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Cloning, Expression and Interaction Studies of the Potential RubisCO Activase CbbQ

by

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May 2014

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ABSTRACT

Carboxysomes are polyhedral microcompartments found in all cyanobacteria and in many chemoautotrophs. Within their shell they contain the enzyme ribulose-1, 5-bisphosphate carboxylase/oxygenase (RubisCO), which fixes CO_2. Downstream of the carboxysome operon there is another gene cluster containing several genes that may enhance carboxysome function. Two of these genes, *cbbQ* and *cbbO*, encode potential RubisCO activases. Using recombinant CbbQ and CbbO protein, and RubisCO isolated from carboxysomes the interaction between these proteins was studied. The CbbO and CbbQ proteins were both His tagged, allowing them to be purified with Ni^{2+}-NTA column chromatography. Each of these tagged proteins was incubated with RubisCO on a Ni^{2+}-NTA column to determine if both proteins could be co-eluted. These studies suggest that recombinant CbbO and CbbQ do not interact individually with RubisCO. Since His-tagged CbbO and untagged CbbQ, when co-expressed in *E. coli*, form a complex, ongoing studies are focused on determining if both potential activases are needed to interact with RubisCO when co-expressed.

Keywords: carboxysomes, affinity column chromotography, cyanobacteria, chemoautotrophs, co-expression, operon, protein purification

ACKNOWLEDGEMENTS

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REVIEW OF RELATED LITERATURE

Global climate change is caused by excessive levels of CO_2, a greenhouse gas in the atmosphere. Human activities, such as burning fossil fuels, release CO_2 into the atmosphere and are major contributors to its unusually high concentrations. The damaging effects of global climate change include rising sea levels, decreasing amounts of precipitation causing droughts, and overall warming in certain parts of the world (Solomon, Plattner, Knutti, & Friedlingstein, 2009). Atmospheric CO_2 levels are decreased through its fixation, or conversion to organic carbon used to build cellular components, by plants, photoautotrophic bacteria, and chemoautotrophic bacteria (Andersson & Backlund, 2008). **Figure 1** illustrates how carbon cycles through the environment, and how there are many more contributors to the release of carbon into the atmosphere as opposed to its fixation.
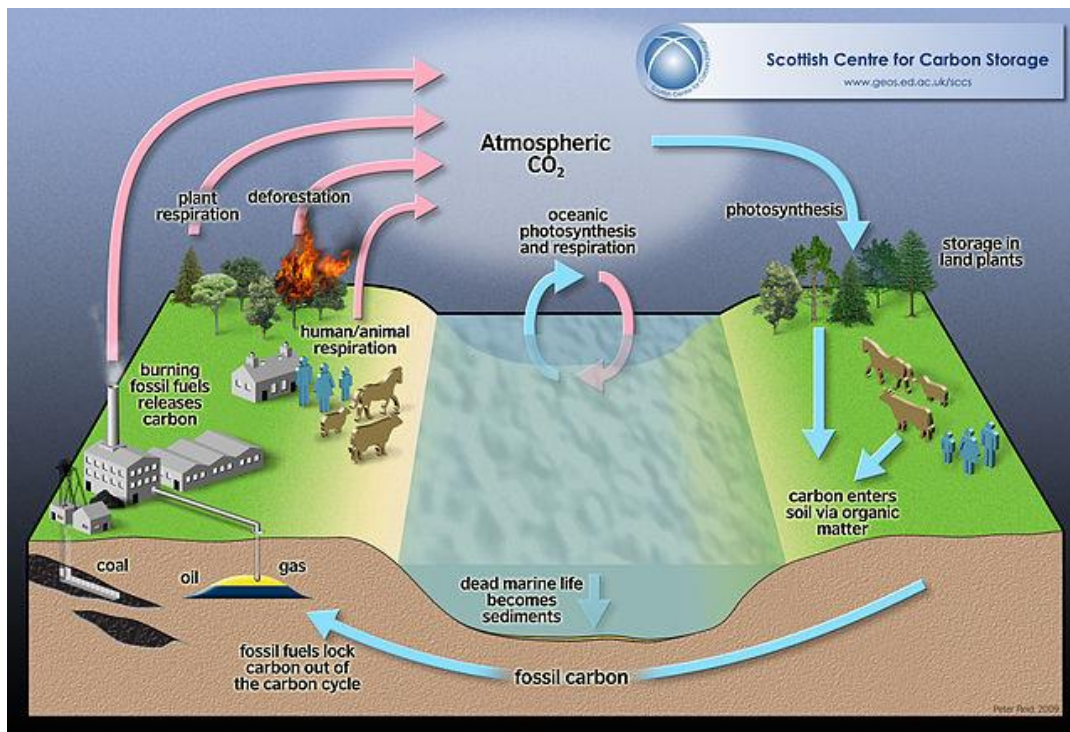
Figure 1: The components of the global carbon cycle. Note that the burning of fossil fuels creates carbon dioxide emissions. Adapted from www.geos.ed.ac.uk/sccs.

The importance of bacteria in the global cycling of carbon in the biosphere is often overlooked in favor of plants, which are typically viewed as the main CO_2 reducers. Both plants and carbon fixing bacteria, however, are essential to the removal of carbon from the atmosphere. Phytoplankton, which consists of bacteria and eukaryotic algae, is responsible for 45% of CO_2 fixation annually (Andersson & Backlund, 2008). Because of their large contribution to global carbon fixation, understanding how bacteria reduce CO_2 will provide essential information on how to increase the efficiency of fixation, thereby reducing the atmospheric levels of carbon and mitigating the damaging environmental effects of high CO_2 levels.

Some chemoautotrophs and all cyanobacteria perform carbon fixation within polyhedral structures, composed entirely of protein, called carboxysomes (**Figure 2**). Carboxysomes were first observed in 1956, and in 1973 were isolated from the sulfur oxidizing chemoautotroph *Halothiobacillus neapolitanus*, shown in **Figure 3**. Carboxysomes are composed of shells, which encapsulate rib<u>u</u>lose 1, 5- <u>bis</u>phosphate <u>c</u>arboxylase/<u>o</u>xygenase (RubisCO), the enzyme responsible for carbon fixation. Their function mimics that of organelles within eukaryotes – they separate portions of the cell, sequester enzymes within their interior, and control the input and output of substrates and other molecules. These structures allow for an increased amount of control over the function of RubisCO (Yeates, Kerfeld, Heinhorst, Cannon, & Shively, 2008).

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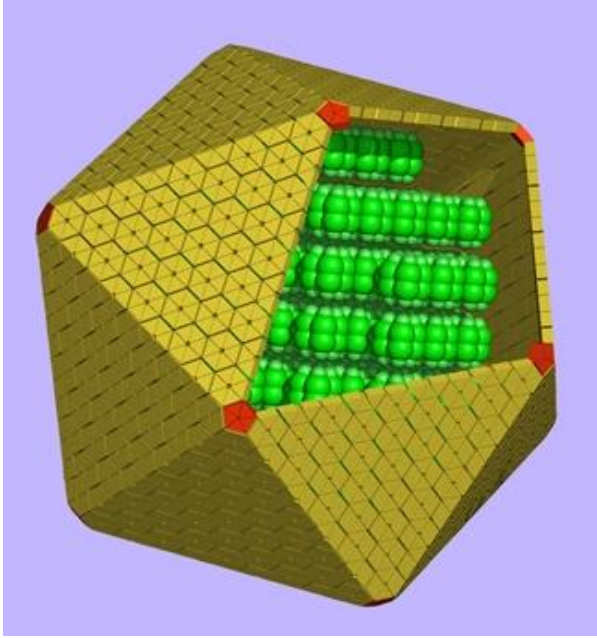
Figure 2: A 3D rendering of the carboxysome, made up of a protein shell that encapsulates RubisCO. Tsai, Y. et al 2007. PLOS 5(6): 1345-1354.
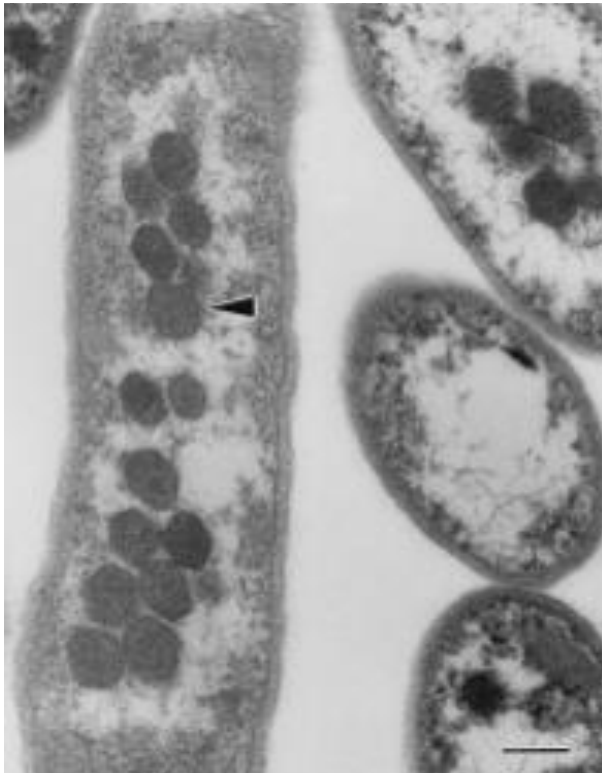
Figure 3: An electron micrograph of *Halothiobacillus neapolitanus*. The arrow points to a carboxysome. Tsai, Y. et al 2007. PLOS 5(6): 1345-1354.

