

University of New Orleans

ScholarWorks@UNO

University of New Orleans Theses and
Dissertations

Dissertations and Theses

8-6-2009

Characterization of Slr1098, a Protein with Similarity to the Bilin Lyase Subunit CpcE from the Cyanobacterium *Synechocystis* sp. PCC 6803

Kali Hicks
University of New Orleans

Follow this and additional works at: <https://scholarworks.uno.edu/td>

Recommended Citation

Hicks, Kali, "Characterization of Slr1098, a Protein with Similarity to the Bilin Lyase Subunit CpcE from the Cyanobacterium *Synechocystis* sp. PCC 6803" (2009). *University of New Orleans Theses and Dissertations*. 979.

<https://scholarworks.uno.edu/td/979>

This Thesis is protected by copyright and/or related rights. It has been brought to you by ScholarWorks@UNO with permission from the rights-holder(s). You are free to use this Thesis in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you need to obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/or on the work itself.

This Thesis has been accepted for inclusion in University of New Orleans Theses and Dissertations by an authorized administrator of ScholarWorks@UNO. For more information, please contact scholarworks@uno.edu.

Characterization of Slr1098, a Protein with Similarity to the Bilin Lyase Subunit CpcE
from the Cyanobacterium *Synechocystis* sp. PCC 6803

A Thesis

Submitted to the Graduate Faculty of the
University of New Orleans
in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biological Sciences

by

Kali Hicks

B.S. Morgan State University, 2000

August 2009

Table of Contents

Abstract.....	v
List of Figures.....	vi
1. Introduction.....	1
1.1 Cyanobacteria: Phycobilisome Role and Composition.....	1
1.1.1 Phycobiliproteins	3
1.1.2 Phycobilins.....	4
1.1.3 Linker Polypeptides	6
1.2 PBS Assembly	9
1.3 PBS Degradation.....	12
1.4 Bilin Lyases and Chromophore Attachment.....	14
1.4.1 E/F Type.....	15
1.4.2 S/U Type	17
1.4.3 T Type.....	18
1.4.4 V Type	18
1.5 Slr1098.....	19
1.6 Purpose of Work	20
2. Materials and Methods.....	21
2.1 Materials	21
2.2 Construction of Recombinant Expression Plasmids	21
2.2.1 Slr1098.....	21
2.2.2 <i>cpcBA</i>	22
2.2.3 <i>cpcS-I and cpcU</i>	23
2.2.4 <i>pcyA/hoIx</i>	27
2.2.5 Transformation.....	27
2.3 Recombinant Protein Production	28
2.4 Slr1098 Purification.....	29
2.5 Phycocyanin Purification	32
2.6 Fluorescence Emission and Absorbance Spectra.....	33
2.7 SDS-PAGE Analysis	33
2.8 Western Blot Analysis	34
2.9 Sucrose Gradients	35
2.10 Pull Down Experiment.....	36
2.11 Binding Assay.....	36
2.12 SEC-HPLC.....	37
3. Results.....	39
3.1 Slr1098 Characteristics and Alignment with Other CpcE Proteins	39
3.2 Expression of Recombinant Proteins Slr1098, HT-sl1098, HT-CpcBA	45
3.3 Purification of <i>Synechocystis</i> sp. PCC 6803 Holo-PC.....	49
3.4 Phycocyanin and Slr1098 Binding Assays	50
3.4.1 Sucrose Density Gradient Ultracentrifugation.....	50
3.4.2 Pull Down Experiment.....	53
3.4.3 Size Exclusion Chromatography of PC-Slr1098 Complex.....	56
3.5 <i>In Vivo</i> Co-Expression of Slr1098 with CpcBA and CpcSU.....	57
Discussion.....	62

References.....	71
Vita.....	77

ACKNOWLEDGEMENTS

To my fiancé Theron Stovall, thank you for the tremendous love and support you give me in all aspects of my life. I will always appreciate the gracious way you listened to all my troubles, turned off the t.v. when I needed to write or study, and never let me be discouraged, no matter what obstacles fell in my path. You have been instrumental to me finishing this stage in my education, and I'm blessed to have you. Words cannot express how important you are to me.

To my mother Maatu King, thank you for being my number one fan and greatest cheerleader. You have given me so much love and support in my life that I couldn't do anything but achieve my goals. You have been a friend and inspiration, and I am proud to call you my mother. To my family, thank you for all the encouragement and for always having a willing ear, even though I know I sounded like I was speaking Greek.

To my advisor Dr. Wendy Schluchter, thank you for inviting me into your lab, and introducing me to the wonderful world of research. I have learned so much from you, and appreciate you standing in my corner these past years. I wish you much continued success.

To my committee members Dr. Mary Clancy and Dr. Soheila Maleki thank you for being on my committee and taking the time to read my thesis.

Thank you to all my fellow lab mates for all your support and assistance, I enjoyed my time sharing a lab with you and wish you all luck in your future endeavors.

Most importantly I want to thank God for allowing me to reach this stage in my life, gifting me with an inquisitive mind and a love for learning, and placing all the above people in my path to help me reach my educational goals.

ABSTRACT

The goal of this research is to investigate the role of the *slr1098* gene in the cyanobacterium *Synechocystis* sp. PCC 6803, a gene with similarity to *cpcE* which encodes a subunit of an enzyme involved in bilin attachment to phycocyanin. This protein is hypothesized to be involved in oligomerization of phycocyanin due to previous results showing the mutant made shorter phycocyanin rods. The recombinant Slr1098 protein was produced and purified from *E. coli* cells. Binding assays showed interaction between Slr1098 and both apo- and holo-phycocyanin, but not to apo-allophycocyanin. Slr1098 blocked bilin addition at Cys-82 on CpcB by the CpcS/CpcU bilin lyase. Size exclusion chromatography and sucrose density gradient analysis of complexes formed suggest that Slr1098 strongly interacts with all intermediate forms of phycocyanin and may be an important checkpoint in the biosynthesis and oligomerization of this protein, but that by itself, Slr1098 does not increase oligomerization of phycocyanin.

Keywords: Bilins, Bilin lyase, Chromophore, Cyanobacteria, Phycobiliproteins, Phycobilisome, Phycocyanin, Slr1098.

List of Figures

Figure 1	2
Figure 2	2
Figure 3	5
Figure 4	8
Figure 5	8
Figure 6	11
Figure 7	24
Figure 8	25
Figure 9	26
Table 1	31
Table 2	37
Figure 10	38
Figure 11	38
Table 3	40
Figure 12	42
Figure 13	43
Figure 14	44
Table 4	46
Figure 15	47
Figure 16	47
Figure 17	48
Figure 18	49
Figure 19	52
Figure 20	52
Figure 21	53
Figure 22	55
Figure 23	55
Figure 24	59
Figure 25	60
Figure 26	61
Figure 27	61

INTRODUCTION

1.1 Cyanobacteria: Phycobilisome Role and Composition

Cyanobacteria are prokaryotic organisms capable of performing oxygenic photosynthesis. The photosynthetic process is a series of radiationless energy transfers, beginning with an antenna complex absorbing a photon (Glazer, 1989). In the final step, excitation energy arrives at the transmembrane, photosynthetic reaction centers. In cyanobacteria, the light-harvesting complexes are called phycobilisomes (PBS). PBS are water-soluble multiprotein complexes responsible for giving cyanobacteria the color for which they were originally named: blue-green algae. PBS allow cyanobacteria to efficiently absorb light in the 460- to 670- nm range, a span that is poorly absorbed by chlorophylls (Glazer, 1989). These flexible structures can adapt to changes in the cell's environment, such as in the case of nutrient depletion, or changes in light conditions (Piven *et al.*, 2005).

Cyanobacterial PBS's are hemidiscoidal protein structures (Fig 1), located on the cytoplasmic face of the thylakoid membrane (Glazer, 1989). They consist of six to eight rods emanating from a core composed of two to five cylinders. The rods are stacks of double discs, 60 x 120-Å in size. The core is made of three cylindrical domains, each one consisting of four 30 x 115-Å discs. PBS are comprised of two types of proteins: phycobiliproteins (PBP) and linker proteins. PBP's have chromophores called bilins covalently attached to Cys (cysteine) residues, giving each PBP its characteristic color. Linker proteins are usually non-pigmented and direct the assembly of the PBS (Glazer, 1989).

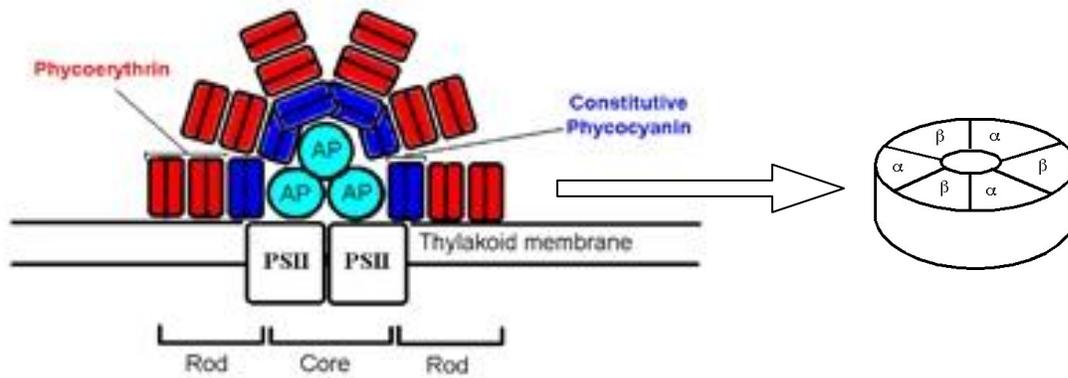


Figure 1: Phycobilisome (PBS) structure (used with permission of D. Kehoe, Indiana Univ) Figure right of arrow shows subunit arrangement on a face-view of the trimeric disc of phycocyanin present in the rods.

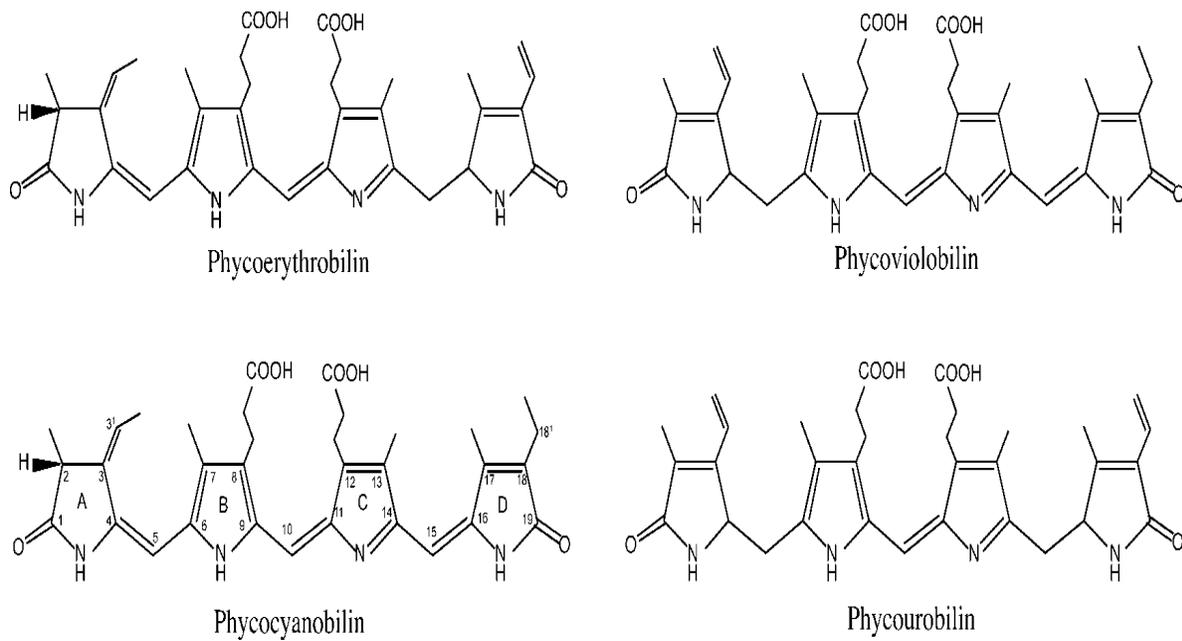


Figure 2: Cyanobacterial bilins: Attachment to Cys residues on phycobiliproteins occurs on ring A at C3¹ (numbering shown in Phycocyanobilin).

1.1.1 Phycobiliproteins

Phycobiliproteins (PBPs) are vividly colored proteins responsible for absorbing and transferring the light energy to the reaction center- namely photosystem II. They comprise 40-50% of the total protein found in cyanobacteria (Glazer, 1989). There are four classes of PBPs, which share similar tertiary and quaternary structures (Glazer, *et al.*, 1995), but are distinguished by their spectral absorbance properties: (1) allophycocyanins (AP: λ_{\max} =650-655 nm), (2) phycocyanins (PC: λ_{\max} =615-640 nm), (3) phycoerythrins (PE: λ_{\max} =565-575 nm), and (4) phycoerythrocyanin (PEC: λ_{\max} = 575 nm) (Sidler, 1994).

Allophycocyanin is the major component of the core of the PBS. Phycocyanin is found in the rod structures that radiate out from the core. Phycoerythrin and phycoerythrocyanin are attached to PC at the ends of the rods, but are not produced by all cyanobacteria. Energy is transferred from PE (or phycoerythrocyanin, most distal) to PC to AP to chlorophyll.

The type and number of bilin chromophores attached to the respective polypeptide and the interaction of the amino acids with the bilin is what gives each phycobiliprotein its spectral properties. Allophycocyanins carry one bilin on each subunit, while phycoerythrocyanin and phycocyanins carry one on the α subunit, and two on the β subunit, and phycoerythrins carry two or three bilins on the α subunit, and three on the β subunit (Glazer *et al.*, 1995).

Each PBP consists of two different polypeptide chains: α is approximately 17 kDa, while β is approximately 18 kDa. One to three bilins is covalently attached to each polypeptide chain at cysteine residues by thioether bonds (Glazer, 1989; Shen *et al.*, 2006). One subunit each of α and β forms a heterodimer referred to as a monomer. This is the basic subunit organization for all phycobiliproteins. Monomers are compiled to form disk-like trimers $(\alpha\beta)_3$ (see Fig 1) that have

a central channel, and two trimers stack face to face to form a hexamer $(\alpha\beta)_6$ that interacts with linker polypeptides to assemble rod and core substructures (Glazer, 1989). The phycobiliproteins are stabilized by the covalent binding of the chromophore, and although this covalent binding is necessary for phycobilisome assembly, some studies have indicated that it is not always required for phycobiliprotein function and cell survival (Scheer and Zhao, 2008).

1.1.2 Phycobilins

Phycobilins (bilins) are open-chain tetrapyrroles that are bound to the phycobiliproteins via covalent thioether bonds. There are four phycobilin isomers found in cyanobacteria (Fig 2), phycocyanobilin (PCB), phycobiliviolin (PVB), phycoerythrobilin (PEB), and phycourobilin (PUB). These bilins differ only in the arrangement of their double bonds (Glazer, 1989; Shen *et al.*, 2006). Their individual spectroscopic properties and interaction with the PBP determine the directional energy transfer (Glazer, 1989). Bilins are bound by one or two thioether bonds to conserved cysteine residues on the polypeptide at C-3¹ and C-18¹ (Scheer and Zhao, 2008; see Fig 2 for numbering). For any given species of cyanobacteria, one to four different bilins can be bound to the individual phycobiliproteins, and there are between eight and twenty individual attachment sites, depending on the organism (Fairchild *et al.*, 1992; Fairchild and Glazer, 1994).

Heme, the cyclic iron-tetrapyrrole, is the precursor to all cyanobacterial bilins. Heme oxygenase cleaves heme (Beale and Cornejo, 1983), forming an opening in the ring at C-5 (Beale and Cornejo, 1984). The resulting open chain tetrapyrrole is biliverdin IX α (BV); this is further reduced by a ferredoxin-dependent enzyme, in a two-step, four electron reaction, to form phycocyanobilin (PCB; Fig 3); this is performed by PcyA and results in the formation of the 3(Z) isomer of PCB (Frankenberg *et al.*, 2001).

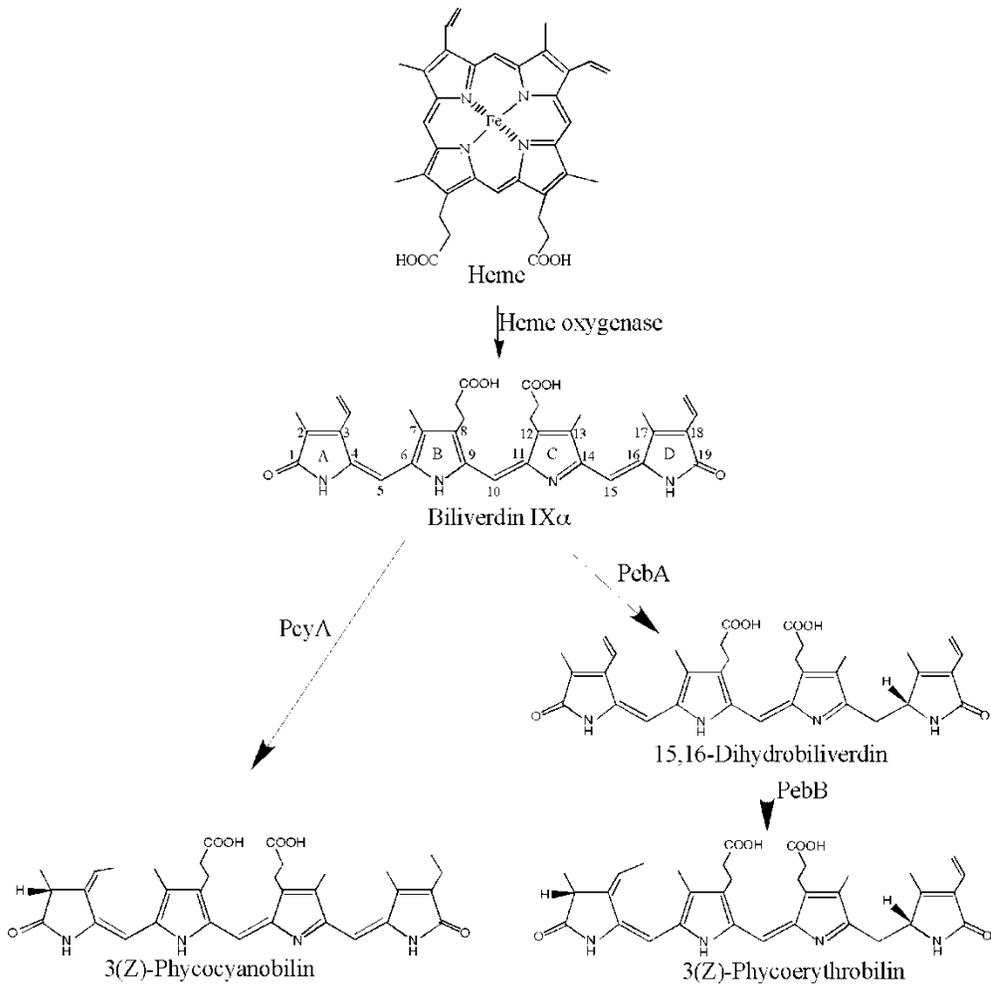


Figure 3: Bilin biosynthetic pathway in cyanobacteria

1.1.3 Linker Polypeptides

Linker polypeptides are proteins responsible for the assembly of the PBS, by mediating the exact locations of the phycobiliproteins within the PBS, and optimizing their spectroscopic properties (Glazer, 1989; Piven *et al.*, 2005). There are four groups of linker proteins, rod-core linkers, rod linkers, small core linkers, and core-membrane linkers (see Fig 4). The rod-core (L_{RC}) linkers attach the peripheral rods to the PBS core. The rod group (L_R) connects PC into the rod segments. Small core linkers (L_C) insert into the biliprotein allophycocyanin trimer central cavity. The core-membrane linker (L_{CM}) contains an allophycocyanin domain and linker domains; it associates with one subunit of AP and binds the trimers of AP together to form the core (Yamanaka *et al.*, 1982; Piven *et al.*, 2005).

