DISSECTING THE ROLES OF CBIZ, A COBYRIC FORMING AMIDOHYDROLASE AND CBIB, AN ADENOSYLCOBINAMIDE-PHOSPHATE SYNTHASE IN THE LAST STEPS OF CORRIN RING BIOSYNTHESIS

by

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(Under the Direction of JORGE ESCALANTE-SEMERENA)

ABSTRACT

Cobamides are organo-metallic tetrapyrroles that are required for all domains in life, These marco-cyclic compounds are an essential component in cellular function. Cobamides are essential to all domains of life. Notably, cobamides are only synthesized *de novo* by some bacteria and archaea. The complexity of the chemical structure of cobamides has challenged chemists, biologists, and biophysicists alike for many decades. In spite of these efforts, our understanding of how this macrocyclic molecule is assembled, remains incomplete. The functions of the enzymes CbiZ (cobyric acid-forming amidohydrolase) and CbiB (cobinamide-phosphate synthase) remain unknown. In most archaea and some bacteria CbiZ functions as a remodeling systems of corrinoids. CbiZ cleaves corrinoids at the amide bond of the amino-propanol moiety, this reaction yields cobyric acid which can re-enter $B_{12}$ biosynthesis pathway and acts as a co-substrate of CbiB. The foci of this work uses *Salmonella enterica* serovar Typhimurium LT2 to dissect the activity of the enzymes and the connections of CbiZ to CbiB.

INDEX WORDS: cobamides, $B_{12}$, CbiZ, CbiB, salvaging

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DEDICATION

To my mother, Rosa L. Enriquez, this one's for you

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

Cobamides (a.k.a. Cbas, $B_{12}$, complete corrinoids) are cobalt-containing cyclic tetrapyrroles that belong to the family of compounds known as 'The Pigments of Life' (e.g. chlorophylls, hemes, coenzyme $F_{430}$) due to their broad distribution in nature and critical role in cell function. Cobamides are essential to many organisms, including humans. A broad array of critical processes in nature depend on the availability of cobamides, for example, methane formation, chlorophyll biosynthesis and propionate detoxification. Notably, cobamides are only synthesized *de novo* by some bacteria and archaea, dedicating a significant portion of genetic information (~30 gene) to the process (1) . The complexity of the chemical structure of cobamides has challenged chemists, biologists, and biophysicists alike for many decades. In spite of these efforts, our understanding of the assembly of cobamides is incomplete.

## IMPORTANCE AND FEATURES

Cobamides, $B_{12}$, complete corrinoids are generic names that describe a family of cobalt-containing cyclic tetrapyrroles (2). The nomenclature in the $B_{12}$ field is complex, and rules for the correct naming of these compounds have been issued by the International Union of Biochemistry (IUB) (3) . A brief definition of the most commonly used terms is presented. Corrinoids: cobalt containing cyclic tetrapyrrole aka corrin ring. Incomplete corrinoids: corrinoids that lack the lower axial base/ligand (Coα) but not the upper axial ligand (Coβ) e.g. cobyric acid (Cby) and cobinamide (Cbi). Cobamide: complete corrinoids containing with lower and upper axial ligands. DMB: 5,6-dimethylbenzimidazole. Cobalamin: Cobamide with Coα as

DMB. AdoCbl (Coenzyme $B_{12}$): Cbl with DMB as the Coα. AdoPseudoCbl: cobamide that has a lower axial base/ligand as adenine.

*The Anatomy of $B_{12}$*

The anatomy of $B_{12}$ contributes and has important implications for the chemistry it performs in the cell. Here it is described in four parts. (i) The cyclic tetrapyrrole containing a cobalt ion center, coordinated via nitrogens to the pyrroles (notably different from the other macrocyclic rings due to its contracted ring), is referred to as the corrin ring. The "D" pyrrole propionamide has (ii) an 1-amino-2 propanyl moiety. This moiety is esterfied to a nucleotide with a 3'-phosphatidyl group (4). Here ribose is connected (iii) a lower base/ligand through an α-N-glycosydic bond. (iv)The upper axial ligand is 5' deoxy-adenosine (Fig. 1.1)

*Relevance*

Cobalamin (vitamin $B_{12}$) is an essential micronutrient (5) for all domains of life but is produced exclusively by prokaryotes. Although human cells do not make this compound, they can convert CNCbl to its coenzymatic form (adenosylcobalamin, AdoCbl, or Coenzyme $B_{12}$). This conversion is due to the specificity of the Intrinsic Factor, a glycoprotein necessary for absorption of $B_{12}$ synthesized by the stomach (6). Humans with $B_{12}$ deficiencies may develop a myriad of $B_{12}$ related illnesses related to deficiencies including pernicious anemia, neurological disorders, and links to osteoporosis (7, 8).

$B_{12}$-DEPENDENT REACTIONS

Cbl is an essential cofactor required of several enzymes involving intramolecular rearrangements (Figure 1.2A), elimination reactions, methyl transfers, and dehalogenations. Examples of theses enzymes include methylmalonyl-CoA mutase (fatty acid catabolism), class II ribonucleotide

reductase (DNA synthesis), methyltransferases (Methionine Synthase Figure 1.2B) and reductive dehalogenases respectively (9-15).

*Methylmalonyl-CoA mutase*

AdoCbl is used to generate a 5'-deoxyadenosyl radical through homolytic cleavage of the C-Co bond (16). AdoCbl binds to the protein in a "base-off" "histidine-on" position, histidine (His) coordinates to the cobalt ion allowing the enzyme to correctly position the active site. The radical abstracts hydrogen from a substrate carbon allowing carbon-skeletal rearrangement(17-20).

*Class II Ribonucleotide Reductase*

AdoCbl binds to the protein in a "base on" configuration. Here AdoCbl generates a thiyl radical using cysteines of the protein, this initiates hydrogen abstraction from a ribonucleotide molecule resulting in the formation of 2-deoxynucleotide (21-26).

*Methionine Synthase (MetH)*

Methionine synthase uses Cbl as a methyl carrier through heterolytic cleavage, generating Co(I)Cbl cycling to MeCbl. A methyl group from 5'methyl tetrahydrofolate is transferred to Co(I)Cbl which is then transferred to the sulfogroup of homocysteine generating methionine (18, 20, 27, 28). Notable Cbl methyltrasnferases are MtaABC complex involved in methanogenesis and AcsAB acetogenesis (29, 30)

*Reductive dehalogenases*

Recent studies, propose mechanisms for dehalogenase reactions. Using a "base- off" configuration halide-Co(II)Cbl complexes via heterolytic or homolytic cleavage of carbon-halide substrate bond generating Co(III)Cbl-halide intermediate (31). One-electron transfers result in the formation of Co(II)Cbl-halide product released from active site (32).

## ADENOSYLCOBALAMIN BIOSYNTHESIS

Some organisms are capable of *de novo* AdoCbl biosynthesis in the presence or absence of oxygen (*Pseudomonas denitrificans* and *S.* Typhimurium respectively). The key difference between the aerobic and anaerobic $B_{12}$ biosynthetic pathways is the timing of cobalt ion chelation (33). In the aerobic pathway, the cobalt ion is inserted into the corrin ring late in the pathway, while in the anaerobic pathway cobalt is inserted very early in the pathway (34). The work described later in *S.* Typhimurium; it is to be noted that while *S.* Typhimurium utilizes the anaerobic pathway, growth under aerobic conditions looking at the late steps of corrin ring synthesis, these intermediates are not sensitive to oxygen (35). To differentiate the different parts of the pathway, a descriptive nomenclature system is the one used in *S.* Typhimurium. In this bacterium, genes encoding functions involved in corrin ring biosynthesis are named *cbi*, to indicate that they catalyze a reaction leading to the synthesis of AdoCbi-P. Genes encoding functions involved in the assembly of the nucleotide loop or the attachment of the upper ligand are referred to as *cob* i.e. CobU. These works will be using the *S.* Typhimurium naming system (34) A thorough review of AdoCbl biosynthesis can be found in (36-43).

### *Synthesis of the corrin ring*

Like the other tetrapyrroles, the corrin ring is derived from the common molecule Uroporphyringen III. Uro III undergoes several modifications. These modifications result in a contracted ring (C1-C19), eight methyl groups, four acetamide groups, and four propionamide groups. My work focuses on the last step in *de novo* corrin ring biosynthesis, the formation of AdoCbi-P (Fig. 1.3), a condensation of 1-amino-2-propanol phosphate and AdoCby by CbiB (EC 6.3.1.10, cobinamide-phosphate synthase) (44).

*Nucleotide loop assembly* (NLA)

CobU, CobS, CobT, and CobC proteins make up the late steps of AdoCbl biosynthesis (45-47). These enzymes make up the nucleotide loop assembly pathway (Fig. 1.3). NLA can be separated into two branches. Branch one is the activation of AdoCbi-P by CobU (EC 2.7.7.62 and EC 2.7.1.156 AdoCbi kinase/AdoCbi-phosphate guanylyltransferase) to yield AdoCbi-GDP (48-53). Branch two is the activation of the lower base/ligand, in this case DMB is activated by CobT (EC 2.4.2.21 (NaMN):5,6-dimethylbenzimidazole phosphoribosyltransferase) to alpha-ribazole phosphate (α-RP). Due to the promiscuous nature of CobT, CobT is capable of activating other base/ligands resulting in a variety of cobamides (54-57). CobS (EC 2.7.8.26, cobalamin-5′-phosphate synthase), the penultimate enzyme, condenses the activated substrates forming AdoCbl-P(46, 58). CobC (EC 3.1.3.73 adenosylcobalamin/α-ribazole-5′-phosphate phosphohydrolase) dephosphorylates this substrate yielding the final product AdoCbl (59).

*Adenosylation of the corrin ring*

As mentioned, the 5'deoxyadenosine (Coβ) of AdoCbl plays a vital role in enzymatic function. The step at which the corrin ring is adenosylated is unclear. Adenosylation is required for *de novo* $B_{12}$ synthesis and assimilation of exogenous corrinoids (60). The mechanism of adenosylation of the corrin ring by CobA (ATP:Co(I)rrinoid adenosyltransferases) is well understood (61-64). Organisms that utilize adenosylated corrinoids must be able to adenosylated them due to the fragile nature (light sensitivity, environmental conditions, acidity) of the cobalt-carbon covalent bond (65-67).

## CORRINOID SCAVENGING

Only some bacteria and archaea synthesize AdoCbl *de novo* (68), but others can salvage the corrin ring from the environment and attach to it the lower ligand and the upper ligand (53, 69-71)12). *De novo* synthesis of adenosylcobalamin (AdoCbl) is genetically and energetically intensive (72). To circumvent the genetic and energy cost some prokaryotes are able to salvage exogenous corrinoids from the environment.

### *Bacterial*

In bacteria, incomplete corrinoids are transported into the cell by $B_{12}$, transport system. Cbi is adenosylated by CobA. CobU phosphorylates AdoCbi to AdoCbi-P (Fig. 1.3) then activates the corrin ring by attaching a guanylyl-diphosphate moiety yielding AdoCbi-GDP which will eventually become AdoCbl through additional enzymatic reactions. Some bacteria are capable of remodeling cobamides. For example, when grown aerobically on acetate, *Rhodobacter sphaeroides* requires AdoCbl (69, 73, 74). Cells fed AdoPseudoCbl are capable of growth because of the presence of CbiZ. CbiZ cleaves AdoPseudoCbl to Cby allowing it to re-enter the pathway and add the appropriate Coα (DMB) to synthesize AdoCbl.

### *Archaeal*

Archaea contain an non-orthologous version of *cobU* gene, *cobY* only encodes for GTP:AdoCbi-P guanylyltransferase activity. Table 1.1 shows the gene encoding GTP:AdoCbi-P transferase, note that both possess homologs for CbiB, CobD, CobT, and CobS. In this instance, exogenous corrinoids are transported into the cell. If the corrinoid is a cobamide the cell cannot use, the cell can remodel the molecule using CbiZ instead of constructing the molecular from scratch. CbiZ will cleave the lower ligand of corrinoids that cannot be used by some organisms, (e.g. adenine the lower ligand of pseudoCbl) generating Cby and thus, reentering the $B_{12}$ pathway

via CbiB. CbiB catalyzes the reaction of AdoCby and 1-amino-2-propanol phosphate to yield AdoCbi-P allowing the correct lower ligand to be added. At present, it is known that CbiB plays an important role in archaea that posses this corrinoid-remodeling enzyme CbiZ (53).

OTHER

*Using S. Typhimurium as a genetic tool for studying $B_{12}$ biosynthesis*

In *S.* Typhimurium, two growth conditions are used to assess production of cobalamin. One growth condition relies on the function of the MetH, a Cbl-dependent methionine synthase (37), this is used as a tool to assess Cbl synthesis (75). Very few $B_{12}$ molecules (~10 per cell) are required to satisfy the methionine needs of the cell; hence this is sensitive method of detection of production of Cbl (76). To take advantage of this a *S.* Tyhimurium strain with a null *metE* allele is used. MetE, the Cbl-independent methionine synthase is inactivated, thus relying on the MetH to regenerate methionine from homocysteine (77). The other growth conditions rely on other AdoCbl-dependent enzymes, ethanolamine ammonia-lyase (EutBC) and 1,2-propanediol dehydratase (PduCDE) (78-81). To use ethanolamine or 1,2-propanediol as carbon and energy sources, *S.* Typhimurium needs to synthesize ~3,000 AdoCbl molecules per cell (82). These two methods provide different conditions that allow for stringency (EutBC), or sensitivity (MetH) for assessing cobalamin production.

*Thesis outline*

Decades of research have been spent on dissecting the role of $B_{12}$ and how this elegant molecule is synthesized. The foci of this thesis are the characterization of the cobyric forming enzyme CbiZ and the enzyme CbiB that uses this substrate to form Cbi-P, the last step in corrin ring biosynthesis. Chapter 2 discusses residues that may be important for catalyzing the amidohydrolase reaction and binding of the substrate. These studies focus on the *R. sphaeroides*

CbiZ, using a modified *S.* Typhimuirum strain to elucidate the role of residues in *Rs*CbiZ. An optimized purification strategy is described; its use for biochemical and structural analyses is discussed Chapter 4. Chapter 3 is a continuation of work described in (44). Using alanine scanning mutagenesis additional residues that appear to contribute to catalytic activity and binding of substrates are discussed. Strategies to elucidate *in vitro* data for both enzymes are discussed in Chapter 4.