The RubisCO within the shells of carboxysomes participates in the first phase of the Calvin-Benson-Bassham cycle, carbon fixation (**Figure 4**) (C. Kerfeld, Heinhorst, & Cannon, 2010). RubisCO is composed of eight large subunits that are catalytically active, and eight small subunits whose function is not fully understood (Spreitzer, 2003). RubisCO fixes CO_2 by combining it with the five carbon molecule ribulose 1, 5- bisphosphate (RuBP) and producing two molecules of 3-phosphoglycerate (3-PGA). RubisCO is inefficient as an enzyme because it is catalytically slow, has a low affinity for CO_2 and participates in a side reaction with O_2 that produces energetically wasteful products. In order to function productively, RubisCO must be in the presence of high local concentrations of CO_2 and limited in its exposure to O_2 (Andersson & Backlund, 2008). The chemoautotrophs and cyanobacteria that contain carboxysomes live in environments where the CO_2 concentrations are too low for RubisCO to function productively. Within the shell of carboxysomes, however, RubisCO is encapsulated in a high CO_2 environment that allows carbon fixation to occur more efficiently (C. Kerfeld et al., 2010).
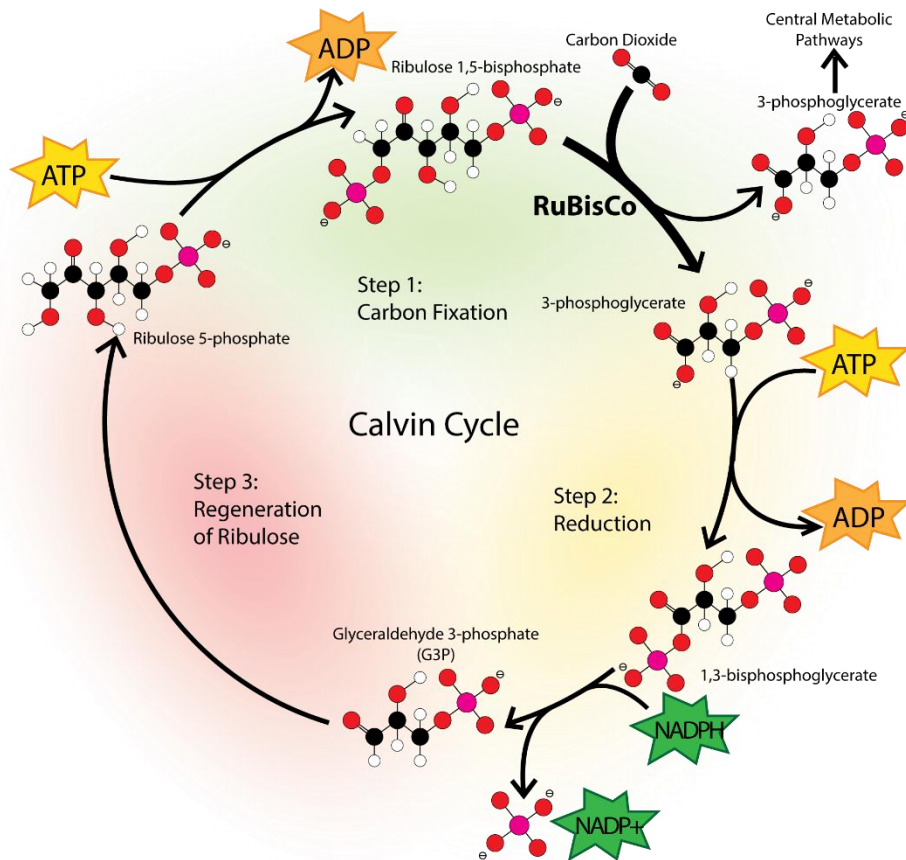
Figure 4: The Calvin-Benson-Bassham Cycle. RubisCO is the key enzyme involved in the first phase of the cycle, carbon dioxide fixation. Adapted from www.ck12.org.

Carboxysomes create high local concentrations of CO_2 for RubisCO, increasing its productivity in the final step of the carbon concentrating mechanism (CCM). In the first step of the CCM dissolved inorganic carbon (DIC) mostly in the form of bicarbonate, enters the carboxysome via transporters in the cell membrane (C. Kerfeld et al., 2010). The second and final step of the CCM occurs within the carboxysome, where the enzyme carbonic anhydrase converts bicarbonate into CO_2 in the vicinity of RubisCO. The carboxysome concentrates CO_2 because of the relative impermeability of the shell to the gas (Yeates et al., 2008). The location of carbonic anhydrase inside the carboxysome is crucial, because through its action CO_2 reaches high concentrations within close proximity of RubisCO (Price & Badger, 1989). Regardless of

the measure taken to improve the output of RubisCO, it is still estimated to be the most abundant

protein on Earth (Andersson & Backlund, 2008). Carboxysomes are just one of the many ways

that carbon fixing organisms have adapted to improve the catalytic performance of RubisCO.

Some bacteria contain RubisCO activases that prevent RubisCO from being deactivated. Others

contain chaperone proteins, which assist in the assembly of the RubisCO holoenzyme.

An example of a RubisCO activase is the CbbX protein of the photosynthetic bacterium

Rhodobacter sphaeroides. RubisCO activation is dependent on the order in which its substrates

and cofactors bind. For RubisCO to become active a non-substrate CO_2 molecule must first bind

its activation site, followed by its Mg^{2+} cofactor, and finally RuBP, which is then carboxylated in

the active site with a substrate CO_2. Premature binding of RuBP deactivates RubisCO and

prevents the enzyme from carboxylating RuBP. CbbX is composed of six subdomains with a

central pore and is thought to function by pulling the C-terminal peptide of RubisCO's large

subunit into its pore and thereby facilitating the release of RuBP. Discharge of the inhibitory

RuBP allows a non-substrate CO_2 molecule to bind and activate RubisCO (Mueller-Cajar et al.,

2011). RubisCO efficiency is also improved through assistance of its assembly from its multiple

subunits.

The RbcX protein in *Synechococcus* has been identified as a chaperone that assists in the

assembly of the RubisCO subunits. Many proteins require the help of chaperones to fold

correctly, and multi-peptide complexes may also use chaperones to assemble their subunits. A

class of chaperones similar to the GroEL/GroES protein folding system was thought to help

assemble RubisCO's eight large and eight small subunits. However, further experimentation

showed that this folding system alone did not yield RubisCO in its fully assembled form,

suggesting that additional chaperones were required. RbcX was identified as the protein that

assists in the assembly of the eight large subunits of RubisCO. After the eight large individual

subunits are assembled via the GroEL/GroES system, RbcX mediates the assembly of the

subunits into the complex. After the dissociation of RbcX the eight small subunits bind, forming

the fully assembled RubisCO holoenzyme (Saschenbrecker et al., 2007).

Potential RubisCO Activases in <u>Halothiobacillus neapolitanus</u>

The proteins that form the carboxysome shell and the RubisCO cargo are all encoded by

genes in a single operon, called *cso*, in *H. neapolitanus*. The first two genes in the operon code

for the large and small subunits of RubisCO, respectively. The shell proteins of the

carboxysome are encoded by the remainder of the genes in the operon. The function of each of

these genes was determined by their deletion and assessment of carbon fixation activity of *H.

neapolitanus* mutants (C. a Kerfeld, Heinhorst, & Cannon, 2010). Recent research with the

cyanobacterium *Prochlorococcus* has shown that the *csoS1D* gene outside of the typical

carboxysome gene region encodes a novel shell protein (Roberts, Cai, Kerfeld, Cannon, &

Heinhorst, 2012). This finding has led researchers to look more closely at genes outside of the

known *cso* operon to determine if they contribute to the function or structure of carboxysomes.

Of particular interest are proteins that could increase the catalytic efficiency of RubisCO.
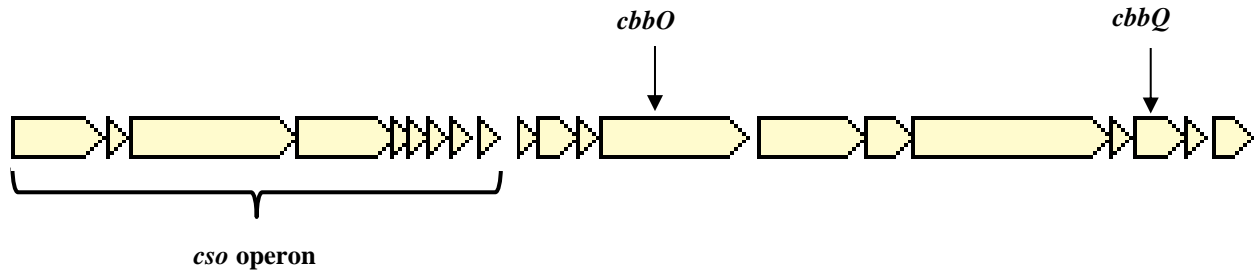
Figure 5: A portion of the *Halothiobacillus neapolitanus* genome. The traditional *cso* operon, *cbbO* and *cbbQ* are labeled.

