Chemically similar yet functionally distinct: Selectivity of methylmalonyl-CoA mutase among naturally diverse cobamide cofactors

By

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Abstract

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Vitamin B_{12} is predominantly associated with human health, and many people are surprised to learn that B_{12} is produced by bacteria. Like humans, bacteria have metabolic enzymes that require B_{12} as a cofactor. B_{12}-dependent enzymes catalyze chemical reactions required for DNA, amino acid, and secondary metabolite synthesis, as well as for use of various carbon and energy sources. Curiously, in addition to producing B_{12}, bacteria and archaea produce and use chemical analogs of B_{12} called cobamides, which share the key structural features of B_{12} but have small chemical differences in a part of the molecule called the lower ligand. Differences in lower ligand structure, while small, are sufficient to make cobamides functionally distinct. Microorganisms are known to prefer certain cobamides over others for growth, which is ecologically important and could also be harnessed as a way to promote or inhibit the growth of specific organisms as an alternative to probiotics or antibiotics. However, the biochemical mechanisms that drive differential cobamide use in bacteria are not understood sufficiently to pursue such an application. Moreover, the response of humans to the diverse cobamides produced by microorganisms has not been fully explored.

I have used a combination of *in vitro* biochemistry and bacterial growth assays to dissect the functional differences between cobamides. The central hypothesis of this work is that a major determinant of the specific cobamide requirements of an organism is the ability of its cobamide-dependent enzymes to use diverse cobamides as cofactors. To better understand the mechanisms by which enzymes are affected by lower ligand structure, I selected a model enzyme, methylmalonyl-CoA mutase (MCM), which is one of the two cobamide-dependent enzymes in humans and is also relatively widespread in bacteria. Using bacterial MCM orthologs from *Sinorhizobium meliloti*, *Escherichia coli*, and *Veillonella parvula*, I found that the major effect of changes in lower ligand structure is alteration of the binding affinity of cobamides for MCM, with smaller effects on enzyme activity. I observed different cobamide-binding selectivity in MCM from different bacteria, which correlated with the cobamides produced by each respective organism, consistent with potential coevolution of cobamide production and use in cobamide-producing bacteria. Importantly, the cobamide-dependent growth of *S. meliloti* was largely consistent with the cobamide selectivity of the MCM enzyme in this organism, supporting the

hypothesis that enzyme selectivity is an important mechanism by which cobamides differentially affect bacterial growth.

In addition to examining bacterial MCM orthologs, I analyzed the ability of human MCM to use cobamides with diverse lower ligands. It is assumed that humans require exclusively B_{12}, but prior to this work the ability of human MCM to use diverse cobamides had not been tested. I found that, in fact, human MCM is able to bind to, and is catalytically active with, several cobamides other than B_{12}, suggesting that other cobamides could be relevant for human physiology. Having discovered this, I tested whether any cobamides besides cobalamin improved the activity of six MCM variants containing disease-associated mutations. Although I did not observe any significant rescue of activity in the mutated MCM enzymes, I did find that, like the WT enzyme, multiple cobamides supported activity of these mutants, reinforcing the potential for application of diverse cobamides in studies of human cobalamin metabolism.

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Dedicated to my family.

Chapter 1

Introduction

Vitamins are micronutrients that humans require in their diet. The largest category of vitamins, B vitamins, are grouped based on their biochemical roles as enzyme cofactors – molecules that bind to particular enzymes and are required by those enzymes to carry out chemical reactions. Major metabolic pathways, including DNA synthesis, sugar metabolism, amino acid metabolism, steroid and fatty acid biosynthesis, and energy production, involve enzymes that use vitamin cofactors. These pathways are not unique to humans; thus, although vitamins are most commonly associated with human health, all life forms, including single-celled organisms like bacteria, require vitamins.

By definition, humans are incapable of producing vitamins. Most vitamins can be synthesized by plants, but one, vitamin B_{12}, is produced exclusively by microorganisms, specifically bacteria and archaea. In addition to its relative structural complexity compared to other cofactors, what makes B_{12} unique is that it is chemically diverse. Bacteria and archaea make more than a dozen different forms of vitamin B_{12} which share common features but have small structural variations. Why microbes produce so many forms of B_{12} remains unknown.

To better understand the biological implications of B_{12} diversity, I have investigated how structural differences between B_{12} forms affect its biochemical function. Part of this dissertation describes a study of the effects of diverse B_{12} variants on the activity of a human B_{12}-dependent enzyme. A large part, however, is devoted to the use of B_{12} vitamins by bacteria themselves. While only two enzymes in humans require vitamin B_{12}, there are over 15 bacterial enzyme families that use this cofactor, and the vast majority of bacteria have at least one B_{12}-dependent enzyme. Despite widespread use of this cofactor in the bacterial domain, only a subset of bacteria can synthesize B_{12}; most rely on other species to obtain this cofactor. Thus, B_{12} is increasingly appreciated not just as an enzyme cofactor, but also as an important driver of microbial associations in natural ecosystems.

I. Introduction to cobalamin and cobamides

B_{12}, hereafter referred to as cobalamin, has been recognized as an essential vitamin for humans for almost a century. Cobalamin is part of a large family of cofactors, all produced exclusively by bacteria and archaea, called cobamides. Recently, the requirement of bacteria for cobalamin and other cobamides has gained appreciation. Shelton et al. reported that, out of 11,000 sequenced bacterial species, 86% have at least one cobamide-dependent enzyme [Shelton et al., 2019]. Cobamides are functionally similar but chemically diverse, and the significance of diversity in this cofactor family for its use by bacteria is not fully understood. This section describes the chemical structure of cobamides and the biological importance of cobamide diversity.

Chemical structure of cobalamin and other cobamides

The chemical structure of cobalamin (Figure 1A) has three main features. First, cobalamin contains a corrin ring macrocyle that coordinates an ion of cobalt. The corrin ring is a tetrapyrrole, related to the porphyrin rings of heme and chlorophyll. Second, a pseudonucleotide with the base 5,6-dimethylbenzimidazole (DMB, blue in Figure 1A) is covalently tethered to the corrin ring.

Third, the central cobalt ion axially coordinates two ligands. The α ("lower") ligand is the DMB base; the β ("upper") ligand (R in Figure 1A) varies. In the vitamin form of cobalamin, the upper ligand is cyanide. This form, cyanocobalamin, is highly stable but only found after isolation of cobalamin in the presence of cyanide. In the biological forms of cobalamin, the upper ligand to the cobalt ion is a methyl group (methylcobalamin), 5'-deoxyadenosine (adenosylcobalamin), or water, in the absence of other ligands. The cobalt ion and upper ligand constitute the chemically reactive part of the cofactor, directly participating in enzyme catalysis.

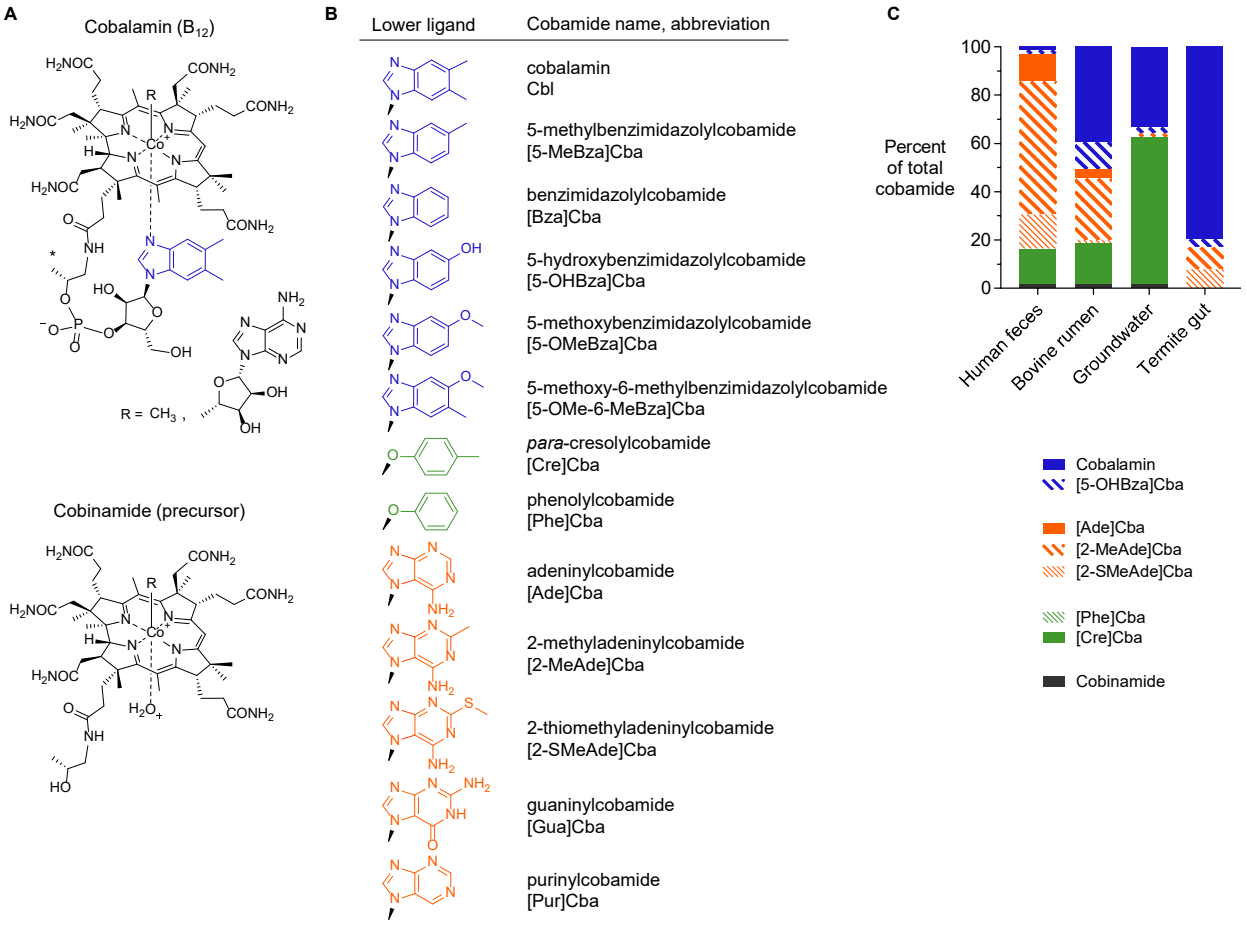


Figure 1: Diverse cobamides produced by bacteria and archaea. (A) The structure of cobalamin (B_{12}). The upper ligand, R, forms the catalytic cobalt-carbon bond of the cofactor. The lower ligand, blue, varies among cobamides. Nor-cobamides lack the methyl group on the propionamide linker (asterisk). Cobinamide, below, is a cobamide precursor that lacks a nucleotide base. (B) Lower ligands of naturally occurring cobamides. Benzimidazoles, blue; phenolics, green; purines, orange. (C) Relative quantities of different cobamides found in various environments [Allen and Stabler, 2008; Girard et al., 2009; Men et al., 2015; Seth, unpublished].

Cobalamin synthesis is complex, and only ~37% of bacteria and a subset of archaea have the genetic potential to produce this cofactor [Shelton et al., 2019]; no other organisms are known to make cobalamin. The longest part of the cobalamin biosynthetic pathway, the synthesis of the precursor cobinamide (Figure 1A), requires ~23 genes; synthesis and attachment of the DMB base requires an additional ~6-10 genes. Interestingly, instead of attaching DMB as a lower ligand,

some bacteria and archaea biosynthesize and attach other benzimidazoles, purines, and phenolics (Figure 1B) [Allen and Stabler, 2008; Hazra et al., 2015; Hoffmann et al., 2000; Renz, 1999; Stupperich et al., 1988; Yan et al., 2018]. Thus, cobalamin belongs to a larger class of cofactors called cobamides which predominantly differ in the structure of the lower ligand. A second more recently discovered structural difference among cobamides is in the nucleotide loop; nor-cobamides are distinguished by the absence of one methyl moiety (starred, Figure 1A) [Keller et al., 2016; Kräutler et al., 2003]. All cobamides, irrespective of the specific features of the nucleotide loop, can share the same catalytic upper ligands.

We infer that diverse cobamides are biologically important based on their abundance in natural environments. For example, quite strikingly, cobalamin is expected to make up less than 5% of the total cobamides in the human gut, while purinyl cobamides are highly abundant [Allen and Stabler, 2008]. Moreover, the cobamide content of different microbial habitats is distinct; the relative amounts of benzimidazolyl, purinyl, and phenolyl cobamides differ between human feces, bovine rumen, groundwater, and the termite gut (Figure 1C) [Allen and Stabler, 2008; Girard et al., 2009; Men et al., 2015; Seth, unpublished]. Some environments also have detectable amounts of cobinamide (Figure 1C). This is consistent with a predicted mechanism of cobamide acquisition called cobinamide salvaging, genetically encoded in ~15% of bacterial genomes, in which organisms scavenge cobinamide and require only a portion of the biosynthetic pathway to complete the synthesis of a cobamide [Escalante-Semerena, 2007; Shelton et al., 2019].

Biological significance of cobamide structural diversity

In principle, all cobamides can catalyze the same types of chemical reactions because the methyl and 5'-deoxyadenosyl upper ligands, which are directly involved in catalysis, are common among cobamides. In practice, however, organisms cannot always use different cobamides interchangeably. For example, several species of eukaryotic algae require cobalamin for growth but cannot grow if [Ade]Cba is available instead [Helliwell et al., 2016]; the organohalide-respiring bacterium *Dehalococcoides maccartyi* grows with some, but not all, benzimidazolyl cobamides [Yi et al., 2012]; *Sporomusa ovata*, which natively produces phenolyl cobamides, cannot grow in certain cobamide-dependent conditions when it is made to overproduce benzimidazolyl cobamides [Mok and Taga, 2013]; and other organisms respond differentially to diverse cobamides as well [Keller et al., 2018; Watanabe et al., 1992; Yan et al., 2018; Yan et al., 2016].

Although some organisms can produce the cobamide they require, most bacteria that require cobamides are auxotrophs, meaning that their growth is dependent on the cobamides present in the environment or produced by neighboring microorganisms [Shelton et al., 2019]. Thus, the requirement of organisms for specific cobamides is important in the context of microbial interactions. For example, specific cobamide requirements drive an obligate association of a cobamide-dependent amoeba with two bacterial partners [Ma et al., 2017], influence the ability of a cobamide-requiring bacterium to colonize the mouse gut [Degnan et al., 2014a], and are implicated in shaping photosynthetic marine microbial communities [Heal et al., 2017; Helliwell et al., 2016] as well as TCE-dechlorinating consortia [Men et al., 2015; Yan et al., 2012].

II. Mechanisms by which cobamides differentially impact bacterial growth

Based on the finding that the sets of cobamides that support growth of different organisms are distinct, we and others have proposed to use cobamides to specifically promote or inhibit the growth of microbes of interest within a community. A major limitation to this vision is that there is currently no way to predict which cobamides are required by a given bacterial species. Our inability to predict cobamide requirements stems from a lack of understanding of the molecular mechanisms by which cobamide structure causes differences in growth. A major challenge surrounding this area of study is that cobamides other than cobalamin are not commercially available and must be purified from large volumes of bacterial cultures. Despite this challenge, there is growing evidence that various molecular processes are affected by cobamide structure. Our current model is that four main processes influence the growth of bacteria in the presence of specific cobamides: cobamide biosynthesis, cobamide import into cells, selectivity of cobamide-dependent enzymes, and cobamide-dependent gene regulation. Evidence for each is summarized in this section.

Cobamide biosynthesis

An organism that synthesizes cobamides should, presumably, produce the cobamide that bests supports its growth. Indeed, cobamide biosynthesis is generally thought of as a selective process, because most isolated, cultured bacteria produce a single cobamide [Shelton et al., 2019]. The molecular basis of specific cobamide production by bacteria has been largely investigated through studies of the substrate specificity of the biosynthetic enzyme CobT. CobT "activates" lower ligands for attachment to cobamides by appending a phospho-α-ribosyl moiety to the lower ligand base that will ultimately be incorporated into a cobamide. Heterologous expression of CobT orthologs from bacteria that produce different cobamides in *Sinorhizobium meliloti* changed the relative incorporation of different lower ligands into cobamides, suggesting that lower ligand selectivity is intrinsic to CobT [Crofts et al., 2013]. Moreover, changing specific residues in *S. meliloti* CobT to those found in other CobT orthologs altered the regiochemistry of lower ligand activation, attributing this additional aspect of cobamide biosynthesis to the CobT enzyme [Crofts et al., 2014].

However, *in vitro* studies of CobT orthologs from bacteria that produce different cobamides challenges this view; the lower ligand selectivity of CobT *in vitro* is very similar across different bacterial species, and CobT is not selective for the lower ligand of the cobamide produced in its native organism [Cheong et al., 2001; Hazra et al., 2013]. A notable exception are ArsA and ArsB, homologs of CobT that act together as a heterodimeric enzyme, which are unique in their ability to activate phenolic lower ligands and are found in all organisms known to produce phenolyl cobamides [Chan and Escalante-Semerena, 2012; Hazra et al., 2013]. The substrate promiscuity of both CobT and ArsAB may be the reason many organisms are capable of guided biosynthesis; providing an exogenous base can often lead to incorporation of that base into cobamides [Anderson et al., 2008; Hazra et al., 2015; Mok and Taga, 2013; Schubert et al., 2019; Yi et al., 2012]. Interestingly, cyanobacteria appear to be unable to incorporate DMB into cobamides, but the recently identified cyanobacterial CobT enzyme does activate DMB *in vitro*; thus, there are unidentified determinants of selective cobamide production in cells [Helliwell et al., 2016; Jeter et al., 2019].

A subset of organisms, including those that do not biosynthesize cobamides, are capable of cobamide remodeling, a process by which the lower ligand of a cobamide is removed and a different lower ligand is attached instead [Gray et al., 2008; Yi et al., 2012]. Remodeling requires the enzyme CbiZ, an amidohydrolase that hydrolyzes the amide moiety in the nucleotide loop, forming adenosyl cobyric acid, a cobamide precursor [Gray et al., 2008]. This enzyme is present in ~9% of sequenced bacterial genomes [Shelton et al., 2019]. Interestingly, CbiZ from *Rhodobacter sphaeroides* cleaves Ado[Ade]Cba *in vitro*, but not AdoCbl (Ado, 5'-deoxyadenosyl upper ligand). This selectivity is likely to be physiologically important, because the growth rate of *R. sphaeroides* in cobamide-dependent conditions is lower in the presence of [Ade]Cba than cobalamin [Gray and Escalante-Semerena, 2009]. Thus, it is advantageous for *R. sphaeroides* to remodel a suboptimal cobamide, while cleaving AdoCbl, a suitable cofactor, might negatively affect growth (although this has not been tested).

Cobamide import

There are three known bacterial cobamide importers: Btu(B)CDF, and the more recently identified ECF-CbrT [Santos et al., 2018] and BtuM [Rempel et al., 2018]. *Bacteroides thetaiotaomicron*, a prominent gut microbe, has three *btuB* homologs and two homologs of the *btuCDF* genes. Degnan *et al.* found that one *btuB* homolog in isolation conferred a competitive growth advantage to *B. thetaiotaomicron* in the presence of cobalamin, [2-MeAde]Cba, [5-OMeBzaCba], and [5-MeBza]Cba, while a second was advantageous in the presence of [Ade]Cba and [Bza]Cba [Degnan et al., 2014a]. This finding suggests that cobamide importers may selectively bind or preferentially transport specific cobamides, although this has not been shown directly. More direct evidence exists for selectivity of transport in *E. coli* BtuCD, which binds cobinamide four times more weakly than cobalamin, and transports cobinamide 1.5 times more slowly than cobalamin, *in vitro* [Mireku et al., 2017]. It remains unknown to what degree lower ligand structure in other cobamides affects import by BtuCD, but this could be tested using the *in vitro* system described by Mireku *et al*. Several organisms have yet unidentified cobamide importers (Chapter 2) or non-canonical proteins implicated in cobalamin uptake [Gopinath et al., 2013], so the overall the contribution of selective cobamide import to the physiology of bacteria remains largely unexplored.

Cobamide-selective gene regulation

The primary mechanisms of regulation of cobamide-dependent metabolisms in bacteria are corrinoid riboswitches [Mukherjee et al., 2019; Nahvi et al., 2004; Rodionov et al., 2003]. Riboswitches are RNA sequences in the 5' untranslated regions of genes that modulate transcription or translation based on their secondary structure, which depends on the presence or absence of a ligand. Cobalamin riboswitches bind cobalamin to turn off the expression of certain genes. Interestingly, the *in vitro* binding affinity of a cobalamin riboswitch regulating cobamide import in *E. coli* was highest for AdoCbl, 3-fold lower for Ado[2-MeAde]Cba, and 8-fold lower for Ado-cobinamide [Gallo et al., 2008], suggesting that lower ligand structure may modulate the binding of different cobamides to cobalamin riboswitches in cells. In support of this possibility, cell-based assays performed by Kristopher Kennedy and Florian Widner (Taga lab, unpublished) reveal that expression of a riboswitch-regulated reporter differs upon addition of structurally

diverse cobamides. Thus, we hypothesize that lower ligand diversity also results in differential gene regulation, additionally influencing the cobamide-dependent growth of bacteria.

Cofactor selectivity of cobamide-dependent enzymes

Enzyme assays and experiments in cell free lysates demonstrate that cobamide-dependent enzyme activity is affected by lower ligand structure. Enzymes that have been studied *in vitro* with regard to cobamide selectivity include methionine synthase [Kolhouse et al., 1991; Tanioka et al., 2010], methylmalonyl-CoA mutase [Lengyel et al., 1960; Poppe et al., 1997], reductive dehalogenase [Keller et al., 2018], glutamate mutase [Barker et al., 1960; Lengyel et al., 1960; Poppe et al., 2000], diol dehydratase, glycerol dehydratase [Poppe et al., 1997], 2-methyleneglutarate mutase, and ethanolamine ammonia lyase [Poppe et al., 2000]. In several enzymes that bind cobamides in the base-off/His-on conformation (see below), including methylmalonyl-CoA mutase, lower ligand structure affects the apparent K_M of cobamides, a value that is interpreted to reflect cobamide affinity [Lengyel et al., 1960; Poppe et al., 1997]. However, the binding affinity of cobamide-dependent enzymes for various cobamides has not been measured directly. In base-on enzymes, [Cre]Cba does not support activity but is a competitive inhibitor of cobalamin, suggesting that it does bind the enzyme despite differences in lower ligand structure [Poppe et al., 2000; Poppe et al., 1997]. In reductive dehalogenases, lower ligand structure appears to also play a role in enzyme maturation and processing (cleavage of the inner membrane translocation signal peptide), which could be due to importance of the lower ligand in proper enzyme folding [Keller et al., 2018]. The primary focus of the research described in this dissertation is further elucidating the importance of lower ligand structure for the biochemistry of cobamide-dependent enzymes.

III. Cobamide-dependent enzymes

Over two dozen cobamide-dependent enzymes have been identified to date. Cobamide-dependent enzymes are involved in many metabolic pathways, including breakdown of various carbon and energy sources [Barker, 1985; Berg et al., 2007; Chang and Frey, 2000; Chen et al., 2001; Erb et al., 2008; Ferguson and Krzycki, 1997; Forage and Foster, 1982; Jeter, 1990; Korotkova et al., 2002; Krasotkina et al., 2001; Ljungdahl, 1986; Scarlett and Turner, 1976; Stupperich and Konle, 1993; Vrijbloed et al., 1999], synthesis of primary metabolites like nucleotides and amino acids [Banerjee et al., 1989; Licht et al., 1996; Miles et al., 2011], and secondary metabolite synthesis [Allen and Wang, 2014; Bridwell-Rabb et al., 2017; Jung et al., 2014; Kim et al., 2017; Marous et al., 2015b; Pierre et al., 2012a; Werner et al., 2011]. The four most common cobamide-dependent enzymes in bacteria are methionine synthase (present in ~30 % of all genomes analyzed by Shelton *et al.*), which transfers a methyl group from methyltetrahydrofolate to homocysteine in the synthesis of methionine [Dorweiler et al., 2003]; epoxyqueuosine reductase, which is involved in the synthesis of an alternate tRNA base, queuosine [Miles et al., 2011]; ribonucleotide reductase, which converts ribonucleotides into deoxyribonuclotides [Licht et al., 1996]; and methylmalonyl-CoA mutase, which interconverts methylmalonyl-CoA and succinyl-CoA (detailed in the next section) [Shelton et al., 2019]. The most recently discovered, and poorly understood, cobamide-dependent enzymes fall into the expansive class of radical *S*-adenosyl-L-methionine (SAM)-B_{12} enzymes. These enzymes are highly versatile and many methylate unactivated carbon centers in the synthesis of natural products

[Bauerle et al., 2015]. In addition to cobamide-dependent enzymes, there is also a cobamide-dependent transcription factor that uses AdoCbl as a light sensing mechanism, as the cobalt-5'-deoxyadenosyl bond of AdoCbl homolyzes upon light exposure [Ortiz-Guerrero et al., 2011]. Recently, a cobalamin-derived chemical probe was synthesized as a tool to search for new cobalamin-binding proteins in cells [Romine et al., 2017; Rosnow et al., 2018].

For several cobamide-dependent enzymes, including methionine synthase, ribonucleotide reductase, and methylmalonyl-CoA mutase, there are cobamide-independent isozymes or alternative pathways present in a subset of bacterial genomes. Interestingly, the cobamide-dependent methionine synthase MetH is more catalytically active than the cobamide-independent isozyme MetE, which could be part of the reason that organisms that do not produce cobamides have retained the cobamide-dependent isozyme [Aretakis et al., 2019; González et al., 1996]. Cobamide-dependent enzymes may also be advantageous in their relative stability to oxidative stress. For example, *Sinorhizobium meliloti* expressing a cobalamin-independent (class I) ribonucleotide reductase, instead of its native cobamide-dependent (class II) isozyme, is able to grow in pure culture but cannot effectively colonize plant root nodules, which requires tolerance to high levels of reactive oxygen species [Taga and Walker, 2010].

Numerous bacterial cobamide-dependent enzymes are listed in Table 1. These enzymes are generally categorized in two ways: by the upper ligand that is required for catalysis, and by the conformation of the lower ligand of the cobamide in the enzyme-bound state. Cobamide-dependent enzymes generally use either the methyl or 5'-deoxyadenosyl upper ligand, although some enzymes do not require an upper ligand (see more below). The upper ligands involved in catalysis in various cobamide-dependent enzymes are listed in Table 1. In terms of the conformation of the lower ligand, cobamide-dependent enzymes fall into three major categories. In "base-on" cobamide-dependent enzymes, the cobamide binds the enzyme with the lower ligand coordinated to the cobalt ion [Larsson et al., 2010; Shibata et al., 2018; Yamanishi et al., 2002]. In "base-off" cobamide-dependent enzymes, the lower ligand is not coordinated to the cobalt ion, and is bound distant from the active site of the enzyme [Bommer et al., 2014; Bridwell-Rabb et al., 2017; Payne et al., 2015a; Payne et al., 2015b]. In a subcategory of base-off enzymes, "base-off/His-on" enzymes, a histidine residue from the protein displaces the lower ligand and coordinates the cobalt ion [Berkovitch et al., 2004; Drennan et al., 1994; Froese et al., 2010; Gruber et al., 2001; Jost et al., 2015; Kurteva-Yaneva et al., 2015; Mancia et al., 1996; Wolthers et al., 2010].

Table 1: Bacterial cobamide-dependent enzymes.

Enzyme	Name	Function	Upper ligand	Lower ligand conformation	Reference(s)
Methionine synthase*	MetH	Methionine synthesis	Me	Base-off/His-on	[Drennan et al., 1994]
Epoxyqueuosine reductase	QueG	tRNA modification	None	Base-off	[Miles et al., 2011; Payne et al., 2015a]
Ribonucleotide reductase	NrdJ	DNA synthesis	Ado	Base-on	[Licht et al., 1996]
Carbon skeleton mutases					
Methylmalonyl-CoA mutase*	MCM	Carbon metabolism; secondary metabolite biosynthesis	Ado	Base-off/His-on	[Banerjee and Chowdhury, 1999]
Ethylmalonyl-CoA mutase	ECM		Ado	Base-off/His-on	[Erb et al., 2008]
Isobutyryl-CoA mutase	ICM, IcmF		Ado	Base-off/His-on	[Cracan et al., 2010; Vrijbloed et al., 1999]
2-Hydroxyisobutyryl-CoA mutase	HCM		Ado	Base-off/His-on	[Yaneva et al., 2012]
Pivalyl-CoA mutase	PCM		Ado	Base-off/His-on	[Cracan and Banerjee, 2012b]
Glutamate mutase	MutES	Glutamate fermentation; secondary metabolite biosynthesis	Ado	Base-off/His-on	[Barker, 1985; Gruber et al., 2001]
Lysine aminomutase		Lysine fermentation	Ado	Base-off/His-on	[Berkovitch et al., 2004; Chang and Frey, 2000]
D-ornithine 4,5-aminomutase		Ornithine fermentation	Ado	Base-off/His-on	[Chen et al., 2001; Wolthers et al., 2010]
SAM-B_{12} proteins	GenK	Biosynthesis of gentamicin			[Kim et al., 2017]
	OxsB	Biosynthesis of oxetanocin A		Base-off	[Bridwell-Rabb et al., 2017]
	Fom3	Biosynthesis of fosfomycin	Me		[Allen and Wang, 2014; Woodyer et al., 2007]
	PhpK	Biosynthesis of phosalacine	Me		[Werner et al., 2011]
	TsrM	Biosynthesis of thiostrepton	Me	Base-off	[Blaszczyk et al., 2016; Pierre et al., 2012b]
	ThnK	Biosynthesis of thienamycin			[Marous et al., 2015a]
	Mmp10	Post-translational modification, methanogenesis			[Radle et al., 2019]
Ethanolamine ammonia lyase	EAL	Ethanolamine metabolism	Ado	Base-on	[Scarlett and Turner, 1976]
Glycerol dehydratase		Glycerol metabolism	Ado	Base-on	[Forage and Foster, 1982]
Diol dehydratase		Propanediol fermentation	Ado	Base-on	[Jeter, 1990; Shibata et al., 1999]
Reductive dehalogenases	PceA	Organohalide respiration		Base-off	[Bommer et al., 2014]
	VcrA		None		[Parthasarathy et al., 2015]
Corrinoid methyltransferases (MT)					
Wood-Ljungdahl corrinoid protein	CFeSP	Acetogenesis	Me	Base-off	[Ragsdale and Pierce, 2008]
O-demethylases		1-carbon metabolism	Me		[Naidu and Ragsdale, 2001; Stupperich and Konle, 1993]
methylamine:Coenzyme M MT		Methylotrophic methanogenesis	Me		[Paul et al., 2000; Sauer and Thauer, 1997; Tallant et al., 2001]
methylamine:THF MT		Methylotrophic growth	Me		[Picking et al., 2019]
Mercury MT	HgcA	Mercury methylation	Me	Base-off/Cys-on†	[Parks et al., 2013]

* Present in humans.
† Predicted; would be a novel cobamide-binding motif, observed only recently in the crystal structure of BtuM [Rempel et al., 2018].
Note: this list of enzymes is not comprehensive.