The core complex of the PBS, which functions as the PBS backbone, has associated on both faces, the complex $AP \cdot L_C^{7,8}$ (Reuter *et al.*, 1999). In order to get a detailed structural analysis of the linkers within a phycobiliprotein-linker complex, and gain insight on the molecular events occurring during linker modulation of the chromophores, researchers used crystallization of the AP complex (Fig 5). The complex is asymmetrical and contains two trimeric AP complexes, associated in a side to side fashion. This association involves both α and β subunits. The linker is placed between two β -subunits, and interacts with the chromophores of those subunits via its N-terminal residues. The binding of the linker involves multiple charged, polar and hydrophobic contacts (Reuter *et al.*, 1999). The crystal structure of linker-free AP has three-fold symmetry, while the presence of a linker causes the trimer to contract and the shape to become more flattened.

This is due to the rotation of the three monomers, bringing the β -chromophores closer to each other. In addition to the conformational change, the charged and polar contacts of the linker binding with the β -chromophore causes a “bathochromic absorbance shift” of the complex to the red end of the spectrum (Reuter *et al.*, 1999). The results of this study indicate that the linker-mediated effects on the complex are responsible for the optimization of the energy transfer within the complex and between the elements of the core.

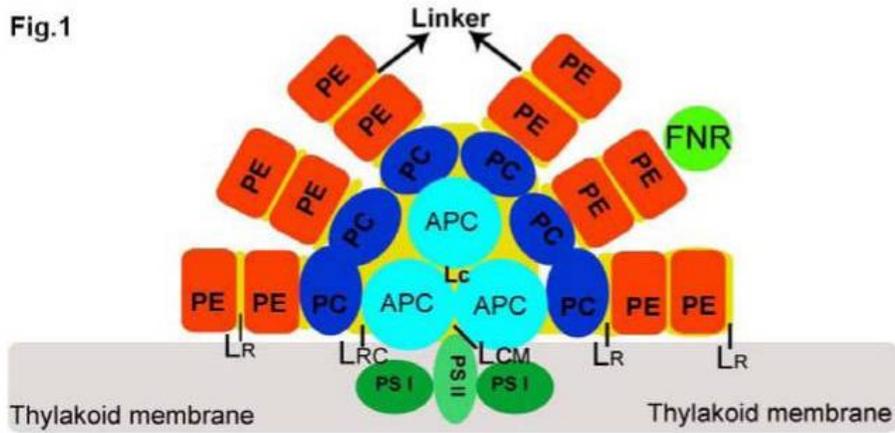


Figure 4: PBS model showing the location of the linker polypeptides (from Guan, X *et. al.*, 2007). APC= allophycocyanin; PC= phycocyanin; PE= phycoerythrin; PS I and PSII= photosystem 1 and 2, respectively.

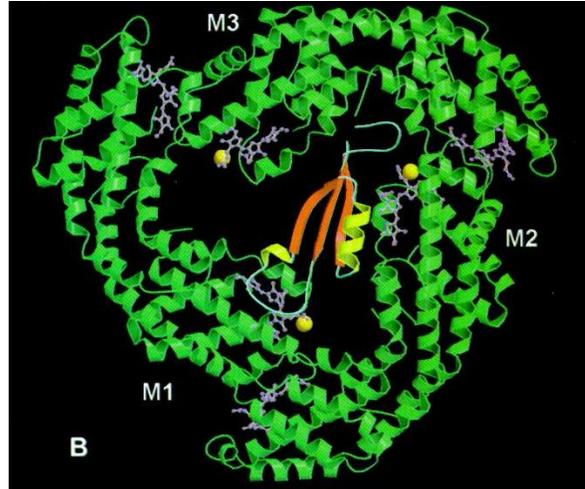


Figure 5: Ribbon plot of AP·L_C^{7.8} complex (From Reuter *et al.*, 1999). M1-3 represent α/β subunits, the chromophores in pink.

1.2 PBS Assembly

All major phycobiliproteins, as mentioned earlier, have the same basic subunit organization, a heterodimer consisting of α and β subunits, called the monomer. The formation of this highly stable monomer is the first step in PC assembly. From here the monomer is integrated into higher order structures which ultimately form phycobilisomes (Plank *et al.*, 1995).

In order to investigate the role of monomer formation in biliprotein assembly, a PC-minus mutant strain of *Synechocystis* sp. PCC 6803, called 4R, was characterized (Plank *et al.*, 1995). 4R contains a mutation in the *cpcB* gene that causes early termination of translation leading to a defective PC β subunit. This results in a complete lack of the α subunit and the rod linker proteins. The authors determined immunologically that the truncation of one subunit resulted in the loss of the other partner subunit. A subunit that is not monomer-bound has an exposed subunit binding domain, and this causes an unstable biliprotein. This exposed domain is susceptible to proteolytic enzymes. The researchers demonstrated that the formation of monomers is a critical stage in biliprotein biosynthesis, and that all of the early stages of assembly must occur in a rapid and precise fashion (Plank *et al.*, 1995).

When a *cpcBA* operon from another *Synechocystis* strain (PCC 6701) was expressed in the *Synechocystis* sp. PCC 6803 4R strain, it compensated for the mutation of the β subunit within this 4R strain (Plank and Anderson, 1995). The foreign α and β subunits exhibited heterologous assembly with the subunits endogenous to the mutant strain and formed functional phycobilisomes. When the foreign operon (6701) was expressed in a strain with intact *cpcBA* (6803), the two species of PC competed for assembly sites, with the endogenous PC favored. This could be due to the 6803 strain affecting translation of the 6701 operon, or homogenous

assembly may be preferred over heterologous due to slight structural differences between the two species (Plank and Anderson, 1995).

Taking the above studies further, Toole *et al.* examined the potential role of bilin attachment in subunit stability (1998). They created mutants containing single and multiple chromophore deletions in the subunits of PC from *Synechocystis* sp. 6701, by replacing each Cys with Ala. One of the two central chromophore binding sites; Cys-82 of CpcB, and Cys-85 of CpcA, or the peripheral site; Cys-153 of CpcB, or a combination of binding sites were missing in the various strains (Fig 6). The mutant subunits were then expressed in the PC-minus strain of *Synechocystis* sp. 6803, 4R. The results showed that bilin deletions affect the ability of PC assembly, with the double mutants (C82A/C153B and C82B/C85A) producing less PC than the single mutants. In addition, PC that lacks bilins is limited in its ability to form into PBS, and the PBS are smaller compared to the control. With the rescue of the 4R strain in mind (Plank and Anderson, 1995), the authors tested if the same concept applied to the PBS of the *cpcA* mutant. They found that, as with 4R, heterologous compounds were formed. Preference was given to bilin-replete heterologous monomers (CpcB 6701: CpcA 6803), over the homologous complex (CpcB 6701: apoCpcA 6701) becoming integrated into phycobilisomes. The loss of a central bilin (β Cys-82 or α -Cys-85) appears to have a greater effect on subunit interactions than loss of a peripheral bilin (β Cys-153). The central bilins are thought to stabilize the subunit structure, as sedimentation data from this study indicate that the loss of one of these bilins makes the monomer weaker, and prone to dissociation (Toole *et al.*, 1998). These studies demonstrate that subunit interactions coupled with bilin binding are critical in biliprotein assembly and stability, the prevention of degradation, and ultimately, PBS formation.

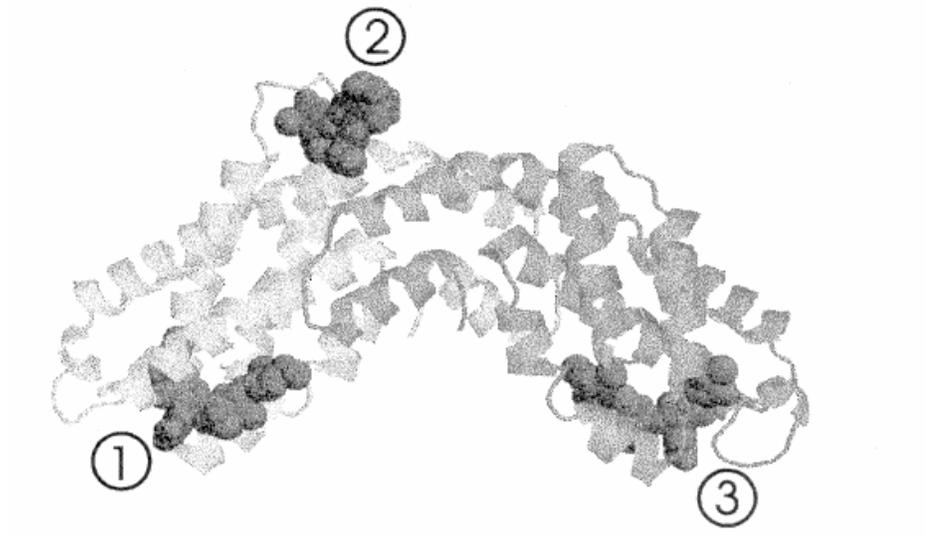


Figure 6: Ribbon plot of PC monomer (from Toole, *et al.*, 1998). 1= β -82, 2= β -153, 3= α -85

1.3 PBS Degradation

Nitrogen and sulfur are necessary for all cell survival, including cyanobacteria. If the cells are starved of either, a process is triggered to rapidly and completely degrade the PBS, and the cells become a chlorotic yellow-green color (Dolganov and Grossman, 1999). Some cyanobacteria can fix nitrogen and avoid this breakdown process. The degradation begins with the PC hexamers in the distal portions of the rods, continuing with the entire rods, followed by the light harvesting core. Cyanobacteria are able to recover some of the missing nutrients through proteolysis, and also prevent the production of harmful reactive oxygen species, which lead to cell damage, by minimizing the absorption of surplus excitation energy. The color change associated with PBS degradation allowed researchers to select for mutant isolates that do not exhibit the acclimation process under nutrient limitation. Screening for these mutants led to the identification of factors that are involved in the control of the proteolytic process. The preliminary phases of this acclimation process are associated with the expression of two genes, *nblA* and *nblB* (Dolganov and Grossman, 1999).

NblA, called the nonbleaching gene, encodes a 59 amino acid polypeptide that is required for phycobilisome degradation. It is so named because inactivation of the gene prevents cells from degrading their PBSs during nitrogen or sulfur-limited conditions (Collier and Grossman, 1994). The first cellular response to nutrient deprivation is the increased transcription of this gene. *NblA* has an affinity for PC and PEC, specifically at the α - and β -subunits (Bienert *et al.*, 2006). After 24 hours of deprivation, wild-type cells showed a two-fold decrease in the PC: AP ratio in the PBS. Yet the *nblA* mutants demonstrated only a 25% decay in PC: AP ratio. In addition, the mutants sustained a greater phycobiliprotein absorbance compared to the wild type (Collier and Grossman, 1994). When researchers increased expression of the sense form of the

gene, the phycobilisomes were degraded completely under phosphorus starvation, a condition that usually signals partial degradation only. These results indicated that *nblA* is required for PBS degradation (Collier and Grossman, 1994).

NblR, a gene of *Synechococcus* sp. strain PCC 7942, encodes a regulator of the *nblA* promoter activity (Dolganov and Grossman, 1999). It is necessary for prolonged cell survival during stressful conditions, and it assimilates the cellular response to a number of different environmental clues. While *nblR* controls *nblA* accumulation, another gene involved in PBS degradation, *nblB*, is constitutively expressed, and not under *nblR*'s control (Dolganov and Grossman, 1999).

Researchers have characterized the nonbleaching mutant complemented by *nblB*. This mutant does not degrade its PBS in either sulfur starvation or high light conditions (Dolganov and Grossman, 1999). The phenotype of this mutant strain demonstrated reduced levels of phycobiliproteins and chlorophyll during sulfur starvation. This was not due to PBS breakdown, but rather, no new PBS synthesis during several cell divisions and growth. In addition, the mutant strain contains higher levels of PC than the wild type when in nutrient replete conditions. These results seem to indicate that *nblB* has a role in PBS acclimation to environmental changes, although its expression is not necessary for PBS degradation. The authors note an intriguing fact that the protein product of the *nblB* gene has a sequence similar to the CpcE bilin lyase subunit, which attaches a bilin to the α subunit of apo-PC (see section 1.4.1). This similarity could be due to both polypeptides' ability to bind chromophores to their respective phycobiliprotein subunits.

Although it is known that *nblA* must be expressed for PBS degradation, its mechanism for initiating the response and how the gene is regulated are inadequately understood. On its

own, NblA has no proteolytic activity, when added to extracts of PBS, the phycobiliproteins were not degraded (Bienert *et al.*, 2006). The protein NblB may expedite the cleavage of the chromophore from the holo-phycobiliproteins once they have been bound to NblA. This hypothesis has merit as the *nblB* negative mutants lacked the ability to breakdown their PBSs and maintained their characteristic coloration (Dolganov and Grossman, 1999).

Recently, two groups of researchers proposed two varying models of NblA's action in PBS degradation. Dines and co-workers (2008) examined NblA's crystal structure, and suggest that NblA may penetrate into a gap in the interface formed between two PC hexamers of the PBS rods. They believe this penetration disrupts the rod structure, therefore making them susceptible to proteolytic degradation (Dines *et al.*, 2008). Karradt and researchers (2008) demonstrated that NblA binds to ClpC (an HSP100/Clp protease) via a conserved motif near its N terminus. They propose that a complex between the PBPs, NblA, and ClpC is necessary for PBS degradation. ClpC may assist in unfolding the PBP, leaving it to be degraded by other proteases (Karradt *et.al.*, 2008).

1.4 Bilin Lyases and Chromophore Attachment

Phycobiliproteins are attached covalently to bilin chromophores via moderately stable thioether bonds. Spontaneous attachment, without the use of lyases, is unlikely *in vivo*, but has been reported *in vitro* with PCB and PEB (Arciero *et al.*, 1988). This form of attachment however, leads to product mixtures and side reactions such as incorrect stereochemistry and oxidized bilins (Arciero *et al.*, 1988; Schluchter and Glazer, 1999). Allophycocyanin E (APE), the core membrane protein of the PBS, is the only example to demonstrate autocatalytic binding,

to PCB (Zhao *et al.*, 2005; A. Biswas and W. M. Schluchter, unpublished results). For correct attachment in most species of cyanobacteria, enzymes called bilin lyases, catalyze the binding of chromophores to the apoprotein in a site- and chromophore-specific manner (Zhao *et al.*, 2007b). For these organisms, a bilin binding site containing a correctly placed cysteine residue is required for binding. Lyases function as chaperones, guiding the chromophore to the correct attachment site in the correct conformation (Arciero *et al.*, 1988; Böhm *et al.*, 2007). There are three main classes of bilin lyases in cyanobacteria, E/F, S/U and T- types. The three groups have different characteristics in their enzyme ability and sites of reaction.

1.4.1 E/F-Type

E/F lyases are responsible for bilin attachment at the cysteine α -84 location on the phycobiliproteins PC and PEC. On the phycocyanin operon (*cpcBACDEF*) of *Synechococcus* sp. PCC 7002, two genes, *cpcE* and *cpcF*, encode proteins that function as site-specific bilin lyases (Fairchild *et al.*, 1992). This was the first cyanobacterial bilin lyase enzyme to be identified and characterized. Zhou *et al.*, (1992) created interposon insertion mutants in either *cpcE* or *cpcF*, and an interposon deletion mutant of both genes. All three mutant strains exhibited a yellow-green color, indicating that they failed to accumulate normal levels of PC. This was confirmed by the absorption spectra, and when the phycobilisomes were purified, the mutant phycobilisomes were significantly smaller than those of the wild type strain. When further biochemical analyses were performed, they discovered that the mutant strains lacked normal chromophorylation on the α -PC subunit only, and the β -subunit carried two PCB chromophores (Zhou *et al.*, 1992).

Fairchild et al. demonstrated that CpcE and CpcF formed a heterodimeric lyase, which attached PCB to the Cys-84 site of the α -subunit of PC (Fairchild *et al.*, 1992). Neither subunit was able to perform the attachment alone. CpcEF was also able to catalyze the reverse reaction, transferring the bilin from the holo- α -subunit of PC to an apo- α -subunit of the same or different species. This implies that the bilin lyase complex is able to access the thioether bond between the protein and bilin. To date, this is the only bilin lyase class that can perform both the binding and detaching reactions. CpcEF can catalyze the binding of PEB to the α subunit of apo-PC as well, with a strong preference for PCB over PEB in affinity and kinetics (Fairchild and Glazer, 1994).

The reversible action of CpcE/F is similar to the function of NblB, the protein mentioned earlier, involved in PBS degradation. NblB is similar to CpcE, and may indicate that the lyase has a role in the degradation of PBSs under stressful environmental conditions (Dolganov and Grossman, 1999).

The cyanobacteria *Anabaena* sp. PCC 7120, and *Mastidocladus laminosus* have three biliproteins. In addition to phycocyanin (PC), and allophycocyanin (AP), they also contain phycoerythrocyanin (PEC). In addition to *cpcE* and *cpcF*, they also carry similar genes *pecE* and *pecF*, located on the *pec* operon (Jung *et al.*, 1995). These genes encode a dual function lyase that attaches PCB to α -PEC, and concurrently isomerizes PCB to phycoviolobilin (PVB) (Zhao *et al.*, 2000). PVB has never been isolated from cyanobacteria in its free form, and it is likely that its isomerization from PCB is the only way it exists *in vivo*. Just like CpcEF, PecEF forms a 1:1 heterodimer, and both subunits are required for correct chromophore attachment. By itself, PecE is able to catalyze the PCB binding, but does not perform isomerization. Alone, PecF is dormant (Zhao *et al.*, 2005).

The E/F type of lyase offered researchers a great model for identifying the lyases corresponding to the binding sites of other apophycobiliproteins due to its ability to form an adduct with bilins other than PCB. Using the genes encoding heme oxygenase (*ho1*) and PCB:ferredoxin oxidoreductase (*pcyA*) the *in vivo* synthesis of α -CPC and α -PEC was reconstructed in *E. coli*. When these genes are co-expressed with *cpcA*, *cpcE*, and *cpcF*, holo- α -CPC is produced, and when *pecA*, *pecE*, and *pecF* are used, holo- α -PEC is the product (Tooley *et al.*, 2001; Tooley and Glazer, 2002). It could be possible to create combinations not found in nature, to be used as a labeling system to further study biliprotein folding and assembly.

1.4.2 S/U-Type

High binding site specificity is the major feature of this class (S/U type) of bilin lyase, as they tend to only bind to one conserved cysteine residue on the apoprotein. PC- and AP-producing organisms have the biliprotein subunit genes and their corresponding bilin linker genes encoded on the same operon. This is not the case for PE producers, the subunit genes (*cpeBA*) and the linker genes are on two different operons. Cobley *et al.* identified the genes for PE-associated linker proteins, on the operon *cpeCDEST*R in *Fremyella diplosiphon* (2002).

