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Analysis of Substrate Specificity in CobT Homologs Reveals Widespread Preference for DMB, the Lower Axial Ligand of Vitamin B_{12}

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SUMMARY

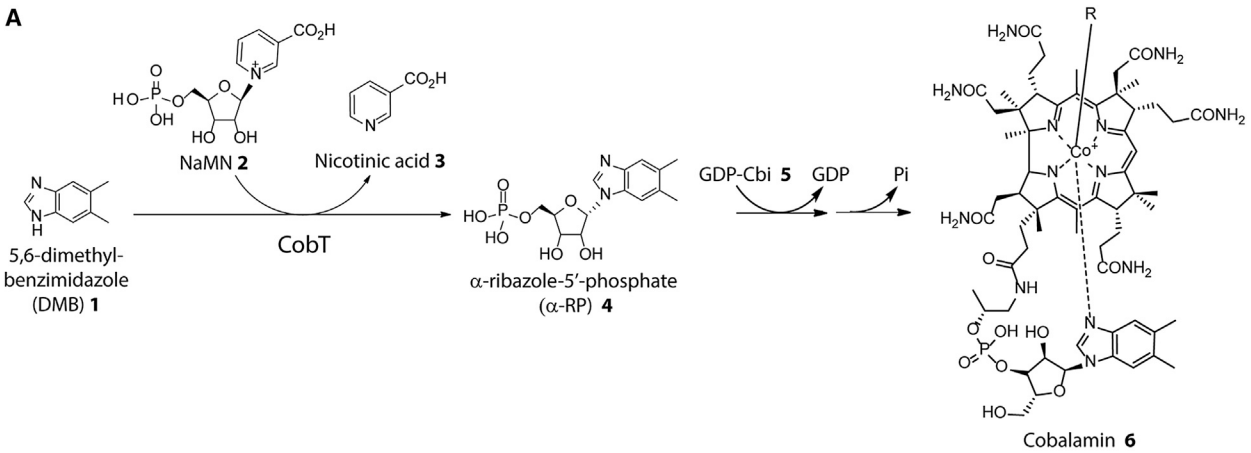
Cobamides such as vitamin B_{12} (cobalamin) are produced exclusively by prokaryotes and used by many other organisms as cofactors for diverse metabolic processes. Cobamides are cobalt-containing tetrapyrroles with upper and lower axial ligands. The structure of the lower ligand varies in cobamides produced by different bacteria. We investigated the biochemical basis of this structural variability by exploring the reactivity of homologs of CobT, the enzyme responsible for activating lower ligand bases for incorporation into cobamides. Our results show that CobT enzymes can activate a range of lower ligand substrates, and the majority of the enzymes tested preferentially attach 5,6-dimethylbenzimidazole (DMB), the lower ligand of cobalamin. This suggests that many bacteria that synthesize cobamides other than cobalamin in pure culture may produce cobalamin in mixed communities by attaching DMB when it is available in the environment.

INTRODUCTION

Vitamin B_{12} (cobalamin **6**, Figure 1A) is an essential cofactor for a wide range of animals, protists, bacteria, and archaea. In humans, cobalamin **6** is an essential micronutrient because of its role as a cofactor for two major enzymes, methylmalonyl CoA mutase and methionine synthase (Banerjee and Ragsdale, 2003). In bacteria, cobalamin **6** facilitates diverse metabolic processes such as the biosynthesis and catabolism of amino acids, modification of tRNA, reductive dehalogenation, and acetogenesis (Frey et al., 1988; Kräutler, 2005; Roth et al., 1996).

Cobalamin **6** is unique among the vitamins because it is synthesized only by prokaryotes (Roth et al., 1996; Warren et al., 2002). The structure of cobalamin **6** consists of a cobalt-containing corrin ring, an upper axial ligand, and a lower axial ligand that is tethered to the corrin macrocycle via the nucleotide loop (Figure 1A) (Roth et al., 1996). Two coenzyme forms of cobalamin **6**, in which the upper ligand is either 5′-deoxyadenosine or a methyl group, catalyze radical and methyl transfer reactions, respectively (Brown, 2005). Cobalamin **6** is a member of the cobamide family of cofactors that have structural variability in the lower ligand and the nucleotide loop (Kräutler et al., 2003; Renz, 1999). The lower ligands of cobamides identified thus far belong to three chemical classes: benzimidazoles, purines, and phenolics (Figure 1B) (Renz, 1999). Cobamide-synthesizing organisms typically produce cobamides with only one or two different lower ligands when grown in pure culture. Although it is known that a variety of cobamides are present in complex microbial communities, important questions remain regarding the molecular factors responsible for specificity in the structure of the lower ligand (Allen and Stabler, 2008; Girard et al., 2009; Herbert et al., 1984).

The function of 5,6-dimethylbenzimidazole (DMB **1**), the lower ligand of cobalamin **6** (the best studied cobamide), has been investigated in several cobamide-dependent enzymes (Kräutler, 1990, 2009). In enzymes such as class II ribonucleotide reductase, diol dehydratase, and ethanolamine ammonia lyase, cobalamin **6** has been shown to bind in the "base-on" form, in which a nitrogen atom in DMB **1** is coordinated to the cobalt center, as shown in Figure 1A (Abend et al., 1999; Lawrence et al., 1999; Martens et al., 2002; Yamanishi et al., 1998). Other enzymes, such as cobalamin-dependent methionine synthase, methylmalonyl CoA mutase, and glutamate mutase, bind cobalamin **6** in the "base-off" form, in which DMB **1** is not coordinated to the cobalt center but remains covalently attached to the nucleotide loop (Drennan et al., 1994; Mancia et al., 1996; Tollinger et al., 2001). In both base-on and base-off enzymes, the structure of the lower ligand may affect the binding and catalytic ability of the cobamide cofactor (Hamza et al., 2005; Kräutler and Ostermann, 2003). Because the structure of the lower ligand affects cobamide function, in some cases bacteria have mechanisms to limit cobamide production to those forms that support their enzymatic functions (Chan and Escalante-Semerena, 2011; Gray and Escalante-Semerena, 2009a; Mok and Taga, 2013). In the accompanying article by Crofts et al. (2013, in this issue of *Chemistry & Biology*), we report that the range of cobamides that can be synthesized in several bacteria is limited by molecular factors encoded by the *cobT* gene. The CobT enzyme catalyzes the activation of the lower ligand base by phosphoribosylation to form an α-ribosylated product (Fyfe and Friedmann, 1969; Trzebiatowski and Escalante-Semerena, 1997). The α-ribosylated product is subsequently incorporated as the lower ligand of the cobamide (Cameron et al., 1991). X-ray crystallographic studies of *Salmonella enterica* CobT showed that several benzimidazoles, purines, and phenolic compounds can be bound in the active site, and all but the phenolic compounds can be used as substrates for phosphoribosylation (Cheong et al., 2001). The inability of *Sa. enterica* to activate phenolic compounds is



B

Lower ligand base	α-riboside phosphate
5-methylbenzimidazole (MeBza) **7**	N^{I} (5′-phospho-α-ribosyl)-5-methylbenzimidazole (MeBza-RP) **8**
5-hydroxybenzimidazole (OHBza) **9**	N^{I} (5′-phospho-α-ribosyl)-5-hydroxybenzimidazole (OHBza-RP) **10**
5-methoxybenzimidazole (OMeBza) **11**	N^{I} (5′-phospho-α-ribosyl)-5-methoxybenzimidazole (OMeBza-RP) **12**
Benzimidazole (Bza) **13**	N^{I} (5′-phospho-α-ribosyl)-benzimidazole (Bza-RP) **14**
Adenine (Ade) **15**	Adenosine-α-monophosphate (Ade-RP) **16**
Phenol (Phe) **17**	O- (5′-phospho-α-ribosyl)-phenol (Phe-RP) **18**
p-cresol (Cre) **19**	O- (5′-phospho-α-ribosyl)-*p*-cresol (Cre-RP) **20**

Figure 1. Activation and Attachment of Cobamide Lower Ligands

(A) The reaction catalyzed by CobT is shown with substrates DMB **1** and NaMN **2**, which form the products nicotinic acid **3** and α-RP **4**. α-RP **4** is subsequently attached to the cobamide precursor, 5′-deoxyadenosylcobinamide-guanosine diphosphate (GDP-Cbi **5**), to form cobalamin **6**.