The types of chemical reactions catalyzed by cobamide-dependent enzymes include methylation reactions, isomerizations, eliminations, and reductive bond cleavage. There are two canonical mechanisms for cobamide-dependent reactions (Figure 2A, B). Methylcobalamin typically acts as a methyl donor in methylation reactions, in a mechanism that involves nucleophilic attack by the substrate and heterolytic cleavage of the cobalt-carbon bond [Dorweiler et al., 2003]. In contrast, adenosylcobalamin initiates catalysis by homolytic cleavage of the cobalt-carbon bond, generating the 5'-deoxyadenosyl radical, which then reacts with the substrate initiating a radical rearrangement [Marsh and Drennan, 2001]. However, with the discovery of new cobamide-dependent enzymes, new mechanisms for cobamide-mediated catalysis have been proposed (Figure 2C, D) [Bridwell-Rabb and Drennan, 2017]. For example, currently accepted mechanisms for the reactions catalyzed by reductive dehalogenases and epoxyqueuosine reductase involve a direct bond between the cobalt and the substrate, and some SAM-B_{12} enzymes are proposed to methylate substrates through radical mechanisms [Kim et al., 2017; Payne et al., 2015a; Payne et al., 2015b; Woodyer et al., 2007]. Thus, our view of the chemical versatility of cobamide cofactors continues to expand.

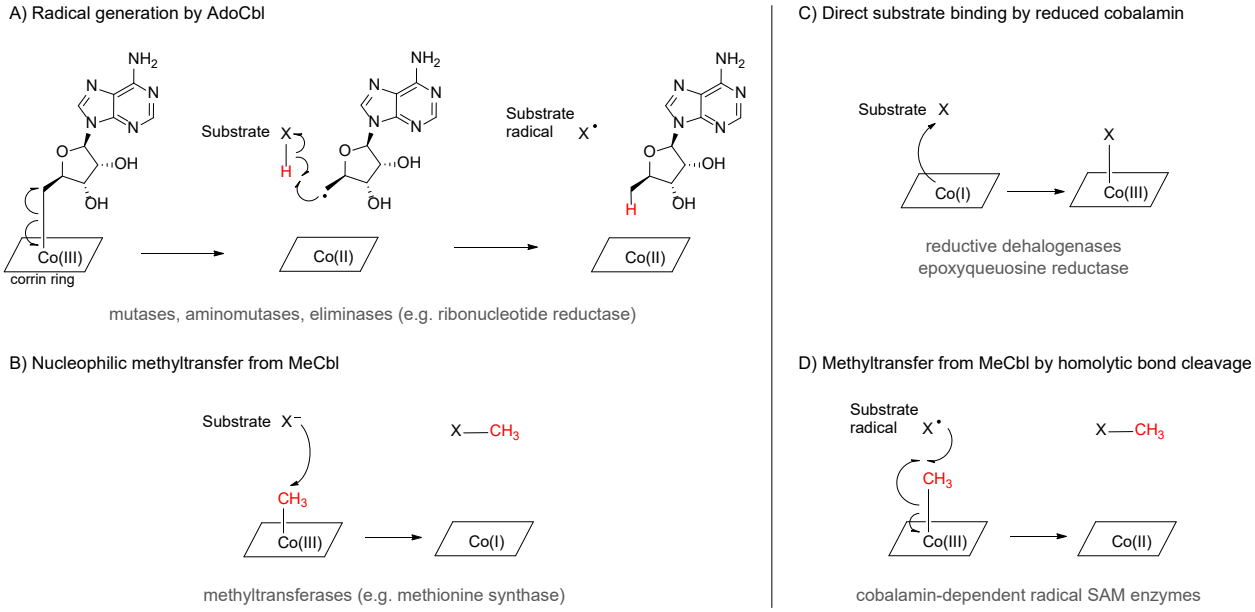


Figure 2: Catalytic mechanisms of cobamides. Left panel: Canonical reactions. (A) Adenosylcobalamin (AdoCbl) generates the 5'-deoxyadenosyl radical through homolytic Co-C bond cleavage, initiating radical chemistry on the substrate. (B) Methylcobalamin (MeCbl) serves as a methyl donor in a nucleophilic substitution reaction. Right panel: Proposed mechanisms for recently discovered cobamide-dependent enzymes. (C) The reduced cobalt center of the cofactor binds the substrate directly. (D) Methyl transfer occurs through homolytic, rather than heterolytic, cleavage of the Co-methyl bond.

IV. Cobalamin in human metabolism

Humans have two cobalamin-dependent enzymes: methylmalonyl-CoA mutase (MCM, MUT in humans), and methionine synthase (MS). The reactions catalyzed by these enzymes are essential, and therefore cobalamin deficiency, or mutations in genes required for cobalamin uptake, trafficking, or use, can lead to serious illness. This section will summarize how humans take up

cobalamin, how cobalamin is trafficked and modified in human cells, the role of this cofactor in human metabolism, and metabolic disorders associated with cobalamin deficiency.

Cobalamin-dependent processes in humans

MUT and MS are the only cobalamin-dependent enzymes in humans. MUT is a mitochondrial enzyme that requires adenosylcobalamin as a cofactor. The reaction catalyzed by MUT is a reversible, radical-initiated isomerization of the metabolite *R*-methylmalonyl-CoA to succinyl-CoA, an intermediate of the TCA cycle. This reaction, detailed in the following section, is essential for the catabolism of branched amino acids, methionine, odd-chain fatty acids, and cholesterol in human cells (Figure 3C) [Fenton et al., 2014]. MS is a cytosolic enzyme that catalyzes the synthesis of L-methionine by nucleophilic methylation of homocysteine. MS requires reduced cobalamin and generates methylcobalamin during its reaction cycle [Kolhouse et al., 1991].

Interestingly, the MS enzyme links several metabolic pathways in human cells (Figure 3D). In addition to being an important building block of proteins, methionine is a precursor to the cofactor *S*-adenosyl-L-methionine (SAM), which has a number of important functions in cells [Landgraf et al., 2016]. The methyl donor in the MS reaction is methyltetrahydrofolate, which is derived from the folate cycle [Froese et al., 2018; Walsh and Tang, 2019]. Because of this, methionine synthesis is additionally linked to DNA synthesis, as a step in pyrimidine biosynthesis is folate-dependent (Figure 3D). Importantly, MS is the only enzyme that de-methylates methyltetrahydrofolate, so in the absence of MS activity the cellular folate pool accumulates as methyltetrahydrofolate, thus depleting other forms of folate (a phenomenon referred to as the "folate trap"), which can adversely affect cells [Froese et al., 2018; Sauer and Wilmanns, 1977; Walsh and Tang, 2019].

Cobalamin import and trafficking

There are at least 10 proteins involved in transporting cobalamin from ingested food to its active sites in MS and MUT (Figure 3). Cobalamin released from food is initially bound by haptocorrin (HC), an acid-stable salivary glycoprotein, in the stomach. Pancreatic enzymes liberate cobalamin from HC in the small intestine, where a higher pH allows the glycoprotein intrinsic factor (IF) to bind cobalamin with femtomolar affinity [Allen et al., 1978; Fedosov et al., 2007]. The IF-cobalamin complex is recognized by the cubulin receptor of ileal cells and internalized [Fyfe et al., 2004]. Once it is exported into the bloodstream [Beedholm-Ebsen et al., 2010], cobalamin is bound mostly by HC and by transcobalamin (TC) [Mørkbak et al., 2006]. Only cobalamin complexed with TC is recognized by the cellular receptor CD320 in tissues throughout the body [Quadros et al., 2009]. The role of HC in the blood is thought to be sequestration of cobalamin degradation products, as HC is distinct from both IF and TC in its high binding affinity for cobinamide and cobamides other than cobalamin [Fedosov et al., 2007; Furger et al., 2013; Kanazawa et al., 1983; Kolhouse and Allen, 1977b].

Cells internalize the TC-cobalamin complex into lysosomes, in which cobalamin is liberated from TC and released through a process involving two membrane proteins, LMBD1 and ABCD4 [Coelho et al., 2012; Rutsch et al., 2009]. The upper ligand of cobalamin (which could be cyanide, an alkyl group, or water) is removed by MMACHC [Hannibal et al., 2009], and cob(II)alamin is transported by MMADHC to the MUT and MS enzymes [Bassila et al., 2017;

Mah et al., 2013]. Mechanistic details of this intracellular transport pathway continue to be elucidated. Recent studies have uncovered a multitude of protein-protein interactions that facilitate cobalamin trafficking [Bassila et al., 2017; Froese et al., 2015] and identified additional proteins that may be involved in cobalamin transport in humans [McDonald et al., 2017].

In mitochondria, MUT is active in a complex with MMAB, the adenosyltransferase enzyme that converts the upper ligand of cobalamin to 5'-deoxyadenosine and transfers adenosylcobalamin (AdoCbl) to MUT, and MMAA, which mediates cofactor transfer, stabilizes MUT against inactivation, and helps remove inactivated cofactor from the MUT enzyme (Figure 3C) [Gherasim et al., 2013; Padovani et al., 2008; Takahashi-Íñiguez et al., 2011]. Similarly, in the cytosol MS is stabilized and reactivated by MSR (methionine synthase reductase), which reduces cob(II)alamin to cob(I)alamin and regenerates cob(I)alamin after occasional oxidation of the cofactor during the MS reaction cycle [Bassila et al., 2017; Yamada et al., 2006].

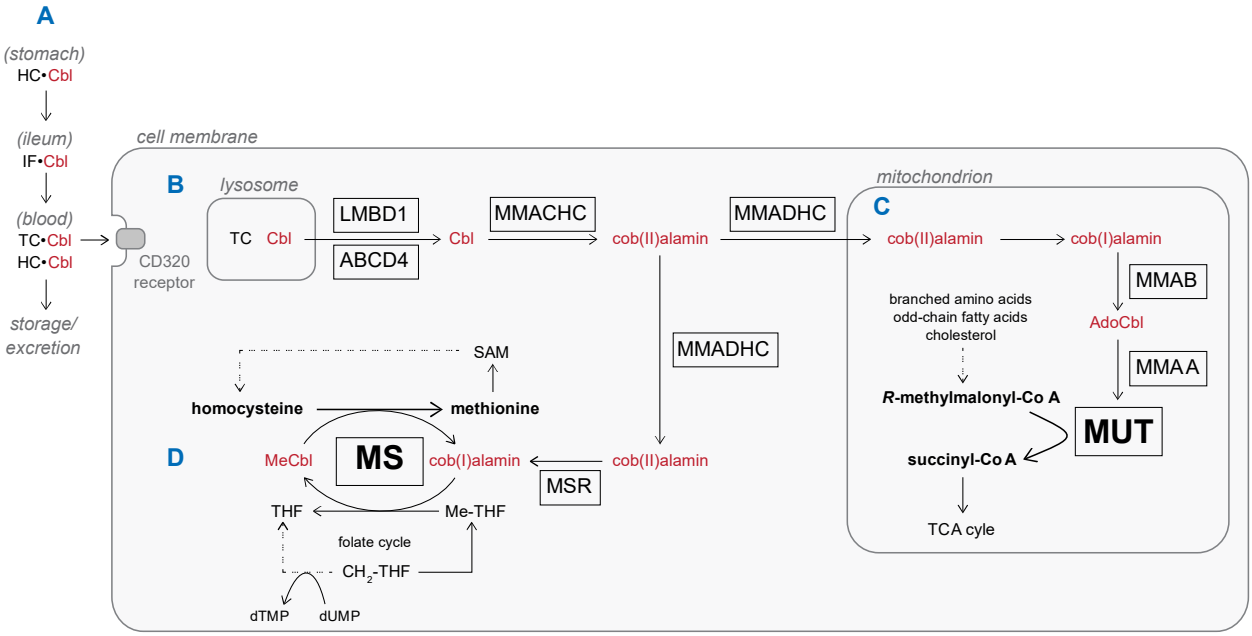


Figure 3: Cobalamin use and trafficking in human cells. (A) Systemic trafficking, (B) intracellular trafficking, (C) MUT pathway, (D) MS pathway. Enzymes involved in intracellular cobalamin trafficking and use are boxed. Cobalamin-dependent reactions (MS and MUT) are bolded. Dashes arrows indicate multiple steps. In red is cobalamin in its various forms as it is modified by trafficking proteins. HC = haptocorrin; IF = intrinsic factor; TC = transcobalamin; Cbl = cobalamin; MeCbl = methylcobalamin; AdoCbl = adenosylcobalamin; THF = tetrahydrofolate; Me-THF = methyltetrahydrofolate; CH_2-THF = methylenetetrahydrofolate; dTMP/dUMP = deoxythymidine/deoxyuridine monophostphate; SAM = *S*-adenosyl-L-methionine.

Metabolic disorders related to cobalamin

There are three types of cobalamin-related disorders: nutritional, acquired, and inborn (inherited). Nutritional deficiency simply results from lack of cobalamin in the diet, which is most common in vegetarians (animal-derived products are primary dietary sources of cobalamin) and in populations experiencing food shortage or decreased meat consumption [Stabler and Allen, 2004]. Acquired disorders include pernicious anemia, an autoimmune condition in which the

immune system attacks IF or the gastric parietal cells that produce IF, which was the deadly illness that fueled the discovery of cobalamin in the early 1900's [Bunn, 2014]. Acquired cobalamin deficiencies typically occur in adulthood [Huemer and Baumgartner, 2019]. Interestingly, bacterial overgrowth in the small intestine has also been correlated with lower cobalamin levels in the human host, potentially because bacteria can compete for cobalamin in the small intestine and remodel some of the cobalamin to cobamides that are not bioavailable to humans [Albert et al., 1980; Brandt et al., 1977; Murphy et al., 1986].

Inborn errors of cobalamin metabolism are inherited, autosomal recessive mutations in genes related to cobalamin absorption, trafficking, and use. Interestingly, the complex intracellular trafficking system of humans illustrated in Figure 3 was elucidated using large collections of cell lines derived from patients with cobalamin-related metabolic disorders [Gravel et al., 1975; Watkins and Rosenblatt, 2013]. These cell lines were experimentally categorized into complementation groups (*cblA-cblG, cblJ, mut*), which corresponded to impairments in distinct steps of cobalamin trafficking (Table 2) [Banerjee, 2006; Froese and Gravel, 2010]. Defects in *cblJ*, *cblF*, or *cblC* result in the metabolic disorders methylmalonic aciduria (MMA) and homocystinuria (HCY), characterized by the accumulation of methylmalonic acid and homocysteine, respectively, in the blood. These mutations impair cobalamin trafficking to both MS and MUT, and therefore the substrates of both enzymes accumulate [Huemer and Baumgartner, 2019; Watkins and Rosenblatt, 2013]. In contrast, *cblG* and *cblE* defects result exclusively in HCY, while *cblA, cblB*, and *mut* defects result in MMA; these defects are specific to either the MS or MUT pathway [Chandler and Venditti, 2005; Gherasim et al., 2013; Watkins and Rosenblatt, 1989]. A unique complementation group, *cblD*, can result in MMA, HCY, or both; mutations in the MMADHC protein can exclusively disrupt its function in the MUT or MS pathway, or disrupt cofactor trafficking to both enzymes [Stucki et al., 2012; Suormala et al., 2004].

Table 2: Inborn errors of cobalamin. MMA = methylmalonic aciduria; HCY = homocystinuria.

Complementation group	Protein affected	Associated disorder(s)	
cblF	LMBD1	MMA and HCY	Shared steps
cblJ	ABCD4	MMA and HCY	
cblC	MMACHC	MMA and HCY	
cblD	MMADHC	MMA, HCY, or both	
cblG	MS (methionine synthase)	HCY	MS-specific
cblE	MSR	HCY	
cblA	MMAA	MMA	MUT-specific
cblB	MMAB	MMA	
mut	MUT (methylmalonyl-CoA mutase)	MMA	

The metabolic disorders associated with cobalamin deficiency result in a wide variety of symptoms. A common manifestation of cobalamin deficiency is megaloblastic anemia, a blood disorder that presents with abnormalities in blood cell size and development. Cobalamin deficiency can also lead to neuropathies (weakness, numbness, tingling sensations, pain from neural damage), psychiatric disorders, weight loss, optic atrophy, and other symptoms that range from mild to severe [Huemer and Baumgartner, 2019; Shevell and Rosenblatt, 2015]. Treatment of cobalamin

deficiency includes oral administration or intramuscular, intravenous, or subcutaneous injections of cobalamin [Huemer and Baumgartner, 2019; Shevell and Rosenblatt, 2015].

V. Methylmalonyl-CoA mutase

Methylmalonyl-CoA mutase (MCM) is a cobamide-dependent enzyme encoded in ~35 % of bacterial genomes and present in humans [Fenton et al., 1982; Shelton et al., 2019]. MCM enzyme homologs are structurally diverse. In humans, and some bacteria including *S. meliloti* and *Escherichia coli*, MCM is a homodimeric protein encoded by a single gene. Each monomer contains a cobamide binding site and is catalytically active [Froese et al., 2010; Haller et al., 2000; Miyamoto et al., 2010; Miyamoto et al., 2003]. In other bacteria and archaea, MCM is a heterodimer, with one catalytic, cobamide-binding subunit, and a second purely structural subunit [Birch et al., 1993; Mancia et al., 1996; Miyamoto et al., 2002; Zhang et al., 1999]; or a dimer of heterodimers, where each heterodimer is composed of two subunits (a large, substrate-binding subunit and a small, cobamide-binding subunit, that together constitute a single MCM enzyme) and binds one cobamide [Han et al., 2012; Yabuta et al., 2015].

MCM catalyzes the reversible isomerization of the central metabolites *(R)*-methylmalonyl-CoA and succinyl-CoA. This reaction involves a radical rearrangement, which is initiated by formation of a 5'-deoxyadenosyl radical by the AdoCbl cofactor (Figure 4). Product release is considered to be the rate-limiting step of the reaction catalyzed by MCM [Meier et al., 1996; Padmakumar et al., 1997]. Several other mutases, including ethylmalonyl-CoA mutase, isobutyryl-CoA mutase, 2-hydroxyisobutyryl-CoA mutase, and pivalyl-CoA mutase, are highly similar in sequence to MCM and catalyze similar types of reactions on related substrates [Cracan and Banerjee, 2012a]. These enzymes can be distinguished from MCM on the basis of several residues in the substrate-binding domain [Yaneva et al., 2012].

MCM is involved in the metabolic pathway by which propionyl-CoA is converted to succinyl-CoA, a TCA cycle intermediate. Branched amino acids, odd-chain fatty acids, and cholesterol are catabolized through this pathway [Fenton et al., 2014; Savvi et al., 2008]. Additionally, the conversion of propionyl-CoA to succinyl-CoA is part of the archaeal 3-hydroxypropionate/4-hydroxybutyrate cycle, which fixes CO_2 and generates acetyl-CoA [Berg et al., 2007; Han et al., 2012; Yaneva et al., 2012], and is part of the pathway for generation of glyoxylate in serine cycle methylotrophic bacteria [Korotkova et al., 2002]. While some microbes, including the pathogen *Mycobacterium tuberculosis*, use MCM for catabolism of propionate [Hosotani et al., 1980; Savvi et al., 2008; Vrijbloed et al., 1999], *Propionibacteria* take advantage of the reverse reaction, converting succinyl-CoA to methylmalonyl-CoA for propionate fermentation [Allen et al., 1964]. In *S. meliloti*, MCM is essential for the degradation of polyhydroxybutyrate, a polymer used by bacteria for carbon storage [Charles et al., 1997]. The diverse uses of the MCM reaction in biology emphasizes the importance of the metabolites methylmalonyl-CoA and succinyl-CoA, and it is therefore not surprising that the MCM reaction has also been used industrially for the production of propionate, bioplastics, biofuels, and antibiotics [Akawi et al., 2015; Aldor et al., 2002; Dayem et al., 2002; Gonzalez-Garcia et al., 2017; Gross et al., 2006; Li et al., 2017; Reeves et al., 2007; Srirangan et al., 2013].

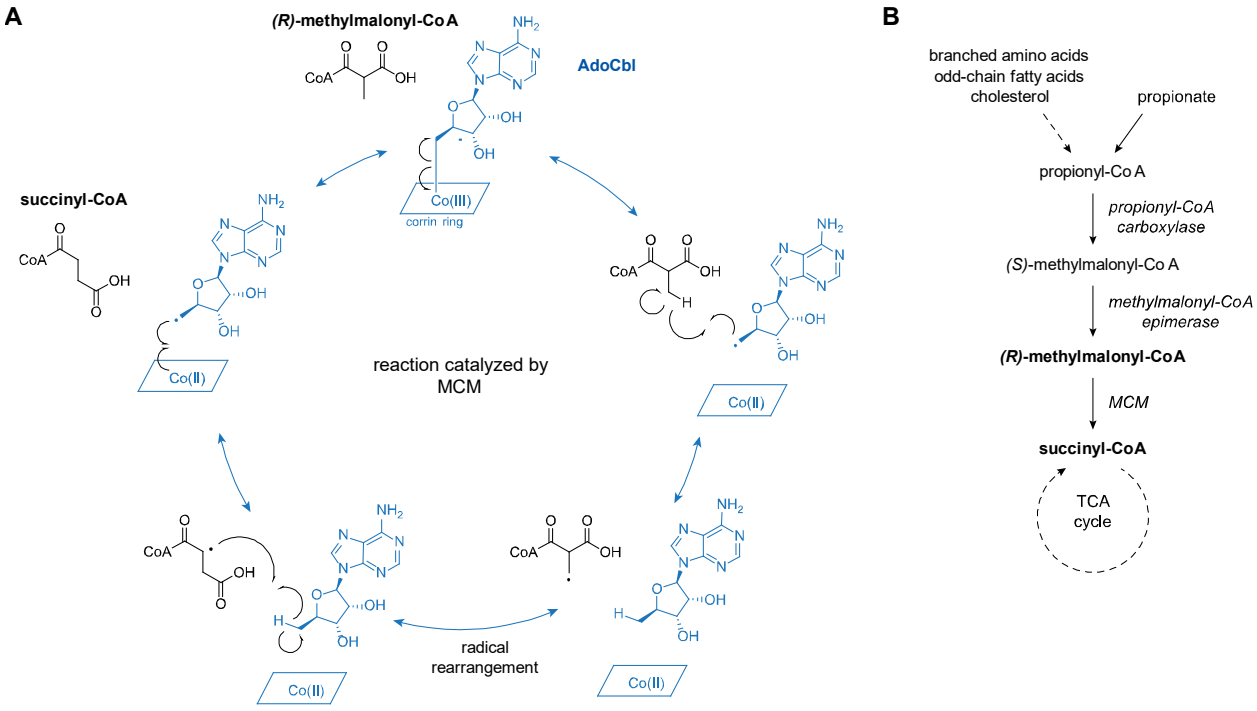


Figure 4: (A) Reaction cycle of MCM. (B) Metabolic pathway involving MCM. Dashed lines indicate multiple steps. CoA, coenzyme A.

About a quarter of sequenced bacterial species that have the MCM gene also have genes encoding the methylcitrate pathway, an alternative, cobamide-independent pathway for conversation of propionyl-CoA to succinate [Horswill and Escalante-Semerena, 1999; London et al., 1999; Shelton et al., 2019; Textor et al., 1997].

In bacteria, the MCM gene is commonly found adjacent to the gene *meaB*, which encodes a P-loop GTPase discovered to act as a chaperone protein for MCM. Disruption of *meaB* in *Methylobacterium extorquens* results in the absence of MCM activity in cell extracts, even in the presence of AdoCbl [Korotkova and Lidstrom, 2004]. Purified MeaB and MCM from *M. extorquens* interact with high affinity [Korotkova and Lidstrom, 2004; Padovani et al., 2006]. MeaB moderately increases the binding affinity of AdoCbl for MCM, and, in a nucleoside-dependent manner, slows the rate of oxidative inactivation of cob(II)alamin during the MCM reaction cycle [Padovani and Banerjee, 2006]. The human ortholog of MeaB, MMAA, has also been found to stimulate MCM activity *in vitro* [Takahashi-Íñiguez et al., 2011]. Similarly to the effects of *meaB* disruption, mutations in MMAA in humans lead to disease (see previous section). Disruption of *meaD*, the adenosyltransferase enzyme in *M. extorquens*, also results in loss of MCM activity, even in the presence of AdoCbl [Korotkova and Lidstrom, 2004]. This is consistent with a role of adenosyltransferase in cofactor loading to MCM, as transfer of AdoCbl from adenosyltransferase to MCM *in vitro* appears to involve direct protein-protein interactions [Padovani et al., 2008]. Thus, physiologically, both the adenosyltransferase and chaperone enzymes are important for MCM activity.

VI. Summary of the following chapters

We chose MCM as a model enzyme to study the mechanisms by which diverse cobamides affect cobamide-dependent enzymes. The distribution of MCM throughout all domains of life and its biochemical tractability are two features that make MCM a practical model. Additionally, since the lower ligand is removed from the enzyme active site in the MCM-cobamide complex (similarly to other base-off/His-on cobamide-dependent enzymes) the importance of lower ligand structure is particularly puzzling. In the context of human biology, MCM selectivity among diverse cobamides is unknown and is important considering that cobamides other than cobalamin are present in food and are produced by gut bacteria. In bacteria, cobamide structure affects growth in biologically significant ways and we hypothesize that enzyme selectivity is a major determinant of cobamide-dependent phenotypes.

A large part of this work involves production of diverse cobamide cofactors, which cannot be purchased. Thus, to study the effects of different cobamides on MCM and on bacterial growth, I produced 14 cobamides by guided biosynthesis in bacteria, including eight that have been previously characterized and six new cobamides that were designed to address specific hypotheses in my work. The following chapters describe my research findings regarding how structural variation among these cofactors affects the function of bacterial and human MCM orthologs; demonstrate the importance of cobamide selectivity for bacterial physiology; and suggest therapeutic applications of diverse cobamides in human biology.

Chapter 2

Cofactor selectivity in methylmalonyl-CoA mutase, a model cobamide-dependent enzyme

This chapter is co-authored by Olga M. Sokolovskaya, Kenny C. Mok, Jong Duk Park, Jennifer L. A. Tran, Kathryn A. Quanstrom, and Michiko E. Taga.

I. Abstract

Cobamides, a uniquely diverse family of enzyme cofactors related to vitamin B_{12}, are produced exclusively by bacteria and archaea but used in all domains of life. While it is widely accepted that cobamide-dependent organisms require specific cobamides for their metabolism, the biochemical mechanisms that make cobamides functionally distinct are largely unknown. Here, we examine the effects of cobamide structural variation on a model cobamide-dependent enzyme, methylmalonyl-CoA mutase (MCM). The *in vitro* binding affinity of MCM for cobamides can be dramatically influenced by small changes in the structure of the lower ligand of the cobamide, and binding selectivity differs between bacterial orthologs of MCM. In contrast, variations in the lower ligand have minor effects on MCM catalysis. Bacterial growth assays demonstrate that cobamide requirements of MCM *in vitro* largely correlate with *in vivo* cobamide dependence. This result underscores the importance of enzyme selectivity in the cobamide-dependent physiology of bacteria.

II. Introduction

Cobalamin, commonly referred to as vitamin B_{12}, is a versatile enzyme cofactor used by organisms in all domains of life. In humans, cobalamin is essential for methionine synthesis and the breakdown of fatty acids, amino acids, and cholesterol [Brodie et al., 1970; Fenton et al., 1982]. Bacteria and archaea additionally use cobalamin and related cofactors, cobamides, for deoxyribonucleotide synthesis [Licht et al., 1996], metabolism of various carbon and energy sources [Barker, 1985; Berg et al., 2007; Chang and Frey, 2000; Chen et al., 2001; Erb et al., 2008; Ferguson and Krzycki, 1997; Forage and Foster, 1982; Jeter, 1990; Korotkova et al., 2002; Krasotkina et al., 2001; Ljungdahl, 1986; Scarlett and Turner, 1976; Stupperich and Konle, 1993; Vrijbloed et al., 1999], synthesis of secondary metabolites [Allen and Wang, 2014; Blaszczyk et al., 2016; Bridwell-Rabb et al., 2017; Jung et al., 2014; Kim et al., 2017; Marous et al., 2015b; Pierre et al., 2012a; Werner et al., 2011], sensing light [Ortiz-Guerrero et al., 2011], and other processes [Barker, 1985; Chang and Frey, 2000; Chen et al., 2001; Cracan and Banerjee, 2012b; Gough et al., 2000; Miles et al., 2011; Parks et al., 2013; Romine et al., 2017; Yaneva et al., 2012]. The finding that 86% of bacterial species encode at least one cobamide-dependent enzyme in their genome [Shelton et al., 2019] demonstrates the prevalence of cobamide-dependent metabolisms. Widespread use of these cofactors can be attributed to their chemical versatility, as they facilitate challenging chemical reactions including radical-initiated rearrangements, methylation reactions, and reductive cleavage of chemical bonds [Banerjee and Ragsdale, 2003; Bridwell-Rabb and Drennan, 2017].