In *Synechococcus* sp. PCC 7002, the paralogous proteins CpcS-I, CpcU, and CpcV share sequence similarity to CpeS from *F. diplosiphon*, and CpcT is similar to CpeT from *F. diplosiphon* (Shen *et al.*, 2004; Shen *et al.*, 2008). Using comparative bioinformatics analyses, Shen and colleagues were able to organize the homologues of CpeS and CpcS into five main groups; A through E. Groups A and B contain proteins found in organisms that are able to synthesize more than one PE. CpeS and CpeU most likely function as phycoerythrobilin (PEB) lyases, and are included in this group. Group C is the largest of the five groups, it is further

subdivided into clades, CpcS-I, CpcS-II and CpcS-III. CpcU is placed in the next group, D, and all organisms that have a protein in the CpcS-I clade also have a protein in this group, the only exception being *Nostoc punctiforme*. The final group, E, contains the protein CpcV, and organisms producing proteins in this group do not have an obvious pigmentation pattern (Shen *et al.*, 2008).

Together with CpcU, CpcS-I forms a 1:1 heterodimeric lyase that attaches PCB to the Cys-82 site of the β -subunit of PC and to the Cys-81 site on the α - and β -subunits of AP (Shen *et al.*, 2008; Saunée *et al.*, 2008).

1.4.3 T-Type

Two of the binding sites in *Synechococcus* sp. PCC 7002 require a heterodimer bilin lyase, CpcEF for Cys-84, and CpcS-I for Cys-82, while the remaining site only requires a single subunit bilin lyase. CpcT is a protein found in this organism, as well as other species that produce PC, that attaches PCB to Cys- β 153 of PC (Shen *et al.*, 2006). The chromophore at this site has the major role of transferring light energy to the chromophore at Cys- β 82, the terminal acceptor. Mutants in which *cpcT* was inactivated had approximately 40% less PC and were more yellow-green colored compared to wild type cells. In addition, the mutant PC transferred less energy to acceptor proteins due to less light absorption.

1.4.4 V-Type

CpcV is found in *Synechococcus* sp. PCC 7002, and is included in the E group of the CpcS and CpeS homologs (Shen *et al.*, 2008). Mutants of this gene demonstrated no obvious phenotypic difference compared to wild type cells. PBP levels were unaffected compared to

wild type and no defects in PBS degradation were seen under nutrient starvation conditions.

When β -AP content of mutant cells was analyzed, the CpcS-I/U/V triple mutant contained half of the content of the CpcS-I/U double mutant, and only 13% of the wild type. This led the authors to speculate that CpcV is involved in AP biosynthesis in the absence of CpcS-I/U. This theory is yet unproven. The gene encoding this lyase is not found among all PC-producing strains of cyanobacteria. *Synechocystis* sp. PCC 6803, for instance, which has the *cpcS-I* and *cpsU* genes does not contain *cpcV*.

1.5 Slr1098

Slr1098 is a protein comprised of 252 amino acids. It has amino acid similarity to CpcE-type bilin lyases, suggesting that it has chromophore addition function. It contains several HEAT-repeat motifs, which are involved in protein-protein interactions. Most of the proteins known to contain these HEAT repeats are involved in protein stabilization and scaffolding functions. Mutants in which the *slr1098* gene was insertionally inactivated were yellow-green in color, instead of the blue-green color of the wild type (Schluchter and Glazer 1999; Schluchter *et al.*, unpublished results). The mutants produced 30-50% of the normal amount of PC, although the phycobiliproteins present were fully chromophorylated. They produced lower levels of chlorophyll than the wild type as well. The mutants were able to breakdown their PBS during nitrogen starvation, implying that Slr1098 is not essential to this process. Although there was no bilin addition activity observed by Slr1098 to HT-CpcBA, the two complexes did interact, and co-purified together (Schluchter *et al.*, unpublished results). The mutant analysis suggests that this protein is involved in PC oligomerization. The exact biosynthetic pathway of PC biogenesis

and complete oligomerization- from monomer to trimer and hexamer assembly is as yet unknown.

1.6 Purpose of Work

The research presented here is focused on investigating the function of the *slr1098* gene in *Synechocystis* sp. PCC 6803. It is believed this gene may be a bilin lyase or involved in the biosynthesis of PC by way of trimer and hexamer formation. It is similar in amino acid sequence to CpcE bilin lyase subunits, and NblB. Previous research has shown that Slr1098 negative mutants make lower amounts of PC and PBS compared to wild-type cells (Schluchter and Glazer, 1999). This phenotypic characteristic is consistent with other bilin lyase mutants, but the protein did not have any bilin addition activity in *in vitro* assays with apo-PC (Schluchter et al., unpublished). One goal of this research was to produce the recombinant protein and perform interaction assays with PC to see if the two interacted and to investigate the size of any complexes formed to determine if Slr1098 has a role in PC oligomerization. The second goal was to determine if Slr1098 could attach bilins to CpcB or CpcA, and to examine any adduct formed, if any, and determine its composition.

MATERIALS AND METHODS

2.1 Materials

Chemicals were purchased either from Sigma Chemical Company (St. Louis, MO) or from Fisher Chemical Company (Houston, TX). Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and Promega Corporation (Madison, WI). BL21 Star (DE3) and BL21 Star (DE3) pLysS *E. coli* competent cells were purchased from Invitrogen (Carlsbad, CA). SDS polyacrylamide gels were purchased from BioRad (Hercules, CA). PVDF membranes were purchased from Amersham Biosciences Corporation (Piscataway, NJ).

2.2 Construction of Recombinant Expression Plasmids

2.2.1 Slr1098

Slr1098 was amplified by polymerase chain reaction (PCR) from the genomic DNA of *Synechocystis* sp PCC6803 using two oligonucleotides (slr1098.5.2 oligo: 5'-GGAAATCATATGGAGAAAATATGGAGCTATGCG- 3'; and slr1098.3 oligo: 5'-CACCCTTTTCTACCCCAGCCTGAGAG- 3'). The resulting 1050-bp product was digested with *NdeI* and *SspI* (after the stop codon within the PCR product) restriction enzymes (*NdeI* site in primer is underlined) and cloned into the *NdeI* and *SmaI* sites of pAED4, a high copy number T7 expression vector. The construct map for this plasmid is shown in Fig 7. To create the HT-slr1098 pBS150v construct, the slr1098/pAED4 clone was digested with *NdeI* and *EcoRI* (*site within vector*) and then sub-cloned into the *NdeI* and *EcoRI* sites of pBS150v to create the slr1098/pBS150v clone. The construct map for this plasmid is shown in Fig 8.

To create the pBASlr1098 plasmid, the slr1098 gene was amplified from slr1098pAED4 using slr10985.2 oligo (above) and slr10983.Xho oligo (5'AACTCGAGTTAGATCAGTCCGTCCATTTCCAGGTCTTC 3'). The PCR product was digested with NdeI and XhoI and cloned into pCDF Duet vector containing HTCpcBA (see below) using the NdeI and XhoI enzymes.

2.2.2 *cpcBA*

The *cpcBA* genes (encoding the β and α subunits of PC, respectively; sll1577 and sll1578) were amplified by PCR from chromosomal DNA of *Synechocystis* sp PCC 6803 using two oligonucleotides (*cpcB*.1: 5'-GGAGATTAATCATATGTTTCG ACGTATTCAC-3' and *cpcA*.3: 5'-CCCAAGCTTCCAGGCCAGCTAGAAT-3'). The 1150-bp product was digested with *NdeI* and *HindIII* restriction enzymes then cloned into the *NdeI* and *HindIII* sites of the expression vector pBS150v. This vector confers resistance to spectinomycin and contains an N-terminal 6 Histidine tag. Both *cpcB* and *cpcA* are expressed from the p_{Trc} promoter and are inducible by the addition of isopropyl 1-thio- β -D-galactopyranoside (IPTG), but only the CpcB protein has the hexa-His-tag (HT). CpcA is co-purified through its association with apo-HT-CpcB. The construct map for the *cpcBA*-pBS150V plasmid is shown in Fig 9.

The *Synechocystis* sp. PCC 6803 *cpcBA* genes were amplified from an existing plasmid called *cpcBA*/pBS150v (described above). The pBS150vNcoF primer (5'-AACCATGGAGATCAGTAACAATAACTCTAGGG-3') which sits within the pBS150v vector sequence and the *cpcAR* (5'-ACTAAAGCTTAATTAGCTGAAGGGCG-3') primer which sits at the 3' end of the *cpcA* gene were used to amplify the product using the PCR. The product was

cloned into the pCDF Duet-1 vector (Novagen, Madison, WI) using the NcoI and HindIII sites (restriction sites in primers are underlined). This expression clone results in the production of histidine-tagged CpcB and non-tagged CpcA. This plasmid is compatible with other Duet plasmids to allow co-expression of multiple genes from up to four plasmids inside *E. coli*.

2.2.3 *cpcS-I* and *cpcU*

The *cpcS-I* and *cpcU* genes were cloned in the pCOLA Duet-1 vector (Novagen, Madison, WI) so that they would be expressed on one mRNA producing HT-CpcU and CpcS, generating the pCpcUS plasmid (see Table 1). First, the *cpcS-I* gene (SYNPCC7002_A1822) was amplified from *Synechococcus* sp. PCC 7002 chromosomal DNA using primers cpcSF (5'-TCCCTGCAGAAGGAGATTTCGATATGCAAAGCTTTGC-3') and cpcSR (5'-ACGGTCGACCTACCAACCGCTAATAGCGTAAAG-3') and cloned into pCOLA Duet-1 after digestion with PstI and Sall (restriction sites in primers are underlined, and the forward primer contains a ribosome binding site) to create the pCpcS plasmid. The *cpcU* gene (SYNPCC7002_A2053) was amplified by PCR from *Synechococcus* sp. PCC 7002 chromosomal DNA using cpcUF (5'-AGGGGGATCCTATGGATATCAATGCCTTTATC-3') and cpcUR (5'-GCCGAATTCTTAGTTACTGGCTTCAGCGGTTAC-3') and cloned into the pCOLA Duet-1 plasmid using the restriction enzymes BamHI and EcoRI (restriction sites underlined in primers) to create the pCpcU plasmid. Then, *cpcU* gene was subcloned into the pCpcS plasmid using BamHI and EcoRI to create the pCpcUS plasmid (A. Biswas and W. Schluchter, unpublished data).

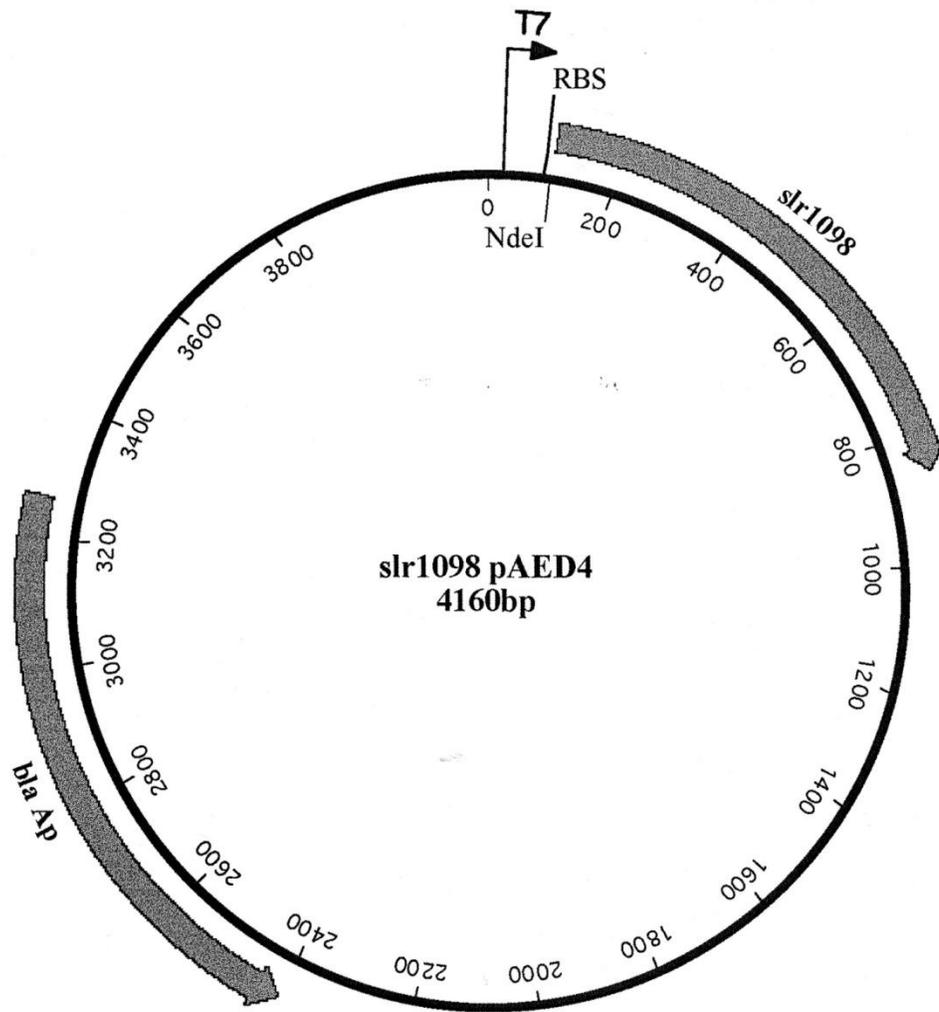


Figure 7: Plasmid map for the *slr1098/pAED4* construct.

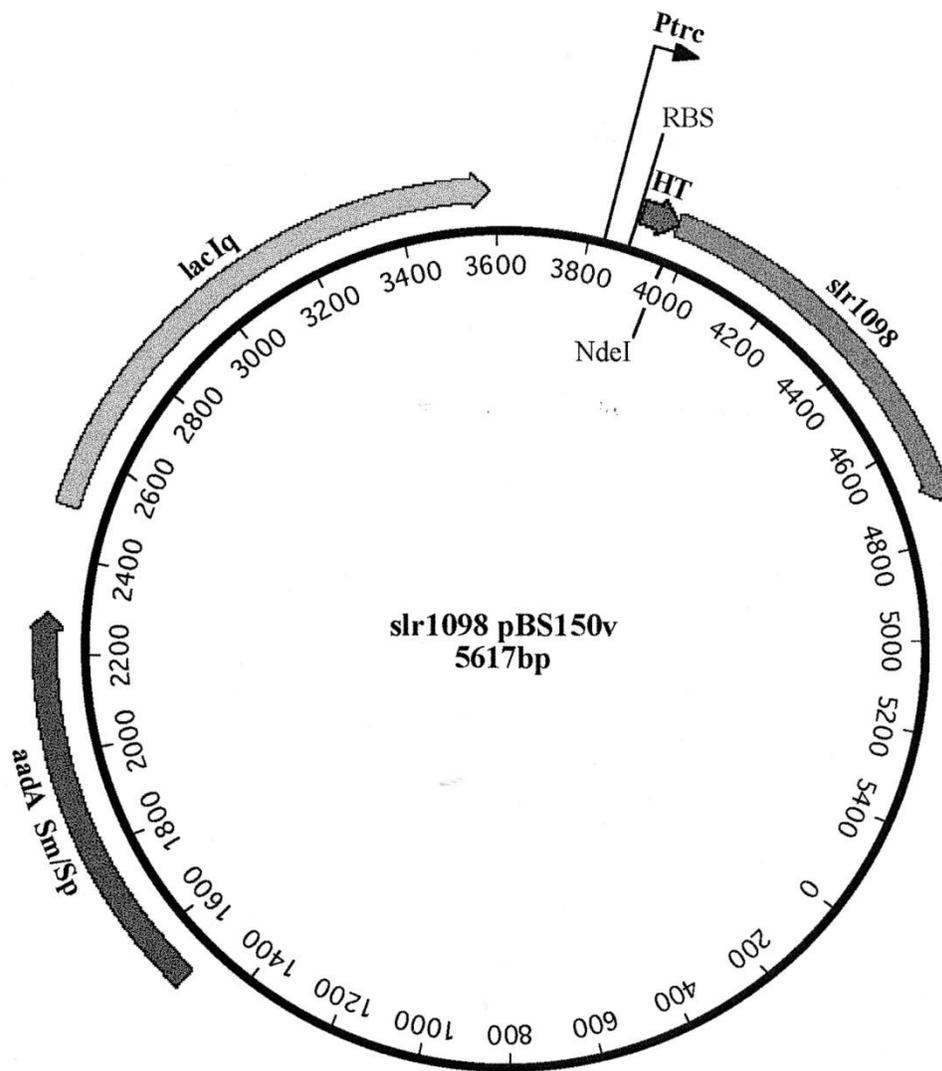


Figure 8: Plasmid map for the *slr1098/pBS150v* construct.

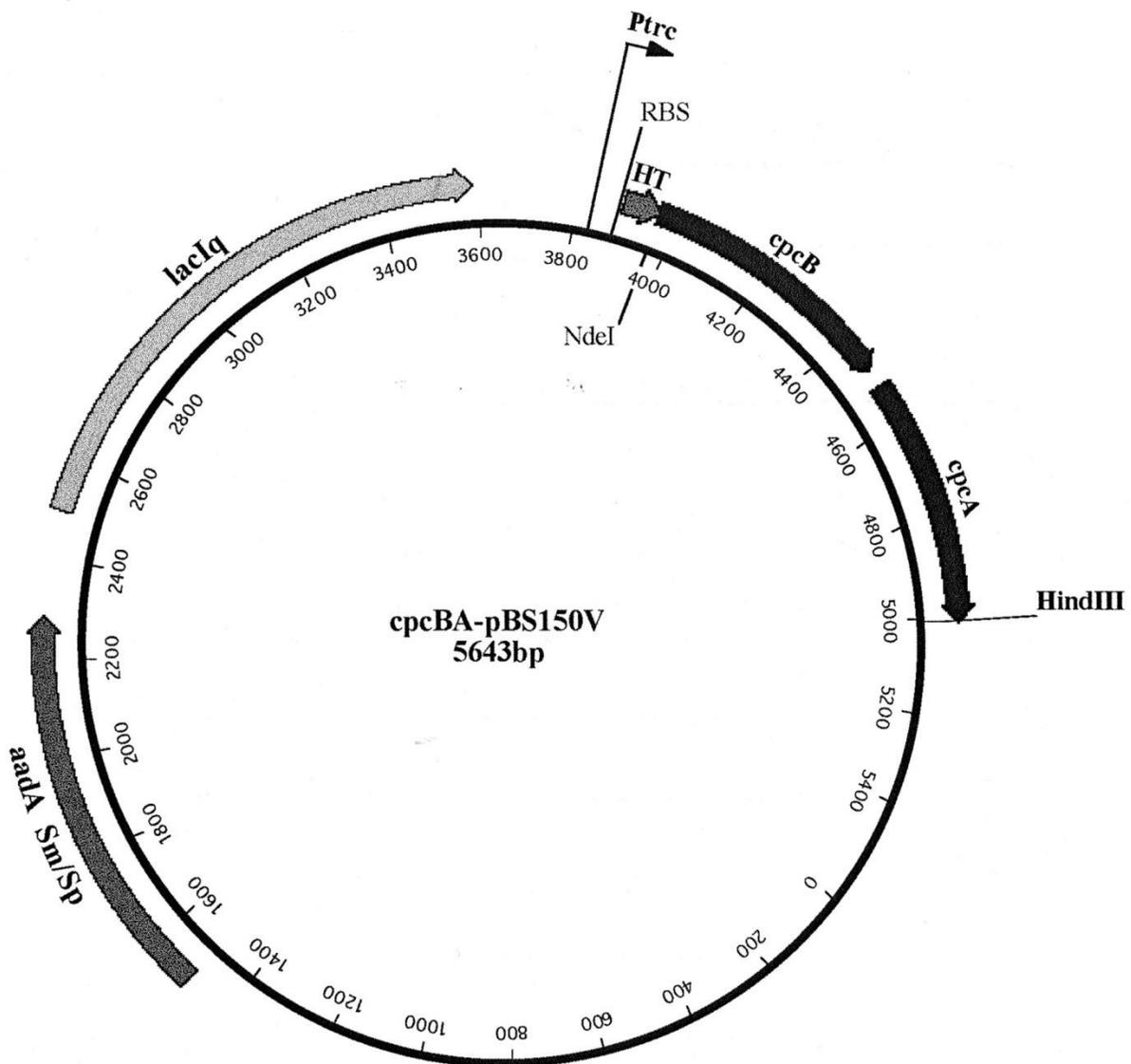


Figure 9: Plasmid map for the *cpcBA-pBS150V* construct.

