12-15-2006

Identification and Characterization of a New Class of Bilin Lyases in Synechococcus sp. PCC 7002

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Identification and Characterization of a New Class of Bilin Lyases in *Synechococcus* sp. PCC 7002

A Thesis

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

Master of Science
in
Biological Sciences

by

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B.S. Texas Christian University, 2002

December, 2006

Table of Contents

ACKNOWLEDGEMENTS

I would like to thank my mother, Peggy Alexander, for all of her love and support. I never would have been able to complete this chapter of my life without her. She has always been my support system and the person that I look up to.

To Dr. Wendy Schluchter, I would like to say thank you for all that you have done for me. Dr. Schluchter, you are a great mentor and friend. Thank you for listening to me complain about anything and everything. Also thank you for the opportunity to go to Penn State. I may have complained about the cold, but it was a great learning experience for me. You are a great boss and I will miss you.

Thank you to the member's of my committee, Dr. Mary Clancy and Dr. Candace Timpte, for all of the help and support throughout this whole process.

I would also like to thank all of the people in the lab, both past and present. I especially want to thank Shervonda Williams and Crystal Miller. You both have helped me tremendously through this entire process. You both listened and let me bounce ideas off of you, and for this I will always be grateful. Thank you for listening to my presentation and reading my thesis, and just for being my friends. I will miss you both very much.

Lastly, I would like to thank Dr. Don Bryant, G. Shen, and all the members of Dr. Bryant's lab at Penn State. You all made a place for me when I was displaced after the hurricane, and made me feel like a part of your lab. Thank you for making me feel welcome and at home, even if it was for only three months.

ABSTRACT

The goal of this research is to identify and characterize the enzymes (lyases) responsible for chromophore (bilin) attachment to phycobiliproteins (light harvesting) in cyanobacteria. Candidates for these lyases were first identified in *Fremyella diplosiphon* as *cpeS* and *cpeT*. In *Synechococcus* sp. PCC 7002, there are three *cpeS*-like genes (named *cpcS, cpcU,* and *cpcV*) and one *cpeT*-like gene (named *cpcT*). These genes were cloned, overexpressed, and purified from *E. coli.* The CpcS and CpcU proteins form a 1:1 complex and catalyze the addition of phycocyanobilin (PCB) to β-82 cysteinyl residue on phycocyanin (PC) *in vitro*. Tryptic digestion and C_{18} RP-HPLC confirmed that CpcSU attached bilin at β-82 cysteine. CpcT was also shown to be a lyase specific for β-153 cysteine as confirmed by tryptic digestion and C_{18} RP-HPLC. CpcSU together and CpcT alone act as phycobiliprotein lyases that added PCB to β-82 and β-153 cysteinyl residues of phycocyanin respectively, making these proteins a new family of phycobiliprotein lyases.

List of Figures and Tables

INTRODUCTION

1.1 Phycobilisome: Structure and Function

Cyanobacteria are gram-negative bacteria that are capable of photosynthesis. Fossil records of cyanobacteria go back over three billion years, which makes cyanobacteria the oldest oxygen-evolving organisms on the planet. Through oxygenic photosynthesis, cyanobacteria played an important role in the development of our oxygen-enriched atmosphere. Through endosymbiosis, ancestors of cyanobacteria are regarded as the precursor of the chloroplasts in oxygen-evolving organisms (including plants and red green and cryptophyan algae) (Bengston, 1994: Sidler, 1994). Plant plastids are believed to have evolved from cyanobacterial genes that migrated to the host nucleus. Thus it is expected that there is an evolutionary relationship between plants and cyanobacteria (Hughes and Lamparter, 1999).

Light harvesting complexes assist in photosynthesis, transferring an increased amount of light energy to the photosynthetic reaction center. Cyanobacterial light harvesting complexes are called phycobilisomes (PBS); (Figure 1), and these were first structurally described by Gantt and Conti in 1966 (Sidler, 1994). Phycobilisomes are found on the stromal side of the thylakoid membrane and transfer energy mainly to photosystem II (PSII) (Figure 2) (Bald *el al*., 1996; Glazer et al., 1983; Sidler, 1994; Wilbanks and Glazer, 1993). Phycobilisomes absorb light in the visible range anywhere from 450-665nm. This wide absorption range allows light to be harvested from between the absorption bands of chlorophylls *a* and *b* (Sidler, 1994). Phycobilisomes are comprised of phycobiliproteins (PBP) that have covalently attached bilin chromophores and other linker polypeptides. Phycobiliproteins absorb light where chlorophyll

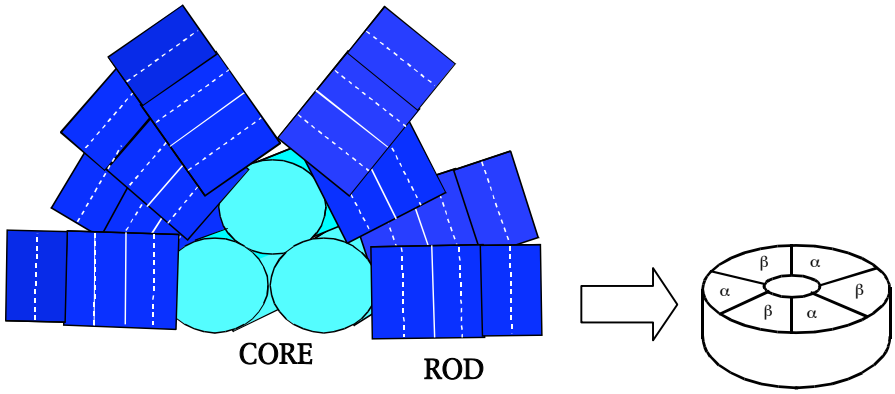


Figure 1: Phycobilisome (PBS) structure

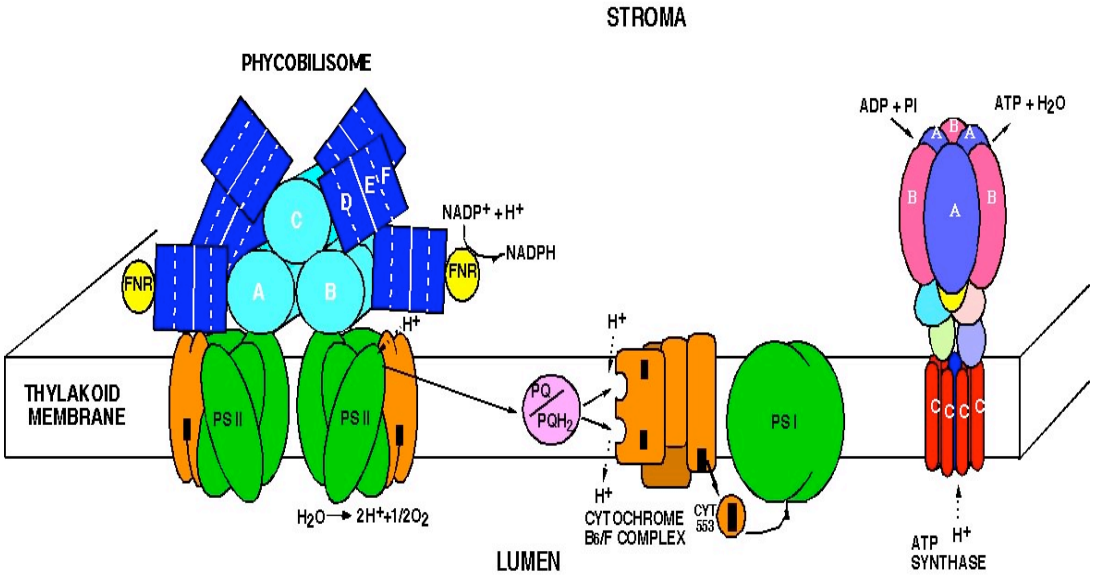


Figure 2: Phycobilisome structure and interaction with PSII and the thylakoid membrane (modified from Beale, 1994).

absorbs weakly, and the excitation energy is transferred with high quantum efficiency (Bald *el* al., 1996; Zhao *et al*., 2006). PBP make up about 80% of the mass of the PBS. Bilins are attached at cysteine residues by thioether bonds (Glazer, 1989). The PBS has two different domains, the core and the rods. The core is composed of three phycobiliprotein structures running parallel to one another. The rods branch out from the core and each rod contains several stacked phycobiliprotein trimer discs (Arciero *et al*., 1988; Arciero, Bryant, and Glazer, 1988; Bald, Kruip, and Rogner, 1996).