All cobamides share the same core structure (Figure 1): a corrin ring that coordinates a cobalt ion, a variable "upper" axial ligand (*R* in Figure 1), and a pseudo-nucleotide that is covalently attached to the corrin ring through an aminopropanol linker [Hodgkin et al., 1956] or

an ethanolamine linker, in the case of nor-cobamides [Keller et al., 2016; Kräutler et al., 2003]. The major differences among cobamides are in the structure of the nucleotide base, more commonly referred to as the lower axial ligand for its ability to coordinate the central cobalt ion. In cobalamin, the lower ligand is 5,6-dimethylbenzimidazole (Figure 1, boxed); in other naturally occurring cobamides, different benzimidazoles, phenolics, and purines constitute the lower ligand (Figure 2C) [Allen and Stabler, 2008; Girard et al., 2009; Men et al., 2015; Renz, 1999; Yan et al., 2018]. Phenolyl cobamides are distinct in that they lack the coordinate bond between the lower ligand and cobalt ion.

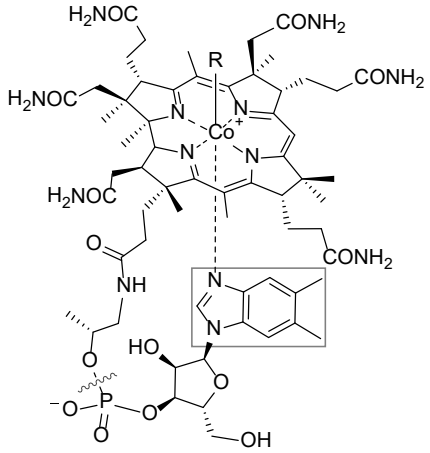


Figure 1: The structure of cobalamin. The lower ligand, boxed, varies in other cobamides. Cobinamide, a cobamide precursor, lacks a nucleotide base (delineated by the wavy line).

While cobamides containing different lower ligands share the same chemically reactive moieties, specifically the cobalt center and methyl or 5'-deoxyadenosyl upper axial ligands, they are nonetheless functionally distinct. Culture-based studies have shown that only a subset of cobamides supports a given bacterial metabolism, and uptake or production of other cobamides can inhibit growth [Helliwell et al., 2016; Keller et al., 2018; Mok and Taga, 2013; Watanabe et al., 1992; Yan et al., 2018; Yan et al., 2016; Yi et al., 2012]. The requirements of bacteria for particular cobamides is notable given the diversity of cobamides present in host-associated and environmental samples [Allen and Stabler, 2008; Girard et al., 2009; Men et al., 2015], coupled with the absence of *de novo* cobamide biosynthesis in more than half of bacteria [Shelton et al., 2019]. Despite the biological relevance of cobamide structure, and the prevalence of cobamide use among bacteria [Degnan et al., 2014a; Rodionov et al., 2003; Shelton et al., 2019; Zhang et al., 2009], little is understood about the biochemical mechanisms by which cobamides differentially impact microbial physiology.

The effect of lower ligand structure on the biochemistry of cobamide-dependent enzymes has been studied to a limited extent. In "base-on" enzymes, the lower ligand base coordinates the central cobalt ion of the cobamide, as drawn in Figure 1 [Larsson et al., 2010; Shibata et al., 2018; Yamanishi et al., 2002]. Because the lower ligand is part of the catalytic center of the enzyme, lower ligand structure can influence catalysis through a variety of mechanisms [Brown, 2006; Conrad et al., 2015; Kozlowski and Zgierski, 2004], and cobamides unable to form an

intramolecular coordinate bond are catalytically inactive in base-on enzymes [Poppe et al., 2000; Poppe et al., 1997]. In contrast, in "base-off" enzymes the lower ligand is bound by the enzyme more than 10 Å away from the active site [Berkovitch et al., 2004; Bommer et al., 2014; Bridwell-Rabb et al., 2017; Drennan et al., 1994; Gruber et al., 2001; Jost et al., 2015; Kurteva-Yaneva et al., 2015; Mancia et al., 1996; Payne et al., 2015a; Payne et al., 2015b; Wolthers et al., 2010]. In a subset of base-off enzymes, referred to as "base-off/His-on," a histidine residue from the protein coordinates the cobalt ion in place of the lower ligand [Drennan et al., 1994; Mancia et al., 1996]. Despite its distance from the reactive center, lower ligand structure affects the activity of base-off enzymes, as evidenced by the cobamide cofactor selectivity of methionine synthase [Tanioka et al., 2010], methylmalonyl-CoA mutase (MCM) [Lengyel et al., 1960; Poppe et al., 1997], reductive dehalogenases [Keller et al., 2018], and other enzymes [Barker et al., 1960; Lengyel et al., 1960; Poppe et al., 2000]. However, the mechanisms by which lower ligand structure affects the biochemistry of base-off cobamide-dependent enzymes remain unclear.

As MCM is one of the most abundant cobamide-dependent enzymes in bacterial genomes [Shelton et al., 2019], and one of the two cobamide-dependent enzymes in humans, we have chosen to study the cobamide selectivity of MCM as a model for base-off/His-on enzymes, all of which share a structurally conserved B_{12}-binding domain [Dowling et al., 2012; Drennan et al., 1994]. MCM catalyzes the interconversion of *(R)*-methylmalonyl-CoA and succinyl-CoA, a bidirectional reaction used in propionate metabolism [Hosotani et al., 1980; Savvi et al., 2008; Vrijbloed et al., 1999], catabolism of branched amino acids and odd-chain fatty acids [Fenton et al., 2014; Savvi et al., 2008], polyhydroxybutyrate degradation [Charles et al., 1997], secondary metabolite biosynthesis [Hunaiti and Kolattukudy, 1984], and autotrophic carbon dioxide fixation [Berg et al., 2007; Han et al., 2012]. MCM-dependent pathways have been harnessed industrially for the bioproduction of propionate, bioplastics, biofuels, and antibiotics [Akawi et al., 2015; Aldor et al., 2002; Dayem et al., 2002; Gonzalez-Garcia et al., 2017; Gross et al., 2006; Li et al., 2017; Reeves et al., 2007; Srirangan et al., 2013].

The presence of a cobamide lower ligand is required for MCM activity, as evidenced by the observation that adenosylcobinamide, a cobamide intermediate lacking a lower ligand (Figure 1), does not support MCM activity *in vitro* [Chowdhury and Banerjee, 1999]. Three studies provide evidence that MCM is selective for cobamides with particular lower ligands. First, MCM from *Propionibacterium shermanii* was found to have different apparent K_M values for cobamides, increasing from AdoCbl to Ado[Bza]Cba to Ado[Ade]Cba (refer to Table 1 for full names of cobamides), and MCM from sheep had a higher apparent K_M for Ado[Bza]Cba than AdoCbl [Lengyel et al., 1960]. Second, *P. shermanii* MCM had a lower apparent K_M for Ado[Cre]Cba than AdoCbl [Poppe et al., 1997]. Third, in *Sinorhizobium meliloti* bacteroids MCM activity was highest with AdoCbl, intermediate with Ado[Bza]Cba, and absent with Ado[Ade]Cba [De Hertogh et al., 1964]. Each of these studies includes only one or two cobamides other than cobalamin, and understandably so; cobamides are difficult to obtain in high quantities and must be purified from large volumes of bacterial cultures. Because of this, the response of MCM orthologs to the full diversity of cobamides has not been explored, and the mechanistic basis of cobamide selectivity remains unclear.

Table 1: Abbreviations used for cobamides and upper axial ligands.

Abbreviation	Cobamide name
Cbl	cobalamin
[5-MeBza]Cba	5-methylbenzimidazolylcobamide
[Bza]Cba	benzimidazolylcobamide
[5-OHBza]Cba	5-hydroxybenzimidazolylcobamide
[Cre]Cba	*para*-cresolylcobamide
[Phe]Cba	phenolylcobamide
[Ade]Cba	adeninylcobamide
[2-MeAde]Cba	2-methyladeninylcobamide
[Pur]Cba	purinylcobamide
[7-MeBza]Cba	7-methylbenzimidazolylcobamide
[7-AmBza]Cba	7-aminobenzimidazolylcobamide
[5-AzaBza]Cba	5-azabenzimidazolylcobamide
[3-DeazaAde]Cba	3-deazaadeninylcobamide
[6-MePur]Cba	6-methylpurinylcobamide
Prefix	Upper ligand
Ado	5'-deoxyadenosine
CN	cyanide

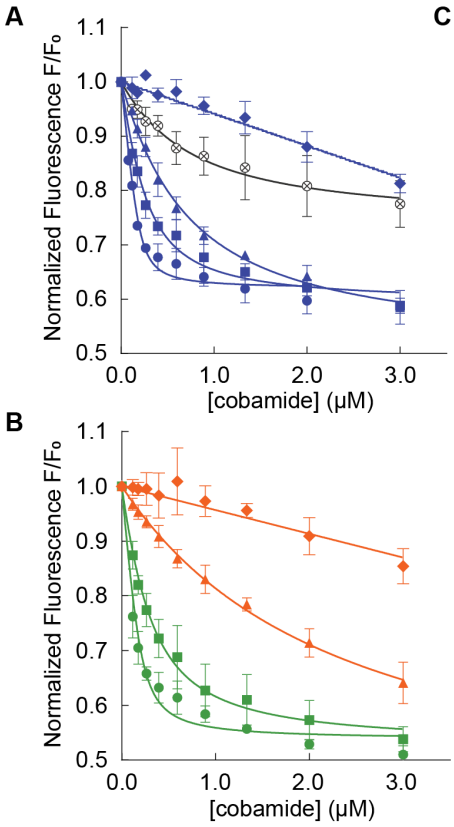
To investigate the mechanisms by which diverse lower ligands affect MCM function, we conducted *in vitro* binding and activity assays with MCM from *S. meliloti* (*Sm*MCM). We discovered major differences in the binding affinities of eight naturally occurring cobamides for *Sm*MCM, while cobamide structure affected enzyme activity to a lesser extent. Using six additional cobamides, five of which are novel analogs that have not been observed in nature or described previously, we identified structural elements of lower ligands that are determinants of binding to *Sm*MCM. To probe the hypothesis that enzyme selectivity influences bacterial growth, we characterized the cobamide dependence of *S. meliloti* growth *in vivo*. By bridging the results of *in vitro* biochemistry of three bacterial MCM orthologs and the cobamide-dependent growth phenotypes of *S. meliloti,* we have elucidated molecular factors that contribute to the cobamide-dependent physiology of bacteria.

III. Results

Lower ligand structure influences cobamide binding to MCM.

We chose *Sm*MCM as a model to examine how lower ligand structure influences MCM function based on previous work demonstrating its activity as a homodimer encoded by a single gene [Charles and Aneja, 1999; Miyamoto et al., 2003]. We purified eight naturally occurring cobamides for *in vitro* studies of this protein, and chemically adenosylated each cobamide to produce the biologically active form used by MCM for catalysis. Previous studies showed that binding of cobamides to *P. shermanii* MCM can be detected *in vitro* by measuring quenching of intrinsic protein fluorescence [Chowdhury and Banerjee, 1999]. We found that the fluorescence of purified, His-tagged *Sm*MCM also decreased in a dose-dependent manner when the protein was reconstituted with increasing concentrations of AdoCbl (Figure 2A). The equilibrium dissociation constant (K_d) derived from these measurements, 0.03 ± 0.02 μM (Figure 2C), is 6-fold lower than the K_d reported for *P. shermanii* MCM [Chowdhury and Banerjee, 1999]. Ado-cobinamide also

bound *Sm*MCM, as was observed with *P. shermanii* MCM [Chowdhury and Banerjee, 1999], albeit with over 10-fold reduced affinity compared to cobalamin (Figure 2A, C).



C

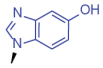
	Lower ligand	K_d (μM)
Ado-cobinamide	none	0.8 ± 0.2
Benzimidazolyl cobamides		
AdoCbl		0.03 ± 0.02
Ado[5-MeBza]Cba		0.10 ± 0.04
Ado[Bza]Cba		0.4 ± 0.2
Ado[5-OHBza]Cba		n. d.
Phenolyl cobamides		
Ado[Cre]Cba		0.05 ± 0.01
Ado[Phe]Cba		0.20 ± 0.03
Purinyl cobamides		
Ado[2-MeAde]Cba		1.7 ± 0.7
Ado[Ade]Cba		n. d.

Figure 2: Binding of diverse cobamides to *Sm*MCM (see also Figure 3). Fluorescence decrease of *Sm*MCM when reconstituted with (A) benzimidazolyl cobamides (blue) and cobinamide (black), and (B) phenolyl (green) and purinyl (orange) cobamides. Data points represent the mean and standard deviation of three technical replicates from a single experiment. (C) K_d values for different cobamides, reported as the average and standard deviation of three or more independent experiments, each consisting of technical triplicates. "n. d.," not determined, indicates that binding was too weak to determine K_d.

We next measured binding of other benzimidazolyl cobamides to *Sm*MCM and found that Ado[5-MeBza]Cba and Ado[Bza]Cba, the cobamides most structurally similar to AdoCbl, also bound the enzyme. However, the absence of one or two methyl groups, respectively, in the lower ligands of these cobamides caused a decrease in binding affinity relative to AdoCbl (Figure 2A, C). Strikingly, no binding of Ado[5-OHBza]Cba to *Sm*MCM was detected at low micromolar concentrations. To rule out the possibility that Ado[5-OHBza]Cba binds *Sm*MCM but does not cause a fluorescence quench, we used an alternative, filtration-based, binding assay and observed little to no binding of Ado[5-OHBza]Cba to *Sm*MCM at micromolar concentrations (Figure 3A, B).

We expanded our analysis of *Sm*MCM-cobamide binding selectivity to include cobamides from other structural classes. Both of the phenolyl cobamides tested, Ado[Cre]Cba and Ado[Phe]Cba, bound *Sm*MCM with affinities similar to those of cobalamin and other benzimidazolyl cobamides (Figure 2B, C). In contrast, the purinyl cobamides Ado[2-MeAde]Cba and Ado[Ade]Cba had lower affinities for *Sm*MCM compared to most benzimidazolyl cobamides

(Figure 2B, C): Ado[2-MeAde]Cba bound *Sm*MCM with ~20-fold lower affinity than cobalamin, and Ado[Ade]Cba did not bind to any significant extent at micromolar concentrations (verified by the filtration assay, Figure 3C). Interestingly, for all three classes of lower ligands, the presence of a methyl substituent promoted binding relative to other cobamides of the same structural class.

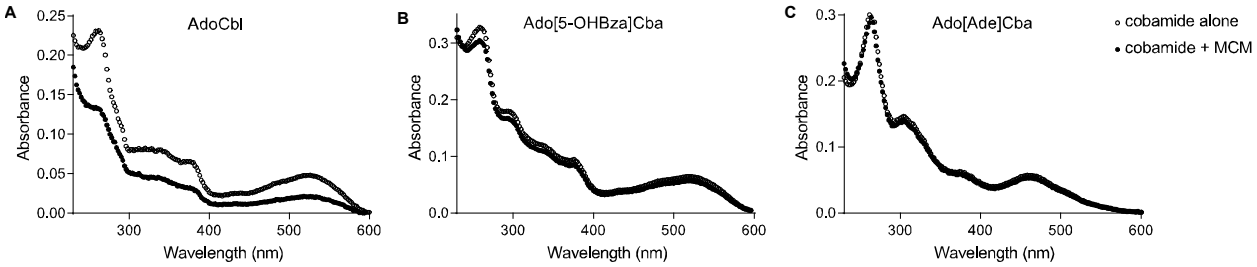
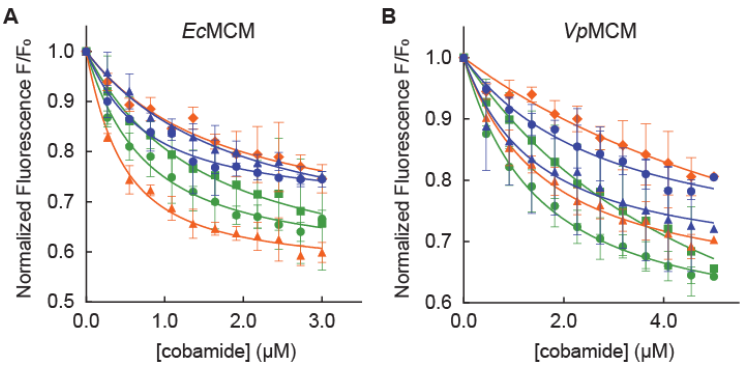


Figure 3: Filtration-based MCM binding assay. UV-Vis spectra of filtrate after pre-incubation of 10 μM (A) AdoCbl, (B) Ado[5-OHBza]Cba, and (C) Ado[Ade]Cba with and without *Sm*MCM (15 μM).

Bacterial MCM orthologs have distinct selectivity.

To test whether cofactor-binding selectivity is a general phenomenon across bacterial MCM orthologs, we compared the cobamide-binding profile of *Sm*MCM to that of MCM orthologs from *Escherichia coli* (*Ec*MCM) and *Veillonella parvula* (*Vp*MCM). Activity of *Ec*MCM with AdoCbl has been reported both *in vivo* and *in vitro*, although its physiological role in *E. coli* remains unclear [Gonzalez-Garcia et al., 2017; Haller et al., 2000]. Annotations for two MCM homologs are present in the genome of *V. parvula*, and we purified the one that exhibits MCM activity when expressed in *S. meliloti* (see Materials and Methods). Because *S. meliloti* produces cobalamin [Kliewer and Evans, 1963], *E. coli* produces [2-MeAde]Cba when provided cobinamide [Hazra et al., 2015], and *V. parvula* produces [Cre]Cba [Crofts et al., 2013], we expected that each ortholog should have distinct cobamide selectivity. Indeed, *Ec*MCM had highest affinity for its native cobamide, Ado[2-MeAde]Cba (Figure 4A, C). All other cobamides bound with 2- to 3-fold reduced affinities relative to Ado[2-MeAde]Cba. Similarly, *Vp*MCM had a higher affinity for Ado[Cre]Cba, its native cobamide, than AdoCbl (Figure 4B, C). *Vp*MCM also bound Ado[2-MeAde]Cba and Ado[Bza]Cba with similar affinity. We observed differences between the total change in fluorescence among cobamides with similar K_d values. This is not unexpected, as protein fluorescence is highly sensitive to local environment and may be affected by subtle conformational differences.

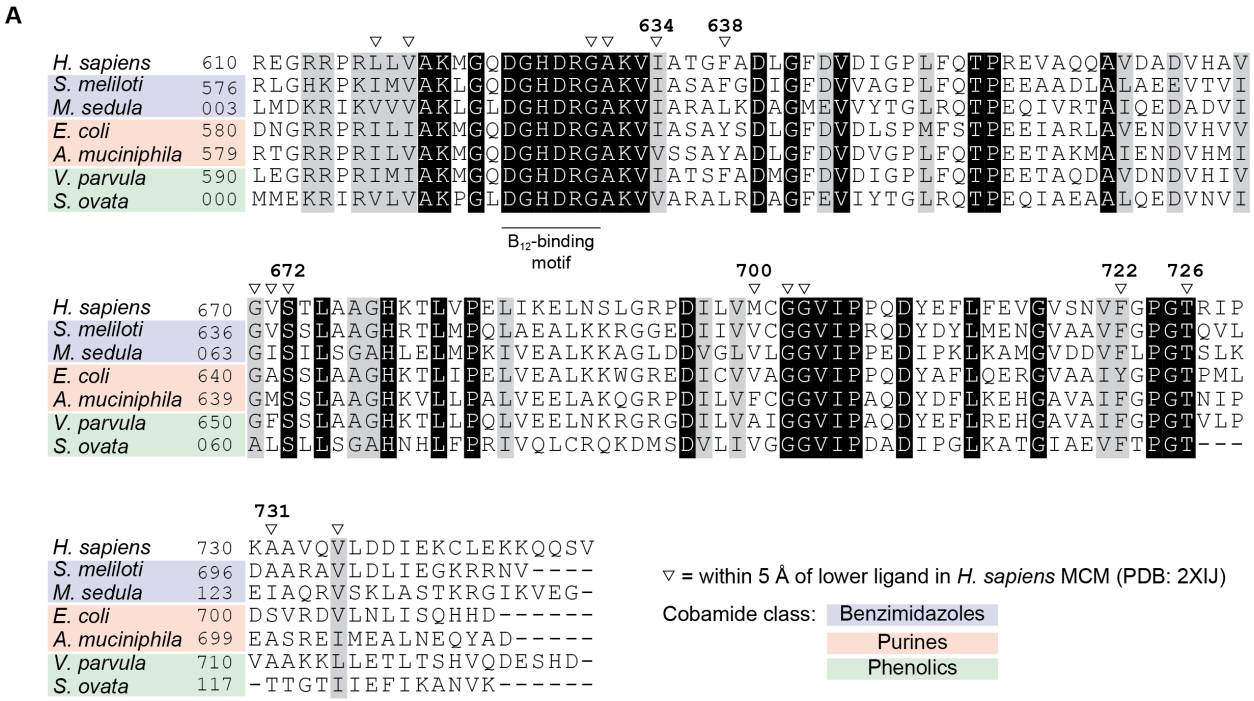


C

	K_d (μM)	
	*Ec*MCM	*Vp*MCM
AdoCbl	0.6 ± 0.1	2.8 ± 0.6
Ado[Bza]Cba	1.8 ± 0.2	1.4 ± 0.3
Ado[Cre]Cba	0.62 ± 0.09	1.3 ± 0.1
Ado[Phe]Cba	1.3 ± 0.2	5 ± 1
Ado[2-MeAde]Cba	0.35 ± 0.04	1.5 ± 0.2
Ado[Ade]Cba	1.6 ± 0.4	9 ± 4

Figure 4: Binding selectivity of diverse MCM orthologs (see also Figure 5). Fluorescence binding assays with (A) *E. coli* MCM and (B) *V. parvula* MCM. Data points and error bars represent the mean and standard deviation, respectively, of technical triplicates from a single experiment; each replicate consisted of an independent cobamide dilution. K_d values from the fitted curves in (A) and (B) are reported in (C); error values reflect the standard error of the curve fit. K_d values for *Vp*MCM binding to Ado[Cre]Cba and AdoCbl and for *Ec*MCM binding to all cobamides were reproduced in independent experiments.

We constructed a sequence alignment of MCM orthologs from diverse organisms known to produce or use various cobamides, in search of amino acid residues that could account for differences in cobamide binding (Figure 5A). The B_{12}-binding domains of diverse MCM orthologs had high overall amino acid identity (38-70%). Cases of low identity correlated with differences in the structural configuration of MCM, which occurs in different organisms as a homodimer [Froese et al., 2010; Haller et al., 2000; Miyamoto et al., 2010; Miyamoto et al., 2003], heterodimer [Birch et al., 1993; Mancia et al., 1996; Miyamoto et al., 2002; Zhang et al., 1999], or heterotetramer [Han et al., 2012; Yabuta et al., 2015] (Figure 5B). We focused our analysis on residues immediately surrounding the lower ligand in the available crystal structure of *Homo sapiens* MCM [Froese et al., 2010] (*Hs*MCM) (Figure 5A, triangles). For the most part, these residues are highly conserved between orthologs. Interestingly, however, *Hs*MCM residues Phe638, Phe722, and Ala731, which are conserved in *Sm*MCM, are substituted with the more polar residues Tyr, Tyr, and Ser, respectively, in *Ec*MCM (Figure 5A), which has a higher affinity for purinyl cobamides. Introducing mutations in *Sm*MCM and *Ec*MCM to test the importance of these residues proved challenging, as it resulted in reduced protein solubility and overall impaired cobamide binding (data not shown). Whether or not these residues co-vary with cobamide selectivity across other MCM orthologs is difficult to interpret because the cobamide selectivity of MCM from most organisms is unknown.



B

% Identity	*H. sapiens*	*S. meliloti*	*M. sedula*	*E. coli*	*A. muciniphila*	*V. parvula*	*S. ovata*
H. sapiens	--	58	37	60	62	66	38
S. meliloti	58	--	47	62	57	57	40
M. sedula	37	47	--	43	39	37	57
E. coli	60	62	43	--	62	63	39
A. muciniphila	62	57	39	62	--	70	42
V. parvula	66	57	37	63	70	--	38
S. ovata	38	40	57	39	42	38	--

Structural composition	
H. sapiens	α_2
S. meliloti	α_2
M. sedula	$\alpha_2\beta_2$
E. coli	α_2
A. muciniphila	$\alpha\beta$
V. parvula	$\alpha\beta$
S. ovata	$\alpha_2\beta_2$

Figure 5: Sequence comparison of the B_{12}-binding domains of MCM orthologs. (A) Sequence alignment of the B_{12}-binding domains of MCM orthologs, generated using the MUSCLE alignment tool from EMBL-EBI. Black and gray shading indicate amino acid identity and similarity, respectively. Sequences are colored by "cobamide class" based on cobamides biosynthesized by the organism or predicted cobamide use [Belzer et al., 2017; Crofts et al., 2013; Han et al., 2012; Hazra et al., 2015; Mok and Taga, 2013]. Residues numbered above the sequence alignment correspond to residues indicated in Figure 10. Locus tags of aligned proteins: *Homo sapiens* AAA59569, *Sinorhizobium meliloti* AAD13665, *Metallosphaera sedula* ABP96195, *Escherichia coli* WP_101348647, *Akkermansia muciniphila* WP_031931429, *Veillonella parvula* WP_004694550, *Sporomusa ovata* WP_021167215. (B) Percent identity matrix of the B_{12}-binding domains aligned in (A), as well as the structural composition of each MCM ortholog: α_2, homodimer; $\alpha\beta$, heterodimer; $\alpha_2\beta_2$, heterotetramer.

The lower ligand of cobamides modulates MCM reaction kinetics.

We reconstituted *Sm*MCM with saturating amounts of each of the four cobamides that bound with highest affinity and measured conversion of (*R*)-methylmalonyl-CoA to succinyl-CoA under steady state conditions. Interestingly, the substrate K_M was nearly invariable among the cobamides tested (Figure 6). Turnover was highest with AdoCbl ($26 \pm 1\ s^{-1}$) and 2- to 3-fold lower with other cobamides. Thus, all of the cobamides tested supported *Sm*MCM catalysis with modest differences in k_{cat}. This finding is consistent with a previous observation that adenosylcobinamide-

GDP, a cobamide precursor with an extended nucleotide loop and a guanine base, supported activity of *P. shermanii* MCM with only slight catalytic impairment compared to AdoCbl [Chowdhury et al., 2001].

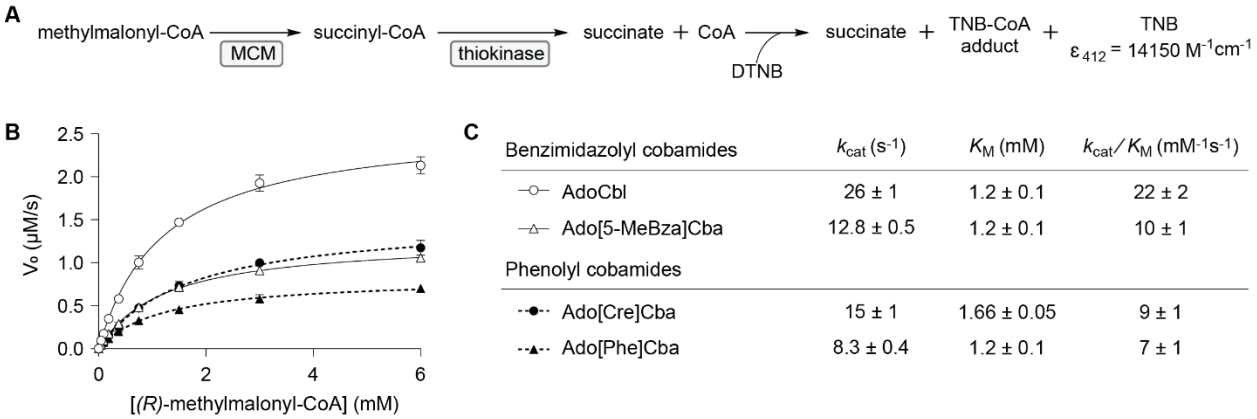


Figure 6: Activity of *Sinorhizobium meliloti* MCM with different cobamide cofactors. (A) Succinyl-CoA formation was detected using a coupled spectrophotometric assay [Taoka et al., 1994]. DTNB, dithionitrobenzoate (Ellman's Reagent); TNB, thionitrobenzoate; CoA, Coenzyme A. (B) Michaelis-Menten kinetic analysis of *Sm*MCM reconstituted with various cobamides. Data points and error bars represent the mean and standard deviation, respectively, of three technical replicates from one experiment; each replicate consisted of an independent substrate dilution. Kinetic constants are presented in (C).

MCM-dependent growth of S. meliloti correlates with the binding selectivity of SmMCM for benzimidazolyl and purinyl cobamides, but not phenolyl cobamides.