2.2.4 *pcyA/hox1x*

The heme oxygenase 1 gene, *hox1* (sll1184) was amplified by PCR from the chromosomal DNA of *Synechocystis* sp. PCC 6803 using *hox1F* (5'-GGTATTTTTTCATATGAGTGTCAACTTAGCTTCC-3') and *hox1R* (5'-AGATATCCTAGCCTTCGGAGGTGGCGAG-3') and cloned into pACYC Duet-1 (Novagen, Madison, WI) using NdeI and EcoRV. The 3Z-Phycocyanobilin: ferredoxin oxidoreductase gene, *pcyA* (SYNPCC7002_A2228) was amplified by PCR from chromosomal DNA of *Synechococcus* sp. PCC 7002 using *pcyAF* (5'-CAGAATTCCATGACTGCCCCTGCAACCAAGC-3') and *pcyAR* (5'-AAGTCGACGATCTAGGCTGGAATATCAAACAGCACC-3'). The gene was cloned into the pACYC Duet-1 vector containing *hox1* using EcoRI and SalI, and the resultant plasmid was named pPcyA (A. Biswas and W. Schluchter, unpublished data). In *E. coli* the expression of these two gene products results in the production of PCB from heme (Frankenberg et al., 2001).

2.2.5 Transformation

For recombinant protein production, plasmids were transformed into either BL21 Star (DE3) or BL21 Star (DE3) pLysS competent *E. coli* cell lines (Invitrogen), according to the manufacturer's directions. Competent cells were thawed on ice, and 1 μ L of the desired plasmid was added. Cells were placed on ice for 30 minutes then in a 42°C water bath for exactly 45 seconds. Cells were immediately placed on ice and 250 μ L of room temperature SOC medium (0.5% Bacto-yeast extract (w/v), 2% Bacto-tryptone (w/v), 2.5 mM KCl, 10 mM NaCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added. Vials with cells were placed in a shaking incubator for one hour at 37°C at 225 rpm. 20 to 200 μ L of the transformation was plated onto

Luria-Bertani (LB) plates containing the appropriate antibiotic to select for the desired vector and insert. To maintain each plasmid, cells were grown in the presence of spectinomycin (100 $\mu\text{g/ml}$), ampicillin (100 $\mu\text{g/ml}$), kanamycin (50 $\mu\text{g/ml}$) and/or chloramphenicol (34 $\mu\text{g/ml}$).

2.3 Recombinant Protein Production

One transformed colony was selected and inoculated into a 50 mL overnight culture (LB plus antibiotic) at 37 °C with shaking. This was used to inoculate 1 L of LB containing antibiotic. Flasks were incubated at 37 °C with shaking at 200 rpm for three hours. Gene expression was induced with 1 mM IPTG (isopropyl β -D-thiogalactoside), and cells were shaken for an additional four hours. For a control, cells containing empty vectors (either pBS150v or pAED4) were grown in the same manner as the described above. Cells were harvested by centrifugation at 7000 x g for 30 minutes in a Sorvall GSA rotor and then frozen at -20°C until needed.

To coexpress *cpcBA*, *slr1098*, *ho1*, *pcyA* and or *cpcSU*, expression plasmids were co-transformed into *E. coli* BL21 *DE3* cells, and colonies were selected on Luria Bertani (LB) plates in the presence of the appropriate combination of antibiotics (see Table 1). To produce PCB using the pPcyA expression plasmid a 50-ml overnight starter culture was added to 1-l of LB medium with the appropriate combination of antibiotics and shaken at 37°C for 4hrs until the absorbance reached $A_{600\text{nm}}=0.6$. Production of T7 RNA polymerase was induced by the addition of 0.5 mM isopropyl β -D thiogalactoside (IPTG). Cells were incubated with shaking at 225 rpm at 30°C for another 4 hrs before cells were harvested by centrifugation at 10,000 x g for ten minutes, and

cell pellets were stored at -20°C. Growth of cells at 30° C after induction with IPTG limited the amount of inclusion bodies produced (data not shown).

2.4 Slr1098 Purification

Following the recombinant Slr1098 protein production in *E. coli*, the thawed cell pellets (on ice) were resuspended in 20 mL of 50 mM Tris-HCl pH 8, 20 mM NaCl and homogenized. This mixture was then passed through a French pressure cell press a total of three times, at 20,000 psi (138 megapascals). Unbroken cells and inclusion bodies were separated from the cell extract by centrifugation at 12,000 x g for 20 minutes in an SS34 rotor (Sorvall). The supernatant was collected and solid ammonium sulfate was added to 40% (w/v), while stirring, at 4°C overnight. The next day the precipitate was centrifuged for 20 minutes at 12,000 x g in an SS34 rotor (Sorvall). Given that the protein was located in the pellet (as judged by SDS-PAGE), the supernatant was discarded, and the pellet was resuspended in 50 mM sodium phosphate, pH 7. To remove the ammonium sulfate, the suspension was dialyzed against the same buffer at 4°C overnight, with stirring, and the dialysis buffer was changed the next day.

Anion exchange chromatography was performed to attain further purification. Approximately, 20 g DEAE powder was added to 400 ml of 50 mM Tris-HCl, pH 8.0, and stirred on a stir plate for 2 hours. In order for the DEAE swell and allow any fines to rise to the top, the solution sat overnight. The fines at the top of the DEAE slurry were poured off. Then a DEAE column (Whatman DE-52: 2.5 x 12.5 cm) was poured and equilibrated with 2 column volumes of 50 mM Tris-HCl, pH 8.0, 1 mM NaN₃ (Buffer A). The Slr1098 solution was loaded onto the column using the BioLogic LP system at room temperature (BioRad, Richmond, CA).

The column program (flow rate of 2 ml/min) is as follows: 0-30 minutes, 100% Buffer A; 30-150 minutes to 100% Buffer B (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM NaN₃); 150-180 minutes, 100% Buffer B; 180-210 minutes 100% Buffer A.

Cells containing Slr1098 with an N-terminal 6 histidine tag (HT-Slr1098) were purified in a similar manner, except they were suspended in buffer O (20 mM Tris-HCl, pH 8.0, 100 mM Na/KCl), and purified by metal affinity chromatography on a Ni-NTA resin column (Qiagen, Inc.). After loading the supernatant onto the column, it was developed with ten column volumes each of buffer A1 (20 mM Tris-HCl, pH 8.0, 100 mM Na/KCl, 20 mM imidazole, 5% glycerol), B (20 mM Tris-HCl, pH 8.0, 1 M Na/KCl), A2 (20 mM Tris-HCl, pH 8.0, 100 mM Na/KCl, 30 mM imidazole) then the histidine-tagged protein was eluted with 2 ml of buffer C (20 mM Tris-HCl, pH 8.0, 100 mM Na/KCl, 200 mM imidazole). The protein was dialyzed against buffer O containing 1mM β -mercaptoethanol.

Table 1: Recombinant protein expression vectors used

<u>Plasmid name</u>	<u>Recombinant proteins produced^a</u>	<u>Parent vector</u>	<u>Anti-biotic^b</u>	<u>Reference</u>
pPcyA	<i>Synechocystis</i> sp. PCC 6803 HO1 and <i>Synechococcus</i> sp. PCC 7002 HT-PcyA	pACYC Duet	Cm	Biswas et al., in prep
pCpcUS	<i>Synechococcus</i> sp. PCC 7002 CpcU and CpcS coexpressed on one mRNA	pCOLA Duet	Km	Biswas et al., in prep
pCpcU	<i>Synechococcus</i> sp. PCC 7002 CpcU	pCOLA Duet	Km	Biswas et al., in prep
pCpcS	<i>Synechococcus</i> sp. PCC 7002 CpcS	pCOLA Duet	Km	Biswas et al., in prep
pCpcBA	<i>Synechocystis</i> sp. PCC 6803 HT-CpcB and CpcA	pCDF Duet	Sp	Biswas et al., in prep
pBASlr1098	<i>Synechocystis</i> sp. PCC 6803 HT-CpcB and CpcA and Slr1098	pCDF Duet	Sp	This thesis
Slr1098/pAED4	<i>Synechocystis</i> sp. PCC 6803 Slr1098	pAED4	Ap	This thesis
Slr1098/pBS150v	<i>Synechocystis</i> sp. PCC 6803 HT-Slr1098	pBS150	Sp	This thesis
CpcBA/pBS150v	<i>Synechocystis</i> sp. PCC 6803 HT-CpcBA	pBS150v	Sp	This thesis

^a Proteins that would be produced as fusions are indicated as HT-

^b Antibiotic resistance used to select for the presence of the plasmid (Ap: ampicillin; Cm: chloramphenicol; Km: kanamycin; Sp: spectinomycin)

2.5 Phycocyanin Purification

PC was purified from *Synechocystis* sp. PCC 6803 using an adaptation of the method described by Glazer and Fang (1973). Cyanobacterial cells were suspended in 50mM sodium acetate pH 5.5 (1 ml per g of wet weight) with 1mM β -mercaptoethanol, and homogenized. The homogenate was passed through a French pressure cell, at 20,000 psi (138 megapascals), at 4°C. The suspension was centrifuged for 30 minutes at 23,000 x g in an SS34 rotor (Sorvall). The supernatant was decanted and saved, and the pellet was washed with sodium acetate buffer. The supernatants were combined, and then fractionated to 65% (w/v) with solid ammonium sulfate overnight at 4°C. The precipitate was collected by centrifugation at 12,000 x g for 20 minutes at 4°C in an SS34 rotor (Sorvall), dissolved in a minimum amount of 5 mM sodium phosphate pH 7 and dialyzed to equilibrium against the same buffer at 4°C. Further purification was achieved through ion exchange chromatography. The dialyzed solution was applied to a DEAE-cellulose (DE-52, Whatman) column (2.5 x 20 cm) pre-equilibrated with 5mM sodium phosphate pH 7 buffer. The column was developed at room temperature using the BioLogic LP chromatography system (BioRad). The program was as follows: 0 to 30 minutes of buffer A (50 mM Tris-HCl, pH 8.0, 1mM NaN₃), 30 to 150 minutes of 0-100% buffer B (50 mM Tris-HCl, pH 8.0, 1M NaCl, 1mM NaN₃), 150 to 180 minutes of buffer B, 180 to 210 minutes of buffer A. Fractions were collected and loaded on a 15% (w/v) SDS- polyacrylamide gel to determine which fractions contained the purest PC. Samples were pooled together, then dialyzed against 5 mM sodium phosphate pH 7 and concentrated using an Amicon YM-10 centrifugal filter unit (Millipore, Billerica, MA). Purified PC was diluted 50- fold in 50 mM phosphate buffer before absorption readings used to determine concentration at 623 nm.

The concentration was calculated using Beer's law; $A = \epsilon c l$. For PC, the extinction coefficient, $E_{623} = 290,000 \text{ M}^{-1} \text{ cm}^{-1}$ was used (Glazer, 1988).

2.6 Fluorescence Emission and Absorbance Spectra

A dual-beam Lambda 35 UV-Visible spectrophotometer (Perkin Elmer, Waltham, MA) was used to gather absorbance spectra. The spectrum of PC was measured from 450 nm to 750 nm. The HT-CpcBA produced in *E. coli* in the presence of the PCB were evaluated by acquiring fluorescence emission spectra using a Perkin Elmer LS55 fluorescence spectrophotometer (Waltham, MA) with slit widths set at 10 nm (excitation and emission). The excitation wavelength was set at 590 nm.

2.7 SDS-PAGE Analysis

Protein separation was performed on 15% Tris-HCl SDS (Sodium dodecyl sulfate)-polyacrylamide gels that were purchased from BioRad (Hercules, CA). Loading dye containing β -mercaptoethanol was added to the protein samples, and the samples were boiled for 5 minutes. The samples were loaded onto the gel, and the gels were run for 12 minutes at 100 volts, followed by an additional 45 minutes at 200 volts. To visualize bilin fluorescence, gels were placed in 10 mM ZnSO_4 solution for 15 minutes and exposed to UV light. Following zinc staining, gels were stained overnight in Coomassie Blue stain: 0.125% (w/v) Coomassie Brilliant Blue, 50% methanol and 10% acetic acid, then destained overnight in 10% methanol, 10% acetic acid. To collect images, gels were photographed on the Gel-Doc (BioRad). Alternatively, some

gels were stained in SYPRO Ruby protein gel stain (BioRad) instead of Coomassie blue. Gels were placed in a fixing solution (50% methanol, 7% acetic acid) for 30 minutes, then in the SYPRO stain overnight, followed by a wash solution (10% methanol, 7% acetic acid) for 30 minutes. Gels were imaged on the FX imager (BioRad).

2.8 Western Blot Analysis

After separation of samples on a 15% SDS- polyacrylamide gel as described above, proteins were transferred to a PVDF membrane. The transfer sandwich included a piece of PVDF membrane cut to the approximate size of the SDS-PAGE gel, two pieces of Whatman paper (3 MM) cut slightly larger than the gel, and two fiber pads. The membrane was soaked in 100% methanol for one minute, and then placed in chilled transfer buffer (25mM Tris-HCL, pH 8.3, 192 mM glycine, 20% (v/v) methanol, 0.1% SDS) for 5 minutes. The sandwich cassette was assembled as follows: fiber pad, filter paper, membrane, gel, filter paper, fiber pad. The apparatus was placed into an electrophoresis holder, and then the holder was put into an SDS gel/transfer tank along with an ice block for cooling, and a stir bar. The cassette was covered with transfer buffer. The transfer was performed for 2 hours at 100 volts with stirring, at room temperature. The resulting blot was placed in blocking buffer (TTBS and 5% (w/v) non-fat dried milk) at room temperature for 30 minutes, or alternately, at 4°C overnight. Following blocking, the blot was washed in TTBS (20 mM Tris-HCL, pH 7.6, 137 mM NaCl, 0.1% Tween 20). The primary antibody, anti-Slr1098, was raised in rabbits (kind gift from Dr. Masato Nakai from Osaka University) and incubation was 1:2000 in TTBS for one hour at room temperature on a benchtop rocker. Incubation with primary antibodies was followed by a brief wash, and then 3

washes for 5 minutes each, with 100 ml of TTBS. Goat anti-rabbit horseradish peroxidase-conjugated antibodies were used as secondary antibodies (BioRad) and were diluted 1:10,000 and incubated with the blot for 1 hour at room temperature. Blots were washed in same manner as after incubation with primary antibodies. Detection involved the Immune-Star™ HRP luminal/enhancer and Immune-Star™ HRP peroxide buffer, purchased from BioRad. The blot was incubated in the chemicals (mixed in a 1:1 ratio with enough volume to cover the blot) for 1 minute, and then placed on the Chemi-Doc System (BioRad) for chemiluminescence detection.

2.9 Sucrose Gradients

Sucrose solutions were made using 0.75 M sodium/potassium phosphate buffer, pH 8, with 1mM sodium azide. Gradients were assembled into 14mm x 18mm centrifuge tubes, in a stepwise fashion in the following order from the bottom; 0.75 mL of 2 M sucrose, 1.0 mL of 1 M sucrose, 2.0 mL of .75 M sucrose, 3.4 mL of .50 M sucrose, 3.4 mL of .25 M sucrose. 150 µL of PC and 250 µL of Slr1098 or empty vector *E. coli* extract (pAED4), as a control, in 50 mM sodium phosphate buffer were incubated for one hour in the dark, at room temperature, and then loaded directly on top of the gradient. The tubes were centrifuged in the Beckman SW41 ultracentrifuge for 18 hours at 37,000 rpm (234,745 x g) at 18°C. Following centrifugation, samples were collected from the tubes and TCA (Trichloroacetic acid) precipitated: an amount equal to the sample was added then samples were placed at -20° C overnight. The next day samples were loaded on a SDS-PAGE gel.

2.10 Pull-Down Interaction Experiment

To test for the interaction of His-tagged Slr1098 (HT-Slr1098) and *Synechocystis* sp. PCC 6803 PC, a test reaction containing 50 µg of HT-Slr1098 and 60 µg PC in 5mM sodium phosphate buffer, pH 7, and a control reaction containing 50 µg of HT-Slr1098 and sodium phosphate buffer alone were incubated on ice for 30- 60 minutes. During this incubation 150-250 µl of Nickel-NTA resin was washed with 5 mM sodium phosphate, pH 7, then the resin was centrifuged for 2 to 5 minutes at 4000 rpm. The supernatant was removed and then the protein mixtures containing a His-tagged subunit were loaded onto the resin, and placed on a rotating spin wheel for 30 minutes at room temperature. The sample was centrifuged at 4000 x g for 2 minutes, and the supernatant was removed. Buffer A1 was added to wash away nonspecific protein interactions. Then 30 µL of buffer C was added to the resin mixture, an equal volume of SDS loading buffer, containing β-mercaptoethanol was added, and the mixture was boiled, subjected to centrifugation, and 15 µl of the supernatant was loaded onto an SDS-PAGE gel.

2.11 Binding Assay

50 µl each of purified *Synechocystis* sp. PCC 6803 PC and partially purified non-tagged Slr1098, or the extract containing empty pAED4 vector as a control, were combined (both contained 50 mM sodium phosphate buffer pH 7). The samples were incubated at room temperature, in the dark, for one hour. The samples were then analyzed by size exclusion chromatography as described below.

2.12 SEC-HPLC

Size exclusion high performance liquid chromatography (HPLC) separations were done with a Waters 600E pump and a photodiode array detector. The binding assay samples were injected onto a Bio-Sil SEC 250 column (BioRad; 300 x 7.8 mm). A guard column (80 x 7.8 mm) of the same material was used. The liquid phase was 50 mM sodium phosphate pH 7 and the flow rate was 0.8 ml min⁻¹. Molecular weight standards (BioRad) and retention times are given in Table 2. The molecular weights were calculated using the equation generated from the standard curve based on the retention times of the standards (Figs 10,11)

Table 2: SEC-HPLC Standards with Retention Time and Molecular Weights

<u>Name</u>	<u>Retention Time</u>	<u>Log MW</u>	<u>Actual Molecular Weight</u>
Thyroglobulin	19.31	5.8261	670,000 Da
γ -Globulin	25.21	5.1987	158,000 Da
Ovalbumin	26.45	4.6435	44,000 Da
Myoglobin	33.11	4.2304	17,000 Da
Vitamin B ₁₂	37.65	3.1303	1350 Da

Molecular Weight Standards 9/29/03

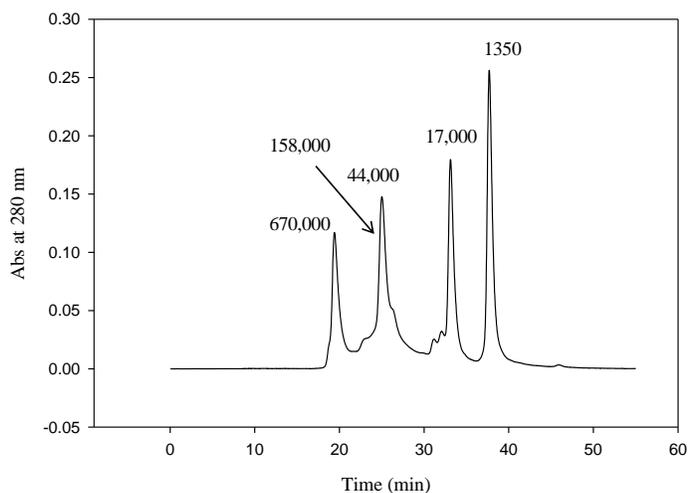


Figure 10: Size Exclusion Chromatogram of Molecular Weight Standards. Molecular weights are indicated for each peak. Standards are thyroglobulin, γ -globulin, myoglobin, and vitamin B₁₂.