(B) CobT catalyzes the activation of several other lower ligand bases to form the corresponding α-riboside phosphate products. The structures of the lower ligand bases used in this study and the corresponding α-riboside phosphate products are shown. The name of each compound is given with abbreviations in parentheses.

significant because *Sa. enterica* is unable to use phenolyl cobamides (Chan and Escalante-Semerena, 2011). In contrast, the ArsAB enzyme of *Sporomusa ovata* can activate both DMB **1** and phenolic compounds, despite the fact that growth of *Sp. ovata* on several substrates is impaired when benzimidazolyl cobamides are produced instead of phenolyl cobamides (Chan and Escalante-Semerena, 2011; Mok and Taga, 2013).

In the accompanying article by Crofts et al. (2013), we describe a genetic analysis of six *cobT* homologs derived from phylogenetically diverse organisms. We showed that the *cobT* gene controls the range of lower ligand bases that can be attached to form cobamides using heterologous expression studies in *Sinorhizobium meliloti* as well as studies in the native organism. This analysis revealed differences in the ability of the CobT homologs to activate lower ligand substrates but did not compare preferences for the different substrates. In the present study, we characterize the substrate specificity of the CobT enzyme homologs in vitro in order to test the hypothesis that

each enzyme has the highest affinity for the lower ligand base corresponding to the cobamide that is produced by the organism. The chosen enzymes are from six phylogenetically diverse bacteria that synthesize cobamides with lower ligands that collectively represent all three structural classes (Figure 1B). The bacteria analyzed include *Si. meliloti*, which produces cobalamin **6** (Kliewer and Evans, 1962); *Sa. enterica*, which produces pseudo-B_{12} ([Ade]Cba) and 2-methyladeninyl cobamide (Keck and Renz, 2000); *Lactobacillus reuteri*, which produces [Ade]Cba (Santos et al., 2007); *Desulfovibrio vulgaris*, which produces guanyl cobamide and hypoxanthyl cobamide (Guimarães et al., 1994); and *Dehalococcoides mccartyi*, which produces benzimidazolyl cobamides only when cultured with a cobamide precursor and a benzimidazole (Yi et al., 2012). The CobT homologs from these five organisms will be abbreviated as *Sm*CobU, *Se*CobT, *Lr*CobT, *Dv*CobT, and *Dm*CobT, respectively. We also analyzed two CobT enzymes of *Veillonella parvula*, which synthesizes cobamides with *p*-cresol (Cre, **19**) and benzimidazole (Bza, **13**) as the lower ligands. One CobT enzyme of *V. parvula*, *Vp*CobT, is unable to activate phenolic compounds, whereas the other, *Vp*ArsAB, is capable of activating phenolic compounds and appears to function as a heterodimer of the *arsA* and *arsB* gene products, similar to the previously characterized ArsAB enzyme of *Sp. ovata* (Chan and Escalante-Semerena, 2011; Newmister et al., 2012, Crofts et al., 2013). Our results show that, surprisingly, six of the seven CobT enzymes tested display a strong preference for DMB **1**, despite the fact that cobalamin **6** is not synthesized by the majority of these bacteria. Moreover, adenine (Ade **15**), which is the lower ligand of the cobamide produced by both *Sa. enterica* and *L. reuteri*, is the least preferred substrate of the CobT homologs of all of the bacteria tested. These results indicate that bacteria may be capable of activating a wide range of lower ligand substrates and that DMB **1** may be preferentially attached as a lower ligand when it is available in the environment.

RESULTS

Characterization of the Formation of α-Ribazole Phosphate by *Sm*CobU

Previous in vitro studies of the biosynthesis of α-ribazole phosphate (α-RP **4**) with cell extracts of *Propionibacterium shermanii* (Friedmann, 1965) and purified *Se*CobT (Trzebiatowski and Escalante-Semerena, 1997) used radioactive thin layer chromatography to resolve ^{14}C-labeled DMB **1** and α-RP **4**. Because we sought to analyze reactions with multiple substrates that are commercially available only in their unlabeled form, we developed a high-performance liquid chromatography (HPLC)-based assay that is capable of resolving all of the substrates and products shown in Figure 1. In the reaction containing purified *Sm*CobU and the substrates DMB **1** and nicotinate mononucleotide (NaMN **2**), the substrates were nearly completely eliminated, and three products were formed (Figure 2A). One product co-elutes with the nicotinic acid **3** standard and was also formed in the reaction lacking DMB **1**, suggesting that hydrolysis of NaMN **2** to nicotinic acid **3** could be achieved by *Sm*CobU in the absence of DMB **1**, as observed previously with *Se*CobT (Cheong et al., 1999) (Figure 2A). The other two products are not present in control reactions lacking either the enzyme or one of the substrates and did not co-elute with any of the standards (Figure 2A).

We performed liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) analysis on the reactions shown in Figure 2A to identify the two reaction products. A compound corresponding to the m/z 359.1 expected for α-RP **4** was detected at 8 min (the same retention time as the second peak in Figure 2A) in the full reaction but was absent in the control reactions (Figure 2B). The total ion spectrum for the peak at 8 min showed a species with an m/z of 359.1 corresponding to α-RP **4** and another species at 381.1 corresponding to its Na-adduct (Figure 2C). An additional in-source fragmentation product with the m/z 147.1 expected for DMB **1** was also observed (Figure 2C). We purified the peak at 8 min by HPLC from a large-scale reaction and analyzed it by one-dimensional ^{1}H NMR. The spectrum obtained and the integration of the proton signals were consistent with the structure of α-RP **4** (Figure 2D). Analysis of the reaction product that eluted at 14 min (Figure 2A) showed that it is likely to have resulted from dephosphorylation of α-RP **4**, as its retention time is consistent with it being less polar than α-RP **4** due to the loss of the phosphate group, and the m/z corresponds to that of α-ribazole (data not shown). This peak appears only in reactions that proceed for 48 hr or longer, suggesting that it is the result of spontaneous nonenzymatic hydrolysis of α-RP **4**.

Previous studies of *Se*CobT have shown that DMB **1** can be activated with multiple mono- and dinucleotide cosubstrates such as NaMN **2**, nicotinamide mononucleotide (NMN **23**), nicotinate adenine dinucleotide (NaAD **21**), and nicotinamide adenine dinucleotide (NAD **25**), although the specific activity of *Se*CobT with NaMN **2** or NMN **23** was higher than with NaAD **21** or NAD **25** (Figure S1; Maggio-Hall and Escalante-Semerena, 2003). As the present study focuses on the lower ligand substrates, we aimed to select one of the four riboside phosphate cosubstrates to use for the analysis of all seven CobT homologs. The reactivity of each cosubstrate was determined for *Sm*CobU and *Se*CobT with DMB **1** as the lower ligand substrate. The corresponding ribosylated products were formed in all four reactions after 48 hr (Figure S1 available online), but product formation occurred after 1 min only in reactions containing NaMN **2** and NMN **23** (data not shown). We further tested the reactivity of *Sm*CobU, *Se*CobT, *Dm*CobT, and *De*CobT with cosubstrates NaMN, NMN, and NAD for the other four benzimidazole bases and adenine and found that NAD did not react with any of the bases tested and only NaMN and NMN gave products for all the bases. Additionally, as observed previously with *Se*CobT, we found that the reaction of *Sm*CobU with NaMN **2** was faster than with NMN **23** (data not shown) (Maggio-Hall and Escalante-Semerena, 2003). Therefore, NaMN **2** was used for all subsequent reactions.

Next, to determine the reactivity of *Sm*CobU with each lower ligand substrate, reactions were performed with NaMN **2** and each of the lower ligand bases shown in Figure 1B. HPLC analysis showed the formation of a product with a unique retention time in each reaction except in those containing phenol (Phe **17**) or Cre **19** (Figure 3A). In all cases, LC-ESI-MS and one-dimensional ^{1}H NMR analysis confirmed the identity of the α-riboside phosphate product corresponding to the lower ligand substrate used in each reaction (Figures S2A–S2E).