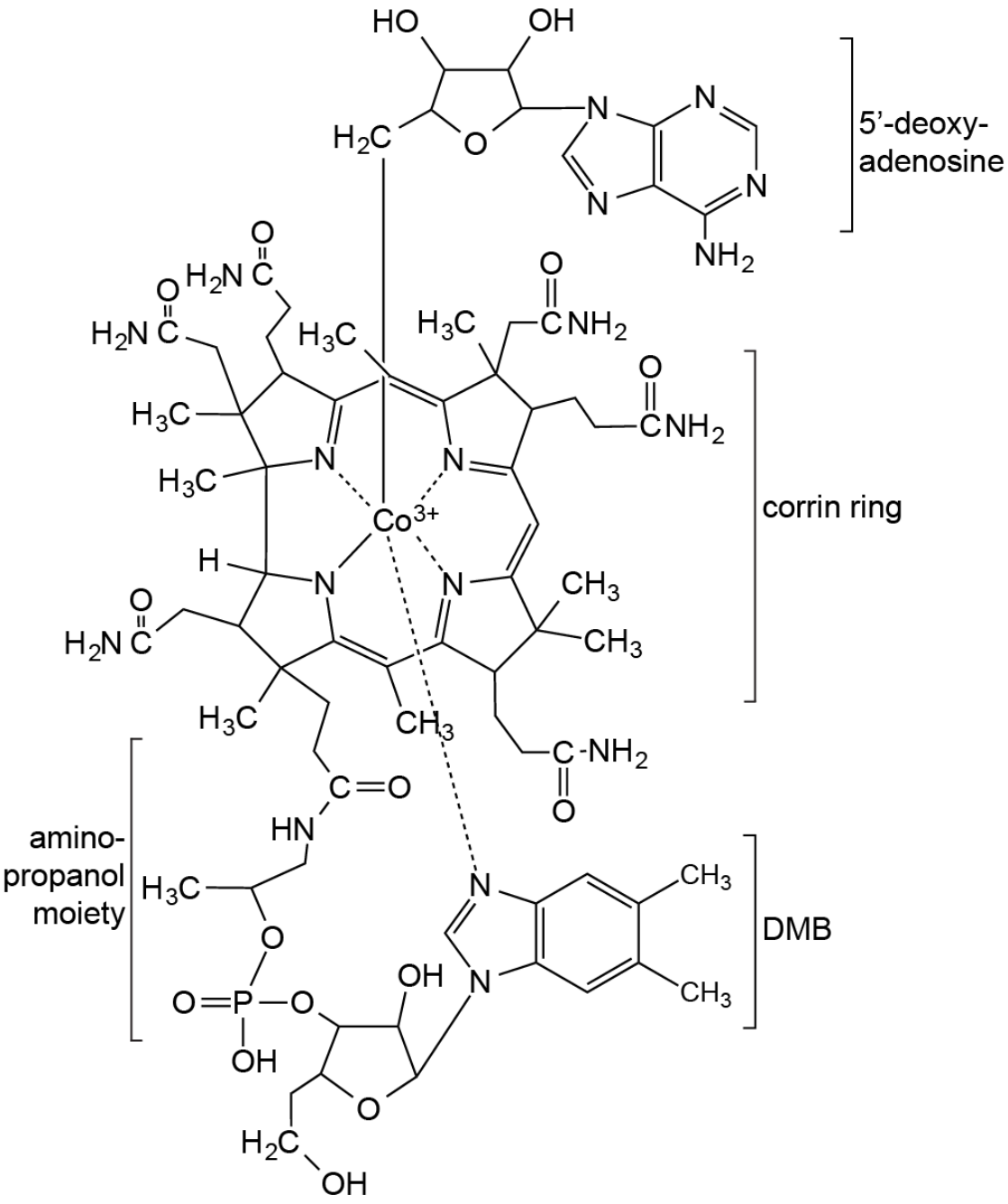


**Figure 1.1**: Chemical structure of Adenosylcobalamin. Key features are labeled and indicated by brackets.

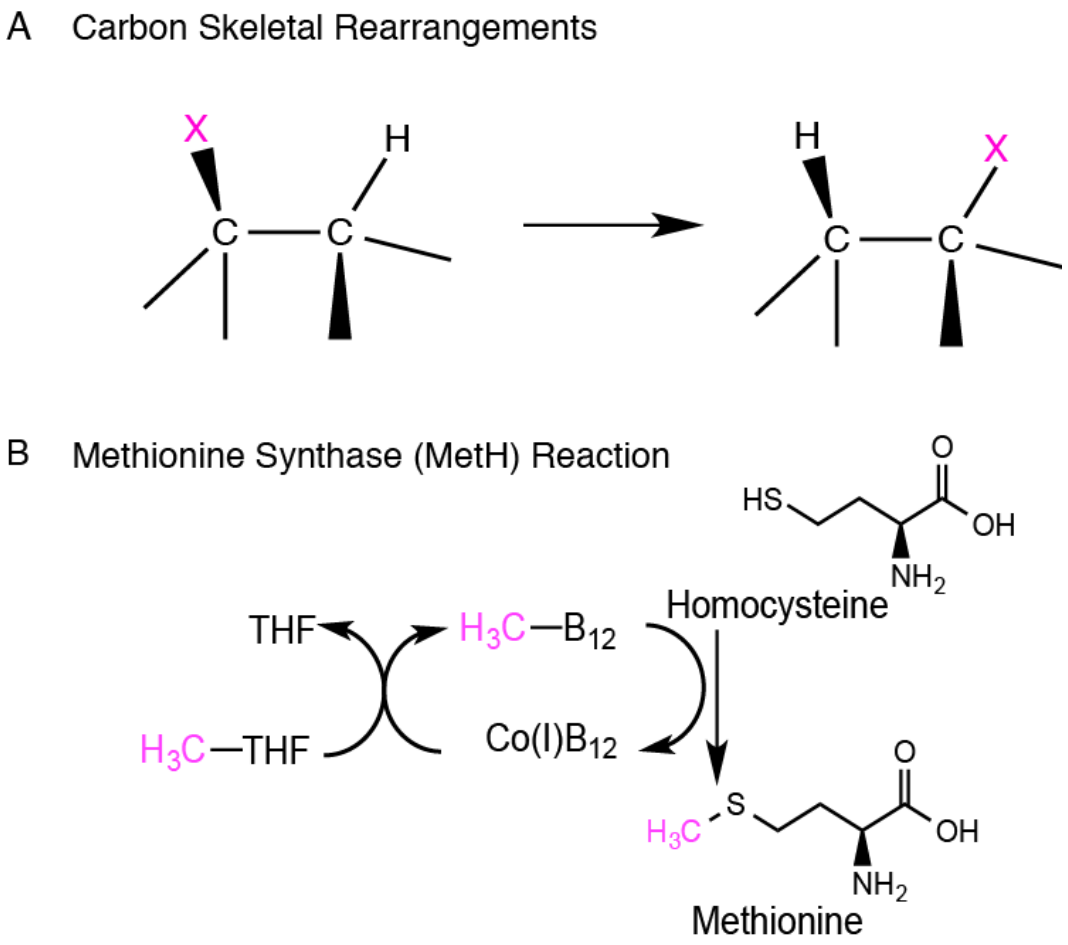


**Figure 1.2**: $B_{12}$-dependent reactions. A. Carbon Skeletal Rearrangements (e.g. methylmalonyl-CoA mutase) B. Reaction mechanism of methionine synthase, a $B_{12}$-depedent methyltransferases.

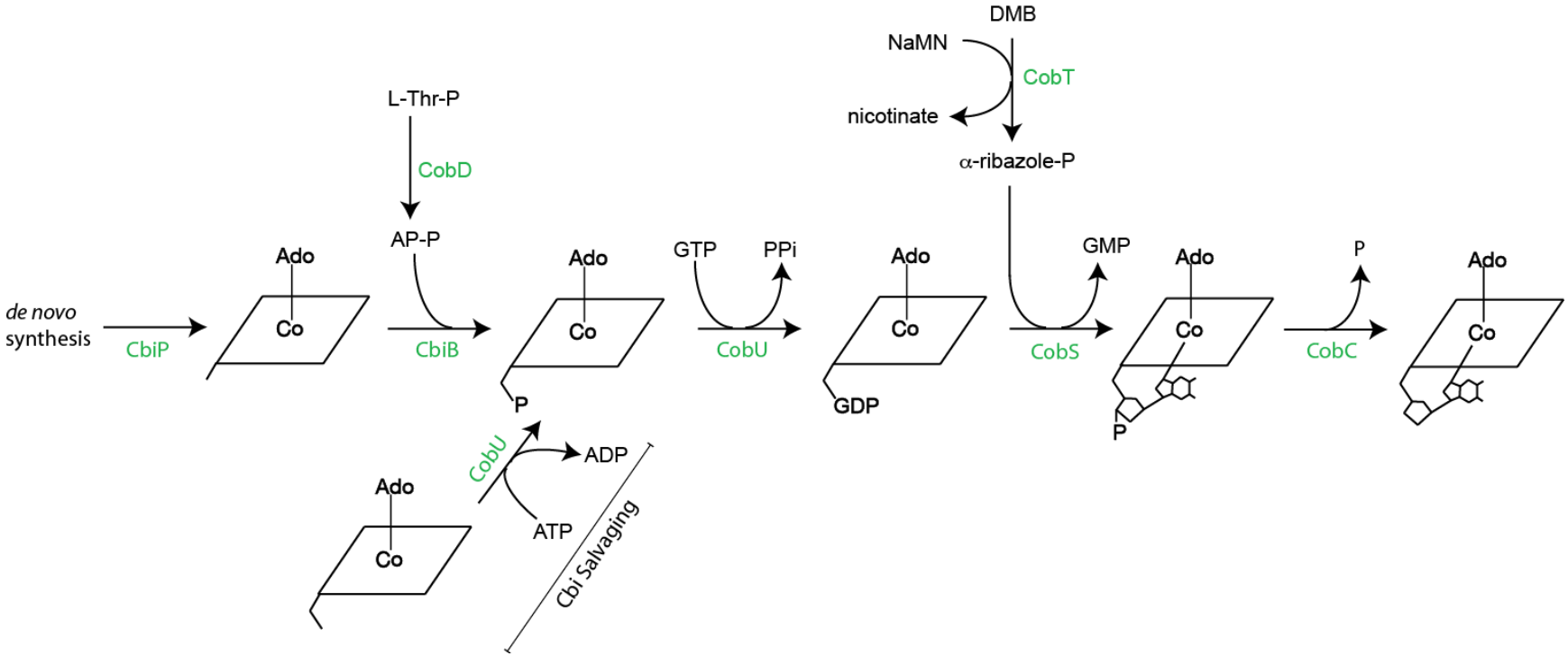


**Figure 1.3**: Late steps of *de novo* synthesis of AdoCbl, starting at the final steps of corrin ring biosynthesis. Enzymes are labeled in green. The trapezoid depicts the corrin ring. The stick protruding from the cobalt center represents the organometallic bond to 5'-deoxyadenosine.

**Table 1.1**: Archaea possess an orthologous guanylytransferase enzyme, CobY used in salvaging, bacteria use the trifunctional enzyme CobU.

| | CbiB | CobD | CobU | CobT | CobS | CobY |
|---|---|---|---|---|---|---|
| *S. enterica* | X[1] | X | X | X | X | |
| *B. halodurans* | X | X | X | X | X | |
| *R. sphaeroides* | X | X | X | X | X | |
| *M. jannaschi* | X | X | | X | X | X |
| *P. furiosus* | X | X | | X | X | X |
| *M. mazei* | X | X | | X | X | X |
| *H. salinarum* | X | X | | X | X | X |

[1]Indicates the organism posses a gene that encodes respective protein

REFERENCES


1. Smith A & Warren M (2009) *Tetrapyrroles : birth, life and death* (Springer, New York, NY).
2. Escalante-Semerena JC & Warren MJ (2008) Biosynthesis and Use of Cobalamin ($B_{12}$). *EcoSal - Escherichia coli and Salmonella: cellular and molecular biology*, eds Böck A, Curtiss III R, Kaper JB, Karp PD, Neidhardt FC, Nyström T, Slauch JM, & Squires CL (ASM Press, Washington, D. C.).
3. Maloy SR & Hughes KT (2007) Strain collections and genetic nomenclature. *Advanced Bacterial Genetics: Use of transposons and phage for genomic engineering* 421:3-8.
4. Jaenicke L (1964) VITAMIN AND COENZYME FUNCTION: VITAMIN B 12 AND FOLIC ACID. *Annual review of biochemistry* 33:287-312.
5. Furger E, Frei DC, Schibli R, Fischer E, & Prota AE (2013) Structural basis for universal corrinoid recognition by the cobalamin transport protein haptocorrin. *J. Biol. Chem.* 288:25466-25476.
6. Hewitt JE, Gordon MM, Taggart RT, Mohandas TK, & Alpers DH (1991) Human gastric intrinsic factor: characterization of cDNA and genomic clones and localization to human chromosome 11. *Genomics* 10(2):432-440.
7. Pruthi RK & Tefferi A (1994) Pernicious anemia revisited. *Mayo Clin. Proc.* 69:144-150.
8. O'Leary F & Samman S (2010) Vitamin B(12) in Health and Disease. *Nutrients* 2(3):299-316.
9. Banerjee R & Ragsdale SW (2003) The many faces of vitamin B12: catalysis by cobalamin-dependent enzymes. *Annu. Rev. Biochem.* 72:209-247.
10. Barker HA (1972) Corrinoid-dependent enzymic reactions. *Ann. Rev. Biochem.* 41:55-90.

11. Bucher D, Sandala GM, Durbeej B, Radom L, & Smith DM (2012) The elusive 5'-deoxyadenosyl radical in coenzyme-B12-mediated reactions. *J. Am. Chem. Soc.* 134:1591-1599.

12. Golding BT & Buckel W (1998) Corrin-dependent reactions. *Comprehensive biological catalysis.*, ed Sinnott SM (Academic Press), Vol 3, pp 239-259.

13. Ludwig ML, Drennan CL, & Matthews RG (1996) The reactivity of B12 cofactors: the proteins make a difference. *Structure* 4:505-512.

14. Stroinski A (1987) Adenosylcobamide-dependent reactions. *Comprehensive B12*, eds Schneider Z & Stroinski A (Walter de Gruyter, Berlin), pp 226-259.

15. Walsh C (1979) Rearrangements dependent on coenzyme B12. *Enzymatic Reaction Mechanisms*, (W. H. Freeman and Company, New York), pp 640-665.

16. Banerjee RV & Matthews RG (1990) Cobalamin-dependent methionine synthase. *FASEB J* 4:1450-1459.

17. Banerjee RV, Harder SR, Ragsdale SW, & Matthews RG (1990) Mechanism of reductive activation of cobalamin-dependent methionine synthase: an electron paramagnetic resonance spectroelectrochemical study. *Biochemistry* 29:1129-1135.

18. Dorweiler JS, Finke RG, & Matthews RG (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.

19. Frey PA (2001) Radical mechanisms of enzymatic catalysis. *Annu. Rev. Biochem.* 70:121-148.

20. Matthews RG (2001) Cobalamin-dependent methyltransferases. *Acc. Chem. Res.* 34:681-689.

21. Booker S, Licht S, Broderick J, & Stubbe J (1994) Coenzyme B12-dependent ribonucleotide reductase: evidence for the participation of five cysteine residues in ribonucleotide reduction. *Biochemistry* 33:12676-12685.

22. Fontecave M (1998) Ribonucleotide reductases and radical reactions. *Cell. Mol. Life Sci.* 54:684-695.

23. Jordan A & Reichard P (1998) Ribonucleotide reductases. *Ann. Rev. Biochem.* 67:71-98.

24. Riera J, Robb FT, Weiss R, & Fontecave M (1997) Ribonucleotide reductase in the archaeon Pyrococcus furiosus: a critical enzyme in the evolution of DNA genomes? *Proc. Natl. Acad. Sci. USA* 94:475-478.

25. Stubbe J (1990) Ribonucleotide reductases: Amazing and confusing. *J. Biol. Chem.* 265:5329-5332.

26. Stubbe J (1998) Ribonucleotide reductases in the twenty-first century. *Proc Natl Acad Sci U S A* 95:2723-2724.

27. Frasca V, Banerjee RV, Dunham WR, Sands RH, & Matthews RG (1988) Cobalamin-dependent methionine synthase from *Escherichia coli* B: electron paramagnetic resonance spectra of the inactive form and the active methylated form of the enzyme. *Biochemistry* 27:8458-8465.

28. Jarrett JT, Huang S, & Matthews RG (1998) Methionine synthase exists in two distinct conformations that differ in reactivity toward methyltetrahydrofolate, adenosylmethionine, and flavodoxin. *Biochemistry* 37(16):5372-5382.

29. DiMarco AA, Bobik TA, & Wolfe RS (1990) Unusual coenzymes of methanogenesis. *Annual review of biochemistry* 59:355-394.

30. Hu SI, Pezacka E, & Wood HG (1984) Acetate synthesis from carbon monoxide by Clostridium thermoaceticum. Purification of the corrinoid protein. *The Journal of biological chemistry* 259(14):8892-8897.

31. Payne KAP*, et al.* (2015) Reductive dehalogenase structure suggests a mechanism for B12-dependent dehalogenation. *Nature* 517:513-516.

32. Liao R-Z, Chen S-L, & Siegbahn PEM (2016) Unraveling the Mechanism and Regioselectivity of the B12-Dependent Reductive Dehalogenase PceA. *Chemistry – A European Journal* 22(35):12391-12399.

33. Roth JR, Lawrence JG, Rubenfield M, Kieffer-Higgins S, & Church GM (1993) Characterization of the cobalamin (vitamin B12) biosynthetic genes of Salmonella typhimurium. *Journal of Bacteriology* 175:3303-3316.

34. Escalante-Semerena JC (2007) Conversion of cobinamide into adenosylcobamide in bacteria and archaea. *J Bacteriol* 189(13):4555-4560.

35. Moore SJ*, et al.* (2013) Elucidation of the anaerobic pathway for the corrin component of cobalamin (vitamin B12). *Proc. Natl. Acad. Sci. U S A* 110:14906-14911.

36. Roth JR, Lawrence JG, & Bobik TA (1996) Cobalamin (coenzyme B12): synthesis and biological significance. *Annu. Rev. Microbiol.* 50:137-181.

37. Roth J, Lawrence J, & Bobik T (1996) COBALAMIN (COENZYME B12): Synthesis and Biological Significance. *Annual Review of Microbiology* 50:137-181.

38. Martens JH, Barg H, Warren MJ, & Jahn D (2002) Microbial production of vitamin $B_{12}$. *Appl. Microbiol. Biotechnol.* 58:275-285.

39. Warren MJ, Raux E, Schubert HL, & Escalante-Semerena JC (2002) The biosynthesis of adenosylcobalamin (vitamin B12). *Nat. Prod. Rep.* 19:390-412.

40. Heldt D*, et al.* (2005) Aerobic synthesis of vitamin B12: ring contraction and cobalt chelation. *Biochem. Soc. Trans.* 33:815-819.

41. Roessner CA & Scott AI (2006) Fine-tuning our knowledge of the anaerobic route to cobalamin (vitamin $B_{12}$). *J. Bacteriol.* 188:7331-7334.

42. Rondon MR, Trzebiatowski JR, & Escalante-Semerena JC (1997) Biochemistry and molecular genetics of cobalamin biosynthesis. *Prog. Nucleic Acid Res. Mol. Biol.* 56:347-384.

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

44. Zayas CL, Claas K, & Escalante-Semerena JC (2007) The CbiB protein of *Salmonella enterica* is an integral membrane protein involved in the last step of the de novo corrin ring biosynthetic pathway. *J. Bacteriol.* 189:7697-7708.

45. Escalante-Semerena JC, Johnson MG, & Roth JR (1992) The CobII and CobIII regions of the cobalamin (vitamin B12) biosynthetic operon of Salmonella typhimurium. *J Bacteriol* 174:24-29.

46. Maggio-Hall LA, Claas KR, & Escalante-Semerena JC (2004) The last step in coenzyme B(12) synthesis is localized to the cell membrane in bacteria and archaea. *Microbiology* 150:1385-1395.

47. O'Toole GA (1994) Biochemistry and genetics of cobalamin nucleotide loop assembly in *Salmonella typhimurium*. Ph.D. Ph.D. (University of Wisconsin, Madison).

48. O'Toole GA & Escalante-Semerena JC (1995) Purification and characterization of the bifunctional CobU enzyme of *Salmonella typhimurium* LT2. Evidence for a CobU-GMP intermediate. *J. Biol. Chem.* 270:23560-23569.

49. O'Toole GA & Escalante-Semerena JC (1993) *cobU*-dependent assimilation of nonadenosylated cobinamide in cobA mutants of *Salmonella typhimurium*. *J. Bacteriol.* 175:6328-6336.

50. Otte MM, Woodson JD, & Escalante-Semerena JC (2007) The thiamine kinase (YcfN) enzyme plays a minor but significant role in cobinamide salvaging in *Salmonella enterica*. *J. Bacteriol.* 189:7310-7315.

51. Thomas MG, Thompson TB, Rayment I, & Escalante-Semerena JC (2000) Analysis of the adenosylcobinamide kinase/adenosylcobinamide-phosphate guanylyltransferase (CobU) enzyme of Salmonella typhimurium LT2. Identification of residue His-46 as the site of guanylylation. *J. Biol. Chem.* 275:27576-27586.

52. Thompson TB, Thomas MG, Escalante-Semerena JC, & Rayment I (1999) Three-dimensional structure of adenosylcobinamide kinase/adenosylcobinamide phosphate guanylyltransferase (CobU) complexed with GMP: evidence for a substrate-induced transferase active site. *Biochemistry* 38:12995-13005.