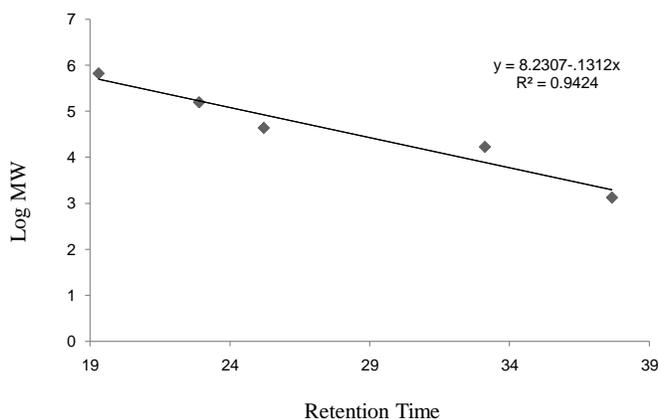


Figure 11: Regression Line of SEC Molecular Weight Standards. The regression line is based on the retention times of the standards. The equation given is used to determine the calculated molecular weights of the protein interaction complexes.

RESULTS

3.1 Slr1098 Characteristics and Alignment with Other CpcE Proteins

Slr1098 from *Synechocystis* sp. PCC 6803 is similar to CpcE-type bilin lyases. A BLAST search revealed that Slr1098 has sequence similarity to 20 other known proteins (Table 3). An alignment was performed to compare its similarity to these proteins (Figs 12- 14). It is 46 % similar to CpcE from *Synechocystis* sp. PCC 6803. Most cyanobacteria seem to have at least two other CpcE-like ORFs, and the range of similarity to these other proteins shown in the alignment was 67.7% to 78.6%, with the proteins from *Microcystis aeruginosa* PCC 7806 (MAE48080), and *Cyanothece* sp. CCY0110 (cce_0658) being the most similar to Slr1098. Previously, *Synechocystis* sp. PCC 6803 mutants, in which *slr1098* was insertionally inactivated, were characterized (Schluchter and Glazer, 1999; Schluchter, *et al.*, unpublished data). These mutants were yellow-green in color, a distinct difference from the blue-green color of the wild type phenotype. Purified phycobilisomes from these mutants contained 30-50% of the normal amount of PC, but all of the phycobiliproteins present appeared to have normal phycocyanobilin content. The mutants were not impaired in phycobiliprotein breakdown during nitrogen starvation, indicating that Slr1098 does not play an essential role in this process (as NblB was shown to do).

The Slr1098 protein was overproduced in *E. coli* and used in binding assays with apo-PC *in vitro*. This protein did not have any bilin addition activity, but Slr1098 did bind and co-purify with apo-HT-CpcB/CpcA (Schluchter, *et al.*, unpublished data). The masses of these complexes were much larger than the monomeric $\alpha\beta$ -PC, and therefore, we hypothesized that Slr1098 may be involved in PC oligomerization into trimers.

Table 3: BLAST search results for Slr1098 sequence similarity

<u>Protein name</u>	<u>Organism name</u>	<u>Description</u>
MAE48080	<i>Microcystis aeruginosa</i> PCC 7806	PBS lyase HEAT-like repeat-containing protein
cce_0658	<i>Cyanothece</i> sp. CCY0110	Importin beta-2 subunit family protein
SYNPCC7002_A1412	<i>Cyanothece</i> sp. PCC 8801	PCB lyase alpha subunit
Ava_1404	<i>Nostoc</i> sp. PCC 7120	PBS lyase HEAT-like repeat
Tery_4484	<i>Trichodesmium erythraeum</i> 1MS101	HEAT domain containing protein
alr4537	<i>Anabaena</i> sp. PCC 7120	Probable serine/threonine- protein phosphatase PP2A regulatory subunit
Npun_F4500	<i>Nostoc Punctiforme</i> ATCC 29133	HEAT domain- containing protein
Synpcc7942_1752	<i>Synechococcus elongatus</i> PCC 7942	PBS lyase HEAT-like repeat
syc2339_d	<i>Synechococcus elongatus</i>	PBS lyase HEAT-like repeat
AM1_2319	<i>Acaryochloris marina</i> MBIC11017	PBS lyase HEAT-like repeat-containing protein
tll0329	<i>Thermosynechococcus elongatus</i> BP-1	Hypothetical protein
CYB-2106	<i>Synechococcus</i> sp. JA-3-3Ab	PBS lyase HEAT-like repeat
CYA_0113	<i>Synechococcus</i> sp. JA-3-3Ab	PBS lyase HEAT-like repeat-containing protein
gll 3007	<i>Gloeobacter violaceus</i> PCC 7421	Hypothetical protein
Syncc6905_2060	<i>Synechococcus</i> sp. CC9605	PBS lyase HEAT-like repeat

SynRcc307_1951	<i>Synechoccus</i> sp. RCC307	PBS lyase HEAT-like repeat-containing protein
PMT 1501	<i>Prochlorococcus marinus</i> MIT9313	Hypothetical protein
Sync9902_0612	<i>Synechoccus</i> sp. CC9902	Hypothetical protein
P9303_04441	<i>Prochlorococcus marinus</i> MIT 9303	Hypothetical protein
Sync_2351	<i>Synechoccus</i> sp. CC9311	Hypothetical protein

Table 3 cont.

Alignment of Slr1098

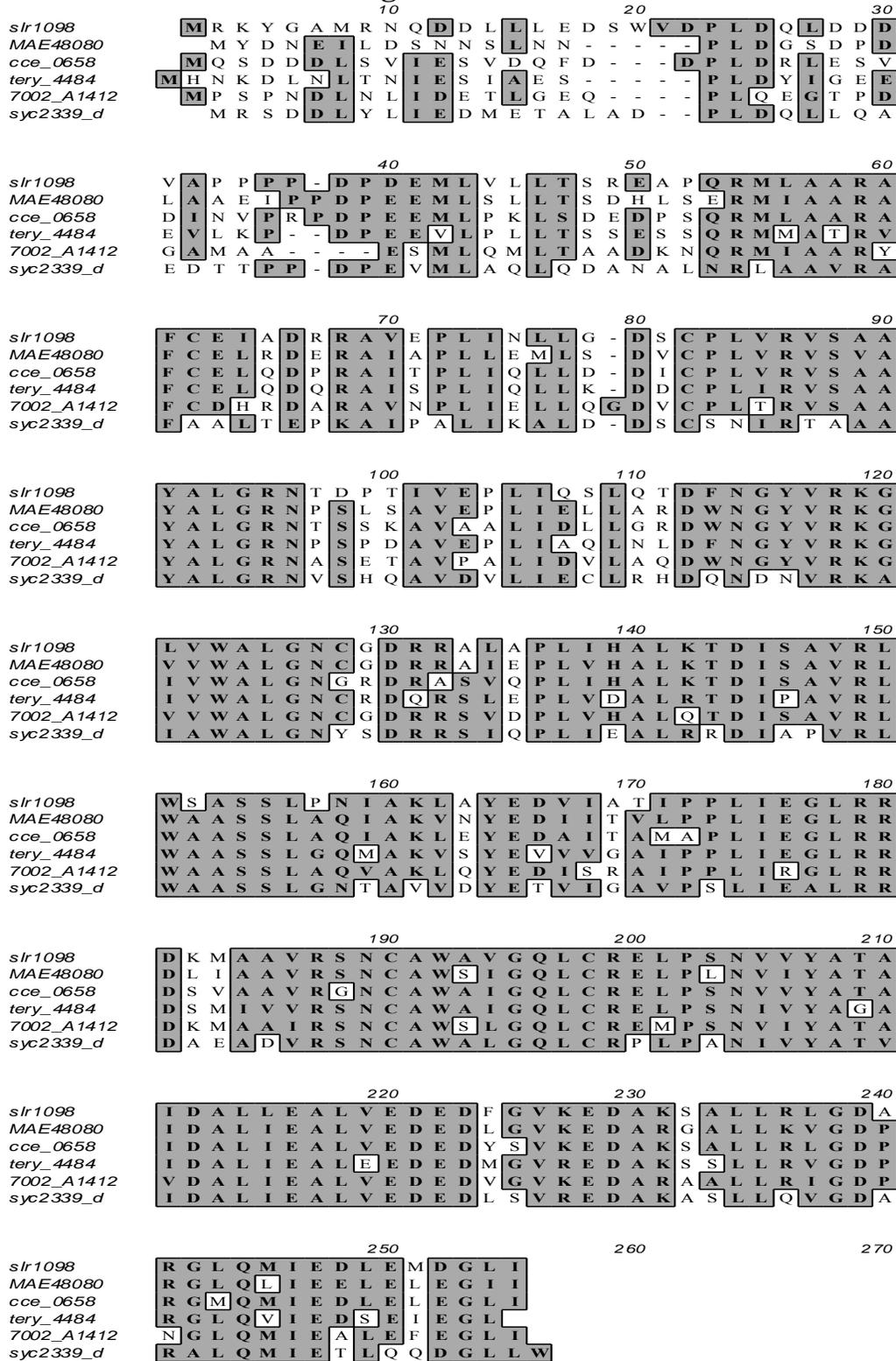


Figure 13: Alignment of Slr1098 with the highest scoring hits from a BLAST search of cyanobacterial genomes. The organism designation can be found in Table 3.

Alignment of Slr1098

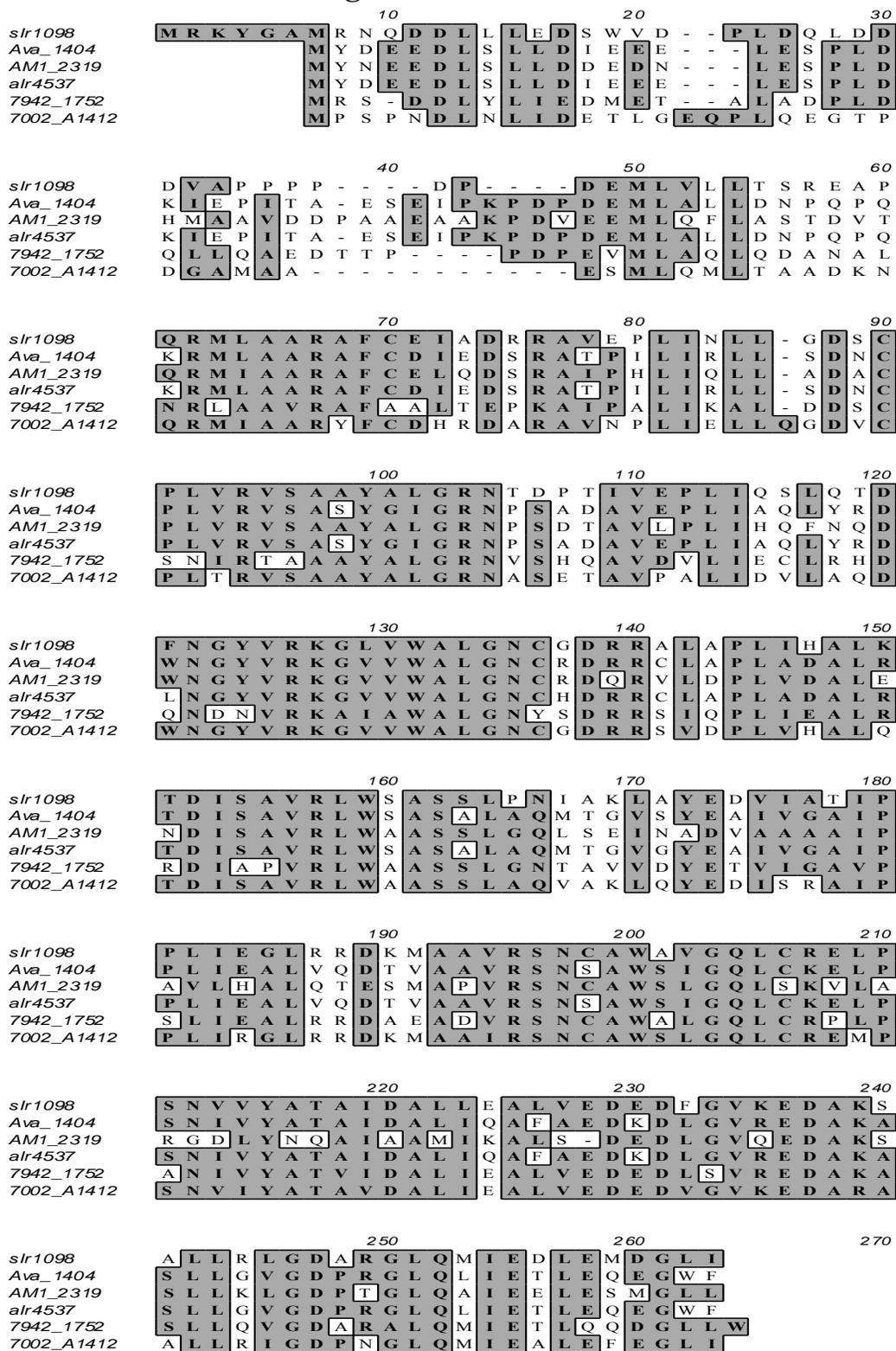


Figure 14: Alignment of Slr1098 with the highest scoring hits from a BLAST search of cyanobacterial genomes. The organism designation can be found in Table 3.

3.2 Expression and Purification of Recombinant Proteins Slr1098, HT-slr1098, HT-CpcBA

In order to assess whether Slr1098 is involved in PC oligomerization, the protein was produced as a his-tagged protein (slr1098/pBS150v) and without a tag (slr1098/pAED4) in *E. coli* cells as described. Expression cells were harvested as described and analyzed by SDS-PAGE (Fig 15). Figure 15 shows a Coomassie-stained gel of the proteins from *E. coli* cells before (lanes 1A, 1,3B) and 4 hours after addition of IPTG (lanes 2A, 2,4B). IPTG inactivates the LacI transcriptional repressor that controls the expression of the T7 RNA Polymerase, allowing for a transcription of the genes with T7 promoters. In panel A, the molecular mass standards are loaded in the lane designated “S” with the masses listed to the left. Lanes 1 and 2 contain *E. coli* extracts with Slr1098 collected before and after IPTG addition, respectively. The position of the Slr1098 protein is indicated by an arrow and is of the expected mass of 27.67 kDa (Table 4). In panel B, lanes 1 and 2 contain *E. coli* extracts with HT-slr1098, before and after IPTG addition, respectively. The molecular mass standards are loaded in the lane designated “S”, with the masses to the left of the figure. Lanes 4 and 5 contain *E. coli* extracts with HT-CpcBA, before and after IPTG addition, respectively. The post induction lanes show that more protein is produced following addition of IPTG. Proteins are indicated by arrows, and each migrated on the SDS-PAGE consistent with its calculated molecular mass (based upon its respective amino acid sequence, see Table 3).

This non-tagged Slr1098 protein was purified using ammonium sulfate fractionation followed by DEAE chromatography as described in the materials and methods. Fig 16 shows the chromatogram of the purification of Slr1098 after DEAE. After running aliquots of fractions collected during this purification on SDS-PAGE, it was determined that the large peak of protein eluting between 40-60 minutes contains Slr1098. The results from this analysis can be seen in

Fig 17 where Slr1098 is most pure in fractions 52, 54, and 56 (lanes 6-8, respectively) but the protein was most abundant in fraction 47 (Lane 5). The most pure fractions were combined, dialyzed to remove the NaCl, and stored until further use.

Table 4: Calculated molecular weights and extinction coefficients

<i>Protein name</i>	<i>Molecular weight</i>	<i>Extinction Coefficient</i>
Slr1098	27,677 Da	$E_{280} = 29,760 \text{ M}^{-1} \text{ cm}^{-1}$
HT-Slr1098	30,555 Da	$E_{280} = 32,320 \text{ M}^{-1} \text{ cm}^{-1}$
6803 HT-CpcBA	38,593 Da	$E_{280} = 29,330 \text{ M}^{-1} \text{ cm}^{-1}$
6803 PC ($\alpha\beta$)	37,477 Da	$E_{623} = 290,000 \text{ M}^{-1} \text{ cm}^{-1}$
Trimeric PC ($\alpha\beta$) ₃	112.4 kDa	
SU1 PC	36.5 kDa	

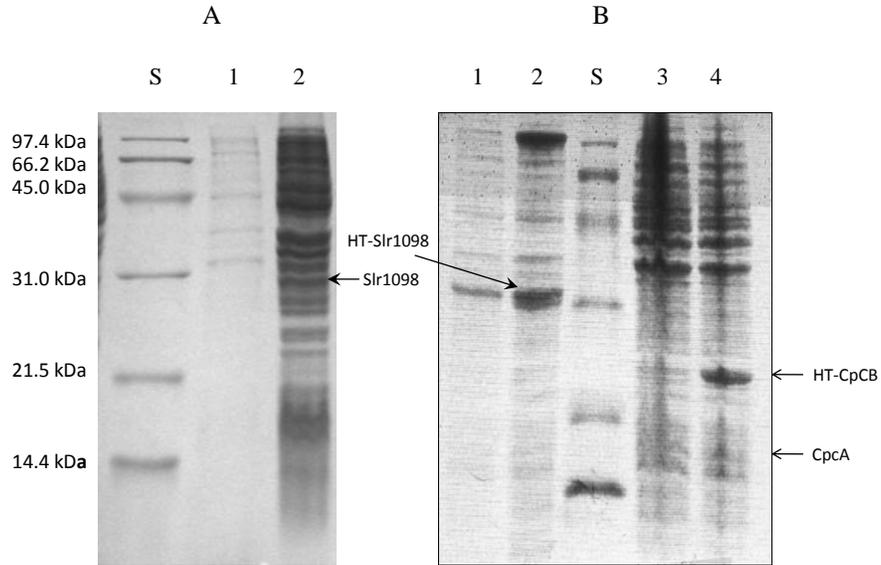


Figure 15: SDS-PAGE analysis of recombinant protein overexpression- Slr1098, HT-slr1098, HT-CpcBA in *E. coli*. One-ml samples of *E. coli* cultures were taken before and 4 hours after induction with IPTG. **A)** Lane S is the molecular mass standards- masses indicated to the left of gel. Lanes 1 and 2 contain Slr1098 before and after induction-respectively. **B)** Lanes 1 and 2 contain HT-slr1098 before and after induction- respectively, lane S is the standard, and lanes 3 and 4 contain HT-CpcBA before and after induction-respectively.