Gene annotation has identified *cbbO* and *cbbQ*, downstream of the carboxysome operon in *H. neapolitanus*, as genes that code for potential RubisCO activases (**Figure 5**). Both of these potential activases are encoded by genes found in the chemolithotrophs *Pseudomonas hydrogenothermophila* (Hayashi, Arai, Kodama, & Igarashi, 1997a), and *Hydrogenovibrio marinus* (Hayashi, Arai, Kodama, & Igarashi, 1999). In *P. hydrogenothermophila* the expression in *E. coli* of the small and large subunit genes of RubisCO, along with *cbbO* and *cbbQ*, dramatically increased the V_{max} of RubisCO (Hayashi et al., 1997a). The *cbbQ* gene of *H. marinus* codes for a protein that has also been shown to increase the activity of RubisCO (Hayashi, Arai, Kodama, & Igarashi, 1999). The functional mechanism of both of these potential RubisCO activases is unknown. One possibility is that they could function in a similar fashion to the chaperone protein, RbcX, which assists in the assembly of the eight large subunits. Another potential function of CbbO and CbbQ is prevention of RubisCO deactivation. The work reported here begins to address these scientific questions by examining whether these two proteins interact with each other and with the carboxysomal RubisCO.

EXPERIMENTAL PROCEDURES

MATERIALS

Media

Luria-Bertani Broth (LB Broth):
10 g/L NaCl
10 g/L Bacto tryptone
5 g/L Bacto yeast extract

Luria-Bertani Agar (LBA):
15 g/L Agar
10 g/L Bacto tryptone
5 g/L Bacto yeast extract

SOC Medium:
20 g/L Bacto tryptone
5 g/L Bacto yeast extract
20 mM Glucose
10 mM NaCl
10 mM $MgCl_2$
10 mM $MgSO_4$
2.5 mM KCl

Dyes

Ethidium Bromide Stock Solution:
0.02 g/mL Ethidium bromide in deionized water

Agarose Gel Tracking Dye:
100 mM EDTA (pH 8.0)
50 % (v/v) Glycerol
1% (w/v) SDS
0.1 % (w/v) Bromophenol blue

Buffers

TAE Buffer, pH 8.0:
40 mM Tris-HCl (pH 7.8)

20 mM Na-acetate
2 mM EDTA

Lysis Buffer, pH 8.0:
50 mM Tris-HCl (pH 8.0)
5 mM 2-mercaptoethanol
1 mM PMSF/PTSF

Phosphate Buffered Saline (PBS), pH 7.4:
137 mM NaCl
2.7 mM KCl
4.3 mM Na_2HPO_4
1.4 mM KH_2PO_4

Affinity Chromatography

pProEx Wash Buffer A/ Column Equilibration Buffer:
20 mM Tris-HCl (pH 8.0)
100 mM KCl
10 % (v/v) Glycerol
20 mM Imidazole
5 mM 2-mercaptoethanol (BME)

pProEX Wash Buffer B:
20 mM Tris-HCl (pH 8.0)
20 mM KCl
10% (v/v) Glycerol
5 mM 2-mercaptoethanol

Elution Buffer:
20 mM Tris-HCl (pH 8.0)
100 mM KCl
10% (v/v) Glycerol
250 mM Imidazole
5 mM 2-mercaptoethanol

Protein Dialysis Buffer for Recombinant Proteins, pH 8.0:
10 mM Tris HCl (pH 8.0)
400 µM PMSF/PTSF

PMSF/PTSF Stock Solution:
100 mM Phenylmethylsulfonylfluoride (PMSF)
100 mM p-toluenesulfonylfluoride (PTSF)
This solution was made with 100% ethanol

Protein Gel Electrophoresis

SDS-PAGE Running Buffer, pH 8.5:
25 mM Tris
192 mM Glycine
1% w/v SDS

4X SDS-PAGE Loading Buffer:
200 mM Tris HCl (pH 6.8)
40% (v/v) Glycerol
8% (w/v) SDS
10 % (v/v) 2-mercaptoethanol

12% Laemmli SDS-PAGE Separating Gel:
2.5 mL 1.5 M Tris-HCl, pH 8.8
4.3 mL of H_2O
3 mL 40% Acrylamide/bis (29:1)
100 µL 10% Ammonium persulfate
7.5 µL Tetramethylethylenediamine (TEMED)

12% Laemmli SDS-PAGE Stacking Gel:
1.25 mL 1.5 M Tris-HCl, pH 6.8
3.18 mL of H_2O
0.5 mL 40% Acrylamide/bis (29:1)
25 µL 10% Ammonium persulfate
5 µL Tetramethylethylenediamine (TEMED)

Antibiotic Stock Solutions

100 mg/mL Ampicillin in H_2O (100 µg/mL final working concentration)
50 mg/mL Kanamycin in H_2O (50 µg/mL final working concentration)
100 mg/mL Spectinomycin in H_2O (100 µg/mL final working concentration)

Plasmids and <u>Escherichia coli</u> Strains

New England Biolabs (NEB) 5-alpha competent *E. coli*
NEB BL21 (DE3) competent *E. coli*
pCR 4Blunt-TOPO vector (Invitrogen)
pET DUET-1 vector (Novagen)
pCDF DUET-1 vector (Novagen)

METHODS

Polymerase Chain Reaction (PCR)

This reaction was performed in a final volume of 50 μL. Each tube contained 100 ng of *Halothiobacillus neapolitanus* chromosomal DNA, 10 μM of the forward primer (5'GGATCCATGACACAAAATGCAGATCAATATCG3'T_m= 59.4 ℃), 10 μM of the reverse primer (5'AAGCTTTTAAAAGAACGTTTTGACGACGG3' T_m= 58.4 ℃), 10 μL of the 5x Reaction Buffer, 1 μL of Deep Vent DNA polymerase (NEB), 2.5 mM dNTPs, and sterilized water. The PCR tubes were placed in a Bio-Rad MyCycler thermal cycler, which was pre-heated to 95 ℃. The initial denaturation step was at 95 ℃ for 3 minutes. Then five repeat cycles of denaturation, annealing and extension were performed at 95 ℃ for 30 seconds, 47.2 ℃ for 30 seconds and 72 ℃ for 2.5 minutes, respectively. This was followed by the main 25 repeat cycles of denaturation (95 ℃ for 30 seconds), annealing (52.2 ℃ for 30 seconds) and extension (72 ℃ for 2.5 minutes). A final extension step at 72 ℃ for 10 minutes followed the cycles. The thermal cycler then remained at 4 ℃ for the hold step. In the initial PCR cycles, the annealing temperature is lower because the primers were designed with restriction sites that do not anneal with the chromosomal DNA. The lower annealing temperature does not take the restriction site into account. In the later cycles the annealing temperature is raised, because the newly copied DNA fragments include the restriction site, and are used as templates. The annealing temperature is adjusted to include the restriction site portion of the primer.

TOPO Cloning and Transformation

The amplified DNA was inserted into the pCR ® BLUNT TOPO II vector using the Invitrogen TOPO PCR Cloning Kit. The protocols used for cloning and transformation were taken from the manual provided with the kit. Cloning was done in a 200 µL microfuge tube containing 1 µL of salt solution, 1 µL of TOPO vector, and 4 µL of the PCR product. This tube was incubated for 30 minutes at room temperature. Two microliters of the cloning mixture were then added to a tube of thawed NEB 5-α chemically competent *E. coli* cells (NEB) and incubated on ice for 30 minutes. The cells were heat shocked for 1 minute at 42 ℃, then immediately transferred to ice. After the addition of 250 µL of S.O.C. medium, the cells were incubated at 37 ℃ with shaking for 1 hour. The transformation mixture was spread on LB plates containing 50 µg/ mL kanamycin and incubated at 37 ℃ overnight.

DNA Plasmid Isolation

A patch plate was made from the colonies on the transformation plate. The patch plate colonies were used to inoculate liquid cultures, containing 3 mL of LB and 50 µg/ mL of kanamycin. The liquid cultures were incubated overnight and the plasmids were then isolated using the protocols and material provided by the QIAgen Spin Miniprep Kit (Qiagen). The concentration of the purified DNA was determined by measuring its absorbance using NanoDrop (Fisher Scientific) based on the ratio of double stranded DNA and protein contamination (260/280 nm).

Restriction Digestion of DNA

Restriction digestion of the pCR ® BLUNT TOPO II vector containing the *cbbQ* insert was done in a 20 µL reaction volume. The reaction consisted of 15 µL of the plasmid (91.7 ng/

µL), 1 µL of 10x NEB Buffer 2, 1 µL of 10x NEB Buffer 3, 0.2 µL of 100x BSA, 2 µL of sterile water, and 0.5 µL of BamHI and HindIII, each. The reaction was mixed briefly and placed in a 37 °C water bath for 1 hour before gel electrophoresis.

Restriction digestion of the pCDF DUET-1 vector was done in a similar fashion. The reaction tube contained 20 µL of pCDF DUET-1 (13.7 ng/µL), 1.5 µL of 10x NEB Buffer 2, 1.5 µL of 10x NEB Buffer 3, 1 µL of BamHI and HindIII each, 5 µL of sterile water, and 0.2 µL of 100x BSA. Following digestion for 1 hour at 37 °C, gel electrophoresis was performed.