To assess whether the cobamide-dependent growth of *S. meliloti* reflects MCM selectivity as observed *in vitro*, we cultured *S. meliloti* under conditions that require MCM activity. Examination of metabolic pathways encoded in the *S. meliloti* genome using the KEGG database [Kanehisa and Goto, 2000] suggests that the degradation of branched amino acids isoleucine and valine to succinyl-CoA, an intermediate of the citric acid cycle, requires MCM. Indeed, growth of *S. meliloti* on L-isoleucine and L-valine as the only carbon sources was dependent on the presence of the *bhbA* gene, which encodes MCM [Charles and Aneja, 1999] (Figure 7).

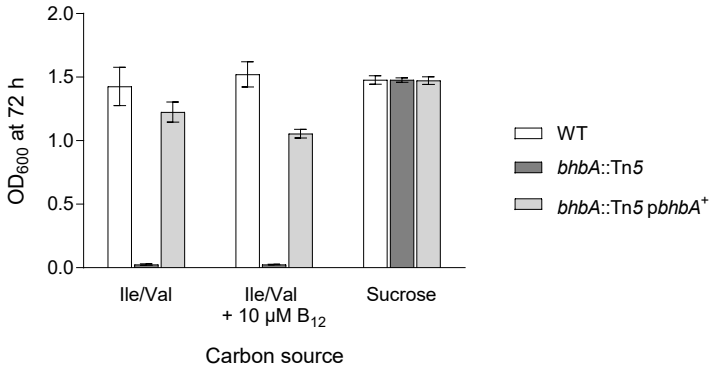


Figure 7: MCM-dependent growth of *S. meliloti*. Final density (OD_{600}), after 72 h of growth in M9 minimal medium, with 4 g/L L-isoleucine and 4 g/L L-valine (Ile/Val) or 2 g/L sucrose. *Sm*MCM is the gene product of the *bhbA* gene. p*bhbA*$^+$, complementation of the *bhbA*::Tn*5* mutation with the *S. meliloti bhbA*

gene expressed in the pTH1227 vector [Cheng et al., 2007]. Plot shows the mean and standard deviation of three biological replicates from a single experiment.

We constructed an *S. meliloti* strain incapable of synthesizing cobalamin and lacking cobamide-dependent enzymes other than MCM to ensure that differential growth could be attributed solely to MCM selectivity for added cobamides (see Materials and Methods). We cultivated this strain with L-isoleucine and L-valine as sole carbon sources in medium supplemented with different cobamides in their cyanylated (CN) forms, which is the form typically used for *in vivo* growth assays. Under these growth conditions, the maximum growth yield (OD_{600}) achieved at high concentrations of all of the cobamides was indistinguishable. However, the concentration of cobamides required to achieve half of the maximal OD_{600} (EC_{50}) differed based on the cobamide provided (Figure 8). Consistent with the binding data, CNCbl had the lowest EC_{50} value. EC_{50} values for CN[Bza]Cba and CN[2-MeAde]Cba were 5-fold higher than CNCbl, and other cobamides had EC_{50} values two orders of magnitude higher than CNCbl.

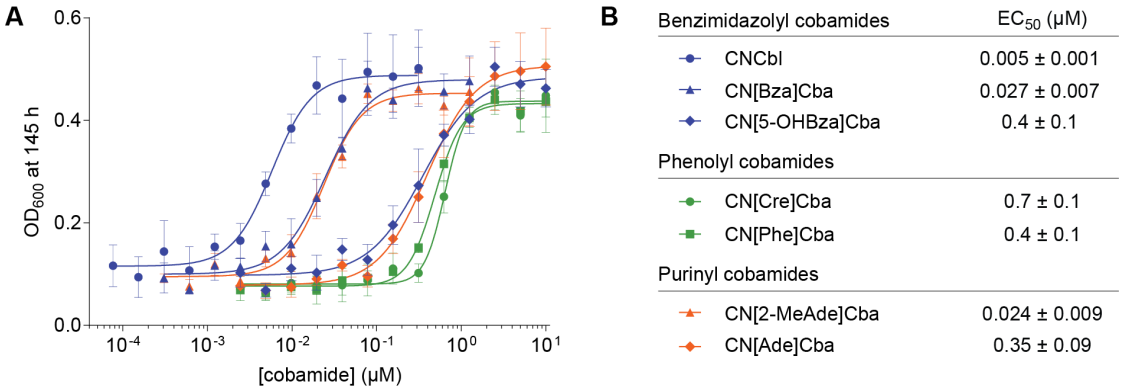


Figure 8: MCM-dependent growth of *S. meliloti cobD*::*gus* Gm^R *metH*::Tn*5* Δ*nrdJ* pMS03-$nrdAB_{Ec}^+$ with various cobamides (see also Figures S3, S4, S5). (A) Dose dependence of growth based on OD_{600} at 145 h. Data points and error bars represent the mean and standard deviation, respectively, of three biological replicates from a single experiment. EC_{50} values reported in (B) are the average and standard deviation of five or more biological replicates across two or more independent experiments.

With the notable exception of the phenolyl cobamides, differences in the EC_{50} values of cobamides *in vivo* qualitatively correlated with the binding selectivity that we observed *in vitro* (Figure 2). Among benzimidazolyl cobamides, EC_{50} values increased from cobalamin to [Bza]Cba to [5-OHBza]Cba, consistent with the relative binding affinities of these cobamides. Similarly, [2-MeAde]Cba, which had an intermediate binding affinity for *Sm*MCM, had a lower EC_{50} value than [Ade]Cba, which did not bind to *Sm*MCM at low micromolar concentrations *in vitro*. The ability of [5-OHBza]Cba and [Ade]Cba to support growth suggests that these cobamides can bind *Sm*MCM at concentrations higher than those tested *in vitro*; a control experiment with an *S. meliloti* strain lacking MCM rules out the possibility that high concentrations of cobamides (10 μM) abiotically enable growth on isoleucine and valine (Figure 7).

We considered the possibility that differences in cobamide internalization by *S. meliloti* could also influence the EC_{50} measurements shown in Figure 8. When *S. meliloti* cultures were supplemented with equimolar amounts of CNCbl, CN[Ade]Cba, or CN[Cre]Cba, the concentration of cobalamin extracted from the cellular fraction was 2- to 3-fold higher than [Ade]Cba and 5- to 6-fold higher than [Cre]Cba (Figure 9A). This result suggests that cobamides

are differentially internalized or retained by the cells. However, MCM-dependent growth does not correlate with intracellular cobamide concentrations, as intracellular concentrations of cobalamin comparable to those of [Ade]Cba and [Cre]Cba supported *S. meliloti* growth to high densities (Figure 9). Therefore, the high EC_{50} of CN[Ade]Cba relative to CNCbl is more likely attributable to enzyme selectivity. Additional factors that could explain the high EC_{50} values of the phenolyl cobamides are considered in the Discussion.

A

Cobamide	Concentration added to medium	Concentration in cells
CNCbl	0.2 μM	25 - 38 μM
	0.1 μM	5 - 17 μM
	0.04 μM	1 - 7 μM
CN[Ade]Cba	0.2 μM	11 - 14 μM
CN[Cre]Cba	0.2 μM	7 - 8 μM

B

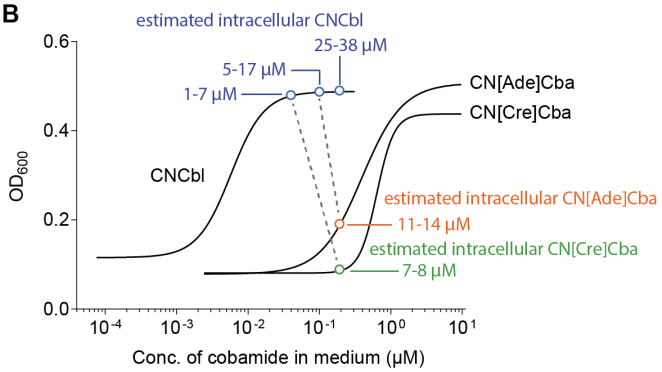


Figure 9: Quantification of cobamides internalized by *S. meliloti cobD*::*gus* Gm^R *metH*::Tn*5* Δ*nrdJ* pMS03-*nrdAB*$_{Ec}^{+}$. (A) Cellular cobamide concentrations following 48 h of growth in M9 sucrose with the indicated concentrations of cobamides added to the medium. The range of concentrations measured in cell pellets was determined by HPLC analysis of corrinoid extractions from two or more independent experiments, each including biological duplicates. (B) A graphic illustrating the concentrations of different cobamides at which intracellular cobamide concentrations are comparable. Internalization data (colored) are overlaid onto dose-response curves from Figure 8 (black). Five points indicate cobamide concentrations at which cobamides were extracted and quantified; dotted lines connect conditions in which intracellular concentrations of different cobamides are approximately equal.

Identification of structural elements that interfere with cobamide binding.

Given the apparent importance of MCM cobamide binding selectivity for the cobamide-dependent growth of *S. meliloti*, we pursued a more mechanistic understanding of how lower ligand structure affects cobamide binding. When cobamides are bound to MCM, the lower ligand is surrounded by protein residues [Froese et al., 2010; Mancia et al., 1996]. Therefore, the reduced affinity of certain cobamides for the enzyme could be a result of exclusion of their lower ligands from this binding pocket because of steric or electrostatic repulsion. We hypothesized that the poor binding of the purinyl cobamides Ado[Ade]Cba and Ado[2-MeAde]Cba is due to the presence of the exocyclic amine (N10CHECK) based on several observations: 1) Ado[5-OHBza]Cba, which also contains a polar functional group, had impaired binding to *Sm*MCM (Figure 2A, C). 2) In the crystal structure of *Hs*MCM [Froese et al., 2010], residues Phe722 and Ala731, which are conserved in *Sm*MCM, would be expected to electrostatically occlude the exocyclic amine of [Ade]Cba (Figure 10A, asterisk). Based on sequence alignment (Figure 5A), polar residues Tyr and Ser would be expected to occupy the corresponding positions in *Ec*MCM, which has higher affinity for purinyl cobamides (Figure 5). 3) Structural modeling of Ado[Ade]Cba bound to *Hs*MCM, which shares 59% amino acid identity to *Sm*MCM in the B_{12}-binding domain, suggests significant displacement of the adenine lower ligand relative to the lower ligand of AdoCbl, in the direction that would be consistent with steric or electrostatic repulsion of the exocyclic amine by surrounding residues (Figure 10B-D).

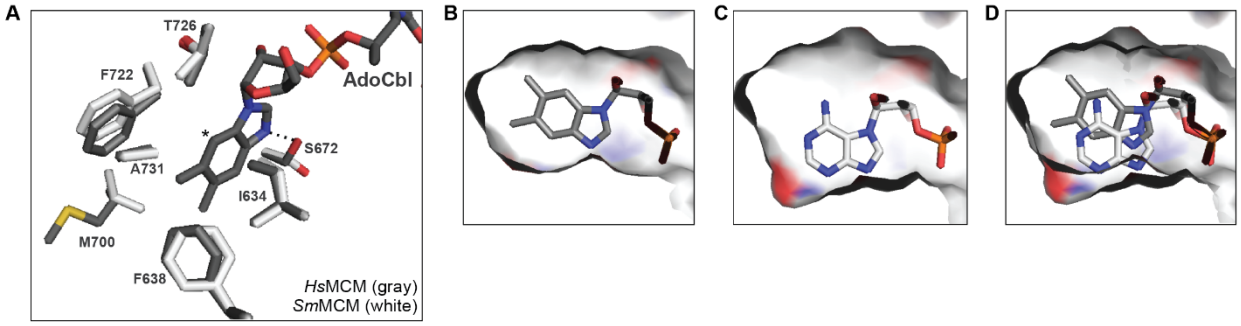


Figure 10: The lower ligand binding pocket of MCM. (A) Residues surrounding the lower ligand of AdoCbl in the X-ray crystal structure of *Homo sapiens* MCM (*Hs*MCM) (PDB: 2XIQ, gray). A model of *Sm*MCM, generated by sequence alignment and threading using Swiss-Prot, is overlaid in white. The asterisk marks the expected position of the exocyclic amine of [Ade]Cba. (B) Surface depiction of the lower ligand binding pocket of *Hs*MCM bound to cobalamin, after performing a constrained energy minimization. As expected, no major differences were observed between the energy minimized model and the original structure. (C) Surface depiction of the lower ligand binding pocket of *Hs*MCM modeled with [Ade]Cba bound, generated by changing the structure of the lower ligand in (B) and performing a constrained energy minimization. (D) Overlay of the structural models in B and C.

To test the importance of the exocyclic amine of adenine in cofactor exclusion, we produced an unsubstituted purinyl cobamide, Ado[Pur]Cba [Yan et al., 2018]. Ado[Pur]Cba also had low affinity for *Sm*MCM (Figure 11A, B), suggesting that the exocyclic amine of adenine is not a major cause of binding exclusion. Consistent with this result, two novel benzimidazolyl cobamides, Ado[7-MeBza]Cba and Ado[7-AmBza]Cba, bound *Sm*MCM with comparable affinities to other benzimidazolyl cobamides (Figure 11A, B), despite being functionalized at the position analogous to N10 of adenine (verified by NMR, Figure X). Rather, these results suggest that the presence of nitrogens in the six-membered ring of the lower ligand interferes with binding. To test this hypothesis directly, we produced three cobamide analogs with at least one nitrogen in the six-membered ring of the lower ligand base. Comparison of the binding of Ado[6-MePur]Cba and Ado[7-MeBza]Cba (Figure 11A, B) supported a role of ring nitrogens in binding inhibition, and comparison of binding affinities between Ado[Bza]Cba and Ado[5-AzaBza]Cba [Schubert et al., 2019] (Figure 2A, C and Figure 11A, B, respectively), and between Ado[7-AmBza]Cba and Ado[3-DeazaAde]Cba (Figure 11A, B), revealed that a single nitrogen atom in the six-membered ring of the lower ligand was sufficient to severely impair binding.

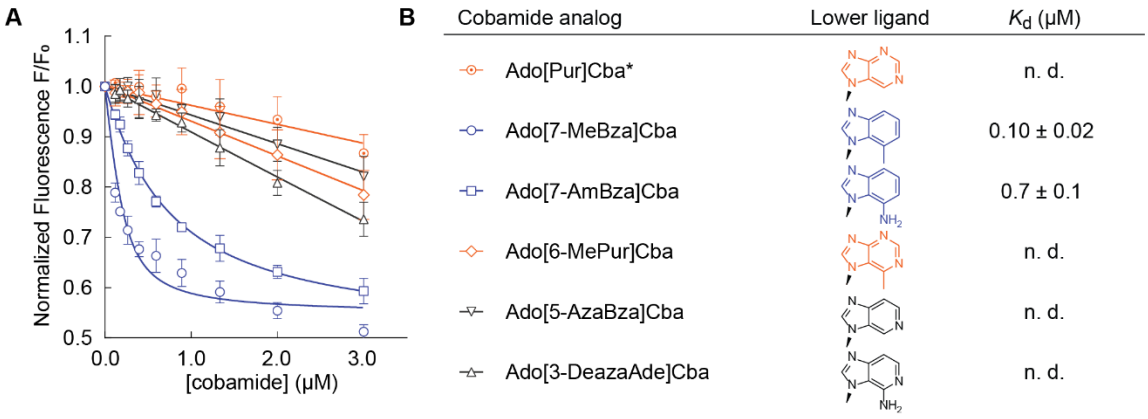


Figure 11: Binding of cobamide analogs to *Sm*MCM (see also Figure 15). (A) Fluorescence decrease of *Sm*MCM when reconstituted with benzimidazolyl (blue), purinyl (orange), and azabenzimidazolyl (black) cobamide analogs. Data points represent the mean and standard deviation of three technical replicates from a single experiment. (B) K_d values for different cobamides, reported as the average and standard deviation of three or more independent experiments, each consisting of technical triplicates. "n. d.," not determined, indicates that binding was too weak to determine K_d. *While it was unreported at the time of our study, [Pur]Cba was discovered to be the cobamide naturally produced by *Desulfitobacterium hafniense* [Yan et al., 2018].

As it was recently discovered to be a naturally occurring cobamide [Yan et al., 2018], we tested the MCM-dependent growth of *S. meliloti* with [Pur]Cba. [Pur]Cba had a high EC_{50} value of 0.6 ± 0.2 μM (Figure 12), further supporting the correlation between binding and growth that we previously observed for benzimidazolyl and purinyl cobamides.

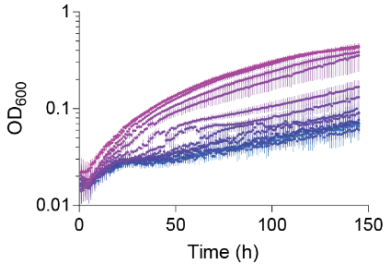


Figure 12: MCM-dependent growth of *S. meliloti cobD*::*gus* Gm^R *metH*::Tn*5* Δ*nrdJ* pMS03-*nrdAB*$_{Ec}^+$ with CN[Pur]Cba. Maximum concentration tested is 10 μM (pink) and decreases by 2-fold dilutions (pink to blue). Plots show the mean and standard deviation of three biological replicates.

IV. Discussion

Cobamides are distinct from other cofactors in their extensive structural diversity, with over a dozen forms that differ in the lower ligand base and nucleotide loop. How cobamide lower ligand structure influences the activity of cobamide-dependent enzymes has not been extensively explored. Here, we report a systematic analysis of the effects of cobamide lower ligand structure on the function of a model cobamide-dependent enzyme, MCM. Our results show that MCM exhibits varied affinities for different cobamides, and that this selectivity is linked to the physiology of the organism.

Our results show that the major determinant of cobamide selectivity in *Sm*MCM is binding, with small changes in the lower ligand capable of dramatically altering the binding affinity of a cobamide. One explanation for these differences is that the chemical compatibility between the lower ligand base and the binding pocket of the protein strongly influences the binding affinity of

cobamides; repulsion of the lower ligand on the basis of electrostatics could reduce the binding affinity of cobamides to MCM. While the structure of *Sm*MCM has not been determined, a model generated by sequence alignment to *Hs*MCM suggested a highly hydrophobic lower ligand binding pocket. Consistent with this, we observed higher affinity of cobamides with hydrophobic lower ligands to *Sm*MCM, as well as interference of ring nitrogens with cobamide binding.

On the other hand, sequence alignments suggested that many of the hydrophobic residues predicted to immediately surround the lower ligand are conserved between diverse MCM orthologs that differ in cobamide selectivity. Assuming that the arrangement of the lower ligand binding pocket is similar across MCM orthologs, this suggests that interactions within the lower ligand binding pocket are not sufficient to account for selectivity. In a similar vein, examination of the residues surrounding the lower ligand in the cobamide-bound structures of reductive dehalogenases does not reveal the basis of exclusion of certain cobamides [Keller et al., 2018]. These observations suggest that the lower ligand may have an unknown role in the binding of cobamides to MCM. Consistent with this idea, studies of the kinetics and pH dependence of AdoCbl binding to *P. shermanii* MCM suggest a pre-association step, wherein a cofactor-protein complex is formed prior to displacement of the lower ligand of the cofactor by a histidine residue in the protein [Chowdhury and Banerjee, 1999]. The nature of this complex is unknown, but potential interactions between the lower ligand and this conformation of the enzyme could provide an opportunity for lower ligand structure to impact the outcome of binding.

Our analysis of MCM orthologs from *E. coli* and *V. parvula* demonstrates that variations in cobamide selectivity have evolved in organisms with different physiologies. The cobamide selectivity patterns in the three MCM orthologs we examined correlate with the physiologies of the bacteria in two ways. First, in all three cases, each MCM ortholog has highest affinity for the native cobamide produced by the organism, suggesting that cobamide biosynthesis and selectivity of cobamide-dependent enzymes have coevolved. Second, *Sm*MCM is more selective than *Ec*MCM and *Vp*MCM, and these differences in selectivity correlate with differences in cobamide biosynthesis, acquisition, and use in these organisms. *S. meliloti* synthesizes cobalamin *de novo* and is incapable of attaching purinyl and phenolyl lower ligands to cobamide precursors [Crofts et al., 2013]. Thus, its cobamide-dependent enzymes have likely evolved to function best with cobalamin. In contrast, *E. coli* does not synthesize cobamides *de novo* and instead relies on the importer BtuBFCD to acquire cobamides from the environment [Borths et al., 2005; Heller et al., 1985]. Alternatively, *E. coli* can produce a variety of benzimidazolyl and purinyl cobamides when provided with precursors [Hazra et al., 2015], making the ability to use multiple cobamides likely advantageous. Like *S. meliloti*, *V. parvula* synthesizes cobamides *de novo*, but can produce both benzimidazolyl and phenolyl cobamides [Crofts et al., 2013; Hazra et al., 2013] and also encodes membrane transport components adjacent to cobalamin riboswitches [Mukherjee et al., 2019], which are likely to be cobamide importers [Nahvi et al., 2004; Rodionov et al., 2003]. Thus, the ability of *Vp*MCM to bind diverse cobamides is similarly consistent with its physiology.

Relative to cobamide binding selectivity, our results suggest that effects of lower ligand structure on the catalytic activity of MCM are minor. Among the cobamides we tested, the maximum differences in *Sm*MCM turnover were 3-fold. We did not observe inhibition of MCM activity with any cobamides, in contrast to the strong inhibition that has been observed with analogs containing variations in the upper ligand or central metal, known as antivitamins [Calafat et al., 1995; Fukuoka et al., 2005; Widner et al., 2016].

In addition to elucidating the biochemical basis of cobamide selectivity in MCM, a major aim of our work was to link biochemical selectivity with cobamide-dependent growth. Our results

with benzimidazolyl and purinyl cobamides support the hypothesis that enzyme selectivity is a major determinant of cobamide-dependent growth. Interestingly, although phenolyl cobamides bound *Sm*MCM with high affinity and supported catalysis *in vitro*, high concentrations were required to support growth of *S. meliloti*. This discrepancy can be partially explained by poorer internalization or retention of these cofactors as compared to cobalamin (Figure 9). The observation that the intracellular cobamide concentrations were 50- to 190-fold higher than the amount added to the growth medium (Figure 9A) suggests that cobamides could be internalized by an uptake mechanism that favors cobalamin, distinct from both BtuBFCD and ECF-CbrT [Rodionov et al., 2009; Santos et al., 2018], both of which are absent from *S. meliloti*. Thus, we propose a model in which the cobamide-dependent growth of bacteria is influenced not only by binding selectivity of cobamide-dependent enzymes, but also by cobamide import (Figure 13). The lower effectiveness of phenolyl cobamides in supporting growth of *S. meliloti* could additionally be explained by inefficient adenosylation of these cobamides *in vivo*, as MCM requires the adenosyl upper axial ligand for activity. Whether or not adenosyltransferase enzymes, specifically CobA and PduO [Escalante-Semerena et al., 1990; Johnson et al., 2001] in *S. meliloti*, are selective with respect to lower ligand structure is unknown.

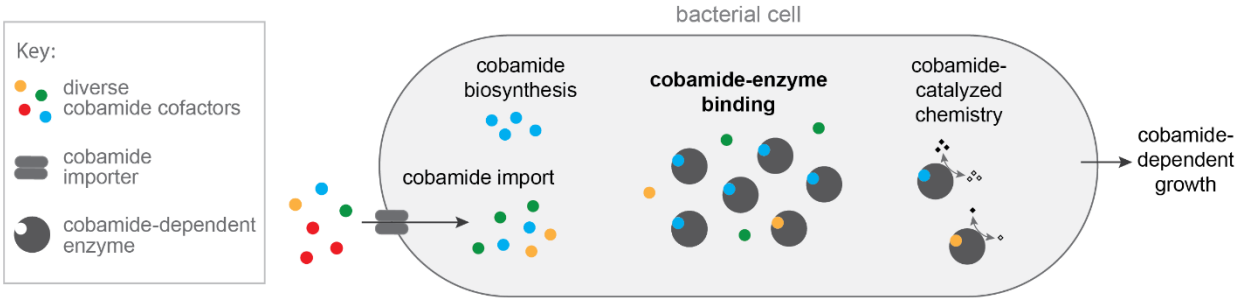


Figure 13: Model describing biochemical determinants of cobamide-dependent growth in bacteria. Cobamides differentially impact bacterial growth due to selective cobamide import and biosynthesis, cobamide-binding selectivity of cobamide-dependent enzymes, and cobamide-dependent catalysis. For MCM-dependent growth of *S. meliloti*, cobamide-binding selectivity is most strongly correlated with cobamide-dependent growth of the organism.

We and others have proposed the possibility of manipulating microbial communities using cobamides by taking advantage of the differential cobamide-dependent growth of bacteria [Abreu and Taga, 2016; Degnan et al., 2014b; Seth and Taga, 2014; Yan et al., 2018]. Cobamides are predicted to mediate microbial interactions that are critical to the assembly of complex communities [Belzer et al., 2017; Degnan et al., 2014a; Heal et al., 2017; Helliwell et al., 2016; Ma et al., 2017; Men et al., 2015; Shelton et al., 2019; Yan et al., 2012], so the ability to selectively inhibit or promote the growth of particular species using corrinoids with various lower ligands could be applied to alter the composition of microbial communities in ways that could promote environmental and human health. This possibility hinges on the ability to predict which cobamides support or inhibit growth of an organism of interest, which requires an understanding of the major biochemical determinants of growth. We observed here that the cobamide binding selectivity of a model base-off cobamide-dependent enzyme correlates with growth to a large extent. Thus, uncovering protein residues that confer selectivity would enable prediction of selectivity in cobamide-dependent enzymes, thereby facilitating prediction of the cobamide requirements of

organisms of interest. Furthermore, our results suggest that additional steps of cobamide trafficking may be important determinants of cobamide-dependent growth. Future studies to understand how these various steps depend on cobamide structure will ultimately allow us to better understand, predict, and manipulate microbial interactions.

V. Materials and Methods

Chemical reagents

Chemicals were obtained from the following sources: 5'-chloro-5'-deoxyadenosine, Santa Cruz Biotechnology; 4-methylbenzimidazole, Accela; 5-methyl-1H-benzimidazole, ACROS Organics; phenol, J. T. Baker; zinc metal, Fisher Scientific; 5-methoxybenzimidazole, purine, and *para*-cresol, Alfa Aesar; methylmalonyl-CoA, methylmalonic acid, coenzyme A, adenosylcobalamin (coenzyme B_{12}), cyanocobalamin, dicyanocobinamide, 6-methylpurine, 1H-imidazo[4,5-c]pyridine-4-amine (3-deazaadenine), benzimidazole, adenine hemisulfate, 5-azabenzimidazole, 1H-benzo[d]imidazol-7-amine (4-aminobenzimidazole), 2-methyl-1H-purine-6-amine (2-methyladenine), and bovine serum albumin (BSA), Sigma.

Molecular cloning, protein expression and purification

*Sm*MCM (locus SM_b20757, *bhbA*) was expressed from the pET28a vector, with an N-terminal hexahistidine (6xHis) tag, in *E. coli* BL21(DE3)pLysS (cloning primers listed in Table 2). The expression strain was grown to an optical density at 600 nm (OD_{600}) of 0.6-0.8 at 37 ˚C, cooled on ice for 15 min, and induced with 1 mM IPTG for 2.5 h at 37 ˚C. Cells were lysed by sonication in 25 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole, with 0.5 mM PMSF, 1 µg/mL leupeptin, 1 µg/mL pepstatin, and 1 mg/mL lysozyme. Clarified lysate was treated with 0.05% polyethyleneimine. An ÄKTA Pure 25 Fast Protein Liquid Chromatography (FPLC) system was used to purify the protein over a GE 5 mL HisTrap HF column, using a gradient of 21 to 230 mM imidazole in the lysis buffer. Purified protein was dialyzed into 25 mM Tris-HCl pH 8.0, 300 mM NaCl, 10% glycerol and concentrated with a Vivaspin 10,000 MWCO protein concentrator. Purity was analyzed by SDS-PAGE (Figure 14), and protein concentration was determined by A_{280} using the theoretical extinction coefficient 55810 M^{-1} cm^{-1} [Fortier et al., 2012]. *Ec*MCM (locus b2917, *scpA*, previously *sbmA*) was expressed with an N-terminal 6xHis tag from a pET28a vector in *E. coli* BL21(DE3), by induction at OD_{600} 0.6-0.8 with 0.1 mM IPTG, for 3.5 h at 30 ˚C. The protein was purified as described above and the final concentration was determined by Coomassie-stained SDS-PAGE (Figure 14), using BSA as a standard.

The *V. parvula* genome has two MCM annotations: a heterotetramer (loci Vpar_RS06295, Vpar_RS06290) and a heterodimer (loci Vpar_RS09005, Vpar_RS09000). The functionality of both homologs was tested by complementation in *S. meliloti*. The two putative *Vp*MCM enzymes were cloned into the pTH1227 vector and transferred by conjugation into an *S. meliloti bhbA*::Tn*5* mutant. Complementation was assessed by growth in M9 liquid medium containing L-isoleucine and L-valine (see "*S. meliloti* growth assays" for additional details). *S. meliloti* co-expressing Vpar_RS09005 and Vpar_RS09000 showed identical growth to a strain expressing *Sm*MCM from pTH1227 and was selected for *in vitro* studies.

The α subunit of *Vp*MCM (encoded by Vpar_RS09005) was expressed with an N-terminal 6xHis tag from the pET-Duet expression vector in *E. coli* BL21(DE3). Protein expression was

induced with 520 µM IPTG for 6 h at 30 ˚C. The protein was batch purified by nickel affinity and subsequently purified by FPLC using a HiTrapQ column with an NaCl gradient from 50 to 500 mM in 20 Tris-HCl pH 8.0, 10% glycerol. The β subunit of *Vp*MCM (encoded by Vpar_RS09000) was expressed separately with an N-terminal 6xHis tag from the pET-Duet expression vector in *E. coli* BL21(DE3). Expression was induced with 1 mM IPTG for 22 h at 16 ˚C and the protein was purified using nickel-affinity chromatography as described for *Sm*MCM. Purified protein was dialyzed into 25 mM Tris-HCl pH 8.0, 300 mM NaCl, 10% glycerol, and 1 mM β-mercaptoethanol. Concentration of pure α and β subunits (Figure 14) was determined by absorbance at 280 nm (A_{280}) using the theoretically calculated extinction coefficients 75290 M^{-1} cm^{-1} and 74260 M^{-1} cm^{-1}, respectively [Fortier et al., 2012]. Equimolar amounts of α and β subunits were combined during the setup of fluorescence binding assays.