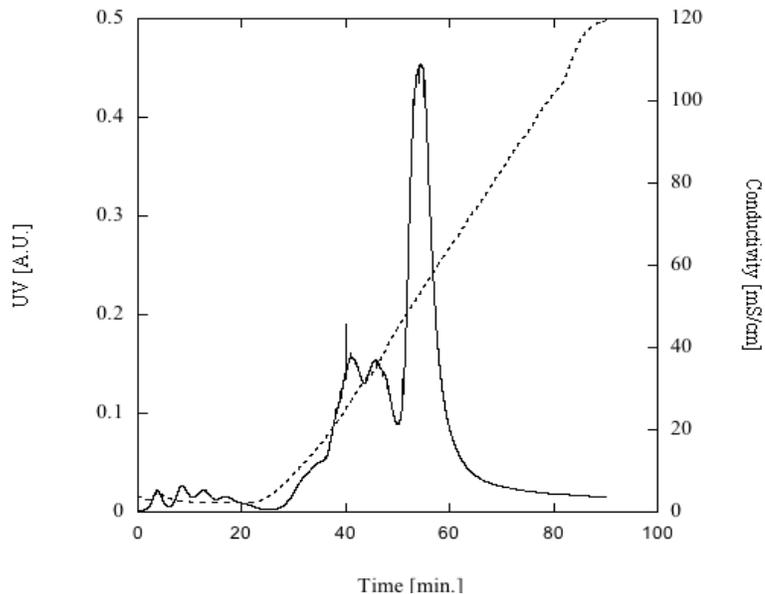


Figure 16: Chromatogram of Slr1098 following DEAE chromatography purification. The solid line shows the proteins absorbance at 280 nm, the dotted line shows conductivity.

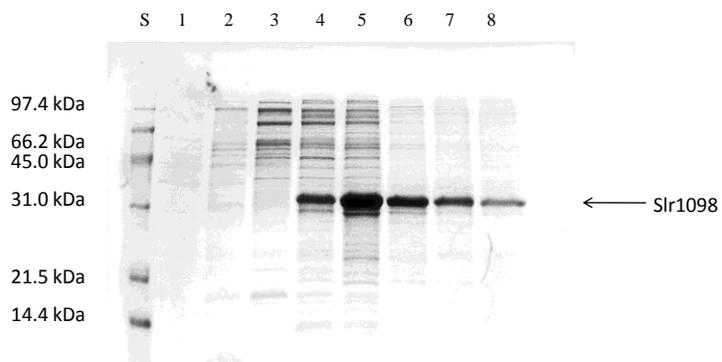


Figure 17: SDS-PAGE analysis of Slr1098 DEAE chromatography purification. Lane S is the molecular mass standards; lanes 1 through 8 contain 10 µl aliquots of fractions 37, 39, 41, 45, 47, 52, 54, and 56, respectively.

3.3 Purification of *Synechocystis* sp. PCC 6803 Holo-PC

Holo-PC was isolated from the cyanobacterium *Synechocystis* sp. PCC 6803 and purified using ammonium sulfate fractionation and DEAE anion exchange chromatography as described. Figure 17 shows the SDS-PAGE analysis of selected fractions collected during ion exchange chromatography. Fig 18A shows the proteins stained with Coomassie blue, while Fig 18B shows zinc enhanced bilin fluorescence of the PC subunits. The purest PC was present in fractions eluting between 10- 25 minutes. These fractions were combined and additionally concentrated as described. Following concentration, the absorbance spectra of the PC was taken to verify the purification and determine the sample's concentration (data not shown).

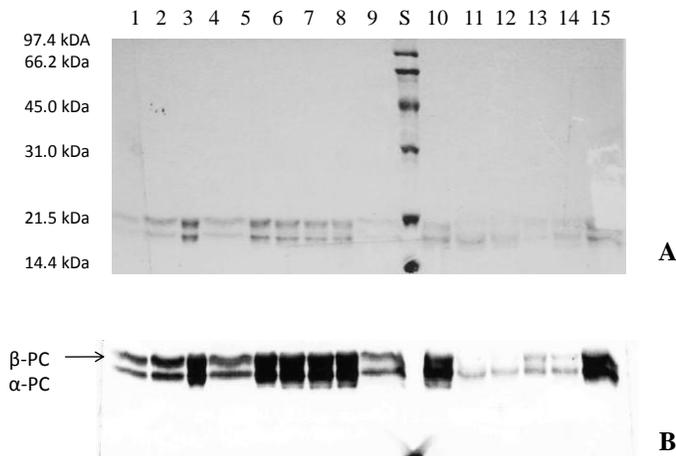


Figure 18: SDS-PAGE analysis of holo-PC purification. A sample from every two fractions (fractions 22-50) was loaded onto a 15% gel. Panel A shows proteins after staining with Coomassie Blue. Panel B shows the bilin-fluorescence of each biliprotein subunit after staining with Zn.

3.4 Phycocyanin and Slr1098 Binding Assays

3.4.1 Sucrose Density Gradient Ultracentrifugation

Before chromophore addition, PC is called apo-PC, whereas after chromophore addition, it is referred to as holo-PC. It has previously been established that Slr1098 and apo-PC interact in a pull-down assay (Schluchter *et al.*, unpublished results). However, it was unknown whether this protein could interact with holo-PC; therefore, the following experiment was performed to test if such an interaction could be detected with holo-PC. The expected molecular mass for one PC ($\alpha\beta$) monomer is 37,477 Daltons (Table 4), and 6803 holo-PC (trimeric) is 112,431 Daltons. Slr1098 and holo-PC were incubated together for one hour and then loaded on top of prepared sucrose density gradients as described in Materials and Methods. The step gradients contain concentrations of sucrose ranging from 2.0 M at the bottom, to 0.25 M at the top. Protein complexes with higher molecular masses and higher densities will travel through the gradient during centrifugation until they reach the point in the gradient at which their density matched that of the surrounding sucrose. As controls, PC plus *E. coli* extract containing empty vector (pAED4 in *E. coli*) or PC and sodium phosphate buffer were incubated and loaded onto the gradients. Following ultracentrifugation, the gradients were removed, and the migration of the PC-containing complexes was measured (Fig 19). PC bands were collected from the gradients, absorbance readings taken, and analyzed by SDS-PAGE (Fig 20). The density of the PC complexed with Slr1098 was slightly greater than both PC treated with the control *E. coli* extract, and PC treated with sodium phosphate buffer, as judged by the relative position within the gradient. The test reaction migrated 0.3 cm further down the gradient than the control, and 0.5 cm further than the control 2 reaction. These results would suggest a Slr1098 and holo-PC form a complex somewhere between a trimer and a hexamer due to the test reaction's relative

distance from both control (trimeric) reactions, but an exact size of this complex cannot be made without appropriate standards.

In order to assess which proteins were present in these complexes, an aliquot of each mixture collected from the gradient was combined with TCA (trichloroacetic acid) to precipitate the protein(s) present. The pellets were solubilized in buffer and subjected to SDS-PAGE. Figure 20 shows the proteins present in the bands collected in sucrose density gradients for the control reaction containing PC and sodium phosphate buffer (lanes 1 and 2), in the test reaction containing PC and Slr1098 (lanes 3 and 4), and in the second control reaction containing PC and pAED4 vector (lanes 5 and 7). The control lanes (1, 2, 5, 7) only contain the α and β -PC subunits, while the test lanes (3, 4) contain Slr1098 as well as α and β -PC (indicated by arrows). This analysis illustrates that the band with the most density present in the sucrose gradient for the test reaction contained both Slr1098 and PC, but no other proteins.

An additional gel with the gradient samples was run along with purified proteins for comparison. These proteins were transferred to a PVDF membrane, and then probed with antibodies against Slr1098, generated in rabbits (Fig 21). Figure 21 shows the antibodies only bind to the Slr1098 present in the test reaction (lanes 1 and 2) and to the purified Slr1098 (lanes 5 and 6), verifying that Slr1098 does interact with holo-PC and forms a complex that appears larger than the trimeric holo-PC ($\alpha\beta$)₃ alone. However, no size standards were loaded to know the exact size of this complex.

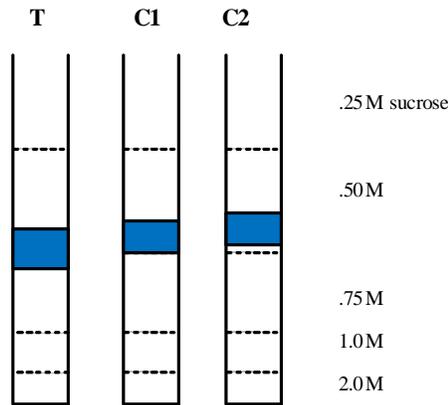


Figure 19: Migration of PC complexes following sucrose gradient ultracentrifugation. Sucrose gradients were poured in a step-wise fashion, most dense (2 M) to least dense (0.25 M), bottom to top. (T) The test contains PC (50 μ L) and Slr1098 (50 μ L). (C) Control 1 contains PC (50 μ L) and pAED4 *E. coli* extract (50 μ L). (C2) Control 2 contains PC (50 μ L) and sodium phosphate buffer (50 μ L). The PC complex isolated from the test gradient (T) demonstrated the highest density based upon its relative migration through the gradient.

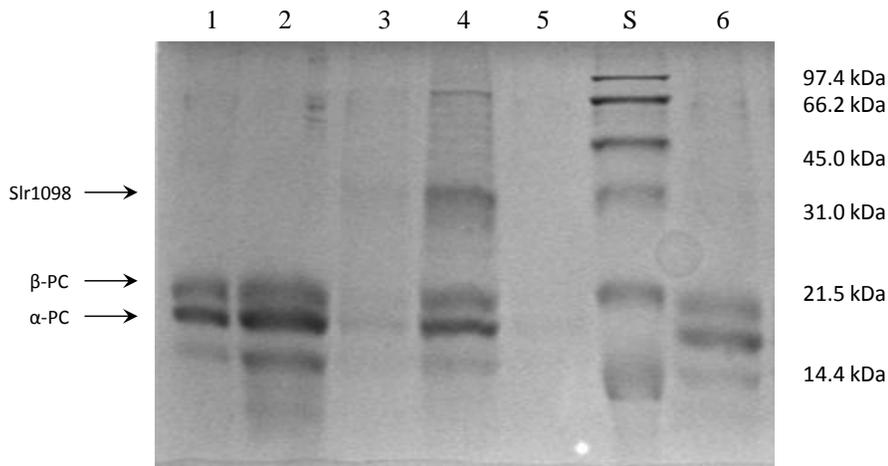


Figure 20: SDS-PAGE analysis of sucrose gradient complexes. Samples were removed and protein precipitated overnight. Lanes are loaded as follows: 1 and 2: 20 μ L and 60 μ L each of control 2 (C2) reaction (PC and sodium phosphate buffer), 3 and 4: 20 μ L and 60 μ L each of test (T) reaction (PC and Slr1098), 5 and 6: 20 μ L and 60 μ L each of control (C) reaction (PC and pAED4 control *E. coli* extract), S: standard, sizes of bands indicated to the right.

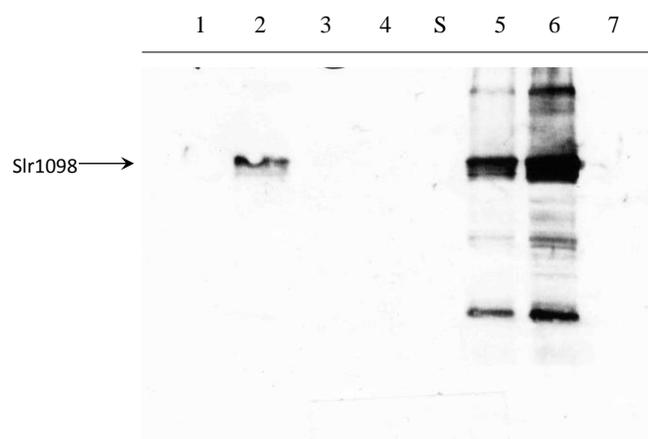


Figure 21: Western blot analysis of sucrose gradient samples. Anti-Slr1098 antibodies generated in rabbits were used to detect Slr1098 within the PC complexes. Lanes 1 and 2 contain 5 μ L and 15 μ L each of the test reaction (Slr1098 and PC) respectively. Lanes 3 and 4 contain 5 μ L and 15 μ L each of the control reaction (PC and pAED4). S contains the mass standards. Lanes 6 and 7 contain 2 μ L and 5 μ L each of purified Slr1098, and lane 7 contains 5 μ L of purified PC.

3.4.2 Pull-Down Experiment

Another experiment was devised to corroborate the previous conclusion that Slr1098 and holo-PC interact and form a complex. In this experiment, a hexa-histidine- tagged form of Slr1098 (HT-Slr1098) and Ni-NTA resin were used. Ni-NTA resin is an agarose resin that binds Nickel ions for high-affinity purification of His-tagged recombinant proteins. Mixtures of PC and HT-Slr1098 (test) or HT-Slr1098 and sodium phosphate buffer (control) were mixed, incubated and loaded onto the resin as previously described. Proteins were eluted by addition of imidazole followed by centrifugation for five minutes. Samples were analyzed by SDS-PAGE (Fig 22). Panel A shows purified PC, used as a comparison (lanes 1 and 2), the control reaction

(lane 3), the test reaction (lane 4), and purified recombinant HT-Slr1098 (lanes 5 and 6) for another comparison. Panel B is the first five lanes of the gel after zinc staining, showing bilin fluorescence. The test lane (4) indicates that the two proteins did interact, as a distinct band can be seen for each (indicated by arrows). Zinc staining verifies that the PC is in holo form, since only covalently bound bilins will fluoresce in the presence of zinc. Figure 23 shows a duplicate gel and subsequent protein transfer to a PVDF membrane, which was probed with antibodies against Slr1098. Figure 22 shows purified HT-Slr1098 (lanes 1 and 5), the test reaction (lane 7) and the control reaction (lane 8). All four lanes, which contain HT-Slr1098, react to the antibodies. This experiment shows that HT-Slr1098 and holo-PC interact, and verifies the result that Slr1098 can co-purify with HT-CpcBA (apo) (Schluchter *et. al.*, unpublished) and that Slr1098 (non-tagged) can form a complex with holo-PC that is larger than that of holo-PC alone (Fig 18).

Since PC and allophycocyanin are similar in primary, secondary and tertiary structure, we wondered whether Slr1098 might also interact with apo-allophycocyanin (HT-

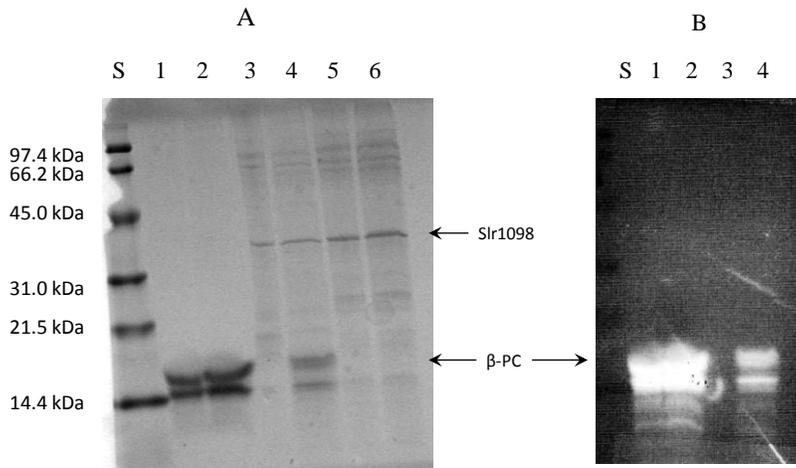


Figure 22: SDS-PAGE analysis of pull-down experiment with HT-Slr1098 and PC. A) S contains the mass standards; masses are indicated to the left, lanes 1 and 2 contain 5 μ g and 15 μ g each of purified PC, lane 3 contains the control reaction (50 μ g HT-Slr1098 and sodium phosphate buffer), lane 4 contains the test reaction (50 μ g HT-Slr1098 and 60 μ g PC), lanes 5 and 6 contain 5 μ g and 15 μ g each of purified recombinant HT-Slr1098. Proteins present are indicated by arrows. B) The same gel after zinc staining, covalently bound bilins are fluorescent.

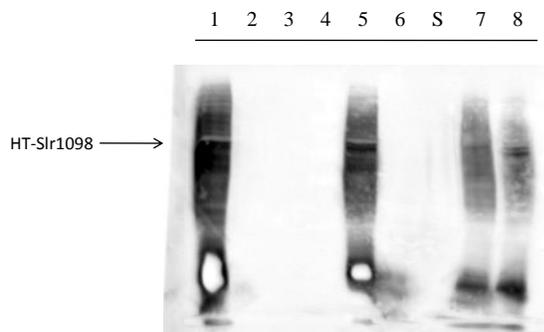


Figure 23: Western blot of pull down experiment samples. Antibodies against HT-Slr1098 were used to detect HT-Slr1098. Lane 1 contains purified HT-slr1098 (15 μ l), lane 2 contains purified PC (15 μ g), lanes 3 and 4 are empty, lane 5 contains purified HT-Slr1098 (5 μ l), lane 6 contains purified PC (5 μ g), S is the low molecular weight standard, lane 7 is the test reaction (HT-Slr1098 and PC), and lane 8 contains the control reaction (HT-Slr1098 and sodium phosphate buffer).

ApcA/ApcB). We attempted to see if Slr1098 would co-purify with HT-ApcA/ApcB. There was no copurification of Slr1098 with HT-ApcA/ApcB (data not shown), providing evidence that the interaction of Slr1098 is specific for PC.

3.4.3 Size Exclusion Chromatography of PC-Slr1098 complex

The next step is to determine the appropriate size of the complex. Size Exclusion Chromatography (SEC) using High Performance Liquid Chromatography (HPLC) was utilized to determine the size of the complex formed by PC and Slr1098 interaction. Two forms of PC were used in the test reactions; purified holo-PC or SU1-PC, which is chromophorylated at one cysteine residue (Cys-82) by the CpcSU lyase (Saunée et al., 2008). Either purified holo-PC (Fig 24) or SU1-PC (Fig 25) and Slr1098 (test reaction) or one form of PC and pAED4 *E. coli* extract (control reaction) were incubated together for one hour at room temperature, and injected onto a size exclusion column as described in Materials and Methods. Figure 24 shows the holo-PC test and control reactions at 280 nm (protein absorbance: panels A and C, respectively) and at 600 nm (bilin absorbance: panels B and D, respectively). Figure 25 shows the SU1-PC test and control reactions at 280 nm (panels A and B) and at 600 nm (panels C and D). Surprisingly, the test reactions for both experiments displayed a longer retention time, and therefore, smaller molecular mass than the controls. The holo-PC control (see Fig 24C) had a peak at 24.26 minutes that also showed absorbance at 600 nm. This peak's calculated molecular weight was 111.6 kDa, which is near to the expected molecular mass of holo-PC; 112.4 kDa. The test's major peak (Slr1098 + PC; see Fig 24A) occurred at 26.3 minutes, and has a calculated molecular weight of 60.3 kDa. This is much smaller than the expected molecular mass of trimeric PC. This suggests that under some conditions, Slr1098 may bind to and dissociate

trimers. Due to the degradation of some of the standards, the molecular weight calculations, unfortunately, are not as accurate as they should be.

The singly chromophorylated SU1-PC has an expected molecular weight of 36.5 kDa. However, the SU1 PC complex eluting at 29.5 minutes in the control run had a calculated molecular weight of 68 kDa (see Fig 25C). This form of PC is chromophorylated at the β -Cys82, position, and it is known that chromophorylation at this position is the most important in order to allow phycobiliprotein incorporation into the PBS (Toole et al., 1998). Although unexpected, it is possible that SU1PC is in the trimeric form already. The size of the complex formed when Slr1098 was added to SU1-PC is smaller than the control with an estimated mass of 20 kDa, consistent with the expected sizes for dissociated alpha and beta PC subunits (see Fig 25A). Our preliminary conclusion is that Slr1098 may be dissociating a SU1-PC complex presumably by binding to the regions of CpcB and CpcA that are important for monomer interface associations.