A BamHI and NcoI double digestion was used to excise the His tag sequence from the pCDF DUET-1 vector containing *cbbQ*. The 20 µL reaction contained 15 µL of plasmid DNA (120.5 ng/ µL), 2 µL of 10x NEB Buffer 3, 1 µL of BamHI, 1 µL of NcoI, and 1 µL of sterile water. The reaction tube was incubated in a 37 °C water bath for 1 hour. After digestion the His tag sequence was separated from the remainder of the plasmid using gel electrophoresis. The linearized plasmid DNA was excised from the gel, the DNA was recovered using the Gene Clean II, and circularized using a ligation reaction (see below).

Agarose Gel Electrophoresis

Agarose gels were prepared using 0.8 % agarose in 50 mL of 1x TBE buffer. The agarose powder and 1x TBE buffer were mixed in a flask then heated in a microwave until the agarose dissolved. The solution was cooled to room temperature then poured into a gel tray containing a well comb. Once the gel solidified, the tray was placed in a gel box, which was then filled with 1x TBE buffer. The samples were prepared in tubes that contained 10 µL of DNA solution, and 1.5µL of 6x loading buffer (NEB). After the DNA was loaded into the wells, the gel was subjected to 100 volts until the dye reached ¾ of the way down the gel. In order to

visualize the DNA, the gel was placed in distilled water containing one drop of ethidium bromide (2mg/ mL) for 10 minutes. In order to analyze the DNA the gel was examined under UV light using VersaDoc (BIO-RAD).

DNA Purification from Agarose Gels

To recover DNA from agarose gel, the fragment of interest was excised using a clean razor blade. The excised fragment was placed in a 1.5 mL microcentrifuge tube, and the Gene Clean II kit protocol was used to recover the DNA. The tube containing the agarose gel fragment was placed on a heat block to slowly melt the agarose. A silica matrix was added to the mixture, which bound to the DNA then wash steps were used to purify the DNA. The salt concentration of the solution was altered to elute the DNA.

DNA Ligation Reaction

A ligation was performed using the pCDF DUET-1 vector and the *cbbQ* gene, which had each been digested with the appropriate restriction enzymes. The reaction was performed in a 20 µL volume, containing 3.5 µL of the pCDF DUET-1 vector (45.2 ng/ µL), 13.5 µL of the *cbbQ* insert (30.1 ng/ µL), 2 µL of T4 DNA Ligase Reaction Buffer, 1 µL of DNA ligase and 1 µL of sterile water. The ligation reaction tubes were incubated overnight at 16 ℃ and 2 µL of the reaction mixture were used to transform OneShot chemically competent BL21 (DE3) *E. coli* cells (NEB).

The linearized pCDF DUET-1 plasmid from which the His tag sequence had been excised by digestion with NcoI and BamHI, was circularized by a ligation. Before the ligation, the overhang caused by the NcoI- BamHI digestion was filled in so that the ends of the DNA could be ligated together. The fill-in reaction was performed using 2.5 mM dNTPs, 1 µL of Deep

Vent DNA polymerase (NEB) and 15 µL of the pCDF DUET- 1 (33.4 ng/ µL) plasmid DNA

purified from the GeneClean II kit. The ligation reaction was performed at a 20 µL reaction

volume containing 15 µL of the linear plasmid (75 ng/ µL), 2 µL of T4 DNA Ligase Reaction

Buffer, 1 µL of DNA ligase and 2 µL of sterile water. The ligation reaction tubes were incubated

overnight at 16 ºC and 2 µL were used to transform OneShot chemically competent BL21 (DE3)

E. coli cells (NEB).

Small Scale Protein Expression

Two milliliters of LB, with 50 µg/ mL of kanamycin, were inoculated with transformants

containing the pCDF DUET-1 vector with a *cbbQ* insert. Two working cultures of each clone,

containing 50 mL of LB and 50 µg/ mL of kanamycin, were inoculated with 0.5 mL of the initial

culture and incubated at 37 ºC with agitation for 2.5 hours or until they reached an A_{600} of 0.6.

One working culture was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG),

while the other remained uninduced; both were incubated at 30 ºC with agitation for 3.5 hours.

One-milliliter samples of each working culture were removed and centrifuged at 10, 000 X g for

3 minutes in a tabletop centrifuge. The supernatant was discarded and the induced and uninduced

cell pellets were placed over ice, then re-suspended in 100 µL of PBS buffer (pH 7.4). To

analyze the samples sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE)

was used. Five microliters of the suspension was combined with 5 µL of 4x Laemmli Loading

Buffer and 10 µL of distilled water, and boiled for 10 minutes. The samples were then loaded

onto a 12 % Laemmli SDS polyacrylamide gel and subjected to electrophoresis at 110 V for 1.5

hours. The gel was then washed in distilled water for 30 minutes, stained with Gel-Code Blue

(Fisher Scientific) overnight, and de-stained in distilled water for 20 minutes.

Large Scale Protein Expression

A 5 mL LB starter culture was inoculated with *E. coli* transformants containing the pCDF DUET- 1 plasmid with *cbbQ* and incubated overnight with shaking at 37 ℃. A 1 liter LB culture was inoculated with all 5 mL of the starting culture and grown at 37 ℃ with agitation for approximately 3 hours or until it reached an A_{600} of 0.6. At this point, IPTG was added to a final concentration of 1 mM, and the culture was incubated at 30 ℃ for 3.5 hours. The culture was poured into 250 mL centrifuge tubes and centrifuged at 8,000 rpm for 10 minutes in a Beckman F250 rotor. The pellet was re-suspended in 40 mL of Lysis Buffer, and the cells were lysed by passing the suspension through a French Pressure Cell three times. The lysed cells were centrifuged at 8,000 rpm for 10 minutes. The crude extract, supernatant and pellet were analyzed by SDS- PAGE, using 5 μL of each.

E. coli co-transformed with the pCDF DUET vector containing the *cbbQ* insert without the His tag, and pET DUET- 1 containing *cbbO* (performed by Dr. Avijit Biswas) was grown and expression and purification of both protein was done in similar conditions to *cbbQ*.

Ni^{2+}- NTA Affinity Column Chromatography

The 40 mL cleared lysate of cells expressing recombinant CbbO and/or CbbQ proteins was incubated with 4 mL of Ni^{2+}- NTA resin slurry, equilibrated in Wash Buffer A, and 0.5 mM PMSF/PTSF at 4 ℃ overnight. After incubation, the resin mixture was poured into a column and allowed to settle. The flow-through fraction containing proteins that did not bind was collected. The column was then washed consecutively with 20 mL each of Wash Buffer A, Wash Buffer B, and Wash Buffer A. The bound protein was eluted using Elution Buffer. The protein samples

were dialyzed overnight in 2 L of 10 mM Tris pH 8. SDS-PAGE was used to analyze the flow-through and eluate fractions.

Affinity column chromatography on Ni^{2+}-NTA resin was also used in the interaction experiments between CbbO and/or CbbQ and RubisCO. Ni^{2+}-NTA resin (1mL) was equilibrated with Wash Buffer A. Three columns were prepared with 300 µL of Ni^{2+}-NTA resin each. In the first column, 250 µL of purified CbbO (0.78 mg/mL) was added, and in the second column 850 µL of RubisCO (1.37 mg/mL) was added. The third column contained recombinant CbbO (250 µL at 0.78 mg/mL) and RubisCO (850 µL at 1.37 mg/mL) in equimolar amounts. The columns were placed at 4 ℃ to incubate overnight. The following day the sample volume was allowed to flow through the column which was then washed with 10 mL of Wash Buffer A, 10 mL of Wash Buffer B, then 10 mL of Wash Buffer A. The column was then eluted with 500 µL of Elution Buffer. The flow through and elution fractions were dialyzed overnight in 2 L of 10 mM Tris pH 8. SDS- PAGE was used to analyze the samples. The same procedure was repeated with purified CbbQ and RubisCO, and with CbbO, CbbQ and RubisCO.

BCA Protein Assay

A Beckman Coulter DU 800 Spectrophotometer was used to perform the assay. Bovine Serum Albumin (BSA) standards were prepared using 1-40 µg of protein in a 100 µL volume. Next, 900 µL of BCA reagent was added, and the samples were incubated at 37 ℃ for 30 minutes. A standard curve was generated using the BSA standards, and recombinant protein samples were measured in the same manner.

