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Investigating the structure and function of CobH and CobB , two consecutive enzymes in the biosynthesis of cobalamin

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MSc Research project

School of Biosciences

University of Kent

Supervisor: Professor Martin Warren

SCHOOL OF BIOSCIENCES

Degree of Master of Science by Research

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Acknowledgement

First and foremost, I would like to express my sincere gratitude to my advisor Prof. Martin Warren for continuous support throughout my study and research and for his patience, motivation, enthusiasm and immense knowledge.

Special profound gratitude goes to my enthusiastic and most dedicated supervisors Dr Evelyne Deery and Dr Andrew Lawrence for their tremendous academic support and guidance. Their support has helped me in all the time of research and writing of my thesis. I could not have imagined having a better supervisors for my Master's degree.

My special thanks also goes to my fellow lab mates: Emi Nemoto-Smith for her support and advice with my research and writing my thesis, Maria Stanley, Naziyat Khan, Joe baker, Rokas Juodeiskis, Mathew Lee for making my experience in laboratory extra special. You have been my family and life time friends your love and support will be with me and I have very fond memories of my time in Warren lab.

Lastly, without unconditional love and support of my family I would not have been where I am today. I am forever in debt to you.

Abstract

Cobalamin (vitamin B_{12}) is the anti-pernicious anaemia factor that is made exclusively by certain prokaryotes. It is constructed along one of the most complex biosynthetic pathways found in nature, involving around 30 enzymes-mediated steps. In the aerobic cobalamin biosynthesis pathway, the central cobalt ion of the corrin component of cobalamin is inserted into a comparatively late pathway intermediate called hydrogenobyrinic acid-*a,c*-diamide (HBAD) to give cobyric acid. This section of the pathway appears to be very sensitive to feedback inhibition and hence there is a lot of interest in how HBAD is made. Surprisingly, there is no structural information about the enzyme that makes HBAD, CobB. This enzyme takes hydrogenobyrinic acid and amidates the *a* and *c* side chains to give HBAD. It is thought that CobB interacts closely with the preceding enzyme in the pathway, CobH, which is known to bind its product, hydrogenobyrinic acid (HBA), very tightly. In this project, attempts have been made to understand the structure and function of CobH and CobB through the application of protein crystallisation and X-ray crystallography. It is thought that CobB may interact with CobH in such way as to release the product and allow its amidation. Herein, the *Allochromatium vinosum* CobH and CobB have been purified and entered into a broad range of different crystal screens. The interaction between the two proteins has also been investigated using standard biochemical techniques. Although the CobB enzyme was observed to be more stable than CobB from other organisms, the CobH was found to be prone to proteolysis. The work suggests that the structure determination of CobB should be possible.

Contents

Chapter 2 Methods

Abbreviations

ALA: Aminolevulinic acid

ALAD: ALA dehydrates

$CaCl_2$: Calcium Chloride

Cbi: Cobalamin

DEAE: Diethylaminoethyl

DNA: Deoxyribonucleic acid

Gm: gram

Hb: Haemoglobin

HBA: Hydrogenobyrinic acid

HBAD: Hydrogenobyrinic acid diamide

IPTG: Isopropyl-beta-D-thiogalactopyranoside

kDa: Kilo Dalton

LB: lurina-Bertani

NaCl: Sodium Chloride

Nm: nanometre

HCl: Hydrochloric acid

OD: Optical density

PAGE: Polyacrylamide gel electrophoresis

PCR: Polymerase chain reaction

Rpm: Revolutions per minute

SAH: S-adenosyl-L- homocysteine

SAM: S-adenosyl-L- Methionine

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide

SUMT: S-adenosyl-L- Methionine uroporphyrinigen III methyltransferase

TEMED: N, N, N', N'- Tetramethylenediamide

Chapter 1

Introduction and overview

1.0 Biosynthesis of tetrapyrroles

Tetrapyrroles are the large macrocyclic compounds which are categorised as the most abundant pigment in nature (Warren & Smith 2009) [3]. These macromolecules are important for living organisms as well as being involved in biochemical reactions. As the name suggests tetrapyrroles contain four pyrrole-derived compounds which are either linked in linear or cyclic form via methine bridges with exception of corrinoids that lack one carbon bridge in their ring system (Scott & Roessner 2002) [6]. The cyclic tetrapyrroles are denoted A-D in a clockwise direction (Warren & Smith 2009) [3].

Cyclic tetrapyrroles include porphyrins which are the most recognised class as molecules such as hemes and chlorophylls belong to this group. The red colour of blood is due to presence of heme subunits in haemoglobin protein and the green colour of plants, algae, and some bacteria is due to presence of chlorophyll. Furthermore, the other tetrapyrrole class known as porphynoids include naturally occurring members such as cobalamin (vitamin B_{12}), siroheme, coenzyme F_{430}, heme d_1 and phytochromobilins (Warren & Smith 2009) [3].

The biosynthesis of tetrapyrroles in eukaryotes is restricted to production of heme, siroheme, chlorophyll and bilins where in prokaryotes more complicated form of

tetrapyrroles such as cobalamin, coenzyme F_{430} and heme *d*$_1$ are produced as shown in figure 1 (Warren & Smith 2009) [3].

The cleavage of cyclic tetrapyrroles results in formation of linear tetrapyrroles hence they only contain three methane bridges. The only exceptions are corrinoids as their ring system don't contain one carbon bridge between the first and fourth pyrrole ring. The cyclic tetrapyrroles are able to chelate variety of metal ions and this important property allows ions such as Fe^{2+}, Mg^{2+}, Co and Ni^{+} to enter the centre of the pyrrole ring (Warren & Smith 2009). The outcome of the metal ion attachment is formation of the tetrapyrrole family which originate from uroporphyrinogen III as shown in figure 1 (Dailey et al. 2013) [21].

Uroporphyrinogen III is the initial cyclic intermediate that can be converted into two products known as coproporphyrinogen III and precorrin-2. Coproporphyrinogen III is used in the synthesis of hemes, chlorophylls and bacteriochlorophylls, whereas precorrin-2 contributes in the production of siroheme, heme *d*$_1$, coenzyme F_{430}, and cobalamin (Warren & Smith 2009) [3].

The synthesis of the complex tetrapyrroles requires conversion of uroporphyrinogen III into precorrin-2 by the action of uroporphyrinogen III methyltransferase. Furthermore, in order to synthesise haem and chlorophyll, uroporphyrinogen III is decarboxylated into coproporphyrinogen III due to the activity of an enzyme known as uroporphyrinogen III decarboxylase (Warren & Smith 2009) [3].

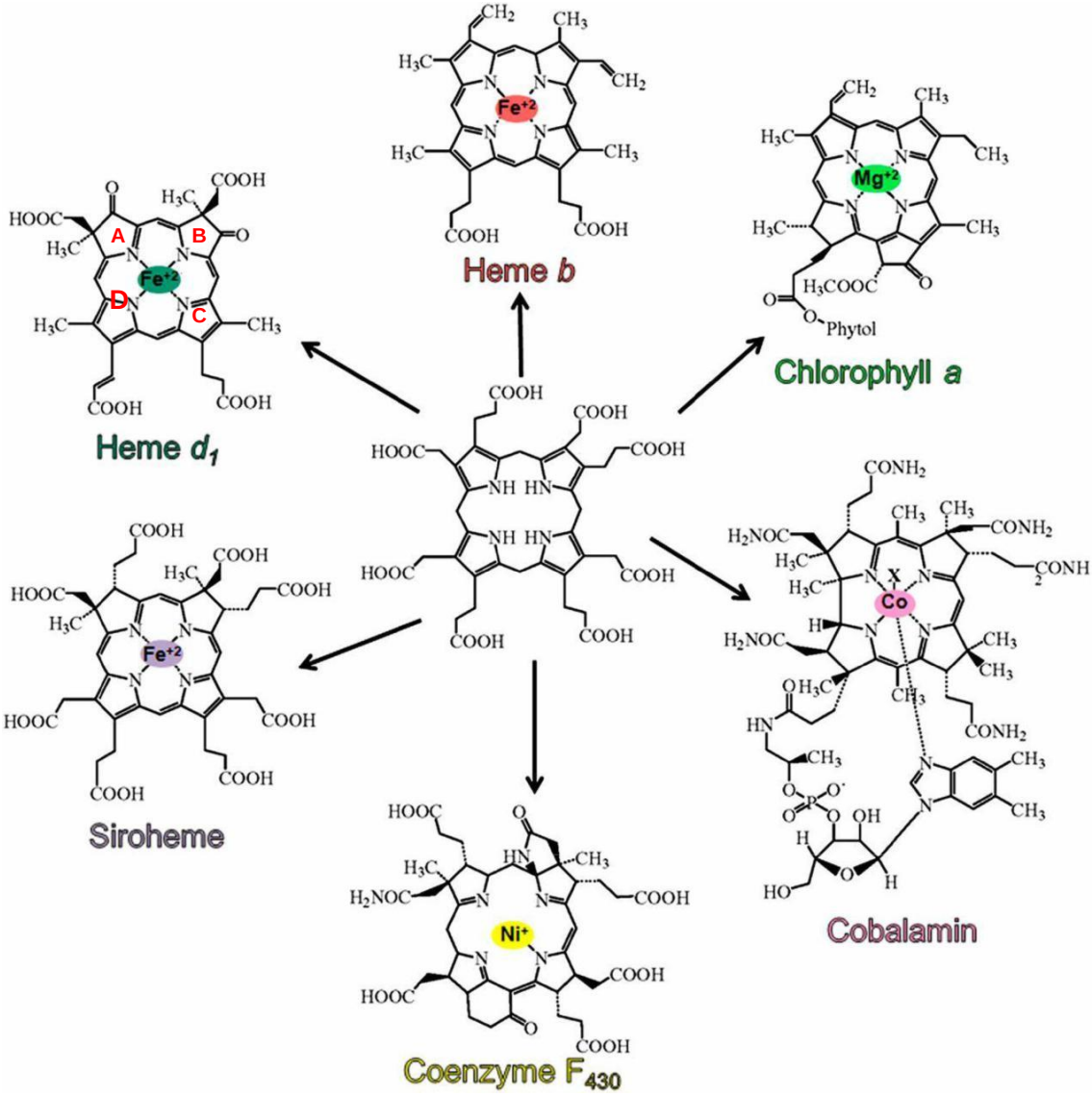


Figure 1: Group of modified tetrapyrroles that are found in nature are driven from uroporphyrinogen III (in the centre) which is the first branch point of the pathway. Chlorophyll is the main component that absorbs light in photosynthetic reactions. Heme is the main requirement in oxygen and electron transport system as well as being the part of enzymes such as catalases and proxidases. Haem b is the most abundant haem that is found in the oxygen transporter proteins such as haemoglobin and myoglobin, heme d_1 is essential in the denitrification of nitrate salts, siroheme plays an important role in sulphur assimilation pathway and nitrite reductases, coenzyme F_{430} is essential in reducing co-enzyme M in methanogenesis and cobalamin functions as a coenzyme in variety of rearrangements, reductions and methylation processes (Warren & Smith 2009)[3] (Raux et al. 2000)[11] (Dailey et al. 2013) [21] .

1.1 Introduction and Overview of Vitamin B_{12}

Vitamin B_{12}, also known as cobalamin, is a modified tetrapyrrole. The architecture of this macromolecule shows the presence of the central cobalt ion that contains an upper and lower ligand. The lower ligand of cobalamin is 5, 6-dimethylbenzimidazole (DMB), which is attached to the corrin ring by a nucleotide loop. The upper ligand in biologically active forms of the nutrient is generally either an adenosyl or methyl group, which are covalently attached to the cobalt ion,forming a unique cobalt-carbon bond (Warren & Smith 2009) [3].

Cobalamin is one of the body's essential vitamins and helps to maintain the regular function of the nervous system, brain and production of red blood cells. This organic compound is not naturally produced by humans, therefore it has to be consumed through diet in order to reach the optimum level that is required for the body to function in good health. Vitamin B_{12} deficiency affects the function of two metabolically important enzymes; methionine synthase and methyl malonyl CoA mutase. Changes to the function of these enzymes result in the rise of conditions such as pernicious anaemia, neurological disorders and other diseases that are associated with methionine metabolism. (Warren & Smith 2009) [3].

Plants and animals are incapable of producing cobalamin as they do not have the cobalamin synthesis enzymes. This fact emphasises the distinctive property of vitamin B_{12} as it is the only vitamin that is produced exclusively by bacteria or archaea, and not by any eukaryote.

The biosynthesis of cobalamin requires several steps which are dependent on the presence of enzymes that initiate the modification of the tetrapyrrole framework by

peripheral methylation, ring contraction, decarboxylation, cobalt insertion, amidations, lower nucleotide loop assembly and attachment, and upper ligand attachment.

Cobalamin is synthesised through two known routes which are referred to as the aerobic and anaerobic pathways.

1.2 The Aerobic and Anaerobic Biosynthesis Pathways

The synthesis of cobalamin requires several enzymes which can be divided into two distinct routes and for this reason the genes that code for the enzymes contributing in aerobic pathway are given the prefix Cob while the genes of the anaerobic pathway have been given the prefix Cbi (Warren & Smith 2009) [3].

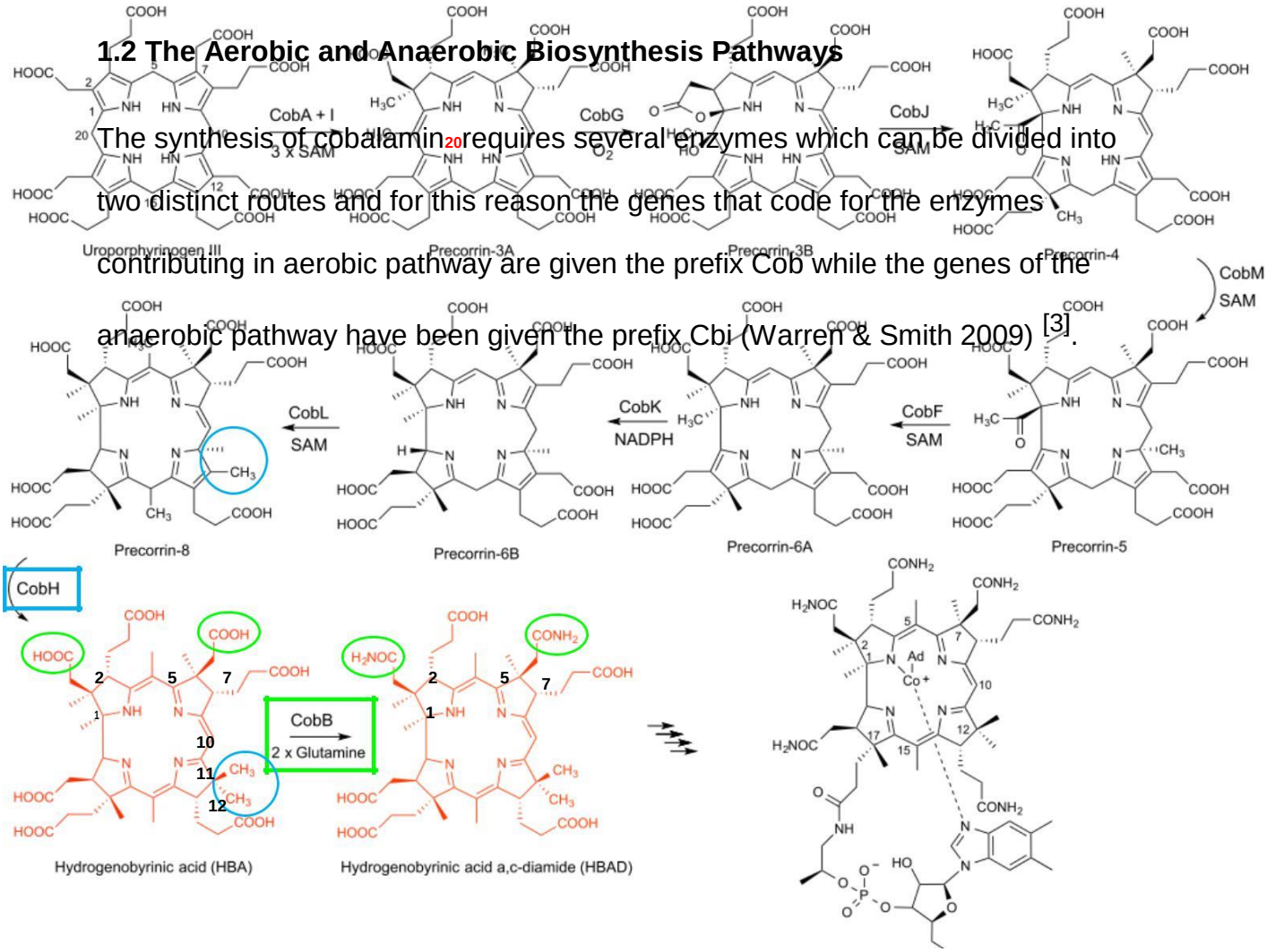


Figure 2: The image above showing the aerobic cobalamin synthesis pathway. The conversion of Uroporphyrinogen III to B_{12} is a process that is catalysed by enzymes that are responsible for the chain of reactions that include peripheral methylation, ring contraction, decarboxylation, cobalt insertion, amidation, lower nucleotide assembly and attachment of an upper ligand. The numbering of the carbon atoms and the order of enzymes appearance during this activity are also shown (Warren and Deery, 2009) [11].

1.2 The Aerobic and Anaerobic Biosynthesis Pathways

The aerobic pathway is known as the early cobalt insertion and anaerobic pathway is the late cobalt inclusion. However, regardless of the differences, the process of methylation and amidation of tetrapyrroles in both pathways are followed in the same order which is an indication of noticeable similarities between the two routes as shown in figure 3 (Deery et al. 2012) [1].

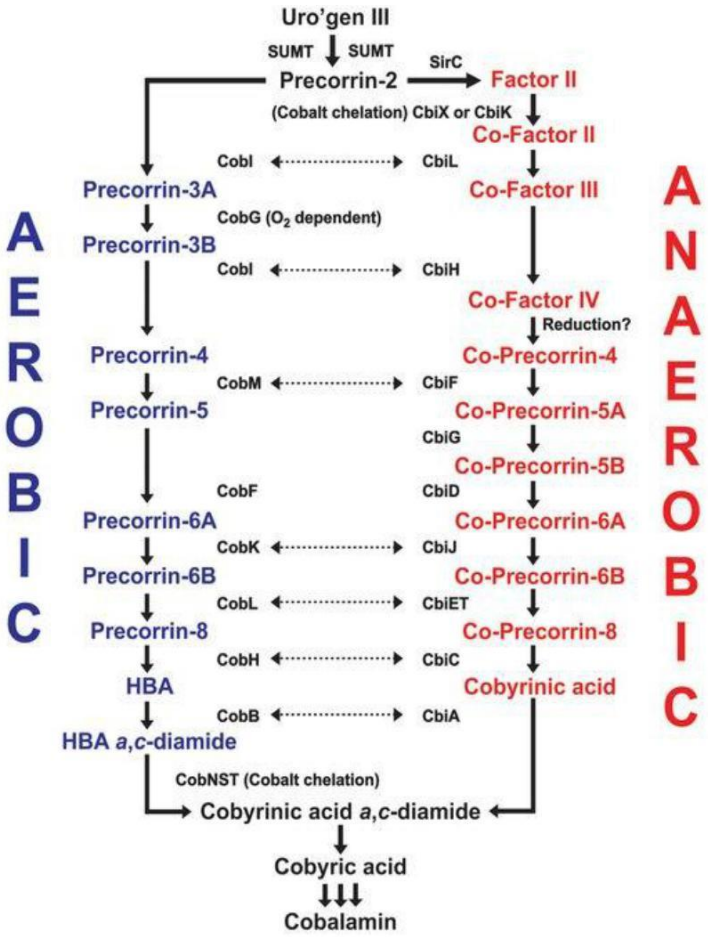


Figure 3: The aerobic (blue) and anaerobic (red) biosynthetic of cobalamin from precorrin-2 to cobyrinic acid *a,c*- diamide. Many biosynthetic steps are predicted from sequence comparison (Moore & Warren 2012) [7].

These pathways are distinguished by the necessity of oxygen. The late insertion pathway requires molecular oxygen as an essential requirement for ring contraction and for this reason it has been termed the aerobic pathway.