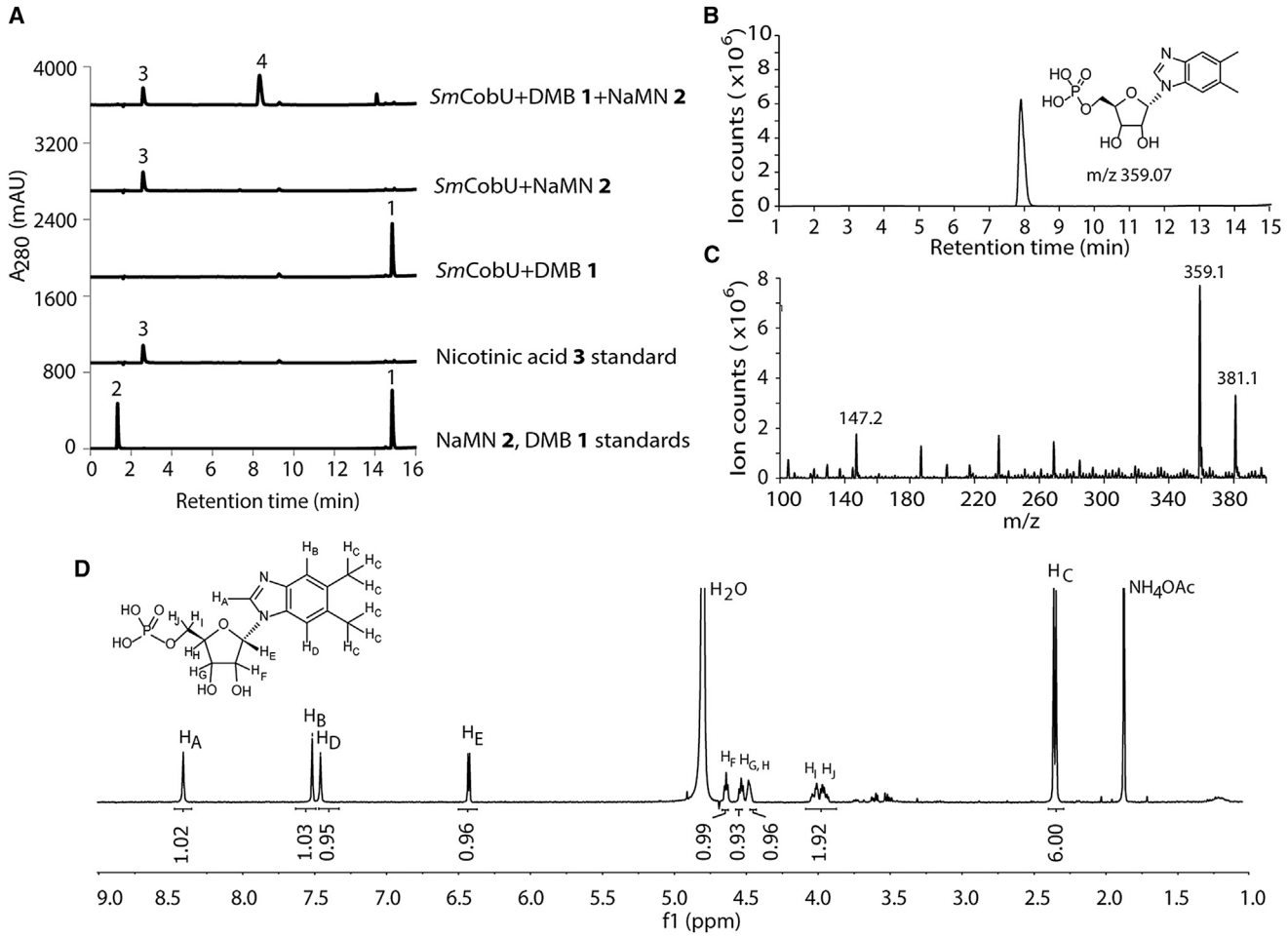


Figure 2. Reaction Catalyzed by *Sm*CobU

(A) HPLC analysis of the reaction containing *Sm*CobU, DMB **1**, and NaMN **2** is shown. Also shown are control reactions lacking each component and the standards for DMB **1**, NaMN **2**, and nicotinic acid **3**.

(B) Positive mode LC-ESI-MS of the reaction containing *Sm*CobU, DMB **1**, and NaMN **2** showing the extracted ion count for m/z 359.1, which corresponds to α-RP **4**.

(C) Total ion spectrum of the α-RP **4** peak at 8 min displays peaks at m/z 359.1, 381.1 and 147.2, which correspond to α-RP **4**, Na-adduct of α-RP **4**, and DMB **1**, respectively.

(D) ^{1}H NMR analysis of purified α-RP **4** is shown. The spectrum shows the ribose ring protons (H_E 6.4 ppm s, H_F 4.6 ppm m, H_G 4.5 ppm m, H_H 4.4 ppm m, H_I, H_J 4.0 ppm dd), the benzimidazole ring protons (H_A 8.4 ppm s, H_B 7.51 ppm s, 7.45 ppm s) and methyl group protons (H_C 2.34 ppm d), consistent with the expected structure of α-RP 4. The residual NH_4OAc buffer peak is visible at 1.91 ppm.

See also Figure S1.

In Vitro Characterization of CobT Homologs Reveals Their Ability to Activate a Range of Lower Ligand Substrates

To compare the reactivity of the other six CobT enzymes, each enzyme was purified and tested in reactions containing NaMN **2** and each of the eight lower ligand bases shown in Figure 1. The reactions were analyzed by HPLC as described above. In addition to the benzimidazole substrates and Ade **15**, our HPLC method also resolved the phenolic substrates and corresponding products, as shown in reactions containing *Vp*ArsAB (Figure 3B). The reactivity of each enzyme was calculated based on the percent depletion of the lower ligand substrate after 48 hr (Table S1). This analysis showed that all of the enzymes were capable of activating all five benzimidazoles and Ade **15**, but only the *Vp*ArsAB enzyme could activate Phe **17** and Cre **19** (Figure 3B; Table S1). The reaction containing purified *Vp*ArsA showed neither NaMN **2** hydrolysis nor formation of α-RP **4** (data not shown), consistent with previous studies (Chan and Escalante-Semerena, 2011; Crofts et al., 2013). Most of the enzymes showed over 90% depletion of the lower ligand substrate (Table S1). The reactions that did not go to completion may indicate a slow rate of conversion or instability of the enzyme over the reaction time. We verified the identity of each α-riboside phosphate product by LC-ESI-MS and one-dimensional ^{1}H NMR (Figures S2A–S2G).

Quantitative Determination of the Relative Preferences of CobT Enzyme Homologs for Lower Ligand Substrates

We next determined the relative preference of each CobT enzyme for each lower ligand substrate by performing reactions

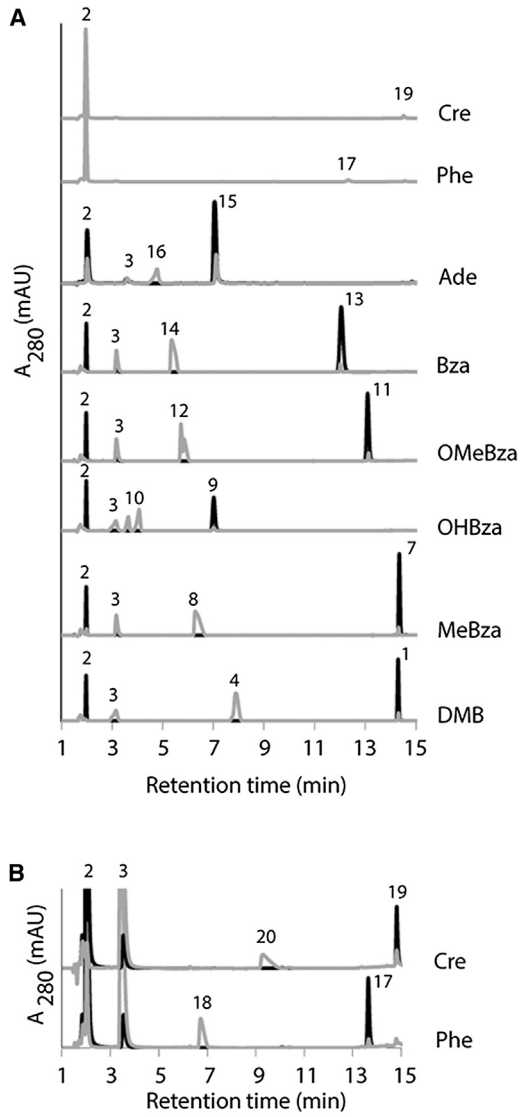


Figure 3. Activation of Lower Ligand Bases by CobT Enzymes

(A) HPLC analysis of reactions containing *Sm*CobU, NaMN **2**, and the indicated lower ligand substrates are shown. Black traces show control reactions in which *Sm*CobU is absent, and gray traces show complete reactions. Numbers correspond to the compounds shown in Figure 1.