53. Woodson JD, Zayas CL, & Escalante-Semerena JC (2003) A new pathway for salvaging the coenzyme $B_{12}$ precursor cobinamide in archaea requires cobinamide-phosphate synthase (CbiB) enzyme activity. *J. Bacteriol.* 185:7193-7201.

54. Chan CH & Escalante-Semerena JC (2011) ArsAB, a novel enzyme from *Sporomusa ovata* activates phenolic bases for adenosylcobamide biosynthesis. *Mol. Microbiol.* 81:952-967.

55. Chan CH*, et al.* (2014) Dissecting cobamide diversity through structural and functional analyses of the base-activating CobT enzyme of *Salmonella enterica*. *Biochim. Biophys. Acta* 1840:464-475.

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

57. Cheong CG, Escalante-Semerena JC, & 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.

58. Maggio-Hall LA & Escalante-Semerena JC (1999) In vitro synthesis of the nucleotide loop of cobalamin by *Salmonella typhimurium* enzymes. *Proc. Natl. Acad. Sci. U S A* 96:11798-11803.

59. O'Toole GA, Trzebiatowski JR, & Escalante-Semerena JC (1994) The *cobC* gene of *Salmonella typhimurium* codes for a novel phosphatase involved in the assembly of the nucleotide loop of cobalamin. *J. Biol. Chem.* 269:26503-26511.

60. Escalante-Semerena JC, Suh SJ, & Roth JR (1990) *cobA* function is required for both de novo cobalamin biosynthesis and assimilation of exogenous corrinoids in *Salmonella typhimurium*. *J. Bacteriol.* 172:273-280.

61. Bauer CB*, et al.* (2001) Three-dimensional structure of ATP:corrinoid adenosyltransferase from *Salmonella typhimurium* in its free state, complexed with MgATP, or complexed with hydroxycobalamin and MgATP. *Biochemistry* 40:361-374.

62. Fonseca MV, Buan NR, Horswill AR, Rayment I, & Escalante-Semerena JC (2002) The ATP:co(I)rrinoid adenosyltransferase (CobA) enzyme of *Salmonella enterica* requires the 2'-OH Group of ATP for function and yields inorganic triphosphate as its reaction byproduct. *J. Biol. Chem.* 277:33127-33131.

63. Moore TC, Newmister SA, Rayment I, & Escalante-Semerena JC (2012) Structural insights into the mechanism of four-coordinate cob(II)alamin formation in the active site of the *Salmonella enterica* ATP:co(I)rrinoid adenosyltransferase (CobA) enzyme: Critical role of residues Phe91 and Trp93. *Biochemistry* 51:9647-9657.

64. Stich TA, Buan NR, Escalante-Semerena JC, & Brunold TC (2005) Spectroscopic and computational studies of the ATP:Corrinoid adenosyltransferase (CobA) from *Salmonella enterica*: Insights into the mechanism of adenosylcobalamin biosynthesis. *J. Am. Chem. Soc.* 127:8710-8719.

65. Hogenkamp HPC, Pailes WH, & Brownson C (1971) Preparation of 5'-deoxyadenosylcobalamin and analogs containing modified nucleosides. *Vitamins and Coenzymes,* Meth. Enzymol., eds McCormick DB & Wright LD (Academic Press, Inc., New York), Vol 18C, pp 57-71.

66. Pailes WH & Hogenkamp HPC (1968) Photolability of Co-alkylcobinamides. *Biochemistry* 7(12):4160-4166.

67. Randaccio L, Geremia S, Demitri N, & Wuerges J (2010) Vitamin B12: unique metalorganic compounds and the most complex vitamins. *Molecules* 15:3228-3259.

68. Woodson JD, Peck RF, Krebs MP, & Escalante-Semerena JC (2003) The *cobY* gene of the archaeon *Halobacterium* sp. strain NRC-1 is required for de novo cobamide synthesis. *J. Bacteriol.* 185:311-316.

69. Gray MJ & Escalante-Semerena JC (2009) In vivo analysis of cobinamide salvaging in *Rhodobacter sphaeroides* strain 2.4.1. *J. Bacteriol.* 191:3842-3851.

70. Woodson JD & Escalante-Semerena JC (2004) CbiZ, an amidohydrolase enzyme required for salvaging the coenzyme $B_{12}$ precursor cobinamide in archaea. *Proc. Natl. Acad. Sci. USA* 101:3591-3596.

71. Yi S*, et al.* (2012) Versatility in corrinoid salvaging and remodeling pathways supports corrinoid-dependent metabolism in *Dehalococcoides mccartyi*. *Appl. Environ. Microbiol.* 78:7745-7752.

72. Roth JR, Lawrence JG, Rubenfield M, Kieffer-Higgins S, & Church GM (1993) Characterization of the cobalamin (vitamin $B_{12}$) biosynthetic genes of *Salmonella typhimurium*. *J. Bacteriol.* 175:3303-3316.

73. Gray MJ & Escalante-Semerena JC (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.

74. Gray MJ, Tavares NK, & Escalante-Semerena JC (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.

75. Jeter RM, Olivera BM, & Roth JR (1984) *Salmonella typhimurium* synthesizes cobalamin (vitamin $B_{12}$) de novo under anaerobic growth conditions. *J. Bacteriol.* 159:206-213.

76. Andersson DI & Roth JR (1989) Mutations affecting regulation of cobinamide biosynthesis in *Salmonella typhimurium*. *J. Bacteriol.* 171:6726-6733.

77. Gonzalez JC, Banerjee RV, Huang S, Sumner JS, & Matthews RG (1992) Comparison of cobalamin-independent and cobalamin-dependent methionine synthases from *Escherichia coli*: two solutions to the same chemical problem. *Biochemistry* 31:6045-6056.

78. Roof DM & Roth JR (1989) Functions required for vitamin B12-dependent ethanolamine utilization in Salmonella typhimurium. *Journal of bacteriology* 171:3316-3323.

79. Bobik TA, Xu Y, Jeter RM, Otto KE, & Roth JR (1997) Propanediol utilization genes (*pdu*) of *Salmonella typhimurium*: three genes for the propanediol dehydratase. *J. Bacteriol.* 179:6633-6639.

80. Brinsmade SR, Paldon T, & Escalante-Semerena JC (2005) Minimal functions and physiological conditions required for growth of *Salmonella enterica* on ethanolamine in the absence of the metabolosome. *J. Bacteriol.* 187:8039-8046.

81. Mori K, Bando R, Hieda N, & Toraya T (2004) Identification of a reactivating factor for adenosylcobalamin-dependent ethanolamine ammonia lyase. *J. Bacteriol.* 186(20):6845-6854.

82. Andersson DI & Roth JR (1989) Mutations affecting regulation of cobinamide biosynthesis in Salmonella typhimurium. *Journal of bacteriology* 171:6726-6733.

# CHAPTER 2

## MUTATIONAL AND FUNCTIONAL ANAYLSIS OF *RS*CBIZ *IN VIVO* AND *IN VITRO*

## INTRODUCTION

$B_{12}$ is a cobalt-containing cyclic tetrapyrrole that belongs to the family of macrocyclic compounds derived from Uroporphyrinogen III (e.g chlorophylls, hemes, coenzyme $F_{430.}$ These molecules are broadly distributed in nature serving a critical role in cell function (1-3). Cobamides are essential to many organisms, including humans. For example, critical processes in nature depend on the availability of cobamides. Notably, cobamides are only synthesized *de novo* by some bacteria and archaea (4-6). These organisms devote a great deal of genetic information to the process. In order to circumvent *de novo* synthesis some archaea and bacteria possess a salvaging pathway or a salvaging and remodeling pathway (7).

In archaea the salvaging pathway involves the uptake of exogenous cobyric acid. Cobyric acid is converted to cobinamide-phosphate (Cbi-P) by the cobinamide phosphate synthase (CbiB), the ring is then activated by the GTP:guanylyransferase, CobY (8). Through additional steps, the final product, cobalamin is reached. Bacteria uptake exogenous precursors using the $B_{12}$ transport system, once in the cytoplasm CobU phosphorylates Cbi to Cbi-P (9). CobU guanylates Cbi-P, forming an activated corrin ring (Cbi-GDP); additional enzymatic steps lead to the final product, Cba (Fig2.1A). Archaea and some bacteria have a remodeling pathway to recycle cobamides (10). Here CbiZ, a cobyric-forming amidohydrolase (11, 12) is capable of remodeling precursors (i.e. incomplete corrinoids) and cobamides. CbiZ, can be found in different cellular compartments, either by itself or fused to other cobamide-related proteins (10)

e.g. BtuD or CobZ (archaeal cobC). CbiZ remodels these corrinoids into adenosylcobyric acid (AdoCby), which enter the biosynthetic pathway. As shown in Figure 2.1B, CbiB converts AdoCby and aminopropanol-phosphate (AP-P) into adenosylcobinamide-phosphate (AdoCbi-P), leading to AdoCba.

Previous studies have examined substrate specificity for CbiZ homologs. Initial substrate characterization of CbiZ was done using *Rhodobacter sphaeroides* CbiZ. *R. sphaeroides* is a bacterium capable of scavenging cobamides and incomplete corrinoids. Previous studies show *Rs*CbiZ prefers AdoPseudoCbl to other corrinoids (10, 11, 13). CbiZ cleaves AdoPseudoCbl to AdoCby allowing it to re-enter the pathway to synthesis AdoCbl. Knowledge of this mechanism remains unknown. Structure and function studies of *Rs*CbiZ are necessary to determine residues involved in catalysis and substrate binding, as well as provide insight into the putative localization of *Rs*CbiZ.

## MATERIALS AND METHODS

### *Bacterial Strains*

All strains used in this work carry a chromosomal null allele *metE* and *ara-9*, an allele that prevents arabinose utilization. JE8268 (*metE ara-9 ΔcobU ΔycfN*) has a deletion of *cobU*, inactivating the salvaging pathway (14-16). Strains used in this work are found in Table 2.1. Using a modified chromosomal gene inactivation system, *cobY*$^{+}$ of *Pyrococcus furiosus* was PCR amplified from pPfCOBY2 (unpublished) with primers *del araA_5'* (5'gaagtatggtttgtgattggcagccagcatttgctgtaggctggagctgcttc) and *3.4 del_ araA* (5'ttaacgtttgaacccgtaatacacctcgttccaggacatgggaattagccatg). The resulting product was extracted and gel purified using Wizard SV Gel and PCR Clean-Up kit (Promega). The remainder of the steps were done as described elsewhere (17), yielding a derivative of *S.* Typhimurium strain

JE8268. JE20530 (Table 2.1) possesses the archaeal gene *cobY* resulting in a chromosomal archaeal salvaging pathway in *S.* Typhimurium (Fig. 2.1B).

*Plasmid Construction*

All plasmids used in this work are listed in Table 2.2. All primers were ordered from Integrated DNA Technologies Inc. (IDT, Coralville, IA). DNA sequencing (Georgia Genomics Facility UGA) was used to verify all plasmids used in this study. *RscbiZ*$^{+}$ was amplified from pRsCbiZ1 and cloned into overexpression vector pTEV6 (18). Mutagenic primers were designed using PrimerX (http://www.bioinformatics.org/primerx/cgi-bin/DNA_1.cgi) (Table 2.3). DNA was mutated using PfuUltra II Fusion DNA polymerase (Stratagene) and mutagenic primers to introduced changes to *RscbiZ*$^{+}$ (pRsCBIZ1) encoding amino acid change of conserved residues to alanine. PCR products were treated with restriction enzyme DpnI (Fermentas) and transformed into chemically competent DH5α (19). Plasmids were prepared and purified using E.Z.N.A Plasmid Mini Kit I (Omega bio-tek). The resulting plasmids were transformed (20, 21) into JE20530 (*metE ara-9 cobU ycfN araA::P.f.cobY*) and tested for amidohydrolase activity *in vivo* under conditions that required functional *Rs*CbiZ activity.

*Growth Conditions*

Overnights of strains were grown in nutrient broth (NB Difco) containing 50 μg/mL ampicillin. A 96-well plate containing 200 μL of No Carbon Essential (NCE) medium (22), supplemented with trace minerals, glycerol (22 mM), $MgSO_4$ (1 mM), 5,6-dimethylbenzimidazole (DMB) (150 μM), ampicillin (50 μg/mL) corrinoids (1 nM of $CN_2Cbi$, $CN_2Cby$ or CNCbl) and L-arabinose (Sigma Aldrich) (0.5 mM when indicated) was inoculated with 1% (v/v) of the overnight. Strains were grown at 37°C for 24-48 hours with shaking and monitored using Gen5 software (BioTek) in an EL808 Ultra or PowerWave XS Microplate

Reader (BioTek instruments). Growth curve data represents a minimum of two independent experiments. Data were analyzed with Prism v6 (GraphPad) analytical software. Error bars represent the standard deviation.

*Purification of RsCbiZ proteins*

Plasmids encoding *cbiZ*$^{+}$ alleles e.g. (pRsCbiZ3) were transformed into *E. coli* C41 (DE3λ) (23). Overnight cultures (20 mL) of the strains were sub-cultured into 1 liter of Terrific Broth containing ampicillin (100 μg /mL). Cells were grown with shaking, 150 rpm, at 37°C for 2 hours. Isopropyl β-D-1-thiogalactopyranoside (IPTG, GoldBio) was added to a final concentration of 0.1 mM, after the addition of IPTG temperature was shifted to 15°C and cells were incubated for 16 hours. Cells were harvested by centrifugation (6,000 x g for 15 minutes). Cells were re-suspended in a 10% (w/v) of (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES (10 mM)), NaCl (200 mM), (3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate) (CHAPS, GoldBio,(12 mM)) at pH 7.6. Cells were lysed at 187,000 kPa using a 1.1 kW TS Series Bench Top cell disruptor (Pressure Biosciences), equipped with a cooling jacket on the disruptor heat to maintain a 15°C temperature using a Neslab ThermoFlex 900 recirculating chiller (ThermoScientific). For every 20 grams of cell paste a crushed 10X Sigma Protease Inhibitor Tablet was added. Lysed cells were centrifuge (40,000 x g for 30 minutes). Supernatant was collected and passed through a 0.45 μm filter and loaded onto AKTA Purifier (GE Healthcare) FPLC that with two connecting 5-mL NiNTA columns. The column was washed with bind buffer containing HEPES (10 mM), NaCl (200 mM), CHAPS (12 mM), imidazole (20 mM) at pH 7.6. The protein was eluted with a 4% to 100% gradient of elution buffer HEPES (10 mM), NaCl (200 mM), CHAPS (12 mM), imidazole (500 mM) at pH 7.6. Fractions were collected and protein was dialyzed into HEPES (10 mM), NaCl (200 mM),

CHAPS (6 mM), pH 7.6 with 5% (w/v) glycerol overnight. A 15-mL bed of amylose resin column was poured and used on the FPLC for a secondary purification. Bound protein was washed with HEPES (10 mM), NaCl (200 mM), CHAPS (6 mM), *tris*(2-carboxyethyl)phosphine (TCEP) (1 mM) , pH 7.6 and 5% (w/v) glycerol, protein was eluted using a gradient 0 to 100% elution buffer that was HEPES (10 mM), NaCl (200 mM), CHAPS (6 mM), maltose (10 mM), TCEP (1 mM) ,pH 7.6 and 5% (w/v) glycerol. Fractions containing protein of interest was pooled. CHAPS was slowly dialyzed away until the final buffer was HEPES (10 mM), NaCl (200 mM), 5% (w/v) glycerol. Protein purity was assessed using Fotodyne imaging system and Foto/Analyst v. 5.00 software (Fotodyne Inc.) TotalLab v.2005 software was used for analysis (Nonlinear Dynamics) Protein purity, after amylose purification was approximately 80% pure. Protein was concentrated using Amicon Ultra-15 10k molecular weight cutoff (MWC). Protein concentrations were assessed using NanoDrop 1000 spectrophotometer (Thermo Scientific), using molecular weights and extinction coefficients predicted by ExPASy Protpram database. After concentrating the protein to 10 mg/mL, protein was passed through a 0.45 μm filter and loaded onto the FPLC with a sizing column (Superdex200 or Superose 12). Fractions that contained protein, as determined by the NanoDrop 1000, were confirmed via SDS-PAGE (24), fractions that had a single band corresponding to protein of interest. After size exclusion chromatography, the purity was assessed at >90% homogeneity. These fractions were pooled and dialyzed into HEPES (10 mM), NaCl (200 mM), 5% (w/v) glycerol and frozen drop-wise into liquid $N_2$ and stored at -80°C until further use. MBP-*Bh*CbiZ was purified in a similar manner for crystallography studies, *in vitro* activity was assayed as described below.

*Analytical gel filtration*

Per run, a 250 μL sample volume at 6 mg/mL of MBP-*Rs*CbiZ$^{+}$ protein was injected onto a Superdex 200 HR 10/30 gel filtration column (GE Healthcare) attached to an AKTA purifier fast protein liquid chromatography (FPLC) system that was equilibrated with buffer (hydroxymethly)aminomethane (tris (20mM, pH 7.5) and NaCl (200 mM)). A calibration standard containing a mixture of molecular masses ranging from 1.35 to 670 kDa (Bio-Rad) was used to generate a standard curve to determine the molecular mass. The standard mixture contained vitamin $B_{12}$ (1.35 kDa), equine myoglobin (17 kDa), chicken ovalbumin (44 kDa), bovine gamma globulin (158 kDa), and thyroglobulin (670 kDa). A flow rate of 0.5 mL min$^{-1}$ was used to develop the column. Elution peak analysis was performed using UNICORN v.4.11 software (GE Healthcare Life Sciences). Data were graphed and analyzed using Prism v6 (GraphPad) analytical software.

*Amidohydrolase Activity Bioassay*

Amidohydrolase activity assays were performed as described (12, 13, 25) with slight modifications (10). Briefly, assays were performed in 200 μL volumes containing 2 μg of recombinant protein, CHES (50 mM, pH 10 at 50°C), dithiothreitol (5 mM), and corrinoid (30 μM). Reactions were incubated at 50°C for 15 minutes. Reactions were stopped by incubation at 95°C for 10 minutes then kept on ice. One microliter of the reaction was spotted onto an agar overlay containing JE20530 and incubated overnight at 37°C. KCN was added to a final concentration of 0.1 M to the remaining reaction, corrinoids were derived under strong light (incandescent) for 15 minutes. Samples were filtered using 0.45 μm Spin-X columns (Corning) before they were resolved by HPLC.