The aerobic pathway requires five S-adenosyl-L-methionine (SAM) dependent methyltransferases in order to introduce six methyl groups which are categorised by combination of oxygen molecule. Due to the activity of an enzyme CobG acts as a monooxygenase as it combines oxygen with carbon 20 before ring contraction of the macrocycle begins. The enzyme CobG also catalyses the combination of oxygen also at position C20 followed by the formation of - lactone in order to produce precorrin-3 hydroxylactone (figure 2) which are the initial requirements for ring contraction (Scott & Roessner 2002) [6].

The intermediates involved in the aerobic biosynthetic pathway from uroporphyrinogen III to adenosylcobalamin have been determined by isolation of genes from *Pseudomonas denitrificans* and the function of the encoded enzymes was allocated by their catalytic activity.

Moreover, specific genes encoding for proteins that are involved in the anaerobic biosynthesis of cobalamin have been isolated from bacteria such as *Salmonella typhimurium*, *Bacillus megaterium* and *Propionibacterium freudenreichii* (Scott & Roessner 2002) [6].

Unlike the aerobic pathway, the catalytic function of the anaerobic enzymes has been determined through experimenting a few gene products and comparison of the genome sequence has allowed identifying their function (Scott & Roessner 2002) [6] .

One feature of cobalamin that distinguishes it from other tetrapyrroles, is the insertion of the central cobalt ion. The biosynthetic pathway to the production of adenosylcobalamin from its five carbon precursor, 5-aminolaevulinic acid (ALA) is divided into two main stages. The initial step is the production of uroporphyrinogen III from ALA which is a common step through both aerobic and anaerobic pathway.

The second step involves conversion of uroporphyrinogen III into ring contracted and deacylated intermediates known as precorrin-6 which is the intermediate of the aerobic pathway or cobalt-precorrin 6 which is the anaerobic equivalent.

1.2.1 Differences between aerobic and anaerobic pathway

The differences between the aerobic and anaerobic pathway is reflected at a step following the ring contraction in which precorrin-4 is converted to precorrin-6 in aerobic pathway and cobalt-precorrin-4 to cobalt-precorrin-6 in the anaerobic pathway (Santander et al. 1997) [19].

This transformation in anaerobic biosynthesis requires opening of the -lactone ring followed by methylation at C11, removal of acetaldehyde and methylation at C-1. Due to the opening of the -lactone ring oxygen is exchanged at C-27 acetate site which results in extrusion of acetaldehyde followed early insertion of the cobalt ion. However in aerobic biosynthesis the oxygen exchange step at C-27 acetate site is not required therefore acetic acid is extrude instead. For this reason the insertion of the cobalt in this pathway occurs after the ring contraction stage is complete (Santander et al. 1997) [19].

Moreover, in anaerobic pathway the conversion of cobalt-precorrin-6B to cobalt -precorrin-8 requires an intermediate step which is catalysed by the action of CbiT followed by CbiE (Negro et al. 2013) [20] .The bifunctional enzyme CbiT converts cobalt-precorrin-6B to cobalt-precorrin-7 by decarboxylating and methylating carbon-15. This event is followed up by the action of CbiE which methylates cobalt-precorrin-7 at carbon-5 in order to produce cobalt-precorrin-8 (Negro et al. 2013) [20].The conversion of precorrin-6B to precorrin-8 in aerobic pathway is a more straight forward process that only requires one bifunctional enzyme known as CobL.

The transformation of the named intermediates to adenosylcobalamin is one of the main differences that distinguishes aerobic pathway from anaerobic pathway. (Scott & Roessner 2002) [6].

1.3 Synthesis of precorrin-8, the substrate for CobH

The intermediates involved in the aerobic biosynthesis are classified as “precorrin-n” in which (n) specifies the number of methyl groups that are attached to the framework at each step of the pathway shown in figure 2.

The first two methyl transfers are catalysed by a homodimer known as S-adenosyl-L-methionine uroporphyrinogen III methyltransferase (SUMT or CobA) shown in figure 1. This enzyme transfers methyl groups donated by S-adenosyl-L-methionine (SAM) to position C2 and C7 of uroporphyrinogen III, producing precorrin-2. This reaction can be inhibited by a competitive inhibitor known as S-adenosyl-L-homocysteine (SAH), the secondary product of this methylation reaction (Warren & Smith 2009) [3]. CobI methylates precorrin-2 at position C20, producing precorrin-3A which is a substrate for the monooxygenase enzyme CobG. This enzyme generates tertiary alcohol at C20 and forms a -lactone with acetate sidechain on ring A (precorrin-3B).

Due to the presence of tertiary alcohol at C20 of this intermediate, precorrin-3B is set to catalyse ring contraction by a SAM dependant precorrin-3B methyltransferase also known as CobJ. This enzyme catalyses C17 methylation by addition of a methyl group from the top plane of the macrocycle unlike other methyl groups at position C1, C2, C7 and C12 which are located on the lower side of the corrin molecule (Negro et al. 2013) [20] . This particular functional property of CobJ suggests that the substrate of this enzyme binds the active site by orientating in the opposite direction and for this reason the acetate and propionate side chains of ring D where C17

methylation takes place are in an inverse orientation (Negro et al. 2013) [20]. The final product of CobJ activity is precorrin-4. This product is substrate for another methyltransferase known as CobM. This enzyme methylates precorrin-4 at position at C 11 of the porphyrin ring to form precorrin-5. Studies have shown that the methyl groups of corrin macrocycle are positioned at C12 and not C11 (Negro et al. 2013) [20]. This specific fact suggests that CobM is a multifunctional enzyme as it methylates C11 in order to allow decarboxylation of acetate side chain which is attached to C12.

The outcome of this reaction contributes in authorizing the function of the subsequent enzyme (Negro et al. 2013) [20]. Further along the pathway is CobF which removes the methyl ketone at position C1 and then methylates the same position and for this reason CobF is best known as a multifunctional enzyme that contains both properties of deacetylase and methyltransferase (Negro et al. 2013) [20]. Both methyltransferases CobM and CobF eliminate acetyl group as acetic acid at position C20 and add the methyl groups at C11 and C1 position. Formation of precorrin-5 and precorrin-6A is the outcome of these reactions (Negro et al. 2013) [20]. Unlike other stages of cobalamin synthesis and the role of enzymes that are involved, CobK causes ring reduction by reducing the double bond between C18 and C19 in presence of the reducing agent NADPH transforming precorrin-6A to precorrin-6B in presence of this reducing agent.

CobL is a multi-functional homo-octameric enzyme which is responsible for methylations at C5 and C15 as well as decarboxylation of the acetate side chain in order to allow the conversion of precorrin-6B into precorrin-8 (Deery et al. 2012) [1].

Data from sequence comparison between the N-terminal and the C-terminal of CobL has determined that the C-terminal region is most likely to take part in decarboxylation and methylation at C15 whereas the N-terminal region hosts the methyltransferase activity at C5 (Warren et al. 2002) [2]. This was subsequently confirmed by biochemical experiments involving dissection of the protein into functional N- and C- terminal domains (Deery et al. 2012 [1], Negro et al. 2013) [20].

1.4 Hydrogenobyrinic Acid (HBA) synthesis

The subsequent step in completing the corrin macrocycle is catalysed by a homodimeric enzyme known as CobH, which converts precorrin-8 into hydrogenobyrinic acid (HBA). This transformation involves the migration of the methyl group attached to C-11 onto C12 through a reaction involving a 1, 5-sigmatropic rearrangement as shown in Figure 2.

CobH was initially discovered as a protein that binds hydrogenobyrinic acid very tightly before it was shown that the enzyme acts as the catalyst that is responsible for methyl group movement within precorrin-8.

The movement of the methyl group has a great effect on the conjugated π-system of the macrocycle which can be detected as the molecule changes colour from yellow to pink due to a visible hypochromic shift.

The CobH protein has recently been crystallised and the structure reveals that CobH is a dimer with two independent active sites as shown in figure 4. This small enzyme has a subunit molecular mass of around 23 kDa with a highly conserved histidine residue that is found in the active site. Previous studies have shown that CobH has high binding affinity for its product hydrogenobyrinic acid (HBA), which binds with a

ratio of 2 protein monomers per 1 molecule of HBA (Thibaut et al. 1992)[4] (Deery et al. 2012)[1].

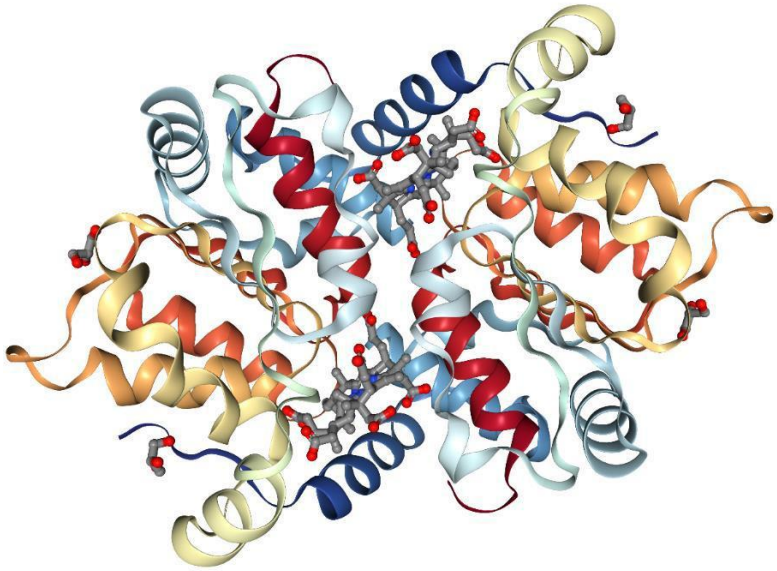


Figure 4: Crystal structure of CobH bound to two molecules of HBA which are located into two separate binding sites located at the central top and bottom of this image.

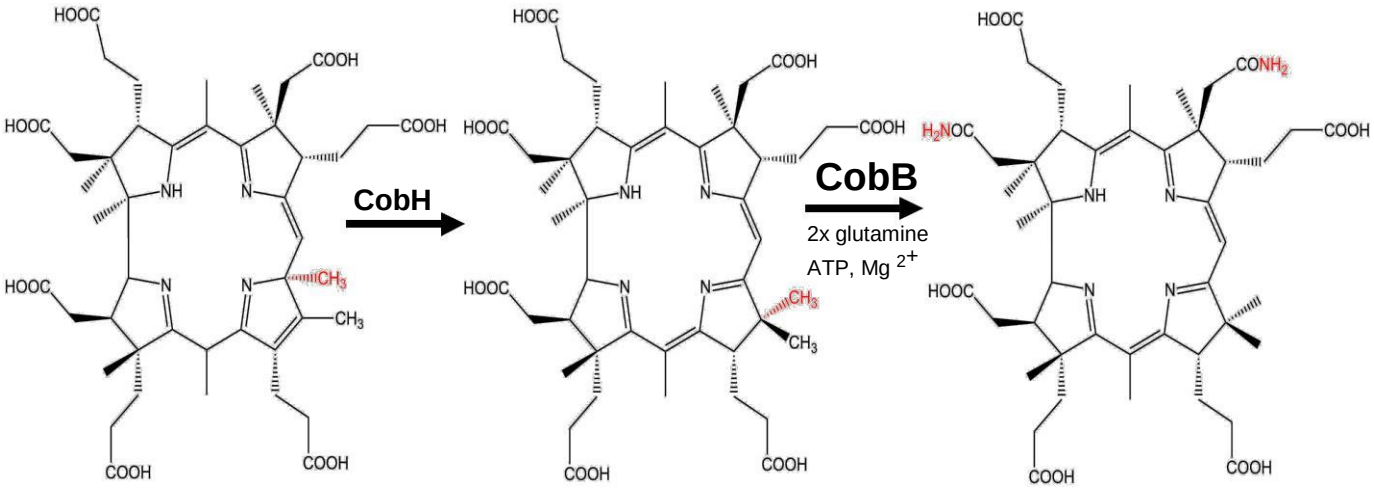
1.5 Hydrogenobyrinic acid *a, c*-diamide synthesis

The enzyme responsible for the conversion of HBA into HBAD is called CobB and was first identified in *P denitrificans* Biochemical characterisation has shown the protein to be a homodimer with a molecular mass of 45 kDa in solution. This enzyme is responsible for amidation of the *a* and *c* side chains of HBA in order to synthesise hydrogenobyrinic acid a,c-diamide.

Generating HBAD is an energy consuming process involving the consumption of Mg^{2+}-ATP for the transfer of the amido group from each of the two glutamine molecules to the acetyl side chain of hydrogenobyrinic acid.

The first amide group is transferred to the side chain of carbon C2 (*a*-chain) and the second amide group is attached to C7 (*c*-chain) of the HBA molecule as shown in

figure 5. The product, hydrogenobyrinic acid *a,c*-diamide (HBAD), is the substrate required by cobaltochelatase, which inserts cobalt into the macrocycle.



Precorrin-8 (PC8) **Hydrogenobyrinic acid (HBA)** **Hydrogenobyrinic acid *a, c* diamide (HBAD)**

Figure 5 : A step in the aerobic (late cobalt insertion) pathway of adenosylcobalamin biosynthesis which shows how CobH catalyses the conversion of precorrin-8 to hydrogenobyrinic acid (HBA) by methyl migration from C11 to C12. CobB generates hydrogenobyrinic acid a,c-diamide, by amidating the *a* and *c* side chain of HBA. The product HBAD is the substrate required by cobaltochelatase, which adds cobalt to the macrocycle.

1.6 Principle of substrate channelling

The principle of substrate channelling defines a mechanistic process in which the intermediate of a reaction is directly delivered from the active site of one enzyme to the active site of a second enzyme without the need of prior dissociation into the solvent. Enzyme channels are referred to as molecular gates which are able to control the transport of substrates through biomolecular systems by monitoring the individual substances before allowing or denying their access to specific sites or within the macromolecules by reversibly switching between open and closed conformation. As a result of this, the product from one active site is actively or passively translocated directly to the active site of another enzyme.

Metabolic channelling aligns the catalytic sites of the enzymes in order to facilitate the rapid movement of intermediates, decreases their transmission time and prevents their loss after diffusing into the surrounding environment.

Substrate channelling can occur in multifunctional enzymes, tightly bound multi enzyme complexes, or transient enzyme complexes.

Aims and objectives

The aim of this project is to investigate the conversion of precorrin-8 into HBAD, focusing on how tightly bound products are released and made available for subsequent enzymes. It has been suggested that substrate channelling is involved in this step of cobalamin biosynthetic pathway. Such direct metabolite transfer from one enzyme to the next must involve some kind of protein-protein interactions. The interaction has to involve conformational change to allow the donating enzyme to release its product and the accepting enzyme must bind it more tightly than the donor. The CobB and CobH enzymes represent an excellent model system in order to study the molecular process.

In this work both CobH and CobB from *A*. *vinosum* were used to study this process. Previous studies on CobBs have shown that these enzymes have difficulty with turning over substrate, which means that the enzyme's catalytic efficiency is low. However, *A. vinosum* CobB was found to be most conformationally stable in comparison to the other studied CobBs. The greater activity of the *A. vinosum* CobB, coupled with its higher stability, suggested that this would be the best enzyme system to work with.

Although the *A. vinosum* CobB had previously been cloned, the CobH had not been characterised from this bacterium. Hence, the work here also involved the cloning and characterisation of the *A. vinosum* CobH. The characterisation of these enzymes

involved the use of a variety of techniques including *in vivo* trapping, *in vitro* binding and crystallography.

Chapter 2 -Methods

2.0 Growth medium

1. LB/LB agar

10 g Tryptone

5 g Yeast extract

5 g NaCl

1 litre dH_2O

+/- 15 g/L agar

Autoclave

2. 2YT medium

2x Yeast Extract Tryptone is a nutritionally rich growth medium which is designed for growth of recombinant strains E.coli. The growth is more rapid in this rich medium as it contains essential nutrients, growth factors that the strain requires.

16 g Tryptone

10 g Yeast extract

5 g NaCl

1 L dH_2O

Autoclave

3. Antibiotics

- Ampicillin at final stock concentration of 100 mg/mL

- Chloramphenicol at final stock concentration of 34 mg/mL in ethanol

4. Addition of glucose

Glucose with final concertation of 20%

2.1 Starter culture and Glycerol stock

One colony from the transformation plate is added to 5 mL of LB containing ampicillin at 100 mg/mL final concentration, chloramphenicol at a final concentration at 34 mg/mL and 50 µL of glucose with a final concentration of 20%. The mixture is incubated overnight at 28 ºC shaking incubator. The glycerol stock of bacteria was made by resuspending 300 µL of overnight starter culture and into 700 µL of 50% glucose. The mixture is immediately frozen at -80 ºC.

2.2 Inoculating starter culture into 1 litre LB

The overnight starter culture was added to one litre LB as well as addition of 1 ml of 100 mg/mL Ampicillin and 34 mg/mL Chloramphenicol. The strain is grown at 28 ºC, 160 rpm incubator until the OD600= 0.3 is reached. The protein production was induced by adding 400 µL of isopropyl 1-thio-β-D – galactopyranoside (IPTG) from 1M stock solution. The litre is incubated at 19 ºC, 160 rpm overnight.

2.3 Harvesting Bacteria

The culture was centrifuged at 4000 rpm for 20 minutes in order to allow separation of the bacterial pellet from the medium.

The bacteria are resuspended in 30 ml of the binding buffer that contained 20 mM Tris-HCl pH 8 + 500 mM NaCl +5 mM imidazole pH 7.

2.4 Protein purification

2.4.1 Cell lysis by sonication

The resuspended cells in binding buffer were used directly for protein purification. Transferred cells into the sonication vessel and that is surrounded by ice water. The Vibracell Ultrasonic processor was set for 30 seconds on 30 seconds off pulses with 50% amplitude for 5 minutes. Once the cells were fully broken up, 50 µL of the protein is collected in the Eppendorf tube for SDS-PAGE. After sonication, the cells were centrifuged at 18000 rpm for 20 minutes and another 50 µL sample was collected for SDS-PAGE.

2.4.2 Purification of recombinant protein using IMAC

Steps in preparing the Nickel column:

1. The column was prepared by loading the empty column with 5 ml of Chelating Sepharose^{TM} and washed with buffer to remove the storage ethanol and allow the resin to pack.

2. The column was charged by using 15 ml of charge buffer 50 mM NiSO4.

3. Equilibrated the column by 20 ml of the binding buffer that was made up of 20 mM Tris-HCl pH 8 + 500 mM NaCl + 5 mM imidazole.

4. It was essential to collect flow- through samples of each step so they can be compared to previous samples by SDS-PAGE

Steps in IMAC purification:

1. The supernatant was loaded onto the column after centrifugation.
2. The first wash required 20 ml of the binding buffer that was used at the prior step,
3. **Wash I:** 10 ml of wash buffer that contained 20 mM Tris-HCl pH 8 + 500 mM NaCl + 50 mM imidazole.
4. **Wash II:** using 5 ml of wash buffer II which was made up of 20 mM TRIS-HCl pH 8 + 500 mM NaCl + 100 mM imidazole.
5. **Protein elution:** 5-10 ml elution buffer containing 20 mM Tris-HCl pH 8 + 500 mM NaCl + 400 mM imidazole.
6. **1 ml fractions** were collected for Bradford assay test as well as SDS-PAGE.
7. **Regeneration of Chelaing Sepherose** by 15 ml of strip buffer made of 20 mM Tris-HCl pH 8 + 500 mM NaCl + 100 mM EDTA.

2.4.3 Bradford Protein Assay

This assay depends on the dye Coomassie blue G250, which is present in the Bradford reagent solution , changing colour from red to blue in presence of protein, to provide a rough estimate quantification of protein concentration in each fraction that had been collected during elution. Drops of 10 µL dH2O were put on a sheet of parafilm to which 5 µL of the Bradfor reagent solution was added and mixed thoroughly by pipetting the mixture up and down. Then 5 µL of each elution fraction was then added to the mixture and depending on the concentration of protein in each fraction the mixture turned blue.

2.4.4 Buffer exchange PD10 column

The most concentrated fractions from the Nickel column were chosen to run on a desalting PD10 column. The protein from prior step contains a high concentration of imidazole, thus applying the sample to this column allowed removing the excess salt.

1. The column was equilibrated by 25 ml of the buffer that contained 20 mM Tris-HCl pH 8 + 100 mM NaCl
2. The total volume of 2.5 ml of the sample was added to the column.
3. Protein was collected by adding 3.5 ml of elution buffer containing 20 mM Tris-HCl pH 8 + 500 mM NaCl + 400 mM imidazole.