(B) Reactions containing *Vp*ArsAB, NaMN **2**, and phenolic substrates are shown. Numbers above peaks represent compounds verified by LC-ESI-MS. See also Figure S2 and Table S1.

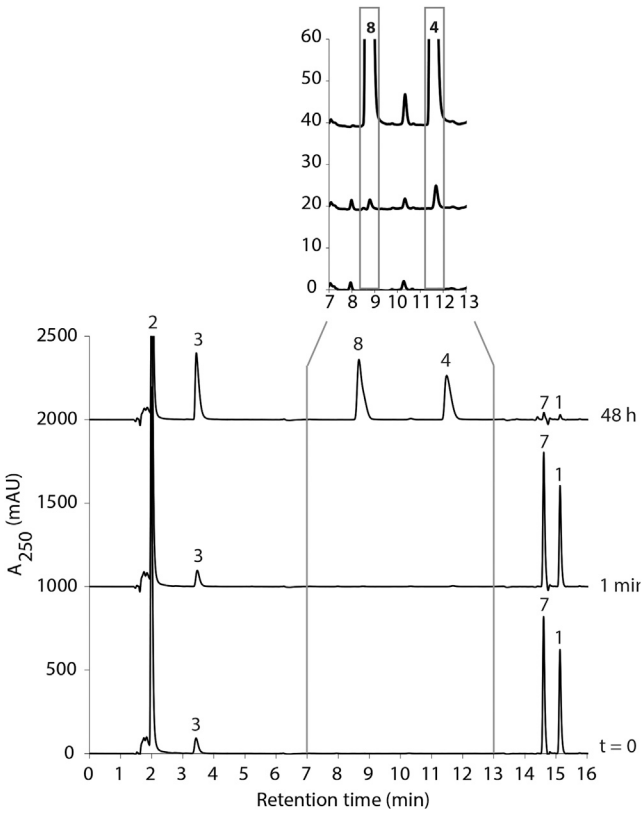


Figure 4. HPLC Analysis of a Competition Reaction between DMB 1 and MeBza 7

Reactions containing *Sm*CobU, NaMN **2**, and equimolar amounts of DMB **1** and MeBza **7** were quenched at the indicated time points. The amplified inset shows the product peaks α-RP **4** and MeBza-RP **8** (indicated by the boxes) that are formed from the corresponding lower ligand substrate. The nicotinic acid **3** present in the t = 0 reaction was formed in the process of quenching the reaction. Numbers above peaks represent compounds verified by LC-ESI-MS. See also Figure S3.

containing equal concentrations of two lower ligand substrates under steady state conditions. These pairwise competition reactions were carried out with all combinations of lower ligand substrates that showed reactivity in Table S1. HPLC analysis of a representative competition reaction containing *Sm*CobU with DMB **1** and 5-methylbenzimidazole (MeBza **7**) is shown in Figure 4. In this reaction, DMB **1** appears to be used preferentially compared to MeBza **7**, because the peak corresponding to the DMB **1** reaction product, α-RP **4**, is substantially larger than 5-methylbenzimidazole riboside phosphate (MeBza-RP **8**) after 1 min (Figure 4). By 48 hr, both substrates were nearly completely converted to the corresponding α-riboside phosphate products (Figure 4).

In order to calculate the ratios of products formed in each competition reaction, the concentrations of the α-riboside phosphate products formed in each reaction after 1 min were determined based on their HPLC peak areas. These compounds are not commercially available for use as standards, and their UV-Vis spectra differ considerably from the corresponding lower ligand base (Figure S3). Therefore, to measure the concentrations of the α-riboside phosphate products formed, the concentration of each purified compound was determined by Electronic REference To access In vivo Concentrations (ERETIC) NMR, and a standard curve was generated for each α-riboside phosphate product as described in Experimental Procedures. The molar ratios calculated for each α-riboside phosphate product formed in the competition reactions containing *Sm*CobU are shown in Table 1. We found that in all reactions containing DMB **1** and another substrate, α-RP **4** was formed in higher amounts than the other α-riboside phosphate product (Table 1). *Sm*CobU also showed distinct preferences among the other lower ligand substrates, with all of the benzimidazoles preferred over

Table 1. Pairwise Competition Reactions with *Sm*CobU

	DMB	MeBza	OMeBza	Bza	OHBza
MeBza	2.4 ± 0.2				
OMeBza	39 ± 3	17.3 ± 0.4			
Bza	163 ± 0.7	55 ± 3	3.6 ± 0.1		
OHBza	172 ± 31	357 ± 21	81 ± 5	32 ± 1	
Ade	400 ± 14	>287[a]	>232[a]	>128[a]	>146[a]

The ratio of products formed after 1 min in reactions containing two lower ligand substrates is shown for *Sm*CobU. The molar ratio of the concentration of the α-riboside phosphate products formed in each pairwise lower ligand substrate competition reaction is given (column:row). Numbers represent the average of three independent experiments with SE. See also Table S2 for the ratio of products formed after 1 min in reactions containing two lower ligand substrates for all the other CobT enzymes.
[a]Ade-RP was not detected. The value given is based on the detection limit of the assay.

Ade **15** (Table 1). The results of pairwise competition reactions performed with the other CobT enzyme homologs are shown in Table S2.

The ratios of products formed in each pairwise competition reaction were used to calculate the overall percent preference of each CobT enzyme for each lower ligand substrate (Figure 5). Surprisingly, the overall preferences of six of the seven enzymes are remarkably similar, with all showing a strong preference for DMB **1** (68%–77%), despite the fact that only one of the CobT homologs, *Sm*CobU, is derived from an organism that synthesizes cobalamin **6** de novo (Figures 5A–5F). In five of these six enzymes, MeBza **7** is the second most preferred substrate, and the preference for the other benzimidazoles totals <10%. The only exception to this trend is *Vp*ArsAB, which shows similar preference for its native lower ligand, Cre **19**, and four of the benzimidazoles (Figure 5G). Interestingly, Ade **15** is the least preferred substrate of all of the CobT homologs.

Competition for Benzimidazoles in *Si. meliloti* Cultures Validates In Vitro Results

We next sought to test whether the substrate preferences observed in vitro reflect the preferences of bacteria for the incorporation of lower ligand bases into cobamides. The *Si. meliloti bluB* mutant was used to assay the relative level of incorporation of DMB **1** and other lower ligand bases into cobamides. The *bluB* mutant is incapable of producing DMB **1** and does not produce a cobamide unless a lower ligand base is provided (Campbell et al., 2006). Only the benzimidazoles were tested because the presence of endogenously synthesized Ade **15** precluded the manipulation of Ade **15** concentrations. Cultures of the *Si. meliloti bluB* mutant strain were supplemented with both DMB **1** and a competing benzimidazole base at molar ratios varying from 10^{-4} to 1, and corrinoids were extracted and analyzed by HPLC to measure the ratios of the cobamides produced. We found that, consistent with the in vitro experiments, DMB **1** was greatly preferred over the other benzimidazoles (Figure 6). The ratio of substrates that resulted in equal concentrations of the two cobamides produced (SD_{50}) was calculated for each benzimidazole (Figure 6). This analysis showed that MeBza **7** was the second most preferred substrate, with Bza **13**, OHBza **9**, and OMeBza **11** incorporated less favorably, similar to *Sm*CobU analyzed in vitro (compare Figures 5A and 6).

DISCUSSION

The synthesis of cobamide cofactors is exclusive to prokaryotes, and cobamide-producing organisms studied to date are known to synthesize only one or two different cobamides (Martens et al., 2002). Cobamides have been characterized extensively for the reactivity conferred by the upper ligand, but the role of the lower ligand in catalysis and the biochemical basis of the diversity in lower ligand structure have been relatively less studied. The accompanying article by Crofts et al. (2013) shows that the range of lower ligand bases that can be attached to form cobamides is limited by the substrate specificity of CobT. In this article, we examine the relative preferences of the CobT enzymes for the different lower ligand bases. We tested seven CobT enzyme homologs in vitro in order to understand how substrate preferences differ among CobT enzymes. These enzymes were chosen because they are derived from bacteria that collectively synthesize a diverse range of cobamides in pure culture. We expected that each enzyme would have highest preference for the lower ligand base found in the cobamide native to each organism. However, we were surprised to find that six of the enzymes exhibit remarkably similar substrate preferences and have a considerably higher preference for DMB **1**. The benzimidazole substrate competition in cultures of *Si. meliloti bluB* also confirmed the preference for DMB **1** in comparison to other benzimidazoles.