RESULTS

The combined results below need biochemical and structural analysis to drive the understanding *Rs*CbiZ. *In vivo* data suggests residues that might contribute to substrate binding and catalytic activity. Bioinformatics were employed to determine candidate residues for alanine scan mutagenesis (Fig. 2.2). *Rs*CbiZ was used as the reference sequence. Residues changed were the following: G63A, T74A, T98A, G100A, G118A, T119A, I120A, A142G, E144A, A145G, G183A, V196A. If a residue was already an alanine, primers were designed to encode a change to a glycine instead.

*Conserved residues contribute to amidohydrolase activity in vivo*

The results of these growth experiments are shown in Table 2.4 and Figure 2.3. Several variants from the alanine scan resulted in growth similar or better then *Rs*CbiZ$^{+}$ (Table 2.4). Residues T74A, G183A, and I120A G183A are important for amidohydrolase activity. Low levels of Cbi result in no growth. In the presence of L-arabinose and 16 nM Cbi poor growth is seen. High levels of Cbi with the presence of arabinose indicate that the variants may play a role to substrate binding. The need for arabinose induction alludes to the importance of the amount of protein presence to allow growth under these conditions. The mutation encoding I120A and G183A result in a no growth phenotype, comparable to empty vector growth, under any condition tested (Fig. 2.1, Table 2.4). Notably, I120A variant has growth comparable to *Rs*CbiZ$^{+}$. Both I120A and G183A contribute to amidohydrolase activity *in vivo*.

*Truncated RsCbiZ is inactive in vivo*

Results from growth analysis of C-terminal truncations of *Rs*CbiZ do not allow growth in a strain requiring production of Cby. The strain carrying pRsCbiZ$^{+}$ (wild-type) was used for comparison to strains containing variants (Fig. 2.4). The strain carrying an empty vector (VOC)

had a growth defect, because of the lack of cobinamide amidohydrolase activity. The plasmid encoding C-terminal truncation, *Rs*cbiZ$^{\Delta 185\text{-}219}$, had a growth defect, even in the presence of L-arabinose (data not shown). This indicates that the growth defect may be a result of mis-localization of enzyme. *Rs*CbiZ is predicted to have a putative transmembrane domain anchoring it to the inner membrane (26).

*CbiZ is active in vitro*

The results of the *in vitro* assay indicate the truncated protein has amidohydrolase activity. $His_6$-MBP-*Rs*CbiZ$^{\Delta 185\text{-}219}$ was overexpressed, purified and assayed to determine *in vitro* activity and compared to purified $His_6$-MBP-*Rs*CbiZ$^{+}$. This result needs to be repeated; peak 2 (Fig. 2.2), which corresponds with $CN_2$Cby, needs to be verified using mass spectrometry. MBP-*Bh*CbiZ is active in vitro when fed $CN_2$Cbi (data not shown).

*RsCbiZ is a dimer or trimer*

Using a three-step purification, $His_6$-MBP-*Rs*CbiZ was purified to homogeneity (Fig. 2.6B). The oligomeric state of *Rs*CbiZ was partially determined using gel filtration chromatography (Fig. 2.7). Membrane prediction software indicates a putative C-terminal single transmembrane helix (TMHMM (27), TMPRED(28)), it was predicted $His_6$-MBP-*Rs*CbiZ would be a monomer (~69 kDa). To test this hypothesis $His_6$-MBP-*Rs*CbiZ was gel filtrated to determine the oligomeric state. Data from two different gel filtration columns indicate that $His_6$-MBP-*Rs*CbiZ is a dimer (*Rs*CbiZ + $His_6$-MBP-*Rs*CbiZ ~93 kDa) or trimer $His_6$-MBP-*Rs*CbiZ +*Rs*CbiZ + *Rs*CbiZ ~118 kDa). The results of this experiment are presented in Figure 2.7 and Figure 2.8A; showing retention times of $His_6$-MBP-*Rs*CbiZ and Bio-Rad protein standards and the elution profile, respectively. Based off the protein standards, the predicted size of a monomer

of *Rs*CbiZ is approximately 25 kDa and dimer/trimer 104 kDa. Other gel filtration techniques were employed and yielded similar results.

## DISCUSSION AND CONCLUSIONS

### *A combination of two residues result in an inactive variant in vivo*

Several variants of *Rs*CbiZ were analyzed using strains encoding mutations in trans. No single residue changes yielded a growth defect when expressed in strains requiring amidohydrolase activity. It is interesting that strains housing plasmids expressing threonine to alanine changes (e.g. T74A, T98A) resulted in a growth defect (Table 2.4). These threonine residues could stabilize the substrate, when one is changed the others compensate for it. Mutants containing single variants I120A and G183A result in doubling times of 3.4 hr and 22.9 hr respectively in the presence of L-arabinose and low Cbi conditions. I120A has a doubling time (dt) greater than wild-type *RscbiZ.* Under high Cbi and L-arabinose, the dt of G183A decrease to 20.2 hr. The mutant strain containing the I120A G183A double variant results in no growth (Fig. 2.3). It is possible that the strain containing double variant inhibits binding of substrate. The mutant containing G183A requires high levels of Cbi and enzyme to elicit a growth response, this could indicate residue G183 contribute to binding of substrate, and the change to G183A disrupts binding. It is interesting that the strain containing variant I120A has a better growth phenotype than wild-type, but in combination with G183A results in no growth. The reasoning behind this could be that I120 and G183 interact or are in close enough proximity that when changed to alanine, there is a hydrophobic pocket made hindering binding of corrinoids.

### *RsCbiZ is likely an oligomer*

$His_6$-MBP-*Rs*CbiZ was affinity purified using NiNTA and Amylose resin (two-steps). The resulting protein was run over a gel filtration column to determine the oligomeric state.

Results from Figure 2.7 indicate that the protein is a dimer or trimer. Figure 2.8 shows the elution profile of purified $His_6$-MBP-*Rs*CbiZ, based off this, it is likely this protein is an oligomer as indicated by the larger peak. Over 80% of $His_6$-MBP-*Rs*CbiZ remains in that fraction. The smaller peak is monomeric *Rs*CbiZ that has likely lost the MBP tag because of degradation. Activity of the peaks was assessed (Fig. 2.8B); both result in product that allowed the growth of JE20530. A second gel filtration column was used to clarify oligomerization of $His_6$-MBP-*Rs*CbiZ, the results were inconclusive (data not shown), but had a similar elution profile to Figure 2.8A, indicating $His_6$-MBP-*Rs*CbiZ to be a dimer or trimer (~104 kDa). While these results were inconclusive, it is likely that $His_6$-MBP-*Rs*CbiZ is a dimer because it is known that several CbiZ homologs (e.g. *Bh*BtuD (CbiZ-BtuD fusion)) are fused to $B_{12}$ synthesis and transport proteins. It is known that BtuD is a dimer, given this it is likely *Rs*CbiZ is a dimer. Analytical ultracentrifugation may elucidate the true oligomeric state of $His_6$-MBP-*Rs*CbiZ (29).

*Precedence of CbiZ localization*

Comparative genomic results from (10) describes CbiZ homologs that are typically fused to $B_{12}$ related proteins (e.g. CobC, BtuD). Of these proteins, several are anchored and localized to the inner membrane. It makes sense for *Rs*CbiZ to be anchored to the membrane given that reaction product is a substrate for CbiB (multi-pass transmembrane protein localized to inner membrane). The enzyme reactions need to be repeated to verify product formed is Cby. If the product is Cby, this C-terminal truncation may serve as a candidate for crystallization studies.

In order to verify these studies, biochemical and structural analysis are required. Having a crystal structural of *Rs*CbiZ is ideal, however due to low protein yields, *Bh*CbiZ has been chosen as a candidate enzyme (several candidates were tested, but yields were too low- data not shown). It is possible overexpression of *Rs*CbiZ$^{\Delta 185\text{-}219}$ could result in higher protein yields if

the putative C-terminal single pass transmembrane domain is what causes the low production. Investigations into the *in vitro* activity of variants are required to confirm mutant phenotypes (Fig. 2.4).

Understanding the mechanism of CbiZ is important. Where the corrinoid binds, and how it binds has implications that could be used to construct CbiZ protein with specific substrate targets. What residues contribute to binding of AdoPseudoCbl in *Rs*CbiZ? Notably, some organisms capable of $B_{12}$-dependent dehalogenation (30) have *cbiZ* such as *Dehalococcoides mccartyi*. *D. mccartyi* has seven putative *cbiZ* genes, three have been confirmed to have amidohydrolase activity *in vivo* (data not shown). It begs the question, why does this organism have so many putative *cbiZ* genes? The different dehalogenases may require different cobamides. The data from this chapter serves as a gateway into understanding what residues to target for binding and structural studies. A continuation of this work is addressed in Chapter 4.

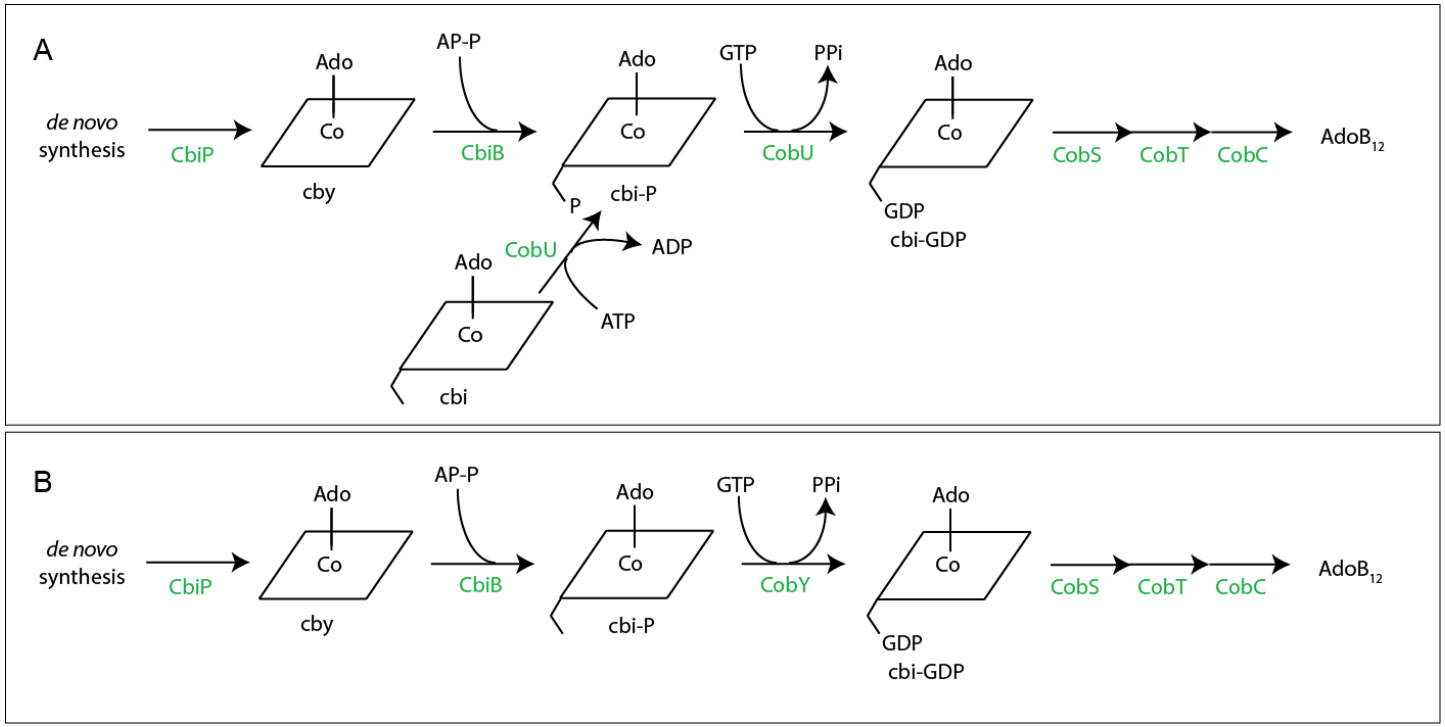


**Figure 2.1**: Comparison of modified bacterial and archaeal salvaging pathways in a *S.* Typhimurium. Trapezoid represents a completed corrin ring. The stick protruding from the Co center is an organometallic covalent bond to 5'- deoxyadenosine (Ado). Green text indicates enzymes. A. Bacterial salvaging in *S.* Typhimurium. B. A modified *S.*Typhimurium strain capable of archaeal salvaging (JE20530)

**Table 2.1**: Strains list of *S. enterica* sv. Typhimurium LT2 derivatives. Strains were constructed during the course of this work unless stated otherwise.

| Strain | Genotype | Reference or Source |
|---|---|---|
| ***Salmonella enterica* LT2** | | |
| **Derivatives of JE6583** | | |
| JE8268 | *cob1315 ycfN112* | (16) |
| JE20530 | *cobU1315 ycfN112 araA1232:: P.f. cobY+* | |
| **Derivatives of JE20530** | | |
| JE20672 | JE20530/pRsCBIZ1 *bla*$^+$ | |
| JE20674 | JE20530/pRsCBIZ11 *bla*$^+$ | |
| JE20675 | JE20530/pRsCBIZ12 *bla*$^+$ | |
| JE20676 | JE20530/pRsCBIZ13 *bla*$^+$ | |
| JE20677 | JE20530/pRsCBIZ14 *bla*$^+$ | |
| JE20678 | JE20530/pRsCBIZ15 *bla*$^+$ | |
| JE20679 | JE20530/pRsCBIZ16 *bla*$^+$ | |
| JE20680 | JE20530/pRsCBIZ17 *bla*$^+$ | |
| JE20681 | JE20530/pRsCBIZ18 *bla*$^+$ | |
| JE20682 | JE20530/pRsCBIZ19 *bla*$^+$ | |
| JE20683 | JE20530/pRsCBIZ20 *bla*$^+$ | |
| JE20684 | JE20530/pRsCBIZ21 *bla*$^+$ | |
| JE20685 | JE20530/pRsCBIZ22 *bla*$^+$ | |
| JE22298 | JE20530/pRsCBIZ43 *bla*$^+$ | |
| | JE20530/pBAD24 *bla*$^+$ | |
| ***Escherichia coli* strains** | | |
| C41 (λDE3) | $F^-$ ompT gal dcm $hsdS_B(r_B^- m_B^-)$ (DE3) | (23) |
| DH5α | *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* | (19) |

**Table 2.2**: Plasmids used in these studies. Plasmids were constructed during the course of this work unless stated otherwise.

| Plasmid | Relevant Genotype | Reference Source |
|---|---|---|
| pRsCBIZ1 | *cbi3 (cbiZ*$^{+}$ cloned from *Rhodobacter sphaeroides)* | (13) |
| pRsCBIZ11 | *cbi4* (encodes CbiZ$^{T74A}$) *bla*$^{+}$ | |
| pRSCBIZ12 | *cbiZ5* (encodes CbiZ$^{T98A}$) *bla*$^{+}$ | |
| pRsCBIZ13 | *cbiZ6* (encodes CbiZ$^{G100A}$) *bla*$^{+}$ | |
| pRsCBIZ14 | *cbiZ57* (encodes CbiZ$^{G118A}$) *bla*$^{+}$ | |
| pRsCBIZ15 | *cbiZ7* (encodes CbiZ$^{T119A}$) *bla*$^{+}$ | |
| pRsCBIZ16 | *cbiZ8* (encodes CbiZ$^{I120A}$) *bla*$^{+}$ | |
| pRsCBIZ17 | *cbiZ9* (encodes CbiZ$^{A142G}$) *bla*$^{+}$ | |
| pRsCBIZ18 | *cbiZ10* (encodes CbiZ$^{E144A}$) *bla*$^{+}$ | |
| pRsCBIZ19 | *cbiZ11* (encodes CbiZ$^{A145G}$) *bla*$^{+}$ | |
| pRsCBIZ21 | *cbiZ12* (encodes CbiZ$^{G189A}$) *bla*$^{+}$ | |
| pRsCBIZ22 | *cbiZ13* (encodes CbiZ$^{V196A}$*) bla*$^{+}$ | |
| pRsCBIZ20 | *cbiZ14* (encodes CbiZ$^{I120A\ G183A}$) *bla*$^{+}$ | |
| pRsCBIZ43 | *cbiZ27* (encodes CbiZ$^{G183A}$) *bla*$^{+}$ | |
| pRsCBIZ30 | *cbiZ20* (encodes truncation CbiZ$^{D185\text{-}219}$) *bla*$^{+}$ | |
| pRsCBIZ3 | *cbiZ3* cloned into pTEV6 *bla*$^{+}$ | (13) |
| pRsCBIZ26 | *cbiZ20* cloned into pTEV17 *bla*$^{+}$ | |
| pBhCBIZ1 | *Bacillus halodurans* cbiZ encoding domain cloned into pMBPparallel1 *bla*$^{+}$ | |
| pBAD24 | Cloning/complementation vector *bla*$^{+}$ | (31) |
| pTEV6 | Cloning/overexpression vector, N-terminal rTEV cleavable His6 MBP tag *bla*$^{+}$ | (18) |
| pTEV17 | Cloning/overexpression vector, N-terminal rTEV cleavable His6 MBP tag *bla*$^{+}$ | (32) |
| pMBPparallel1 | Cloning/overexpression vector, N-terminal rTEV cleavable MBP tag *bla*$^{+}$ | (33) |