2.4.5 SDS-PAGE

10% Acrylamide separation gel		**12.5% Acrylamide separation gel**		**5% Acrylamide stacking gel**	
dH_2O (ml)	4.7	dH_2O (ml)	3.4	dH_2O (ml)	3.4
30% Acrylamide (ml)	5	30% Acrylamide (ml)	6.3	30% Acrylamide (ml)	1.5
1.5M Tris-HCl, pH 8.8 (ml)	3.8	1.5M Tris-HCl, pH 8.8 (ml)	3.8	0.5 M Tris-HCl, pH 8.8 (ml)	1.9
10% SDS (ml)	1.5	10% SDS (ml)	1.5	10% SDS (ml)	0.75
10% APS (ml)	0.15	10% APS (ml)	0.15	10% APS (ml)	0.075
TEMED (ml)	0.01	TEMED (ml)	0.01	TEMED (ml)	0.01

Table 1: Polyacrylamide gels were set up as outlined in above table. The gels were run at 200 volts for 1 hour in order to allow separation of protein subunits as it travels along the gel.

1. Approximately 50 µL of the fractions that had been collected at different stages of the purification are mixed with 50 µL of 2 times laemmli buffer and boiled for 5 to 10 minutes.
2. The gel was securely placed into the tank and addition of 10x running buffer filled up the wells.
3. 10 µL of individual samples were loaded into each well of the SDS-gel
4. The gel was run at 200 volts for 1 hour and stained with coomassie blue stain
5. The gel was destained by several washes with dH_2O or overnight in dH_2O on a rocking platform.

2.5 Gel Filtration- FPLC

This method is used for size exclusion of the protein which is purified through the Superdex ^{TM} 200 10/300 GL resin.

The column was washed with Milli Q water or double distilled (ddH_2O) and then equilibrated with a buffer containing 20 mM Tris-HCl pH 8 + 100 mM NaCl. Around 1.5 ml of the most concentrated protein sample is injected into the column and 12 fractions were collected at the end of the run. The generated evaluation graph indicated the highly concentrated fragment by showing the highest absorbance peak at 280 nm

The most concentrated fraction was picked to for purity check through SDS-PAGE before being used for crystallography.

The column was then cleaned by another wash with Milli Q water and final equilibration in 20% ethanol to prevent microbial growth.

2.6 HBA purification

2.6.1 DEAE - Ion exchange chromatography (HBA –ve, DEAE +ve)

Around 1 ml -1.5 ml of DEAE Sephadex column solution is loaded into a syringe. After the resin has settled, it must be equilibrated with 25 mL of 20 mM Tris-HCl pH8 + 100 mM NaCl in order to reach pH8 before loading the supernatant. The supernatant of the HBA producing culture is applied to the column and washed several times with increasing concentration of NaCl. This separates any impurities from HBA.The bright orange substrate, HBA is ready to be eluted when the concentration of salt reaches 300 mM (figure 6).

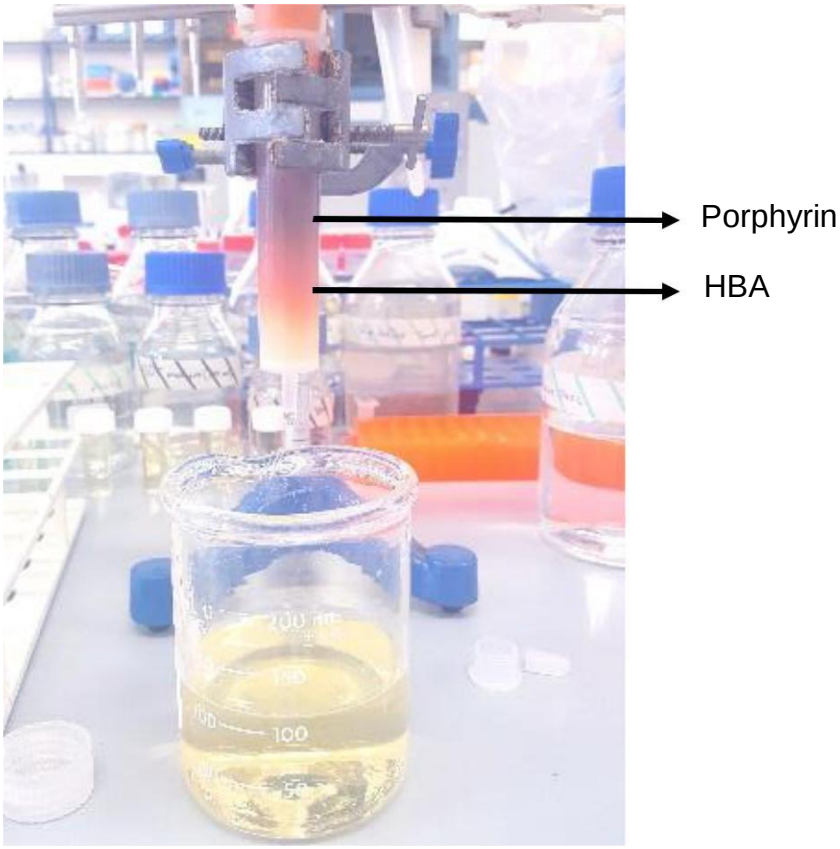


Figure 6: the DEAE column after applying the HBA supernatant to the resin. The separation of the materials takes place as the concentration of salt gradually increases. HBA elutes after the addition of 300 mM NaCl and porphyrin is lastly removed by addition of 1M salt.

2.6.2 RP18 desalting column

The samples had to be acidified with 0.5 M HCl to a pH below 4 after elution from DEAE column. The sample was then centrifuged at 18000 rpm for 20 minutes in order to allow separation of the impurities from the sample.

RP18 column preparation

Washed column with 3 ml of acetonitrile

Equilibrate column with 10-15 ml of 0.1%

TFA Applied the sample of HBA

Wash sample

Wash I: 0.1% TFA

Wash II : 5% ethanol in 0.1% TFA

Wash III: 10% ethanol in 0.1% TFA (around 10mls)

Wash V: 20% ethanol in 0.1% TFA (collect fractions, keep adding until all HBA is removed)

Eluted with 50% ethanol

The column can be cleared by urea if column was washed with 5% ethanol

2.7 Crystallography

The protein was concentrated using an Amicon Ultra-4 centrifugal filter to the desired concentration. Approximately 1mL of purified CobB with initial concentration of 2.5 mg/mL was concentrated to 6 mg/mL by continuous centrifugation. The sample is then used for carrying out the crystallisation process.

Conditions were screened using a structural screen 1 and 2 kit from Molecular dimensions. The hanging drop vapour diffusion method was used, in which 500 µL of molecular dimension condition solutions were pipetted into a 24 well invro plate.

Each well was sealed with a glass cover slip that had two drops on in order to examine the crystallisation process of protein in presence and absence of the substrate. The ratio of 5:1 of HBA is added to the protein. For this purpose, 2 µL of the well condition was added on top of 1 µL of 6 mg/ mL protein + 1 µL of HBA (3.53mM) is put on each glass covers.

Orange drop – HBA or HBAD (1 µL of 3.52 mM stock) + protein (1µL of 6 mg/mL)

White drop – Protein only: 2 µL CobB (6 mg/mL)

For enhancing the chances of crystal formation the protein of interest is mixed with other Co-factors which include ATP, magnesium and glutamine.

Magnesium 10x protein cencentration

ATP 5x protein concentration

Glutamine 5x protein concentration

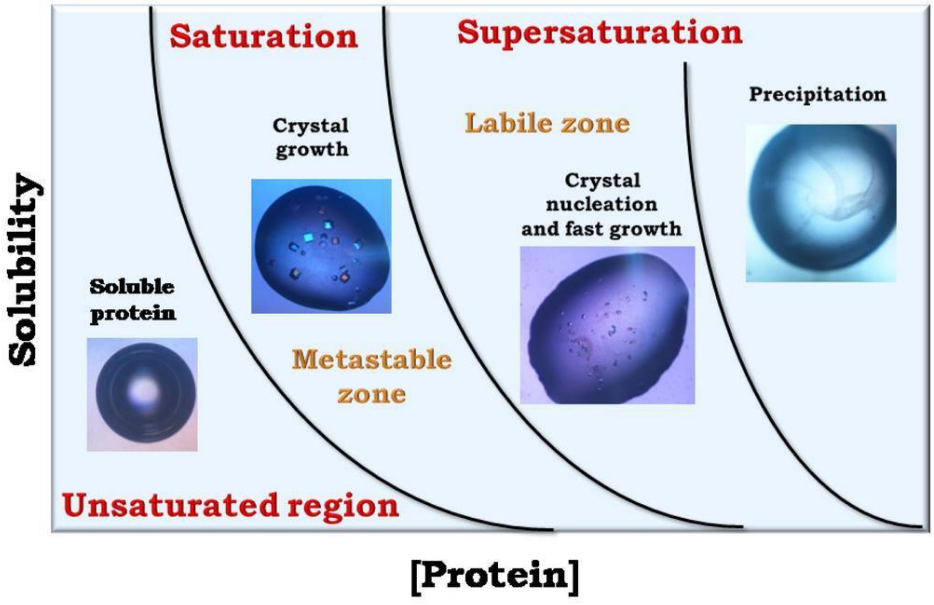


Figure 7: The two-dimensional phase diagram shows the changes in protein concentration against the precipitating agent concentration. The solubility curve divides the changes in state of protein concentration into two regions known as unsaturated and supersaturated region. The supersaturated area includes the metastable, nucleation and precipitation zones.

2.8 Thrombin Cleavage

10x Thrombin cleavage	1x Thrombin cleavage
200 mM Tris-HCl pH 8.4 1.5 M NaCl 25 mM $CaCl_2$	20 mM Tris-HCl pH 8.4 150 mM NaCl 2.5 mM $CaCl_2$

Depending on the concentration of protein 1 unit of thrombin is used for 1 mg of protein. The mixture could either be kept at 25 ^{o}C room temperature or kept in the fridge overnight (4 ^{o}C).

Once the His-tag was cleaved from the protein it had to be filtered through Nickel column. This ensures that the His-tag is fully cleaved as the protein was eluted straight after being loaded on to the column. Then the sample was also run through PD10 column to further purify the protein and make sure that it is in the right buffer before the gel filtration purification.

2.9 Cloning

Plasmid table

Plasmid	Description	Source
pET3a-AIGJFMKL	Contains all genes that code for PC8	ED
pET3a-AIGJFMKL+ His-tagged $cobH^{Alvin}$	His-tagged $cobH^{Alvin}$ +HBA	BJ
pET14b-AlvinCobB	His-tagged $cobB^{Alvin}$	BJ
pET3a-AIGJFMKLH+ His-tagged $cobB^{Alvin}$	His-tagged $cobB^{Alvin}$ +HBAD	BJ

Bacterial strain

E.coli strain	Genotype /phenotype	Source
JM109	*end*A1, *rec*A1, *gyr*A96, *thi,* *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F´ *tra*D36, *pro*AB, *laq*I^qZΔM15]	Promega
BL21*(DE3) plysS	High-Efficiency Protein Expression from Gene with T7 Promoter and Ribosome Binding Site Genotype : F–, *omp*T, *hsd*S_B (r_B–, m_B–), *dcm*, *gal*, λ(DE3), pLysS, Cm^r.	Promega

PCR amplification of CobH gene

PCR	A -	B +	C
FSHF buffer x10	5	5	5
DMSO	0	(1 µl)up to 5 µl	4
Forward primer @ 10 µM	2	2	2
Reverse primer @ 10 µM	2	2	2
dNTPS @ 2 mM	5	5	5
Template (genomic DNA diluted 1:100)	1	1	1
H_2O	34.5	(33.5) up to 50 µl	30.5

FSHF enzyme (no1 in freezer)	0.5	0.5	0.5
2 mins at 96˚C			
30 cycles of	30 s at 96˚C 30 s at 55˚C x min at 72˚C (x = 1000 bp/min)		40 s @72˚C
7 mins at 72˚C			

Agarose Gel for separating DNA from buffer

PCR samples are loaded by the following order shown below and left for an hour at 120V in order to allow the samples to separate in size as it reaches the end of agarose gel.

Marker	A+2 µl of (x 5) loading Buffer	B+2 µl of (x 5) loading buffer	C+ 2 µl of (x 5) loading buffer
5 µL	**60 µL**	**60 µL**	**60 µL**

As the run on the agarose gel ends, the appropriate bands that correspond to the expected size of DNA are required to be cut out of the gel.The cobH band for the cloning experiment was cut at the band that gave 4149 bp. The sample is placed into a sterile Eppendor for process of gel extraction using the QIAquick PCR purification kit.

2.9.1 Creating initial vector and insert

pET3a-AIGJFMKL (PC8 genes)

pET3a-cobH ^{Alvin} (non-His tagged)

pET14b- cobH ^{Alvin} (His-tagged)

The initial step was to create two plasmids; one containing all the genes up to and including *cobH* and His-tagging the *cobH* ^{Alvin} and one that only expressed the CobH enzyme. Based on the position of Histag appropriate restriction enzymes were chosen to cut the plasmids. The insert pET3a- cobH ^{Alvin} was cut by *Xba*I and *Eco*RI and the same restriction enzymes were used to cut pET14b- cobH ^{Alvin} that contained His-tag. The vector pET3a-AIGJFMKL was cut by *Spe*I and *Eco*RI.

During ligation, *Spe*I and *Xba*I restriction sites, both lose nucleotide bases from each site which allows sealing and locking of the plasmid. The JM109 competent cell was transformed with the ligation sample onto LB + agar + glucose and Ampicillin plate.

2.9.2 Cloning pET3a-AIGJFMKL + His-tagged cobH ^{Alvin}

Designing a plasmid that contained genes coding for His-tagged cobH ^{Alvin} required the vector fragment that expressed all genes for PC8 and an insert that expressed His-tagged cobH ^{Alvin}. The vector was cut with restriction enzymes *Eco*RI and *Spe*I and the insert was cut with *Xba*I and *Eco*RI.

2.9.3 Cloning pET3a-AIGJFMKLH ^{Alvin} and pET14b- cobB ^{Alvin}

The aim of this cloning is to create a plasmid that co-expresses genes that code for the production of HBA as well as the genes that code for HBAD production. In order to achieve this, appropriate restriction enzymes are selected so a vector and an insert with correct size of base pairs are cut and ligated together to form a complete

plasmid. The vector fragment was created by cutting the pET3a-AIGJFMKLH using restriction enzymes *Xba*I and *Spe*I whereas the insert pET14b-cobB Alvin was formed by restriction enzymes *Xba*I and *Eco*RI. The ligated fragments will form a plasmid that contains genes coding for CobH and the succeeding enzyme CobB. The vector of this plasmid contains all the genes that code for precorrin-8 and the enzyme cobH that catalyses the production of HBA. However, the insert of interest is only expressing the His-tagged CobB that in combination with the prior genes in the vector catalyses the conversion of HBA to HBAD. After ligation, a starter culture is made using a single colony from the agar plate and then grown in 5 ml LB at 28 °C. The bacterial DNA was extracted from the bacterial pellets by QIAprep spin Mini-prep kit.

2.9.4 Miniprep QIAprep Spin Miniprep Kit Using a Microcentrifuge

The DNA of the bacterial strain is isolated by using the QIAprep spin miniprep kit. The eluted DNA is then used for the process of restriction digest.

2.9.5 Restriction digests and agarose gels

In order to test the vector and insert of interest the purified miniprep DNA of both plasmids are mixed with following:

- **Digest:**

In one Eppendorf tube:

Miniprep DNA	5 µL (pET3a –CobB $_{Alvin}$ or pET14b-*Alvin* CobH)
Buffer NEB2.1 (x 10)	1 µL
Restriction enzyme *Eco*RI	0.5 µL
Restriction enzyme *Nde*I	0.5 µL

> Incubate at 37 degrees Celsius for the restriction enzyme activity for 1 to 2 hours.

2.9.6 DNA agarose gel electrophoresis

0.8 grammes of Agarose powder was weighed and put into the 250 ml conical flask. Then 80 ml of 1 x TAE buffer was added and mixed with the agarose. The mixture was then fully dissolved and its temperature was reduced by gently swirling the conical flask under the tap water. One drop of ethidium bromide (10 mg/ml) was added following by a gentle swirl to mix. The gel was poured into the tray and the comb was inserted. The gel was left to set for 30 minutes.

Loading the agarose gel

5 µL of the DNA marker

Added 2 µL of DNA loading buffer (x5) to each digest (mixed thoroughly) The sample was run at 110 volts for 1 hour.

The gel was placed under the UV transilluminator in order to identify and cut the insert and vector of interest for gel extraction and PCR clean up.

2.9.7 Isolation of DNA from agarose gel electrophoresis

The DNA band was cut out of the agarose gel and then weighed after placing in the Eppendorf tube. By using the QIAquick column the DNA of interest is isolated for further use in the process of DNA ligation.

2.9.8 DNA ligation

The extracted and purified DNA of the inserts and vector were ligated together in order to link the fragments and form a plasmid that contains the gene of interest. Ligations were set up as below in Table 2.

2x ligase buffer	5 µL
vector	2.25 µL (extracted & purified from 5 µL digested miniprep)
insert	2.25 µL (extracted & purified from 5 µL digested miniprep or digested & purified PCR)
T4 DNA Ligase	0.5 µL
Either overnight in the fridge or 2 hours at room temperature.	

Table 2: The process of ligation is summarised and clarified in the table above.

2.9.9 Transformation of *E Coli*

The competent cells were transformed by incubating 0.5 µL of this plasmid is put into 50 µL of competent cells. The sample is then put on ice for 15 minutes, heat shocked for 1 minute at 42 °C and then placed on ice for two minutes. SOC medium (200 µL) is then added to the cells, which are then incubated at 37 °C in a water bath (no shaking) for 1 hour. Once the incubation time is over the sample is transferred onto LB-agar plate containing the following: Chloramphenicol at stock concentration of 34 mg/mL, Ampicillin at stock concentration of 100 mg/mL and glucose 20%.The plate is incubated at 37 ºC overnight in order to allow bacterial colonies to grow.

In case of expressing genes that require BL21 star (DE3)plysS competent cells agar plate must contain Ampicillin at stock concentration of 100 mg/mL and glucose whereas the molecular biology samples require agar plate that contains Ampicillin at stock concentration of 100 mg/mL ,Chloramphenicol at stock concentration of 34 mg/mL and glucose 20%.

2.10.0 HPLC-MS

High Performance Liquid Chromatography Mass spectrometry (HPLC-MS) allows applying the sample mixtures in a mobile phase by using the C 18 column.

The sample is pumped with high pressure as it requires passing through a stationary phase that contains the packing material. This column is able to separate and categorise the components in the sample.

The sample retention time is dependent on the interaction between the molecule of interest and the stationary phase, hence the samples that have the least interaction with the stationary phase will exit the column faster in comparison to other analytes that have different polarities and force of attraction to the stationary phase. As the components elute from the column, they are then introduced to a mass spectrometer by two interfaces known as the electrospray ionisation and the atmospheric pressure chemical ionisation interface in order to further analyse the chromatographic separation.

Results

Chapter 3

In the cobalamin biosynthetic pathway many enzymes tightly bind their products (Deery et al. 2012) [1], therefore it is hypothesized that some form of interaction between the enzyme-product bound and the following pathway enzyme must occur in order to release the intermediate and allow the biosynthesis to continue. In the following chapters, the function of CobH and CobB is investigated in order to observe the interaction using enzyme trapping methodologies and X-ray crystallography.

3.0 HBA binding to CobB

The purified substrate (HBA) was run on the UV visible spectrophotometer in order to measure absorbance at 328 nm and by using the extinction coefficient at 328 nm, the concentration of HBA can be calculated. The extinction coefficient value for this substrate is 50000 M^{-1} cm^{-1} and by following the following formula the concentration of purified HBA was calculated. The absorbance at 328 nm is around 0.349 as shown in figure 8.