The ability of CobT homologs to activate a limited range of lower ligand bases is important because not all cobamides are functionally equivalent. For example, the methionine synthase of *Arthrospira platensis* shows a higher affinity for [Ade]Cba than cobalamin **6**, whereas *Rhodobacter sphaeroides* requires cobalamin **6** and is incapable of using [Ade]Cba (Gray and Escalante-Semerena, 2009b; Tanioka et al., 2010). In addition, we recently found that *Sp. ovata* requires phenolyl cobamides when using certain carbon sources and that only three benzimidazolyl cobamides can be utilized by *Dc. mccartyi* (Mok and Taga, 2013; Yi et al., 2012). Similarly, in the accompanying article by Crofts et al. (2013), we find that the degree to which different cobamides support the growth of *Si. meliloti* varies. These differences in cobamide function may suggest adaptations of cobamide-dependent enzymes for specific cobamides on the basis of their lower ligands.

Based on these observations that cobamide-dependent enzymes in different bacteria are optimized for the use of different cobamides, it is surprising that the CobT enzyme homologs tested in this study show a nearly universal preference for DMB **1**. Our results suggest that bacteria that preferentially activate DMB **1** may produce cobalamin **6** when DMB **1** is available in the environment, as is the case for *Sa. enterica* (Anderson et al., 2008). The preference of CobT for DMB **1** may indicate that cobalamin **6** functions more effectively than other cobamides in a wide variety of organisms. However, the production of cobalamin **6** by the incorporation of exogenous DMB **1** may not benefit some bacteria, as we have observed in *Sp. ovata* (Mok and Taga, 2013). It remains unclear whether the use of

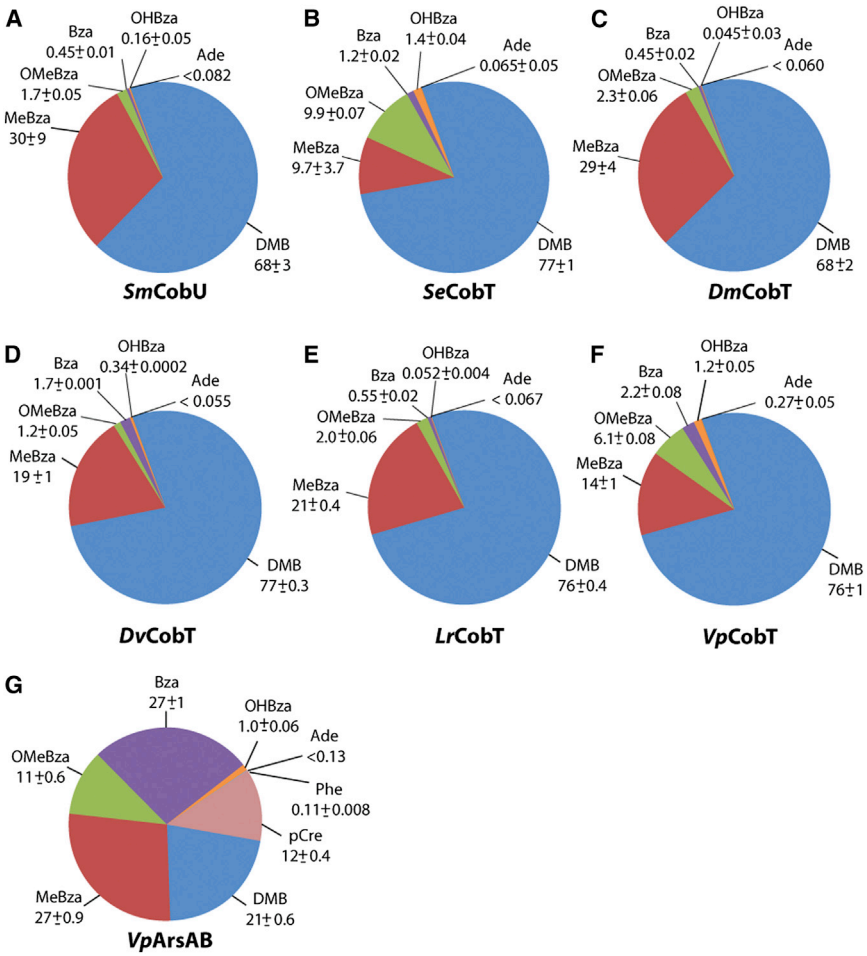


Figure 5. Overall Lower Ligand Substrate Preferences for Each CobT Enzyme Homolog under Conditions Assayed

The relative percent preference for each substrate was calculated from the ratios of α-riboside phosphate products in pairwise competition reactions with all combinations of lower ligand substrates for each purified CobT enzyme homolog under the same assay conditions. The ratios of products formed in each set of pairwise competition reactions are presented in Tables 1 and S2.

cobamides other than cobalamin **6** is an early or relatively recent evolutionary adaptation.

Our analysis showed that *Vp*ArsAB has distinct substrate preferences compared to the other six CobT enzymes tested and is the only enzyme in this study that is capable of activating phenolic compounds. The function of *Vp*ArsAB is similar to the well-characterized ArsAB enzyme from *Sp. ovata* in its ability to catalyze the phosphoribosylation of both phenolic compounds and benzimidazoles to form O-linked and N-linked glycosidic bonds, respectively. In contrast, phenolic compounds inhibit the hydrolysis of NaMN **2** in the other six CobT homologs, possibly due to their binding in the active site in an orientation such that water is excluded, as previously observed in a structural study of *Se*CobT (Cheong et al., 2001, 2002). The ability of ArsAB to activate phenolic compounds is likely due to structural features unique to the ArsAB heterodimer and the identities and positions of key active site residues (Cheong et al., 1999; Newmister et al., 2012). Despite the apparent specialization of ArsAB for the activation of phenolic compounds, it is interesting to note that *Vp*ArsAB has slightly higher preference for three of the benzimidazoles compared to the phenolic compounds. The fact that *V. parvula* also possesses the *Vp*CobT enzyme that has a higher preference for benzimidazoles suggests that the ability of *Vp*ArsAB to activate benzimidazoles could be a vestigial function (Näsvall et al., 2012).

*Dv*CobT was included in this study because *Dv. vulgaris* is the only organism reported to synthesize guanyl and hypoxanthyl cobamides (Guimarães et al., 1994). However, we were surprised to find that neither guanine nor hypoxanthine could be used as a substrate for *Dv*CobT in vitro or in a *Si. meliloti* strain expressing *Dv. vulgaris cobT* (data not shown). It is possible that *Dv. vulgaris* possesses an enzyme other than *Dv*CobT that activates guanine and hypoxanthine for incorporation into cobamides, analogous to *V. parvula*, which has two CobT enzymes with different substrate specificities. Although no other *cobT* homolog appears to be present in the *Dv. vulgaris* genome, a novel gene may be responsible for this activity.

The results of this in vitro study are largely consistent with our analysis of lower ligand attachment in the accompanying article (Crofts et al., 2013). The lower ligand attachment profiles of the CobT homologs measured in *Si. meliloti* and in vitro are parallel except that Ade **15** can be used as a substrate by all of the CobT enzymes in vitro, whereas [Ade]Cba is produced only by some of the *Si. meliloti* strains expressing *cobT* homologs (Crofts et al., 2013). Based on our studies of the reactivity of the CobT homologs with various lower ligand substrates (Table S1) and the competition assays indicating that Ade **15** is far less preferred relative to the other substrates (Figure 5), it is likely that [Ade]Cba is synthesized by all of the *Si. meliloti* strains, but in some cases at quantities that could not be detected by our methods.

A possible explanation for the preference of the CobT enzymes for benzimidazoles over Ade **15** is that cobamides other than [Ade]Cba function more efficiently as cofactors in these organisms. If this is the case, the lower affinity of CobT enzymes for Ade **15** may serve to prevent cells from incorporating Ade **15,** which is available in the cell, when more favorable lower ligand bases are present. This hypothesis is consistent with previous observations that *Sa. enterica* attaches DMB **1** instead of Ade **15** when DMB **1** is available and that both cobalamin **6** and [Ade]Cba function in *Sa. enterica* (Anderson et al., 2008). It is also consistent with our results in *Si. meliloti*, but not in *L. reuteri*, which is unable to incorporate benzimidazoles into cobamides (Santos et al., 2007; Crofts et al., 2013).