**Table 2.3**: Mutagenic primers used to introduce changes to RscbiZ$^{+}$ (pRsCbiZ1)

| Plasmid | Residue Changed | Forward Primer Sequence | Reverse Primer Sequence |
|---|---|---|---|
| pRsCBIZ11 | T74A | GTGGGGATGCTCGCGTCGCGCACGCTC | GAGCGTGCGCGACGCGAGCATCCCCAC |
| pRSCBIZ12 | T98A | CCTGTCTGGCGGCGGTCGGGCTCGG | CCGAGCCCGACCGCCGCCAGACAGG |
| pRsCBIZ13 | G100A | CTGGCGACCGTCGCACTCGGCAATGC | GCATTGCCGAGTGCGACGGTCGCCAG |
| pRsCBIZ14 | G118A | GCGGGCGCTCGCAACGATCAACCTCC | GGAGGTTGATCGTTGCGAGCGCCCGC |
| pRsCBIZ15 | T119A | CTCGGCGCGATCAACCTCCTTGTGG | CCACAAGGAGGTTGATCGCGCCGAG |
| pRsCBIZ16 | I120A | CGGCACGGCCAACCTCCTTGTGGCG | CGCCACAAGGAGGTTGGCCGTGCCG |
| pRsCBIZ17 | A142G | CGATTGGAGTCGAGGCGCGGACGGC | GCCGTCCGCGCCTCGACTCCAATCG |
| pRsCBIZ18 | E144A | CGTCGCAGCGCGGACGGCTTCGGTG | CACCGAAGCCGTCCGCGCTGCGACG |
| pRsCBIZ19 | A145G | CGAGGGACGGACGGCTTCGGTGCTG | CAGCACCGAAGCCGTCCGTCCCTCG |
| pRsCBIZ21 | G189A | CACGGCGGCAGCTGAGGCGATCGGC | GCCGATCGCCTCAGCTGCCGCCGTG |
| pRsCBIZ22 | V196A | GCTGCCGCATGCGCAGCGGTGGGTC | GACCCACCGCTGCGCATGCGGCAGC |
| pRsCBIZ43 | G183A | CCGCTACGCGGCACTCCACACGGCG | CGCCGTGTGGAGTGCCGCGTAGCGG |

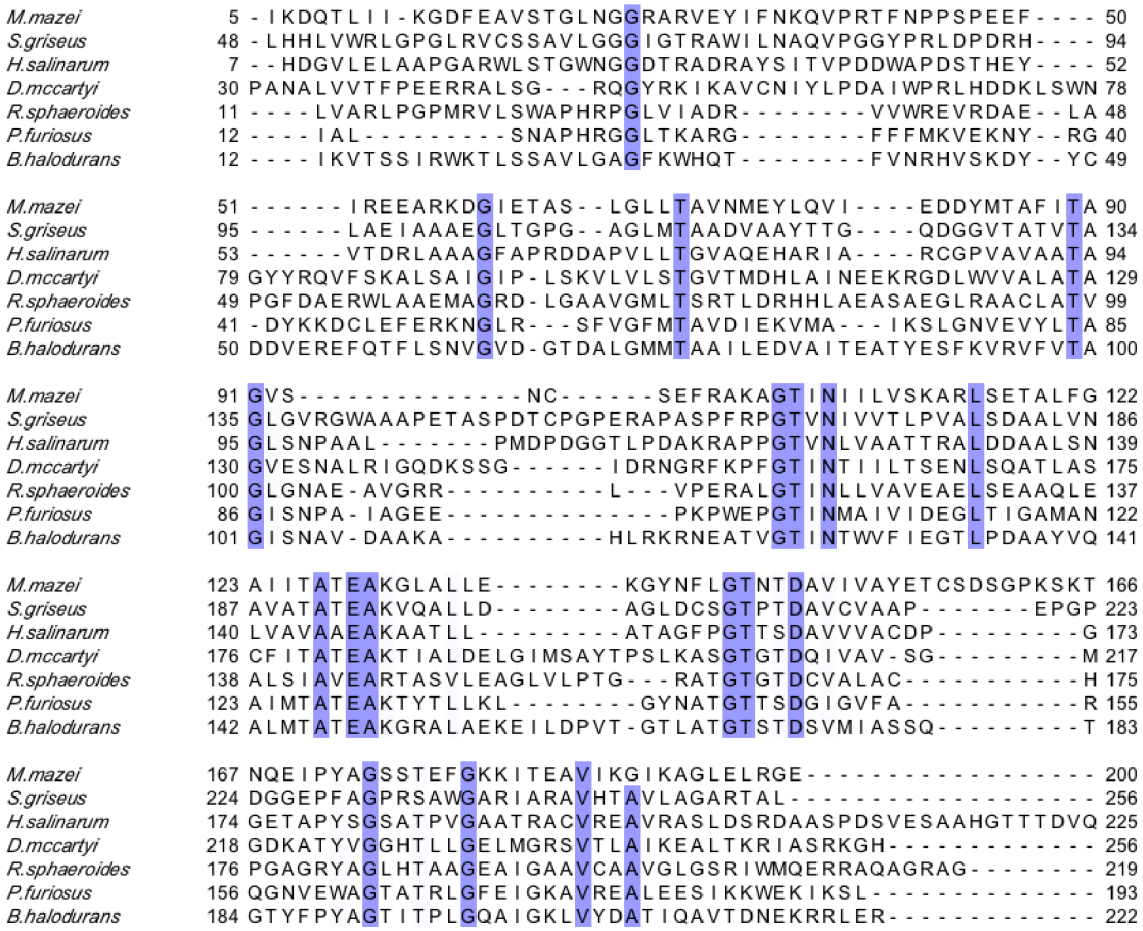


**Figure 2.2**: Alignment of archaeal and bacterial CbiZ homologs. Shaded regions indicate conserved residues and potential candidates for alanine scan mutagenesis.

**Table 2.4**: Doubling time (hours) of the indicator strain JE20530 carrying alleles of *cbiZ* encoding the indicated variant in minimal medium containing the indicated concentration of $CN_2Cbi$.

| | 1 nM | | | 2 nM | |
|---|---|---|---|---|---|
| **Arabinose[1]** | **-** | **+** | | **-** | **+** |
| **Variant** | | | **Variant** | | |
| WT | 3.2 | 6.6 | WT | 3.4 | 2.4 |
| T74A | NG | 18.6 | T74A | NG | 13.9 |
| T98A | 19.7 | 5.9 | G118A | 3.4 | 2.02 |
| G100A | 4.9 | 5 | T119A | 21.5 | 14 |
| I120A | 3.9 | 2.4 | A142G | 1.9 | 1.9 |
| I120A G183A | NG | NG | E144A | 3.1 | 2.05 |
| G183A | NG | NG | A145G | 3.0 | 1.98 |
| | | | I120A G183A | NG | NG |
| | | | G183A | NG | 22.9 |
| | | | G189A | 2.98 | 1.8 |
| | | | V196A | 3.6 | 2.03 |

[1]Cells grown with (+) or without (-) L-arabinose

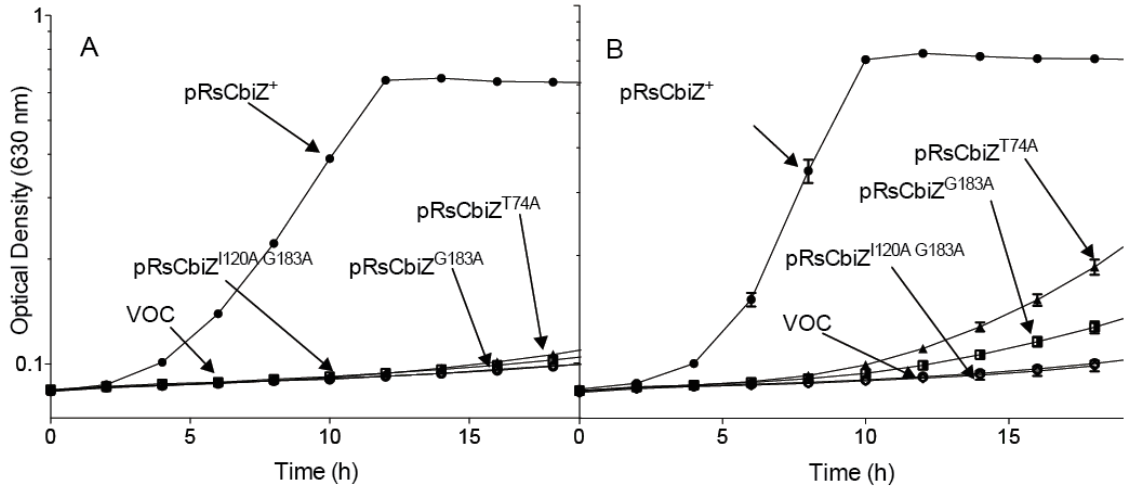


**Figure 2.3:** Growth results of *Rs*CbiZ variants. VOC, p*Rs*cbiZ$^+$, and mutants were transformed into the indicator strain (*metE205 ara-9 cobU1315 ycfN112 araA1232::P.f. cobY*$^+$). A. Resulting strains were grown in NCE supplemented with trace minerals, glycerol (22 mM), DMB (150 μM), ampicillin (50 μg/mL) and $CN_2Cbi$ (16 nM). Doubling time in hours: WT (3) T74A, I120A G183, and G183A (no growth, NG) B. Strains in presence of L-arabinose WT (1.7), T74A (9.9), I120A G183 (NG), and G183A (20.2).

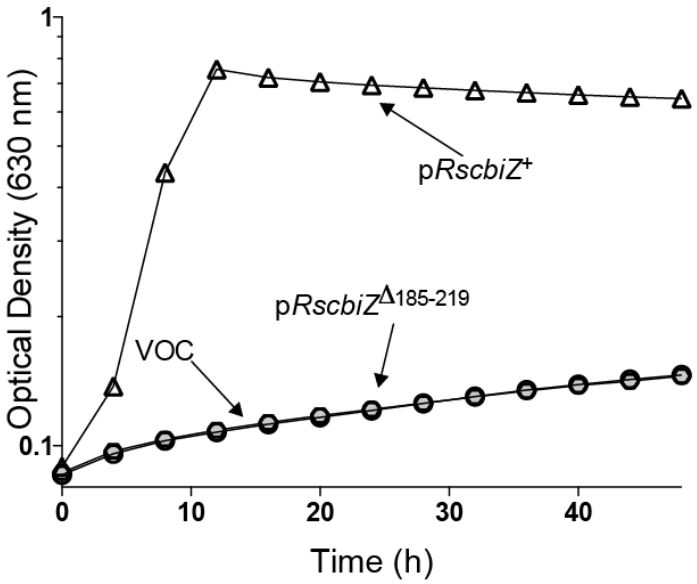


**Figure 2.4**: Growth results of a C-terminal truncation of *Rs*CbiZ. VOC, pRscbiZ$^{+}$, and p*Rs*cbiZ$^{\Delta 185\text{-}219}$ were transformed into the indicator strain (*metE205 ara-9 cobUycfN araA::P.f. cobY*$^{+}$). Resulting strains were grown in conditions requiring amidohydrolase function. Doubling time in hours: WT (2.3), Δ185-219 (NG), VOC (NG)

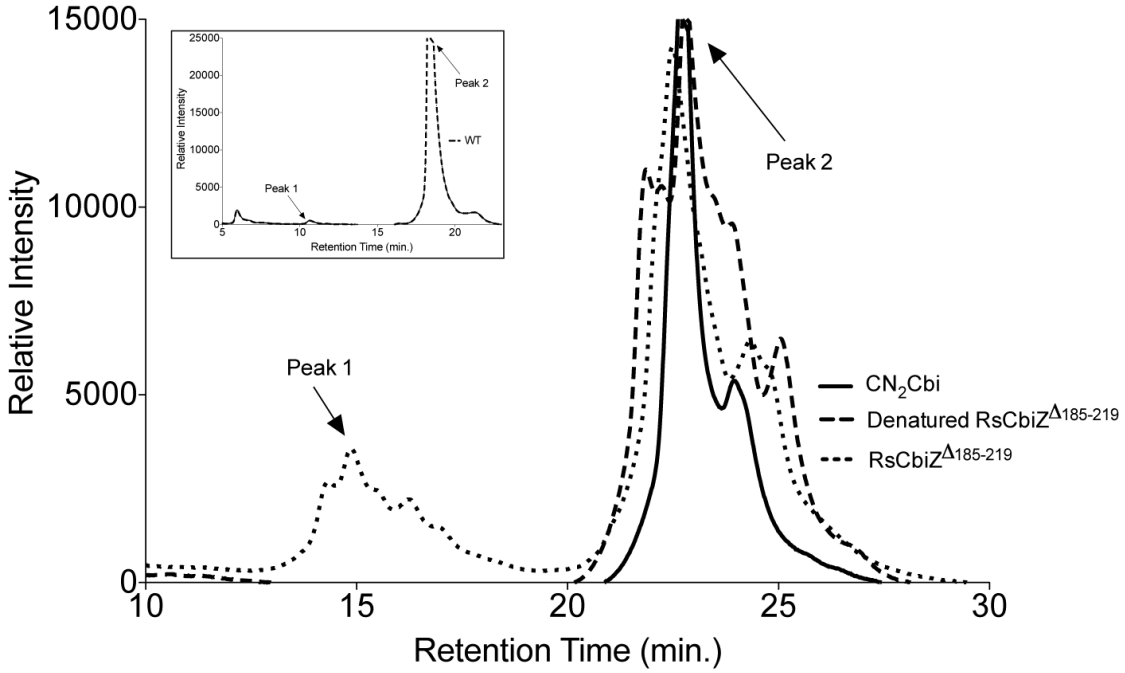


**Figure 2.5**: *In vitro* activity of *Rs*CbiZ $^{\Delta 185\text{-}219}$. Activity assays were set up as described in material and methods. Inset panel represents a previous reaction set up with *Rs*CbiZ$^{+}$. Corresponding peaks are labeled.

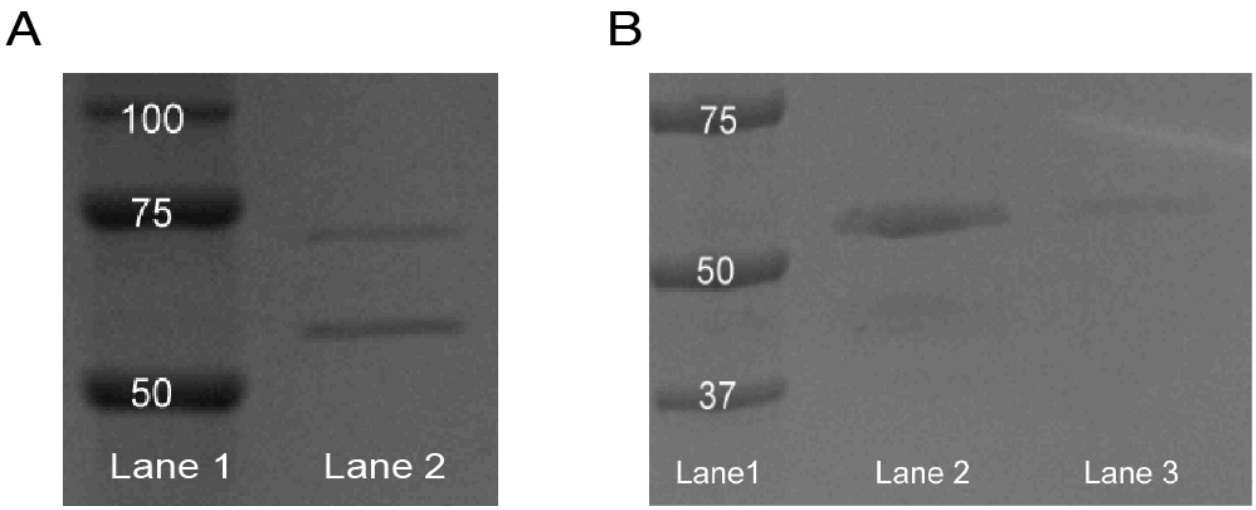


**Figure 2.6**: Multistep-Purification of CbiZ Homologs. A. Two-step purification of MBP-*Bh*CbiZ. Lane 1 represents BioRad Ladder standards (kDa). Lane 2 is gel filtration of amylose purified MBP-BhCbiZ. B. Three-step purification $His_6$-MBP-*Rs*CbiZ to homogeneity Lane 1 represents BioRad Ladder standards (kDa). Lane 2 after two-step purification, Lane 3 after third-step purification (gel filtration).

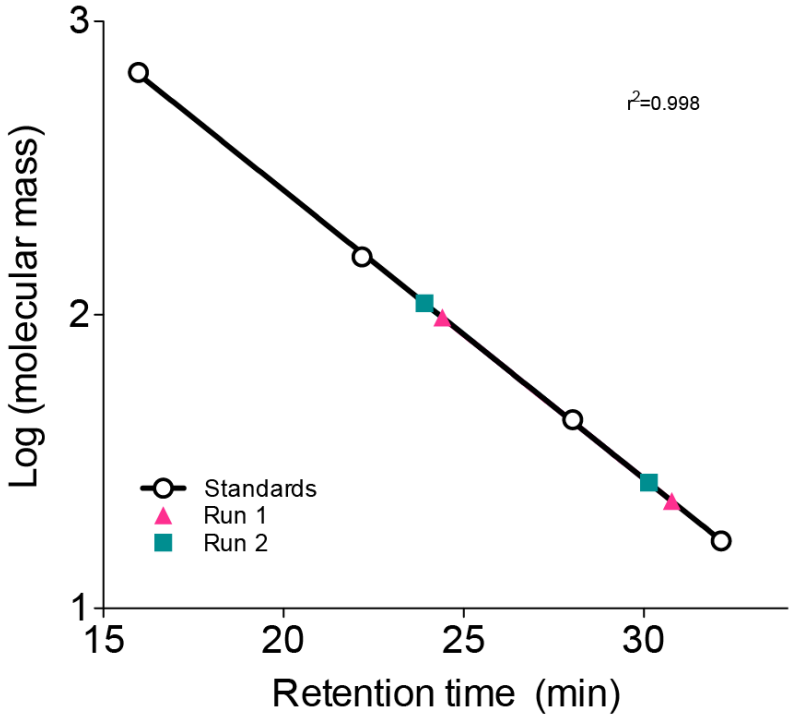


**Figure 2.7**: Oligomerization of $His_6$-MBP-*Rs*CbiZ. Oligomeric state of $His_6$-MBP-*Rs*CbiZ was determined using gel filtration chromatography.