$C = \varepsilon / AL$

A = Absorbance (no unit)

ε = Extinction coefficient → M^{-1} cm^{-1}

C = Concentration → units in M or mole / L

L = Path length of 1 ml → unit is Cm

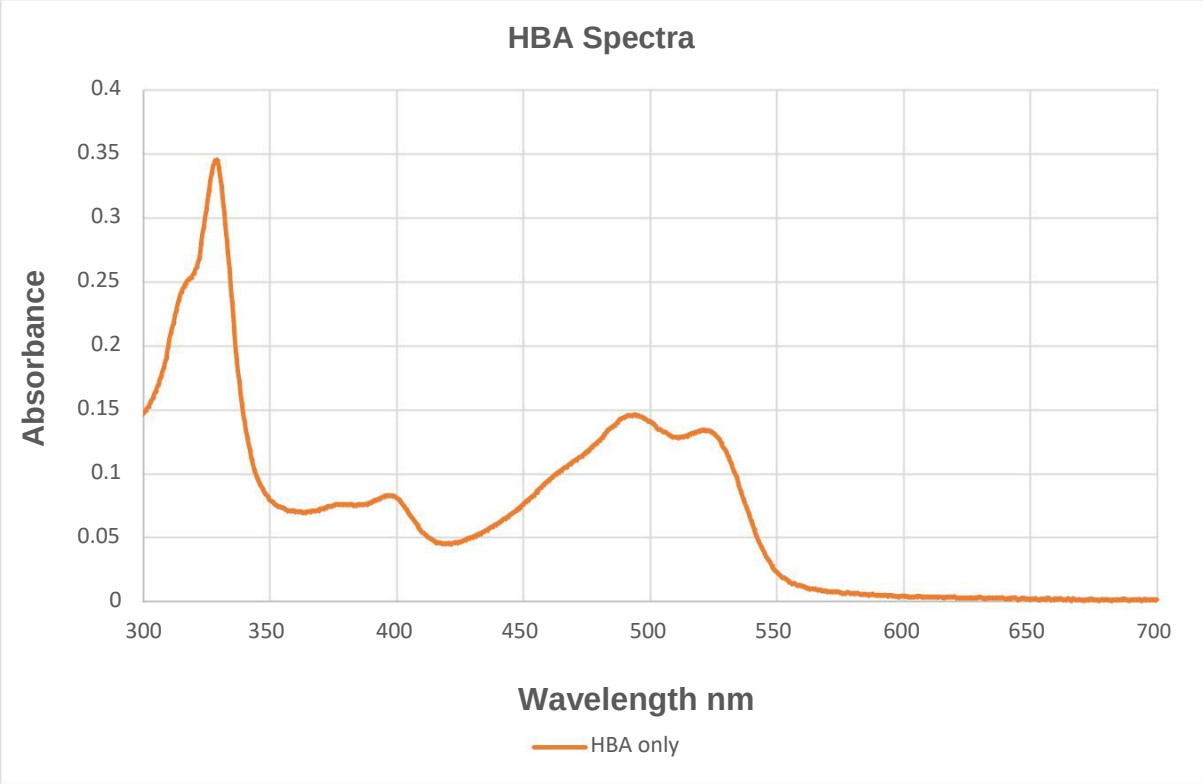


Figure 8: The purified HBA from RP18 column was examined by UV visible spectrophotometer in order to compare the spectra with the data that had been published in literature and make sure that the reading is following the same absorbance pattern.

HBA, the product of CobH, is the substrate for CobB which amidates sidechains *a* and *c* to produce HBAD. At the early stage of project it is vital to observe how CobB behaves in presence of HBA in a mixture in order to observe the binding affinity of the substrate to the enzyme. The absorbance spectrum of the mixture clearly shows the close interaction of HBA and CobB spectra at 329 nm (figure 9). This spectra was taken at the initial stages of the experimenting CobB in order to become more familiar with its function and behaviour when the substrate is unbound from CobH.

Figure 9: The interaction between HBA and CobB *Alvin* is shown as the spectra of HBA (orange) is in close proximity of CobB curve which suggests that the enzyme and substrate binding interaction takes place.

3.1 Identifying the oligomeric state of CobB *Alvin*

Gel filtration is a commonly used technique for determining the size and the molecular weight of the protein of interest in a more precise and wider range. In order to measure accurately, the oligomeric state of CobB is estimated by comparing the retention time with a known range of known protein standards as shown in calibration curve in figure 10. The CobB elution volume is at 15.5 mAU which suggests that the oligomeric state of CobB protein is a dimer (figure 11).

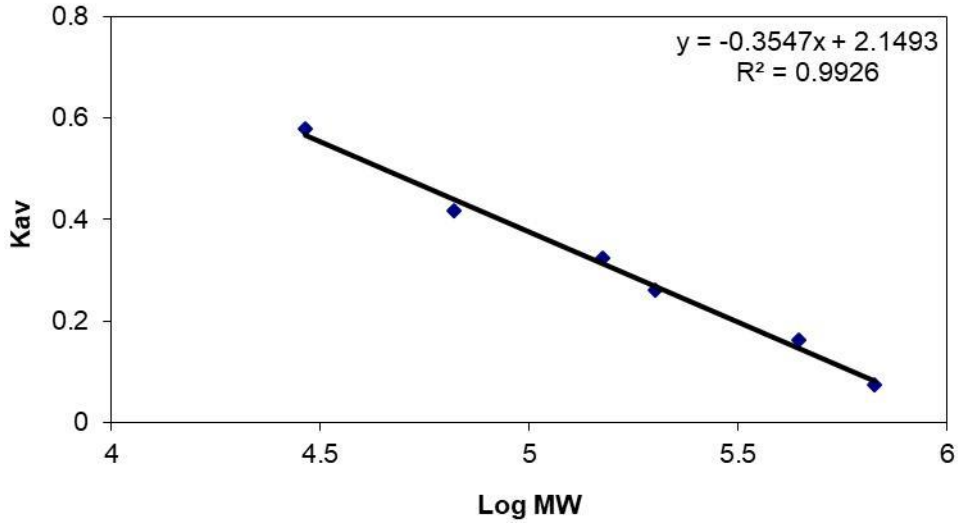


Figure 10 : the calibration curve is plotted by using the gel-phase distribution coefficient (Kav) versus the logarithm of the molecular weight (Log MW). The straight line is the calibration curve drawn from the data for obtaining the standard molecular weight (R^2= 0.9926). This data is then used to determine the concentration of the CobB by comparing the highest elution peak with the known concentration of known standard samples.

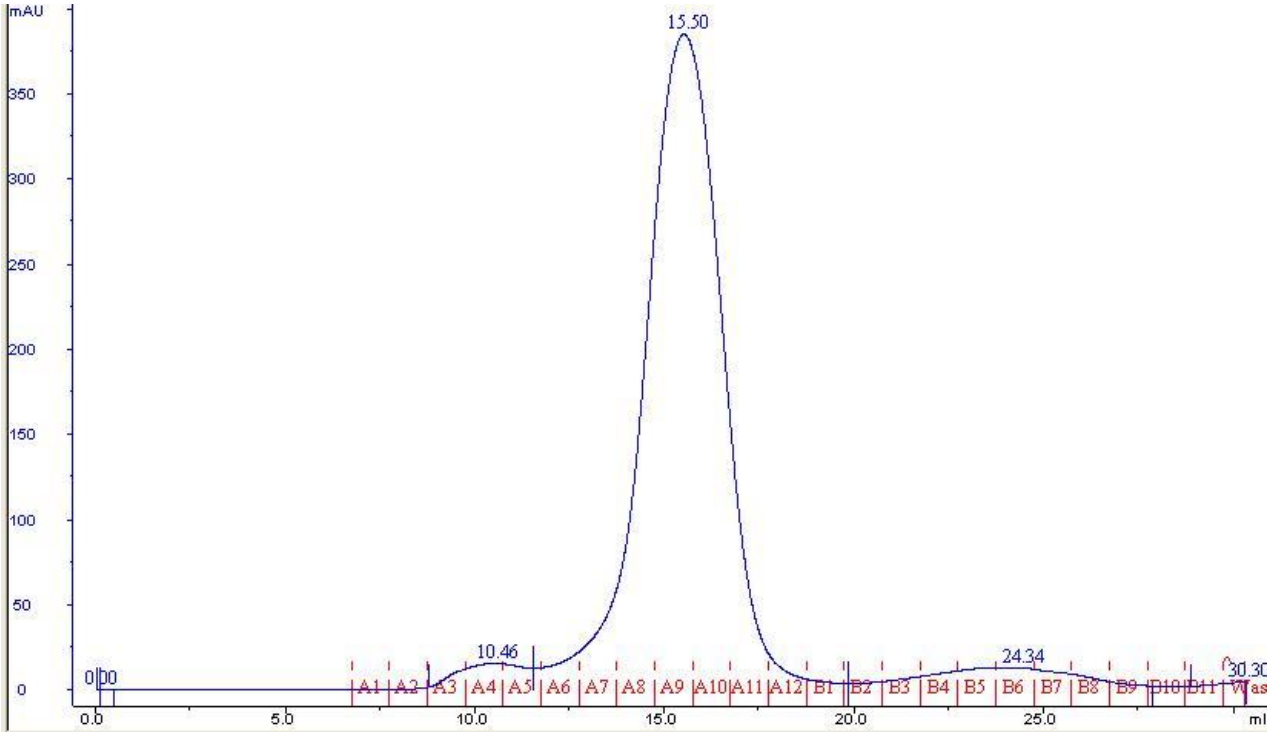


Figure 11: The elution volume of CobB is used to determine the oligomeric state of this enzyme. By comparing the value of the highest peak (15.50 mAU) with the standard protein range, it confirms that CobB is a dimer.

The concentration of this protein is determined by taking a UV visible spectrophotometer read at 280 nm. The extinction coefficient of CobB is equal to 27515 M^{-1} cm^{-1}. This value is obtained from Protparam. Using the obtained values the final concentration of CobB can be determined.

3.2 Thrombin cleavage of His-tag from CobB

The main purpose of removing the His-tag from CobB is to aid crystallisation. This approach may also enhance the formation of crystals as the absence of His-tag enables the protein to fold more and rotate more freely. The labelled elution peak in figure 12 suggest that removal of His-tag has minor effect on the overall volume of the CobB when the His-tag is intact. This change is also noticeable as after the cleavage of the His-tag, the protein moves further down along the SDS-PAGE figure 13 .This result provides an important evidence in terms of protein folding flexibilities when it comes to further investigating this protein at the crystallography stage.

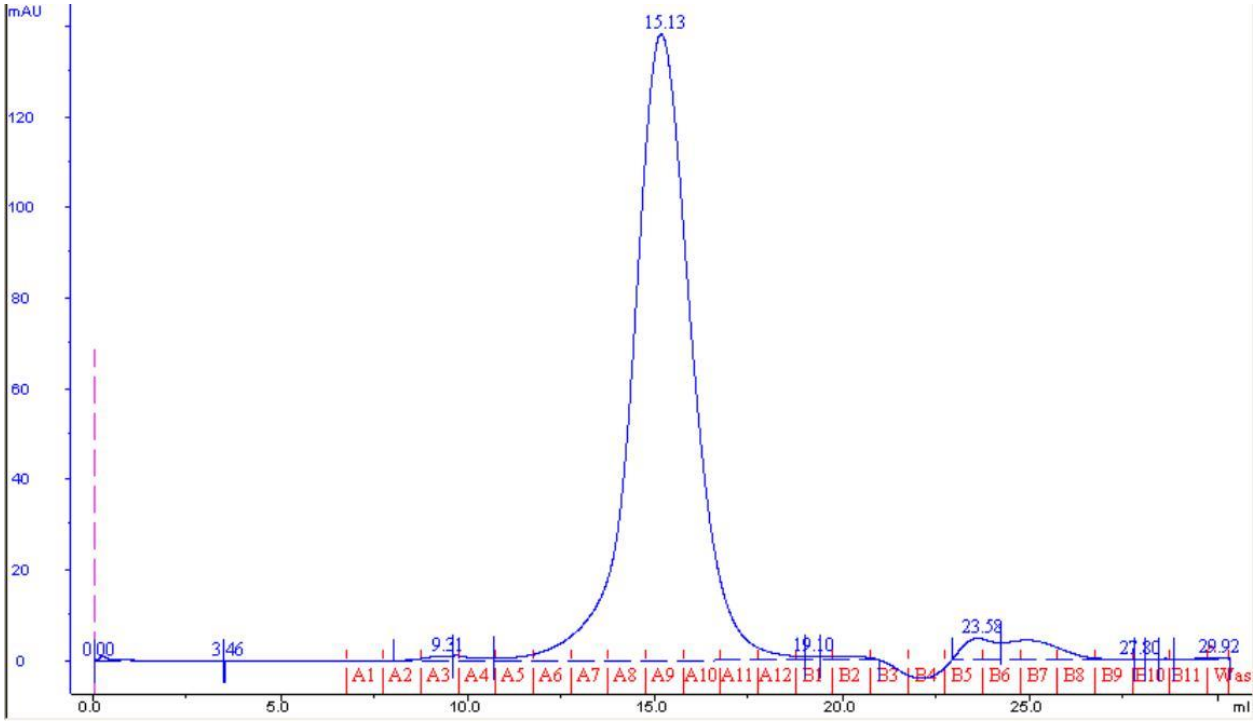


Figure 12 : After removing the His-tag from CobB, a slight changes in the elution volume is observed.

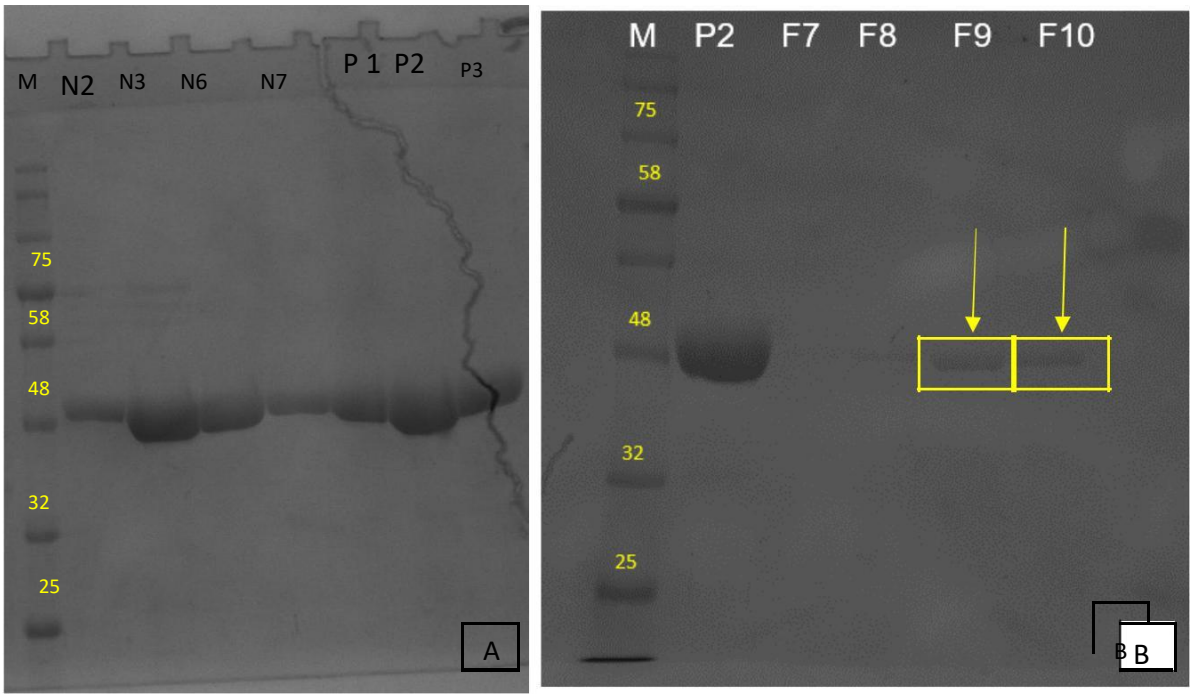


Figure 13 : The SDS-PAGE (A) is showing the CobB elution fractions that were collected during Nickel column and PD10 purification steps. The SDS-PAGE (B) is showing the non-His tagged CobB samples from FPLC elution fractions. The concentration of the protein in image B has reduced as the bands become fader and thinner in comparison to the image A. slight difference in the location of the bands suggests that removal of the His-tag has been successful.

3.3 Trapping HBA with His-tagged CobH **Alvin**

The way of isolating CobH with its trapped product, HBA, is to express all the biosynthesis genes up to CobH including in one plasmid.

All the genes necessary to produce precorrin-8 were overexpressed in *E coli* at the same time as overexpressing a His-tagged version of CobH.

The resultant plasmid pET3a-AIGJFMKL-His-cobH contains all the genetic information to allow for the production of HBA as shown in figure 14.

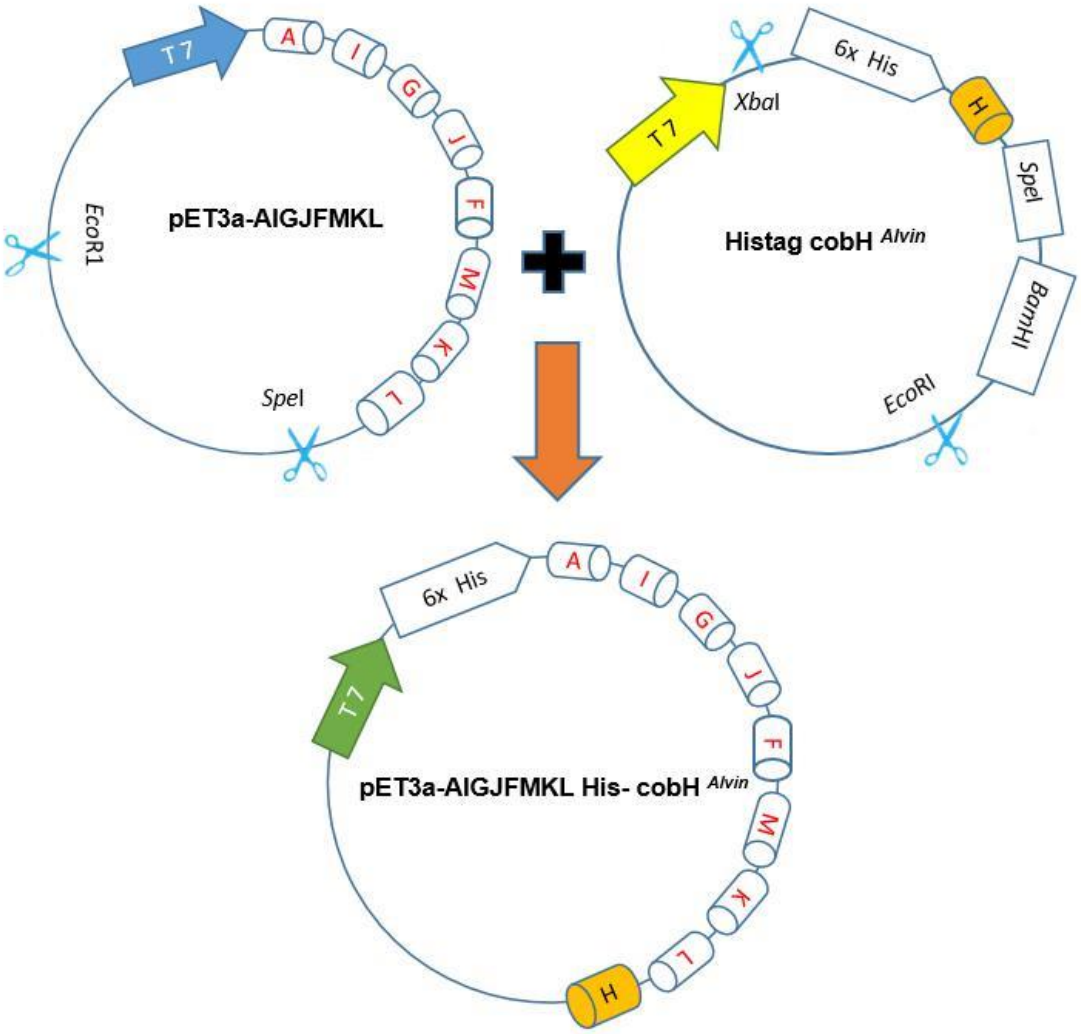


Figure 14: The plasmid containing the genes encoding for the enzyme required for precorrin-8 was digested by *Eco*RI and *Spe*I endonuclease enzymes. Additionally, the plasmid containing the RBS His-tagged cobH ^{Alvin} was digested with *Eco*RI and *Xba*I. The two DNA fragments pET3a-AIGJFMKLH and RBS His-CobH were then ligated together in order to generate a plasmid that could be used to produce both His-tagged CobH ^{Alvin} and precorrin-8 at the same time.