A phycobiliprotein is composed of equimolar amounts of two polypeptides, α and β, which have roughly 162 and 172 amino acid residues respectively (Arciero, Bryant, and Glazer, 1988; Glazer, 1989; Sidler, 1994). The αβ units of the phycobiliproteins are arranged in doughnut-shaped trimers $(\alpha\beta)_3$ or hexamers $(\alpha\beta)_6$ (Swanson and Glazer, 1990; Bald, Kruip, and Rogner, 1996). The way the phycobiliproteins are arranged assists a downhill transfer of energy from the rods to the core. In *Synechococcus* sp. PCC 7002, the light energy is transferred from phycocyanin (~620 nm) to allophycocyanin (~650 nm) to PSII (~680 nm).

αβ linker proteins hold trimers and hexamers together. These proteins control the absorption characteristics of the phycobiliproteins, but they do not bind chromophores with the exception being ApcE and some marine cyanobacteria that contain γ linker subunits, which are chromophorylated (Bald, Kruip, and Rogner, 1996). Each α and β polypeptide has covalently attached prosthetic groups, called bilins. These bilins are open-chained linear tetrapyroles that are derived from heme and are covalently attached to cysteine residues within the α and β subunits (Frankenburg *et al*., 2001; Glazer, 1989). There can be as many as three bilins bound to a single α or β polypeptide. Bilin chromophores are bound at specific conserved cysteine

residues usually through a thioether linkage at a cysteinyl residue to the vinyl group ring A of the phycobiliprotein (Sidler, 1994).

There are nine naturally occurring known bilins but only four bilin groups are found in cyanobacteria: phycocyanobilin (PCB), phycoviolobilin (PVB; also known as phycobiliviolin), phycoerythrobilin (PEB), and phycourobilin (PUB); (Figure 3) (Glazer, 1989; Sidler, 1994). The colors of the phycobiliproteins are due to the type of bilin present and its interaction with the protein. Different absorption characteristics of bilins are due to the variation of the number of conjugated double bonds and to the side chains in the prosthetic groups (Glazer, 1994; Sidler, 1994).

Synechocystis sp. PCC 6803 and *Synechococcus* sp. PCC 7002 contain only phycocyanobilin. In *Synechococcus* sp. PCC 7002, phycocyanobilin is seen in two isomers: R and S isomers at the 3^1 carbon (Figure 4) (Bishop, Nagy, and Rapoport, 1991; Shen *et al*., 2006). According to the CIP system of configuration assignments for 3-D structures (named for chemists R.S. Cahn, C.K. Ingold, and V. Prelog), each chiral center is given a prefix (R or S) depending on if it is right or left handed. By assigning priority to the groups adjacent to the carbon center, the handedness can be determined. If the highest priority bonded group to the next highest and then the next highest is a clockwise turn, then the configuration is R. If the highest priority group is a counterclockwise turn to the next highest priority group, then the configuration is S (Bloch, 2006).

1.2 Phytochromes, the other Biliprotein

Phytochromes were discovered first in plants in 1959. Phytochromes are the photoreceptor that controls the growth rate and development of plants during periods of long-

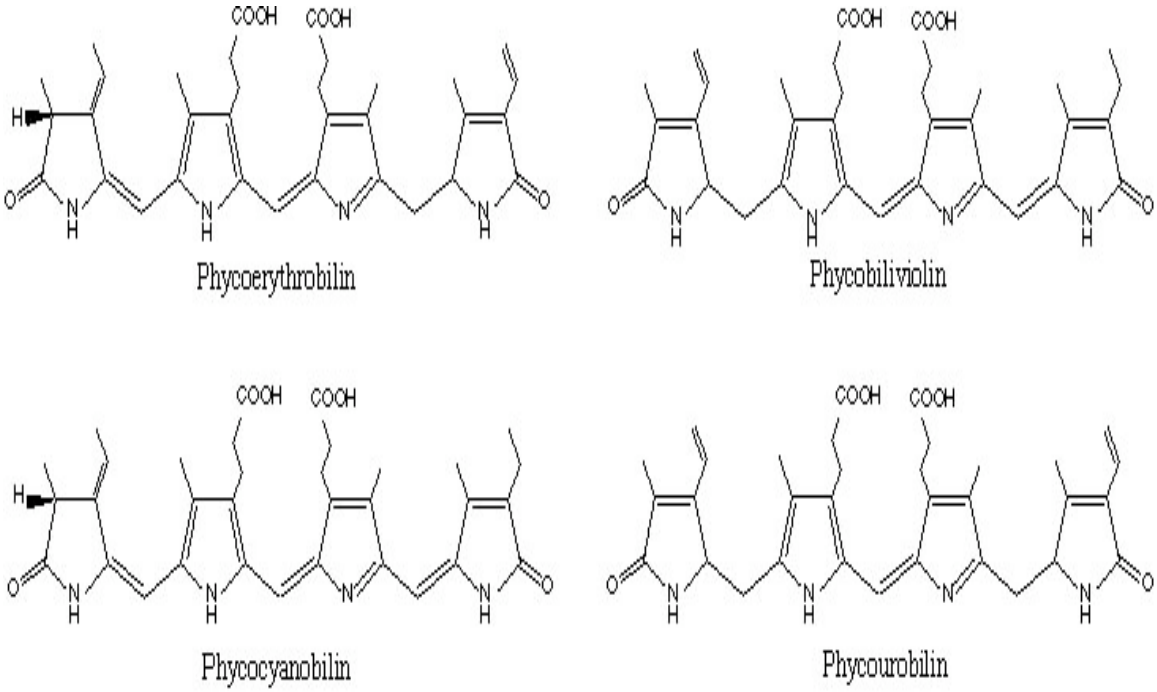
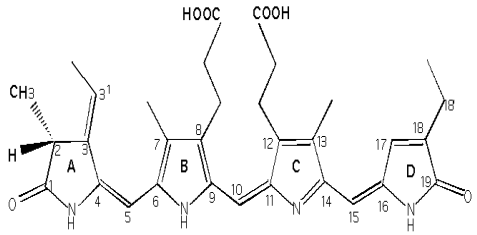
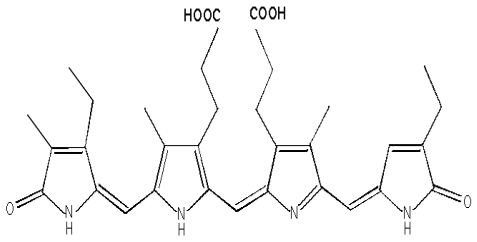


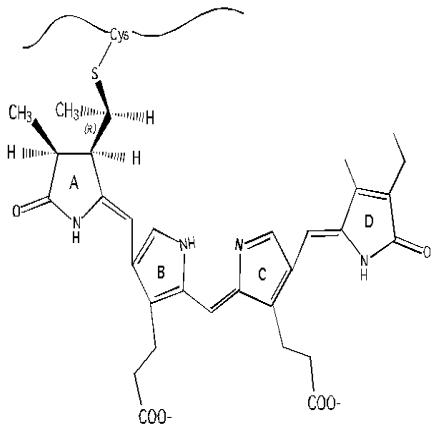
Figure 3. Bilins in cyanobacteria



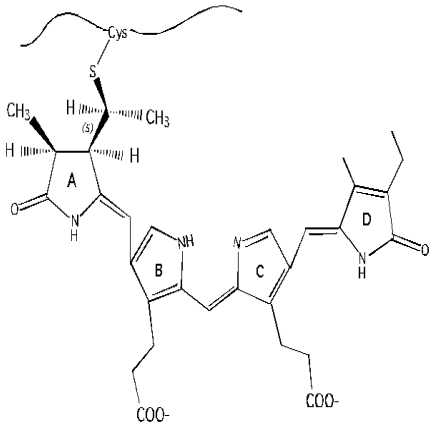
Phycocyanobilin



Mesobiliverdin



3^1*(R)*-Cys-PCB



3^1*(S)*-Cys-PCB

Figure 4. Structure of phycocyanobilin (PCB) and mesobiliverdin (MBV) (from Shen *et al.*, 2006).

wavelengths of light (Davis, 2004; Hanzawa *et al*., 2000; Hughes and Lamparter, 1999; Lamparter, Esteban, and Hughes, 2001; Rockwell, Su, and Lagarias, 2006). Seed germination, stem elongation, chloroplast movement, etc. are all mediated by phytochromes (Lamparter, 2004). Recognition of light by phytochromes requires photoisomerization of a double bond, which is covalently attached to a bilin. Phytochromes carry phycochromobilin (PϕB) or phycocyanobilin as their chromophore, both of which are derived from biliverdin. The phytochrome reaction is reversible so that members can convert between two forms: one that absorbs red light (λ_{max}~660 nm) and the other that absorbs at far-red light (λ_{max}~730 nm) (Wu and Lagarias, 2000; Lamparter , Esteban, and Hughes, 2001; Kami *et al*., 2004; Lamparter *et al*., 2003).

