamine ammonia-lyase enzyme could be evolutionarily adapted for binding cobamides with a substituent in this position.

The existence of cobamide isomers with lower ligands attached at either of two nitrogen atoms, as we describe here, has previously been proposed based on the appearance of a second cobamide species in extracts of native cobamides containing asymmetric benzimidazole lower ligands.[31,44−46] These cobamides, comprising up to 20% of the total cobamide content of some bacteria and archaea,[44] were not characterized structurally, and their biological activities were not determined. The present work provides a likely explanation for this observation, as the CobT homologues of these organisms could direct the formation of two isomers that may both function as cofactors.

This work and previous studies have demonstrated that the substrate specificity and regiospecificity of CobT can be altered by structure- and homology-guided design of active site variants by introducing a small number of point mutations.[10,13,37] Because of the success of such manipulations, CobT is emerging as a model for engineering altered specificity in enzyme active sites. For example, a recent study demonstrated the successful design of a gain-of-function variant of $CobT_{Se}$ with the ability to phosphoribosylate *p*-cresol, an activity that is naturally restricted to ArsAB homologues.[37] In the present work, we have shown that a single point mutation in the same enzyme results in relaxed regiospecificity. The ability to produce mutants that can accommodate an expanded range of substrates, or act on additional sites of a single substrate, is a desired outcome of efforts to engineer bacterial enzymes for the biosynthesis of novel natural product variants.

ASSOCIATED CONTENT

Supporting Information

Additional figures including data for the *in vitro* characterization of the α-5-OHBza-RP, α-6-OHBza-RP, [5-OHBza]Cba, and [6-OHBza]Cba and 1D and 2D NMR analysis of the ribosides and cobamides is provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

HPLC, high-performance liquid chromatography; UV−vis, ultraviolet−visible; LC-MS, liquid chromatography-tandem mass spectrometry; EIC, extracted ion chromatogram; NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple-bond correlation; DMB, 5,6-dimethylbenzimidazole; 5-OHBza, 5-hydroxybenzimidazole; 5-OMeBza, 5-methoxybenzimidazole; 5-MeBza, 5-methylbenzimidazole; 5-OHBza-RP, α-5-hydroxybenzimidazole-riboside phosphate; 6-OHBza-RP, α-6-hydroxybenzimidazole-riboside phosphate; 5-OMeBza-RP, α-5-methoxybenzimidazole-riboside phosphate; 6-OMeBza-RP, α-6-methoxybenzimidazole-riboside phosphate; 5-OHBza-R-OH, α-5-hydroxybenzimidazole-riboside; 6-OHBza-R-OH, α-6-hydroxybenzimidazole-riboside; 5-OMeBza-R-OH, α-5-methoxybenzimidazole-riboside; 6-OMeBza-R-OH, α-6-methoxybenzimidazole-riboside; Cba, cobamide; [5-OHBza]Cba, 5-hydroxybenzimidazolylcobamide; [6-OHBza]CbA, 6-hydroxybenzimidazolylcobamide; [5-OMeBza]Cba, 5-methoxybenzimidazolylcobamide; [6-OMeBza]Cba, 6-methoxybenzimidazolylcobamide; Cbi, cobinamide

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