3.3.1 Transformation, growth and purification of trapped CobH ^{Alvin}

E. coli BL21 star (DE3) plysS competent cells were transformed with the pET3a-AIGJFMKL-His cobH plasmid. The resulting recombinant strain carrying the plasmid was grown in the 2YT medium at 28 ^{o}C for 24 hours and the overproduction of protein was induced by the addition of IPTG after 7 hours of growth.

The bacteria were harvested by centrifugation and were subsequently resuspended in binding buffer composed of 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 5 mM imidazole.

After sonication to lyse the cells, the debris was separated from the soluble fraction by centrifugation. The soluble fraction was then applied to a chelating Sepharose charged with $NiSO_4$ in order to purify the His-tagged CobH (abbreviated to His-CobH ^{Alvin}). The column was equilibrated with binding buffer (20 mM Tris-HCl, pH 8.0, containing 500 mM NaCl and 5 mM imidazole) and the unbound protein was washed with wash buffer I and II, which contain 50 mM and 100 mM imidazole respectively.

The His-tagged protein was eluted from the column by application of elution buffer, which contains 500 mM imidazole. The His-tagged CobH protein was eluted as a pink protein as shown in Figure 15B, which indicated that the protein had product bound to it. The protein was then applied to a PD10 buffer exchange column that had previously been equilibrated with low salt buffer (20 mM Tris-HCl pH 8.0, containing 100 mM NaCl). The purification of the protein was followed by SDS-PAGE, and the results are shown in Figure 16, which the purified protein showing a molecular mass consistent with that of His-CobH^{Alvin} .

3.3.2 Product of pET3a-AIGJFMKL + His-tagged cobH^{Alvin}

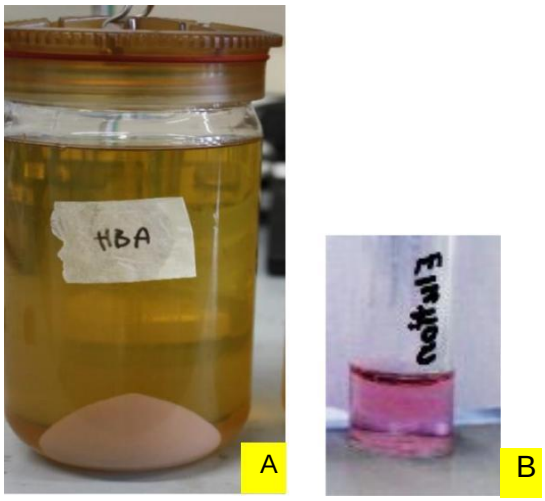


Figure 15: Bacteria containing pET3aAIGJFMKL + His tagged cobH ^{Alvin} were grown in 2YT medium (A) and the His-CobH ^{Alvin} was purified through a Nickel column as shown in the image on the right (B). The elution fraction of His-CobH ^{Alvin} contained pink pigments, consistent with the presence of bound HBA.

3.3.3 SDS-PAGE of the purified His-tagged CobH ^{Alvin}

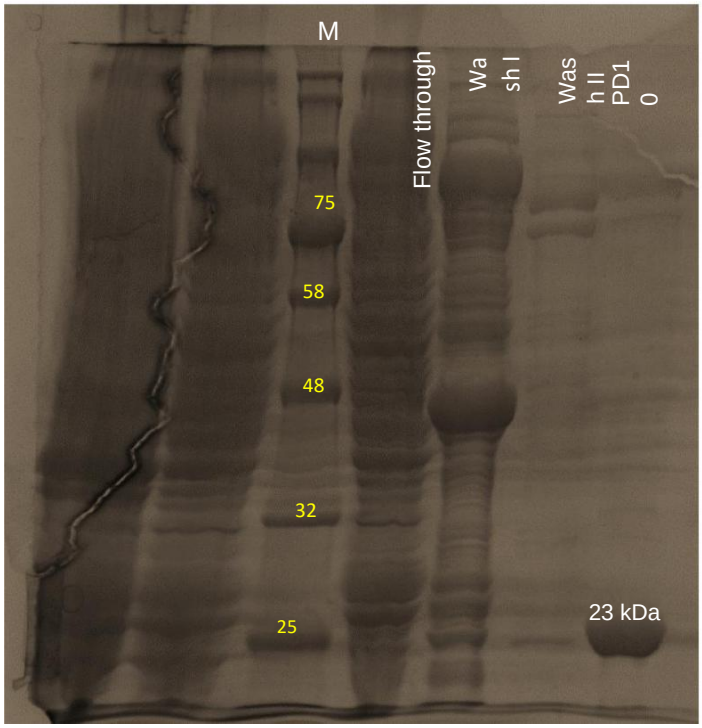


Figure 16: The collected fractions from each step of the purification were run analysed by SDS-PAGE. As shown on the image the trapped His-tag CobH^{Alvin} runs with the expected molecular mass for this enzyme (23kDa), as calculated from the amino acid sequence found in KEGG pathway database.

Conclusion

The results show that transformation of *E.coli* BL21 star (DE3) plysS with the pET3a-AIGJFMKL-His-cobH^{Alvin} has been successful as the purified His-CobH^{Alvin} protein eluted with a pink colour. This suggests that the His-CobH^{Alvin} is able to strongly bind its product, HBA.

3.4 *In vitro* binding of CobH to HBA

Previous studies have shown that the *Rhodobacter capsulatus* CobH also has a high binding affinity for its product HBA (Deery et al 2012) [1] (Thibaut et al. 1992) [6]. The binding interaction between HBA and CobH has been investigated *in vitro* previously by titrating enzyme into a known concertation of HBA. To determine the ratio of HBA that binds to His-CobH^{Alvin} 1 mL of 4.2 µM HBA was pipetted into a cuvette and 5 µL of CobH ^{Alvin}, at a concentration of 5 µM, was added to the HBA solution. The absorbance readings were recorded on a UV visible spectrophotometer. This process involved the gradual and consecutive addition of 5 µL aliquots of His-CobH^{Alvin} into the cuvette containing HBA. This procedure was repeated until the enzyme was fully saturated with HBA as shown in Figure 17.

This titration allowed observations in the changes of the HBA spectrum in response to increases in the concentration of CobH enzyme. The results showed that the UV visible spectrum of HBA is reduced as more protein is added. The absorbance values from 328 nm and 700 nm were selected in order to calculate the binding ratio of enzyme to HBA.

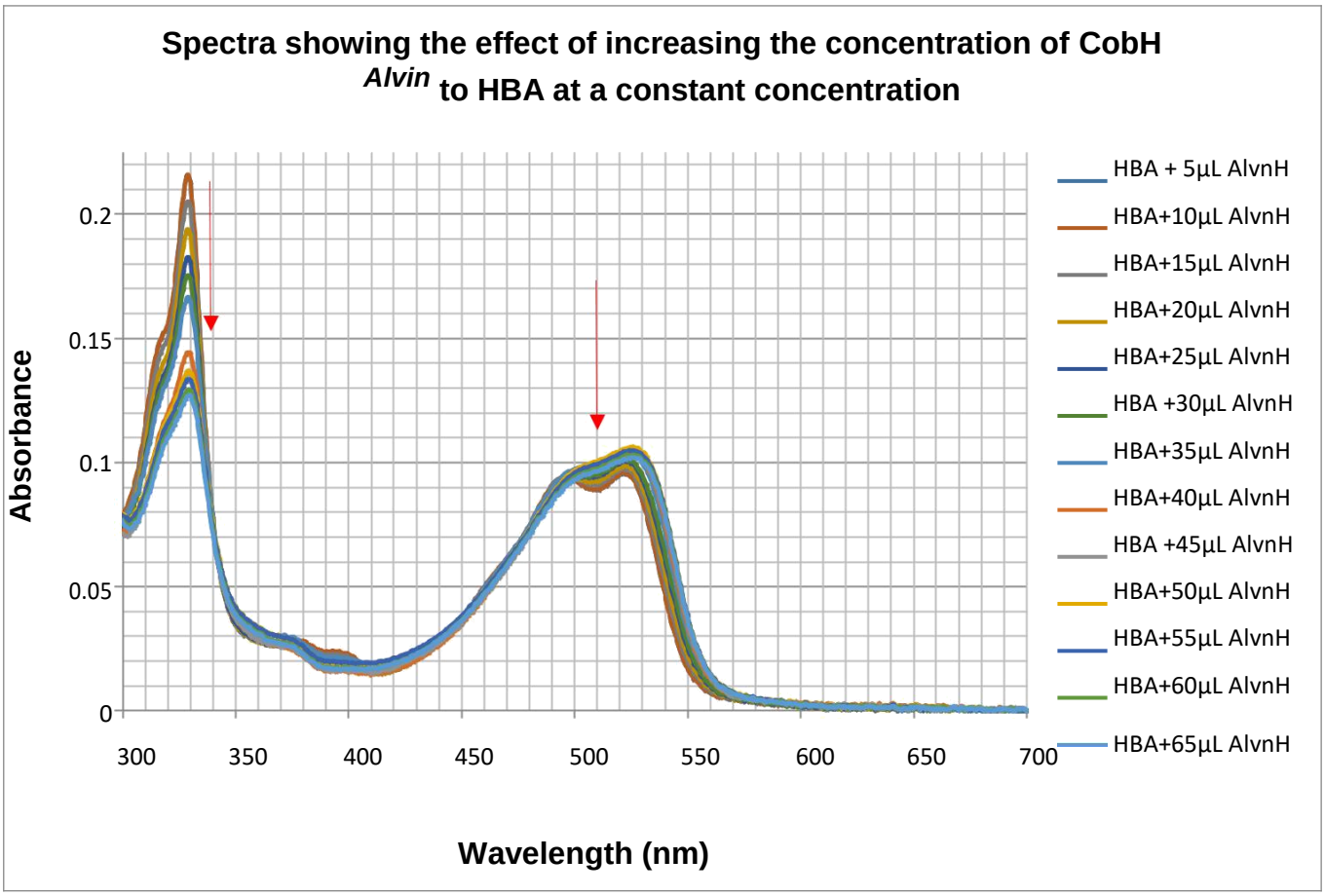


Figure 17: The interaction between CobH and HBA is measured as the concentration of protein is increased. The curve shows that the in presence of CobH the HBA peak gradually decreases as the enzyme becomes saturated. This spectrum was recorded over the range of 300 nm to 700 nm.

3.4.1 Binding ratio of CobH to its product HBA

The UV visible spectroscopy data collected from the titration was plotted on a graph by selecting the readings taken at 328 nm in order to determine the binding ratio between CobH and HBA. The absorbance readings at 328 nm were selected because they showed the most significant change during the course of the titration. The binding curve is presented in figure 18.

The graph clearly demonstrates that there is a relationship between the concentration of protein and the absorbance of HBA at 328 nm.

The lines drawn on the graph indicate the point of saturation, where the HBA is fully bound to the protein. The data show that when the concentration of protein is around 8 µM which indicates that the protein is a dimer interacts with 4.2 µM of HBA. The obtained values suggest that the binding ratio of CobH to HBA is 2:1, which suggest that only one HBA molecule is bound per protein dimer.

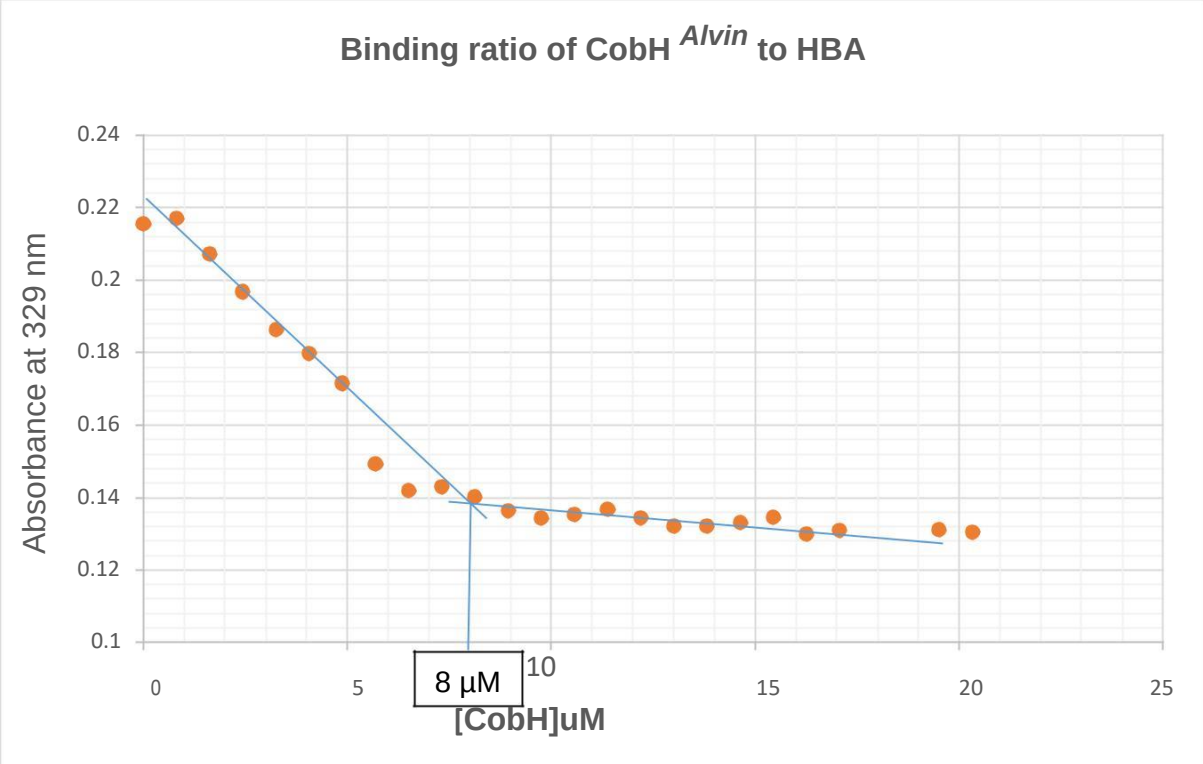


Figure 18: The binding ratio of HBA to enzyme (CobH *Alvin*) is estimated at the point where the line flattens. This corresponds to the point of saturation where 4.2 µM HBA is bound completely by CobH. From reading this point on the X-axis it shows that the concentration of CobH is around 8 µM. Therefore, the binding ratio of CobH to HBA is 2:1.

Conclusion

By undertaking a titration of HBA with purified CobH ^{Alvin} it was possible to determine the ratio of product that binds to the enzyme. The binding ratio of the CobH to the HBA was found to be similar to what had been observed previously and reported in the literature (Thibaut et al. 1992) [6] , which is 2:1 ratio.

CobH is known to be a homodimeric enzyme (Warren et al. 2002) [2]. The results here indicate that one molecule of HBA binds per dimer of protein.

Although the protein is known to have two active sites per dimer it is clear from this titration that only one site is occupied per dimer. This suggests that there is some gating mechanism whereby when one site is occupied the other site becomes closed so as not to allow another HBA molecule to bind. It is not immediately obvious why this should be so, but it does suggest that the enzyme has regulatory mechanism in place in order to limit the activity of the enzyme. Alternatively, the dimer may be able to bind two molecules of product, but the second site must have a very low affinity for HBA.

3.5 Trapping HBA/ HBAD with the His tagged CobB ^{Alvin}

Constructing a His-CobB ^{Alvin} trapping plasmid involved cloning the inserts from pET3a-AIGJFMKLH and pET14b-cobB ^{Alvin} together in order to express a non His-tagged CobH and a His-tagged CobB ^{Alvin} at the same time, as outlined in Figure 15. To achieve this, the pET14b-His-cobB^{Alvin} plasmid was cut with the restriction enzymes *Xba*I and *Eco*RI to generate the insert His-cobB ^{Alvin} .The pET3a-AIGJFMKLH plasmid was cut with restriction enzymes *Xba*I and *Spe*I and then the His-cobB Alvin insert was ligated into the larger plasmid to generate pET3a-AIGJFMKLH-His-cobB^{Alvin}.

The cloned plasmid has the ability to produce both HBAD as well as a His-tagged version of CobB ^{Alvin} as shown in figure 19.

3.5.1 Trapping CobH with His-tagged CobB ^{Alvin}

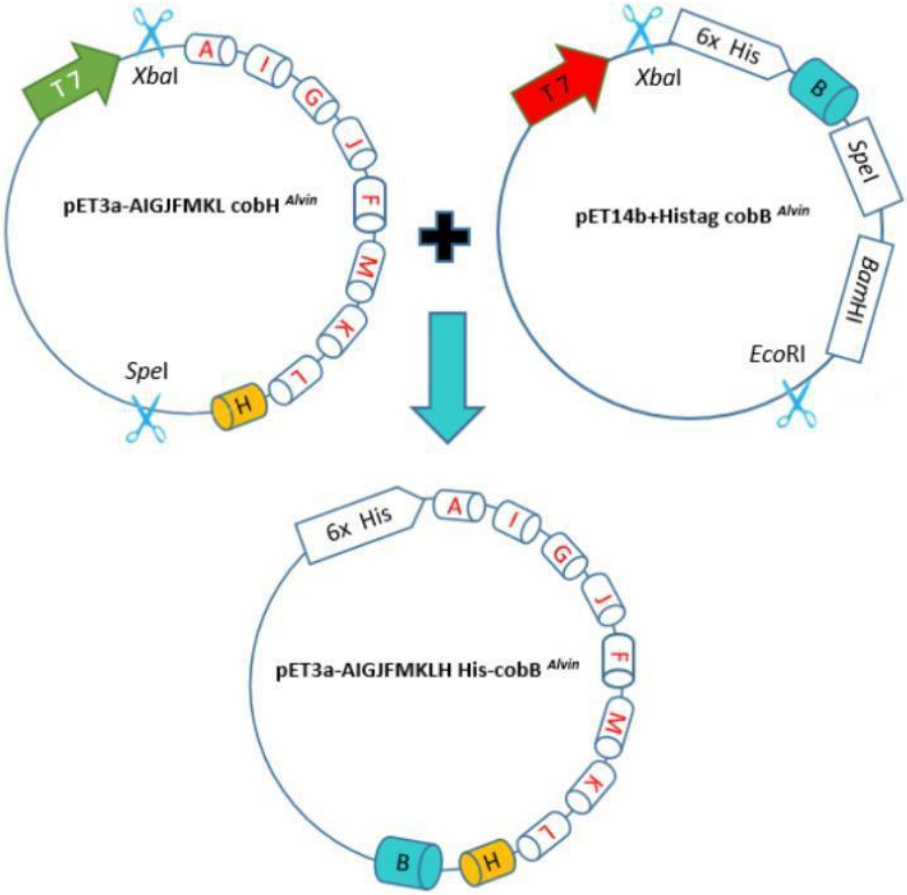


Figure 19: The plasmid containing genes encoding the enzymes for precorrin-8 production and CobH was digested by *Xba*I and *Spe*I restriction endonucleases. Additionally, the plasmid containing His-tagged-CobB^{Alvin} was digested with *Eco*RI and *Xba*I. The two fragments are ligated together in order to obtain a plasmid that was able to produce His-CobB^{Alvin} and HBAD.

3.5.2 Transformation, growth and purification of protein with trapped intermediates

E. coli BL21 cells containing pLysS were transformed with pET3a-AIGJFMKLH-His-cobB^{Alvin}. The resultant strain was then grown in order to determine if a product-trapped version of CobB could be purified. The trapped His-CobB^{Alvin} was purified following the same procedures as described for purification of the trapped His-CobH^{Alvin} as described in Section 2.4. The trapped His-CobB^{Alvin} eluted with a pink/orange colour, as shown in Figure 20. The purification of the protein was followed by SDS-PAGE, where the purified protein was found to migrate with a molecular mass of 48 kDa, which is consistent with its predicted value (Figure 21).