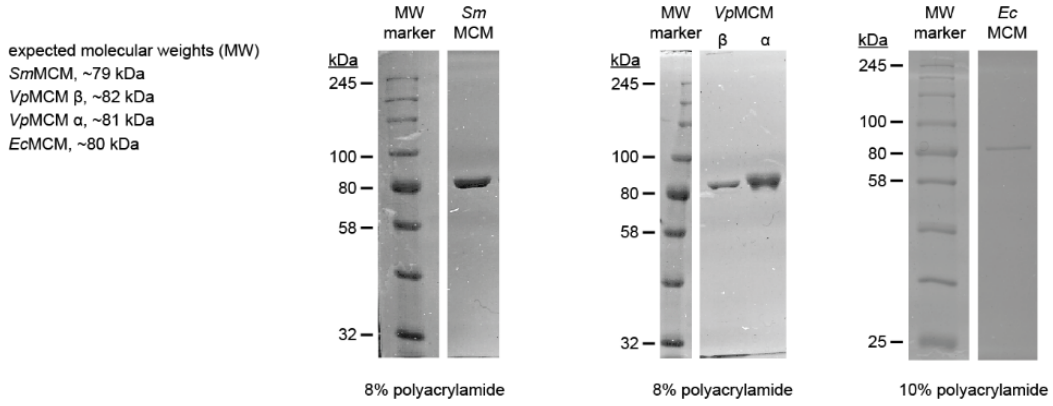


Figure 14: SDS-PAGE analysis of purified MCM orthologs. Gels are Coomassie-stained.

Table 2: Cloning primers. Vector-derived sequences are in lower case letters.

Construct	Primer sequence
pET28a-*bhbA* (*Sm*MCM)	ccgcgcggcagccatatggctagcACCGAAAAGACCATCAAGGACTG aagcttgtcgacggagctcgaattcTTACACGTTTCGCCGCTTGC
pET28a-*scpA* (*Ec*MCM)	cgcgcggcagccatatgATGTCTAACGTGCAGGAGTGG gtgcggccgcaagcttTTAATCATGATGCTGGCTTATCAG
pTH1227-Vpar_RS06295-Vpar_RS06290	ttcacctcgagatctatcgatgcatACCTGTTTTTTTTAGGAGGATGATGAAAAC gcttgaattcgagctcccgggtaccTTATTTTACGTTTTCTTTAATAAAGTTAACGATATCGC
pTH1227-Vpar_RS09005-Vpar_RS09000	ttcacctcgagatctatcgatgcatGGTTTCGAGTTGAAAAGGAGGCA gcttgaattcgagctcccgggtaccTCAGTCATGAGACTCGTCTTGAAC
pETDuet-Vpar_RS09005 (*Vp*MCM α)	cattggatcctATGTCTGACAAAAAGAC ctaactcgagTCAGTCATGAGACTCGTC
pETDuet- Vpar_RS09000 (*Vp*MCM β)	cattggatcctATGTTTAAAAATC ctaactcgagTCAGTCATGAG

E. coli thiokinase containing an N-terminal 6xHis tag was expressed from a vector provided by Gregory Campanello from the laboratory of Ruma Banerjee. Expression was induced with 1 mM IPTG in *E. coli* BL21(DE3)pLysS at 28 ˚C for 3 h. The protein was purified as a heterodimer using nickel-affinity chromatography as described above. His-tagged *Rhodopseudomonas palustris* MatB [Crosby et al., 2012] was expressed from a pET16b expression plasmid provided by Omer Ad from the laboratory of Michelle Chang. The protein was overexpressed in *E. coli* BL21(DE3) at 16 ˚C overnight, after induction with 1 mM IPTG, and

purified by nickel affinity chromatography as indicated above. Thiokinase and MatB concentrations were determined by Coomassie-stained SDS-PAGE, using BSA as a standard.

Guided biosynthesis, extraction, and purification of cobamides

Sporomusa ovata strain DSM 2662 was used for the production of its native cobamide, [Cre]Cba, and for production of [Phe]Cba, [5-MeBza]Cba, [Bza]Cba, [5-OHBza]Cba, [7-MeBza]Cba, and [7-AmBza]Cba, by guided biosynthesis as previously described [Mok and Taga, 2013]. 5-OHBza was synthesized as described [Crofts et al., 2013]. *Salmonella enterica* serovar Typhimurium strain LT2 and *Propionibacterium acidipropionici* strain DSM 20273 were used for production of [Ade]Cba [Hoffmann et al., 2000; Yi et al., 2012]. [2-MeAde]Cba, [Pur]Cba, [5-AzaBza]Cba, [3-DeazaAde]Cba, and [6-MePur]Cba were produced by guided biosynthesis in *P. acidipropionici*. Cobamides were extracted as previously described [Yi et al., 2012] and purified by High Performance Liquid Chromatography (HPLC) using previously published methods [Crofts et al., 2014; Crofts et al., 2013; Yi et al., 2012] as well as additional methods listed in Table 3. In many cases more than one method was required to achieve high purity. Identity of cobamides was confirmed by Liquid Chromatography (LC) coupled to Mass Spectrometry (MS) using an Agilent 1260 LC/6120 quadrupole MS instrument. Lower ligand orientation in the novel cobamides [7-MeBza]Cba, [7-AmBza]Cba, [3-DeazaAde]Cba, and [6-MePur]Cba was inferred based on their absorbance spectra, which reveal a base-on conformation in the cyanylated form in acidic conditions (Figure 15). The orientation of the lower ligands in [Pur]Cba and [5-AzaBza]Cba was not determined.

Table 3: HPLC methods for purification of cobamides.

Compounds purified	Mobile Phases	Flow (mL/min)	Temp (˚C)	Gradient
Phenolyl, non-polar benzimidazolyl, and adenosylated cobamides	A: 0.1% formic acid in water B: 0.1% formic acid in methanol	2	30	25% B, 2 min 25 – 60% B, 24 min
Purinyl cobamides and azabenzimidazolyl cobamides	A: 0.1% formic acid in water B: 0.1% formic acid in methanol	1.5	15	10 – 30% B, 3 min 30% B, 13.9 min 30 – 37% B, 2.1 min
Purinyl cobamides and azabenzimidazolyl cobamides	A: 0.1% formic acid in water B: 0.1% formic acid in methanol	2	30	10 – 42% B, 20 min
[5-OHBza]Cba	A: 0.1% formic acid in water B: 0.1% formic acid in methanol	2	25	18 – 25% B, 2.5 min 25%, 22.5 min
β-adenosylcobinamide[Widner, 2013]	A: 10 mM sodium phosphate pH 7 B: acetonitrile	2	25	2 – 23% B, 40 min

Column: Zorbax Eclipse Plus C18, 9.4 x 250 mm, 5µm

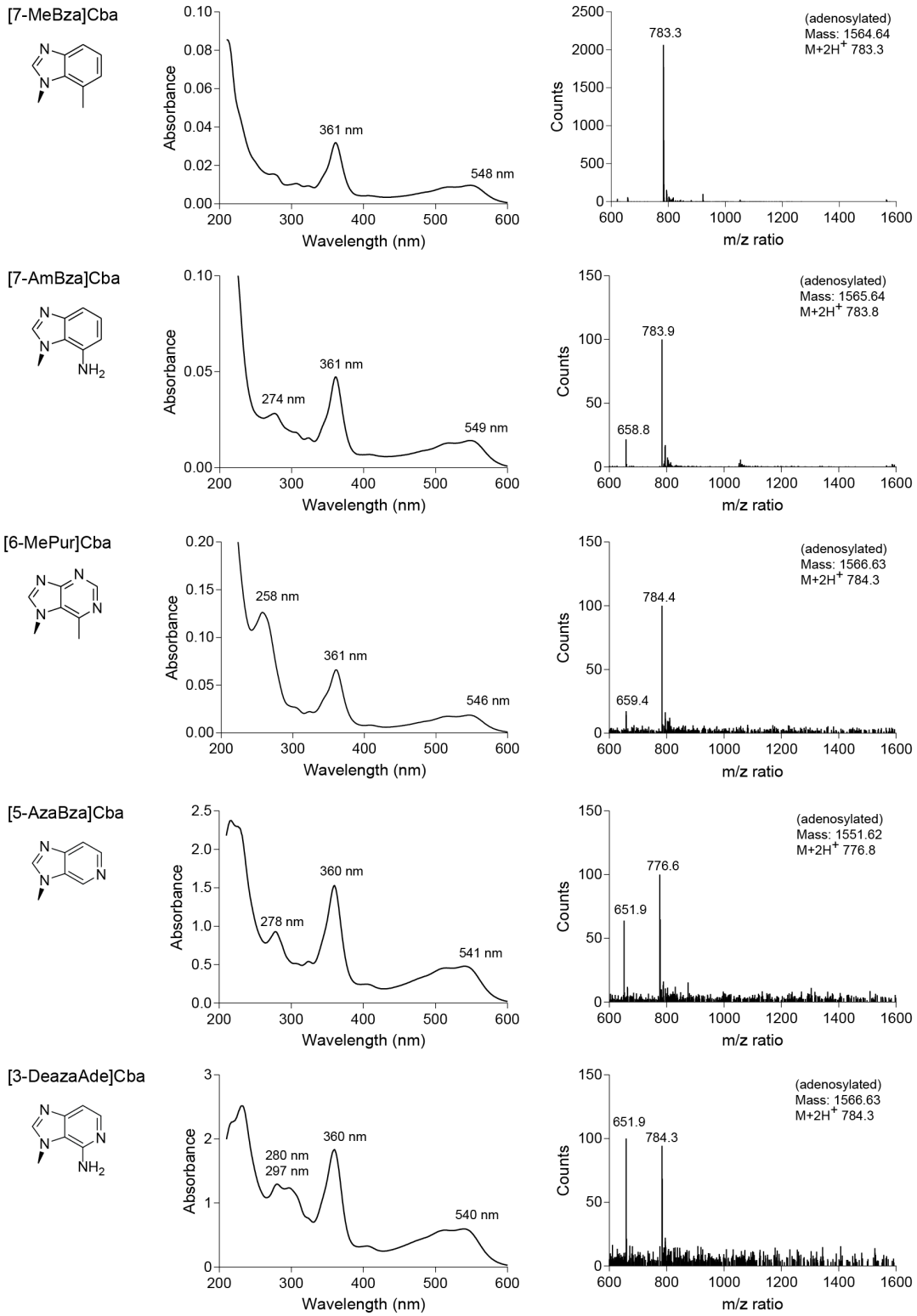


Figure 15: Spectral analysis of novel cobamide analogs. Left: Absorbance spectra of cyanylated cobamides, recorded during HPLC analysis of crude extracts. Labels indicate local absorbance maxima. Right: Mass spectra from HPLC-MS analysis of cobamides following adenosylation. Cobamides containing impurities were further purified by HPLC.

Chemical adenosylation of cobamides

Cobamide adenosylation was performed as previously described [Brown and Zou, 1999; Crofts et al., 2014]. Briefly, cobamides at concentrations 0.5 – 1 mM were reduced with activated zinc metal under anaerobic conditions, with vigorous stirring for 0.5 – 2 h. 5'-chloro-5'-deoxyadenosine was added and adenosylation was allowed to proceed for 1 – 3 h in the dark. The progress of the reaction was monitored by HPLC. Following adenosylation, cobamides were desalted using a C18 SepPak (Waters), purified by HPLC, desalted again, dried, and stored at -20 ˚C or -80 ˚C.

Cobamide quantification

Purified cobamides were dissolved in water and quantified by UV-Vis spectrophotometry on a BioTek Synergy 2 plate reader using the following extinction coefficients: for cyanylated benzimidazolyl cobamides, ε_{518} = 7.4 x 10^3 M^{-1} cm^{-1} [Hogenkamp, 1975]; for cyanylated purinyl cobamides, ε_{548} = 7.94 x 10^3 M^{-1} cm^{-1} [Schneider and Stroinski, 1987]; for cyanylated phenolyl cobamides, ε_{495} = 9.523 x 10^3 M^{-1} cm^{-1} [Stupperich et al., 1988]; for adenosylated benzimidazolyl cobamides (AdoCbl, Ado[5-MeBza]Cba, Ado[Bza]Cba, Ado[5-OHBza]Cba, Ado[7-MeBza]Cba, and Ado[7-AmBza]Cba), which are predominantly base-on in water, ε_{522} = 8.0 mM^{-1} cm^{-1} [Hogenkamp, 1975]; for adenosylated purinyl cobamides (Ado[Ade]Cba, Ado[2-MeAde]Cba, and Ado[Pur]Cba), which are predominantly base-off in water, and phenolyl cobamides (Ado[Cre]Cba and Ado[Phe]Cba), which are base-off, ε_{458} = 8.8 mM^{-1} cm^{-1} [Schneider and Stroinski, 1987]; for adenosylated azabenzimidazolyl cobamides (Ado[3-DeazaAde]Cba, Ado[5-AzaBza]Cba and Ado[6-MePur]Cba), which are a mixture of base-on and base-off in water, the concentration was estimated from the average of concentrations calculated using the extinction coefficients above.

Fluorescence Binding Assays

An *in vitro* assay previously described for measuring binding of AdoCbl to *P. shermanii* MCM [Chowdhury and Banerjee, 1999] was adapted to a 96-well format: MCM (0.2 μM) was combined with a range of cobamide concentrations (as specified in each experiment) in a black 96-well plate in 50 mM potassium phosphate pH 7.5 with 1 mM DTT, on ice. All steps involving cobamides were conducted in the dark. The plate was centrifuged for 1 min at 3800 rpm to level the surface of the liquid in each well. The plate was then incubated for 40 min at 30 ˚C to allow binding, with a brief shaking step after 30 min. Preliminary experiments showed that this time is sufficient for equilibration. Following incubation, fluorescence emission at 340 nm (5 nm slit width) was measured upon excitation at 282 nm (5 nm slit width) using a Tecan Infinite M1000 PRO Plate Reader. Fluorescence, normalized to the initial value, was plotted as a function of cobamide concentration, and fit to the following equation [Warner and Copley, 2007]:

$$\frac{F}{F_0} = 1 + \frac{\Delta F_{max}}{F_0} \frac{([E] + [L] + K_d) - \sqrt{([E] + [L] + K_d)^2 - 4[E][L]}}{2[E]}$$

where F is fluorescence, F_0 is initial fluorescence, $[E]$ is total enzyme concentration, $[L]$ is total ligand concentration, and K_d is the binding dissociation constant.

Filtration binding assay

Cobamides (10 μM) with and without MCM (15 μM) were incubated in 50 mM Tris-phosphate buffer pH 7.5 at 30 ˚C for 40 min, transferred to Nanosep 10K Omega centrifugal devices (Pall Corporation), and centrifuged for 5 minutes at 13,900 x g to separate unbound cobamides from enzyme-bound cobamides. The UV-Vis spectra of the filtrates were recorded on a BioTek Synergy 2 plate reader.

Structural modeling

A model of *Sm*MCM was generated using the Swiss-Model software [Guex et al., 2009] based on the known crystal structure of *Homo sapiens* MCM (*Hs*MCM) (PBD ID: 2XIJ) [Froese et al., 2010]. No major differences were observed in the B_{12}-binding domain between *Sm*MCM models generated from *Hs*MCM and *Propionibacterium freudenrichii* MCM (PDB ID: 4REQ) [Mancia et al., 1996].

Maestro [Schrödinger Release 2015: Maestro] was used to generate a model of *Hs*MCM bound to [Ade]Cba. The initial structure of *Hs*MCM bound to cobalamin (PDB ID: 2XIQ) [Froese et al., 2010] was prepared using standard methods. A constrained energy minimization (atoms within 10 Å of cobalamin freely moving; atoms within a second 10 Å shell constrained by a force constant of 200; remaining structure frozen) was performed using MacroModel [Schrödinger Release 2015: MacroModel]. The structure of the lower ligand was then modified to adenine, and the constrained energy minimization was repeated to generate a model of the lower ligand binding pocket bound to [Ade]Cba.

Enzymatic synthesis of (R)-methylmalonyl-CoA

(*R)*-methylmalonyl-CoA synthesis reactions contained the following in 10 mL: 100 mM sodium phosphate pH 7.5, 20 mM $MgCl_2$, 5 mM ATP, 10 mM methylmalonic acid, 2 mM coenzyme A, 5 mM β-mercaptoethanol, and 1.5 μM purified MatB protein. After combining ingredients on ice, the reaction was incubated at 37 ˚C for 1 h. The reaction was then frozen in liquid nitrogen and lyophilized. To purify (*R)*-methylmalonyl-CoA, the dried reaction mixture was resuspended in 3.2 mL water and the protein was precipitated with 200 μL trichloroacetic acid; precipitate was pelleted; supernatant was neutralized with 200 μL of 10 M NaOH; and salts and remaining starting materials were removed using a C18 SepPak column (Waters) (loaded in 0.1% formic acid, washed with water, methylmalonyl-CoA eluted with 50% methanol in water). Formation of (*R)*-methylmalonyl-CoA was initially verified by ^{1}H NMR and in subsequent preparations by HPLC (Table 4). The concentration of (*R)*-methylmalonyl-CoA was determined using an extinction coefficient of 12.2 mM^{-1} cm^{-1} at 259 nm.

Table 4: HPLC methods for analysis and purification of methylmalonyl-CoA

Column	Mobile Phases	Flow (mL/min)	Temp (˚C)	Gradient
Eclipse XBD-C18 4.6 x 150 mm, 5μm	A: 100 mM acetic acid, 100 mM sodium phosphate, pH 4.6 B: Solution A, 18% methanol	0.75	40	44% B, 14 min
Zorbax Eclipse Plus C18 9.4 x 250 mm, 5μm	A: 0.1% formic acid in water B: acetonitrile	3	25	0 – 10% B, 30 min 10 – 70% B, 3 min

MCM activity assays

A thiokinase-coupled, spectrophotometric MCM activity assay was adapted from previous work [Taoka et al., 1994], except that ADP was used instead of GDP, and the experiment was conducted in 96-well plates. Final concentrations of reagents in the assays are as follows: Tris-phosphate buffer pH 7.5, 100 mM Tris, 50 mM phosphate; DTNB, 400 µM; ADP, 1 mM; $MgCl_2$, 10 mM; (*R)*-methylmalonyl-CoA, 0 – 4 mM; thiokinase, 5 µM; MCM, 50 nM; and cobamides, 2 µM. Preliminary experiments were conducted to ensure that concentrations of thiokinase, DTNB, and cobamides were not rate limiting.

Three separate mixes were prepared, all in 1X Tris-phosphate buffer: an assay mix containing DTNB, ADP, and $MgCl_2$, a substrate mix containing (*R)*-methylmalonyl-CoA, and an enzyme mix containing thiokinase, MCM, and cobamides. All steps involving cobamides were conducted in the dark. The assay and enzyme mixes were prepared as a master mix and aliquoted into 96-well plates; substrate mixes were prepared in individual wells, in triplicate. All components were incubated at 30 ˚C for 40 minutes to equilibrate temperature and allow pre-binding of cobamides and MCM. After incubation, one replicate at a time, the substrate mix was added to the assay mix, followed by the enzyme mix. Absorbance at 412 nm (A_{412}) was recorded immediately after addition of enzyme and for 1-3 minutes, every 3 seconds, on a BioTek Synergy 2 plate reader. The increase in A_{412} in reactions lacking substrate was subtracted from all readings, to account for reactivity of DTNB with thiols on protein surfaces. A_{412} values were converted to concentration of free CoA using a pathlength correction determined for the reaction volume and extinction coefficient of 14150 M^{-1} cm^{-1}.

S. meliloti growth assays

MCM-dependent growth experiments were performed with *S. meliloti* strain Rm1021 *cobD*::*gus* Gm^R *metH*::Tn*5* Δ*nrdJ* pMS03-$nrdAB_{Ec}^+$, which lacks cobamide-dependent enzymes other than MCM and does not synthesize cobalamin. *cobD* is required for cobalamin biosynthesis [Campbell et al., 2006], *metH* encodes methionine synthase [Banerjee et al., 1989; Campbell et al., 2006; Sato et al., 1974], and *nrdJ* encodes ribonucleotide reductase [Cowles et al., 1969]. Because *nrdJ* is essential, the *E. coli* cobamide-independent ribonucleotide reductase encoded by *nrdA* and *nrdB* was expressed from the pMS03 plasmid [Taga and Walker, 2010]. The strain was pre-cultured in M9 medium [Maniatis et al., 1989] (modified concentration of $MgSO_4$: 1 mM) containing 0.1% sucrose, 2 g/L isoleucine, 2 g/L valine, 1 g/L methionine, and 20 µg/mL gentamycin, shaking at 30 ˚C. After two days, cells were washed and diluted to an OD_{600} of 0.02 into M9 medium containing 4 g/L isoleucine, 4 g/L valine, 1 g/L methionine, 20 µg/mL gentamycin, and cobamides at various concentrations as indicated for each experiment, in 384-well plates. The plates were incubated at 30 ˚C for 145 h in a Biotek Synergy 2 plate reader with linear shaking at 1140 cpm. OD_{600} was measured in 1 h increments.

For quantification of intracellular cobamides in *S. meliloti*, the strain above was pre-cultured as described, diluted into 50 mL of M9 medium containing 0.2% sucrose and various cobamides, and grown for 48 h (until OD_{600} 0.6-0.8). Cobamides were extracted from cell pellets as previously described [Yi et al., 2012], using 5 mL of methanol containing 500 µg of potassium cyanide, and including a partial purification by means of a wash step with 20% methanol in water during the SepPak desalting procedure. Extracted cobamides were quantified by HPLC using peak

areas at 525 nm and external standard curves, and cellular cobamide concentrations were calculated assuming 8 x 10^8 cells/mL at OD_{600} 1.0 and cellular volume of 1 μm^3.

Chapter 3

Diverse naturally occurring cobamides as cofactors for human methylmalonyl-CoA mutase

I. Abstract

Cobalamin, commonly known as vitamin B_{12}, is an essential micronutrient for humans. Cobalamin and related cofactors, cobamides, are produced exclusively by bacteria and archaea. In bacteria, cobamide-dependent enzymes are selective for particular cobamides, and in some cases are more active with alternate cobamides than with cobalamin. Humans have two cobamide-dependent enzymes, methylmalonyl-CoA mutase (MUT) and methionine synthase, but the ability of these enzymes to use diverse cobamides produced by microorganisms in food and in the human gut is not fully explored. Here, we characterize the biochemical cofactor selectivity of MUT in binding diverse cobamides, and the effects of cobamide structure on MUT activity. We find that while MUT is selective among cobamides, it is able to bind to and catalyze reactions with several cobamides other than cobalamin. We screened cobamide use in six disease-associated variants of MUT, each containing a single amino acid substitution rendering the enzyme largely inactive, and found that while several cobamides could support activity, none rescued activity to the level of WT. Overall, our studies call into question the assumption that humans require exclusively cobalamin, and instead suggest that several other cobamides may be relevant for human physiology and useful as therapeutics as well.

II. Introduction

Vitamins are diet-derived micronutrients that are essential for human health. Humans require vitamin B_{12} (cobalamin) because it is a cofactor for two metabolic enzymes: methionine synthase (MS) and methylmalonyl-CoA mutase (MCM, MUT in humans) (Figure 1A) [Kolhouse and Allen, 1977b]. MS is a cytosolic enzyme that catalyzes the synthesis of methionine by methylation of homocysteine. In addition to its importance in the methionine cycle, MS is linked to folate metabolism, as the methyl moiety in the MS-catalyzed reaction is derived from methyltetrahydrofolate (Me-THF) [Froese et al., 2018]. MUT is a mitochondrial enzyme that catalyzes the reversible isomerization of *(R)*-methylmalonyl-CoA and succinyl-CoA, an intermediate of the TCA cycle. This reaction is part of the propionate catabolism pathway in humans, and is required for the breakdown of branched amino acids, odd-chain fatty acids, and cholesterol [Fenton et al., 2014]. Mutations in MUT or MS result in the metabolic disorders methylmalonic aciduria (MMA) and homocystinuria (HCY), respectively, which are characterized by the accumulation of methylmalonate or homocysteine in the blood [Chandler and Venditti, 2005; Watkins and Rosenblatt, 1989]. Mutations in other enzymes involved in cobalamin absorption, trafficking, or activation in humans can also cause MMA and HCY [Huemer and Baumgartner, 2019; Watkins and Rosenblatt, 2014]. Inherited disorders of cobalamin metabolism, as well as nutritional cobalamin deficiency or issues with cobalamin absorption into the body (e.g. pernicious anemia, cobalamin malabsorption) can result in illnesses ranging from mild anemia to severe neurological dysfunction [Huemer and Baumgartner, 2019; Shevell and Rosenblatt, 2015].

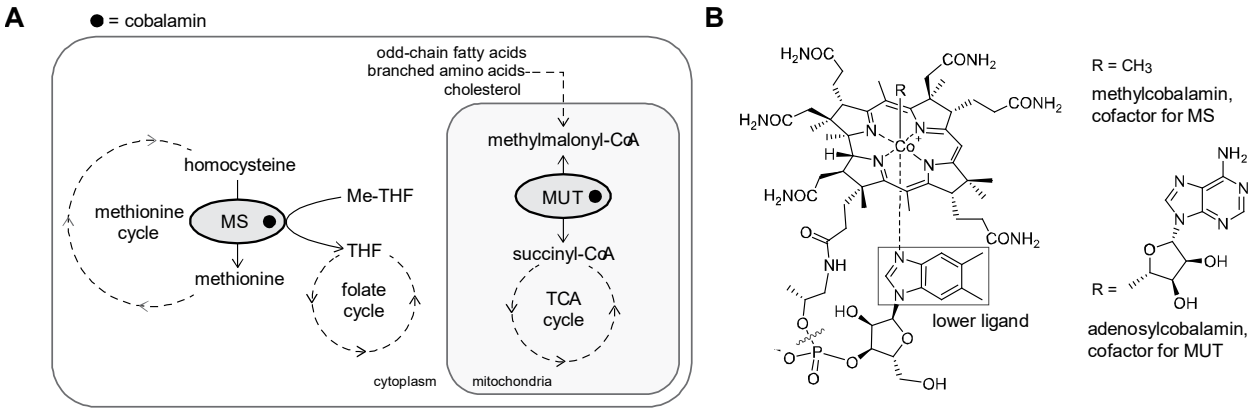


Figure 1: Cobalamin in human metabolism. (A) Diagram of metabolic pathways involving cobalamin in human cells. Dotted arrows indicate multiple reactions. MS and MUT are the only cobalamin-dependent enzymes in humans. SAM, *S*-adenosylmethionine; THF, tetrahydrofolate; Me-THF, methyltetrahydrofolate. (B) The structure of cobalamin. The upper ligand, R, varies for different enzymes. MS, methionine synthase; MUT, methylmalonyl-CoA mutase. The cobamide precursor cobinamide is delineated by the wavy line. The lower ligand, boxed, is different in other cobamides.

Although it is found in animal tissues, cobalamin is produced exclusively by a subset of prokaryotes [Shelton et al., 2019]. In addition to producing cobalamin, many bacteria and archaea produce cobalamin analogs that share the same general structure: a corrin ring that coordinates a cobalt ion, a pseudo-nucleotide that is covalently connected to the corrin ring through a propionamide sidechain, and interchangeable reactive upper ligands (Figure 1B). Cobalamin and its analogs, collectively termed cobamides, differ in the structure of the nucleotide base, commonly referred to as the lower ligand (Figure 1B, boxed), which can be a benzimidazole, purine, or phenolic [Allen and Stabler, 2008; Hazra et al., 2015; Hoffmann et al., 2000; Renz, 1999; Stupperich et al., 1988; Yan et al., 2018]. Importantly, lower ligand structure affects the biochemistry of cobamides; bacterial and mammalian cobamide-dependent enzymes, including both MCM and MS, are selective among cobamides with different lower ligands [Barker et al., 1960; De Hertogh et al., 1964; Keller et al., 2018; Lengyel et al., 1960; Poppe et al., 2000; Poppe et al., 1997; Tanioka et al., 2010]. It is noteworthy that in some environments, including the human gut, purinyl or phenolyl cobamides can be significantly more abundant than cobalamin [Allen and Stabler, 2008; Men et al., 2015].

It is widely accepted that humans are unable to use cobamides other than cobalamin, in part because of the intricate cobalamin uptake and trafficking system in humans, which is thought to be highly selective. Human intrinsic factor (IF), a glycoprotein that captures cobalamin in the intestine with femtomolar affinity and mediates uptake into ileal cells, has been reported to bind Ado[Ade]Cba and Ado[2-MeAde]Cba six orders of magnitude more weakly than AdoCbl [Fedosov et al., 2007; Kolhouse and Allen, 1977a], and to have low affinity for base-off cobamides including [Cre]Cba and the cobamide precursor cobinamide (Figure 1B) [Stupperich and Nexø, 1991]. Human transcobalamin (TC), which subsequently binds cobalamin that has entered the bloodstream and facilitates uptake into various tissues, is also highly selective against cobinamide, but less selective than IF for AdoCbl over other cobamides [Fedosov et al., 2007; Kolhouse and Allen, 1977a; Stupperich and Nexø, 1991]. The selectivity of both proteins against cobinamide and other cobamide degradation products [Kondo et al., 1982; Watanabe et al., 1998] is considered important, since cobinamide is not a suitable cofactor for MCM [Chowdhury and Banerjee, 1999].