Research has shown that other organisms contain phytochrome. Cyanobacteria, anoxygenic photosynthetic bacteria, and fungi all have been found to contain phytochrome like proteins. Finding phytochrome in fungi shows that this family of proteins is not limited to organisms that photosynthesize (Lamparter, 2004; Rockwell, Su, and Lagarias, 2006). Some cyanobacterial strains undergo complementary chromatic adaptation (CCA), a process in which at certain light wavelengths the cyanobacteria adjust the bilins they use to absorb the most amount of light possible. This photoreversible reaction may be regulated by a similar phytochrome receptor found in plants. The first gene found in cyanobacteria that was a phytochrome is Cph1 from *Synechocystis* sp. PCC 6803. Other phytochrome-like genes have been in *Fremyella diplosiphon*, *Calothrix*, *Rhodospirillum centenum*, and *Synechococcus* sp. PCC 7942 (Wu and Lagarias, 2000; Lamparter, Esteban, and Hughes, 2001; Lamparter *et al*., 2003; Lamparter, 2004).

1.3 Phycocyanobilin:Ferredoxin Oxidoreductase (PcyA)

Heme is the precursor molecule for all bilins. The synthesis of phycocyanobilin is catalyzed by a enzyme called phycocyanobilin:ferredoxin oxidoreductase (PcyA). This enzyme is dependent on ferredoxin for its source of electrons to perform a four-electron reduction of biliverdin (Figure 5 and 6). All bilins are derived from biliverdin, which is their precursor in the biosynthetic pathway. Recombinant PcyA forms a very stable complex with biliverdin (Frankenburg *et al*., 2001; Frankenburg and Lagarias, 2003).

1.4 Bilin Attachment to Phycobiliproteins

Previous research showed that phycocyanobilins were covalently attached to phycocyanin at three positions: one on the α subunit at cysteine 84 (α-82) and two on the β subunit at cysteine 82 (β-82) and 153 (β-153) (reviewed in Glazer, 1989). Only over the past 15 years have researchers begun to understand how bilins are attached to phycobiliproteins. The most uncomplicated way to attach bilins would be through spontaneous addition as occurs with phytochrome, but *in vitro* experiments with bilin addition offered support for the existence of a class of enzymes called lyases. These lyases function to covalently attach bilins to the apobiliprotein at the correct cysteine residue (Glazer, 1994).

Arciero *et al.* (1988a&b) hypothesized that the addition of phycocyanobilin to phycocyanin was a spontaneous event, one not requiring enzymes. To test their theory, *in vitro* addition experiments were carried out. The genes encoding the α and β polypeptides (*cpcA* and *cpcB* respectively) of *Synechococcus* sp. PCC 7002 were cloned into a plasmid so that *Escherichia coli* would manufacture the α and β apoproteins (proteins without bilins attached). The apoproteins were copurified and was shown to be a monomer (αβ), and this purified form

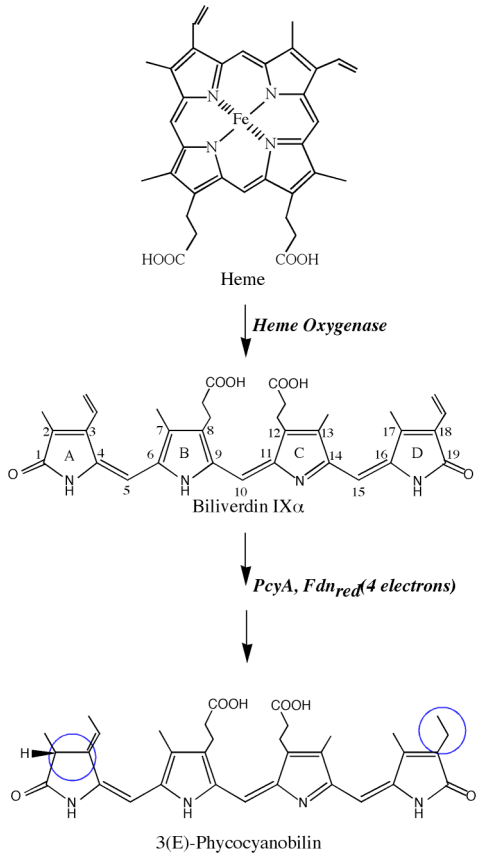


Figure 5: Conversion of heme to phycocyanobilin.

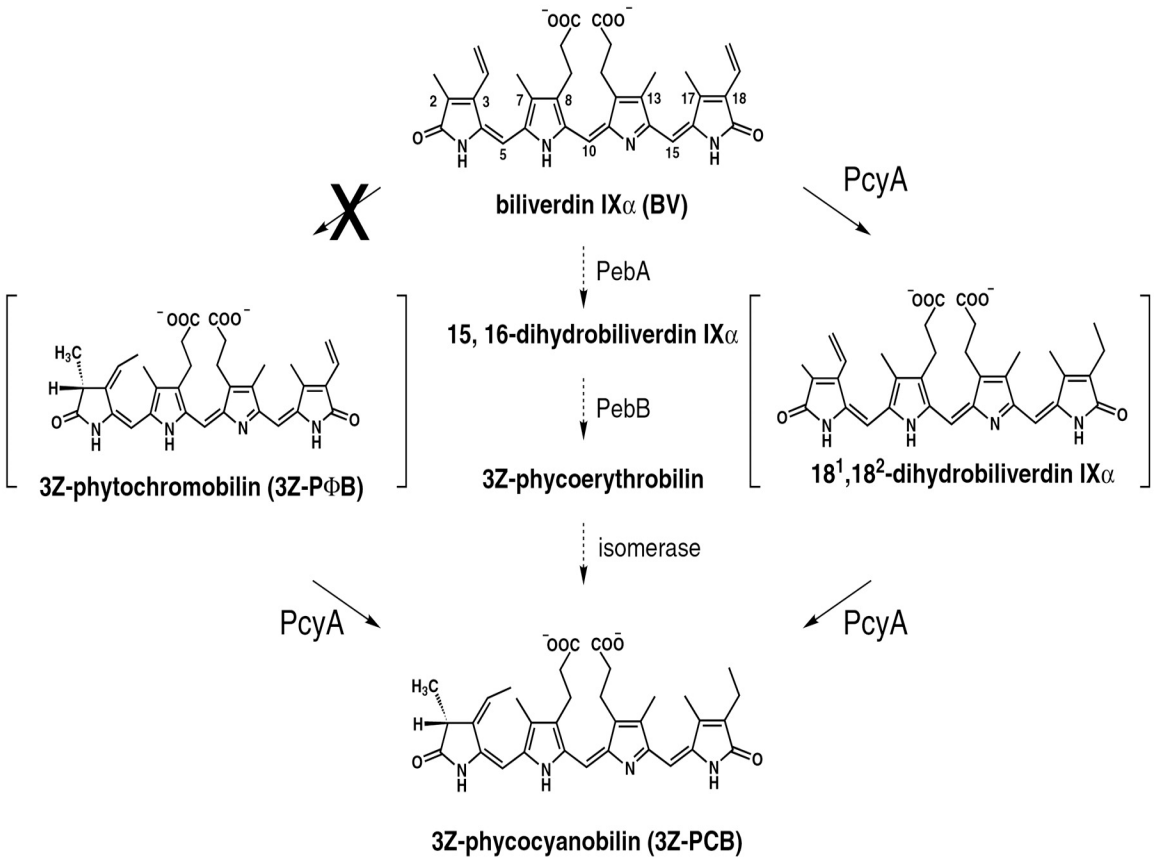


Figure 6: The reduction of biliverdin by PcyA (from Frankenburg and Lagarias, 2003). Reduction of biliverdin IXα by PcyA could result in the formation of the intermediate 18^1, 18^2-dihydrobiliverdin IXα or 3Z-phytochromobilin. Frankenburg and Lagarias (2003) showed that 18^1, 18^2-dihydrobiliverdin IXα is an intermediate of 3Z-phycocyanobilin production mediated by PcyA. The center of the figure shows an alternative pathway to form 3Z-phycocyanobilin in *C. caldarium,* by the reduction of biliverdin IXα to the intermediates 15, 16-dihydrobiliverdin IXα and 3Z-phycoerythrobilin. This is accomplished by PecA and PecB and an isomerase that has not been identified yet (Frankenburg and Lagarias, 2003).