3.5.3 Isolation of trapped His-CobB^{Alvin}

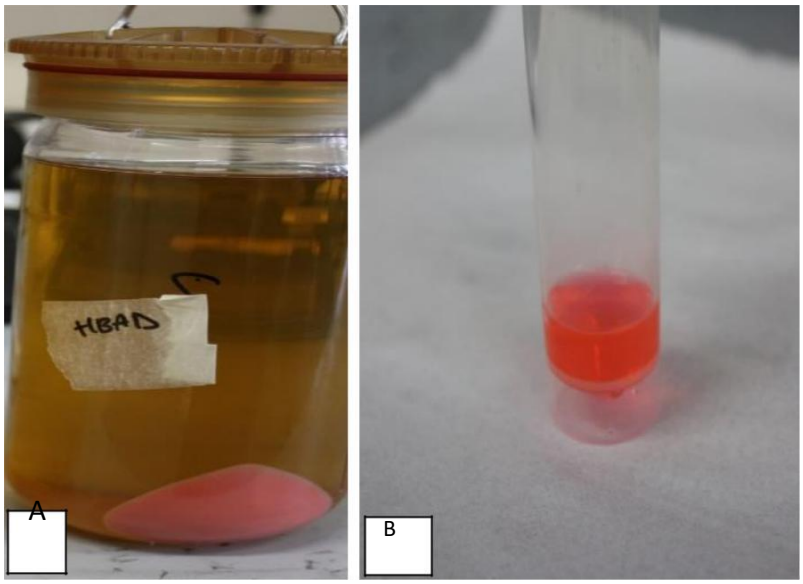


Figure 20: The pellet of the CobB-trapping strain and the purified His-CobB^{Alvin}.The pET3a-AIGJFMKLH-His-cobB was grown in 2YT medium (Figure A) and purified by IMAC, as shown in Figure B.

3.5.4 SDS-PAGE of purified trapped His-CobB^{Alvin}

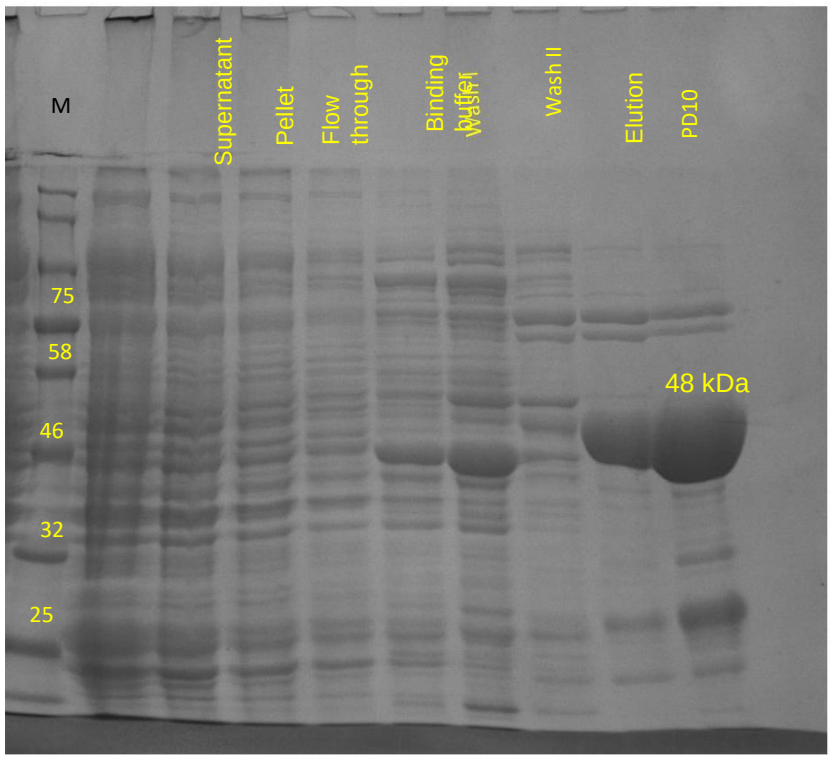


Figure 21: The fractions at each stage of the purification of the His-CobB^{Alvin} were run on an SDS-PAGE. The molecular mass of the trapped His-CobB^{Alvin} is 48 kDa, which is consistent with its gene-derived molecular mass.

Conclusion

The colonies obtained from the transformation of *E. coli* BL21 star (DE3) pLysS with the cobB-trapping plasmid were grown in 2YT medium. The pink colour of the bacterial pellet was an indication that the plasmid was producing a corrin of some description, as this is the colour of HBA/HBAD. The purified protein eluted with a strong pink colour, which could be either HBA/HBAD, as the spectra of HBA/HBAD have identical UV visible spectra. The His-CobB^{Alvin} was therefore purifying with a bound material that was likely to be either is substrate of its product. This pink product was identified by HPLC-MS spectrometry.

3.6 Identifying the spectrometry of coloured pigment by HPLC- MS

A sample of purified His-CobB Alvin obtained after nickel affinity column purification (Figure 20 B) was heated to 80 ºC in order to denature the protein and release the bound pigment from it. This released pigment was then analysed by HPLC-MS in order to determine the nature of the intermediate, substrate or product (Figure 22-25).

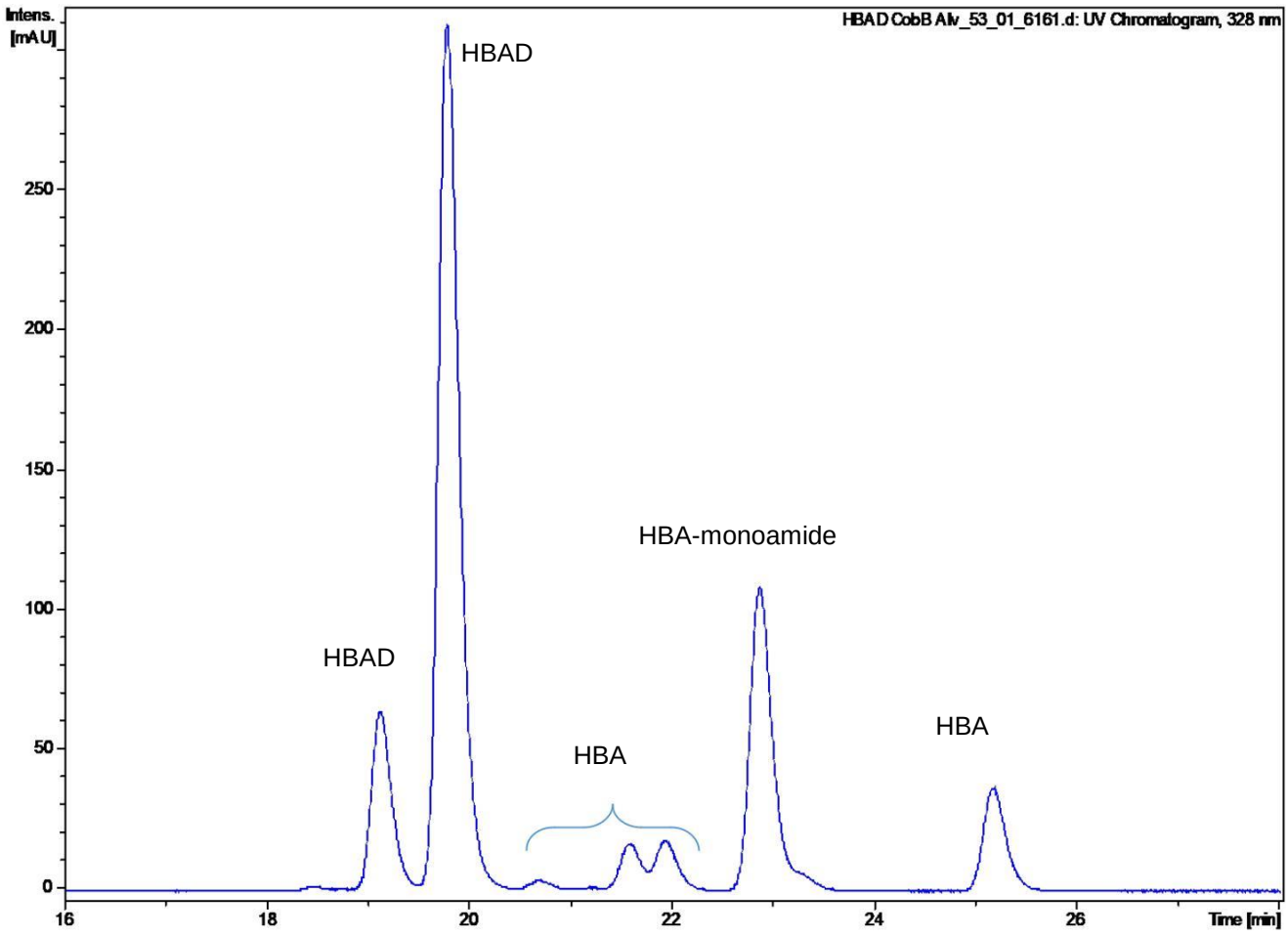


Figure 22: Pigment analysis of the purified His-CobB^{Alvin}. As shown in the trace above, HBAD is the most abundant product of the enzyme trapping experiment. However, other peaks are present which represent the substrate, HBA, as well as the partially amidated intermediate HBA-monoamide were also present.

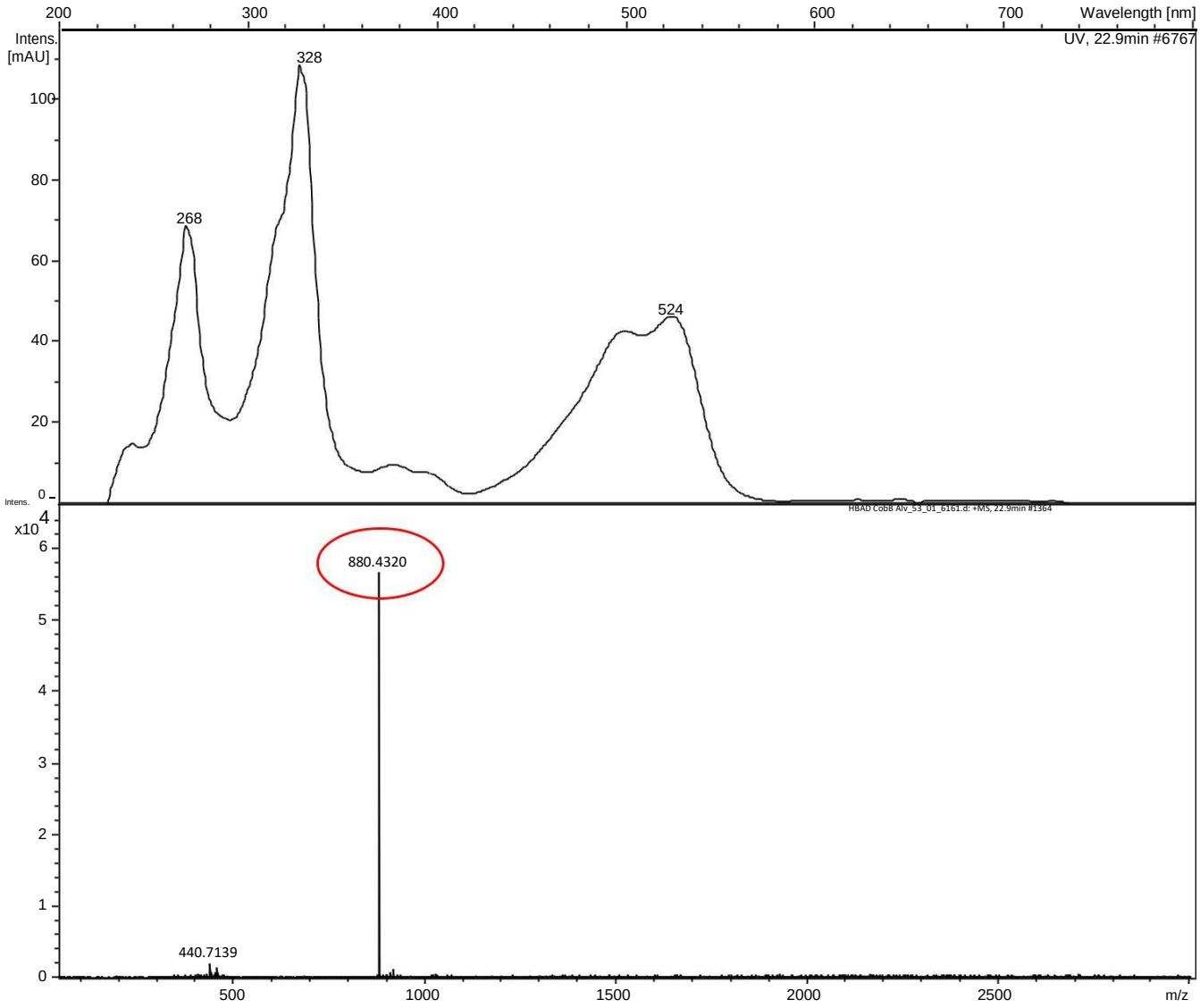


Figure 23: The HPLC MS showing the absorbance at 328 nm after 22.9 minutes. The number (m/z) 880 (M^+H) is an indication of HBA-monoamide being present

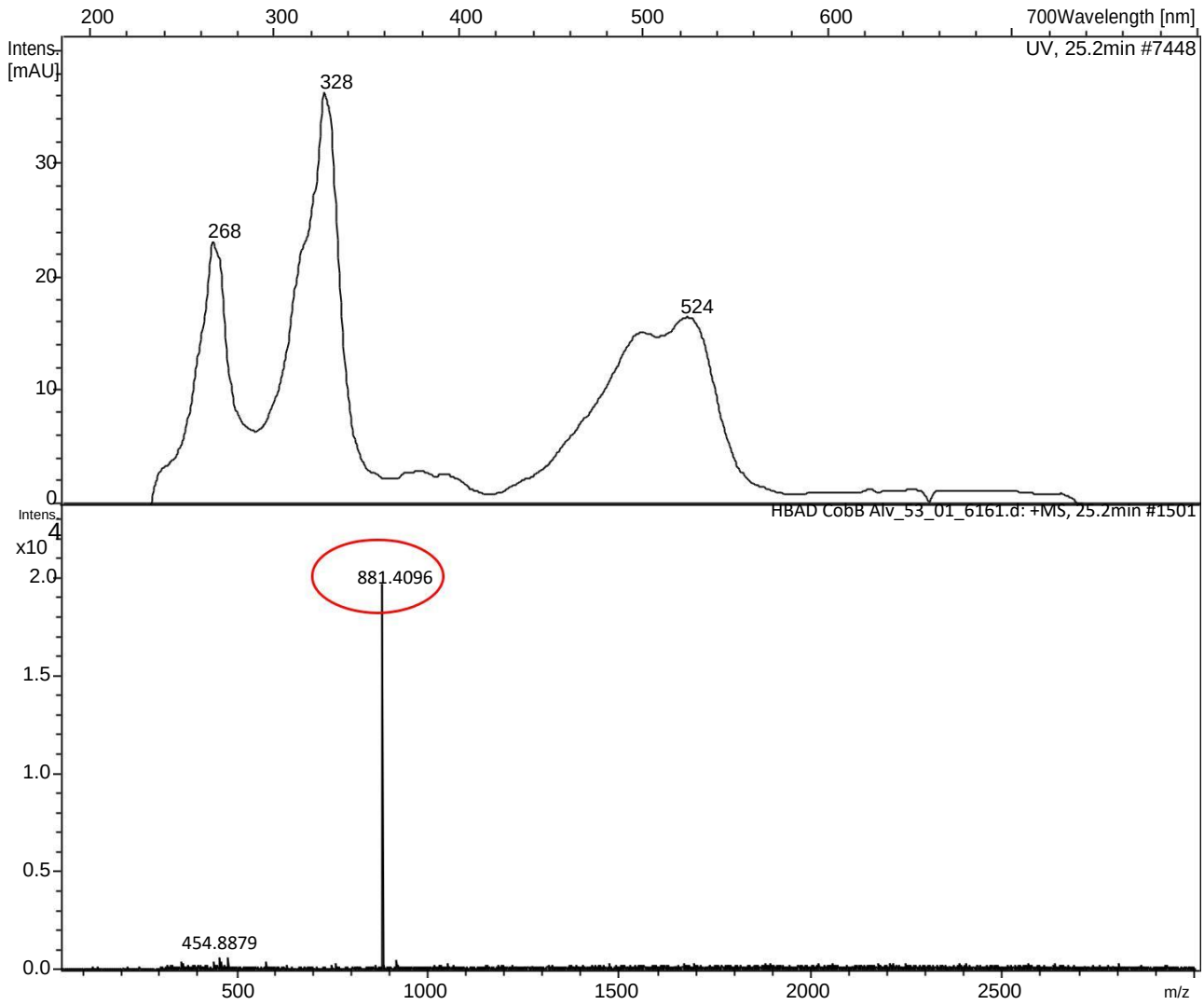


Figure 24: The HPLC MS trace showing an absorbance at 328 nm after 25.2 minutes. The number (m/z) 881 (M^+H) is an indication of HBA being present.

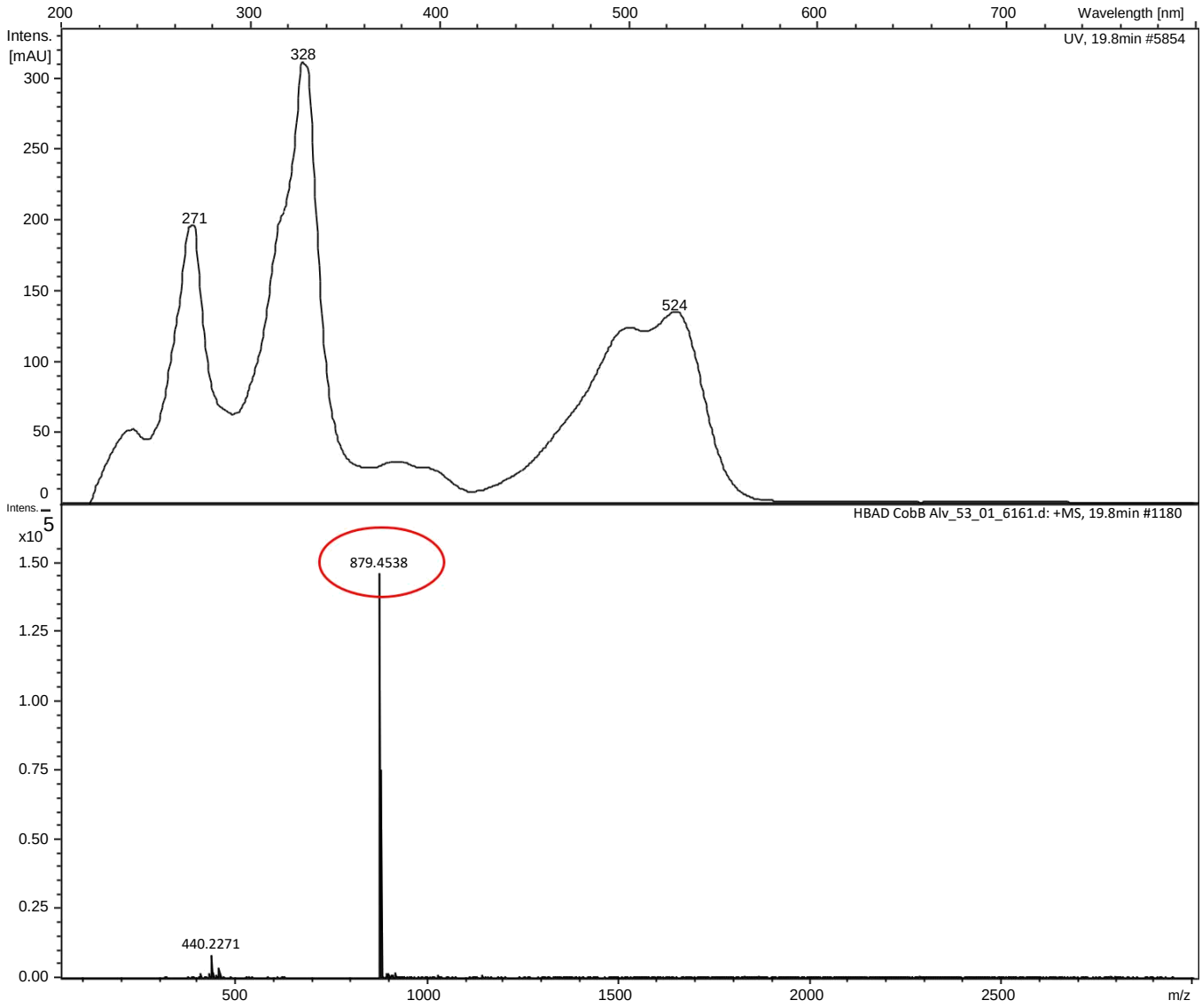


Figure 25: The HPLC MS showing an absorbance at 328 nm after 19.8 minutes. The number (m/z) 879 (M^+H) is an indication of HBAD being present.

HPLC analysis reveals that His-CobBAlvin is able to bind its substrate HBA, HBA monoamide as well as its major product HBAD. The major pigment bound to the enzyme appears to be HBAD.