The structural basis for IF and TC selectivity has been suggested by comparison of X-ray crystal structures of these proteins to the crystal structure of haptocorrin, a relatively promiscuous cobamide transport glycoprotein that binds cobamides and cobinamide in the upper GI tract and in the blood [Furger et al., 2013].

Despite the apparent selectivity of the human cobamide uptake and trafficking proteins, several lines of evidence suggest that cobamides besides cobalamin may reach human tissues. Human IF was found to bind [5-MeBza]Cba indistinguishably from cobalamin, and IF affinity for [Bza]Cba and [5-OHBza]Cba was within 2-fold of cobalamin [Kolhouse and Allen, 1977a; Stupperich and Nexø, 1991]. Thus, these cobamides are likely to enter the ileum. Although their identities are unknown, cobalamin analogs have been reported in patient serum samples [Kanazawa et al., 1983], and orally and subcutaneously administered cobamide analogs, including [Bza]Cba, can be found in rat and rabbit tissues [Kolhouse and Allen, 1977a; Stabler et al., 1991]. Considering these findings, it is important to understand how alternative cobamides impact the biochemistry of cobamide-dependent enzymes in humans.

Interestingly, *in vitro* studies suggest that human MS is active with cobamides containing structural variations in the corrin ring and lower ligand, and even with cobinamide, which lacks a lower ligand entirely (Figure 1B) [Dorweiler et al., 2003; Kolhouse et al., 1991]. Although the *in vitro* reconstitution of MUT with any cobamides besides cobalamin has not been reported, studies with other mammalian MCM orthologs suggest that MUT may bind several cobamides. For example, one study reports that MCM purified from sheep kidney is active both with AdoCbl and Ado[Bza]Cba (although the apparent K_M for Ado[Bza]Cba is 10-fold higher than for AdoCbl) but not with Ado[Ade]Cba [Lengyel et al., 1960], and a study in rats suggests that [Bza]Cba may be inhibitory to MCM activity, although this result is based on subcutaneous injections of cobamides and may therefore be influenced by numerous biological factors [Kolhouse et al., 1991]. Thus, the cobamide selectivity of MUT remains largely unknown.

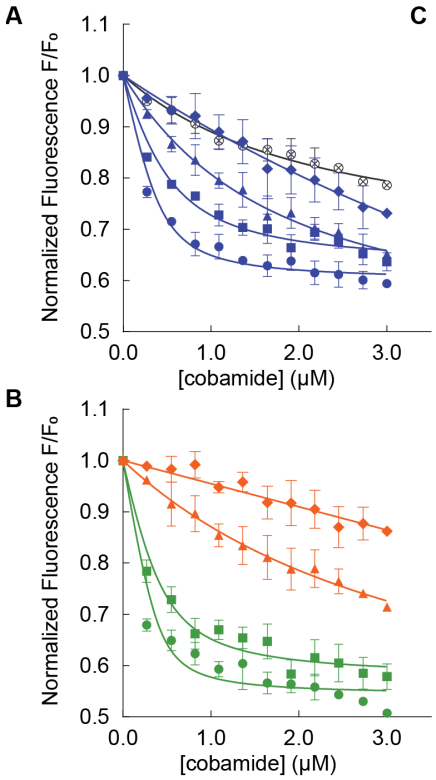
Here, we describe our work to characterize the activity of purified MUT with eight structurally diverse, naturally occurring cobamides. We found that MUT binds to, and is active with, several benzimidazolyl and phenolyl cobamides *in vitro*. Interestingly, in a study aimed at elucidating biochemical manifestations of disease-causing mutations in MUT, Forny and Froese *et al*. identified a set of MUT mutants with reduced apparent affinity for, and activity with, AdoCbl [Forny et al., 2014]. We investigated whether cobamides other than cobalamin could enhance the activity of six of these MUT mutants, which would be possible if any mutants had altered lower ligand selectivity compared to the WT enzyme. Although we found that the activity of MUT mutants with different cobamides remained well below that of the wildtype enzyme, this investigation demonstrated that both WT and mutant human MUT variants are able to use several cobamides as cofactors, contrary to previous assumptions that cobalamin is the only suitable cobamide for humans.

III. Results

MUT binds diverse cobamides.

Using a previously described fluorimetric assay [Chowdhury and Banerjee, 1999] (Chapter 2), we tested the cofactor binding selectivity of MUT *in vitro*. The equilibrium binding constant (K_d) determined for AdoCbl by this method (0.09 ± 0.03 μM, Figure 2) is close a previously published K_d of 0.2 μM [Campanello et al., 2018]. Using this assay, we found that MUT was not exclusively selective in binding AdoCbl. With the exception of Ado[5-OHBza]Cba, other

benzimidazolyl cobamides bound MUT. However, the absence of one or both methyl groups was sufficient to lower the binding affinities of Ado[5-MeBza]Cba and Ado[Bza]Cba by 3- and 17-fold, respectively, compared to AdoCbl. Interestingly, both phenolyl cobamides, Ado[Cre]Cba and Ado[Phe]Cba, had binding affinities similar to AdoCbl. In contrast, purinyl cobamides Ado[Ade]Cba and Ado[2-MeAde]Cba had low affinity for MUT. Overall, the cobamide-binding selectivity of MUT strongly resembles that of MCM from the α-proteobacterium *S. meliloti* (Chapter 2).



C

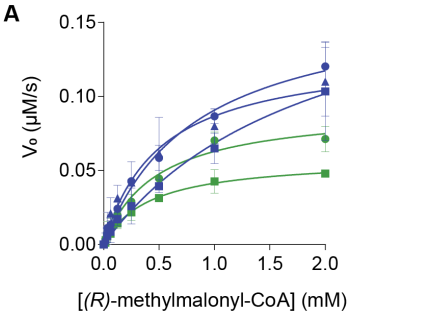
	Lower ligand	K_d (μM)
Ado-cobinamide	none	1.9 ± 0.4
Benzimidazolyl cobamides		
AdoCbl		0.09 ± 0.03
Ado[5-MeBza]Cba		0.27 ± 0.06
Ado[Bza]Cba		1.5 ± 0.2
Ado[5-OHBza]Cba		10 ± 4
Phenolyl cobamides		
Ado[Cre]Cba		0.05 ± 0.03
Ado[Phe]Cba		0.15 ± 0.05
Purinyl cobamides		
Ado[2-MeAde]Cba		3.3 ± 0.6
Ado[Ade]Cba		n. d.

Figure 2: Binding of structurally diverse cobamides to MUT. Fluorescence decrease of MUT (OS prep) when reconstituted with (A) benzimidazolyl cobamides (blue) and cobinamide (gray), and (B) phenolyl (green) and purinyl (orange) cobamides. Data points represent the mean and standard deviation of three technical replicates from a single experiment. (C) K_d values derived by curve fitting (see Methods). "n. d.," not determined, indicates that binding was too weak to determine K_d.

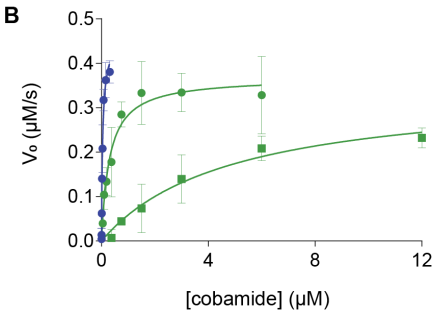
Benzimidazolyl and phenolyl cobamides support MUT activity with different apparent K_M values.

Our previous study of bacterial MCM showed that lower ligand structure does not significantly affect MCM activity. Similarly, using a coupled MCM activity assay described previously (Chapter 2) [Taoka et al., 1994], we found that MUT was active when reconstituted with AdoCbl, Ado[5-MeBza]Cba, Ado[Bza]Cba, Ado[Cre]Cba, and Ado[Phe]Cba (Figure 3A). Interestingly, however, the apparent K_M of the cobamides, determined by varying the concentration of cofactor, rather than substrate, differed drastically between cobamides. $K_{M,\,app}$ of AdoCbl was 0.046 ± 0.010 μM, in agreement with a value reported by another group [Janata et al., 1997]; $K_{M,\,app}$ for Ado[Cre]Cba was 10-fold higher, and for Ado[Phe]Cba was 100-fold higher (Figure 3B).

Thus, while several cobamides supported MUT activity, the concentration of cofactor required for activity varied significantly.



Benzimidazolyl cobamides	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($mM^{-1}s^{-1}$)
AdoCbl	3.4 ± 0.2	0.8 ± 0.1	4.3 ± 0.6
Ado[5-MeBza]Cba	4.0 ± 0.4	1.9 ± 0.4	2.1 ± 0.5
Ado[Bza]Cba	5.2 ± 0.4	0.50 ± 0.09	10 ± 2
Phenolyl cobamides			
Ado[Cre]Cba	1.8 ± 0.1	0.47 ± 0.09	4.0 ± 0.8
Ado[Phe]Cba	1.16 ± 0.02	0.41 ± 0.03	2.8 ± 0.2



Benzimidazolyl cobamides	k_{cat} (s^{-1})	$K_{M,\ app}$ (μM)
AdoCbl	46 ± 3	0.046 ± 0.010
Phenolyl cobamides		
Ado[Cre]Cba	37 ± 2	0.30 ± 0.05
Ado[Phe]Cba	30 ± 10	5 ± 3

Figure 3: MUT activity with different cobamides. (A) Michaelis-Menten kinetic analysis of MUT (OS prep) reconstituted with various cobamides at concentrations 10-fold higher than the measured K_d reported in Figure 2. (B) MUT (Oxford prep) activity with varying cobamide concentrations. The k_{cat} values in (A) and (B) differ because the experiments were performed with different enzyme preparations. Data points and error bars represent the mean and standard deviation, respectively, of three technical replicates from one experiment.

Screening for cobamides that enhance activity of MUT mutants.

Although we found that AdoCbl was the most effective cofactor for wild-type (WT) MUT, we saw a possibility that MUT mutants might have different selectivity from WT. If it were the case that a different cobamide better stabilized these mutants, or had higher affinity than AdoCbl, administration of that cobamide could be a potential disease therapy. We therefore decided to screen our library of 14 cobamides for the ability to enhance the activity of six MMA-causing MUT variants [Forny et al., 2014]. We specifically focused on mutations that result in a K_M defect, as this defect could potentially be associated with cofactor binding. Several such mutations (G648D, V633G, G717V) are located in the B_{12}-binding domain of the enzyme, although others (P86L, Y100C, Y231N) are located in the substrate-binding domain near the cofactor or near the MUT dimer interface [Forny et al., 2014; Janata et al., 1997].

We performed an activity screen in which purified MUT variants were reconstituted with each cobamide and product formation was detected after 30 minutes of activity. We reasoned that improvements in activity could occur due to increased stability, lower cofactor K_M, or improved catalysis. Consistent with our previous results, WT MUT was active with AdoCbl, Ado[5-MeBza]Cba, and Ado[Bza]Cba (Figure 4, compounds A, B, C), as well as phenolyl cobamides (Figure 4, compounds G, H), and was additionally active with three unnatural benzimidazolyl cobamide analogs (Figure 4, compounds J, K, P). No activity was observed with purinyl cobamides

(Figure 4, compounds E, F, M, N), likely due to low binding affinity (Figure 2), or with aza-benzimidazolyl cobamides (Figure 4, compounds L, O). We did not observe rescue of mutant activity to levels comparable to WT activity with any cobamides (Figure 4). However, there were several interesting results: 1) Activity of Y100C appears to be slightly higher with Ado[Cre]Cba (G) than with AdoCbl (A); 2) Activity of V633G with Ado[Cre]Cba (G) is relatively high, and surpassed activity with AdoCbl at a later time point (data not shown); 3) While WT MUT retains significant activity with benzimidazolyl cobamides Ado[5-MeBza]Cba (B), Ado[Bza]Cba (C), Ado[4-MeBza]Cba (J), and Ado[4-AmBza]Cba (K), some mutants, most notably V633G and G648D, lose activity with those cobamides relative to AdoCbl. Interestingly, the same mutants also lose activity with Ado[Phe]Cba (H) relative to Ado[Cre]Cba (G). Thus, in both cases, cobamides with lower ligands lacking methyl groups at the analogous positions of the 5- and 6-methyl moieties of AdoCbl result in a significant loss of activity in the mutants. All of these results warrant further investigation.

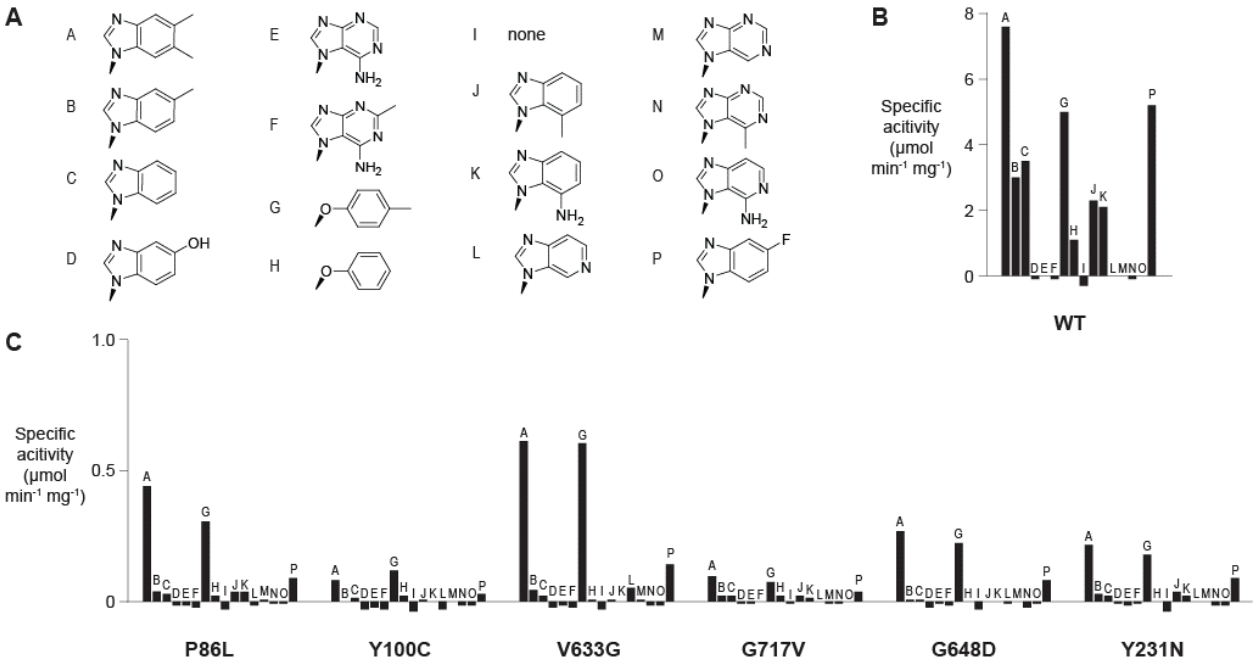


Figure 4: Activity screen of MUT WT and mutants. (A) Lower ligands of cobamides screened in this experiment. Specific activity of MUT WT (B) and mutants (C) reconstituted with different cobamides and cobinamide (1 µM) was determined after 30 minutes of activity. Each letter (A – P) corresponds to a cobamide, as assigned in (A). Note the difference in the y-axis scale between WT and mutant proteins. Data are from a single experiment with one replicate. Results for WT MUT were qualitatively reproduced in a second experiment.

IV. Discussion

Two human enzymes, MS and MUT, require cobalamin as a cofactor. The metabolic functions of these enzymes are essential, and cobalamin deficiency can result in mild to severe illness [Huemer and Baumgartner, 2019; Shevell and Rosenblatt, 2015]. Thus, humans have evolved a complex, high affinity uptake system for cobalamin, which is generally thought to be selective against other structurally related compounds [Fedosov et al., 2007; Kolhouse and Allen,

1977a; Stupperich and Nexø, 1991]. One hypothesis regarding selectivity in cobamide importers is that selectivity has evolved to protect human cells from importing cobamides that are unsuitable as cofactors for MS and MUT. Indeed, cobalamin precursors or degradation products can be inhibitory to cobamide-dependent enzyme activity [Stabler et al., 1991]. However, whether or not cobamides with diverse lower ligands are suitable cofactors for MUT and MS has only been investigated to a limited extent.

Here, we discovered that MUT binds, and is active with, several benzimidazolyl cobamides other than cobalamin, in addition to phenolyl cobamides. Interestingly, although WT MUT had similar binding affinities (K_d values) for AdoCbl, Ado[Cre]Cba, and Ado[Phe]Cba, the apparent K_M values of these cofactors in activity assays differed by orders of magnitude. The apparent K_M of cobamides is expected to be equal to the K_d if MUT catalyzes multiple consecutive turnovers while remaining bound to the cobamide cofactor in the base-off/His-on conformation, which is the current model for MUT activity with AdoCbl [Padovani and Banerjee, 2006; Takahashi-Íñiguez et al., 2011]. However, it is possible that MUT cycles between multiple cobamide-bound states, and the rate at which these states interconvert is affected by lower ligand structure. Such differences in rate could alter K_M relative to K_d [Klinman and Matthews, 1985]. Despite its high apparent K_M, [Cre]Cba supported MUT specific activity comparably to cobalamin in our activity screen, both for the WT and mutant enzymes. Thus, [Cre]Cba and other cobamides are potentially suitable cofactors for MUT.

Our results related to cobamide selectivity in MUT are consistent with what we observed for bacterial MCM orthologs: MCM is selective with respect to cobamide binding but is active with many cobamides (Chapter 2). The cobamide-binding selectivity of MUT is remarkably similar to that of *Sinorhizobium meliloti* MCM, considering the evolutionary distance between these organisms and their different mechanisms of cobamide acquisition. Nonetheless, out of the bacteria whose MCM orthologs were characterized in Chapter 2, *S. meliloti* is most closely related to human MUT, as it is an α-proteobacterium, and mitochondria are derived from an α-proteobacterial ancestor (MUT is a mitochondrial enzyme). Orthologs of MCM from *E. coli* (a γ-proteobacterium) and *Veillonella parvula* (phylum Firmicutes), differed from MUT in cobamide-binding selectivity. Interestingly, although *S. meliloti* MCM and MUT share high amino acid identity in the B_{12}-binding domain (58%), MUT has even higher sequence identity to *E. coli* MCM (60%) and *V. paruvla* MCM (66%) in this region. Thus, looking for residues that are similar between MUT and *S. meliloti* MCM but differ in the other orthologs may elucidate potential selectivity determinants in these enzymes.

A question pertinent to this work is how humans may be exposed to diverse cobamides. Cobamides produced by the gut microbiota are thought to be largely inaccessible to humans because cobalamin is absorbed in the small intestine [Seetharam and Alpers, 1982], while the majority of bacteria reside in the large intestine [Donaldson et al., 2016]. However, bacteria that reside in the small intestine have been found to produce cobalamin and potentially other cobamides [Albert et al., 1980], which would be spatially suited for absorption into human tissues. Thus, small intestinal bacteria could be a source of diverse cobamides. Additionally, certain foods are known to have high content of cobamides other than cobalamin [Watanabe et al., 2013], and bacterial species associated with the production of fermented foods such as yogurt synthesize alternate cobamides [Santos et al., 2007]. Thus, diverse cobamides derived from the diet may also be present in the small intestine, where IF is found.

In a study similar to ours, Kolhouse *et al.* demonstrated that, *in vitro*, human MS was active with multiple cobalamin analogs, including [Bza]Cba, [Ade]Cba, and [2-MeAde]Cba [Kolhouse

et al., 1991]. Importantly, however, subcutaneous administration of most of these analogs in rats did not support MS activity *in vivo*, as evidenced by high serum homocysteine levels compared to a cobalamin control [Stabler et al., 1991]. The apparent disagreement between the *in vitro* results and experiments in live animals (with the caveat that the MS enzymes in the two studies are from different organisms, albeit both mammals) could be explained by additional factors that function upstream of MS being inhibited by these cobamide analogs. Indeed, a large number of proteins and enzymes are known to interact with cobalamin prior to its use as an enzyme cofactor, either as escort proteins or as enzymes that modify and activate the cofactor [Gherasim et al., 2013; Huemer and Baumgartner, 2019]. In our discussion of our data on cobamide-dependent growth of *S. meliloti* we suggest that enzymatic addition of the 5'-deoxyadenosyl upper ligand to cobamides may be influenced by lower ligand structure (Chapter 2). Thus, although diverse cobamides are suitable cofactors for MUT *in vitro*, it is important to understand the activity of cobamide trafficking enzymes on these analogs if they are to be administered *in vivo*.

Administration of specific cobamides to humans could be considered as possible therapies if cobamides other than cobalamin were preferentially used by MUT mutants associated with disease. Hundreds of deleterious mutations have been found in the MUT gene in patients with MMA, distributed throughout the enzyme [Forny et al., 2014; Froese and Gravel, 2010]. Any mutation that changes the cobamide-binding selectivity of the enzyme could potentially be compensated by a cobamide other than cobalamin. Among the six MUT mutants that we tested, none of the 14 cobamides in our collection rescued activity to a significant extent. Interestingly, however, like WT MUT, all of the mutants were active with more than one cobamide. Moreover, mutants had altered relative specific activity with different cobamides compared to the WT enzyme, suggesting some alteration of cobamide selectivity. Thus, it is possible that mutants exist which would be preferentially active with cobamides other than cobalamin, which would be important both from a therapeutic perspective and also in elucidation of residues important for cobamide-selectivity in MCM.

V. Materials and Methods

Protein purification

The work in this chapter includes experiments with two different preparations of WT MUT. One, referred to as OS prep, was prepared as follows: An expression plasmid containing N-terminally hexahistidine (6xHis)-tagged MUT was provided by María Elena Flores [Takahashi-Íñiguez et al., 2011]. A 2.5 L culture of *E. coli* BL-21(DE3)pLysS cells containing the 6xHis-MUT expression plasmid was grown at 37 ˚C to OD_{600} 0.65. The culture was cooled on ice for 15 min, and protein expression was induced with 1 mM IPTG and proceeded at 20 ˚C overnight. Cells were lysed by sonication in 50 mM sodium phosphate pH 8.0, 100 mM NaCl, containing 0.5 mM PMSF, 1 µg/mL leupeptin, 1 µg/mL pepstatin, and 1 mg/mL lysozyme. The enzyme was batch-bound to Ni-NTA resin, loaded to a column, washed with 50 mM sodium phosphate pH 8.0, 300 mM NaCl, and 30 mM imidazole, and eluted with 50 mM sodium phosphate pH 8.0, 100 mM NaCl, 250 mM imidazole. Purified protein was dialyzed overnight into 25 mM Tris-HCl pH 8.0, 300 mM NaCl, and 10% glycerol. Due to the presence of a minor contaminant, the dialyzed protein was re-purified by Ni-affinity the following day and dialyzed again before freezing. Final protein concentration was determined by A_{280} using the theoretical extinction coefficient 72310 M^{-1} cm^{-1} and confirmed by Bradford assay (Bio-Rad).

Another prep of MUT, referred to as Oxford prep, was purified and sent to us by collaborator Wyatt Yue (University of Oxford). This prep contains a C-terminal 6xHis tag. MUT mutants were purified and sent by collaborator Sean Froese (University Children's Hospital, Zurich).

Enyzme assays

Cobamides were purified and adenosylated as previously described (Chapter 2). Cobamide binding was assayed using a fluorescence-based assay described in earlier work (Chapter 2), except that the concentration of MUT used in the binding assay was 0.4 μM. Enzyme kinetics were measured using a previously described coupled spectrophotometric assay (Chapter 2). The concentration of MUT used was 50 nM (OS prep), and cobamides were added at concentrations 10-fold higher than the K_d, as determined in Figure 2. To determine apparent K_M values of cobamides, the same assay was modified as follows: the concentration of substrate was fixed at 4 mM; cobamide concentration was varied across samples; MUT concentration was 10 nM (Oxford prep).

A modified version of the thiokinase-coupled activity assay was used to screen activity of mutant enzymes. The enzymes were pre-incubated with cobamides, at 1.25X final concentration, in 100 mM Tris, 50 mM phosphate pH 7.5, on ice, for 30 minutes [Chowdhury and Banerjee, 1999] in a 384-well plate. The plate was transferred to 30 ˚C and a 5X mixture containing thiokinase, $MgCl_2$, ADP, and methylmalonyl-CoA was added to initiate the reaction. Final concentrations of reagents are as previously described (Chapter 2), with the following adjustments: cobamide concentration, 1 μM (a saturating concentration of AdoCbl for WT activity); (*R*)-methylmalonyl-CoA, 2 mM; MUT WT, 0.01 μM; MUT G717V 1 μM; all other MUT mutants, 0.1 μM. DTNB was omitted from the reaction mixture, as it was found to inhibit protein activity on timescales longer than those used to measure initial rates. After 30 minutes, a sample of the reaction mixture was removed and immediately combined with DTNB (2.5 mM). A_{412} was measured on a BioTek Synergy 2 plate reader and concentration of CoA was calculated as previously described (Chapter 2).

Conclusion

Cobamides, including vitamin B_{12}, are enzyme cofactors used by organisms in all domains of life. Cobamides are structurally diverse, and microbial growth and metabolism vary based on cobamide structure. Understanding cobamide preference in microorganisms is important given that cobamides are widely used and appear to mediate microbial interactions in host-associated and aquatic environments. Until now, the biochemical basis for cobamide preferences was largely unknown. In the research projects described here, I identified mechanisms by which structural diversity of cobamides affects a model cobamide-dependent enzyme, methylmalonyl-CoA mutase (MCM). The results of this work emphasize the importance of cobamide-dependent enzyme selectivity in bacterial growth, and suggest new applications of diverse cobamides in human biology.

A major challenge ahead of these projects is identification of amino acid residues in MCM that confer cobamide selectivity. The ability to correlate primary sequence with biochemical selectivity would enable prediction of cobamide requirements of unculturable organisms, which would be a powerful way to generate hypotheses related to molecular interactions that might occur in complex microbial communities based on genomic information. One of the major barriers to identifying sequence determinants of selectivity is that the selectivity of most enzymes is unknown, and therefore functionally grouping protein sequences to identify conserved residues is not possible.

I supervised two junior colleagues on research projects that sought to identify amino acids involved in cobamide selectivity in MCM. Based on MCM orthologs with known selectivity, coupled with bioinformatic predictions of cobamide biosynthesis in different organisms, Jong Duk Park initiated a project to test site-specific MCM mutants for altered cobamide selectivity. Jong identified a promising mutation in the B_{12}-binding domain of *S. meliloti* MCM that appears to result in higher affinity for purinyl cobamides compared to the wildtype enzyme. At the same time, I constructed MCM chimeras to test the hypothesis that selectivity is conferred by the B_{12}-binding domain. In a complementary approach, Kathryn Quanstrom optimized conditions for random mutagenesis of the B_{12}-binding domain of MCM, with the goal of performing an enrichment for mutations that alter selectivity. Kenny Mok and I explored different approaches to high-throughput screening or evolution of enzyme selectivity *in vivo*. In the future, growth-based approaches would be greatly facilitated by identification of bacterial strains that grow robustly in MCM-dependent conditions, as *Sinorhizobium meliloti* was challenging to work with in this respect.

A second perspective that was highlighted by this work is that understanding the selectivity of cobamide modification and trafficking proteins is important for predicting cobamide-dependent growth. Cobamide selectivity of MCM only partially explained the differential cobamide-dependent growth of *S. meliloti*, and I hypothesize that the adenosyltransferase enzyme that activates cobamides for use by MCM may also be selective with respect to lower ligand structure. Differential transport of cobamides into *S. meliloti* may have contributed to the phenotypes I observed as well, and much remains unknown about the effects of lower ligand structure on cobamide import into bacterial cells. Similarly, proteins involved in cobalamin trafficking and modification in humans are likely selective with respect to lower ligand structure, but most have not been studied in this capacity. Thus, future work on cobamide selectivity can be extended to a multitude of proteins both in bacteria and in humans. Combined together, the results of such studies would paint a more complete picture of the physiological significance of structural diversity in this unique family of enzyme cofactors.

I return to a question that I mentioned in introducing this work: why do organisms incorporate diverse lower ligands into cobamide cofactors? The answer remains unknown. One speculation is that production of a cofactor that is unusable by surrounding microorganisms could be a way for a particular species to gain a competitive advantage in its environment. Although I observed cobamide selectivity in MCM enzymes, every MCM ortholog that I tested could use multiple cobamides as cofactors. This, along with the fact that most organisms studied to date are able to grow with multiple cobamides, suggests that this competitive model may be overly simple. Perhaps, through future work towards understanding how cobamide diversity impacts different aspects of metabolism, more refined models explaining the importance of the diversity of these cofactors can be generated.

References

Abreu, N.A., and Taga, M.E. (2016). Decoding molecular interactions in microbial communities. FEMS Microbiol Rev *40*, 648-663.

Akawi, L., Srirangan, K., Liu, X., Moo-Young, M., and Perry Chou, C. (2015). Engineering *Escherichia coli* for high-level production of propionate. J Ind Microbiol Biotechnol *42*, 1057-1072.

Albert, M.J., Mathan, V.I., and Baker, S.J. (1980). Vitamin B_{12} synthesis by human small intestinal bacteria. Nature *283*, 781-782.

Aldor, I.S., Kim, S.W., Prather, K.L., and Keasling, J.D. (2002). Metabolic engineering of a novel propionate-independent pathway for the production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in recombinant *Salmonella enterica* serovar Typhimurium. Appl Environ Microbiol *68*, 3848-3854.

Allen, K.D., and Wang, S.C. (2014). Initial characterization of Fom3 from *Streptomyces wedmorensis*: The methyltransferase in fosfomycin biosynthesis. Archives of biochemistry and biophysics *543*, 67-73.