was used for further reactions with phycocyanobilin. Phycocyanobilin was added to a solution containing apo-phycocyanin and allowed to react at room temperature in the dark for 16 hours (Arciero *et al*., 1988a). There was a noticeable difference in the visible absorption spectra of the *in vitro* produced phycocyanin and native phycocyanin. Additional analysis of the *in vitro* product demonstrated that addition was occurring at cysteinyl residues: α-84 and β-82, but not at β-153. NMR spectra of the *in vitro* products showed that the actual covalent bilin product was more oxidized than wild-type phycocyanobilin (Arciero *et al*., 1988a). The unnatural product was later determined to be 3'cysteinylmesobiliverdin, which differs from biliverdin by the addition of a double bond between carbons 2 and 3 of ring A (Figure 4) (Arciero *et al*., 1988b). This additional double bond makes mesobiliverdin absorb at a longer wavelength than phycocyanobilin; in addition, this bilin has only 10% of the normal fluorescence displayed by native phycocyanobilin bound to phycocyanin. These results indicate that the addition of phycocyanobilin to apo-phycocyanin is most likely an event that requires enzymes to be engaged, and not a spontaneous event (Arciero *et al*., 1988a and b). Arciero *et al*. (1988) also concluded that the spontaneous addition of bilins to apo-phycocyanin does not occur with the positional selectivity *in vitro* that is shown *in vivo* as no addition occurred at β-153 (Arciero *et al*., 1988c).

1.5 Lyases for α Subunit - CpcE, CpcF, PecE, PecF

The first phycobiliprotein lyases were identified in *Synechococcus* sp. PCC 7002. *CpcE* and *cpcF* were found in the *cpc* operon, which also contains *cpcA, cpcB, cpcC,* and *cpcD*, encoding structural proteins found in phycocyanin rods (Zhou *et al*., 1992*)*. These genes, *cpcE* and *cpcF*, were not structural components of the phycobilisome. To study the function of

cpcE/F, mutants were created that had *cpcE* and/or *cpcF* insertionally interrupted with an antibiotic resistance gene. The color of the mutant strains was yellow-green, while the wild type was blue-green in color. The mutants grew at half the rate of wild-type and had very low levels of phycocyanin. Further study indicated that the mutant strains had normal phycocyanin β subunits with bilins at both positions, but 90% of the phycocyanin produced lacked a bilin on the α subunit. These mutants were unable to attach phycocyanobilin correctly to the α subunit of phycocyanin (Swanson *el al.*, 1992), which suggested that the CpcE and CpcF polypeptides form a phycocyanobilin lyase specifically required for chromophorylation of the α subunit of phycocyanin (Zhou *et al.,* 1992; Swanson *et al*., 1992).

In order to confirm that CpcE and CpcF were lyases, Fairchild *et al.* (1992) overproduced them, and reactions were set up where CpcE, CpcF, and CpcE/F were added to apo-α-phycocyanin bound resin. When phycocyanobilin was added to apo-α-phycocyanin-bound resin and CpcE, CpcF, or CpcE/F, bilin addition occurred. The bilin adduct was distinguished, through fluorescence emission spectra, from mesobiliverdin, the non-enzymatic reaction product. HPLC separation showed one polypeptide with a chromophore attached, which corresponded to α-84 residue. CpcE/F also was shown to be able to transfer bilins to the apo-α-phycocyanin bound resin, which is the reverse reaction from bilin addition. These enzymes together catalyze the transfer reaction from holo-α phycocyanin to apo-α phycocyanin. Neither CpcE nor CpcF could transfer a bilin alone; only with both enzymes was the addition or transfer reaction possible. The research done by Fairchild *et al.* (1992) demonstrated that CpcE/CpcF catalyze a specific addition of phycocyanobilin to the cysteinyl residue of apo-α-phycocyanin. It was also the first decisive identification of a protein required for the attachment of bilin to a phycobiliprotein. It also showed that the forward (bilin addition) and reverse reactions (transfer

to apo-α phycocyanin) need both CpcE and CpcF to function properly and the reactions are specific to a single addition site, α-Cys-84 (Fairchild *at al.*, 1992). Later work demonstrated that CpcE and CpcF form an enzymatic 1:1 active complex that functions to attach phycocyanobilin to α-phycocyanin, while having no affect on the β subunit of phycocyanin (Fairchild and Glazer, 1994).

The pathway of α-phycocyanin biosynthesis was recreated inside *E.coli* cells, encoded on two plasmids. One plasmid contained all the essential genes for bilin biosynthesis, including enzymes that convert heme to phycocyanobilin. The second plasmid contained the genes for apo-α-phycocyanin (*cpcA*) and the lyases *cpcE* and *cpcF*. After induction of the products, one third of the apo-CpcA was converted into holo-CpcA. SDS-PAGE and spectroscopic analysis showed that the *in vitro* produced holo-CpcA had similar absorbance and fluorescent properties as the native protein found in cyanobacteria. The study also showed no bilin addition occurred in *E. coli* lacking both the *cpcE* and *cpcF* genes; mesobiliverdin was not even detected. The authors thought that the mesobiliverdin CpcA product was not produced because of the reducing environment within the *E. coli* cell (Tooley, Cai, and Glazer, 2001).

To further study the lyase activity of CpcE and CpcF, Zhao *et al.* (2006) studied CpcE and CpcF in *Mastigocladus laminosus* PCC 7603 and showed that it has 1:1 stoichiometry. Deletion and site-directed mutants of the two lyases were created, and the proteins were overexpressed in *E. coli* cells. These mutants served to probe for functional domains of the lyases. In CpcE, if the N-terminus was truncated 41 amino acids or the C-terminus was truncated 39 amino acids, the lyase became inactive. CpcF lost activity when 20 amino acids were deleted from the N-terminus and the loss of 53 amino acids from the C-terminus prohibited complex formation with CpcE, most probably because of misfolding. This study showed that

phycocyanobilin transfer ability is associated with the activity of the lyase and that the phycocyanobilin bound to CpcE/CpcF is only an intermediate of the reaction. It is believed that CpcE/CpcF form a complex that binds phycocyanobilin and modifies the conformation of phycocyanobilin; then phycocyanobilin is subsequently transferred to CpcA (Zhao *et al*., 2006).

In *Anabaena* sp. PCC 7120, the phycoerythrocyanin (*pec*) operon consists of five different genes (*pecB, pecA, pecC, pecE, pecF*). The *pecE* and *pecF* genes show 47% similarity to the *cpcE* and *cpcF* genes. The β subunit of both phycocyanin and phycoerythrocyanin carries phycocyanobilin at cysteinyl residues β-82 and β-155, while the α subunit of phycoerythrocyanin contains phycobiliviolin at cysteinyl residue α-84. Jung *et al*. (1995) created insertional mutants that inactivated *pecE*, *pecF*, and *pecE/F*. While the *cpcE* and *cpcF* mutants synthesized less phycocyanin, the three *pec* mutants produced significantly less phycoerythrocyanin, and all showed the wrong bilin addition at the α subunit pf phycoerythrocyanin compared to the wild type. When both *pecE/F* were inactivated, the α-phycoerthrocyanin contained phycocyanobilin at the phycoerythrocyanin α subunit, instead of the normally present phycobiliviolin. These results corroborated that *pecE* and *pecF* encode a phycoerythrocyanin α subunit phycobiliviolin lyase and also showed that the phycobiliprotein bilin lyases are selective for the substrate and the bilin (Jung, Chan, and Glazer, 1995).