Conclusion:

The purified trapped His-CobB^{Alvin} was a pink colour. In order to identify the tetrapyrole bound to the His-CobB^{Alvin} the pigment was extracted and run on a MS-HPLC system. The resulting chromatograms showed that CobB binds HBAD, HBA-monoamide and HBA. However, the most abundant material was found to be HBAD.

3.7 *In vitro* binding of His-CobB^{Alvin} to HBA and HBAD

The *in vitro* binding of His-Cob ^{Alvin} to HBA or HBAD was investigated by varying the concentration of His-CobB^{Alvin} (0, 2.5 µM, 5 µM, 7.5 µM, 10 µM and15 µM) to a fixed concentration of HBA or HBAD, during which the UV-vis spectrum was recorded. The aim of this titration was to see how the presence of CobB could modify the HBA/HBAD spectrum as shown in Figures 26 and 27.

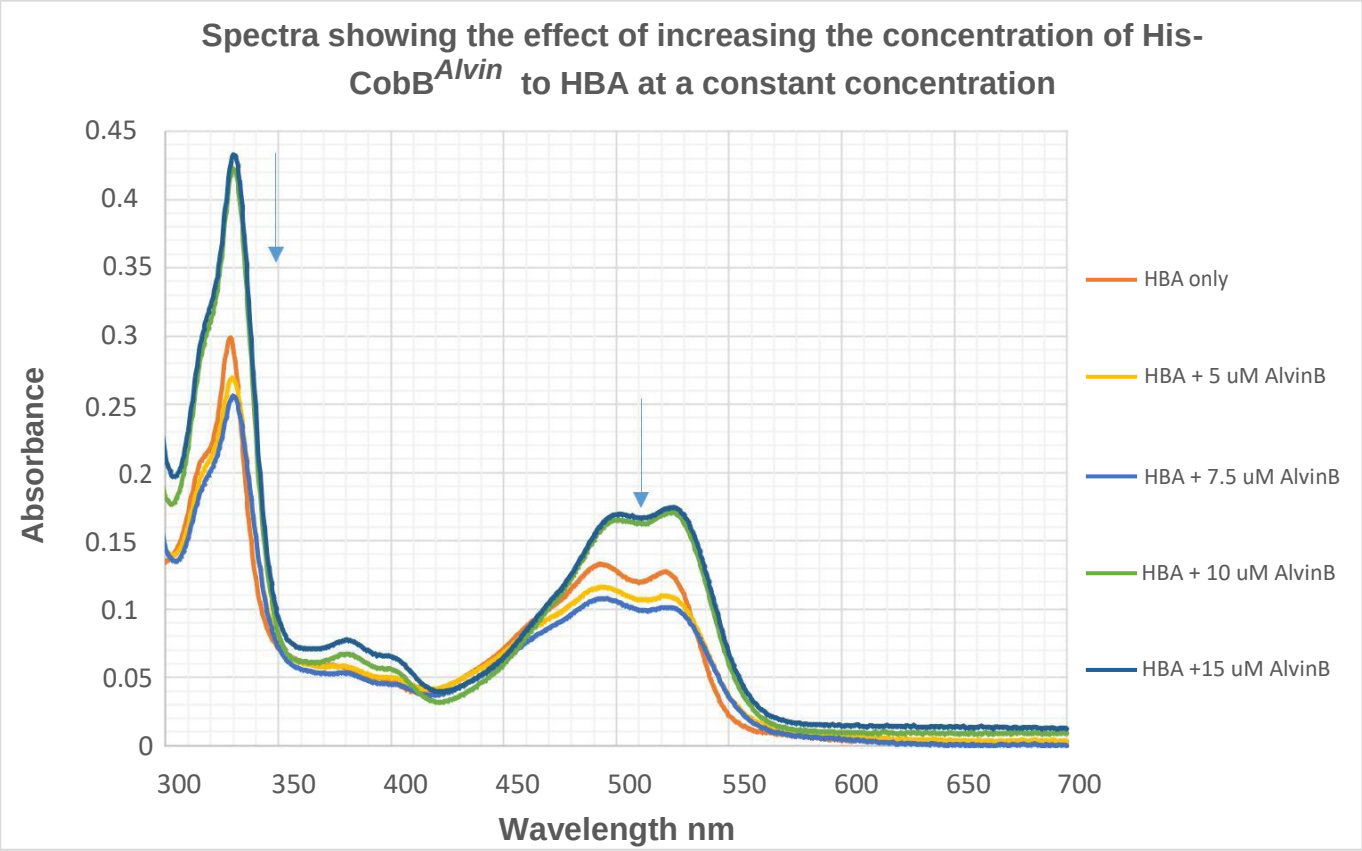


Figure 26: Spectra showing the UV-vis absorbance readings from 300 nm to 700 nm in which the concentration of HBA was kept constant and the concentration of His-CobB^{Alvin} varied from 0, 2.5, 5, 7.5,10 and 15 µM.

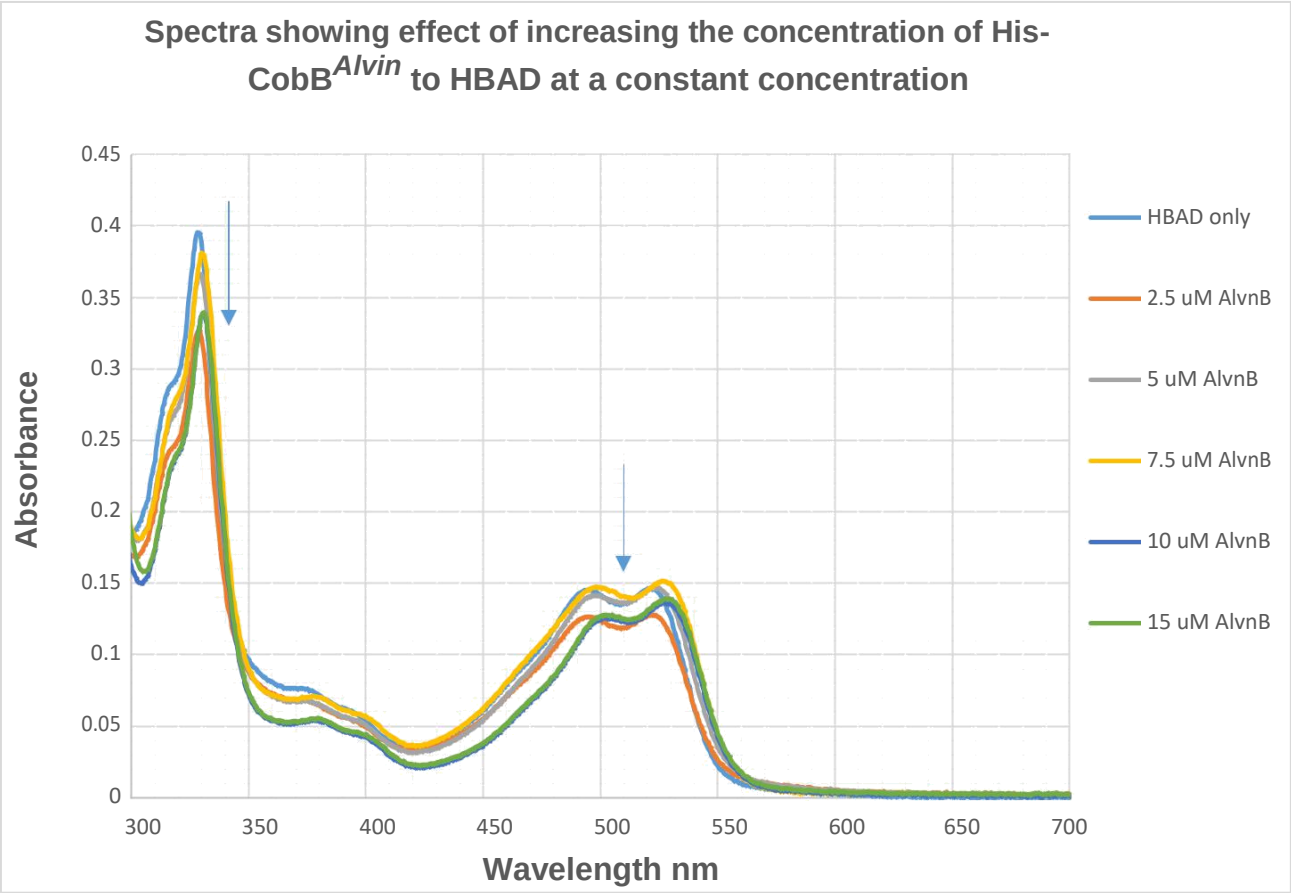


Figure 27: Spectra showing the absorbance readings from 300 nm to 700nm during a His-CobB^{Alvin} titration with HBAD. The concentration of HBAD was kept constant and the concentration of His-CobB^{Alvin} was varied from: 0, 2.5, 5, 7.5,10 and 15 µM.

Conclusion

The modification to the HBA and HBAD spectra when His-CobB^{Alvin} was titrated was not conclusive in that it was difficult to observe a saturation point. This is could be due to a number of reasons, including pipetting errors or uneven mixing of the protein to HBA or HBAD. Due to time restrictions this experiment was not repeated. Nonetheless, the fact that clear changes in the spectrum were observed suggested that the technique could be used to determine the binding stoichiometry.

3.8 *In vitro* binding of His-CobB^{Alvin} to CobH-HBA

Another method regarding the *in vitro* binding of CobB to HBA was undertaken by using Native-PAGE and SDS-PAGE. In this experiment, His-CobB^{Alvin} was added to the bound complex of CobH-HBA. The reason for carrying this test was to investigate if CobB was able to release the HBA from CobH, as would be expected if direct metabolite transfer was in operation.

To a fixed concentration of CobH-HBA variable concentrations of His-CobB^{Alvin} (0, 2.5, 3.75, 5, 6.25, 7.5, 8.75, 10, 12.5 and15 µM) were added. This test also required a number of controls including non-mixed samples for CobB and CobH-HBA.

The interaction between the two enzymes was observed when they were run on the Native-PAGE as shown in Figure 28. CobH with bound HBA and apo CobH migrate differently on the native gel.

3.8.1 Native–PAGE of His-CobH^{Alvin} +HBA interaction with His-CobB^{Alvin}

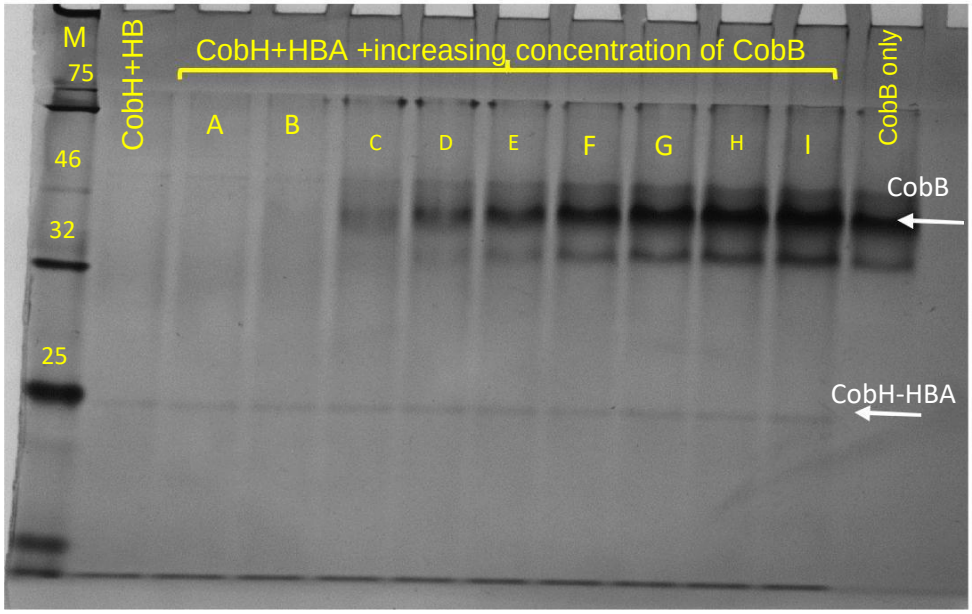


Figure 28: The Native-PAGE gel shows the un-denatured mixture of CobH ^{Alvin} -HBA + CobB $_{Alvin}$ in order to determine whether HBA has been released from CobH. This would result in uptake of this product by His –CobB ^{Alvin} varied from 2.5 µM (A), 3.75 µM (B), 5 µM (C), 6.25 µM (D), 7.5 µM (E), 8.75 µM (F), 10 µM (G), 12.5 µM (H) and15 µM (I)

The same procedure towards preparing the samples was used for the denaturing gel SDS-PAGE. The CobB concentrations of 0, 2.5, 3.75, 5, 6.25, 7.5, 8.75, 10, 12.5 and 15 µM were mixed with a constant concentration of CobH-HBA (5.8 mg/mL) and boiled for 5 to 10 minutes before loading onto the SDS-PAGE gel.

3.8.2 SDS-PAGE of CobH *Alvin* +HBA interaction with CobB *Alvin*

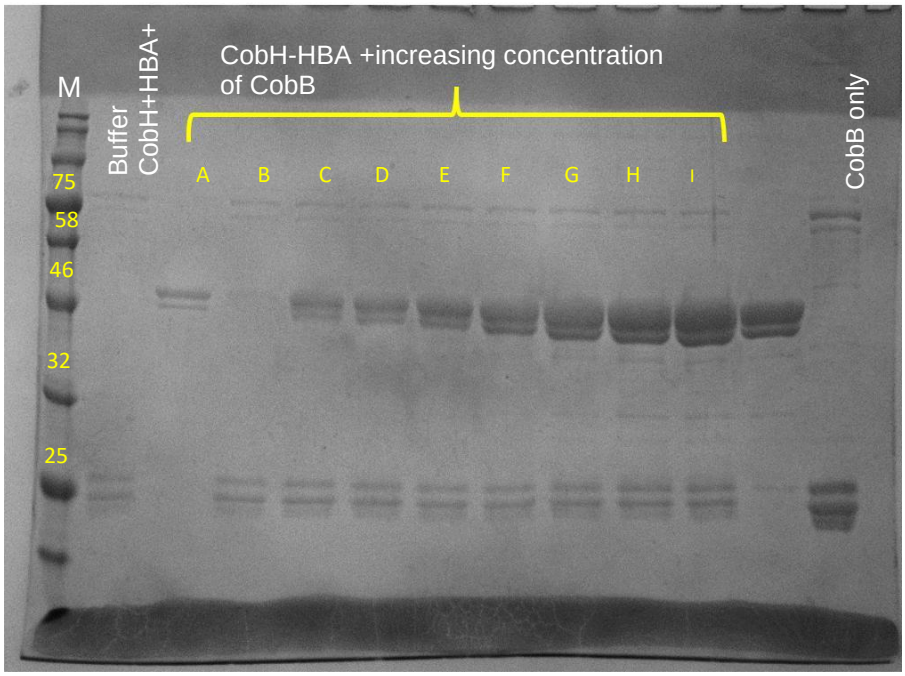


Figure 29: The SDS-PAGE gel showing the denatured mixture of CobH^{Alvin} HBA + CobB^{Alvin} in order to confirm the presence of both CobH and CobB, with increasing concentration of CobB. 2.5 µM (A), 3.75 µM (B), 5 µM (C), 6.25 µM (D), 7.5 µM (E), 8.75 µM (F), 10 µM (G), 12.5 µM (H) and15 µM (I).

Conclusion:

The results from both the Native PAGE (Figure 28) and the SDS-PAGE (Figure 29) did not show strong signs of interaction between protein and the substrate. The weak interaction in both cases suggests that no complex is made between CobH-HBA and CobB. However, as the protein bands were still present, it could mean that the concentration of CobH-HBA was too low hence the possibility of interaction with CobB was reduced. Moreover, the gel suggest that a certain amount of proteolysis has taken place, as both CobH and CobB ran as multiple bands. This investigation needs to be redone in the presence of a higher concentration of CobH-bound HBA.

3.9 Crystallography of CobH/ CobB *Alvin* in presence and absence of HBA

The process of identifying structural properties of macromolecules via X-ray crystallography involves a multistep procedure that includes choosing a suitable target molecule, cloning, purifying and crystallising the pure soluble protein. The protein needs to be crystallised under conditions that allow the protein to fall out of solution in a controlled and ordered fashion. These conditions may alter if substrates are also present.

Crystallography provides an opportunity to learn more about the active site of the enzyme and the binding site of the substrate in molecular detail. Kits from Molecular Dimensions, screens 1 and 2 were used to investigate conditions for crystallisation of both CobB and CobH through the method known as hanging drop vapour diffusion. In this method the drop containing the protein, its product and the reagent are equilibrated with the liquid reservoir.

When the concentration of buffer in the drop and the reservoir reaches equilibrium the concentration of precipitant and protein increases in the drop and should the protein be in an appropriate crystallisation buffer, crystal growth will occur in the drop. The condition of interest that sustains crystal growth is subtly altered by screen optimisation that involves changing the concentration of chemicals that were in the condition. The screen conditions are designed by using the Hampton research tray maker program which allows inserting a different range of variable values. The following favourable conditions that allowed CobB protein to crystallise is as follow: Condition: MD1-01 tube 23 0.2 M Calcium chloride dihydrate (0, 0.1, 0.2, 0.3) 0.1 M Sodium HEPES pH 7.5 (constant) 28 % v/v PEG 400 (22, 24, 26, 28, 30, 32).

The pH of the solution is constant using appropriate buffer. However, other factors such as the percentage of PEG400 and the concentration of calcium chloride dihydrate were changed in respect to the volume from the original screen. The total volume of 500 µL is achieved by addition of water to the mixture as shown in table 3.

0 M $CaCl_2$ 22 % PEG 400 0.1 M HEPES pH7.5	0.1 M $CaCl_2$ 22 % PEG 400 0.1 M HEPES pH7.5	0.2 M $CaCl_2$ 22 % PEG 400 0.1 M HEPES pH7.5	0.3 M $CaCl_2$ 22 % PEG 400 0.1 M HEPES pH7.5
0 M $CaCl_2$ 24 % PEG 400 0.1 M HEPES pH7.5	0.1 M $CaCl_2$ 24 % PEG 400 0.1 M HEPES pH7.5	0.2 M $CaCl_2$ 24 % PEG 400 0.1 M HEPES pH7.5	0.3 M $CaCl_2$ 24 % PEG 400 0.1 M HEPES pH7.5
0 M $CaCl_2$ 26 % PEG 400 0.1 M HEPES pH7.5	0.1 M $CaCl_2$ 26 % PEG 400 0.1 M HEPES pH7.5	0.2 M $CaCl_2$ 26 % PEG 400 0.1 M HEPES pH7.5	0.3 M $CaCl_2$ 26 % PEG 400 0.1 M HEPES pH7.5
0 M $CaCl_2$ 28 % PEG 400 0.1 M HEPES pH7.5	0.1 M $CaCl_2$ 28 % PEG 400 0.1 M HEPES pH7.5	0.2 M $CaCl_2$ 28 % PEG 400 0.1 M HEPES pH7.5	0.3 M $CaCl_2$ 28 % PEG 400 0.1 M HEPES pH7.5
0 M $CaCl_2$ 30 % PEG 400 0.1 M HEPES pH7.5	0.1 M $CaCl_2$ 30 % PEG 400 0.1 M HEPES pH7.5	0.2 M $CaCl_2$ 30 % PEG 400 0.1 M HEPES pH7.5	0.3 M $CaCl_2$ 30 % PEG 400 0.1 M HEPES pH7.5
0 M $CaCl_2$ 32 % PEG 400 0.1 M HEPES pH7.5	0.1 M $CaCl_2$ 32 % PEG 400 0.1 M HEPES pH7.5	0.2 M $CaCl_2$ 32 % PEG 400 0.1 M HEPES pH7.5	0.3 M $CaCl_2$ 32 % PEG 400 0.1 M HEPES pH7.5

Table 3: The condition of which the crystal growth took place is closely examined by screening around the variables that made up the condition. The Hampton research tray design has allowed calculating the volume of essential ingredients required for each well so the accuracy of the measurements is ensured before the experiment was carried out. The volume of $CaCl_2$ is varied from 0 M-0.3 M, HEPES pH 7.5 remained unchanged and lastly PEG400 percentage is varied between 22%- 32%.

The tray was kept at 18 °C and the percentage of precipitants were examined 1 hour after placing the tray. The following examination of the precipitant took place 3 days later in order to allow the process of crystallisation to complete. The crystal samples picked from condition with higher percentage of PEG 400 mostly contained a sticky layer which is referred to as “skin”.