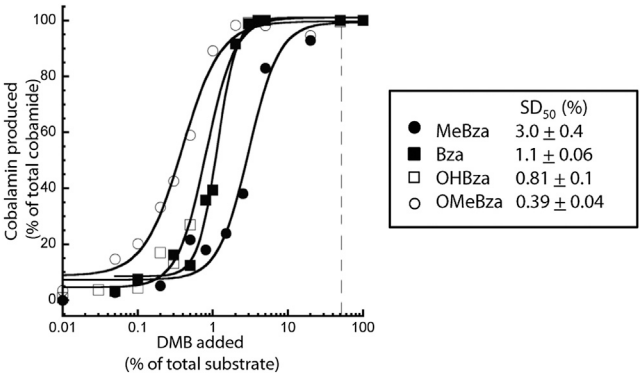


Figure 6. Competition for Lower Ligand Attachment in *Si. meliloti*

The *Si. meliloti bluB* mutant was cultured with DMB **1** and another benzimidazole at different ratios prior to corrinoid extraction and HPLC analysis. Each point shows the relative amount of cobalamin **6** present in a culture containing DMB **1** and another benzimidazole at the ratio indicated on the x-axis, expressed as a percentage of the total cobamides in the culture. The calculated SD_{50} values, which represent the percent DMB **1** added to cultures that would result in the production of equal levels of the two cobamides, are given for each competing benzimidazole. The dashed vertical line is drawn at the point representing equal levels of DMB **1** and the competing benzimidazole.
See also Table S3.

The substrate preferences we report here are based on in vitro reactions in which two lower ligand substrates are in direct competition for a limited amount of CobT enzyme. These reactions may mimic environmental conditions in which multiple substrates for CobT are available. CobT presumably encounters multiple potential substrates in bacteria that synthesize benzimidazolyl or phenolyl cobamides because other bases such as Ade **15** are also present in the cell. Consistent with this idea, we have detected free benzimidazoles in microbial communities, indicating that benzimidazole bases are available in some environments (Y. Men, E.C. Seth., S. Yi, T.S.C., R.H. Allen, M.E.T., and L. Alvarez-Cohen, unpublished data). Based on this observation, it is possible that benzimidazolyl cobamides are produced in the environment by bacteria that are incapable of synthesizing benzimidazoles. However, [Ade]Cba has also been found in significant proportions in several environments, indicating that a subpopulation in the community produces [Ade]Cba even when DMB **1** is present (Allen and Stabler, 2008; Girard et al., 2009; Y. Men, E.C. Seth., S. Yi, T.S.C., R.H. Allen, M.E.T., and L. Alvarez-Cohen, unpublished data).

The cobamide family of cofactors is unique in that the structure of the lower ligand, a site removed from the catalytic center, varies depending on the organism. Although structural variability has been observed in cofactors such as heme, NAD/NADP, flavins, and pterins, extensive organism-specific variability has only been observed in cobamides (Bowman and Bren, 2008; Carugo and Argos, 1997; Kritsky et al., 2010; Li et al., 2011). Cobamides are also unique in that their biosynthesis is restricted to less than half of the bacteria that use them and requires the investment of a significant proportion of the genome (Lawrence and Roth, 1996; Zhang et al., 2009). For these reasons, bacteria may have evolved to synthesize and use specific cobamides as a means of preventing competitive sequestration of cobamides by other organisms or promoting cooperative interactions with organisms that require specific cobamides. Further studies of the functions of structurally diverse variants of these cofactors will be necessary to understand their roles in catalysis and in the environment.

SIGNIFICANCE

Vitamin B_{12} (cobalamin) is a member of the cobamide family of cofactors that function in diverse biological processes with applications in natural product biosynthesis, bioremediation, biofuels production, and medicine. Cobamides consist of a cobalt-containing corrin ring and upper and lower axial ligands. The lower ligand is structurally variable in the cobamides produced by different bacteria. Here, we have explored the biochemical basis of this structural variability. We analyzed the substrate specificity of seven homologs of CobT, the enzyme that catalyzes the activation of the lower ligand base prior to incorporation into a cobamide. Based on the results of competition assays with pairs of lower ligand substrates, we found that six of the seven CobT enzymes have a higher preference for 5,6-dimethylbenzimidazole (DMB), the lower ligand of cobalamin, compared to the other seven substrates tested. This nearly universal preference for DMB is surprising because most bacteria produce cobamides other than cobalamin when cultured in the absence of an added lower ligand base. This preference for DMB suggests that in some bacteria, DMB may be attached when it is available in the environment and could be an indication that cobalamin functions more efficiently than their native cobamide. Our results further show that adenine is the least preferred substrate for all of the CobT enzymes, including two derived from bacteria that produce [Ade]Cba when cultured without an added lower ligand base. Together, our results suggest that the cobamide produced by bacteria in pure culture may often differ from the cobamide produced in the environment.

EXPERIMENTAL PROCEDURES

Molecular Techniques

To construct plasmids for the expression of CobT homologs fused to an N-terminal hexahistidine tag, each *cobT* open reading frame was amplified by PCR and cloned into the vector pET28a. For *L. reuteri cobT*, a codon-optimized version in plasmid pMP220+P_{trp} (Crofts et al., 2013) was used as the template for PCR. For *V. parvula arsAB*, the *arsA* and *arsB* genes were amplified together from *V. parvula* genomic DNA, and only *arsA* was fused to the His tag. *Escherichia coli* strain DH5α was used for all cloning procedures. Each plasmid was introduced into *E. coli* BL21(DE3) for expression and purification.

Overexpression and Purification of CobT Enzyme Homologs

E. coli strain BL21(DE3) containing each CobT expression plasmid was grown in LB medium containing 25 μg ml^{-1} kanamycin at 37°C with aeration at 180 rpm until the optical density at 600 nm (OD_{600}) reached 0.6. Protein overexpression was induced at 25°C with aeration at 90 rpm for 16 hr by the addition of isopropyl-β-D-thiogalactopyranoside at a final concentration of 250 μM. The cells were harvested by centrifugation, and the resulting cell pellets were stored at −80°C. Cell pellets from 1 l of culture were resuspended in 30 ml lysis buffer (50 mM sodium phosphate, 100 mM NaCl, pH 8.0) and lysed by sonication. The resulting cell lysate was clarified by centrifugation, and the CobT protein was loaded onto a Ni-NTA column and purified according to the manufacturer's instructions (BioRad). After elution, the protein was transferred into

50 mM Tris-HCl pH 7.0 or 7.5 containing 20% glycerol using a 10-DG resin (BioRad) and stored at −80°C.

In Vitro Assay for CobT Enzyme Activity

Reactions were performed with 10 μM of the CobT enzyme, 2 mM NaMN **2**, 10 mM $MgCl_2$, and 250 μM of lower ligand base in 50 mM Tris-HCl pH 7.5 for the indicated time. Control reactions lacking enzyme, NaMN **2**, or lower ligand base were also conducted when each substrate was tested for the first time. Reactions were quenched by the addition of formic acid at a final concentration of 4% at 100°C for 1 min. The quenched reaction was then immediately placed on ice, and NaOH was added to each reaction at a final concentration of 0.75 mM to raise the pH to 5. The precipitated protein was removed by centrifugation prior to analysis by reverse-phase HPLC.

Analysis of the reactions was performed on an Agilent Technologies 1200 series HPLC system equipped with a 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% to 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.

Chemical Analysis of α-Riboside Phosphate Products

LC-ESI-MS analysis of the reactions was performed on an Agilent Technologies 6410 liquid chromatograph-triple quadrupole mass spectrometer using the chromatography method described above. Samples were analyzed using an Agilent Eclipse XDB C-18 column (5 μm, 4.6 × 150 mm) or a Zorbax SB-Aq column (5 μm, 4.6 × 150 mm) and separated at 1 ml min^{-1} using the method described above. The α-riboside phosphate products were detected using an MS2 scan (gas flow rate 8.5 l min^{-1}, temperature 325°C). To prepare samples for one-dimensional ^1H NMR analysis, *Se*CobT was used to generate each of the α-riboside phosphate products except phenolyl-riboside phosphate (Phe-RP **18**) and p-cresolyl-riboside phosphate (Cre-RP **20**), and *Vp*ArsAB was used to produce Phe-RP **18** and Cre-RP **20**, in 3 ml reactions. The products were separated by HPLC as described above, and fractions were collected using an Agilent 1200 series fraction collector connected in line with the HPLC. The purified products were then lyophilized to dryness and resuspended in 100% D_2O. The pH was measured to be 6–6.5 for all of the samples.