Later, Zhao *et al.* (2000) overexpressed the genes for *pecE* and *pecF* in *E. coli*. Apo-α-phycoerythrocyanin was incubated with phycocyanobilin and PecE and PecF, with the resulting product being holo-phycoerythrocyanin. Upon spectroscopic analysis, the *in vitro* produced holo-phycoerythrocyanin was identical to the naturally formed product. However, in the absence of PecE, PecF, or PecE/F, an unnatural chromoprotein (mesobiliverdin) is produced. These results indicate that PecE and PecF perform both the addition of phycocyanobilin to α-

phycoerythrocyanin and the following isomerization reaction to phycobiliviolin (Zhao *et al.*, 2000). Consequently, these lyases must catalyze the attachment of the bilin before the isomerization reaction to the correct bilin can occur. PecE functions in binding the bilin while PecF functions in the isomerization of the chromophore (Storf *et al.*, 2001; Zhao *et al*., 2002).

The biosynthetic pathway for holo-α-phycoerythrocyanin was engineered in *E. coli* cells with two plasmids. The genes that are required for the conversion of heme to phycocyanobilin were expressed from one plasmid. The α-phycoerythrocyanin (*pecA*) gene and the lyase/isomerase *pecE* and *pecF*, were expressed from a separate plasmid. After induction with IPTG, roughly two-thirds of the apo-PecA had been converted to holo-PecA. The *in vitro* holo-PecA produced had analogous absorbance and fluorescence properties as native holo-PecA. No attachment was seen in engineered plasmids that were deficient in either *pecE* or *pecF* (Tooley and Glazer, 2002).

1.6 Phycocyanobilin Addition to the β Subunit of Phycocyanin (CpcB)

Phycocyanin is the phycobiliprotein in *Synechococcus* sp. PCC 7002 that carries phycocyanobilin on three cysteinyl residues: α-84, β-82, and β-153. As stated above, there is considerable knowledge about bilin addition to the α-subunit of phycocyanin. Studies have shown that CpcE/CpcF form a complex that catalyzes the addition of bilin to the α-subunit, while PecE/PecF works together to catalyze the addition of bilin and isomerize it to the correct chromophore (Arciero *et al.*, 1988abc; Tooley *et al.*, 2001; Zhao *et al*., 2006; Zhao *et al.*, 2000; Storf *et al.*, 2001; Zhao *et al*., 2002; Jung *et al.*, 1995). Conversely, there is little known about any β-subunit lyase or about the addition of phycocyanobilin to the β-subunit in general.

Cobley *et al*. (2002) found a protein in *Fremyella diplosiphon* (CpeR) that activated the expression of the phycoerythrin operon. This operon (*cpeBA*) contains both the α and β phycoerythrin subunits. CpeR is found in the phycoerythrin linker polypeptide (PE) operon. This operon also contains *cpeCDESTR*. The *cpeS* and *cpeT* genes have been found in a conserved gene cluster in other cyanobacterial species, even species that lack phycoerythrin (Cobley *et al*., 2002). A transposon mutant lacking *cpeT* yielded no phycoerythrin (Cobley *et al*., 2002). Cobley *et al*. (2002) believed that this protein is involved in the regulation of transcription for the PE operon. The exact function of *cpeT* and *cpeS* are not known (Cobley *et al*., 2002). Through comparative genomics, genes similar to *cpeT* and *cpeS* are also found in *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803, neither of which produces phycoerythryin. Therefore, theses genes were reasonable candidates for β-phycobilprotein lyases.

1.7 Characteristics of *Synechococcus* sp. PCC 7002

The cyanobacteria *Synechococcus* sp. PCC 7002, previously known as *Agmenellum quadruplicatum*, grows in brackish and marine environments. This cyanobacteria is unicellular or short filaments (usually of two to four cells) and grows optimally at 38°C. *Synechococcus* sp. PCC 7002 is a facultative photoheterotroph that uses glycerol as substrate. It has a doubling time of around 3.5 hours, which makes it one of the fasting growing cyanobacteria. Phycobilisomes in this species contain only phycocyanin and allophycocyanin (Herdman *et al*., 1979a&b; Van Baalen, 1962). Because *Synechococcus* sp. PCC 7002 is naturally transformable and has a relatively small genome size (about $3*10^6$ bp), this organism was ideal for genome sequencing

and mapping. The genome has been sequenced and it has a G+C content of 49.1% (Li et al., unpublished results; Chen and Widger, 1993).

Because the genome of *Synechococcus sp. PCC 7002* has been sequenced, it was possible to search for genes that were similar to *cpeS* and *cpeT* by performing a BLAST search. There were two main criteria used to determine paralogs of these genes: 1) these genes should only be found in cyanobacteria because they are required in phycobiliprotein synthesis, thus should only be found in organisms that synthesize phycobiliproteins 2) genes found should be located near other phycobiliprotein genes, like their counterparts in *Fremyella* (Shen *et al*., 2006; unpublished data from Schluchter lab). One gene was found to be a paralog of *cpeT* and the Schluchter lab designated this gene *cpcT* (because *Synechococcus sp. PCC 7002* has phycocyanin and not phycoerythrin). There were three genes found to be paralogs of *cpeS*, and they were named *cpcS, cpcU, cpcV* (Shen *et al*., 2006; unpublished data from Schluchter lab).

1.8 Purpose of this Work

The research presented here focuses on the phycobiliprotein biosynthesis in *Synechococcus* sp. PCC 7002. The goal of this research was to determine if there are enzymes that are involved in the biosynthesis of phycobiliproteins, and if so, characterize them. Past evidence reported above shows that enzymes are required to get the correct bilin adduct and not a more oxidized unnatural product. The first goal of this research was to clone the *cpeS* and *cpeT*-like genes (named *cpcS, cpcU, cpcV,* and *cpcT*) from *Synechococcus* sp. PCC 7002 into plasmids, and sequence these genes to make sure they were correct. Recombinant proteins were then overexpressed and purified using various methods. The second goal was to perform bilin addition reactions with apo-phycocyanin to determine if these proteins are in fact adding a bilin

to the β-subunit of phycocyanin. Protein-protein complex interactions between the different proteins were also examined. Biochemical analysis was used to determine if the correct bilin addition product occurred in the presence of these lyases.

MATERIALS AND METHODS

Materials:

Chemicals were purchased from either Fisher Chemical Company (Houston, TX) or Sigma Chemical Company (St. Louis, MO). Restriction Enzymes were purchased from both New England Biolabs (Beverly, MA) and Promega Corporation (Madison, WI). The Gene Clean kits were purchased from Qbiogene (Carlsbad, CA). PCR primers were purchased from Qiagen (Alameda, CA). QIAquick PCR purification kit and QIAprep spin miniprep kit were purchased from Qiagen Incorporated (Valencia, CA). DH5α and BL21*DE3* cells were purchased from Invitrogen (Carlsbad, CA). BL21 and BL21-T1^{r} cells were purchased from Sigma-Aldrich Incorporated (St. Louis, MO). Plasmid midiprep kit and SDS-PAGE gels were purchased from Bio-Rad Laboratories (Hercules, CA). Sep-pak cartridges were purchased from Waters Corporation (Milford, MA). Big Dye was purchased from Applied Biosystems (ABI) (Foster City, CA.)

2.1 Construction of Recombinant Expression Plasmids

2.1.1 Polymerase Chain Reaction (PCR)

A BLAST search was performed to determine if there were genes comparable to *cpeS* and *cpeT* in other cyanobacterial species. Three genes were found that were similar to *cpeS,* and one *cpeT* ortholog was discovered in the genome. All PCR reactions were performed with 40 pmol of each primer (see Table 1), 10 ng of 7002 chromosomal DNA, and 25 μl of PCR master mix (which contains dNTPs, T4 polymerase, etc) from Promega Corporation (Carlsbad, Ca.). All PCR reactions were performed in the W.M. Keck Foundation Lab using a Thermocycler (i-cycler, Bio-Rad). The PCR amplification program consisted of: 1) an initial denaturing step of 4 minutes at 95°C, 2) a second denaturing step for 30 seconds at 95°C, 3) an annealing step for 30 seconds at 50°C, 4) an extension step for 1 minute at 72°C (this cycle of steps 2-4 was repeated 35 times), 5) 7 minutes at 72°C, 6) the cycle was held at 4°C as long as needed. QIAquick PCR purification kit was used to remove any unincorporated primers and dNTPs. Following manufacturer guidelines the PCR product was absorbed to a silica-membrane while any other contaminants passed through the column and did not bind. The pure PCR product was eluted in a low salt buffer.