RESULTS

Amplification of the *H. neapolitanus cbbQ* gene and insertion into pCR ® BLUNT TOPO II

The purpose of this experiment was to amplify the *cbbQ* gene by PCR, insert it into a vector and confirm its sequence. Primers were designed for amplification of the *cbbQ* gene using polymerase chain reaction (PCR). Gel electrophoresis on a 0.8% agarose gel was used to analyze the PCR product. A band corresponding to the gene of interest (*cbbQ*) migrated between the 0.5 and 1 kb markers (**Figure 6**). The amplified DNA was inserted into the pCR ® BLUNT TOPO II vector. Chemically competent *E. coli* cells were then transformed with the *cbbQ* plasmid. The DNA from ten of the transformant colonies was isolated and subjected to a BamHI and HindIII digestion to confirm the insertion of *cbbQ*. The DNA fragments migrated to approximately 4 kb and 0.8 kb, corresponding to the vector and the insert, respectively (**Figure 7**). The sequence of clones 3, 4, 7, 8, and 9 was determined to ensure that no errors had occurred during the amplification. Clone 8 matched the known *H. neapolitanus* sequence of *cbbQ* and was used for all subsequent experiments.
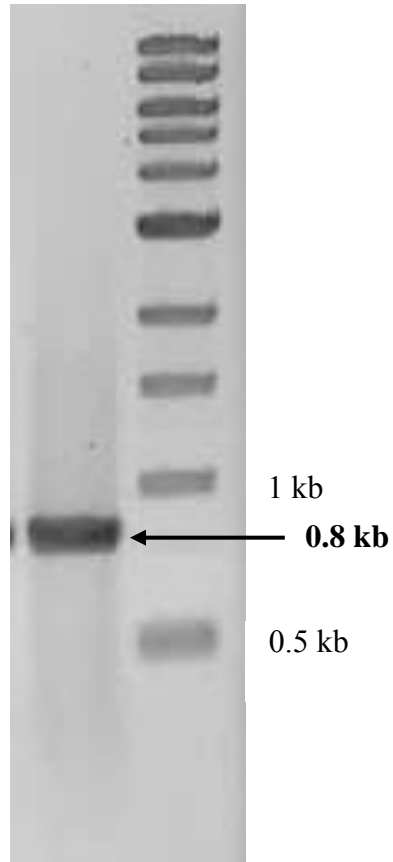
Figure 6: The *cbbQ* gene was amplified using PCR. A small aliquot of the product was analyzed using gel electrophoresis (0.8% agarose gel), and as indicated by the arrow, the amplified *cbbQ* gene had the expected length of approximately 0.8 kb.
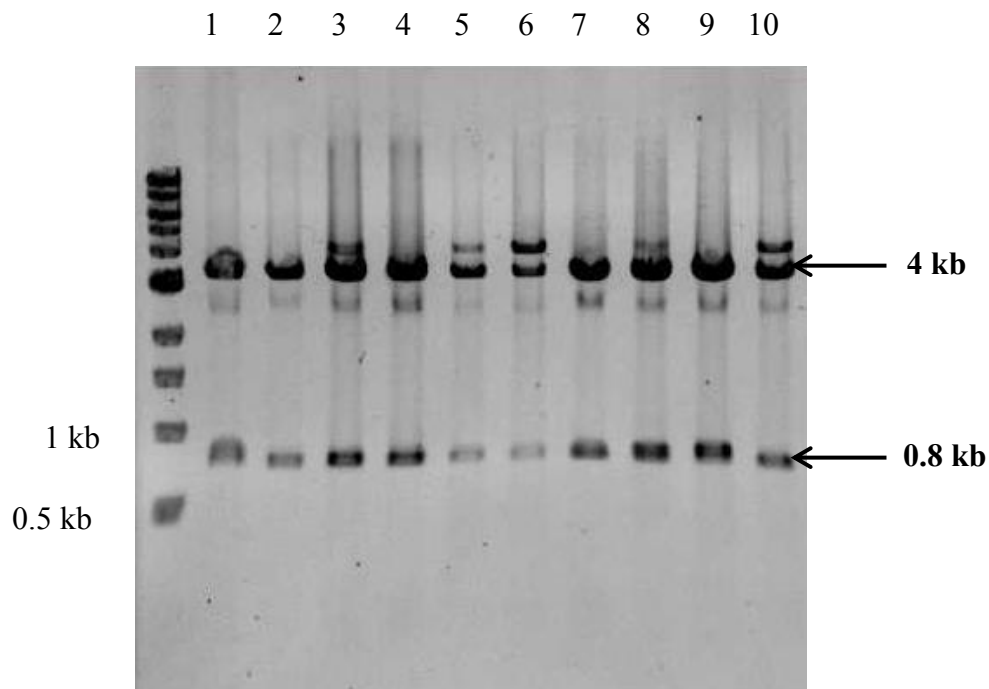
Figure 7: The *cbbQ* PCR product was inserted into the pCR ® BLUNT TOPO II vector, then a transformation was performed in which plasmid DNA was introduced into competent *E. coli* cells. Plasmid DNA was isolated from selected transformants and digested with the restriction enzymes BamHI and HindIII. The results of the enzyme digest are shown above. The 4 kb band identifies the vector and the 0.8 kb band identifies *cbbQ*. Clones 3, 4, 7, 8, and 9 were sent for sequencing. The sequence of clone 8 matched the published sequence of *H. neapolitanus cbbQ*.

Insertion of *cbbQ* into an Expression Vector

In order to express protein, the *cbbQ* gene was moved from the pCR ® BLUNT TOPO II vector into an expression vector. The insert was separated from the pCR ® BLUNT TOPO II vector using a BamHI and HindIII digestion. The pCDF DUET-1 expression vector was also digested using BamHI and HindIII and ligated with the *cbbQ* gene to create the expression construct. This expression construct was used to transform chemically competent *E. coli* cells. To confirm the success of the ligation, the plasmid DNA was isolated from ten transformant colonies and digested using BamHI and HindIII. All 10 colonies showed two bands: the 4 kb

fragment corresponding to the vector (3.781kb), and a band at 0.8 kb which corresponds to the
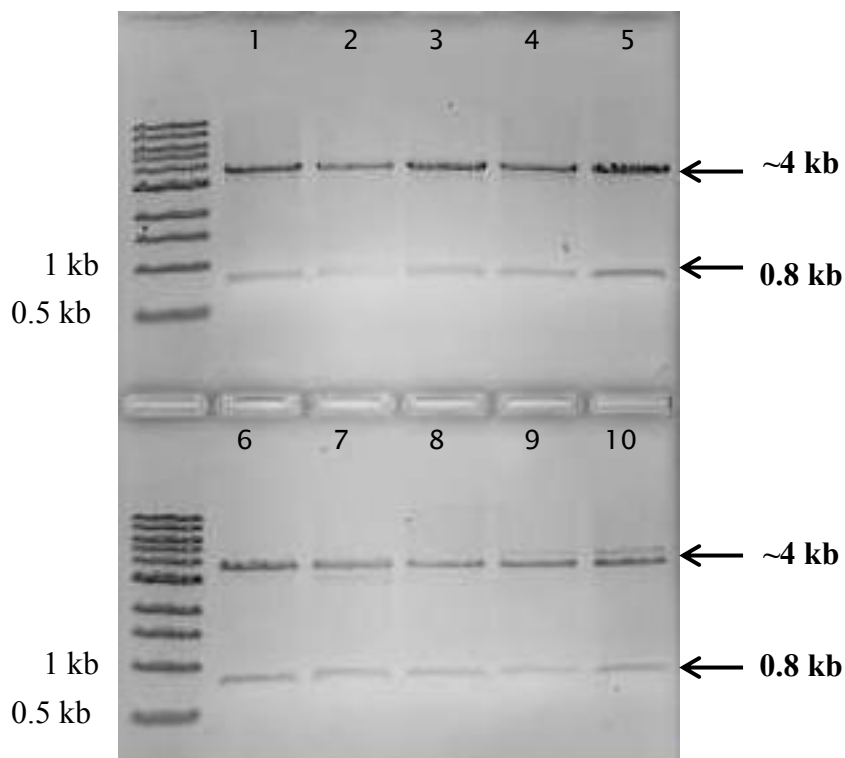
insert (0.804) (**Figure 8**).

Figure 8: The *cbbQ* insert was cut from the pCR ® BLUNT TOPO II vector using the BamHI and HindIII restriction sites and purified from the gel using the GeneClean II kit. An expression vector, pCDF DUET-1, was also cut using the same restriction sites and purified using the same procedure. A ligation was performed between *cbbQ* and the linearized expression vector, followed by a transformation of *E. coli*. The plasmid DNA of selected transformants was screened to confirm the insertion of the *cbbQ* using restriction digests at the BamHI and HindIII sites. The arrows identify *cbbQ* at 0.8 kb and the pCDFDUET-1 vector at approximately 4 kb.

Small and Large Scale Expression of CbbQ

To determine if recombinant CbbQ could be expressed, a small scale induction was

performed. The *E. coli* clones 6, 7, 8, 9 and 10 (**Figure 8**) were grown at 30 °C for

approximately four hours or until they reached log phase. At this time IPTG was added, which

allowed for the overexpression of CbbQ. Cell extracts of all five clones displayed the expected

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protein band at 32 kDa in the induced but not the uninduced samples. The induced samples all showed that CbbQ, at approximately 32 kDa, expressed well at 30 °C in 3.5 hours as indicated by the heavy bands (**Figure 9**).