Determination of the Concentration of the α-Riboside Phosphate Products

To determine the concentrations of the α-riboside phosphate products, 0.7–1.8 mg of each α-riboside phosphate product was purified by HPLC from large-scale CobT reactions. Each product was then subjected to ERETIC NMR (Akoka et al., 1999). The electronic calibration signal for the concentration was generated with standard samples of 50 mM nicotinic acid **3** in triplicate. The concentration of each sample was determined by comparison to the electronic reference by using the nonexchangeable H_D proton doublet signal and the H_A proton singlet signal. These samples were used to generate standard curves with the HPLC method described above. The concentrations of α-riboside phosphate reaction products analyzed by HPLC were determined by comparison of the peak areas with the standard curves.

In Vitro Competition Assays

Reactions were performed with 1 μM of enzyme, 2 mM NaMN **2**, and two lower ligand substrates, each at 250 μM, in 50 mM Tris buffer pH 7.5 with 10 mM $MgCl_2$. Aliquots were quenched following 0 min, 1 min, 5 min, and 48 or 72 hr and analyzed by HPLC using a Zorbax SB-Aq column (5 μm, 4.6 × 150 mm) with the method described above.

In reactions in which one of the α-riboside phosphate products was not detectable at 1 min, reactions were performed with the concentration of one substrate increased to 5 mM.

To calculate the percent preference of each enzyme for DMB **1**, the ratios of the competing α-riboside phosphate products to α-RP **4** formed in each competition reaction containing DMB **1** were calculated. The reciprocal of the sum of these ratios plus the ratio of products formed if DMB **1** were competed with itself (1) was expressed as a percent, indicating the preference for DMB **1**. The preferences for each of the other lower ligand substrates were calculated similarly based on the ratios of the products of the remaining competition reactions.

Competition Assay to Determine Relative Preferences for Benzimidazoles in *Si. meliloti*

The *Si. meliloti bluB*::*gus* Gm^R strain (Campbell et al., 2006) was used for in vivo competition experiments. M9 minimal medium was supplemented with 1 mg ml^{-1} methionine, 10 ng ml^{-1} biotin, and 10 μM $CoCl_2$ (Maniatis et al., 1982). For analysis of cobamides in the *Si. meliloti* cultures containing two benzimidazoles, 5 ml of M9 was inoculated with a colony of *Si. meliloti* grown on LBMC (Maniatis et al., 1982) and incubated for 40–50 hr at 30°C with aeration. These cultures were diluted to an OD_{600} of 0.02 to 0.04 in 250 ml M9 containing both DMB **1** and another benzimidazole at concentrations totaling 5 μM. Cells were harvested following 48–60 hr of growth at 30°C with aeration, and corrinoids were extracted and analyzed by HPLC as described (Crofts et al., 2013). Concentrations of each cobamide were calculated based on integrated peaks areas at 550 nm as compared to a standard curve of cyanocobalamin. SD_{50} values were determined by fitting the data to a sigmoidal curve using the KaleidaGraph 4.0 (Synergy Software). The equation used for the sigmoidal fit is $y = m1 + (m2 - m1)/(1 + (x/m3)\wedge m4)$, where m1 and m2 are the maximum and minimum normalized y-axis values (B_{12} produced) respectively, m3 is the SD_{50}, and m4 is the slope of the curve. The values for each individual fit are reported in Table S3.

SUPPLEMENTAL INFORMATION

Supplemental Information contains three figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2013.08.007.

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REFERENCES

Abend, A., Bandarian, V., Nitsche, R., Stupperich, E., Rétey, J., and Reed, G.H. (1999). Ethanolamine ammonia-lyase has a "base-on" binding mode for coenzyme B(12). Arch. Biochem. Biophys. *370*, 138–141.

Akoka, S., Barantin, L., and Trierweiler, M. (1999). Concentration measurement by proton NMR using the ERETIC method. Anal. Chem. *71*, 2554–2557.

Allen, R.H., and Stabler, S.P. (2008). Identification and quantitation of cobalamin and cobalamin analogues in human feces. Am. J. Clin. Nutr. *87*, 1324–1335.

Anderson, P.J., Lango, J., Carkeet, C., Britten, A., Kräutler, B., Hammock, B.D., and Roth, J.R. (2008). One pathway can incorporate either adenine or dimethylbenzimidazole as an alpha-axial ligand of B12 cofactors in *Salmonella enterica*. J. Bacteriol. *190*, 1160–1171.

Banerjee, R., and Ragsdale, S.W. (2003). The many faces of vitamin B12: catalysis by cobalamin-dependent enzymes. Annu. Rev. Biochem. *72*, 209–247.

Bowman, S.E.J., and Bren, K.L. (2008). The chemistry and biochemistry of heme c: functional bases for covalent attachment. Nat. Prod. Rep. *25*, 1118–1130.

Brown, K.L. (2005). Chemistry and enzymology of vitamin B12. Chem. Rev. *105*, 2075–2149.

Cameron, B., Blanche, F., Rouyez, M.C., Bisch, D., Famechon, A., Couder, M., Cauchois, L., Thibaut, D., Debussche, L., and Crouzet, J. (1991). Genetic

analysis, nucleotide sequence, and products of two Pseudomonas denitrificans cob genes encoding nicotinate-nucleotide: dimethylbenzimidazole phosphoribosyltransferase and cobalamin (5′-phosphate) synthase. J. Bacteriol. *173*, 6066–6073.

Campbell, G.R.O., Taga, M.E., Mistry, K., Lloret, J., Anderson, P.J., Roth, J.R., and Walker, G.C. (2006). *Sinorhizobium meliloti bluB* is necessary for production of 5,6-dimethylbenzimidazole, the lower ligand of B12. Proc. Natl. Acad. Sci. USA *103*, 4634–4639.

Carugo, O., and Argos, P. (1997). NADP-dependent enzymes. I: Conserved stereochemistry of cofactor binding. Proteins *28*, 10–28.

Chan, C.H., and Escalante-Semerena, J.C. (2011). ArsAB, a novel enzyme from *Sporomusa ovata* activates phenolic bases for adenosylcobamide biosynthesis. Mol. Microbiol. *81*, 952–967.

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

Cheong, C.G., Escalante-Semerena, J.C., and Rayment, I. (2001). Structural investigation of the biosynthesis of alternative lower ligands for cobamides by nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase from *Salmonella enterica*. J. Biol. Chem. *276*, 37612–37620.

Cheong, C.-G., Escalante-Semerena, J.C., and Rayment, I. (2002). Capture of a labile substrate by expulsion of water molecules from the active site of nicotinate mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase (CobT) from *Salmonella enterica*. J. Biol. Chem. *277*, 41120–41127.

Crofts, T.S., Seth, E.C., Hazra, A.B., and Taga, M.E. (2013). Cobamide structure depends on both lower ligand availability and CobT substrate specificity. Chem. Biol. *20*. Published online September 19, 2013. http://dx.doi.org/10.1016/j.chembiol.2013.08.006.

Drennan, C.L., Huang, S., Drummond, J.T., Matthews, R.G., and Ludwig, M.L. (1994). How a protein binds B12: A 3.0 A X-ray structure of B12-binding domains of methionine synthase. Science *266*, 1669–1674.

Friedmann, H.C. (1965). Partial purification and properties of a single displacement trans-N-glycosidase. J. Biol. Chem. *240*, 413–418.

Frey, B., McCloskey, J., Kersten, W., and Kersten, H. (1988). New function of vitamin B12: cobamide-dependent reduction of epoxyqueuosine to queuosine in tRNAs of *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. *170*, 2078–2082.

Fyfe, J.A., and Friedmann, H.C. (1969). Vitamin B12 biosynthesis. Enzyme studies on the formation of the alpha-glycosidic nucleotide precursor. J. Biol. Chem. *244*, 1659–1666.

Girard, C.L., Santschi, D.E., Stabler, S.P., and Allen, R.H. (2009). Apparent ruminal synthesis and intestinal disappearance of vitamin B12 and its analogs in dairy cows. J. Dairy Sci. *92*, 4524–4529.

Gray, M.J., and Escalante-Semerena, J.C. (2009a). The cobinamide amidohydrolase (cobyric acid-forming) CbiZ enzyme: a critical activity of the cobamide remodelling system of *Rhodobacter sphaeroides*. Mol. Microbiol. *74*, 1198–1210.