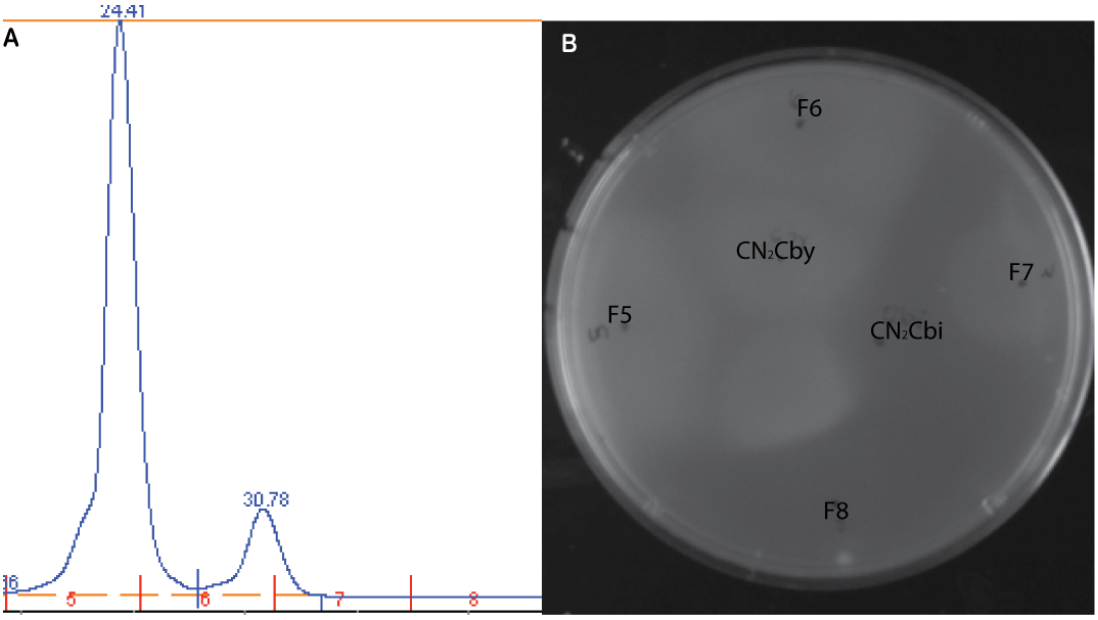


**Figure 2.8**: Purification and bioassay of *Rs*CbiZ. A. Chromatogram showing elution profile of $His_6$-MBP-*Rs*CbiZ run on a Superdex 75 column B. overlay of JE20530 (*metE205 ara-9 cobUycfN araA::P.f. cobY*$^+$) with reactions containing protein from fractions from panel A. $CN_2Cby$ is spotted as a positive control. $CN_2Cbi$ is spotted as a negative control

REFERENCES


1. Battersby AR, McDonald E, Cornforth JW, & Frydman B (1976) Biosynthesis of Porphyrins and Corrins [and Discussion]. *Phil. Transac. Royal Soc. London. Series B, Biol. Sci.* 273(924):161-180.
2. Friedmann HC & Thauer RK eds (1992) *Macrocyclic tetrapyrrole biosynthesis in bacteria* (Academic Press, Inc., New York), Vol 3, pp 1-19.
3. Smith A & Warren M (2009) *Tetrapyrroles : birth, life and death* (Springer, New York, NY).
4. Moore SJ*, et al.* (2013) Elucidation of the anaerobic pathway for the corrin component of cobalamin (vitamin B12). *Proc. Natl. Acad. Sci. U S A* 110:14906-14911.
5. Moore SJ & Warren MJ (2012) The anaerobic biosynthesis of vitamin B12. *Biochem. Soc. Trans.* 40:581-586.
6. Roth JR, Lawrence JG, Rubenfield M, Kieffer-Higgins S, & Church GM (1993) Characterization of the cobalamin (vitamin $B_{12}$) biosynthetic genes of *Salmonella typhimurium*. *J. Bacteriol.* 175:3303-3316.
7. Escalante-Semerena JC (2007) Conversion of cobinamide into adenosylcobamide in bacteria and archaea. *J. Bacteriol.* 189:4555-4560.
8. Newmister SA, Otte MM, Escalante-Semerena JC, & Rayment I (2011) Structure and mutational analysis of the archaeal GTP:AdoCbi-P guanylyltransferase (CobY) from *Methanocaldococcus jannaschii*: Insights into GTP binding and dimerization. *Biochemistry* 50:5301-5313.
9. Thompson TB, Thomas MG, Escalante-Semerena JC, & Rayment I (1998) Three-dimensional structure of adenosylcobinamide kinase/adenosylcobinamide phosphate

guanylyltransferase from Salmonella typhimurium determined to 2.3 A resolution. *Biochemistry* 37(21):7686-7695.

10. Gray MJ, Tavares NK, & Escalante-Semerena JC (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.

11. Gray MJ & Escalante-Semerena JC (2009) In vivo analysis of cobinamide salvaging in *Rhodobacter sphaeroides* strain 2.4.1. *J. Bacteriol.* 191:3842-3851.

12. Woodson JD & Escalante-Semerena JC (2006) The *cbiS* gene of the archaeon *Methanopyrus kandleri* AV19 encodes a bifunctional enzyme with adenosylcobinamide amidohydrolase and alpha-ribazole-phosphate phosphatase activities. *J. Bacteriol.* 188:4227-4235.

13. Gray MJ & Escalante-Semerena JC (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.

14. Maggio-Hall LA & Escalante-Semerena JC (1999) In vitro synthesis of the nucleotide loop of cobalamin by *Salmonella typhimurium* enzymes. *Proc. Natl. Acad. Sci. U S A* 96:11798-11803.

15. O'Toole GA & Escalante-Semerena JC (1993) *cobU*-dependent assimilation of nonadenosylated cobinamide in cobA mutants of *Salmonella typhimurium*. *J. Bacteriol.* 175:6328-6336.

16. Otte MM, Woodson JD, & Escalante-Semerena JC (2007) The thiamine kinase (YcfN) enzyme plays a minor but significant role in cobinamide salvaging in *Salmonella enterica*. *J. Bacteriol.* 189:7310-7315.

17. Datsenko KA & Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* 97:6640-6645.

18. Rocco CJ, Dennison KL, Klenchin VA, Rayment I, & Escalante-Semerena JC (2008) Construction and use of new cloning vectors for the rapid isolation of recombinant proteins from *Escherichia coli*. *Plasmid* 59:231-237.

19. Woodcock DM*, et al.* (1989) Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucl. Acids Res.* 17:3469-3478.

20. Ryu J & Hartin RJ (1990) Quick transformation in *Salmonella typhimurium* LT2. *Biotechniques* 8:43-45.

21. Tsai SP, Hartin RJ, & Ryu J (1989) Transformation in restriction-deficient *Salmonella typhimurium* LT2. *J. Gen. Microbiol.* 135:2561-2567.

22. Berkowitz D, Hushon JM, Whitfield HJ, Jr., Roth J, & Ames BN (1968) Procedure for identifying nonsense mutations. *J. Bacteriol.* 96:215-220.

23. Miroux B & Walker JE (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J. Mol. Biol.* 260:289-298.

24. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

25. Woodson JD & Escalante-Semerena JC (2004) CbiZ, an amidohydrolase enzyme required for salvaging the coenzyme $B_{12}$ precursor cobinamide in archaea. *Proc. Natl. Acad. Sci. USA* 101:3591-3596.

26. Claros MG & von Heijne G (1994) TopPred II: an improved software for membrane protein structure predictions. *Comput. Appl. Biosci.* 10:685-686.

27. Sonnhammer EL, von Heijne G, & Krogh A (1998) A hidden Markov model for predicting transmembrane helices in protein sequences. *Proceedings. International Conference on Intelligent Systems for Molecular Biology* 6:175-182.

28. Hofmann K & Stoffel W (1993) TMbase - A database of membrane spanning protein segments. *Biol. Chem.* 374:166.

29. Schuck P, Taraporewala Z, McPhie P, & Patton JT (2001) Rotavirus nonstructural protein NSP2 self-assembles into octamers that undergo ligand-induced conformational changes. *The Journal of biological chemistry* 276(13):9679-9687.

30. Men Y*, et al.* (2014) Sustainable growth of *Dehalococcoides mccartyi* 195 by corrinoid salvaging and remodeling in defined lactate-fermenting consortia. *Appl. Environ. Microbiol.*:In press.

31. Guzman LM, Belin D, Carson MJ, & Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 177(14):4121-4130.

32. VanDrisse CM & Escalante-Semerena JC (2016) New high-cloning-efficiency vectors for complementation studies and recombinant protein overproduction in Escherichia coli and Salmonella enterica. *Plasmid* 86:1-6.

33. P S, S G, & Z D (1998) Overcoming Expression and Purification Problems of RhoGDI Using a Family of "Parallel" Expression Vectors. *Protein Expression and Purification* 15:34-39.

# CHAPTER 3

# CYOTPLASMIC AND PERIPLASMIC RESIDUES ARE CRITICAL FOR CBIB ACTIVITY *IN VIVO*

## INTRODUCTION

Cobamides are part of the family of macrocyclic compounds, referred to as tetrapyrroles (1). Cbl is essential nutrient and cofactor for many organisms including humans. Cbl has been industrially produced yielding CNCbl. Although human cells do not make this compound, they can convert CNCbl to its coenzymatic form (adenosylcobalamin, AdoCbl, or $CoB_{12}$) (2, 3). Some organisms are capable of *de novo* AdoCbl biosynthesis in the presence or absence of oxygen. The key difference between the aerobic and anaerobic $B_{12}$ biosynthetic pathways is the timing of cobalt insertion (4). In the anaerobic pathway cobalt is inserted very early in the pathway (5). *Salmonella enterica* serovar Typhimurium LT2 (hereafter *S.* Typhimurium) is a model organism for studying complete biosynthesis of AdoCbl, committing a significant portion of its genome (>20 genes) to this process (4, 6). Genes leading to the formation of AdoCbi-P are abbreviate with *cbi*, genes involved thereafter are indicate using *cob,* e.g CobU (5).

Our understanding of the last step of the *de novo* coring ring biosynthetic branch of the pathway is limited. In this reaction, the carboxylate of the propionate substituent of ring D is attached to an 1-amino-2-propanol-phosphate (AP-P) moiety via an amide bond. The enzyme that catalyzes this reaction is known as the AdoCbi-P synthase or CbiB (7). Interestingly, CbiB is an integral membrane protein. CbiB is predicted to be 35.2 kDa (319 residues), and to traverse the membrane six times as shown in Figure 3.1 (2)(8). CbiB remains largely uncharacterized. For

example, we do not understand the biochemistry underpinning the reaction, molecular details of mechanism of function are unknown, its three-dimensional structure has not been reported, and the identity of possible interacting partners remains unknown. In this chapter, I address residues that may contribute to catalytic and binding activity and affects of CbiB production.

## MATERIALS AND METHODS

### *Bacterial Strains*

All strains used in this worked were derivatives of a null allele *metE* (requiring synthesis of $B_{12}$) and *ara-9* an allele preventing arabinose utilization and a null allele of *cbiB* (JE8185). Strains used in this work are found in Table 3.1.

### *Plasmid Construction*

All plasmids used in this work are listed in Table 3.2. All primers were ordered from Integrated DNA Technologies Inc. (IDT, Coralville, IA). DNA sequencing (Georgia Genomics Facility UGA) was used to verify all plasmids used in this study. *cbiB*$^+$ was amplified from pCBIB44 and cloned into overexpression vectors pTEV6 and pYES2NTC using restriction enzyme cloning (9). In order to construct pCBIB97, *cbiB* was PCR amplified using primers SeCbiB_KpnI 5' (nnggtaccgatgacgattcttgcc) and SeCbiB_EcoRI NS 3' (nngaattcggccacgccagataac). The amplified fragment was cloned into pBAD-mCherry2 using restriction enzyme cloning. Mutagenic primers were designed using PrimerX (http://www.bioinformatics.org/primerx/cgi-bin/DNA_1.cgi) (Table 3.3). DNA was mutated using PfuUltra II Fusion DNA polymerase (Stratagene) and mutagenic primers to introduced changes to *cbiB*$^+$ (pCBIB67) encoding amino acid changes of conserved residues to alanine, or glycine where appropriate. PCR products were treated with the restriction enzyme DpnI (Fermentas) and transformed into chemically competent DH5α (10). Plasmids were prepared and

purified using E.Z.N.A Plasmid Mini Kit I (Omega bio-tek). The resulting plasmids were transformed into JE8185 and tested for activity *in vivo* under conditions that required functional AdoCbi-P synthase activity.

*Growth conditions*

Overnight cultures were prepared by inoculating strains into Nutrient Broth ( NB Difco) supplemented with 100 μg/mL ampicillin. A 96-well plate containing 200 μL of No Carbon Essential (NCE) medium, trace minerals (11), supplemented with glycerol (22 mM), $MgSO_4$ (1 mM), dimethylbenzimidazole (DMB 150 μM), ampicillin (50 μg/mL), corrinoids (1 nM (unless indicated otherwise) of $CN_2$Cbi or $CN_2$Cby or CNCbl,) and L-arabinose (0.5 mM when indicated) was inoculated with 1% (v/v) of the overnight. Strains were grown at 37°C for 24-48 hours with intermediate shaking and monitored using Gen5 software (BioTek) in an EL808 Ultra or PowerWave XS Microplate Reader (BioTek instruments). Growth curve data represents a minimum of two independent experiments. Data were analyzed using Prism v6 (GraphPad) analytical software. Error bars represent the standard deviation.

*Overexpression and solubilization of CbiB*

*E. coli* C41 (DE3λ) (12) cells carrying pCBIB44 encoding N-terminally $His_6$ tagged *cbiB*$^+$ allele from *S. enterica* (13) were grown as previously described in (7). Overexpressed protein was not detected. A detergent screen as described in (14) was performed. Briefly ectopically expressed CbiB (pCBIB44) was harvested at 6000 x g for 15 minutes. Pellet was split and solubilized in (hydroxymethly)aminomethane (Tris-HCl, 20 mM, pH 7.4) with additions of Triton X (Fisher), fos-choline 16 (Avanti), or n-Dodecyl β-D-maltoside (DDM, Anatrace). Whole cell lysate, solubilized and insoluble fractions were visualized by SDS-PAGE stained with coomaisse. Cell lysates were prepared using a modified membrane enrichment protocol

described in (15). Prepared lysates were affinity purified over a 2-mL bed of NiNTA resin (Thermo scientific). Fractions were run on 12% SDS-PAGE and visualized using coomaisse blue staining. Bands from the gel were excised and sent off for matrix assisted laser desorption ionization-TOF-MS (MALDI). Western blots were performed using anti-histidine (1:5000) primary antibodies. Alkaline Phosphate conjugated antibodies (1:10,000) were used as secondary antibodies. Westerns were visualized similarly as described in (16).

*Heterologous Expression systems of CbiB*

Several expression systems have been developed to aid in the purification of membrane proteins. One method was *Rhodospirillum rubrum* expression system uses a mutant strain of *R. rubrum* (H2) capable of expressing intra cytoplasmic membrane (ICM) (17). In *E. coli* or *S.* Typhimurium overexpression of membrane proteins stresses the cell resulting in lower protein expression. The ICM provides additional surface area for the insertion of *Se*CbiB, allowing high levels of overexpression and higher protein yields. Attempts using this system were unsuccessful. Using a mammalian derived system, Human Embyronic Kidney cells were transfected with a plasmid containing $His_6$-GFP C-terminally tagged *Homo sapiens* codon optimize *cbiB* (GenScript) (18). Cells expressing the plasmid were grown, extracted and lysed using 10 mL of Tris-HCl (25 mM) pH 8 at 4°C with NaCl (150 mM), NP-40 (0.5%) and phenylmethane sulfonyl fluoride (PMSF (0.2 mM protease inhibitor)). Cells were incubated on ice for 30 minutes and pelleted for five minutes at 7000 x g. Cells were lysed using sonication at 50% duty, on for 2s and off 2s for a total of 60 seconds. Cell debris was cleared by spinning for five minutes at 2000 x g. Supernatant was incubated with NiNTA resin. Western blots were done using mouse-anti-histidine primary antibodies (1:5000) and Alkaline Phosphate conjugated goat-anti rabbit secondary antibodies (1:10,000). Westerns were visualized similarly as

described in (16). An empty vector and plasmids encoding N or C-terminally tagged *Se*CbiB (pCBIB87/88) were transformed into chemically competent *Saccharomyces cerevisiae* (strain BY4742) as described in (19) and plated on minimal medium without uracil.

*Detection of CbiB using fluorescence microscopy*

Overnight cultures of JE8185 (*ΔcbiB*) with empty vector or pCBIB97, *in trans*, were grown in nutrient broth supplemented with 100 μg/mL. Fresh NB was inoculated with 1:100 of each strain and incubated with shaking (200 rpm) at 37°C for four hours. Cells were harvested at 4000 x g for 5 minutes, the supernatant was removed; cells were then re-suspended in 1:10 volume of buffered saline. Ten microliters of cells and 5 μL of 1 mM L-arabinose were spotted on to a slide pretreated with a 0.1% (v/w) of poly-L-lysine (Sigma Aldrich). Cultures on slides were visualized using fluorescence microscopy.

RESULTS

*Identification of residues critical for CbiB function*

The active site of CbiB is still unknown. To address this problem of the location of the active site, conserved residues were targeted for site-directed mutagenesis (SDM). Variants were categorized based off of the mutant phenotypes. Figure 3.2 depicts represented growth results from plasmids encoding residues *in trans*. Table 3.4 is a summary of doubling times of strains with synthesized *cbiB* variants. Residues that abolished activity under any conditions tested were D181, R130, E150A, D154. It is unknown if the growth impairment was a result of impaired activity or instability of the protein. Increasing concentrations of Cby show an increase growth of mutant strains possessing variants, included residues A143V, P159A, R211A, and Y268F. Subsequently in the presence of L-arabinose these strains were further rescued. Residues P159A, and Y268F resulted in growth comparable to wild-type CbiB.