The skin traps the crystal inside the drop hence it becomes difficult to get hold of the crystal or due to the several attempts the crystal can break or get dissolved in the solution of drop. PEG is known to act as a protective factor in crystallography but it can also be considered as a disadvantage when its concentration exceeds a certain percentage.

For this reason the crystals that were found in lower percentage of PEG 400 were collected for X-ray diffraction examination. Other factors such as lower concentration of calcium chloride dihydrate seemed to have least effect on the quality and quantity of the crystals hence the picked samples were selected from 0.2 and 0.3 M $CaCl_2$.

Another set of screening was performed for the following condition in which the CobB protein crystallised in presence of its substrate HBA.

MD1-02 tube 8: 0.2 M Ammonium phosphate monobasic $(NH_4)_3$ PO_4, 0.1 M Tris, pH 8.5, 50 % v/v MPD (highlighted in table 4)

The concentration of each chemical that made up this condition was varied in order to enhance the chances of obtaining crystals therefore for this reason two separate screens were set up. In order to see the effect of change in pH and $(NH_4)_3$ PO_4 concentration, the pH ranged between 8.1-8.5 and $(NH_4)_3$ PO_4 ranged from 0.14 M to 0.24 M as shown in table 4.

Tris pH	Ammonium phosphate concentration					
	0.14	0.16	0.18	0.2	0.22	0.24
8.1	70µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 130 µl H_2O	80µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 120µl H_2O	90µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 110 µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 100µl H_2O	110µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 90 µl H_2O	120µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 80 µl H_2O
8.3	70µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 130µl H_2O	80µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 120 µl H_2O	90µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 110µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 100 µl H_2O	110µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 90 µl H_2O	120µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 80 µl H_2O
8.5	70µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 130 µl H_2O	80µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 120 µl H_2O	90µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 110µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 100 µl H_2O	110µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 90 µl H_2O	120µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 80 µl H_2O
8.7	70µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 130 µl H_2O	80µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 120 µl H_2O	90µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 110 µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 100 µl H_2O	110µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 90 µl H_2O	120µl $(NH_4)_3PO_4$ 50µl Tris-HCl 250 µl MPD 50% 80 µl H_2O

Table 4: CobB bound HBA protein crystal was obtained from the condition containing 0.2 M $(NH_4)_3$ PO_4, 0.1 M Tris, pH 8.5, 50 % v/v MPD which is highlighted in pink. The optimization includes changing the $(NH_4)_3$ PO_4 concentration shown on the x-axis of the table and the pH of Tris as shown on the Y-axis of the table. The 50% MPD remained constant at this stage.

The second optimised screen was focused on changing the percentage of MPD at different pH of Tris while the $(NH_4)_3$ PO_4 concentration is kept constant (table5). As shown in table 6, the crystals picked from the suitable condition and frozen so the sample is pryo-protected for X-ray analysis in order to determine the structure of CobB when bound to the substrate HBA.

Tris pH	30% MPD	40 % MPD	60% MPD	70% MPD
8.1	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 150 MPD 30% 200 µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 200 MPD 40% 150 µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 300 MPD 60% 50 µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris- HCl 350 MPD 70% Non µl H_2O
8.3	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 150 MPD 30% 200 µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris- HCl 200 MPD 40% 150 µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 300 MPD 60% 50 µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 350 MPD 70% Non µl H_2O
8.5	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 150 MPD 30% 200 µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 200 MPD 40% 150 µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 300 MPD 60% 50 µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 350 MPD 70% Non µl H_2O
8.7	100µl $(NH_4)_3PO_4$ 50µl Tris- HCl 150 MPD 30% 200 µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 200 MPD 40% 150 µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris-HCl 300 MPD 60% 50 µl H_2O	100µl $(NH_4)_3PO_4$ 50µl Tris- HCl 350 MPD 70% Non µl H_2O

Table 5: the optimised screen based on variables such as MPD % (30%-70%) and Tris-pH (8.1-8.7). The concentration of $(NH_4)_3 PO_4$, remained constant (0.2 M).

1	+HBA	CobB	30% MPD at pH 8.5 and 100mM Ammonium Phosphate
2	-HAB	CobB	30% MPD at pH 8.5 and 100mM Ammonium Phosphate
3	+HBA	CobB	70% MPD at pH 8.1 and 100mM Ammonium Phosphate
4	+HBA	CobB	60% MPD at pH 8.3 and 100mM Ammonium Phosphate
5	+HBA	CobB	60% MPD at pH 8.3 and 100mM Ammonium Phosphate

Table 6: the cob-HBA crystals from the optimised screen were selected and picked from the listed conditions in this table.

3.9.1 Crystallising CobH^{Alvin} +/- HBA

In order to crystallise CobH^{Alvin} in presence of its substrate HBA, the concentration of protein Cob ^{Alvin} was measured at 280nm giving a value around 6 mg/mL and the concentration of HBA at 328 nm was determined to be 5 mg/mL. For crystallography screens HBA was added to the protein in a ratio of 5:1.

The protein formed crystals in the following condition:

CobH^{Alvin} without HBA crystals:

2M Sodium-acetate Trihydrate, 0.1 M Imidazole pH 6.5

CobH^{Alvin} + HBA crystals (figure 30):

0.2 M Sodium-citrate tribasic dihydrate, 0.1 M Tris pH8.5, 30 % v/v PEG 400

The optimisation of these conditions is still under investigation as this experiment requires more time for growing the crystals in these favourable conditions.

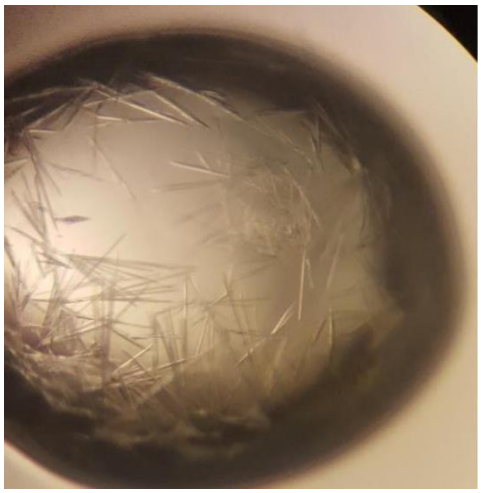


Figure 30: By using the crystallisation structure screens 1 and 2 provided by Molecular Dimensions, CobH ^{Alvin} was found to crystallise in the absence of HBA.

3.9.2 Crystallising CobB^{Alvin} +/- HBA and HBAD

CobB^{Alvin} was also tested using the Molecular Dimension screening kits.

The initial attempt towards crystallising CobB^{Alvin} was done in presence and absence of HBA. A significant amount of precipitation was seen due to the high concentration of the protein. Hence in the subsequent attempts the concentration of protein was reduced. Favourable conditions that generated crystals were optimised by further screening around these conditions. The resultant crystals were sent for X-ray diffraction analysis. Disappointingly, the results from the X-ray crystallography indicated that the crystals were formed from salt and not protein.

Further attempts to crystallise the protein were undertaken in order to find alternative conditions, which included the addition of coenzymes such as ATP, non-hydrolysable ATP, glutamine and Mg^{2+}. Each of the named coenzymes and substrates can enhance crystal development by increasing the stability of enzyme.

Depending on the total concentration of the purified protein, the addition of ATP (5x) and Mg^{2+} (10x) excess was required. As mentioned before, the ratio of 5:1 HBA to protein was added to the conditions where the protein and its substrate were in the drop. The optimised screens showed an increase in the number of crystals in conditions where the protein was not in mixture with the substrate.

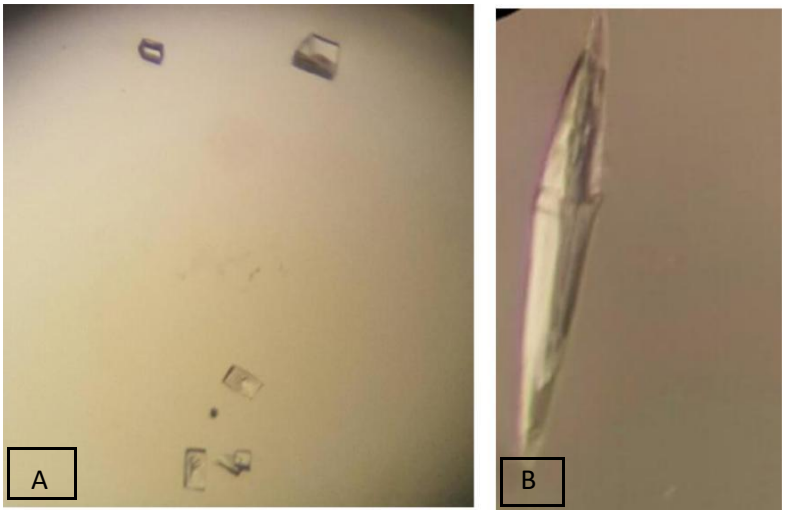


Figure 31: Figure A shows the crystals in a condition where protein (CobB) and substrate (HBA) were in the mixture drop. Figure B shows a crystal of CobB on its own. The shape of the protein crystal changed with the addition of additives such as ATP, Mg^{2+} and glutamine.

The presence of heavy precipitates in conditions where the substrate was included in the mixture suggested that further improvements were required to enhance crystallisation. The His-tag on the protein also has the potential to interfere with the crystallisation process. Hence, the N-terminus His-tag of the protein was removed by thrombin cleavage.

The His-tag free protein drops contained the least precipitate and the crystals were found to have smoother surfaces and edges in comparison to the crystals that were obtained before removing the His-tag. However, CobB did not seem to interact with HBA therefore no crystals were seen in the mixture drop of protein and HBA.

Temperature is a significant variable in the crystallisation of macromolecules as it influences nucleation and crystal growth by controlling the solubility and supersaturation of the sample. For this reason, another approach aimed at reducing the rate of precipitation in the drop was undertaken by lowering the temperature in which the crystal was set and stored. This resulted in the formation of smaller crystals in comparison to the crystals that were grown at room temperature. However, a significant reduction in the formation of heavy precipitant was seen (Figure 32).

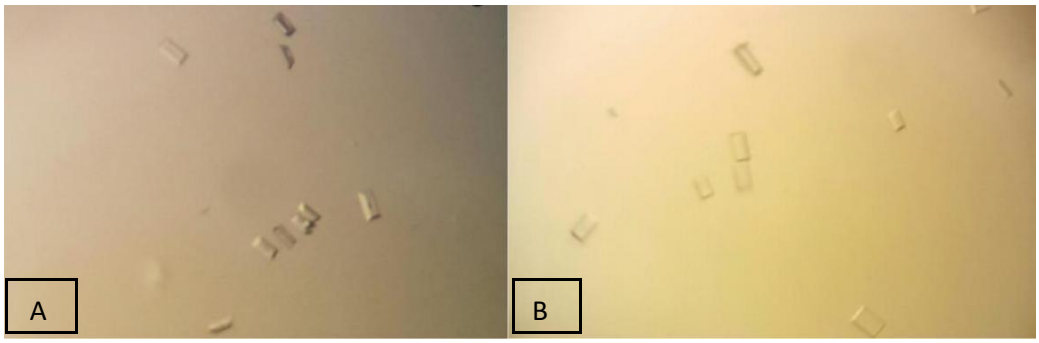


Figure 32: Both figures A and B show crystals of CobB in the presence of HBA in the mixture. The screen was set and stored at lower temperature and this factor has affected the rate of growth, size and shape of the crystals.

The X-ray crystallography results showed that due to the unstable conformation of the protein crystals it was not possible to see a diffraction pattern when the crystals were placed in the X-ray beam (Figure 31 and 32). Since methods such as His-tag cleavage and reduction in temperature did not result in crystallisation of the protein of interest, future screens need to be carried out .

The addition of certain chemicals that affect hydration and intermolecular interactions between protein molecules or protein molecules and solvent, and even ligands, may assist in this process.

The following CobB protein crystals were obtained by addition of additives as well as coenzymes.

The CobB protein crystals were picked from the following optimised condition:

MD1-02 condition 23

1.6 M Ammonium sulphate

0.1 M MES 6.5

10 % v/v 1,4-Dioxane

In order to optimise this condition, the concentration of certain chemicals that made up the buffer of interest were changed. For instance, the crystal required 1.6 M ammonium sulphate but for the purpose of optimisation, this concentration was varied from 1 to 2 M. The pH of 0.1 M MES was kept constant because changes in pH could result in the formation of a heavy precipitate. Lastly, the concentration of 1, 4-Dioxane was varied from 6 -12 %.

As shown in Figure 33(A) the CobB drops are at the stage known as phase separation, which likely represents a metastable transition where protein crystals may form. Hence the condition was optimised as shown in Figure 33(B). At the final stage of optimisation (Figure 33 C) a crystal was picked for X-ray analysis.

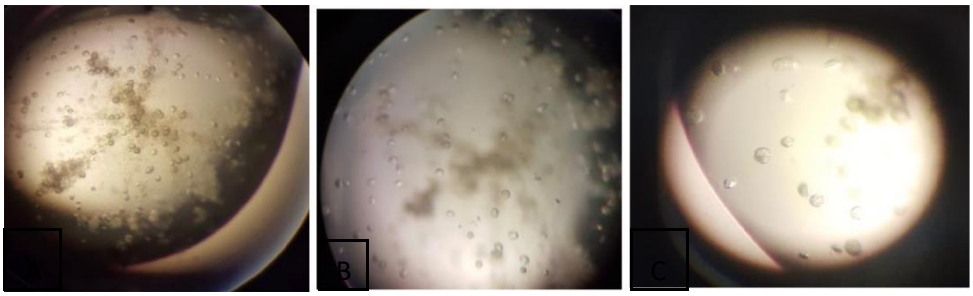


Figure 33: The CobB crystal formed in condition 23 of structure screen 1. The original condition (A) is optimised in the following steps (B and C) in order to increase the quantity and quality of crystal formation.

Conclusion

The results have shown that it is possible to crystallise the CobB protein both in the absence and presence of HBA. Unfortunately, the crystals that have been obtained so far did not diffract.

Chapter 4

Discussion and Conclusion

This report describes attempts to understand how metabolites are passed between enzymes within the cobalamin (vitamin B_{12}) biosynthetic pathway. Of the thirty enzymes linked with cobalamin biosynthesis this investigation has focussed on the potential interaction between CobH and CobB. Specifically, the project has looked at these enzymes from *A. vinosum*, as preliminary research on the *A. vinosum* CobB had shown that this enzyme is more active and more stable than previously studied enzymes. The method of communication between CobH and CobB relates to the transfer of HBA, which is the product of the reaction that is catalysed by CobH. Previous publications had provided convincing evidence that there is a tight interaction between CobH and HBA (Thibaut et al. 1992) (Deery et al. 2012). Indeed, characterisation of the *A. vinosum* CobH also showed that this protein is able to bind HBA tightly, revealing a ratio of one HBA bound per CobH dimer. The fact that the *A. vinosum* His-CobH purifies from a HBA-producing strain with bound HBA indicates the strength of the interaction.

Additionally, the sequence comparison between the solved 3D-structure of *Rodobacter capsulatus* CobH-HBA and *Alvin*-CobH which is provided at the end of this chapter shows the regions of similarity between the two sequences. This comparison shows an indication of functional and structural relationship that exists between the named bacterial strains. This level of similarity and identity could also mean that crystallising the $CobH^{Alvin}$ can be made possible.

The key question underlying this research project is, given that CobH binds HBA so tightly, how CobB recognises the product of CobH and how this product (HBA) is released from the protein? It would make sense that an interaction with the next

enzyme in the pathway, CobB, would lead to a conformational change and the release of the product to allow it to bind to CobB. In this respect it can be thought that the interaction between CobH and CobB would weaken the interaction between CobH and HBA, allowing the released intermediate to bind with CobB. To investigate this further a number of experiments were undertaken with a view to gaining some insight into the interaction between CobH and CobB. These experimental approaches included attempts to look at the release of HBA by native gel electrophoresis and the crystallisation of both enzymes. The latter approach would provide the necessary molecular detail to determine if the proteins were able to interact with each other.

As mentioned above, it was possible to demonstrate that the *A. vinosum* His-CobH is able to bind HBA in a HBA-producing strain. This enzyme-trap approach allows for the isolation of large amounts of HBA-bound CobH. Using a similar enzyme-trap approach it was also possible to show that the *A. vinosum* His-CobB also could be purified with a bound pigment. However, with CobB was not clear whether the extracted enzyme had bound its substrate or its product. Thus the pigment bound to CobB was released after the protein was denatured and the material analysed by HPLC-MS. This revealed that CobB had bound HBAD, HBA and HBA monoamide. However, the major component isolated in this study was HBAD, suggesting that CobB also holds onto its product tightly.

Returning to the question of how CobB get hold of its product from CobH, we sought to devise a way to follow the release of HBA from CobH in the presence of CobB. One idea was to try and follow this process by using Native PAGE. Previous research had shown that CobH runs differently on a native gel to CobH with bound HBA. We therefore though that it may be possible to follow the release of HBA from CobH by looking for a shift in the mobility of CobH with increasing concentrations of

CobB. Similarly, it may be that CobB would also show a differential migration in the presence of a bound substrate. However, when this gel shift analysis was undertaken there was no apparent shift in either CobH or CobB. However, both proteins showed some evidence of degradation indicating that the proteins were perhaps not as stable as previously thought. However, the experimental approach also really needs to be done again in the presence of substrate and other cofactors including ATP and glutamine as the absence of these may have contributed to the instability of CobH and CobB.

Obtaining crystal structures of both CobH and CobB would improve our understanding of the binding sites of the enzymes of interest. Ideally, a structure of a CobH/CobB complex would provide unprecedented insights into how these proteins interact. As a start to this overall aim protein crystallography was undertaken on the individual enzymes, CobH and CobH. Attempts were made to gain crystals of these enzymes in the presence and absence of HBA, as well as the other substrates associated with the CobB enzyme. A wide range of conditions were explored, mainly using the kits available from Molecular Dimensions. Removal of the His-tag was also explored as a way to try and grow improved crystals. However, despite the wide range of conditions that were explored, no diffraction quality crystals were obtained.

Bioinformatics analysis of CobB performed by others suggests that the protein is composed of two domains, representing a domain that releases ammonia from glutamine and a domain that amidates the carboxylic acid side chain of HBA. Movement between these domains may mean that the protein is quite flexible and this flexibility may prevent the formation of suitable crystals. Therefore, future work may have to involve the generation of mutants of CobB that are not able to turn over, which

will be used to bind the substrate without catalysing the reaction in a more static conformation.

In conclusion, although it has been possible to crystallise CobH and CobB diffraction quality crystals still need to be formed, a process that requires more time. CobH binds HBA very tightly but this does not prevent the interaction between CobH-HBA and CobB. CobB also binds its product very tightly. All of this provides further evidence that the B_{12} biosynthetic enzymes must work by substrate channelling.

Sequence comparison between the solved 3D-structure of *Rhodobacter Capsulatus* CobH-HBA and *Alvin*-CobH

Length: 210

Identity: 126/210 (60.0%)

Similarity: 154/210 (73.3%)

Gaps: 3/210 (1.4%)

Score: 601

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EYNRDAHDIYRQSFAIIRAEADLSRIPADLEALAVRVIHSCGMPEIA 49 |.

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EMBOSS_001 1

MPHEYEKDGAKIYVQSFATIRAEADLARFTPEEEVVVVRMIHAAGMVGLE 50

EMBOSS_001 50

AELEFSPGAGAAGCAALESGATILCDSRMVSEGITRARMPADNPIVCTLH 99

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EMBOSS_001 51

NHVRFAPGMAIAARAALEAGAPILCDARMVSEGITRARLPAKNEVICTLQ 100

EMBOSS_001 100

DPSVPALARELGNTRTAAALELWRPHLAGSIVVVGNAPTALFRLLEMLDE 149

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EMBOSS_001 101

DPRVPALAQEMGNTRSAAALELWRPKLEGAVVAIGNAPTALFHLLNMLED 150

EMBOSS_001 150 GA-

PKPALILGFPVGFVGAVESKEALAADSRGVPFVAVRGRRGGSAMAAA 198

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EMBOSS_001 151 PACPRPAAIIGCPVGFIGAAESKAALAV-

ANPVPWVIVEGRLGGSAITVA 199

EMBOSS_001 199 AVNALGREHS 208

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EMBOSS_001 200 AVNALACRKE 209

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