Allen, R.H., Seetharam, B., Podell, E., and Alpers, D.H. (1978). Effect of proteolytic enzymes on the binding of cobalamin to R protein and intrinsic factor: in vitro evidence that a failure to partially degrade R protein is responsible for cobalamin malabsorption in pancreatic insufficiency. The Journal of clinical investigation *61*, 47-54.

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.

Allen, S.H., Kellermeyer, R.W., Stjernholm, R.L., and Wood, H.G. (1964). Purification and properties of enzymes involved in the propionic acid fermentation. Journal of bacteriology *87*, 171-187.

Anderson, P.J., Lango, J., Carkeet, C., Britten, A., Krautler, B., Hammock, B.D., and Roth, J.R. (2008). One pathway can incorporate either adenine or dimethylbenzimidazole as an alpha-axial ligand of B_{12} cofactors in *Salmonella enterica*. J Bacteriol *190*, 1160-1171.

Aretakis, J.R., Gega, A., and Schrader, J.M. (2019). Absolute measurements of mRNA translation in *Caulobacter crescentus* reveal important fitness costs of vitamin B_{12} scavenging. mSystems *4*.

Banerjee, R. (2006). B_{12} trafficking in mammals: A case for coenzyme escort service. ACS chemical biology *1*, 149-159.

Banerjee, R., and Chowdhury, S. (1999). Methylmalonyl-CoA mutase. In Chemistry and Biochemistry of B12, R. Banerjee, ed. (New York: John Wiley & Sons, Inc.), pp. 707-729.

Banerjee, R., and Ragsdale, S.W. (2003). The many faces of vitamin B_{12}: Catalysis by cobalamin-dependent enzymes. Annu Rev Biochem *72*, 209-247.

Banerjee, R.V., Johnston, N.L., Sobeski, J.K., Datta, P., and Matthews, R.G. (1989). Cloning and sequence analysis of the *Escherichia coli metH* gene encoding cobalamin-dependent methionine synthase and isolation of a tryptic fragment containing the cobalamin-binding domain. J Biol Chem *264*, 13888-13895.

Barker, H.A. (1985). beta-Methylaspartate-glutamate mutase from *Clostridium tetanomorphum*. Methods in enzymology *113*, 121-133.

Barker, H.A., Smyth, R.D., Weissbach, H., Toohey, J.I., Ladd, J.N., and Volcani, B.E. (1960). Isolation and properties of crystalline cobamide coenzymes containing benzimidazole or 5,6-dimethylbenzimidazole. J Biol Chem *235*, 480-488.

Bassila, C., Ghemrawi, R., Flayac, J., Froese, D.S., Baumgartner, M.R., Gueant, J.L., and Coelho, D. (2017). Methionine synthase and methionine synthase reductase interact with MMACHC and with MMADHC. Biochim Biophys Acta Mol Basis Dis *1863*, 103-112.

Bauerle, M.R., Schwalm, E.L., and Booker, S.J. (2015). Mechanistic diversity of radical *S*-adenosylmethionine (SAM)-dependent methylation. J Biol Chem *290*, 3995-4002.

Beedholm-Ebsen, R., van de Wetering, K., Hardlei, T., Nexo, E., Borst, P., and Moestrup, S.K. (2010). Identification of multidrug resistance protein 1 (MRP1/ABCC1) as a molecular gate for cellular export of cobalamin. Blood *115*, 1632-1639.

Belzer, C., Chia, L.W., Aalvink, S., Chamlagain, B., Piironen, V., Knol, J., and de Vos, W.M. (2017). Microbial metabolic networks at the mucus layer lead to diet-independent butyrate and vitamin B_{12} production by intestinal symbionts. mBio *8*, e00770-00717.

Berg, I.A., Kockelkorn, D., Buckel, W., and Fuchs, G. (2007). A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in Archaea. Sci *318*, 1782-1786.

Berkovitch, F., Behshad, E., Tang, K.H., Enns, E.A., Frey, P.A., and Drennan, C.L. (2004). A locking mechanism preventing radical damage in the absence of substrate, as revealed by the x-ray structure of lysine 5,6-aminomutase. Proceedings of the National Academy of Sciences of the United States of America *101*, 15870-15875.

Birch, A., Leiser, A., and Robinson, J.A. (1993). Cloning, sequencing, and expression of the gene encoding methylmalonyl-coenzyme A mutase from *Streptomyces cinnamonensis*. J Bacteriol *175*, 3511-3519.

Blaszczyk, A.J., Silakov, A., Zhang, B., Maiocco, S.J., Lanz, N.D., Kelly, W.L., Elliott, S.J., Krebs, C., and Booker, S.J. (2016). Spectroscopic and electrochemical characterization of the iron-sulfur and cobalamin cofactors of TsrM, an unusual radical S-adenosylmethionine methylase. Journal of the American Chemical Society *138*, 3416-3426.

Bommer, M., Kunze, C., Fesseler, J., Schubert, T., Diekert, G., and Dobbek, H. (2014). Structural basis for organohalide respiration. Science *346*, 455-458.

Borths, E.L., Poolman, B., Hvorup, R.N., Locher, K.P., and Rees, D.C. (2005). In vitro functional characterization of BtuCD-F, the *Escherichia coli* ABC transporter for vitamin B_{12} uptake. Biochemistry *44*, 16301-16309.

Brandt, L.J., Bernstein, L.H., and Wagle, A. (1977). Production of vitamin B_{12} analogues in patients with small-bowel bacterial overgrowth. Annals of internal medicine *87*, 546-551.

Bridwell-Rabb, J., and Drennan, C.L. (2017). Vitamin B_{12} in the spotlight again. Current opinion in chemical biology *37*, 63-70.

Bridwell-Rabb, J., Zhong, A., Sun, H.G., Drennan, C.L., and Liu, H.W. (2017). A B_{12}-dependent radical SAM enzyme involved in oxetanocin A biosynthesis. Nature *544*, 322-326.

Brodie, J.D., Burke, G.T., and Mangum, J.H. (1970). Methylcobalamin as an intermediate in mammalian methionine biosynthesis. Biochemistry *9*, 4297-4302.

Brown, K.L. (2006). The enzymatic activation of coenzyme B_{12}. Dalton Transactions, 1123-1133.

Brown, K.L., and Zou, X. (1999). Thermolysis of coenzymes B_{12} at physiological temperatures: activation parameters for cobalt-carbon bond homolysis and a quantitative analysis of the perturbation of the homolysis equilibrium by the ribonucleoside triphosphate reductase from *Lactobacillus leichmannii*. J Inorg Biochem *77*, 185-195.

Bunn, H.F. (2014). Vitamin B_{12} and pernicious anemia - the dawn of molecular medicine. The New England journal of medicine *370*, 773-776.

Calafat, A.M., Taoka, S., Puckett, J.M., Semerad, C., Yan, H., Luo, L., Chen, H., Banerjee, R., and Marzilli, L.G. (1995). Structural and electronic similarity but functional difference in methylmalonyl-CoA mutase between coenzyme B_{12} and the analog 2',5'-dideoxyadenosylcobalamin. Biochemistry *34*, 14125-14130.

Campanello, G.C., Ruetz, M., Dodge, G.J., Gouda, H., Gupta, A., Twahir, U.T., Killian, M.M., Watkins, D., Rosenblatt, D.S., Brunold, T.C*., et al.* (2018). Sacrificial cobalt-carbon bond homolysis in coenzyme B_{12} as a cofactor conservation strategy. Journal of the American Chemical Society *140*, 13205-13208.

Campbell, G.R., 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 B_{12}. Proceedings of the National Academy of Sciences of the United States of America *103*, 4634-4639.

Chan, C.H., and Escalante-Semerena, J.C. (2012). ArsAB, a novel enzyme from *Sporomusa ovata* activates phenolic bases for adenosylcobamide biosynthesis. Mol Microbiol *81*, 952-967.

Chandler, R.J., and Venditti, C.P. (2005). Genetic and genomic systems to study methylmalonic acidemia. Molecular genetics and metabolism *86*, 34-43.

Chang, C.H., and Frey, P.A. (2000). Cloning, sequencing, heterologous expression, purification, and characterization of adenosylcobalamin-dependent D-lysine 5,6-aminomutase from *Clostridium sticklandii*. J Biol Chem *275*, 106-114.

Charles, T.C., and Aneja, P. (1999). Methylmalonyl-CoA mutase encoding gene of *Sinorhizobium meliloti*. Gene *226*, 121-127.

Charles, T.C., Cai, G.Q., and Aneja, P. (1997). Megaplasmid and chromosomal loci for the PHB degradation pathway in *Rhizobium (Sinorhizobium) meliloti*. Genetics *146*, 1211-1220.

Chen, H.P., Wu, S.H., Lin, Y.L., Chen, C.M., and Tsay, S.S. (2001). Cloning, sequencing, heterologous expression, purification, and characterization of adenosylcobalamin-dependent D-ornithine aminomutase from *Clostridium sticklandii*. J Biol Chem *276*, 44744-44750.

Cheng, J., Sibley, C.D., Zaheer, R., and Finan, T.M. (2007). A *Sinorhizobium meliloti minE* mutant has an altered morphology and exhibits defects in legume symbiosis. Microbiology (Reading, England) *153*, 375-387.

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.

Chowdhury, S., and Banerjee, R. (1999). Role of the dimethylbenzimidazole tail in the reaction catalyzed by coenzyme B_{12}-dependent methylmalonyl-CoA mutase. Biochemistry *38*, 15287-15294.

Chowdhury, S., Thomas, M.G., Escalante-Semerena, J.C., and Banerjee, R. (2001). The coenzyme B_{12} analog 5'-deoxyadenosylcobinamide-GDP supports catalysis by methylmalonyl-CoA mutase in the absence of trans-ligand coordination. J Biol Chem *276*, 1015-1019.

Coelho, D., Kim, J.C., Miousse, I.R., Fung, S., du Moulin, M., Buers, I., Suormala, T., Burda, P., Frapolli, M., Stucki, M.*, et al.* (2012). Mutations in *ABCD4* cause a new inborn error of vitamin B_{12} metabolism. Nat Genet *44*, 1152-1155.

Conrad, K.S., Jordan, C.D., Brown, K.L., and Brunold, T.C. (2015). Spectroscopic and computational studies of cobalamin species with variable lower axial ligation: implications for the mechanism of Co-C bond activation by class I cobalamin-dependent isomerases. Inorg Chem *54*, 3736-3747.

Cowles, J.R., Evans, H.J., and Russell, S.A. (1969). B_{12} coenzyme-dependent ribonucleotide reductase in *Rhizobium* species and the effects of cobalt deficiency on the activity of the enzyme. J Bacteriol *97*, 1460-1465.

Cracan, V., and Banerjee, R. (2012a). Novel B_{12}-dependent acyl-CoA mutases and their biotechnological potential. Biochemistry *51*, 6039-6046.

Cracan, V., and Banerjee, R. (2012b). Novel coenzyme B_{12}-dependent interconversion of isovaleryl-CoA and pivalyl-CoA. J Biol Chem *287*, 3723-3732.

Cracan, V., Padovani, D., and Banerjee, R. (2010). IcmF is a fusion between the radical B_{12} enzyme isobutyryl-CoA mutase and its G-protein chaperone. J Biol Chem *285*, 655-666.

Crofts, T.S., Hazra, A.B., Tran, J.L., Sokolovskaya, O.M., Osadchiy, V., Ad, O., Pelton, J., Bauer, S., and Taga, M.E. (2014). Regiospecific formation of cobamide isomers is directed by CobT. Biochemistry *53*, 7805-7815.

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. Chemistry & biology *20*, 1265-1274.

Crosby, H.A., Rank, K.C., Rayment, I., and Escalante-Semerena, J.C. (2012). Structure-guided expansion of the substrate range of methylmalonyl coenzyme A synthetase (MatB) of *Rhodopseudomonas palustris*. Appl Environ Microbiol *78*, 6619-6629.

Dayem, L.C., Carney, J.R., Santi, D.V., Pfeifer, B.A., Khosla, C., and Kealey, J.T. (2002). Metabolic engineering of a methylmalonyl-CoA mutase-epimerase pathway for complex polyketide biosynthesis in *Escherichia coli*. Biochemistry *41*, 5193-5201.

De Hertogh, A.A., Mayeux, P.A., and Evans, H.J. (1964). The relationship of cobalt requirement to propionate metabolism in *Rhizobium*. Journal of Biological Chemistry *239*, 2446-2453.

Degnan, P.H., Barry, N.A., Mok, K.C., Taga, M.E., and Goodman, A.L. (2014a). Human gut microbes use multiple transporters to distinguish vitamin B_{12} analogs and compete in the gut. Cell host & microbe *15*, 47-57.

Degnan, P.H., Taga, M.E., and Goodman, A.L. (2014b). Vitamin B_{12} as a modulator of gut microbial ecology. Cell metabolism *20*, 769-778.

Donaldson, G.P., Lee, S.M., and Mazmanian, S.K. (2016). Gut biogeography of the bacterial microbiota. Nat Rev Microbiol *14*, 20-32.

Dorweiler, J.S., Finke, R.G., and Matthews, R.G. (2003). Cobalamin-dependent methionine synthase: probing the role of the axial base in catalysis of methyl transfer between methyltetrahydrofolate and exogenous cob(I)alamin or cob(I)inamide. Biochemistry *42*, 14653-14662.

Dowling, D.P., Croft, A.K., and Drennan, C.L. (2012). Radical use of Rossmann and TIM barrel architectures for controlling coenzyme B_{12} chemistry. Annu Rev Biophys *41*, 403-427.

Drennan, C.L., Huang, S., Drummond, J.T., Matthews, R.G., and Ludwig, M.L. (1994). How a protein binds B_{12}: A 3.0 A X-ray structure of B_{12}-binding domains of methionine synthase. Science *266*, 1669-1674.

Erb, T.J., Retey, J., Fuchs, G., and Alber, B.E. (2008). Ethylmalonyl-CoA mutase from *Rhodobacter sphaeroides* defines a new subclade of coenzyme B_{12}-dependent acyl-CoA mutases. J Biol Chem *283*, 32283-32293.

Escalante-Semerena, J.C. (2007). Conversion of cobinamide into adenosylcobamide in bacteria and archaea. J Bacteriol *189*, 4555-4560.

Escalante-Semerena, J.C., Suh, S.J., and Roth, J.R. (1990). *cobA* function is required for both *de novo* cobalamin biosynthesis and assimilation of exogenous corrinoids in *Salmonella typhimurium*. Journal of Bacteriology *172*, 273-280.

Fedosov, S.N., Fedosova, N.U., Krautler, B., Nexo, E., and Petersen, T.E. (2007). Mechanisms of discrimination between cobalamins and their natural analogues during their binding to the specific B_{12}-transporting proteins. Biochemistry *46*, 6446-6458.

Fenton, W.A., Gravel, R.A., and Rosenblatt, D.S. (2014). Disorders of propionate and methylmalonate metabolism. In The Online Metabolic and Molecular Bases of Inherited Disease, A.L. Beaudet, B. Vogelstein, K.W. Kinzler, S.E. Antonarakis, A. Ballabio, K.M. Gibson, and G. Mitchell, eds. (New York, NY: The McGraw-Hill Companies, Inc.).

Fenton, W.A., Hack, A.M., Willard, H.F., Gertler, A., and Rosenberg, L.E. (1982). Purification and properties of methylmalonyl coenzyme A mutase from human liver. Archives of biochemistry and biophysics *214*, 815-823.

Ferguson, D.J., Jr., and Krzycki, J.A. (1997). Reconstitution of trimethylamine-dependent coenzyme M methylation with the trimethylamine corrinoid protein and the isozymes of methyltransferase II from *Methanosarcina barkeri*. J Bacteriol *179*, 846-852.

Forage, R.G., and Foster, M.A. (1982). Glycerol fermentation in *Klebsiella pneumoniae:* functions of the coenzyme B_{12}-dependent glycerol and diol dehydratases. J Bacteriol *149*, 413-419.

Forny, P., Froese, D.S., Suormala, T., Yue, W.W., and Baumgartner, M.R. (2014). Functional characterization and categorization of missense mutations that cause methylmalonyl-CoA mutase (MUT) deficiency. Hum Mutat *35*, 1449-1458.

Fortier, A., Grosdidier, A., Hernandez, C., Baratin, D., Kuznetsov, D., de Castro, E., Gasteiger, E., Csardi, G., Rossier, G., Stockinger, H.*, et al.* (2012). ExPASy: SIB bioinformatics resource portal. Nucleic acids research *40*, W597-W603.

Froese, D.S., Fowler, B., and Baumgartner, M.R. (2018). Vitamin B_{12}, folate, and the methionine remethylation cycle - biochemistry, pathways, and regulation. J Inherit Metab Dis.

Froese, D.S., and Gravel, R.A. (2010). Genetic disorders of vitamin B_{12} metabolism: eight complementation groups - eight genes. Expert Rev Mol Med *12*, e37.

Froese, D.S., Kochan, G., Muniz, J.R., Wu, X., Gileadi, C., Ugochukwu, E., Krysztofinska, E., Gravel, R.A., Oppermann, U., and Yue, W.W. (2010). Structures of the human GTPase MMAA and vitamin B_{12}-dependent methylmalonyl-CoA mutase and insight into their complex formation. J Biol Chem *285*, 38204-38213.

Froese, D.S., Kopec, J., Fitzpatrick, F., Schuller, M., McCorvie, T.J., Chalk, R., Plessl, T., Fettelschoss, V., Fowler, B., Baumgartner, M.R*., et al.* (2015). Structural insights into the MMACHC-MMADHC protein complex involved in vitamin B_{12} trafficking. J Biol Chem *290*, 29167-29177.

Fukuoka, M., Nakanishi, Y., Hannak, R.B., Krautler, B., and Toraya, T. (2005). Homoadenosylcobalamins as probes for exploring the active sites of coenzyme B_{12}-dependent diol dehydratase and ethanolamine ammonia-lyase. Febs j *272*, 4787-4796.

Furger, E., Frei, D.C., Schibli, R., Fischer, E., and Prota, A.E. (2013). Structural basis for universal corrinoid recognition by the cobalamin transport protein haptocorrin. J Biol Chem *288*, 25466-25476.

Fyfe, J.C., Madsen, M., Hojrup, P., Christensen, E.I., Tanner, S.M., de la Chapelle, A., He, Q., and Moestrup, S.K. (2004). The functional cobalamin (vitamin B_{12})-intrinsic factor receptor is a novel complex of cubilin and amnionless. Blood *103*, 1573-1579.

Gallo, S., Oberhuber, M., Sigel, R.K., and Krautler, B. (2008). The corrin moiety of coenzyme B_{12} is the determinant for switching the btuB riboswitch of *E. coli*. Chembiochem *9*, 1408-1414.

Gherasim, C., Lofgren, M., and Banerjee, R. (2013). Navigating the B_{12} road: assimilation, delivery, and disorders of cobalamin. J Biol Chem *288*, 13186-13193.

Girard, C.L., Santschi, D.E., Stabler, S.P., and Allen, R.H. (2009). Apparent ruminal synthesis and intestinal disappearance of vitamin B_{12} and its analogs in dairy cows. Journal of dairy science *92*, 4524-4529.

Gonzalez-Garcia, R.A., McCubbin, T., Wille, A., Plan, M., Nielsen, L.K., and Marcellin, E. (2017). Awakening sleeping beauty: production of propionic acid in *Escherichia coli* through the *sbm* operon requires the activity of a methylmalonyl-CoA epimerase. Microbial cell factories *16*, 121.

González, J.C., Peariso, K., Penner-Hahn, J.E., and Matthews, R.G. (1996). Cobalamin-independent methionine synthase from *Escherichia coli*: A zinc metalloenzyme. Biochemistry *35*, 12228-12234.

Gopinath, K., Venclovas, C., Ioerger, T.R., Sacchettini, J.C., McKinney, J.D., Mizrahi, V., and Warner, D.F. (2013). A vitamin B_{12} transporter in *Mycobacterium tuberculosis*. Open Biol *3*, 120175.

Gough, S.P., Petersen, B.O., and Duus, J.O. (2000). Anaerobic chlorophyll isocyclic ring formation in *Rhodobacter capsulatus* requires a cobalamin cofactor. Proceedings of the National Academy of Sciences of the United States of America *97*, 6908-6913.

Gravel, R.A., Mahoney, M.J., Ruddle, F.H., and Rosenberg, L.E. (1975). Genetic complementation in heterokaryons of human fibroblasts defective in cobalamin metabolism. Proceedings of the National Academy of Sciences of the United States of America *72*, 3181-3185.

Gray, M.J., and Escalante-Semerena, J.C. (2009). 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., Tavares, N.K., and Escalante-Semerena, J.C. (2008). The genome of *Rhodobacter sphaeroides* strain 2.4.1 encodes functional cobinamide salvaging systems of archaeal and bacterial origins. Mol Microbiol *70*, 824-836.

Gross, F., Ring, M.W., Perlova, O., Fu, J., Schneider, S., Gerth, K., Kuhlmann, S., Stewart, A.F., Zhang, Y., and Muller, R. (2006). Metabolic engineering of *Pseudomonas putida* for methylmalonyl-CoA biosynthesis to enable complex heterologous secondary metabolite formation. Chemistry & biology *13*, 1253-1264.

Gruber, K., Reitzer, R., and Kratky, C. (2001). Radical shuttling in a protein: ribose pseudorotation controls alkyl-radical transfer in the coenzyme B_{12} dependent enzyme glutamate mutase. Angewandte Chemie (International ed in English) *40*, 3377-3380.

Guex, N., Peitsch, M.C., and Schwede, T. (2009). Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: a historical perspective. Electrophoresis *30 Suppl 1*, S162-173.

Haller, T., Buckel, T., Rétey, J., and Gerlt, J.A. (2000). Discovering new enzymes and metabolic pathways: Conversion of succinate to propionate by *Escherichia coli*. Biochemistry *39*, 4622-4629.

Han, Y., Hawkins, A.S., Adams, M.W., and Kelly, R.M. (2012). Epimerase (Msed_0639) and mutase (Msed_0638 and Msed_2055) convert *(S)*-methylmalonyl-coenzyme A (CoA) to succinyl-CoA in the *Metallosphaera sedula* 3-hydroxypropionate/4-hydroxybutyrate cycle. Appl Environ Microbiol *78*, 6194-6202.

Hannibal, L., Kim, J., Brasch, N.E., Wang, S., Rosenblatt, D.S., Banerjee, R., and Jacobsen, D.W. (2009). Processing of alkylcobalamins in mammalian cells: A role for the MMACHC (cblC) gene product. Molecular genetics and metabolism *97*, 260-266.

Hazra, A.B., Han, A.W., Mehta, A.P., Mok, K.C., Osadchiy, V., Begley, T.P., and Taga, M.E. (2015). Anaerobic biosynthesis of the lower ligand of vitamin B_{12}. Proceedings of the National Academy of Sciences of the United States of America *112*, 10792-10797.

Hazra, Amrita B., Tran, Jennifer L.A., Crofts, Terence S., and Taga, Michiko E. (2013). Analysis of substrate specificity in CobT homologs reveals widespread preference for DMB, the lower axial ligand of vitamin B_{12}. Chemistry & biology *20*, 1275-1285.

Heal, K.R., Qin, W., Ribalet, F., Bertagnolli, A.D., Coyote-Maestas, W., Hmelo, L.R., Moffett, J.W., Devol, A.H., Armbrust, E.V., Stahl, D.A.*, et al.* (2017). Two distinct pools of B_{12} analogs reveal community interdependencies in the ocean. Proceedings of the National Academy of Sciences of the United States of America *114*, 364-369.

Heller, K., Mann, B.J., and Kadner, R.J. (1985). Cloning and expression of the gene for the vitamin B_{12} receptor protein in the outer membrane of *Escherichia coli*. J Bacteriol *161*, 896-903.

Helliwell, K.E., Lawrence, A.D., Holzer, A., Kudahl, U.J., Sasso, S., Krautler, B., Scanlan, D.J., Warren, M.J., and Smith, A.G. (2016). Cyanobacteria and eukaryotic algae use different chemical variants of vitamin B_{12}. Curr Biol *26*, 999-1008.

Hodgkin, D.C., Kamper, J., Mackay, M., Pickworth, J., Trueblood, K.N., and White, J.G. (1956). Structure of vitamin B_{12}. Nature *178*, 64-66.

Hoffmann, B., Oberhuber, M., Stupperich, E., Bothe, H., Buckel, W., Konrat, R., and Kräutler, B. (2000). Native corrinoids from *Clostridium cochlearium* are adeninylcobamides: Spectroscopic analysis and identification of pseudovitamin B_{12} and factor A. J Bacteriol *182*, 4773-4782.

Hogenkamp, H.P.C. (1975). The chemistry of cobalamins and related compounds. In Cobalamin Biochemistry and Pathophysiology, B.M. Babior, ed. (John Wiley and Sons, Inc.), p. 55.

Horswill, A.R., and Escalante-Semerena, J.C. (1999). *Salmonella typhimurium* LT2 catabolizes propionate via the 2-methylcitric acid cycle. Journal of bacteriology *181*, 5615-5623.

Hosotani, K., Yokota, A., Nakano, Y., and Kitaoka, S. (1980). The metabolic pathway of propionate in *Euglena gracilis* z grown under illumination. Agricultural and Biological Chemistry *44*, 1097-1103.

Huemer, M., and Baumgartner, M.R. (2019). The clinical presentation of cobalamin-related disorders: From acquired deficiencies to inborn errors of absorption and intracellular pathways. J Inherit Metab Dis *42*, 686-705.

Hunaiti, A.A., and Kolattukudy, P.E. (1984). Source of methylmalonyl-coenzyme A for erythromycin synthesis: Methylmalonyl-coenzyme A mutase from *Streptomyces erythreus*. Antimicrobial agents and chemotherapy *25*, 173-178.

Janata, J., Kogekar, N., and Fenton, W.A. (1997). Expression and kinetic characterization of methylmalonyl-CoA mutase from patients with the *mut*$^{-}$ phenotype: evidence for naturally occurring interallelic complementation. Human Molecular Genetics *6*, 1457-1464.

Jeter, R.M. (1990). Cobalamin-dependent 1,2-propanediol utilization by *Salmonella typhimurium*. Journal of general microbiology *136*, 887-896.

Jeter, V.L., Mattes, T.A., Beattie, N.R., and Escalante-Semerena, J.C. (2019). A new class of phosphoribosyltransferases involved in cobamide biosynthesis is found in methanogenic archaea and cyanobacteria. Biochemistry *58*, 951-964.

Johnson, C.L., Pechonick, E., Park, S.D., Havemann, G.D., Leal, N.A., and Bobik, T.A. (2001). Functional genomic, biochemical, and genetic characterization of the *Salmonella pduO* gene, an ATP:cob(I)alamin adenosyltransferase gene. J Bacteriol *183*, 1577-1584.

Jost, M., Cracan, V., Hubbard, P.A., Banerjee, R., and Drennan, C.L. (2015). Visualization of a radical B_{12} enzyme with its G-protein chaperone. Proceedings of the National Academy of Sciences of the United States of America *112*, 2419-2424.

Jung, W.S., Kim, E., Yoo, Y.J., Ban, Y.H., Kim, E.J., and Yoon, Y.J. (2014). Characterization and engineering of the ethylmalonyl-CoA pathway towards the improved heterologous production of polyketides in *Streptomyces venezuelae*. Applied microbiology and biotechnology *98*, 3701-3713.

Kanazawa, S., Herbert, V., Herzlich, B., Drivas, G., and Manusselis, C. (1983). Removal of cobalamin analogue in bile by enterohepatic circulation of vitamin B_{12}. Lancet (London, England) *1*, 707-708.

Kanehisa, M., and Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. Nucleic acids research *28*, 27-30.

Keller, S., Kunze, C., Bommer, M., Paetz, C., Menezes, R.C., Svatoš, A., Dobbek, H., and Schubert, T. (2018). Selective utilization of benzimidazolyl-norcobamides as cofactors by the tetrachloroethene reductive dehalogenase of *Sulfurospirillum multivorans*. Journal of Bacteriology.

Keller, S., Treder, A., von Reuss, S.H., Escalante-Semerena, J.C., and Schubert, T. (2016). The SMUL_1544 gene product governs norcobamide biosynthesis in the tetrachloroethene-respiring bacterium *Sulfurospirillum multivorans*. J Bacteriol *198*, 2236-2243.

Kim, H.J., Liu, Y.N., McCarty, R.M., and Liu, H.W. (2017). Reaction catalyzed by GenK, a cobalamin-dependent radical *S*-adenosyl-L-methionine methyltransferase in the biosynthetic pathway of gentamicin, proceeds with retention of configuration. Journal of the American Chemical Society *139*, 16084-16087.

Kliewer, M., and Evans, H.J. (1963). Identification of cobamide coenzyme in nodules of symbionts & isolation of the B_{12} coenzyme from *Rhizobium meliloti*. Plant physiology *38*, 55-59.

Klinman, J.P., and Matthews, R.G. (1985). Calculation of substrate dissociation constants from steady-state isotope effects in enzyme-catalyzed reactions. Journal of the American Chemical Society *107*, 1058-1060.

Kolhouse, J.F., and Allen, R.H. (1977a). Absorption, plasma transport, and cellular retention of cobalamin analogues in the rabbit. Evidence for the existence of multiple mechanisms that prevent the absorption and tissue dissemination of naturally occurring cobalamin analogues. The Journal of clinical investigation *60*, 1381-1392.

Kolhouse, J.F., and Allen, R.H. (1977b). Recognition of two intracellular cobalamin binding proteins and their identification as methylmalonyl-CoA mutase and methionine synthetase. Proceedings of the National Academy of Sciences *74*, 921-925.