A large scale protein expression was performed to purify large amounts of recombinant CbbQ for antibody generation and crystallization trials. The *E. coli* clones 6 and 7 were grown and induced with IPTG under the same conditions as in the small scale expression. Ni^{2+}-NTA affinity chromatography was used to separate the His-tagged CbbQ protein from other proteins in the crude extract. In the flow through fractions, non-His tagged proteins were found. The two E lanes represent the eluate fractions, which contained protein of the mass expected for CbbQ (32 kDa) (**Figure 10**). Significant amounts of recombinant protein were generated, as indicated by the high intensity of the stained CbbQ bands in elution fractions. A BCA assay was used to determine that the concentration of the purified recombinant protein was approximately 4.18 mg/ mL. Background bands indicate that the protein was not completely pure. Antibodies have also been generated for CbbQ, from recombinant protein that was expressed and purified by Emily Bustin and Dr. Avijit Biswas.
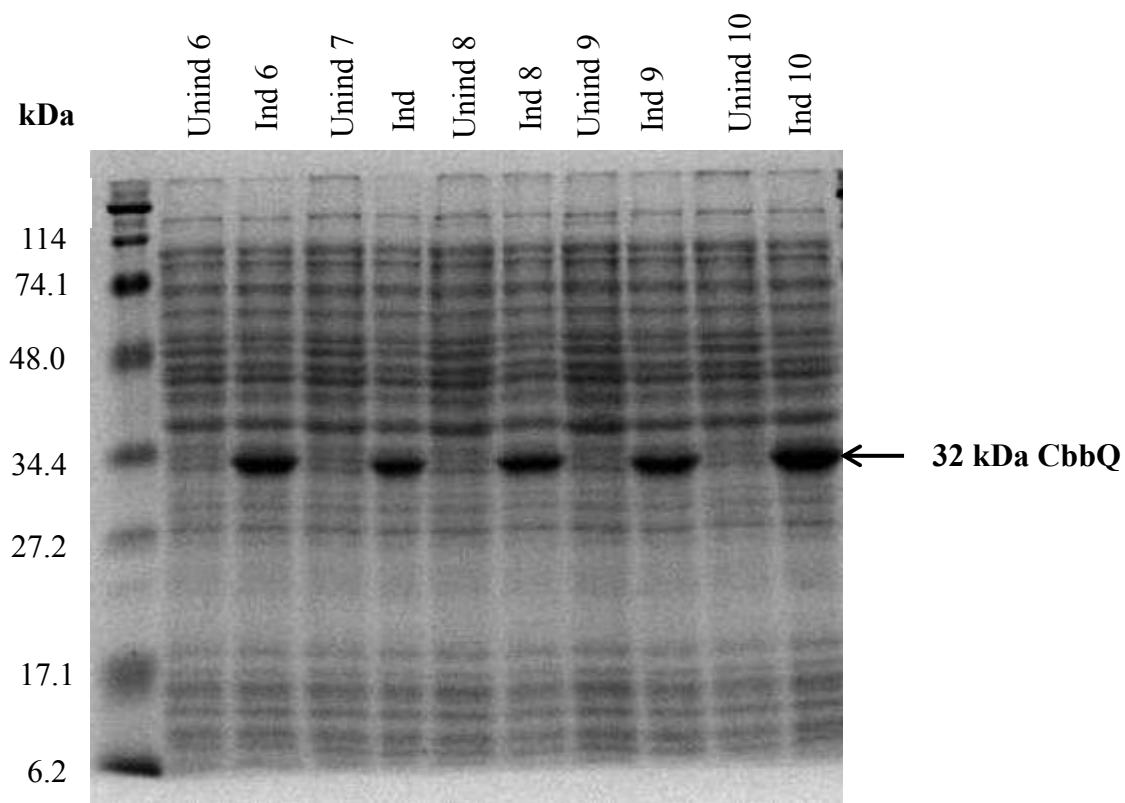
Figure 9: A small scale expression was performed with *E. coli* clones 6, 7, 8, 9 and 10 (Figure 8) to determine if they expressed recombinant CbbQ protein. Transcription of the *cbbQ* gene was induced by addition of IPTG. Cell extracts of all five clones displayed the expected protein band at 32 kDa in the induced but not the uninduced samples.
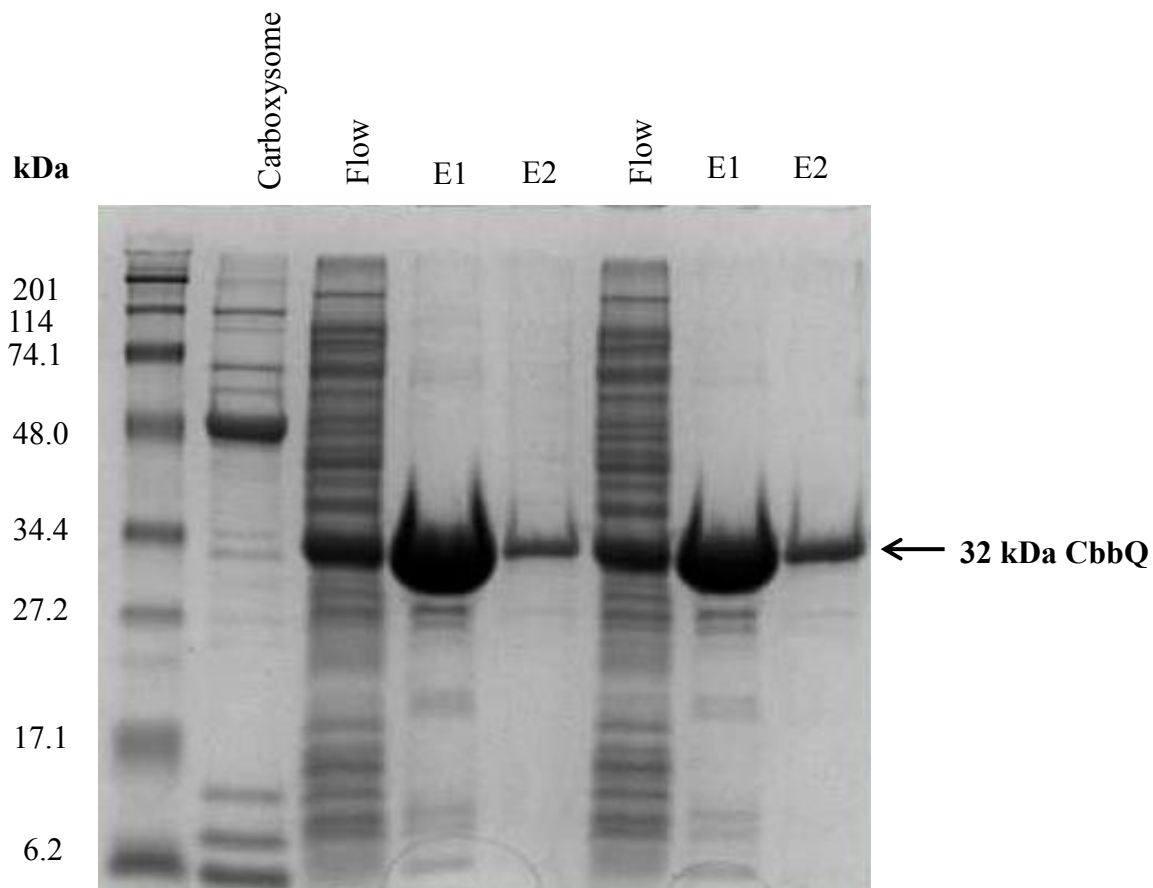
Figure 10: A large scale protein expression was performed to purify large amounts of recombinant CbbQ for antibody generation and crystallization trials. The *E. coli* clones 6 and 7 were grown, induced and purified using Ni^{2+}-NTA affinity chromatography. The two E lanes represent the eluate fractions, which contained protein of the mass expected for CbbQ (32 kDa).

Interaction Studies between CbbQ, CbbO and RubisCO

Annotation of the *cbbQ* and *cbbO* genes indicates that they are potential RubisCO activases and therefore have the potential to associate with RubisCO. To test the possibility that recombinant CbbQ and CbbO interact with RubisCO, interaction studies were performed. Recombinant CbbO was obtained from cloning and expression that was performed by a previous undergraduate student (Bustin, 2013). First, an interaction study was performed with purified recombinant CbbO and RubisCO isolated from carboxysomes. Three columns were prepared by

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the addition of Ni^{2+}- NTA resin. One column contained His-tagged CbbO, while another column contained RubisCO. The third column contained equimolar amounts of CbbO and RubisCO, with respect to its holoenzyme form. The mixture was incubated overnight. The Ni^{2+}- NTA resin bound to His tagged proteins, while proteins that interacted with the His tagged proteins could also be co- eluted. In **Figure 11**, lane 1 shows the RubisCO purified from carboxysomes and lane 2 shows recombinant CbbO. Lanes 3 and 4 show the flow through and eluate, respectively, of the first column. As expected, CbbO migrated as an approximately 100 kDa protein and was identified in the eluate. In lanes 5 and 6, the flow through and eluate of the second column are shown. As expected, most of the RubisCO polypeptides, were visible as 50 kDa (large subunit) and a 10 kDa (small subunit) bands, in the flow through fraction. RubisCO was not His tagged and therefore did not bind to the Ni^{2+} NTA resin. The final lanes, 7 and 8, show the flow through and eluate, respectively, of the third column. If CbbO interacted with RubisCO, forming a complex, then CbbO and RubisCO would co-elute. CbbO would bind to the Ni^{2+}- NTA resin through its His tag and RubisCO would co-elute only through its interaction with CbbO. RubisCO appeared in the flow through fraction and not the eluate, suggesting that it did not interact with recombinant CbbO (**Figure 11**). The interaction between CbbQ and RubisCO was tested using the same procedure. The first two lanes are controls and showing RubisCO and CbbQ. Lanes 3 (flow through) and 4 (eluate) show that as expected, CbbQ migrated as an approximately 32 kDa protein, and appeared in the elution fraction. Lanes 5 and 6 represent the flow through and eluate of the RubisCO column and, as expected RubisCO appeared mostly in the flow through and was visible as an approximately 50 kDa protein, corresponding to the large subunit, and a 10 kDa protein corresponding to the small subunit. Lanes 7 and 8 showed the flow through and elution fractions of the column containing both CbbQ and RubisCO. Most of the