3.5 *In Vivo* Co-Expression of Slr1098 with HTCpcBA and CpcSU

To determine if Slr1098 would affect PCB chromophore addition by CpcSU to HTCpcB, *E. coli* cells were transformed with three plasmids (ho1/pcyA/pACYC Duet; cpcS/cpcU/ pCOLA Duet; and either cpcBA/slr1098/pCDF Duet or cpcBA/pCDF Duet; see Table 1). Both cultures were blue in color due to chromophorylation of CpcB by CpcSU, but the culture containing Slr1098 has less blue color (data not shown; Saunée *et al.*, 2008). Absorbance spectra of the HT-CpcBA purified from these two cultures are shown in Fig 26. Panel A shows the absorbance

spectrum of the HT-CpcBA synthesized in the absence of Slr1098, and it shows a sharp peak at 620 nm as expected. Panel B shows the absorbance spectrum of the HT-CpcBA purified from cells expressing Slr1098; the peak is broader and its maxima is at 616 nm. The extract from the HTCpcBA, from the Slr1098-expressing cells was separated by SDS-PAGE (see Fig 27, lane 1); after purification of HT-CpcBA, one can see that Slr1098 is also copurifying with HT-CpcB/CpcA (Fig 27, lane 2). This analysis suggests that Slr1098 is binding to HT-CpcB and therefore inhibiting its ability to be chromophorylated by CpcSU, but further experiments will be required to further examine this inhibition of chromophorylation by Slr1098.

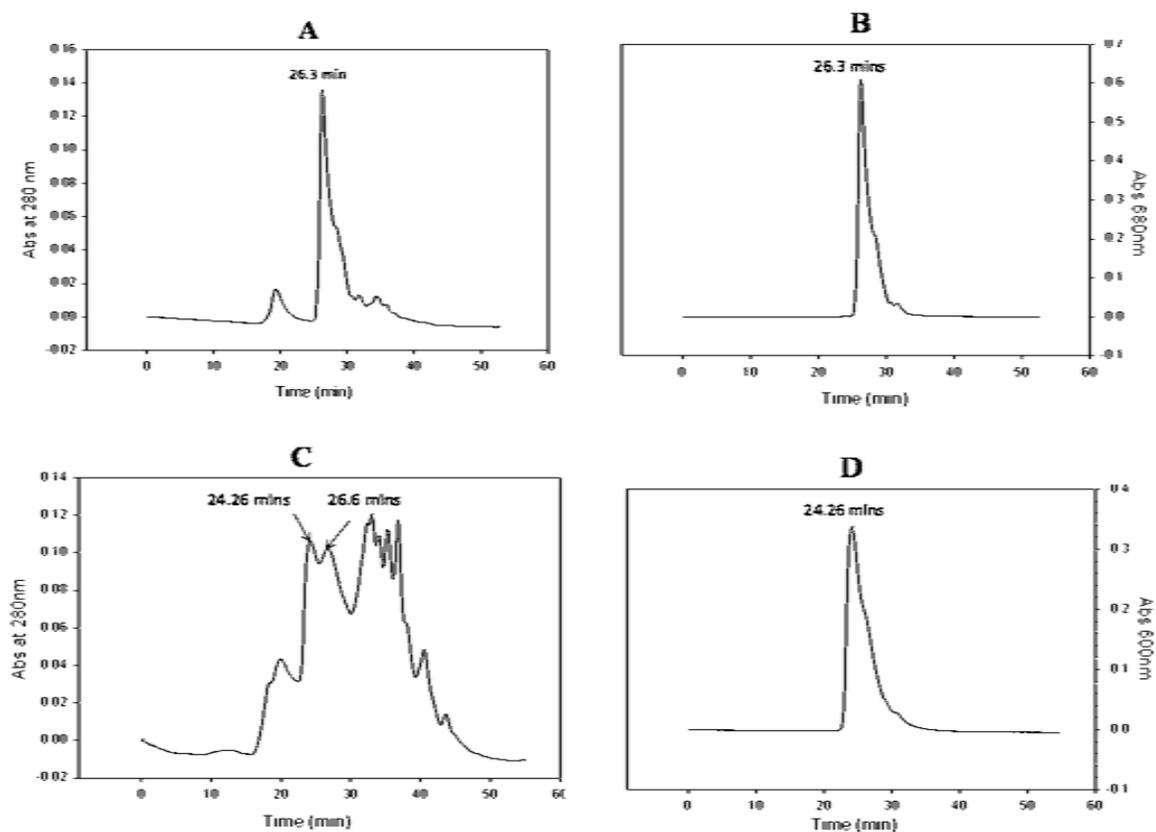


Figure 24: Size exclusion chromatograms of holo-PC and Slr1098. Panel A shows the test reaction (holo-PC and Slr1098) at 280 nm. Panel B shows the test reaction at 600 nm. The peak at 26.3 minutes has a calculated molecular weight of 60.3 kDa. Panel C shows the control reaction (holo-PC and pAED4 *E. coli* extract) at 280 nm. Panel D shows the control reaction at 600 nm. The peak at 24.26 minutes has a calculated molecular weight of 111.6 kDa.

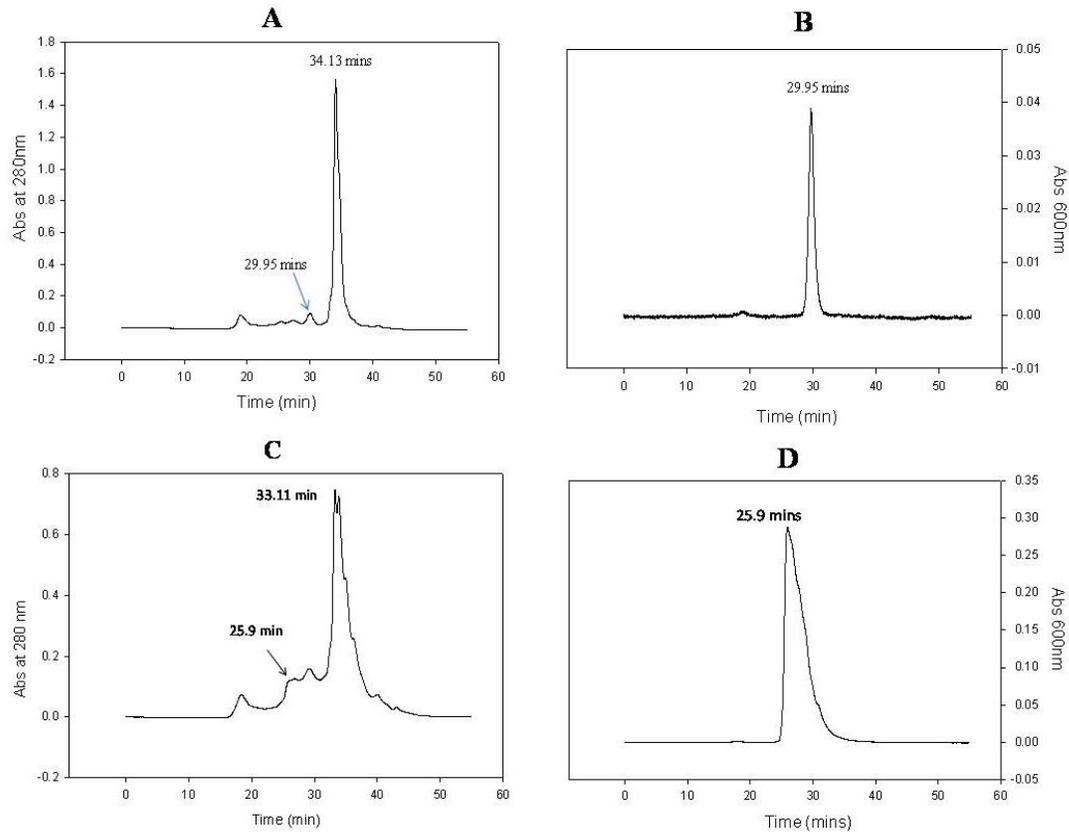


Figure 25: Size exclusion chromatograms of SU1-PC (PC chromophorylated at one cysteine residue) and Slr1098. Panel A shows the test reaction (SU1-PC and Slr1098) at 280 nm. The peak 29.95 minutes had a calculated molecular weight of 20 kDa. Panel B shows the test reaction at 600 nm. Panel C shows the control reaction (SU1-PC and pAED4 *E. coli* extract) at 280 nm. Panel D shows the control reaction at 600 nm. The peak at 25.9 minutes had a calculated molecular weight of 68 kDa.

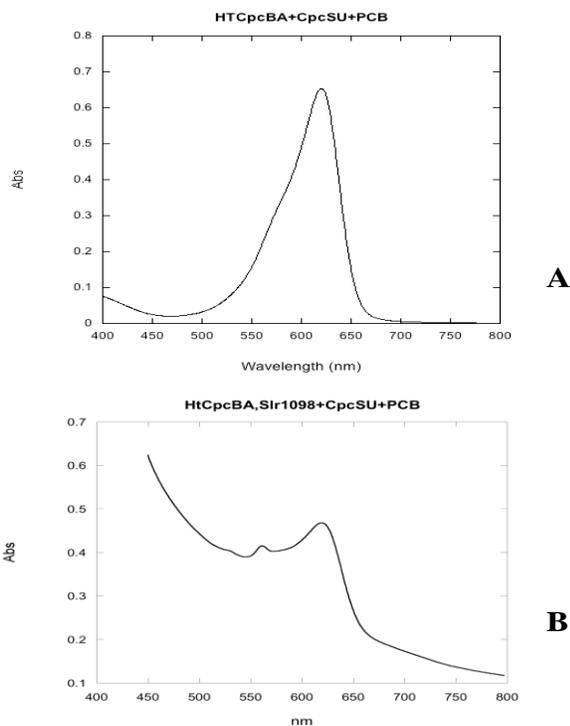


Figure 26: Absorbance spectra of HT-CpcBA synthesized in the presence and absence of Slr1098. Panel A shows the spectra of HT-CpcBA in the absence of Slr1098, with the expected peak at 620 nm. Panel B shows the spectra in the presence of Slr1098, the peak at 620 is diminished and is much broader in the presence of Slr1098.

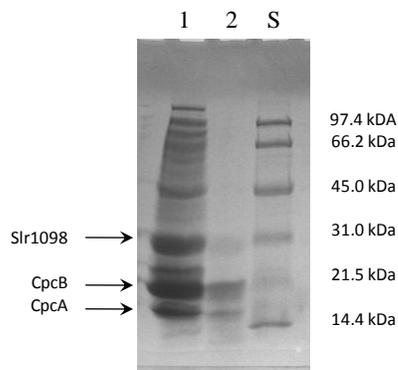


Figure 27: SDS-PAGE analysis of HT-CpcBA synthesized in the presence and absence of Slr1098. Lanes 1 contains an *E. coli* extract after expressing Slr1098, cpcBA, ho1, pcyA, and cpcSU. Lane 2 shows the eluate after purification on a Ni-NTA column. Molecular mass standards are loaded in the lane designated “S”, and their masses are indicated to the right.

DISCUSSION

Phycobiliprotein Practical Uses

Phycobiliproteins have a major biotechnological application as naturally occurring fluorescent tags. They are used in histochemistry, flow cytometry, immunoassay and fluorescence activated cell sorting (Glazer 1994). The advantages of PBP's over other organic dyes include; low occurrence of non-specific binding to intact cells, the ability to store them for long periods of time in aqueous solution, and the numerous functional groups present on the PBP surface that can be easily coupled to a variety of small molecules, such as monoclonal antibodies. The study of phycobiliproteins and the mode of bilin attachment to these proteins, have importance in the use of diagnostic assays and research applications.

Importance of Linker Polypeptides

Linker polypeptides are responsible for the assembly of the PBS by mediating the exact location of the phycobiliproteins within the PBS. They are also responsible for optimizing each phycobiliprotein's spectroscopic properties (Glazer, 1989; Piven *et al.*, 2005). The rod-core junction of phycobilisomes, where AP of the core interacts with PC of the rods, is an important location in the structure and energy transfer pathway of the PBS (Glauser *et al.*, 1993).

Researchers isolated and characterized three rod-core linker complexes from the PBS of *Mastigocladus laminosus*: $(\alpha\beta)_3^{\text{PC}} \cdot \text{L}_{\text{RC}}^{31}$ (IV), $(\alpha\beta)_3^{\text{PC}} \cdot \text{L}_{\text{RC}}^{29.5}$ (V), and $(\alpha\beta)_6^{\text{PC}} \cdot \text{L}_{\text{RC}}^{29.5} \cdot (\alpha\beta)_3^{\text{AP}} \cdot \text{L}_{\text{C}}^{8.9}$ (VI) (Glauser *et al.*, 1993). Complex VI is composed of a hexamer of PC from the rods, associated with a rod-core linker, as well as a trimer of AP from the core associated with the small core linker. This complex was able to be reconstituted from its sub-complexes- $(\alpha\beta)_3^{\text{PC}} \cdot \text{L}_{\text{RC}}^{29.5}$ and $(\alpha\beta)_3^{\text{AP}} \cdot \text{L}_{\text{C}}^{8.9}$. The native and reconstituted complexes showed nearly

identical spectral properties. The PC-AP interaction in Complex VI is mediated by the $L_{RC}^{29.5}$ linker, and this mediation is specific, since the necessary sub-complex cannot be substituted by similar sub-complexes. The N-terminus of the rod-core linker is responsible for its association to PC, while the C-terminal contains the AP binding domains (Glauser *et al.*, 1993).

Another group of researchers were able to isolate the core complex $(\alpha\beta)_3^{AP} \cdot L_C^{8.9}$ from the same organism, and overproduce the linker polypeptide in *E. coli* cells, from its *apcC* gene (Betz *et al.*, 1993). Using the isolated AP complex, the original core complex could be reconstituted with either the isolated or recombinant linker. Both reconstituted complexes and the native complex had the same spectral properties. The isolation of linker polypeptides is difficult due to the linkers' high susceptibility to proteolytic degradation. This degradation is common even in the presence of protease inhibitors. This causes the yield of pure linker polypeptides to be very low, about 15% of the total isolated protein (Betz *et al.*, 1993). The results of this study offer researchers an alternative method to studying the mechanisms of linker-mediated trimetric and hexametric complex assembly, and the role they play in fine tuning the spectral properties of those complexes.

Piven and associates demonstrated with immunological techniques that some linker proteins within the assembled PBS of *Synechocystis* sp. PCC 6803 are permanently phosphorylated, in particular, L_{CM} , L_R^{33} , L_R^{35} , and L_{RC} (Piven *et al.*, 2005). The researchers employed the same immunological assay using two mutants with changes in their PBS to verify that the phosphorylation signal was coming from the PBS linkers as opposed to co-migrating contaminant polypeptides. One mutant (CK) lacked rods and the rod linkers, resulting in a truncated PBS. The other mutant (PAL) fully lacked the PBS due to the deletion of the core-membrane linker and AP genes. They also observed that dephosphorylation of linker proteins

only occurred in partially disassembled phycobilisomes, and the linkers in intact PBSs are not accessible. In addition, they noted that this dephosphorylation promoted the further disassembly of the PBS subunits. These results suggest that this dephosphorylation of linkers has a role in the mechanism that directs the changes in the PBS under conditions of nutrient limitation and high intensity light (Piven *et al.*, 2005).

As mentioned before, a few of these changes in the PBS are proteolysis and subsequent PBS breakdown, changes in gene expression, and PBS remodeling. Once the PBS complex starts to breakdown, linker dephosphorylation seems to mark the biliproteins degradation. This is supported by the observation that changes in protein phosphorylation modify protein folding, as seen in higher plant chloroplasts. The authors observed that under stress conditions, phosphorylation of the linkers decreases. Perhaps once a level of dephosphorylation is reached, the protein refolds, making it more accessible to proteolytic enzymes and further breakdown. This study gives further insight into what is required in the PBS degradation mechanism.

Bilin Lyases

Bilin lyases are responsible for the correct attachment of bilin chromophores in many species of cyanobacteria. There are three main classes of lyases, E/F, S/U and T- types. This area of study has seen many advances in recent years. CpcEF was the first lyase to be identified and characterized. Inactivated mutants were yellow- green in color, similar to Slr1098 mutants. *CpcS-I* and *cpcU* mutants demonstrated a chlorotic yellow green color indicating a reduced PBP content compared to wild type cells. Additionally, they exhibited a slower growth rate and higher sensitivity to high light intensity. The single and double mutant strains displayed dramatic PBS assembly impairment, and triple mutants- *cpcS-I/U/T*- were

unable to produce intact PBS at all. The *cpcS* and *cpcU* mutant PBS contained a β -PC* that was smaller than the wild type β -PC. The β -PC* polypeptide had an absorption maxima of PC that was blue-shifted compared to the wild type, at 605 nm instead of the expected 625 nm. This is indicative of a missing PCB chromophore at position Cys-82 of the β -subunit. The mutants also demonstrated a greatly diminished ability to harvest light and inefficiency in excitation energy transfer with the PBS. When either bilin lyase subunit (CpcS or CpcU) was missing, PCB would associate with the Cys-82 site non-covalently, allowing the slow autocatalytic attachment to occur in a small amount of PC (Shen *et al.*, 2008).

In vitro bilin addition assays verified the CpcS/CpcU bilin lyase's function. Either CpcS, HT-CpcU, or CpcSU, were added to recombinant CpcBA with PcyA-generated PCB. Only the reaction containing the CpcSU heterodimer exhibited the absorption maximum similar to the expected maximum for PCB bound at Cys-82 on β -PC (Saunée *et al.*, 2008). The investigators were able to reproduce the results when PcyA-generated PCB was replaced with purified PCB. A similar assay was done to test the bilin addition to apo-AP. Although all incubations (CpcS-I, CpcU, apo HT-AP as control) produced a similar absorbance maximum consistent with that expected for holo-monomeric AP, only the heterodimer-dependent reaction product was fluorescent and exhibited the expected emission maximum.

Synechococcus sp. PCC 7002 has three chromophore binding sites; Cys-84 located on α -PC, and Cys-153 and Cys-82 on β -PC. The Cysteine-82 binding site is common to all phycobiliproteins, and PCB chromophore attachment to this site encourages proper subunit folding and stability to the $\alpha\beta$ monomer (Toole *et al.*, 1998). In addition, this site is the terminal energy accepting or fluorescing bilin within PC trimers, hexamers, and rods. PCB attachment to this site is therefore the most important step in PBP biogenesis. Based upon the lyase needed to

catalyze PCB attachment to Cys-82 on the β subunit of PC, cyanobacteria can all be divided into two groups. One group requires a heterodimeric lyase for proper attachment, while the other group only needs a single subunit-type lyase. It is interesting that although *cpeS* and *cpcS-I* are homologous, the first is able to function alone, while the latter requires a partner to be an active lyase. The genes of the two species are in the same class but different subgroups. The identification of all three obligatory bilin lyases for PC biogenesis in *Synechococcus* sp. PCC 7002 was finalized with the characterization of the CpcS-I/U lyase, in addition to CpcT (see below).