Kolhouse, J.F., Utley, C., Stabler, S.P., and Allen, R.H. (1991). Mechanism of conversion of human apo- to holomethionine synthase by various forms of cobalamin. J Biol Chem *266*, 23010-23015.

Kondo, H., Binder, M.J., Kolhouse, J.F., Smythe, W.R., Podell, E.R., and Allen, R.H. (1982). Presence and formation of cobalamin analogues in multivitamin-mineral pills. The Journal of clinical investigation *70*, 889-898.

Korotkova, N., Chistoserdova, L., Kuksa, V., and Lidstrom, M.E. (2002). Glyoxylate regeneration pathway in the methylotroph *Methylobacterium extorquens* AM1. Journal of Bacteriology *184*, 1750-1758.

Korotkova, N., and Lidstrom, M.E. (2004). MeaB is a component of the methylmalonyl-CoA mutase complex required for protection of the enzyme from inactivation. J Biol Chem *279*, 13652-13658.

Kozlowski, P.M., and Zgierski, M.Z. (2004). Electronic and steric influence of trans axial base on the stereoelectronic properties of cobalamins. The Journal of Physical Chemistry B *108*, 14163-14170.

Krasotkina, J., Walters, T., Maruya, K.A., and Ragsdale, S.W. (2001). Characterization of the B_{12}- and iron-sulfur-containing reductive dehalogenase from *Desulfitobacterium chlororespirans*. J Biol Chem *276*, 40991-40997.

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-B_{12}, a new type of a natural corrinoid, Vol 86.

Kurteva-Yaneva, N., Zahn, M., Weichler, M.T., Starke, R., Harms, H., Muller, R.H., Strater, N., and Rohwerder, T. (2015). Structural basis of the stereospecificity of bacterial B_{12}-dependent 2-hydroxyisobutyryl-CoA mutase. J Biol Chem *290*, 9727-9737.

Landgraf, B.J., McCarthy, E.L., and Booker, S.J. (2016). Radical S-adenosylmethionine enzymes in human health and disease. Annu Rev Biochem *85*, 485-514.

Larsson, K.M., Logan, D.T., and Nordlund, P. (2010). Structural basis for adenosylcobalamin activation in AdoCbl-dependent ribonucleotide reductases. ACS chemical biology *5*, 933-942.

Lengyel, P., Mazumder, R., and Ochoa, S. (1960). Mammalian methylmalonyl isomerase and vitamin B_{12} coenzymes. Proceedings of the National Academy of Sciences of the United States of America *46*, 1312-1318.

Li, J., Zhu, X., Chen, J., Zhao, D., Zhang, X., and Bi, C. (2017). Construction of a novel anaerobic pathway in *Escherichia coli* for propionate production. BMC biotechnology *17*, 38-38.

Licht, S., Gerfen, G.J., and Stubbe, J. (1996). Thiyl radicals in ribonucleotide reductases. Science *271*, 477-481.

Ljungdahl, L.G. (1986). The autotrophic pathway of acetate synthesis in acetogenic bacteria. Annual review of microbiology *40*, 415-450.

London, R.E., Allen, D.L., Gabel, S.A., and DeRose, E.F. (1999). Carbon-13 nuclear magnetic resonance study of metabolism of propionate by *Escherichia coli*. J Bacteriol *181*, 3562-3570.

Ma, A.T., Beld, J., and Brahamsha, B. (2017). An amoebal grazer of cyanobacteria requires cobalamin produced by heterotrophic bacteria. Applied and Environmental Microbiology *83*, e00035-00017.

Mah, W., Deme, J.C., Watkins, D., Fung, S., Janer, A., Shoubridge, E.A., Rosenblatt, D.S., and Coulton, J.W. (2013). Subcellular location of MMACHC and MMADHC, two human proteins central to intracellular vitamin B_{12} metabolism. Molecular genetics and metabolism *108*, 112-118.

Mancia, F., Keep, N.H., Nakagawa, A., Leadlay, P.F., McSweeney, S., Rasmussen, B., Bosecke, P., Diat, O., and Evans, P.R. (1996). How coenzyme B_{12} radicals are generated: the crystal structure of methylmalonyl-coenzyme A mutase at 2 A resolution. Structure (London, England : 1993) *4*, 339-350.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1989). Molecular cloning: a laboratory manual (Cold spring harbor laboratory press).

Marous, D.R., Lloyd, E.P., Buller, A.R., Moshos, K.A., Grove, T.L., Blaszczyk, A.J., Booker, S.J., and Townsend, C.A. (2015a). Consecutive radical *S*-adenosylmethionine methylations form the ethyl side chain in thienamycin biosynthesis. Proceedings of the National Academy of Sciences *112*, 10354-10358.

Marous, D.R., Lloyd, E.P., Buller, A.R., Moshos, K.A., Grove, T.L., Blaszczyk, A.J., Booker, S.J., and Townsend, C.A. (2015b). Consecutive radical S-adenosylmethionine methylations form the ethyl side chain in thienamycin biosynthesis. Proceedings of the National Academy of Sciences of the United States of America *112*, 10354-10358.

Marsh, E.N., and Drennan, C.L. (2001). Adenosylcobalamin-dependent isomerases: new insights into structure and mechanism. Current opinion in chemical biology *5*, 499-505.

McDonald, M.K., Fritz, J.A., Jia, D., Scheuchner, D., Snyder, F.F., Stanislaus, A., Curle, J., Li, L., Stabler, S.P., Allen, R.H.*, et al.* (2017). Identification of ABC transporters acting in vitamin B_{12} metabolism in *Caenorhabditis elegans*. Molecular genetics and metabolism *122*, 160-171.

Meier, T.W., Thoma, N.H., and Leadlay, P.F. (1996). Tritium isotope effects in adenosylcobalamin-dependent methylmalonyl-CoA mutase. Biochemistry *35*, 11791-11796.

Men, Y., Seth, E.C., Yi, S., Crofts, T.S., Allen, R.H., Taga, M.E., and Alvarez-Cohen, L. (2015). Identification of specific corrinoids reveals corrinoid modification in dechlorinating microbial communities. Environmental microbiology *17*, 4873-4884.

Miles, Z.D., McCarty, R.M., Molnar, G., and Bandarian, V. (2011). Discovery of epoxyqueuosine (oQ) reductase reveals parallels between halorespiration and tRNA modification. Proceedings of the National Academy of Sciences of the United States of America *108*, 7368-7372.

Mireku, S.A., Ruetz, M., Zhou, T., Korkhov, V.M., Krautler, B., and Locher, K.P. (2017). Conformational change of a tryptophan residue in BtuF facilitates binding and transport of cobinamide by the vitamin B_{12} transporter BtuCD-F. Sci Rep *7*, 41575.

Miyamoto, E., Tanioka, Y., Nishizawa-Yokoi, A., Yabuta, Y., Ohnishi, K., Misono, H., Shigeoka, S., Nakano, Y., and Watanabe, F. (2010). Characterization of methylmalonyl-CoA mutase involved in the propionate photoassimilation of *Euglena gracilis* Z. Archives of microbiology *192*, 437-446.

Miyamoto, E., Watanabe, F., Charles, T.C., Yamaji, R., Inui, H., and Nakano, Y. (2003). Purification and characterization of homodimeric methylmalonyl-CoA mutase from *Sinorhizobium meliloti*. Archives of microbiology *180*, 151-154.

Miyamoto, E., Watanabe, F., Yamaji, R., Inui, H., Sato, K., and Nakano, Y. (2002). Purification and characterization of methylmalonyl-CoA mutase from a methanol-utilizing bacterium, *Methylobacterium extorquens* NR-1. Journal of nutritional science and vitaminology *48*, 242-246.

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.

Mørkbak, A.L., Hvas, A.M., Lloyd-Wright, Z., Sanders, T.A., Bleie, O., Refsum, H., Nygaard, O.K., and Nexø, E. (2006). Effect of vitamin B_{12} treatment on haptocorrin. Clin Chem *52*, 1104-1111.

Mukherjee, S., Das Mandal, S., Gupta, N., Drory-Retwitzer, M., Barash, D., and Sengupta, S. (2019). RiboD: A comprehensive database for prokaryotic riboswitches. Bioinformatics (Oxford, England).

Murphy, M.F., Sourial, N.A., Burman, J.F., Doyle, D.V., Tabaqchali, S., and Mollin, D.L. (1986). Megaloblastic anaemia due to vitamin B_{12} deficiency caused by small intestinal bacterial overgrowth: possible role of vitamin B_{12} analogues. British journal of haematology *62*, 7-12.

Nahvi, A., Barrick, J.E., and Breaker, R.R. (2004). Coenzyme B_{12} riboswitches are widespread genetic control elements in prokaryotes. Nucleic acids research *32*, 143-150.

Naidu, D., and Ragsdale, S.W. (2001). Characterization of a three-component vanillate O-demethylase from Moorella thermoacetica. J Bacteriol *183*, 3276-3281.

Ortiz-Guerrero, J.M., Polanco, M.C., Murillo, F.J., Padmanabhan, S., and Elias-Arnanz, M. (2011). Light-dependent gene regulation by a coenzyme B_{12}-based photoreceptor. Proceedings of the National Academy of Sciences of the United States of America *108*, 7565-7570.

Padmakumar, R., Padmakumar, R., and Banerjee, R. (1997). Evidence that cobalt−carbon bond homolysis is coupled to hydrogen atom abstraction from substrate in methylmalonyl-CoA mutase. Biochemistry *36*, 3713-3718.

Padovani, D., and Banerjee, R. (2006). Assembly and protection of the radical enzyme, methylmalonyl-CoA mutase, by its chaperone. Biochemistry *45*, 9300-9306.

Padovani, D., Labunska, T., and Banerjee, R. (2006). Energetics of interaction between the G-protein chaperone, MeaB, and B_{12}-dependent methylmalonyl-CoA mutase. J Biol Chem *281*, 17838-17844.

Padovani, D., Labunska, T., Palfey, B.A., Ballou, D.P., and Banerjee, R. (2008). Adenosyltransferase tailors and delivers coenzyme B_{12}. Nature chemical biology *4*, 194-196.

Parks, J.M., Johs, A., Podar, M., Bridou, R., Hurt, R.A., Jr., Smith, S.D., Tomanicek, S.J., Qian, Y., Brown, S.D., Brandt, C.C.*, et al.* (2013). The genetic basis for bacterial mercury methylation. Science *339*, 1332-1335.

Parthasarathy, A., Stich, T.A., Lohner, S.T., Lesnefsky, A., Britt, R.D., and Spormann, A.M. (2015). Biochemical and EPR-spectroscopic investigation into heterologously expressed vinyl chloride reductive dehalogenase (VcrA) from *Dehalococcoides mccartyi* strain VS. Journal of the American Chemical Society *137*, 3525-3532.

Paul, L., Ferguson, D.J., and Krzycki, J.A. (2000). The trimethylamine methyltransferase gene and multiple dimethylamine methyltransferase genes of *Methanosarcina barkeri* contain in-frame and read-through amber codons. Journal of Bacteriology *182*, 2520-2529.

Payne, K.A., Fisher, K., Sjuts, H., Dunstan, M.S., Bellina, B., Johannissen, L., Barran, P., Hay, S., Rigby, S.E., and Leys, D. (2015a). Epoxyqueuosine reductase structure suggests a mechanism for cobalamin-dependent tRNA modification. J Biol Chem *290*, 27572-27581.

Payne, K.A., Quezada, C.P., Fisher, K., Dunstan, M.S., Collins, F.A., Sjuts, H., Levy, C., Hay, S., Rigby, S.E., and Leys, D. (2015b). Reductive dehalogenase structure suggests a mechanism for B_{12}-dependent dehalogenation. Nature *517*, 513-516.

Picking, J.W., Behrman, E.J., Zhang, L., and Krzycki, J.A. (2019). MtpB, a member of the MttB superfamily from the human intestinal acetogen *Eubacterium limosum*, catalyzes proline betaine demethylation. J Biol Chem.

Pierre, S., Guillot, A., Benjdia, A., Sandstrom, C., Langella, P., and Berteau, O. (2012a). Thiostrepton tryptophan methyltransferase expands the chemistry of radical SAM enzymes. Nature chemical biology *8*, 957-959.

Pierre, S., Guillot, A., Benjdia, A., Sandström, C., Langella, P., and Berteau, O. (2012b). Thiostrepton tryptophan methyltransferase expands the chemistry of radical SAM enzymes. Nature chemical biology *8*, 957.

Poppe, L., Bothe, H., Bröker, G., Buckel, W., Stupperich, E., and Rétey, J. (2000). Elucidation of the coenzyme binding mode of further B_{12}-dependent enzymes using a base-off analogue of coenzyme B_{12}, Vol 10.

Poppe, L., Stupperich, E., Hull, W.E., Buckel, T., and Retey, J. (1997). A base-off analogue of coenzyme-B_{12} with a modified nucleotide loop; ^{1}H-NMR structure analysis and kinetic studies with *(R)*-methylmalonyl-CoA mutase, glycerol dehydratase, and diol dehydratase. European journal of biochemistry *250*, 303-307.

Quadros, E.V., Nakayama, Y., and Sequeira, J.M. (2009). The protein and the gene encoding the receptor for the cellular uptake of transcobalamin-bound cobalamin. Blood *113*, 186-192.

Radle, M.I., Miller, D.V., Laremore, T.N., and Booker, S.J. (2019). Methanogenesis marker protein 10 (Mmp10) from *Methanosarcina acetivorans* is a radical *S*-adenosylmethionine methylase that unexpectedly requires cobalamin. J Biol Chem.

Ragsdale, S.W., and Pierce, E. (2008). Acetogenesis and the Wood-Ljungdahl pathway of CO_2 fixation. Biochim Biophys Acta *1784*, 1873-1898.

Reeves, A.R., Brikun, I.A., Cernota, W.H., Leach, B.I., Gonzalez, M.C., and Weber, J.M. (2007). Engineering of the methylmalonyl-CoA metabolite node of *Saccharopolyspora erythraea* for increased erythromycin production. Metabolic engineering *9*, 293-303.

Rempel, S., Colucci, E., de Gier, J.W., Guskov, A., and Slotboom, D.J. (2018). Cysteine-mediated decyanation of vitamin B_{12} by the predicted membrane transporter BtuM. Nat Commun *9*, 3038.

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: John Wiley & Sons, Inc.), pp. 557-566.

Rodionov, D.A., Hebbeln, P., Eudes, A., ter Beek, J., Rodionova, I.A., Erkens, G.B., Slotboom, D.J., Gelfand, M.S., Osterman, A.L., Hanson, A.D*., et al.* (2009). A novel class of modular transporters for vitamins in prokaryotes. Journal of Bacteriology *191*, 42.

Rodionov, D.A., Vitreschak, A.G., Mironov, A.A., and Gelfand, M.S. (2003). Comparative genomics of the vitamin B_{12} metabolism and regulation in prokaryotes. J Biol Chem *278*, 41148-41159.

Romine, M.F., Rodionov, D.A., Maezato, Y., Anderson, L.N., Nandhikonda, P., Rodionova, I.A., Carre, A., Li, X., Xu, C., Clauss, T.R*., et al.* (2017). Elucidation of roles for vitamin B_{12} in regulation of folate, ubiquinone, and methionine metabolism. Proceedings of the National Academy of Sciences of the United States of America *114*, E1205-e1214.

Rosnow, J.J., Hwang, S., Killinger, B.J., Kim, Y.M., Moore, R.J., Lindemann, S.R., Maupin-Furlow, J.A., and Wright, A.T. (2018). A cobalamin activity-based probe enables microbial cell growth and finds new cobalamin-protein interactions across domains. Appl Environ Microbiol *84*.

Rutsch, F., Gailus, S., Miousse, I.R., Suormala, T., Sagne, C., Toliat, M.R., Nurnberg, G., Wittkampf, T., Buers, I., Sharifi, A*., et al.* (2009). Identification of a putative lysosomal cobalamin exporter altered in the cblF defect of vitamin B_{12} metabolism. Nat Genet *41*, 234-239.

Santos, F., Vera, J.L., Lamosa, P., de Valdez, G.F., de Vos, W.M., Santos, H., Sesma, F., and Hugenholtz, J. (2007). Pseudovitamin B_{12} is the corrinoid produced by *Lactobacillus reuteri* CRL1098 under anaerobic conditions. FEBS Lett *581*, 4865-4870.

Santos, J.A., Rempel, S., Mous, S.T.M., Pereira, C.T., ter Beek, J., de Gier, J.-W., Guskov, A., and Slotboom, D.J. (2018). Functional and structural characterization of an ECF-type ABC transporter for vitamin B_{12}. eLife *7*, e35828.

Sato, K., Inukai, S., and Shimizu, S. (1974). Vitamin B_{12}-dependent methionine synthesis in *Rhizobium meliloti*. Biochemical and biophysical research communications *60*, 723-728.

Sauer, H., and Wilmanns, W. (1977). Cobalamin dependent methionine synthesis and methyl-folate-trap in human vitamin B_{12} deficiency. British journal of haematology *36*, 189-198.

Sauer, K., and Thauer, R.K. (1997). Methanol:coenzyme M methyltransferase from *Methanosarcina barkeri*. European journal of biochemistry *249*, 280-285.

Savvi, S., Warner, D.F., Kana, B.D., McKinney, J.D., Mizrahi, V., and Dawes, S.S. (2008). Functional characterization of a vitamin B_{12}-dependent methylmalonyl pathway in *Mycobacterium tuberculosis*: Implications for propionate metabolism during growth on fatty acids. Journal of Bacteriology *190*, 3886.

Scarlett, F.A., and Turner, J.M. (1976). Microbial metabolism of amino alcohols. Ethanolamine catabolism mediated by coenzyme B_{12}-dependent ethanolamine ammonia-lyase in *Escherichia coli* and *Klebsiella aerogenes*. Journal of general microbiology *95*, 173-176.

Schneider, Z., and Stroinski, A. (1987). Comprehensive B_{12}: Chemistry, Biochemistry, Nutrition, Ecology, Medicine. In, W.d. Gruyter, ed.

Schrödinger Release 2015: MacroModel, S., LLC, New York, NY, 2015.

Schrödinger Release 2015: Maestro, S., LLC, New York, NY, 2015.

Schubert, T., von Reuss, S.H., Kunze, C., Paetz, C., Kruse, S., Brand-Schon, P., Nelly, A.M., Nuske, J., and Diekert, G. (2019). Guided cobamide biosynthesis for heterologous production of reductive dehalogenases. Microb Biotechnol *12*, 346-359.

Seetharam, B., and Alpers, D.H. (1982). Absorption and transport of cobalamin (vitamin B_{12}). Annual review of nutrition *2*, 343-369.

Seth, E. (unpublished). Analysis of cobamides in *Zootermopsis* sp. worker gut.

Seth, E.C., and Taga, M.E. (2014). Nutrient cross-feeding in the microbial world. Frontiers in microbiology *5*, 350.

Shelton, A.N., Seth, E.C., Mok, K.C., Han, A.W., Jackson, S.N., Haft, D.R., and Taga, M.E. (2019). Uneven distribution of cobamide biosynthesis and dependence in bacteria predicted by comparative genomics. The ISME journal *13*, 789-804.

Shevell, M.I., and Rosenblatt, D.S. (2015). The neurology of cobalamin. Canadian Journal of Neurological Sciences / Journal Canadien des Sciences Neurologiques *19*, 472-486.

Shibata, N., Masuda, J., Tobimatsu, T., Toraya, T., Suto, K., Morimoto, Y., and Yasuoka, N. (1999). A new mode of B_{12} binding and the direct participation of a potassium ion in enzyme catalysis: X-ray structure of diol dehydratase. Structure (London, England : 1993) *7*, 997-1008.

Shibata, N., Sueyoshi, Y., Higuchi, Y., and Toraya, T. (2018). Direct participation of a peripheral side chain of a corrin ring in coenzyme B_{12} catalysis. Angewandte Chemie (International ed in English) *57*, 7830-7835.

Srirangan, K., Akawi, L., Liu, X., Westbrook, A., Blondeel, E.J., Aucoin, M.G., Moo-Young, M., and Chou, C.P. (2013). Manipulating the sleeping beauty mutase operon for the production of 1-propanol in engineered *Escherichia coli*. Biotechnology for biofuels *6*, 139.

Stabler, S.P., and Allen, R.H. (2004). Vitamin B_{12} deficiency as a worldwide problem. Annual review of nutrition *24*, 299-326.

Stabler, S.P., Brass, E.P., Marcell, P.D., and Allen, R.H. (1991). Inhibition of cobalamin-dependent enzymes by cobalamin analogues in rats. The Journal of clinical investigation *87*, 1422-1430.

Stucki, M., Coelho, D., Suormala, T., Burda, P., Fowler, B., and Baumgartner, M.R. (2012). Molecular mechanisms leading to three different phenotypes in the cblD defect of intracellular cobalamin metabolism. Hum Mol Genet *21*, 1410-1418.

Stupperich, E., Eisinger, H.J., and Krautler, B. (1988). Diversity of corrinoids in acetogenic bacteria. *p*-cresolylcobamide from *Sporomusa ovata*, 5-methoxy-6-methylbenzimidazolylcobamide from *Clostridium formicoaceticum* and vitamin B_{12} from *Acetobacterium woodii*. European journal of biochemistry *172*, 459-464.

Stupperich, E., and Konle, R. (1993). Corrinoid-dependent methyl transfer reactions are involved in methanol and 3,4-dimethoxybenzoate metabolism by *Sporomusa ovata*. Appl Environ Microbiol *59*, 3110-3116.

Stupperich, E., and Nexø, E. (1991). Effect of the cobalt-N coordination on the cobamide recognition by the human vitamin B12 binding proteins intrinsic factor, transcobalamin and haptocorrin. European journal of biochemistry *199*, 299-303.

Suormala, T., Baumgartner, M.R., Coelho, D., Zavadakova, P., Kozich, V., Koch, H.G., Berghauser, M., Wraith, J.E., Burlina, A., Sewell, A.*, et al.* (2004). The cblD defect causes either isolated or combined deficiency of methylcobalamin and adenosylcobalamin synthesis. J Biol Chem *279*, 42742-42749.

Taga, M.E., and Walker, G.C. (2010). *Sinorhizobium meliloti* requires a cobalamin-dependent ribonucleotide reductase for symbiosis with its plant host. Mol Plant Microbe Interact *23*, 1643-1654.

Takahashi-Íñiguez, T., García-Arellano, H., Trujillo-Roldán, M.A., and Flores, M.E. (2011). Protection and reactivation of human methylmalonyl-CoA mutase by MMAA protein. Biochemical and biophysical research communications *404*, 443-447.

Tallant, T.C., Paul, L., and Krzycki, J.A. (2001). The MtsA subunit of the methylthiol:coenzyme M methyltransferase of *Methanosarcina barkeri* catalyses both half-reactions of corrinoid-dependent dimethylsulfide: coenzyme M methyl transfer. J Biol Chem *276*, 4485-4493.

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

Taoka, S., Padmakumar, R., Lai, M.T., Liu, H.W., and Banerjee, R. (1994). Inhibition of the human methylmalonyl-CoA mutase by various CoA-esters. J Biol Chem *269*, 31630-31634.

Textor, S., Wendisch, V.F., De Graaf, A.A., Muller, U., Linder, M.I., Linder, D., and Buckel, W. (1997). Propionate oxidation in *Escherichia coli*: evidence for operation of a methylcitrate cycle in bacteria. Archives of microbiology *168*, 428-436.

Vrijbloed, J.W., Zerbe-Burkhardt, K., Ratnatilleke, A., Grubelnik-Leiser, A., and Robinson, J.A. (1999). Insertional inactivation of methylmalonyl coenzyme A (CoA) mutase and isobutyryl-CoA mutase genes in *Streptomyces cinnamonensis*: influence on polyketide antibiotic biosynthesis. J Bacteriol *181*, 5600-5605.

Walsh, C.T., and Tang, Y. (2019). The chemical biology of human vitamins (Royal Society of Chemistry).

Warner, J.R., and Copley, S.D. (2007). Pre-steady-state kinetic studies of the reductive dehalogenation catalyzed by tetrachlorohydroquinone dehalogenase. Biochemistry *46*, 13211-13222.

Watanabe, F., Abe, K., Fujita, T., Goto, M., Hiemori, M., and Nakano, Y. (1998). Effects of microwave heating on the loss of vitamin B_{12} in foods. Journal of Agricultural and Food Chemistry *46*, 206-210.

Watanabe, F., Nakano, Y., and Stupperich, E. (1992). Different corrinoid specificities for cell growth and cobalamin uptake in *Euglena gracilis* Z, Vol 138.

Watanabe, F., Yabuta, Y., Tanioka, Y., and Bito, T. (2013). Biologically active vitamin B_{12} compounds in foods for preventing deficiency among vegetarians and elderly subjects. J Agric Food Chem *61*, 6769-6775.

Watkins, D., and Rosenblatt, D.S. (1989). Functional methionine synthase deficiency (cblE and cblG): clinical and biochemical heterogeneity. American journal of medical genetics *34*, 427-434.

Watkins, D., and Rosenblatt, D.S. (2013). Lessons in biology from patients with inborn errors of vitamin B_{12} metabolism. Biochimie *95*, 1019-1022.

Watkins, D., and Rosenblatt, D.S. (2014). Inherited disorders of folate and cobalamin transport and metabolism. In The Online Metabolic and Molecular Bases of Inherited Disease, A.L. Beaudet, B. Vogelstein, K.W. Kinzler, S.E. Antonarakis, A. Ballabio, K.M. Gibson, and G. Mitchell, eds. (New York, NY: The McGraw-Hill Companies, Inc.).

Werner, W.J., Allen, K.D., Hu, K., Helms, G.L., Chen, B.S., and Wang, S.C. (2011). In vitro phosphinate methylation by PhpK from *Kitasatospora phosalacinea*. Biochemistry *50*, 8986-8988.

Widner, F.J. (2013). Synthesis and characterization of the Rh analog of the cobalt-corrin coenzyme B_{12}: Probing the role of the metal ion. In Institute of Organic Chemistry, Center for Molecular Biosciences Innsbruck (Leopold-Franzens-University Innsbruck).

Widner, F.J., Lawrence, A.D., Deery, E., Heldt, D., Frank, S., Gruber, K., Wurst, K., Warren, M.J., and Krautler, B. (2016). Total synthesis, structure, and biological activity of adenosylrhodibalamin, the non-natural rhodium homologue of coenzyme B_{12}. Angewandte Chemie (International ed in English) *55*, 11281-11286.

Wolthers, K.R., Levy, C., Scrutton, N.S., and Leys, D. (2010). Large-scale domain dynamics and adenosylcobalamin reorientation orchestrate radical catalysis in ornithine 4,5-aminomutase. J Biol Chem *285*, 13942-13950.

Woodyer, R.D., Li, G., Zhao, H., and van der Donk, W.A. (2007). New insight into the mechanism of methyl transfer during the biosynthesis of fosfomycin. Chem Commun (Camb), 359-361.

Yabuta, Y., Kamei, Y., Bito, T., Arima, J., Yoneda, K., Sakuraba, H., Ohshima, T., Nakano, Y., and Watanabe, F. (2015). Functional and structural characteristics of methylmalonyl-CoA mutase from *Pyrococcus horikoshii*. Biosci Biotechnol Biochem *79*, 710-717.

Yamada, K., Gravel, R.A., Toraya, T., and Matthews, R.G. (2006). Human methionine synthase reductase is a molecular chaperone for human methionine synthase. Proceedings of the National Academy of Sciences of the United States of America *103*, 9476-9481.

Yamanishi, M., Yunoki, M., Tobimatsu, T., Sato, H., Matsui, J., Dokiya, A., Iuchi, Y., Oe, K., Suto, K., Shibata, N.*, et al.* (2002). The crystal structure of coenzyme B_{12}-dependent glycerol dehydratase in complex with cobalamin and propane-1,2-diol. European journal of biochemistry *269*, 4484-4494.

Yan, J., Bi, M., Bourdon, A.K., Farmer, A.T., Wang, P.H., Molenda, O., Quaile, A.T., Jiang, N., Yang, Y., Yin, Y.*, et al.* (2018). Purinyl-cobamide is a native prosthetic group of reductive dehalogenases. Nature chemical biology *14*, 8-14.

Yan, J., Ritalahti, K.M., Wagner, D.D., and Loffler, F.E. (2012). Unexpected specificity of interspecies cobamide transfer from *Geobacter* spp. to organohalide-respiring *Dehalococcoides mccartyi* strains. Appl Environ Microbiol *78*, 6630-6636.

Yan, J., Simsir, B., Farmer, A.T., Bi, M., Yang, Y., Campagna, S.R., and Loffler, F.E. (2016). The corrinoid cofactor of reductive dehalogenases affects dechlorination rates and extents in organohalide-respiring *Dehalococcoides mccartyi*. The ISME journal *10*, 1092-1101.

Yaneva, N., Schuster, J., Schäfer, F., Lede, V., Przybylski, D., Paproth, T., Harms, H., Müller, R.H., and Rohwerder, T. (2012). Bacterial acyl-CoA mutase specifically catalyzes coenzyme B_{12}-dependent isomerization of 2-hydroxyisobutyryl-CoA and *(S)*-3-hydroxybutyryl-CoA. J Biol Chem *287*, 15502-15511.

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, W., Yang, L., Jiang, W., Zhao, G., Yang, Y., and Chiao, J. (1999). Molecular analysis and heterologous expression of the gene encoding methylmalonyl-coenzyme A mutase from rifamycin SV-producing strain *Amycolatopsis mediterranei* U32. Applied Biochemistry and Biotechnology *82*, 209-225.

Zhang, Y., Rodionov, D.A., Gelfand, M.S., and Gladyshev, V.N. (2009). Comparative genomic analyses of nickel, cobalt and vitamin B_{12} utilization. BMC genomics *10*, 78.