RubisCO was identified in the flow through fraction, while CbbQ was seen only in the eluate, suggesting that there was no interaction between them (**Figure 12**). The final interaction study was performed using a combination of His-tagged recombinant CbbO and CbbQ, and RubisCO. Lanes 1, 2 and 3 are control lanes. Lanes 4 and 5 show the flow through and eluate of CbbQ. Some of the CbbQ appeared in the flow through, but the majority appeared in the eluate and migrated as an approximately 32 kDa protein. Lanes 6 and 7 contained the flow through and elution fractions of CbbO. The eluate lane contained a band that migrated to 100 kDa corresponding to CbbO. In Lanes 9 and 10, the flow through and eluate of RubisCO, respectively, are shown. As expected the majority of the RubisCO was found in the flow through as bands were visible at 50 kDa, for the large subunit, and at 10 kDa for the small subunit. The final lanes, 10 and 11, show the eluate and flow through of the CbbQ, CbbO and RubisCO combination. The RubisCO appeared in the flow through and its polypeptides migrated as approximately 50 kDa (large subunit) and 10 kDa (small subunit) proteins, while CbbO and CbbQ both appeared in the elution fraction because they were His tagged. The eluate contained both His tagged proteins, but the absence of RubisCO suggested that there was no interaction between both proteins and RubisCO (**Figure 13**).
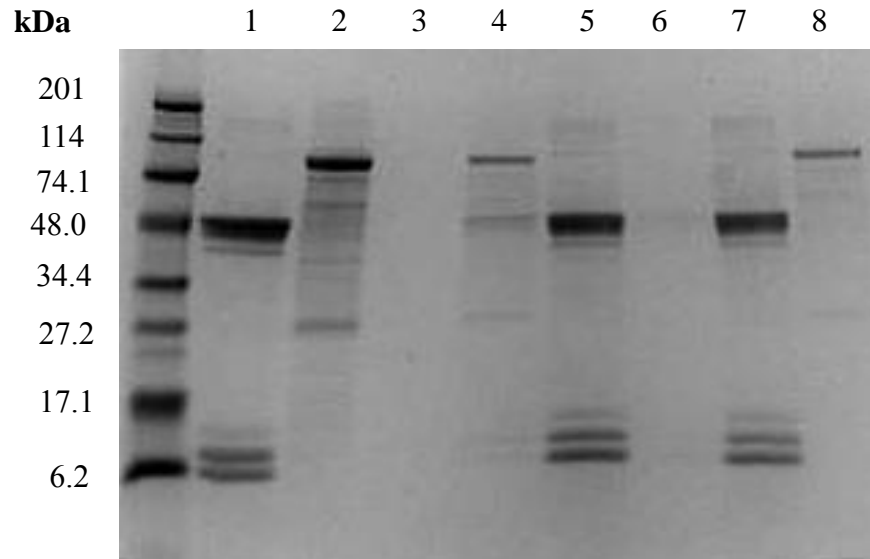
Figure 11: An interaction study was performed with recombinant CbbO and RubisCO. Lanes 3 and 4 show the flow through and eluate, respectively, of CbbO by itself. Lanes 5 and 6 show the flow through and the eluate of the RubisCO, respectively. As indicated by the flow through (lane 7) and eluate (lane 8) of the column co-incubated with CbbO and RubisCO, no interaction between the two proteins was observed.
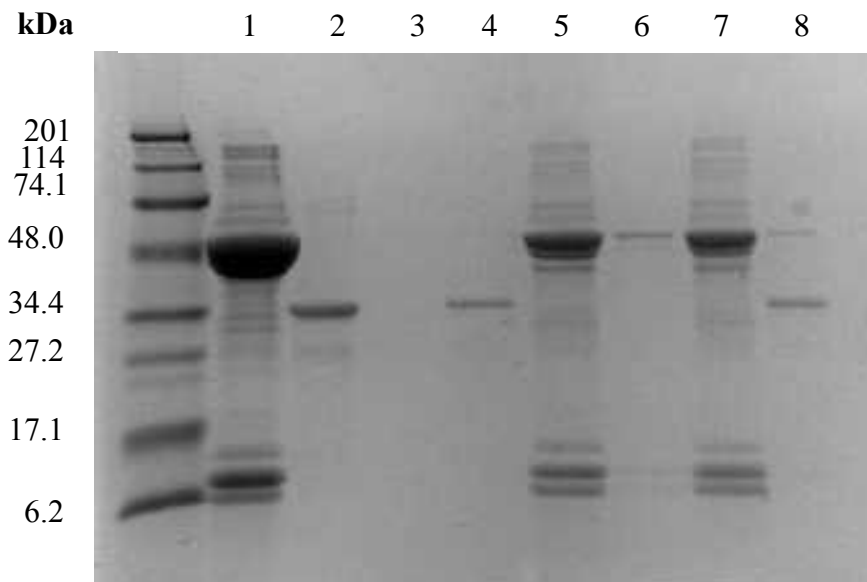

Figure 12: A similar interaction study was performed using CbbQ and RubisCO. Lanes 3 and 4 show the flow through and eluate, respectively, of the CbbQ column. Lanes 5 and 6 show the flow through and the eluate of the column containing RubisCO by itself, respectively. The final two lanes show the flow through (lane 7) and eluate (lane 8) of

column containing both recombinant CbbQ and RubisCO. Most of the RubisCO was identified in the flow through fraction, while CbbQ is seen only in the eluate, suggesting that there was no interaction between them.
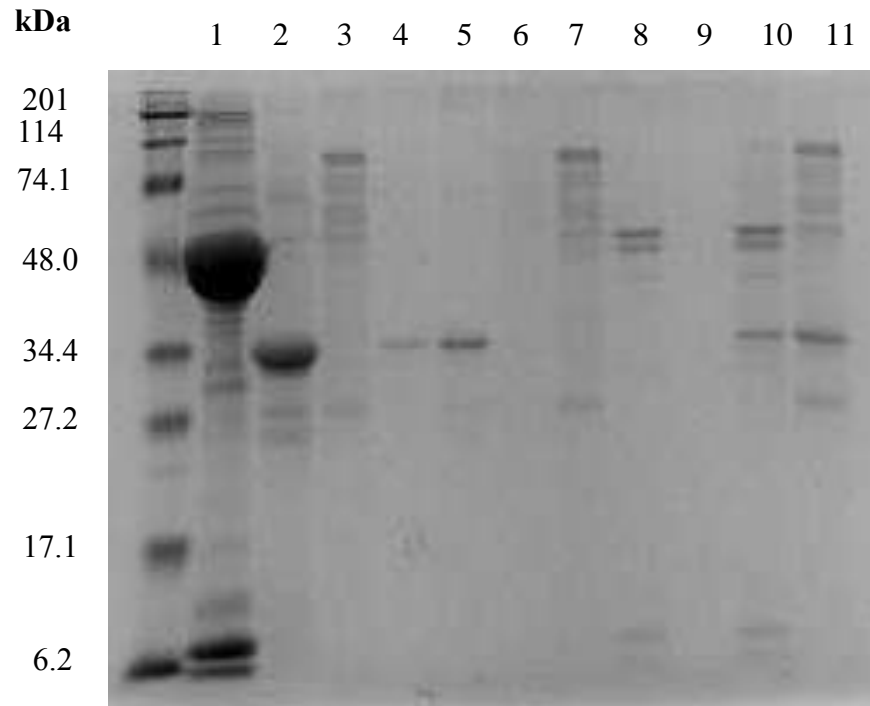

Figure 13: The final interaction study was done between CbbO, CbbQ and RubisCO. Lanes 4 and 5 show the flow though and eluate of the CbbQ column. Lanes 6 and 7 show the flow through and eluate of the CbbO column and lanes 8 and 9 show the flow through and eluate of the RubisCO column. The final two lanes show the flow through (lane 10) and eluate (lane 11) of the column containing CbbQ, CbbO and RubisCO. The RubisCO appears in the flow through at approximately 50 kDa (large subunit) and 10 kDa (small subunit), while CbbO and CbbQ both appear in the elution fraction lane, as was expected for His tagged proteins. This suggests there was no interaction of CbbO and CbbQ with RubisCO.