*Solubilization of CbiB presents a bottleneck*

Ideal solubilization of extracts was seen at low concentrations of DDM or fos-choline 16 (~1%)(14). Purification of N-terminally $His_6$-CbiB was unsuccessful, because the N-terminus appears localized in the membrane, inhibiting contacts with column resin. A C-terminal $His_6$ tagged CbiB was constructed into a pET28b vector. Knowing that OmpA/F was a potential problem in rich medium, overexpression was performed in minimal medium containing 25 nM cobyric acid in an *S.e* strain lacking *cbiB*. Growth in the medium would indicate the expression and activity of *cbiB*. NiNTA resin purification was unsuccessful. Denatured samples were passed over nickel resin. Bands were present in the elution samples and sent off for MALDI-TOF-MS. CbiB was not detected; OmpA/F was the top-hit (20, 21). Alternative expressions systems were used to express CbiB. Plasmids pCBIB87/88 were unsuccessfully transformed into S. cerevisiae. The empty vector resulted in transformants. The inability for BY4742 to uptake pCBIB87/88 may correlate to toxicity of CbiB for membranes. Additional optimization for the expression of $cbiB^+$ in HEK cells is needed.

*Fluorescence microscopy can detect the presence of CbiB*

This experiments provides foundation steps to look at reassessing the topology of CbiB, specifically loop four. Figure 3.3 shows cells, *ΔcbiB* with an empty vector vs. Δ*cbiB* with pCBIB97. Cells expressing $cbiB^+$ (pCBIB97) fluoresce, as indicated by red cells (rods).

DISCUSSION AND CONCLUSIONS

*Residues important for in vivo activity*

Strains harboring alleles encoding variants indicate the residues located in loop three and loop four are important in CbiB function (Fig 3.1). When concentrations of Cby are increased, doubling time in strains containing plasmids encoding residues A143V, P159A, R211A and

Y268F, is decreased. This suggests that these residues may play a role in binding of Cby. Alternatively, some of these residues could play a role in binding AP-P. It was previously reported that a complete loss of CbiB activity came from strains expressing plasmids encoding residue substitutions D181N and E150A (5, 22). It was speculated that loss activity for D181N was do to a loss of an interaction partner from a negative-positive interaction or that D181N led to a change in membrane topology resulting in loss of activity. Plasmids encoding variants D181E (conserved) and D181A resulted in no growth (data not shown). It is unlikely that the loss of activity is because of a negative-positive interaction (23). Residues D154 and D181 may contribute to catalytic activity of CbiB. Residue R130 could contribute to the stabilization of an ATP molecule, supporting the hypothesis that CbiB could be a synthetase, or the residue could stabilize the phosphate group of the product, biochemical data is needed to verify. If CbiB is a synthetase, residues in loop four may contribute to binding of the metal cofactor, magenesium. If this is the case, topology of CbiB should be reassessed. It is peculiar that residues located in loop four would impact activity because the substrates for CbiB are in the cytoplasm and $B_{12}$ is synthesized in the cytoplasm. It is possible that loop four is houses residues that make up the active site. An alternative idea is that AP-P and Cby are transported out to the periplasm by CbiB, the reactions takes place and then the product is stabilized by loops three and five and passed to CobU. Questions remain as to what role loop four plays in CbiB activity. Strategies for reassessment are discussed in Chapter 4.

*Overexpression of CbiB stresses the membrane*

Several methods were employed and unsuccessful in purifying and detecting CbiB. Lysate from cells grown in minimal medium requiring functional recombinant CbiB resulted in a visible increase of a protein corresponding to the size of $His_6$-CbiB. Mass spectrometry results

reveal the proteins expressed are OmpA and OmpF, proteins that are typically expressed because of disruption of the cell membrane. In *Escherichia coli*, OmpA functions in structural stability of the outer-membrane(24), suggesting that overexpression of CbiB *in trans* destabilizes the outer-membrane. OmpF is associated with BtuB (cobalamin transporter), when colicins (bacteriocins) are present (25, 26). Anitbiotics and environmental factors may induce expression of colicins affecting levels of OmpF. This is not the first case in which a protein is expressed to stabilize bacterial membranes. When CobS (cobalamin 5' phosphate synthase) is overexpressed, PspA (phage shock protein A) expression relieves membrane stress associated with CobS, it acts as a biological membrane plug (27). Alternatives methods were used to express CbiB. The absence of CbiB containing *S. cerevisiae* transformants supports the idea that expressing CbiB may be toxic to the cell.

*Using fluorescence for detection of CbiB*

A different approach was used to track and detect the production of CbiB. Detection of expressed *cbiB* and active CbiB is essential before characterizing variants to determine if *in vitro* activity is abolished due to inactivity or lack of protein stability. Fusion tags can be used to visualize CbiB without disrupting function (data not shown). Cells containing C-terminally tagged CbiB-mCherry$^{+}$ were visualized using fluorescence microscopy, indicating this is a viable approach to confirm stability of CbiB variants, specifically in loop four. Future directions for this chapter are discussed in chapter 4.

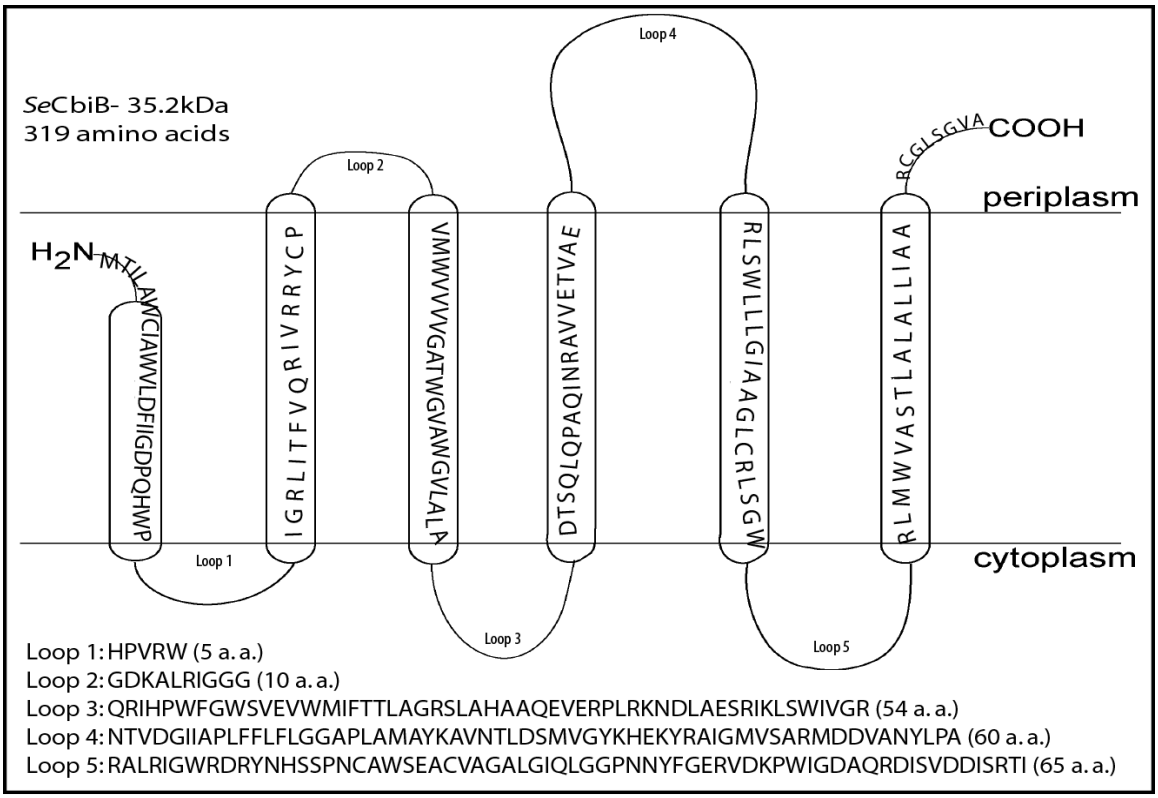


**Figure 3.1**: Topology of *Se*CbiB. Loops are labeled with amino acid sequence below diagram.

**Table 3.1**: Strains list of *S. enterica* sv. Typhimurium strain LT2 derivatives. Strains were constructed during the course of this work unless stated otherwise.

| Strain | Relevant Genotype | Reference Source |
|---|---|---|
| ***Salmonella enterica*** | | |
| JE8185 | *metE209 ara*-9 *ΔcbiB1309* | (7) |
| **Derivatives of JE8185** | | |
| JE19549 | JE8185/pMkCBIB1 *bla*$^+$ | |
| JE19606 | JE8185/pCBIB52 *kan*$^+$ | |
| JE21386 | JE8185/pCV1 *bla*$^+$ | |
| JE21387 | JE8185/pCBIB67 *bla*$^+$ | |
| JE21388 | JE8185/pCBIB69 *bla*$^+$ | |
| JE21389 | JE8185/pCBIB70 *bla*$^+$ | |
| JE21390 | JE8185/pCBIB71 *bla*$^+$ | |
| JE21391 | JE8185/pCBIB72 *bla*$^+$ | |
| JE21392 | JE8185/pCBIB73 *bla*$^+$ | |
| JE21393 | JE8185/pCBIB74 *bla*$^+$ | |
| JE21394 | JE8185/pCBIB75 *bla*$^+$ | |
| JE21807 | JE8185/pCBIB76 *bla*$^+$ | |
| JE21808 | JE8185/pCBIB77 *bla*$^+$ | |
| JE21809 | JE8185/pCBIB78 *bla*$^+$ | |
| JE21810 | JE8185/pCBIB79 *bla*$^+$ | |
| JE21811 | JE8185/pCBIB80 *bla*$^+$ | |
| JE21812 | JE8185/pCBIB81 *bla*$^+$ | |
| JE21813 | JE8185/pCBIB82 *bla*$^+$ | |
| JE21817 | JE8185/pCBIB93 *bla*$^+$ | |
| JE21818 | JE8185/pCBIB94 *bla*$^+$ | |
| JE21713 | JE8185/pCBIB87 *bla*$^+$ | |
| JE21714 | JE8185/pCBIB88 *bla*$^+$ | |
| JE21715 | JE8185/pYES2/NTC *bla*$^+$ | |
| | JE8185/pCBIB68 *bla*$^+$ | |
| | JE8185/pCBIB89 *bla*$^+$ | |
| | JE8185/pCBIB97 *bla*$^+$ | |
| | JE8185/pBAD-mCherry2 *bla*$^+$ | |
| ***Escherichia coli*** | | |
| C41 (DE3) | $F^-$ ompT gal dcm $hsdS_B(r_B^- m_B^-)$(DE3) | (28) |
| DH5α | *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* | (10) |
| ***Saccharomyces cerevisiae*** | | |
| BY4742 | MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* | (29) |

**Table 3.2**: Plasmids list. Plasmids were constructed during the course of this work unless stated otherwise.

| Plasmid | Relevant Genotype | Reference Source |
|---|---|---|
| pCBIB44 | *cbiB38* cloned into pTEV16 *bla*$^+$ | |
| pCBIB52 | *cbiB45* (encodes CbiB$^{E150A}$) *kan*$^+$ | |
| pCBIB67 | *cbiB37* (encodes CbiB$^+$) *bla*$^+$ | |
| pCBIB68 | *cbiB63* (encodes CbiB$^{\Delta 2\text{-}12}$) *bla*$^+$ | |
| pCBIB69 | *cbiB57* (encodes CbiB$^{D202A}$) *bla*$^+$ | |
| pCBIB70 | *cbiB47* (encodes CbiB$^{P159A}$) *bla*$^+$ | |
| pCBIB71 | *cbiB54* (encodes CbiB$^{Y186A}$) *bla*$^+$ | |
| pCBIB72 | *cbiB48* (encodes CbiB$^{T179A}$) *bla*$^+$ | |
| pCBIB73 | *cbiB39* (encodes CbiB$^{D13E}$) *bla*$^+$ | |
| pCBIB74 | *cbiB52* (encodes CbiB$^{M183A}$) *bla*$^+$ | |
| pCBIB75 | *cbiB60* (encodes CbiB$^{Y268F}$) *bla*$^+$ | |
| pCBIB76 | *cbiB41* (encodes CbiB$^{P25A}$) *bla*$^+$ | |
| pCBIB77 | *cbiB43* (encodes CbiB$^{A143V}$) *bla*$^+$ | |
| pCBIB78 | *cbiB46* (encodes CbiB$^{D154A}$) *bla*$^+$ | |
| pCBIB79 | *cbiB49* (encodes CbiB$^{D181A}$) *bla*$^+$ | |
| pCBIB80 | *cbiB53* (encodes CbiB$^{G185A}$) *bla*$^+$ | |
| pCBIB81 | *cbiB55* (encodes CbiB$^{G195A}$) *bla*$^+$ | |
| pCBIB82 | *cbiB56* (encodes CbiB$^{A199G}$) *bla*$^+$ | |
| pCBIB87 | *cbiB38* cloned into pYES2/NT *URA*$^+$ *bla*$^+$ | |
| pCBIB88 | *cbiB38* cloned into pYES2/CT *URA*$^+$ *bla*$^+$ | |
| pCBIB89 | *cbiB64* (encodes CbiB$^{\Delta 297\text{-}319}$) *bla*$^+$ | |
| pCBIB93 | *cbiB51* (encodes CbiB$^{D181N}$) *bla*$^+$ | |
| pCBIB94 | *cbiB58* (encodes CbiB$^{R211A}$) *bla*$^+$ | |
| pCBIB97 | *cbiB38* cloned into pBAD-mCherry2 *bla*$^+$ | Laboratory Collection |
| pMkCBIB1 | *Methanopyrus kandleri cbiB* cloned into pCV1 *bla*$^+$ | |
| pBAD-mCherry2 | C-terminal mCherry tag cloned into pBAD24 *bla*$^+$ | |
| pCV1 | Cloning/complementation vector *bla*$^+$ | (13) |
| pTEV16 | Cloning/overexpression vector, N-terminal rTEV cleavable $His_6$ tag *bla*$^+$ | (13) |
| pYES2/NTC | *S. cerevisiae* expression vector | Invitrogen |

**Table 3.3**: Mutagenic primers used to introduce changes to *cbiB*

| Plasmid | Residue Changed | Primers Forward and Reverse (5' to 3') |
|---|---|---|
| pCBIB69 | D202A | GCGAACTATCTTGCTGCCCGACTGAG<br>CTCAGTCGGGCAGCAAGATAGTTCGC |
| pCBIB70 | P159A | GTTGACGGCATTATCGCGGCGCTCT<br>GGAAAAAGAGCGCCGCGATAATGCCG |
| pCBIB71 | Y186A | CCCTGGATTCAATGGTGGGCGCCAAACATG<br>CATGTTTGGCGCCCACCATTGAATCCAGGG |
| pCBIB72 | T179A | GCCTACAAAGCCGTCAATGCCCTGG<br>CCATTGAATCCAGGGCATTGACGGCTTTGTAG |
| pCBIB73 | D13E | GTGTATCGCCTGGGTGCTGGAGTTTATC<br>GATAAACTCCAGCACCCAGGCGATACAC |
| pCBIB74 | M183A | CAATACCCTGGATTCAGCGGTGGGCTACAAACATG<br>CATGTTTGTAGCCCACCGCTGAATCCAGGGTATTG |
| pCBIB75 | Y268F | GGCCCAAATAACTTCTTTGGCGAGCGTG<br>CACGCTCGCCAAAGAAGTTATTTGGGCC |
| pCBIB76 | P25A | CAACACTGGCCCCATGCGGTACGCTGGATAG<br>CTATCCAGCGTACCGCATGGGGCCAGTGTTG |
| pCBIB77 | A143V | CAGATCAATCGCGTCGTGGTGGAAACG<br>CGTTTCCACCACGACGCGATTGATCTG |
| pCBIB78 | D154A | GAAAACACCGTTGCCGGCATTATCGCG<br>CGCGATAATGCCGGCAACGGTGTTTTC |
| pCBIB79 | D181A | GTCAATACCCTGGCTTCAATGGTGGGC<br>GCCCACCATTGAAGCCAGGGTATTGAC |
| pCBIB80 | G185A | CCTGGATTCAATGGTGGCGTACAAACATGAAAAATA<br>GTATTTTTCATGTTTGTACGCCACCATTGAATCCAGG |
| pCBIB81 | G195A | GATTGGTATGGTCAGCGGACGTATGGACGACGTAG<br>CTACGTCGTCCATACGTCCGCTGACCATACCAATC |
| pCBIB82 | A199G | GTCAGCGCCCGTATGGCAGACGTAGCGAACTATC<br>GATAGTTCGCTACGTCTGCCATACGGGCGCTGAC |
| pCBIB90 | R130A | CTCCTGGATCGTCGGGGCAGATACGTCGCAACTTC<br>GAAGTTGCGACGTATCTGCCCCGACGATCCAGGAG |
| pCBIB91 | E150A | GAAACGGTTGCAGCAAACACCGTTGACG<br>CGTCAACGGTGTTTGCTGCAACCGTTTC |
| pCBIB93 | D181N | CCGTCAATACCCTGAATTCAATGGTGGGC<br>GCCCACCATTGAATTCAGGGTATTGACGG |
| pCBIB94 | R211A | CGAACTATCTTCCTGCCGCACTGAGCTGG<br>CCAGCTCAGTGCGGCAGGAAGATAGTTCG |
| pCBIB95 | Y268A | ATCCAGCTCGGTGGCCCAAATAACGCCTTTGGCGAGC<br>GTGTGGACAAGC |

**Table 3.4**: Doubling time (hours) of strains synthesizing cbiB variants in increasing concentrations of $CN_2Cby$

| | 1 nM | | 2 nM | | 4 nM | | 8 nM | |
|---|---|---|---|---|---|---|---|---|
| **arabinose** | - | +[1] | - | + | - | + | - | + |
| Variant | | | | | | | | |
| WT | 2.6 | 2.4 | 1.7 | 1.7 | 1.5 | 1.5 | 1.5 | 1.4 |
| R130A | NG[2] | NG | NG | NG | NG | NG | NG | NG |
| A143V | NG | NG | NG | 20.6 | NG | 13.3 | 20.9 | 8.8 |
| D154A | NG | NG | NG | NG | NG | NG | NG | NG |
| P159A | NG | 3.8 | 16.3 | 2.4 | 10.3 | 1.9 | 6.9 | 1.6 |
| D181N | NG | NG | NG | NG | NG | NG | NG | NG |
| A199G | NG | NG | NG | NG | NG | NG | NG | NG |
| R211A | NG | 8.3 | NG | 5.7 | NG | 4.8 | 13.5 | 4.3 |
| Y268F | 16.8 | 3.2 | 10 | 2.2 | 7 | 1.8 | 5.1 | 1.7 |