Gray, M.J., and Escalante-Semerena, J.C. (2009b). In vivo analysis of cobinamide salvaging in *Rhodobacter sphaeroides* strain 2.4.1. J. Bacteriol. *191*, 3842–3851.

Guimarães, D.H., Weber, A., Klaiber, I., Vogler, B., and Renz, P. (1994). Guanylcobamide and hypoxanthylcobamide-corrinoids formed by *Desulfovibrio vulgaris*. Arch. Microbiol. *162*, 272–276.

Hamza, M.S.A., Zou, X., Banka, R., Brown, K.L., and van Eldik, R. (2005). Kinetic and thermodynamic studies on ligand substitution reactions and base-on/base-off equilibria of cyanoimidazolylcobamide, a vitamin B12 analog with an imidazole axial nucleoside. Dalton Trans. *4*, 782–787.

Herbert, V., Drivas, G., Manusselis, C., Mackler, B., Eng, J., and Schwartz, E. (1984). Are colon bacteria a major source of cobalamin analogues in human tissues? 24-hr human stool contains only about 5 micrograms of cobalamin but about 100 micrograms of apparent analogue (and 200 micrograms of folate). Trans. Assoc. Am. Physicians *97*, 161–171.

Keck, B., and Renz, P. (2000). *Salmonella typhimurium* forms adenylcobamide and 2-methyladenylcobamide, but no detectable cobalamin during strictly anaerobic growth. Arch. Microbiol. *173*, 76–77.

Kliewer, M., and Evans, H.J. (1962). Effect of cobalt deficiency on the B12 coenzyme content of *Rhizobium meliloti*. Arch. Biochem. Biophys. *97*, 427–429.

Kräutler, B. (1990). Chemistry of methylcorrinoids related to their roles in bacterial C1 metabolism. FEMS Microbiol. Rev. *7*, 349–354.

Kräutler, B. (2005). Vitamin B12: chemistry and biochemistry. Biochem. Soc. Trans. *33*, 806–810.

Kräutler, B. (2009). Organometallic chemistry of B12 coenzymes. In Metal Ions in Life Sciences, R.K. Sigel, S. Astrid, and S. Helmut, eds. (Cambridge, UK: Royal Society of Chemistry), pp. 1–51.

Kräutler, B., and Ostermann, S. (2003). The Porphyrin Handbook. (San Diego: Elsevier Science).

Kräutler, B., Fieber, W., Ostermann, S., Fasching, M., Ongania, K.-H., Gruber, K., Kratky, C., Mikl, C., Siebert, A., and Diekert, G. (2003). The cofactor of tetrachloroethene reductive dehalogenase of *Dehalospirillum multivorans* is norpseudo-B12, a new type of a natural corrinoid. Helv. Chim. Acta *86*, 3698–3716.

Kritsky, M.S., Telegina, T.A., Vechtomova, Y.L., Kolesnikov, M.P., Lyudnikova, T.A., and Golub, O.A. (2010). Excited flavin and pterin coenzyme molecules in evolution. Biochemistry. Biokhimiia *75*, 1200–1216.

Lawrence, J.G., and Roth, J.R. (1996). Evolution of coenzyme B12 synthesis among enteric bacteria: evidence for loss and reacquisition of a multigene complex. Genetics *142*, 11–24.

Lawrence, C.C., Gerfen, G.J., Samano, V., Nitsche, R., Robins, M.J., Rétey, J., and Stubbe, J. (1999). Binding of Cob(II)alamin to the adenosylcobalamin-dependent ribonucleotide reductase from Lactobacillus leichmannii. Identification of dimethylbenzimidazole as the axial ligand. J. Biol. Chem. *274*, 7039–7042.

Li, T., Bonkovsky, H.L., and Guo, J.T. (2011). Structural analysis of heme proteins: implications for design and prediction. BMC Struct. Biol. *11*, 13.

Maggio-Hall, L.A., and Escalante-Semerena, J.C. (2003). Alpha-5,6-dimethylbenzimidazole adenine dinucleotide (alpha-DAD), a putative new intermediate of coenzyme B12 biosynthesis in *Salmonella typhimurium*. Microbiology *149*, 983–990.

Mancia, F., Keep, N.H., Nakagawa, A., Leadlay, P.F., McSweeney, S., Rasmussen, B., Bösecke, P., Diat, O., and Evans, P.R. (1996). How coenzyme B12 radicals are generated: the crystal structure of methylmalonyl-coenzyme A mutase at 2 A resolution. Structure *4*, 339–350.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Plainview, NY: Cold Spring Harbor Laboratory Press).

Martens, J.H., Barg, H., Warren, M.J., and Jahn, D. (2002). Microbial production of vitamin B12. Appl. Microbiol. Biotechnol. *58*, 275–285.

Mok, K.C., and Taga, M.E. (2013). Growth inhibition of *Sporomusa ovata* by incorporation of benzimidazole bases into cobamides. J. Bacteriol. *195*, 1902–1911.

Näsvall, J., Sun, L., Roth, J.R., and Andersson, D.I. (2012). Real-time evolution of new genes by innovation, amplification, and divergence. Science *338*, 384–387.

Newmister, S.A., Chan, C.H., Escalante-Semerena, J.C., and Rayment, I. (2012). Structural insights into the function of the nicotinate mononucleotide:-phenol/p-cresol phosphoribosyltransferase (ArsAB) enzyme from *Sporomusa ovata*. Biochemistry *51*, 8571–8582.

Renz, P. (1999). Biosynthesis of the 5,6-dimethylbenzimidazole moiety of cobalamin and of the other bases found in natural corrinoids. In Chemistry and Biochemistry of B12, R. Banerjee, ed. (New York: Wiley), pp. 557–576.

Roth, J.R., Lawrence, J.G., and Bobik, T.A. (1996). Cobalamin (coenzyme B12): synthesis and biological significance. Annu. Rev. Microbiol. *50*, 137–181.

Santos, F., Vera, J.L., Lamosa, P., de Valdez, G.F., de Vos, W.M., Santos, H., Sesma, F., and Hugenholtz, J. (2007). Pseudovitamin B12 is the corrinoid produced by *Lactobacillus reuteri* CRL 1098 under anaerobic conditions. FEBS Lett. *581*, 4865–4870.

Tanioka, Y., Miyamoto, E., Yabuta, Y., Ohnishi, K., Fujita, T., Yamaji, R., Misono, H., Shigeoka, S., Nakano, Y., Inui, H., and Watanabe, F. (2010). Methyladeninylcobamide functions as the cofactor of methionine synthase in a Cyanobacterium, *Spirulina platensis* NIES-39. FEBS Lett. *584*, 3223–3226.

Tollinger, M., Eichmüller, C., Konrat, R., Huhta, M.S., Marsh, E.N., and Kräutler, B. (2001). The B12-binding subunit of glutamate mutase from *Clostridium tetanomorphum* traps the nucleotide moiety of coenzyme B12. J. Mol. Biol. *309*, 777–791.

Trzebiatowski, J.R., and Escalante-Semerena, J.C. (1997). Purification and characterization of CobT, the nicotinate-mononucleotide:5,6-dimethylbenzimidazole phosphoribosyltransferase enzyme from *Salmonella typhimurium* LT2. J. Biol. Chem. *272*, 17662–17667.

Warren, M.J., Raux, E., Schubert, H.L., and Escalante-Semerena, J.C. (2002). The biosynthesis of adenosylcobalamin (vitamin B12). Nat. Prod. Rep. *19*, 390–412.

Yamanishi, M., Yamada, S., Muguruma, H., Murakami, Y., Tobimatsu, T., Ishida, A., Yamauchi, J., and Toraya, T. (1998). Evidence for axial coordination of 5,6-dimethylbenzimidazole to the cobalt atom of adenosylcobalamin bound to diol dehydratase. Biochemistry *37*, 4799–4803.

Yi, S., Seth, E.C., Men, Y.-J., Stabler, S.P., Allen, R.H., Alvarez-Cohen, L., and Taga, M.E. (2012). Versatility in corrinoid salvaging and remodeling pathways supports corrinoid-dependent metabolism in *Dehalococcoides mccartyi*. Appl. Environ. Microbiol. *78*, 7745–7752.

Zhang, Y., Rodionov, D.A., Gelfand, M.S., and Gladyshev, V.N. (2009). Comparative genomic analyses of nickel, cobalt and vitamin B12 utilization. BMC Genomics *10*, 78.