2.1.2 DNA Purification/Agarose Gels

DNA separation on agarose gel for PCR and purification of digested products was performed using DNA fragment purification grade agarose. Agarose gels were 0.8% agarose in 1X Tris acetate EDTA (TAE) buffer (0.4 M Tris-acetate, pH 8.0, 1 mM EDTA). Gels were then stained in 10 mg/ml ethidium bromide solution for 10 minutes and visualized under ultraviolet

(UV) light. The DNA bands were excised from the gel and purified using manufacturer guidelines in the Geneclean Kit.

2.1.3 Ligation

The cleaned PCR product and the vector were added together and incubated overnight at room temperature. Two different vectors were used: pAED4 and pBS150v. The pAED4 vector has an Ampicillin resistance gene and a T7 RNA polymerase cloned into it. The pBS150v vector contains a section of DNA that codes for an extra 23 amino acids at the N-terminus, which includes six histidines. This row of histidines, called a his-tag (HT), makes the protein easy to purify by metal affinity chromatography (nickel-NTA resin). The pBS150v vector has a lac fusion promoter that allows the protein to be expressed when lactose (or IPTG) is present. The ligations were composed of: 200 ng of digested PCR product, 50 ng of digested vector (pAED4 or pBS150v), 1 μl of T4 DNA ligase, 1 μl of 10x ligase buffer, and ddH_2O to give a total of 10 μl. Ligations were incubated overnight at 16°C.

2.1.4 Transformation

After incubation, the ligations were transformed into DH5α cells using the method suggested by supplier. Transformation was obtained by adding 2-5 μl of ligation to 50 μl of cells. The mixture was put on ice for 30 minutes and then heat shocked at 42°C for 45 seconds. After the heat shock, the cells were placed on ice for 2 minutes and 900 μl of SOC (0.5% Bacto-yeast extract (w/v), 2% Bacto-tryptone (w/v), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 1 mM NaOH, 20 MM glucose) was added. Then the cells were placed in at 37°C water bath for 1 hour.

Luria–Bertani (LB) plates with either Ampicillin or Spectinomycin were used to plate the transformations. The appropriate antibiotic was used to select for plasmids that contained both vector and insert. Ampicillin selected for the presence of the pAED4 vector, while Spectinomycin selected for the pBS150v vector. Colonies were grown in 5 ml of LB (1% NaCL (w/v), 1% Bacto-tryptone (w/v), 0.5% Bacto yeast extract) with the appropriate antibiotic (Ampicillin (100μg/ml) or Spectinomycin (100μg/ml)) overnight at 37°C for midiprep.

2.1.5 Minipreps and Midipreps

Minipreps were performed on each colony (colony grown in 5 ml LB with antibiotic) using manufacturer guidelines from the miniprep kit from Qiagen Incorporated (Valencia, Ca.). After a 0.8% agarose gel confirmed the correct size of the transformed molecule, a large scale midiprep (from a 50 ml culture) was performed from the midiprep kit, again following manufacturer guidelines. Midiprep DNA was used for sequencing.

2.1.6 *cpcS, cpcU, cpcV, cpcT,* and *cpcBA*

The *cpcS* gene was amplified by polymerase chain reaction (PCR) from wild type *Synechococcus* sp. PCC 7002 chromosomal DNA using two primers 7002 cpeS1.6 and 7002 cpeS1.3.2 (see Table 1). All clones were made from 7002 chromosomal DNA unless otherwise specified. After PCR and subsequent purification, the *cpcS* gene, along with vector pAED4, was digested with restriction enzymes *NdeI* and *XhoI*. The digestion products were run on a 0.8% agarose gel, and the bands were extracted. The vector and insert was ligated together. Ligations were transformed into DH5α cells and plated on LB plates with Ampicillin (LBAp). Colonies

Table 1: Primers used for PCR amplification. Restriction enzyme sites are underlined.

Primer	Sequence (5' to 3')	Use
7002 cpeT1.6	AGCAATTTCATATGTCCCACTCTACCGATGCC	Amplification of *cpcT*
7002 cpeT1.4	CTTTCTCGAGTTTAGCCGCCATAATTTTTGCTTCTCTCC	Amplification of *cpcT*
7002 cpes1.6	AATTTTTCCATATGCAAAGCTTTGCGGATGCC	Amplification of *cpcS*
7002 cpeS1.3.2	TTGACTCGAGCAACACGGATATCTCTGTGGG	Amplification of *cpcS*
7002 cpes2.6	GTAACTGTTCATATGGATATCAATGCCTTTATCG	Amplification of *cpcU*
7002 cpeS2.3	CTAAAAGCTTTCGTTAGTTACTGGCTTCAGCGG	Amplification of *cpcU*
7002 Cpes3.5	GCTCTTCGCATATGAATTTACTTGCGAC	Amplification of *cpcV*
7002 cpeS3.3	TTTAAGCTTACTAAAGACGCGTTTCTAAATACTGCGC	Amplification of *cpcV*
7002 cpcB.5	GAGATAAACATATGTTTGATATTTTTACCCGGGTTG	Amplification of *cpcB*
SyncpcA162	ACTAAGCTTAATTAGCTGAGGGCG	Amplification of *cpcA*
pAED45.2	AGCAGCCGGATCTGATATCATCG	Sequencing of *cpcS, cpcT, cpcV, cpcBA*
pAED43.2	TAACGGATCCAAGGAGATATACATATG	Sequencing of *cpcS, cpcT, cpcV, cpcBA*
pBS150NcoI	CAGACCATGGGTCATCATCATCATCATCACG	Sequencing of *cpcU*
pBS150V.3	AAACTGCAGGGCCAGTGCCAAGCTT	Sequencing of *cpcU*

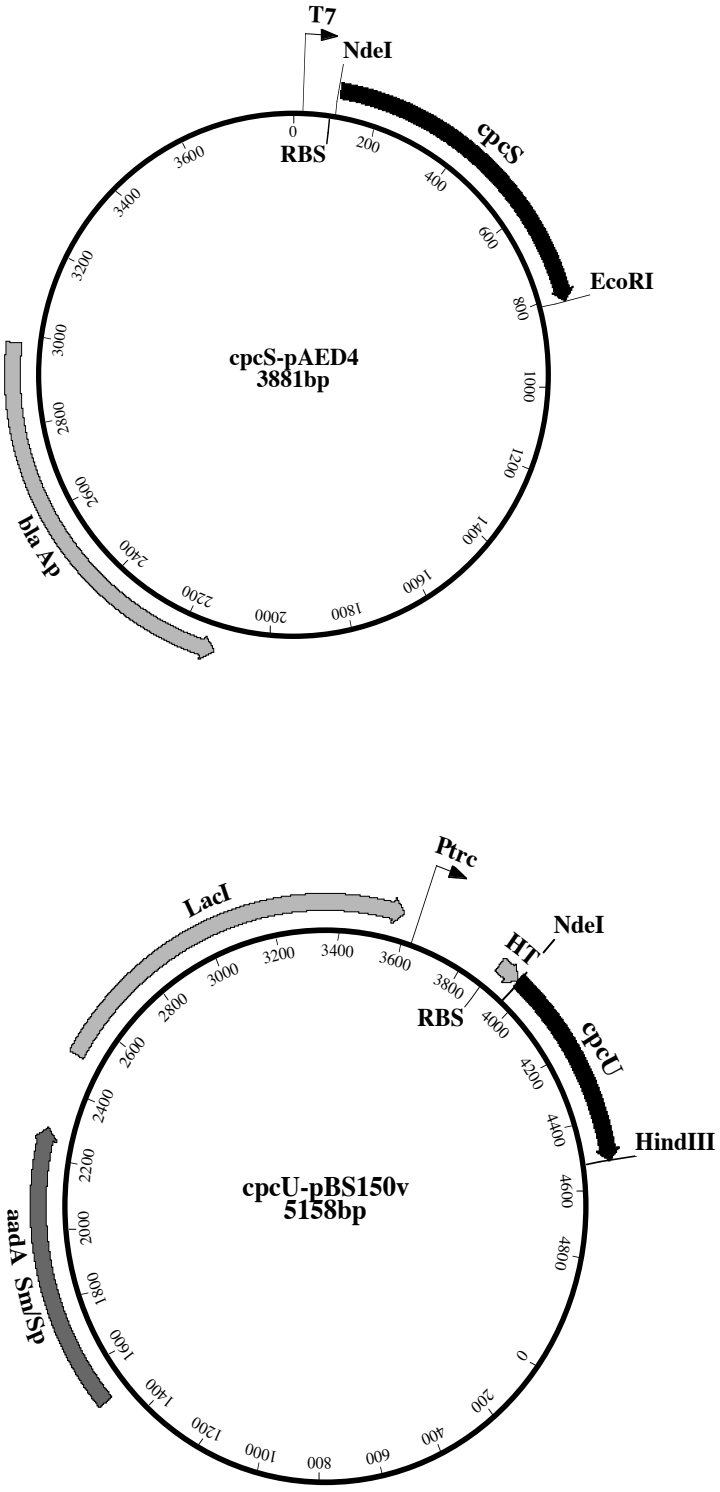


Figure 7: Plasmid maps for clones *cpcS*/pAED4 (upper) and *cpcU*/pBS150v (lower)

were grown and minipreps were made. DNA with vector and insert had large scale midipreps made. Plasmid construct maps of cpcS/pAED4 were made (Figure 7 upper).