Co-expression of *cbbO* and *cbbQ*

The chemoautotrophic organisms that contain *cbbO* and *cbbQ*, such as *H. neapolitanus*, always contain both genes in tandem, suggesting that their protein products may interact. The interaction of proteins that form a complex often occurs during the translational phase (Duncan & Mata, 2011). Before the interaction studies were performed the His tag sequence was removed

from the *cbbQ* pCDF DUET-1 expression construct. An NcoI and BamHI digestion was used to excise the His tag sequence. The construct was then re- circularized using a ligation. *E. coli* was transformed with the *cbbQ* expression plasmid (without His tag) and the *cbbO* expression construct in order to co- express the proteins. Within the *E. coli* clones that contain both plasmids, the synthesis of CbbO and CbbQ could be induced simultaneously. Since both genes are being expressed within the same cells, the proteins are able to interact with one another during their translation.

The expression of the His tagged CbbO and non-tagged CbbQ was performed under similar conditions to the expression of His tagged CbbQ and the proteins were then purified using Ni^{2+}- NTA affinity column chromatography. **Figure 14** shows the SDS PAGE gel of the fractions collected from the purification through chromatography. A control was performed for the expression of *cbbO*, and the lanes labelled HT- CbbO show the flow through and eluates (E1- E4) of the column used to purify the protein. As expected, CbbO was purified through its His tag and is visible as a band that migrated as an approximately 100 kDa protein. The *cbbQ* plasmid was expressed and purification was performed using affinity column chromatography. Lanes E1- E4 represent the elution fractions of the column used to purify CbbQ. Due to the lack of a His tag on CbbQ, there was no interaction with the Ni^{2+}- NTA beads, and no elution. In the HT- CbbO - NT- CbbQ lanes, the co-expression results are shown. The untagged CbbQ co-eluted with His tagged CbbO, suggesting that the proteins form a complex when co-expressed. The NT-CbbQ lanes confirm that untagged CbbQ is not purified through Ni^{2+}- NTA affinity column chromatography, meaning that in order to elute during the co-expression, CbbQ would have to associate with His tagged CbbO. This suggests that CbbO and CbbQ formed a complex during their synthesis within *E.coli*.
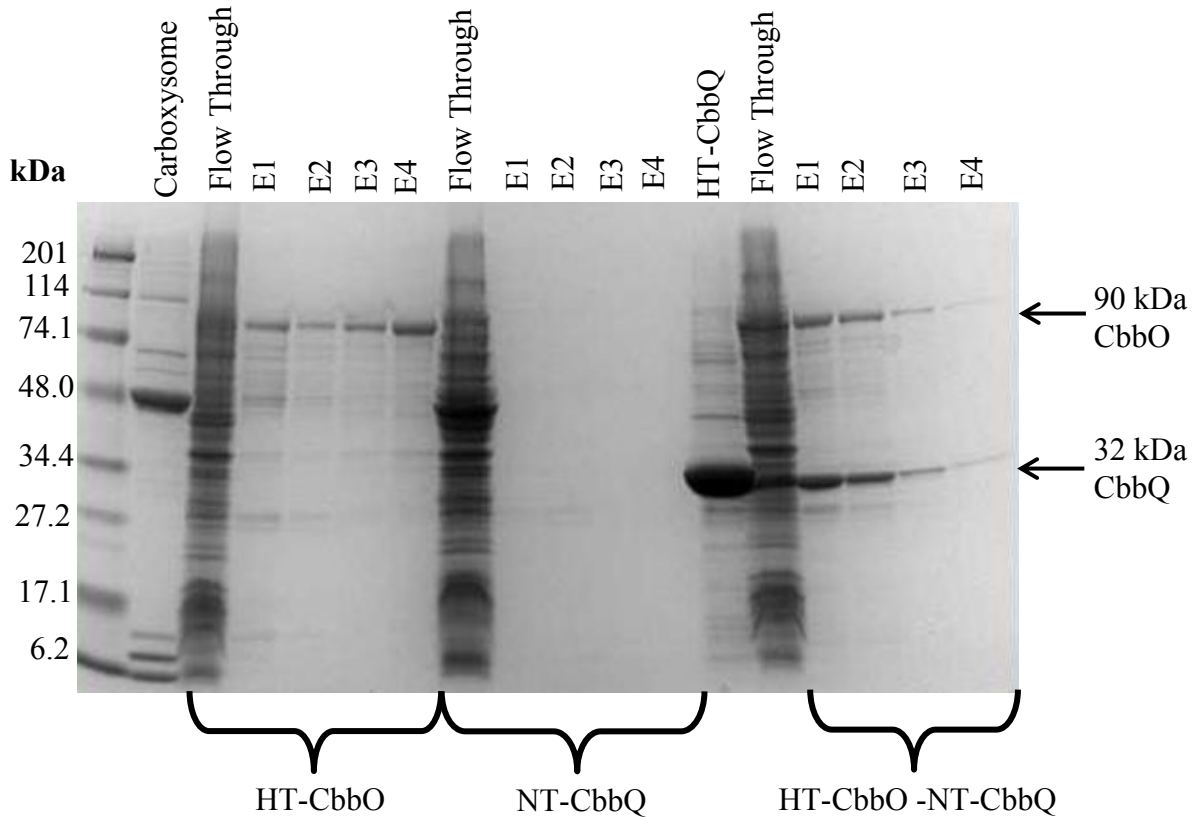
Figure 14: A co-expression experiment was performed between *cbbO* and *cbbQ*. The *cbbQ* pCDF DUET-1 plasmid, from which the His tag sequence had been excised, and the *cbbO* pET DUET-1 plasmid were used to co-transform *E. coli*. Expression of the proteins was induced using IPTG. The HT- CbbO- NT CbbQ lanes show the flow through and elution (E1-E4) fractions. The non-His tagged CbbQ, at approximately 32 kDa, co-eluted with tagged CbbO, at about 100 kDa, suggesting that the proteins interacted with one another during their synthesis. The HT-CbbO lanes are a control for the expression of HT CbbO. Since the CbbO is tagged, and it is visualized in the lanes E1-E4, which are the eluate lanes. The NT-CbbQ lanes are a control for the expression and purification of non- His tagged CbbQ. Since the protein is not tagged, it is not visualized in the eluate E1-E4 lanes.

DISCUSSION AND CONCLUSIONS

The *cbbQ* gene of *H. neapolitanus* is located in a region downstream of the *cso* operon (**Figure 5**). It is of interest because it is suspected to encode a RubisCO activase. Also located in this downstream region is *cbbO,* another gene suspected to encode a protein that interacts with RubisCO. In order to study the function of these genes they were first cloned and expressed. Using the recombinant CbbQ and CbbO proteins, interaction studies were performed with RubisCO. CbbQ and CbbO were also co-expressed to determine if they formed a complex.

Interaction studies were performed using recombinant CbbO and CbbQ and free RubisCO purified from carboxysomes. The purpose of these experiments was to determine if RubisCO co-eluted with CbbO and CbbQ on a Ni^{2+}- NTA column. Co-elution of RubisCO with one or both of the potential RubisCO activases would indicate that they interacted. The results suggest that there was no interaction between recombinant CbbO and CbbQ and free RubisCO. These studies were performed with proteins that were fully folded. The recombinant CbbO and CbbQ that were purified were in their native conformation, and the RubisCO isolated from ruptured carboxysomes was in its active folded form. Another similar interaction experiment could be performed using RubisCO deactivated with RuBP (Mueller-Cajar et al., 2011). CbbO and CbbQ could activate RubisCO by releasing a bound RuBP repressor. If they are involved in the disassociation of RuBP then they would interact with RubisCO in the inactivate state rather than in the active state. Another potential interaction experiment could be done with the addition of ATP. RubisCO activases typically have ATPase activity (Mueller-Cajar et al., 2011) (Saschenbrecker et al., 2007) and may require ATP in order to perform their activase function.

The *cbbO* and *cbbQ* genes occur in tandem in the genome of *H. neapolitanus*, and in all other chemoautotrophs that contain the potential activases. The consistent presence of both genes with one another implies that their protein products interact. In order to determine if CbbO and CbbQ form a complex during their folding, a co-expression experiment was performed between CbbO and CbbQ. *E. coli* was transformed with His tagged *cbbO*, and non-His tagged *cbbQ* and after induction, the proteins of interest were eluted using Ni^{2+}- NTA column chromatography. The eluate contained both the CbbO and CbbQ, suggesting that the non-tagged CbbQ formed a complex with the His tagged CbbO during their synthesis. Future experimentation would involve co-expressing *cbbO* and *cbbQ* with RubisCO to observe any interaction. In *Hydrogenophilus thermoluteolus* (formerly known as *Pseudomonas hydrogenothermophilia*) *cbbO* and *cbbQ* co-expression with RubisCO led to increased enzymatic activity of RubisCO through an increase of its V_{max} (Hayashi, Arai, Kodama, & Igarashi, 1997). This suggests that *cbbO* and *cbbQ* are both involved in activating RubisCO when they are in associated in a complex.

In conclusion, CbbO and CbbQ are both suspected RubisCO activases found within *H. neapolitanus*. In-vitro interaction experiments between recombinant CbbO and/or CbbQ and active RubisCO indicate that the proteins do not interact. It is possible that the potential activases may require ATP to function, or they may act on RubisCO while it's inactivated by RuBP. A complex between the two suspected activases was formed when *cbbQ* and *cbbO* were co-expressed. Future experimentation would involve the co-expression of *cbbO* and/or *cbbQ* with RubisCO.

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