In *Anabaena (Nostoc)* sp. PCC7120, open reading frame alr0617, denoted by the authors as CpeS1, is a bilin lyase with nearly universal ability (Zhou *et al.*, 2007a). CpcS-III is a more accurate nomenclature than CpeS1 because this species does not naturally produce biliproteins with PEB chromophores (Shen *et al.*, 2008). In all phycobiliproteins, the cysteine-84 binding site is conserved. This lyase is able to correctly attach PCB to Cys-84 of all allophycocyanin subunits, and to the β subunits of PC and PEC, *in vivo* and *in vitro* (Zhao *et al.*, 2007a). In addition, it binds PEB to both the α - and β -subunits of phycoerythrin (PE) at Cys-82. CpeS1 is able to catalyze bilin addition to the apoprotein for the three groups of phycobiliproteins in cyanobacteria, AP, PC, and PE. In all cases, it demonstrated high binding site specificity by only binding to Cys-84. This bilin lyase is able to bind the chromophore quickly and reversibly. The reaction to transfer it to the apoprotein is irreversible and a good deal slower (Scheer and Zhao, 2008). The singly chromophorylated β -subunits of PC and PEC, at Cys-82, were able to be synthesized using *E. coli* with CpeS1 (Zhao *et al.*, 2006). Unlike the E/F lyases, however, CpeS1 is able to catalyze binding alone, while CpcEF and PecEF must form a heterodimer in order to be active. Another difference is that the E/F lyases are encoded by genes located on the

same operon as the apoproteins, but *cpeSI* is not located near the genes encoding PC or PEC.

Similar to the CpcS-I/CpcU study, CpcT mutants produce a PC*. This PC has a red-shifted absorption maximum indicating a missing PCB from Cys-153. Using recombinant CpcB/CpcA, and CpcT, the investigators performed bilin addition assays. When PCB was added to reactions with CpcT present, the mixture turned from blue to purple within minutes. The control reaction had no color change after two hours. The product containing CpcT was highly fluorescent, while the control was nearly non-fluorescent by comparison. This lack of fluorescence, along with the absorbance spectra and emission maximum of the control reaction, are properties consistent with the behavior of MBV adducts.

This study was the first to characterize a bilin lyase in this class (Shen *et al.*, 2006) as CpcT does not show sequence similarity to the E/F type or the S/U type bilin lyase subunits. Later, it was found that this recombinant protein lyase in *Nostoc (Anabaena)* PCC 7120 was able to catalyze the attachment of β -PC (CpcB) and β -phycoerythrocyanin (PecB) to PCB at Cys-155 of both apobiliproteins (Zhao *et al.*, 2007b). Although this lyase operates in a regiospecific manner, it does not differentiate between the two phycobiliproteins. When paired with CpcS from the same organism, these lyases catalyze the attachment of PCB to both CPC and PEC at β Cys-84 for CpcS and β Cys-155 for CpcT. This binding activity is also order specific; CpcT must be first since PCB binding at β Cys-84 impeded the attachment at β Cys-155. When bound to β Cys-84, PCB is in the *R*-configuration, but when it is bound to β Cys-155, it is found as the *S*-isomer (Shen *et al.*, 2008). Interestingly enough, these lyases catalyze binding equally well with CpcB and PecB from two species, *Nostoc* and *M. laminosus*. The T-type lyases exhibit high site specificity and some specificity for the substrate phycobiliprotein. This was the first report of an

order-specific requirement for the correct attachment of biliprotein subunits having multiple chromophores (Zhao *et al.*, 2007b).

Research Conclusions

All CpcE proteins contain a HEAT repeat motif –a short sequence containing two alpha helices, generally involved in protein-protein interactions (Scheer and Zhao 2008). Slr1098 has several HEAT repeat motifs in its sequence, and was found to form a complex with the IscA homolog, Slr1565, also found in *Synechocystis* sp. PCC 6803 (Morimoto *et al.*, 2002). IscA homologs are involved in iron-sulfur cluster formation, and the binding of Slr1098 stabilized the iron-sulfur cluster in Slr1565. These results suggest another function of Slr1098, a function related to protein stabilization and scaffolding as we hypothesized.

Although Slr1098 shares amino acid similarity to characterized bilin lyases such as CpcE, it exhibits no chromophore addition activity on CpcB or CpcA (Schluchter *et al.*, unpublished data). *Slr1098* null mutants demonstrated a yellow-green color instead of the blue-green color typical of wild type cells; this color difference was attributed to a reduction in the amount of PC, rather than an impairment in bilin content, because the PBS contained smaller rods as judged by sucrose density gradient ultracentrifugation (Schluchter and Glazer, 1999). It is also not a candidate for involvement in PBS degradation like the role suggested for NblB, another ORF similar to both CpcE and Slr1098, since the *slr1098* mutants were capable of breaking down their PBS during nitrogen starvation.

Even though Slr1098 exhibited no bilin addition activity with apo-HTCpcBA, it did co-purify with this apo-PC and formed larger complexes as judged by SEC-HPLC (Schluchter *et al.*, unpublished data). When partially purified recombinant Slr1098 was added to holo-PC (see Fig

19) and analyzed by sucrose gradient centrifugation, the PC and Slr1098 mixture migrated further through the sucrose than PC alone, suggesting that Slr1098 interacted with holo- PC and formed a larger complex. Holo-PC is isolated as a trimer ($\alpha\beta$)₃ so we hypothesized that this larger complex may be a hexamer.

A subsequent pull-down experiment with HT-Slr1098 and PC also suggested that Slr1098 can interact and copurify with holo-PC. This interaction was shown to be PC-specific, as there was no co-purification of non-tagged recombinant Slr1098 with HT-apo-allophycocyanin (HT-ApcA/ApcB, data not shown).

The sucrose density gradient results indicated that Slr1098 and holo-PC interact and copurify together, and the band migration suggested that the size of the complex was slightly larger than the control complex for trimeric holo-PC. However, the conclusions for the size-exclusion chromatography were different. When Slr1098 was allowed to interact with holo-PC before injection on SEC-HPLC, the PC complex eluted before the control PC complex (without Slr1098) suggesting that Slr1098 under some conditions may disrupt trimers into monomers of holo-PC. The SU1-PC has a chromophore attached to Cys-82 of the β -subunit, the most important site to be chromophorylated within a trimer of PC (Toole et al, 1998; Anderson and Toole, 1998). Although apo-PC will only form monomers, no one had ever looked at whether the presence of the chromophore at Cys-82 allowed for trimerization of this SU1-PC. Therefore, we were surprised to see that this PC eluted with the mass more appropriate for a trimer than a monomer. PC trimers are known to be more compact and migrate on SEC-HPLC at a smaller size than expected (Cai *et al.*, 2001). It appears that Slr1098 can interact with this partially chromophorylated PC and that it dissociates the trimer into a smaller complex, presumably back to a monomer or even individual subunits. One possible role for Slr1098 may be in preventing

association of trimers before complete chromophorylation. Slr1098 may bind to the regions of CpcB and/or CpcA important for oligomerization, and can disrupt or aid this oligomerization process, depending upon the conditions, and potentially, the proteins present.

In vivo co-expression of Slr1098 with CpcBA and CpcSU yielded cultures with less chromophorylation than cells expressed in the absence of Slr1098. Slr1098 interacted with CpcBA as seen in previous experiments, and this binding appeared to inhibit complete chromophorylation by CpcSU. This analysis again indicates that Slr1098 is able to interact with either apo or holo-PC, presumably near the Cys-82 binding site.

Together with the above mentioned mutant analysis, the results presented in this thesis suggest that Slr1098 does bind and interact with both apo-PC and holo-PC, and in addition, partially chromophorylated PC. Its HEAT repeats may be important in aiding protein-protein interactions required for these interactions. It appears to be specific for PC as apo-AP did not co-purify with Slr1098, and the *slr1098* mutants did not make less AP trimers; only PC rod length was affected (Schluchter et al., unpublished).

Future experiments to investigate the interaction of Slr1098 and PC could include incubating Slr1098 with PC containing one or more binding site deletions. These results could shed light on the interaction location of the proteins. Another experiment could reconstruct the *in vivo* synthesis of holo-PC in the presence and absence of Slr1098 in *E. coli*. The resulting cultures could be analyzed through various means to determine any interaction and possible oligomerization.

REFERENCES

- Anderson, L.K., and Toole, C.M., (1998) A model for early events in the assembly pathway of cyanobacterial phycobilisomes. *Molec Micro* **30**: 467-474.
- Arciero, D.M., Bryant, D.A., Glazer, A.N., (1988) *In vitro* attachment of bilins to apophycocyanin. *J Biol Chem* **263**: 18343-18349.
- Beale, S. I., Cornejo, J., (1983) Biosynthesis of phycocyanobilin from exogenous labeled biliverdin in *Cyanidium caldarium*. *Arch Biochem Biophys* **227**: 279-286.
- Betz, M., Rügsegger, U., Esteban, A.M., Sidler, W.A., Zuber, H., (1993) Reconstitution of the core complex $(\alpha\beta)_3^{APC} \cdot L_C^{8,9}$ of the phycobilisome from *Mastigocladus laminosus* using the $L_C^{8,9}$ linker polypeptide overexpressed in *Escherichia coli*. *Biol Chem Hoppe-Seyler* **374**: 435-443.
- Bienert, R, Baier, K., Volkmer, R., Lockau, W., and Heinemann, U., (2006) Crystal structure of NblA from *Anabaena* sp. PCC 7120, a small protein playing a key role in phycobilisome degradation. *J Biol Chem* **281**: 5216-5223.
- Böhm, S., Endres, S., Scheer, H., Zhao, K.H., (2007) Biliprotein chromophore attachment: chaperone-like function of the PecE subunit of α -Phycoerythrocyanin lyase. *J Biol Chem* **282**: 25357-25366
- Cai, Y.A., Murphy, J.T., Wedemayer, G.J., Glazer, A.N., (2001) Recombinant Phycobiliproteins: Recombinant C Phycocyanins equipped with affinity tags, oligomerization and biospecific recognition domains. *Anal. Biochem.* **290**; 186-204.
- Collier, J.L., Grossman, A.R., (1994) A small polypeptide triggers complete degradation of light-harvesting phycobiliproteins in nutrient deprived cyanobacteria. *EMBO* **13**: 1039-1047

- Cobley, J., Clark, A., Weersurya, S., Queseda, F., Xiao, J., Bandrapali, N., D'Silva, I., Thounaojam, M., Oda, J., Sumiyoshi, T., Chu, M., (2002) CpeR is an activator required for expression of the phycoerythrin operon (*cpeBA*) in the cyanobacterium *Fremyella diplosiphon* and is encoded in the phycoerythrin linker-polypeptide operon (*cpeCDEST*). *Molec Micro* **44**:1517-1531.
- Dines, M., Sendersky, E., David, L., Schwarz, R., Adir, N., (2008) Structural, functional and mutational analysis of the NblA protein provides insight into possible modes of interaction with the phycobilisome. *J Biol Chem* **283**:30330-30340.
- Dolganov, N., and Grossman, A., (1999) A polypeptide with similarity to phycoerythrin α -subunit phycoerythrin lyase involved in degradation of phycobilisomes. *J Bacteriol* **181**: 610-617.
- Fairchild, C.D., Zhao, J., Zhou, J., Colson, S., E., Bryant, D., A., and Glazer, A.N., (1992) Phycoerythrin α -subunit phycoerythrin lyase. *Proc Natl Acad Sci USA* **89**: 7017- 7021.
- Fairchild, C.D., and Glazer, A.N., (1994) Oligomeric structure, enzyme kinetics and substrate specificity of the phycoerythrin α -subunit phycoerythrin lyase. *J Biol Chem* **269**: 8686-8694.
- Frankenburg, N., Mukougawa, K., Kohchi, T., Lagarias, J.C., (2001) Functional genomic analysis of the HY2 family of ferredoxin-dependent bilin reductases from oxygenic photosynthetic organisms. *Plant Cell* **13**: 965-978.
- Glauer, M., Sidler, W., Zuber, H., (1993) Isolation, characterization, and reconstitution of phycobiliprotein rod-core linker polypeptide complexes from the phycobilisome of *Mastigocladus laminosus*. *Photochem Photobiol* **57**: 344-351.
- Glazer, A.N., (1988) Phycobilisomes. In Methods in Enzymology. Packer, L., Glazer, A.N.,

- (eds.). San Diego: Academic Press, vol. 167, pp. 304-312.
- Glazer, A.N., (1989) Directional energy transfer in a photosynthetic antenna. *J Biol Chem* **264**: 1-4.
- Glazer, A.N., (1994) Phycobiliproteins- a family of valuable, widely used fluorophores. *J Appl Phycol* **6**:105-112.
- Glazer, A.N., Fairchild, L.J., Chan, C.F., (1995) Phycobiliproteins: studies of bilin attachment. In Photosynthesis: from Light to Biosphere. Mathis, P, (ed.). Netherlands: Kluwer Academic Publishers, pp. 3-9.
- Glazer, A.N., Fang, S., (1973) Chromophore content of blue-green algal phycobiliproteins. *J Biol Chem* **248**: 659-662.
- Guan X., Qin, S., Zhao, F., Zhang, X., Tang, X., (2007) Phycobilisomes linker family in cyanobacterial genomes: divergence and evolution. *Int J Biol Sci* **3**: 434-445.
- Jung, J.J., Chan, C.F., Glazer, A.N., (1995) Candidate genes for the phycoerythrocyanin α subunit lyase. *J Biol Chem* **270**: 12877-12884.
- Karradt, A., Sobanski, J., Mattow, J., Lockau, W., Baier, K., (2008) NblA, a key protein of phycobilisome degradation, interacts with CplC, a HSP100 chaperone partner of a cyanobacterial Clp protease. *J Biol Chem* **283**: 32394-32403.
- Moritmoto, K., Nishio, K., Nakai, M., (2002) Identification of a novel prokaryotic HEAT-repeats-containing protein which interacts with a cyanobacterial IscA homolog. *FEBS let* **519**: 123-127.
- Piven, I., Ajlani, G., and Sokolenko, A., (2005) Phycobilisome linker proteins are phosphorylated in *Synechocystis* sp. PCC 6803. *J Biol Chem* **280**: 21667-21672.
- Plank, T., and Anderson, L.K., (1995) Heterologous assembly and rescue of stranded

- phycocyanin subunits by expression of a foreign *cpcBA* operon in *Synechocystis* sp. strain 6803. *J Bacteriol* **177**: 6804-6809.
- Plank, T., Toole, C., and Anderson, L.K., (1995) Subunit interactions and protein stability in the cyanobacterial light-harvesting proteins. *J Bacteriol* **177**: 6798-6803.
- Reuter, W., Wiegand, G., Huber, R., Than, M., (1999) Structural analysis at 2.2 Å of orthorhombic crystals presents the asymmetry of the allophycocyanin-linker AP·L_C^{7,8} from phycobilisomes of *Mastigocladus laminosus*. *Proc Natl Acad Sci USA* **96**: 1363-1368.
- Saunée, N.A., Williams, S.R., Bryant, D.A., and Schluchter, W.M., (2008) Biogenesis of phycobiliproteins: II. CpcS-I and CpcU comprise the heterodimeric bilin lyase that attaches phycocyanobilin to Cys-82 of β-phycocyanin and Cys-81 of allophycocyanin subunits in *Synechococcus* sp. PCC 7002. *J Biol Chem* **283**: 7513-7522.
- Scheer, H., and Zhao, K.H., (2008) Biliprotein maturation: the chromophore attachment. *Molec Microbiol* **68**: 263-276.
- Schluchter, W.M., and Glazer, A.N., (1999) Biosynthesis of phycobiliproteins in cyanobacteria. In *The Phototrophic Prokaryotes*. Peschek, G.A., Löffelhardt, W., and Schmetterer, G. (eds). New York: Kluwer/Plenum Press, pp. 83-95.
- Shen, G., Saunée, N.A., Gallo, E., Begovic, Z., Schluchter, W.M., and Bryant, D.A., (2004) in *PS 2004 Light Harvesting Systems Workshop*. Niederman, R.A., Blankenship, R.E., Frank, H., Robert, B., van Grondelle, R. (eds). Saint Adele, Quebec, pp. 14-15.
- Shen, G., Saunée, N.A., Williams, S.R., Gallo, E.F., Schluchter, W.M., and Bryant, D.A., (2006) Identification and characterization of a new class of bilin lyase: the *cpcT* gene encodes a bilin lyase responsible for attachment of phycocyanobilin to Cys-153 on the β-subunit of phycocyanin in *Synechococcus* sp. PCC 7002. *J Biol Chem* **281**: 17768-17778.

- Shen, G., Schluchter, W.M., and Bryant, D.A., (2008) Biogenesis of phycobiliproteins: I. *cpcs-I* and *cpcU* mutants of the cyanobacterium *Synechococcus* sp. PCC 7002 define a heterodimeric phycocyanobilin lyase specific for β -phycocyanin and allophycocyanin subunits. *J Biol Chem* **283**: 7503-7512.
- Sidler, W.A., (1994) Phycobilisome and phycobiliprotein structures. In *The Molecular Biology of Cyanobacteria*. Bryant, D.A. (ed). Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 139-216.
- Toole, C.M., Plank, T. L., Grossman, A.R., Anderson, L.K., (1998) Bilin Deletions and subunit stability in cyanobacterial light-harvesting proteins. *Molec Micro* **30**: 475-486.
- Tooley, A.J., Cai, Y.A., Glazer, A.N., (2001) Biosynthesis of a fluorescent cyanobacterial C-phycocyanin holo- α subunit in a heterologous host. *Proc Natl Acad Sci USA* **98**: 10560-10565.
- Tooley, A.J., Glazer, A.N., (2002) Biosynthesis of the cyanobacterial light-harvesting polypeptide phycoerythrocyanin holo- α subunit in a heterologous host. *J Bacteriol* **184**: 4666-4671.
- Yamanaka, G., Lundell, D.J., Glazer, A.N., (1982) Molecular Architecture of a light- harvesting antenna. Isolation and characterization of phycobilisome subassembly particles. *J Biol Chem* **25**: 4077-4086.
- Zhao, K.H, Deng, M., Zheng, M., Zhou, M., Parbel, A., Storf, M., Meyer, M., Strohamn, B., Scheer, H., (2000) Novel activity of a phycobiliprotein lyase: both the attachment of phycocyanobilin and the isomerization to phycoviolobilin are catalyzed by the proteins PecE and PecF encoded by the phycoerythrocyanin operon. *FEBS Lett* **469**: 9-13.
- Zhao, K.H., Su, P., Böhm, S., Song, B., Zhou, M., Bubenzer, C., Scheer, H., (2005)

- Reconstitution of phycobilisome core-membrane linker L_{CM}, by autocatalytic chromophore binding to ApcE. *Biochim Biophys Acta* **1706**: 81-87.
- Zhao, K.H., Su, P., Li, J., Tu, J., Zhou, M., Bubenzer, C., and Scheer, H., (2006) Chromophore attachment to phycobiliproteins β -subunits: Phycocyanobilin: cysteine- β phycobiliproteins lyase activity of CpeS- like protein from *Anabaena* sp. PCC7120. *J Biol Chem* **281**: 8573-8581.
- Zhao, K., Su, P., Tu, J., Wang, X., Liu, H., Plöscher, M., Eichacker, L., Yang, B., Zhou, M., and Scheer, H., (2007a) Phycobilin: cysteine-84 biliprotein lyase, a near universal lyase for cysteine-84-binding sites in cyanobacterial phycobiliproteins. *Proc Natl Acad Sci USA* **104**: 14300-14305.
- Zhao, K.H., Zhang, J., Tu, J.M., Böhm, S., Plöscher, M., Eichacker, L., Bubenzer, C., Scheer, H., Wang, X., and Zhou, M., (2007b) Lyase activities of CpcS- and CpcT- like proteins from *Nostoc* PCC7120 and sequential reconstitution of binding sites of phycoerythrocyanin and phycocyanin β - subunits. *J Biol Chem* **282**: 34093-34103.
- Zhou, J., Gasparich, G.E., Stirewalt, V.L., de Lorimier, R., Bryant, D.A., (1992) The cpcE and cpcF genes of *Synechococcus* sp. PCC 7002. *J Biol Chem* **267**: 16138-16145.

VITA

Kali Hicks was born in East Orange, New Jersey. She received her B.S. in Biology from Morgan State University in Baltimore, Maryland.