The *cpcU* gene was amplified by PCR using two primers 7002 cpeS2.6 and cpeS2.3 (Table 1). After PCR, the *cpcU* gene, along with vector pBS150v, was digested with restriction enzymes *NdeI* and *HindIII*. The pBS150v vector includes a histidine tag to ease the purification of the protein. The digestion products were ligated together. Ligations were transformed into DH5α cells and plated on LB plates with Spectinomycin (LBSp). Minipreps and midipreps were made for vector with insert in it. Plasmid construct maps of cpcU/pBS150v were made (Figure 7 lower).

The *cpcT* gene was amplified by PCR using primers 7002 cpeT1.6 and cpeT1.4 (Table 1). After PCR amplification, the *cpcT* gene, along with vector pAED4, was digested with restriction enzymes *NdeI* and *XhoI*. The digestion products were ligated together after gel purification of fragments. Ligations were transformed, and DNA was extracted from transformants by minipreps. Plasmid construct maps of cpcT/pAED4 were made (Figure 8 upper).

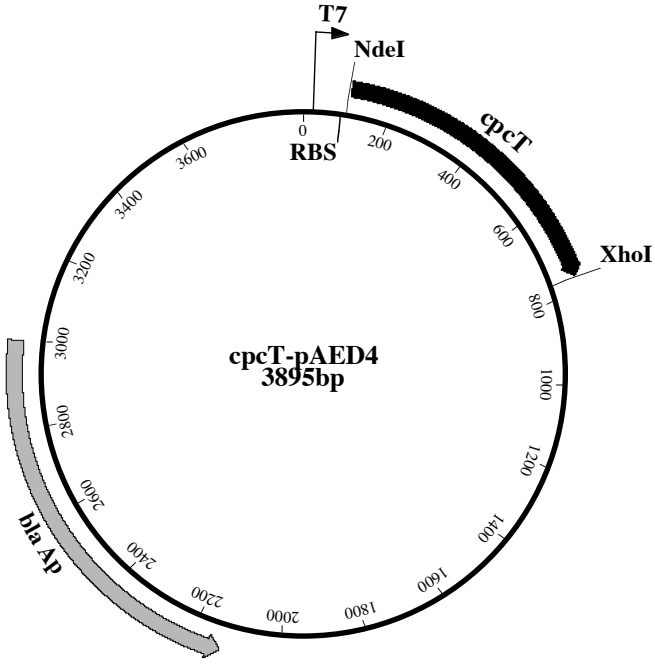
The *cpcV* gene was amplified by PCR using primers 7002 cpeS3.5 and cpeS3.3 (Table 1). After PCR amplification, the *cpcV* gene, along with vectors, was digested with restriction enzymes *NdeI* and *HindIII*. *CpcV* was the only gene that was ligated to two different vectors (pAED4 and pBS150v). The digestion products were ligated together and transformed into DH5α cells. Minipreps were performed. Plasmid construct maps of cpcV/pAED4 (Figure 8 lower) and cpcV/pBS150v were made (Figure 9 upper).

The *cpcB* and *cpcA* genes were amplified together by PCR using oligonucleotides 7002 CpcB.5 and syncpcA162 (Table 1). The *cpcBA* gene PCR products, along with vector pAED4,

were digested with restriction enzymes *NdeI* and *HindIII*. The digestion products were ligated together. Ligations were transformed, and minipreps were performed to check constructs. Plasmid construct maps of cpcBA/pAED4 were made (Figure 9 lower).

2.2 Sequencing

The clones were sequenced at the W.M. Keck Conservation and Molecular Genetics lab to confirm that the clones were correct and that no mutations had been introduced. To sequence these genes for confirmation, 2 μl of template (about 300-500ng) was added to 1 μl of primer (0.8 pmol/μl) and 2 μl of Big Dye from ABI (containing fluorescently labeled ddNTPs, dNTPs, and a polymerase). The cycle for amplification of the sequencing reactions was as follows: a initial denaturing step, a second denaturing step at 96°C for two seconds, an annealing phase for 15 seconds at 50°C, and an extension phase for 4 minutes at 60°C. These three steps were repeated 25 times. To remove unincorporated dNTPs, a slurry of Sephadex G-50 was used. A 2.0 ml eppendorf tube with a mini-column (USA Scientific) was used to clean up the reactions. 650 μl of Sephadex slurry was added to the 2.0ml tube and centrifuged for 3 minutes at 14,000 * g. The column was moved to a new 1.5 ml eppendorf tube, and the sample was then added to the column and centrifuged for 3 minutes at 14,000 *g. The cleaned samples were then dried in a speed vac for 8 minutes. 20 μl of hi di (formamide) was added to each sample, and they were then loaded onto the ABI PRIZM 3100 Genetic Analyzer. The results of the sequencing reaction were then examined using the program Macvector (Accelrys, San Diego, CA.) to align the sequence to the known expected gene sequence to determine if the constructs were correct and if they had any mutations.



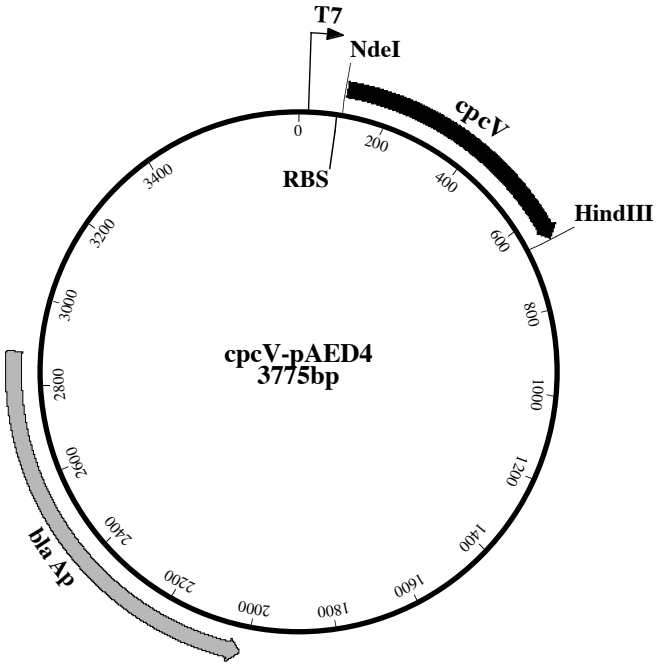
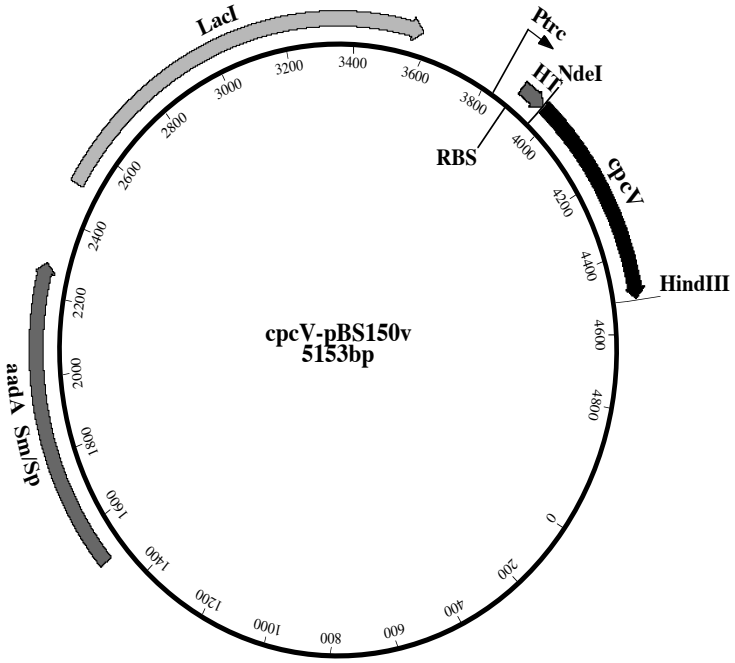