[1]Doubling times in the presence of 0.5 mM arabinose
[2]NG indicates no significant growth

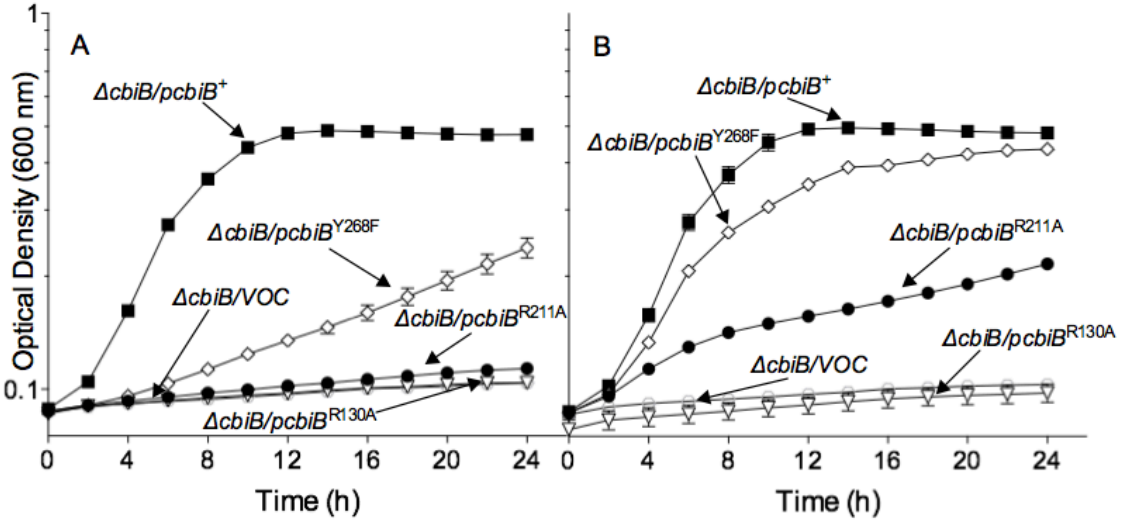


**Figure 3.2**: Complementation of a *cbiB S.* Typhimurium strain. Growth analysis of *cbiB* mutants (in trans) grown aerobically at 37°C in NCE minimal medium supplemented with A. Glycerol (22 mM), $MgSO_4$ (1 mM), Cby (1 nM) DMB (150 μM) Ampicillin (50 μg/mL) or B. in the presence of L-arabinose (0.5 mM) Refer to table 3.4 for doubling times

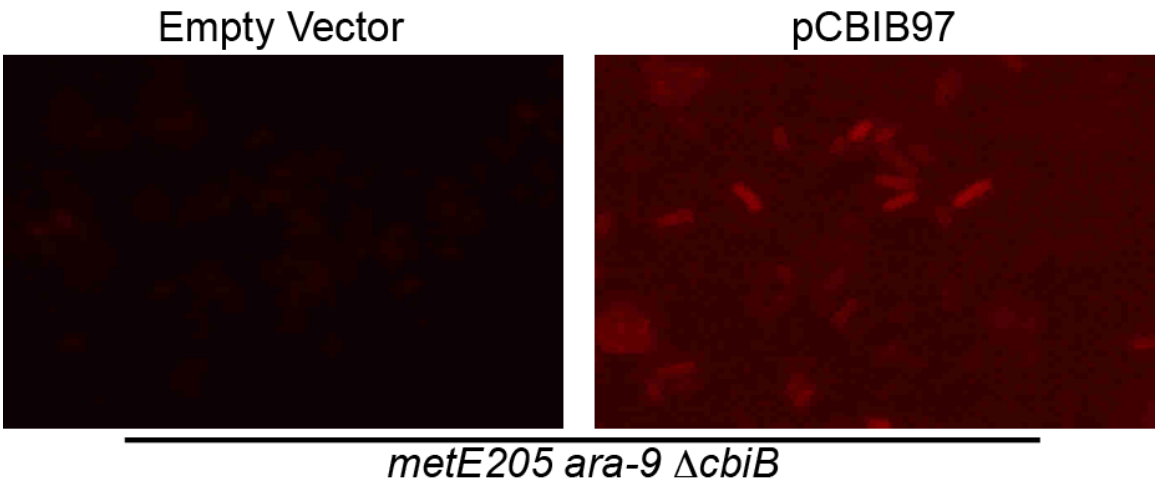


**Figure 3.3**: Fluorescence microscopy of *S.* Typhimurium *cbiB* strains. *cbiB* mutant are expressing mCherry$^{+}$ (Empty Vector) or with cbiB- mCherry$^{+}$ (pCBIB97) in the presence of 1 mM arabinose

REFERENCES


1. Warren MJ & Smith AG (2009) *Tetrapyrroles: Birth, Life and Death* (Springer) p 422.
2. Roth J, Lawrence J, & Bobik T (1996) COBALAMIN (COENZYME B12): Synthesis and Biological Significance. *Annual Review of Microbiology* 50:137-181.
3. Gray MJ, Tavares NK, & Escalante-Semerena JC (2008) The genome of Rhodobacter sphaeroides strain 2.4.1 encodes functional cobinamide salvaging systems of archaeal and bacterial origins. *Molecular microbiology* 70:824-836.
4. Roth JR, Lawrence JG, Rubenfield M, Kieffer-Higgins S, & Church GM (1993) Characterization of the cobalamin (vitamin B12) biosynthetic genes of Salmonella typhimurium. *Journal of Bacteriology* 175:3303-3316.
5. Escalante-Semerena JC (2007) Conversion of cobinamide into adenosylcobamide in bacteria and archaea. *J Bacteriol* 189(13):4555-4560.
6. Smith A & Warren M (2009) *Tetrapyrroles : birth, life and death* (Springer, New York, NY).
7. Zayas CL, Claas K, & Escalante-Semerena JC (2007) The CbiB protein of *Salmonella enterica* is an integral membrane protein involved in the last step of the de novo corrin ring biosynthetic pathway. *J. Bacteriol.* 189:7697-7708.
8. Wilkins MR*, et al.* (1999) Protein identification and analysis tools in the ExPASy server. *Methods Mol. Biol.* 112:531-552.
9. Rocco CJ, Dennison KL, Klenchin VA, Rayment I, & Escalante-Semerena JC (2008) Construction and use of new cloning vectors for the rapid isolation of recombinant proteins from *Escherichia coli*. *Plasmid* 59:231-237.

10. Woodcock DM*, et al.* (1989) Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucl. Acids Res.* 17:3469-3478.

11. Berkowitz D, Hushon JM, Whitfield HJ, Jr., Roth J, & Ames BN (1968) Procedure for identifying nonsense mutations. *J. Bacteriol.* 96:215-220.

12. Miroux B & Walker JE (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J. Mol. Biol.* 260:289-298.

13. VanDrisse CM & Escalante-Semerena JC (2016) New high-cloning-efficiency vectors for complementation studies and recombinant protein overproduction in Escherichia coli and Salmonella enterica. *Plasmid* 86:1-6.

14. Gabrielsen M*, et al.* (2011) High-throughput identification of purification conditions leads to preliminary crystallization conditions for three inner membrane proteins. *Molecular membrane biology* 28:445-453.

15. Hohenfeld IP, Wegener AA, & Engelhard M (1999) Purification of histidine tagged bacteriorhodopsin, pharaonis halorhodopsin and pharaonis sensory rhodopsin II functionally expressed in Escherichia coli. *FEBS Letters* 442(2–3):198-202.

16. Blake MS, Johnston KH, Russell-Jones GJ, & Gotschlich EC (1984) A rapid, sensitive method for detection of alkaline phosphate-conjugated anti-body on Western blots. *Anal. Biochem.* 136:175-179.

17. Butzin NC, Owen HA, & Collins ML (2009) A new system for heterologous expression of membrane proteins: *Rhodospirillum rubrum*. *Protein Expr. Purif.*

18. Subedi GP, Johnson RW, Moniz HA, Moremen KW, & Barb A (2015) High Yield Expression of Recombinant Human Proteins with the Transient Transfection of HEK293 Cells in Suspension. *Journal of visualized experiments : JoVE* (106):e53568.

19. Gietz RD, Schiestl RH, Willems AR, & Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast (Chichester, England)* 11(4):355-360.

20. Cai SJ & Inouye M (2002) EnvZ-OmpR interaction and osmoregulation in Escherichia coli. *The Journal of biological chemistry* 277:24155-24161.

21. Wang Y (2002) The Function of OmpA in Escherichia coli. *Biochemical and Biophysical Research Communications* 292(2):396-401.

22. Anonymous (2007) Thesis. ed Zayas CL.

23. Zayas CL & Escalante-Semerena JC (2007) Reassessment of the late steps of coenzyme $B_{12}$ synthesis in *Salmonella enterica*: Evidence that dephosphorylation of adenosylcobalamin-5'-phosphate by the CobC phosphatase is the last step of the pathway. *J. Bacteriol.* 189:2210-2218.

24. Sonntag I, Schwarz H, Hirota Y, & Henning U (1978) Cell envelope and shape of Escherichia coli: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. *Journal of Bacteriology* 136(1):280-285.

25. Spector J*, et al.* (2010) Mobility of BtuB and OmpF in the Escherichia coli outer membrane: implications for dynamic formation of a translocon complex. *Biophysical journal* 99:3880-3886.

26. Zakharov Stanislav D, Sharma O, Zhalnina M, Yamashita E, & Cramer William A (2012) Pathways of colicin import: utilization of BtuB, OmpF porin and the TolC drug-export protein. *Biochemical Society Transactions* 40:1463-1468.

27. Maggio-Hall LA, Claas KR, & Escalante-Semerena JC (2004) The last step in coenzyme B(12) synthesis is localized to the cell membrane in bacteria and archaea. *Microbiology* 150:1385-1395.

28. Miroux B & Walker JE (1996) Over-production of proteins in Escherichia coli: Mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *Journal of Molecular Biology* 260:289-298.

29. CB B*, et al.* (1998) Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast (Chichester, England)* 14:115-132.

# CHAPTER 4

# SUMMARY AND FUTURE DIRECTIONS

## SUMMARY

AdoCbl biosynthesis has been extensively studied (1-4). These works represent the studies of late steps of biosynthesis. Even though this pathway has been studied for decades, knowledge gaps remain in understanding the reaction mechanism of proteins, in particular CbiZ and CbiB (5, 6). Chapter 2 and 3 establishes the importance of certain residues, a need for protein structures and an activity assay. Here I summarize the next steps to further study these enzymes.

## FUTURE DIRECTIONS

### *What are the metal requirements of CbiZ?*

CbiZ belongs to the enzyme superfamily of amidohydrolases (7). Typical characteristics include having metallic cofactors that contribute to catalytic activity. In order to determine this purified CbiZ needs to be screened against various monovalent and divalent metal cofactors. Determining the native cofactor will be important for kinetic analysis.

### *What is the activity of RsCbiZ and variants in vitro*

In order to understand the phenotypes of *Rs*CbiZ variants, *in vitro* techniques must be used to study enzyme activity, substrate binding and protein stability. These include using methods to assess activity One approach is using isothermal calorimetry (8) to measure binding of substrate to protein. In this case AdoPseudoCbl, AdoCbl, AdoCbi and AdoCby would need to be assessed using wild-type *Rs*CbiZ and then comparing results to variants to determine what role those residues play. To determine if variants result in mis-folded protein Differential

Scanning Calorimetry (DSC) (9) can be employed and compared to the thermal signature of $His_6$-MBP-*Rs*CbiZ$^+$.

*A Crystal structure of CbiZ is needed*

Several homologs were overexpressed to determine the best candidate to submit for crystal screening. MBP-*Bh*CbiZ was the top candidate because of the quantity of protein expressed. Unpublished data shows that the CbiZ domain of the fusion protein *Bh*CbiZ-BtuD is active *in vitro* when $CN_2$Cbi is the substrate. It remains unknown the specific substrate *Bh*CbiZ is able to use, it's function is secondary to having a crystal structure. Currently MBP-*Bh*CbiZ is being submitted to the Rayment Labaratory. If the submission of this protein results in a crystal structure, purification of MBP-*Bh*CbiZ in complex with $CN_2$Cbi would help shed light on where the corrinoids are binding.

*Does CbiZ interact with CbiB?*

Previous studies show that CbiB function is necessary in scavenging cobamides and archaeal salvaging. Is it known that the product of CbiZ reactions is Cby, the substrate that reenters the $B_{12}$ biosynthesis pathway via CbiB yielding Cbi-P, which is then reintegrate into the nucleotide loop assembly pathway resulting in the formation of AdoCba. In order to continue these studies, tagged CbiB that contains antibody specific epitopes can be used to track changes using immunoblots. One approach is using *in vivo* crosslinking where a *S.* Typhimurium strain expressing *RsCbiZ* and fluorescently tagged CbiB (10-12). Antibodies against fluorescent tag and *Rs*CbiZ would be used to track protein weight changes in an immunoblot.

*Reassessment of CbiB topology*

LacZ-PhoA fusions were used to elucidate experimental topology of CbiB (5). Data from Chapter 3 shows that three out of four residues that are the result of a null allele are located in

loop 4, that was determined to be exposed in the periplasm (5). This is of interest because the product is the substrate of the cytoplasmic enzyme CobU. The product of the pathway, AdoCbl, is also found in the cytoplasm. To verify this loop is exposed to the periplasm, pstI cut sites constructed in *cbiB* plasmids, as described in (5) will be used to insert gene-encoding GFP. Using GFP fusions is advantageous because it is a fraction of the size of LacZ and PhoA proteins (13, 14). It is a possibility that using these larger proteins may have disrupted the topology/orientation of CbiB. Using fluorescent microscopy, the orientation of the loop four can be verified. If loop four remains in the periplasm, it begs the question, why? Are the residues in that loop contributing to protein stability and not involved directly in the catalytic mechanism? Constructing mutants encoding conserved mutations of the null alleles and testing phenotypes would help determine if lack of activity is a result of residues changed to alanine or if they are important residues for activity.

*Is CbiB a synthase or synthetase?*

It has been shown that a diverse superfamily of enzymes is capable of ATP-dependent carboxylate-amine/thiol ligase activity (15). The proposed mechanism of CbiB coincides with the mechanism seen from this superfamily of enzymes (16). These reactions combine a carboxylate and amine to form an amide (5). In this instance, AdoCby is the carboxylate and AP-P is the amine resulting in AdoCbi-P. From a chemical standpoint, an amine is too weak of a nucleophile and a hydroxyl, a poor leaving group. ATP-dependent ligases form an acyl-phosphate intermediate to activate the carbonyl group. The amine can then attack the intermediate, displace the phosphate group and form the final product, an amide. From an energetics standpoint having an activated intermediate (AdoCby-P) would mean CbiB is a

synthetase and not a synthase, an activity assay must be established to assess the energy source of the reaction, if any.

*Confirming membrane toxicity from CbiB production*

To verify membrane toxicity from the overexpression and production of CbiB, *E. coli* mutants, single and double of *ompA* and *ompF*, respectively should be constructed. Cellular arrest in these deletion strains would indicate that expressing cbiB$^{+}$ damages the bacterial membrane.

REFERENCES


1. Frank S*, et al.* (2005) Anaerobic synthesis of vitamin B12: characterization of the early steps in the pathway. *Biochem. Soc. Trans.* 33:811-814.
2. Heldt D*, et al.* (2005) Aerobic synthesis of vitamin B12: ring contraction and cobalt chelation. *Biochem. Soc. Trans.* 33:815-819.
3. Martens JH, Barg H, Warren MJ, & Jahn D (2002) Microbial production of vitamin $B_{12}$. *Appl. Microbiol. Biotechnol.* 58:275-285.
4. Warren MJ, Raux E, Schubert HL, & Escalante-Semerena JC (2002) The biosynthesis of adenosylcobalamin (vitamin B12). *Nat. Prod. Rep.* 19:390-412.
5. Zayas CL, Claas K, & Escalante-Semerena JC (2007) The CbiB protein of *Salmonella enterica* is an integral membrane protein involved in the last step of the de novo corrin ring biosynthetic pathway. *J. Bacteriol.* 189:7697-7708.
6. Gray MJ & Escalante-Semerena JC (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.
7. Seibert CM & Raushel FM (2005) Structural and Catalytic Diversity within the Amidohydrolase Superfamily. *Biochemistry* 44(17):6383-6391.
8. Velazquez-Campoy A & Freire E (2006) Isothermal titration calorimetry to determine association constants for high-affinity ligands. *Nat .Protoc.* 1:186-191.
9. Novokhatny V & Ingham K (1997) Thermodynamics of maltose binding protein unfolding. *Protein science : a publication of the Protein Society* 6(1):141-146.

10. Melcher K (2004) New chemical crosslinking methods for the identification of transient protein-protein interactions with multiprotein complexes. *Curr. Protein Pept. Sci.* 5:287-296.

11. Studdert CA & Parkinson JS (2004) Crosslinking snapshots of bacterial chemoreceptor squads. *Proc. Natl. Acad. Sci. U S A* 101:2117-2122.

12. Kataoka K, Schoeberl UE, & Mochizuki K (2010) Modules for C-terminal epitope tagging of Tetrahymena genes. *Journal of Microbiological Methods* 82(3):342-346.

13. Drew D*, et al.* (2002) Rapid topology mapping of *Escherichia coli* inner-membrane proteins by prediction and PhoA/GFP fusion analysis. *Proc. Natl. Acad. Sci. USA* 99:2690-2695.

14. Michaelis S, Inouye H, Oliver D, & Beckwith J (1983) Mutations that alter the signal sequence of alkaline phosphatase in Escherichia coli. *J. Bacteriol.* 154:366-374.

15. Galperin MY & Koonin EV (1997) A diverse superfamily of enzymes with ATP-dependent carboxylate-amine/thiol ligase activity. *Protein Science* 6:2639-2643.

16. Fawaz MV, Topper ME, & Firestine SM (2011) The ATP-grasp enzymes. *Bioorganic chemistry* 39(5-6):185-191.