Figure 8: Plasmid maps for clones *cpcT*/pAED4 (upper) and *cpcV*/pAED4 (lower)



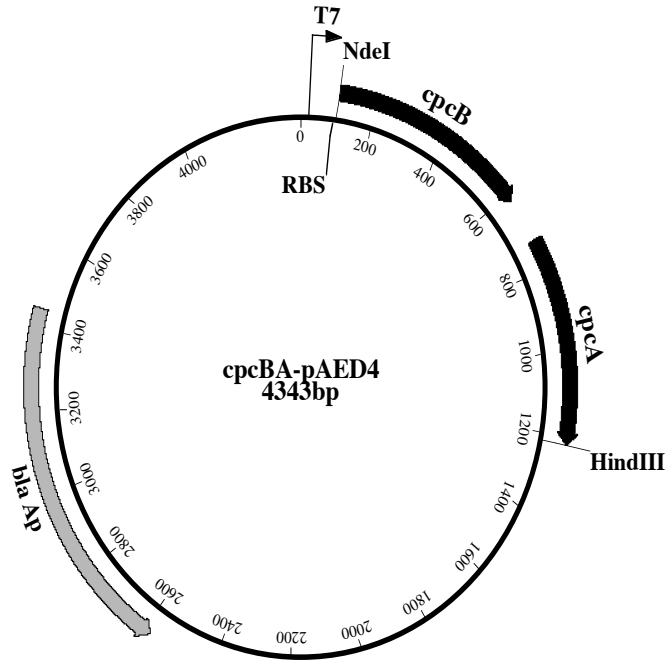


Figure 9: Plasmid maps for clones *cpcV*/pBS150v (upper) and *cpcBA*/pAED4 (lower)

2.3 SDS-PAGE Analysis

Protein gels were run on 15% Tris-HCl SDS-PAGE gels purchased from BioRad. SDS-PAGE gels were run as follows: 12 minutes at 100 volts followed by 45 minutes at 200V. Gels were generally stained overnight in Coomassie blue stain and destained overnight in 10% methanol, 10% acetic acid. For bilin addition assays (explained later in Materials and Methods), zinc ions were added to bind to bilin for detection by UV fluorescence. 100 mM $ZnSO_4$ (about 100 ml) was added to SDS-PAGE gel and rocked for 2-3 minutes. The gel was then put on the FX machine and images were taken.

2.4 Protein Overexpression and Purification

After sequencing confirmed the clones, *cpcS, cpcT, cpcV,* and *cpcBA* containing plasmids were transformed into BL21 *DE3* cells (followed same protocols as DH5α cells above), and colonies were selected on LB plates in the presence of Ampicillin (100 μg/ml). *CpcU* and *cpcV* plasmids were transformed into BL21 cells, and colonies were selected on LB plates with Spectinomycin (100 μg/ml). For production of proteins, a 50-ml overnight starter culture was added to 1-L LB with 100 mg of the correct antibiotic and grown for 4 hours. *CpcS* and *cpcT* cells were grown at 30°C while *cpcU*, *cpcV*, and HT-*cpcV* (HT=histidine tag) cells were grown at 37°C. Protein expression was induced by the addition of 0.5 mM IPTG (isopropyl β-D-thiogalactoside), and cells were grown for another four hours. For a control, pAED4 transformed BL21 *DE3* cells were grown like the experimental genes at 37°C: 4 hours growth, induction, 4 hours growth. *CpcBA* was expressed at 30°C in LB with Ampicillin. Molecular weight of all proteins can be found in Results (Table 2).

The c*pcBA* genes were expressed in 1L of LB by the addition of a 50-ml starter culture and grown for 11 hours with no induction with IPTG. This procedure gave the most amount of correctly folded protein. Growing this gene as described above resulted in the production of inclusion bodies. CpcBA were coexpressed because the subunits are not stable when expressed alone. All expression cells were harvested and spun down by centrifugation (30 minutes at 3500 rpm) and frozen at -20°C until later.

2.4.1 CpcS Protein Purification

To purify CpcS, expression cells were resuspended in 50 mM Tris-HCl, pH 8.0, homogenized and passed through a French Pressure Cell at 20,000 psi three times. Whole cell extracts were centrifuged at 15,000 rpm (26,000 * g) for 25 minutes to pellet unbroken cells and inclusion bodies. The supernatant was brought to 40 % (w/v) ammonium sulfate and was left at 4°C overnight. The ammonium sulfate precipitation was centrifugated at 15,000 rpm (26,000 * g) for 20 minutes. The CpcS protein was in the pellet so the supernatant was discarded. The pellet was resuspended in a small amount (about 10 ml) of 50 mM Tris-HCl, pH 8.0, and dialyzed at 4°C against the same buffer to remove the ammonium sulfate.

Further purification was attained through anion exchange chromatography. 200 g of DEAE powder was added to 400 ml of 50 mM Tris-HCl, pH 8.0, and stirred on a stir plate for 2 hours. The solution sat overnight to let the DEAE swell and allow any fines to rise to the top. 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 NaN_3 (Buffer A). 10-ml aliquots of the CpcS solution were 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 NaN_3); 150-180 minutes, 100% Buffer B; 180-210 minutes 100% Buffer A. Fractions with ≥ 0.08 Absorbance at 280 nm were collected and examined for purity by SDS-PAGE. Fractions containing CpcS were pooled and dialyzed against 50 mM Tris-HCl, pH 8.0 overnight at 4°C. The buffer was changed and the sample was dialyzed for another 4 hours. CpcS was concentrated using Amicon YM-10 concentrators and stored in 2 ml aliquots at -20°C until needed. The purification chromatograph can be seen in Results (Figure 15) as well as the SDS-PAGE gel of purified fractions in Results (Figure 16).

2.4.2 CpcT Protein Purification

CpcT was purified by resuspending the expression cells in 20mM Tris-HCL, pH 8.0, 50 mM NaCl, 1 mM DTT (dithiothreitol), by homogenizing and passing them three times through a French Pressure Cell at 20,000 psi. The cell suspension was centrifuged at 15,000 rpm (26,000 * g) for 25 minutes. The supernatant was brought to 30% (w/v) ammonium sulfate and left at 4°C overnight. After centrifugation for 30 minutes at 15,000 rpm (26,000 * g), the supernatant containing the CpcT protein was brought to 50% (w/v) ammonium sulfate and again left overnight at 4°C. The solution was centrifuged for 30 minutes at 15,000 rpm, and the CpcT protein was now in the pellet. The pellet was resuspended in the smallest amount possible of 50 mM Tris-HCl, pH 8.0, and dialyzed as for CpcS against 50 mM Tris-HCl, pH 8.0. Further purification was achieved following the same procedure as purification of CpcS by anion-exchange chromatography (DEAE). The procedure was carried out on a BioLogic LP system, and fractions with $A_{280\ nm} \geq 0.05$ were collected and analyzed by SDS-PAGE. Pooled fractions

were dialyzed as above against 50 mM Tris-HCl, pH 8.0, and concentrated (using Amicon YM-10) four-fold. 2.0 ml aliquots were frozen at -20°C until further use. The purification chromatograph (Results; Figure 17) as well as SDS-PAGE gel analysis of purified fractions can be found in Results (Figure 18).

2.4.3 Purification of HT-CpcU and HT-CpcV

Because CpcU and CpcV were cloned into a vector that has a histidine tag, they are easily purified by metal affinity Chromatography using Ni-NTA resin. *E. coli* cells expressing CpcU and CpcV were resuspended in Buffer O (20 mM Tris-HCl, pH 8.0, 100 mM Na/KCl) homogenized and passed three times through a French Pressure Cell Press at 20,000 psi. The extracts were centrifuged at 15,000 rpm (26,000 * g) for 25 minutes. 10 ml of Ni-NTA resin was washed with 25 ml of buffer O and centrifuged for 5 minutes at 5000 *g. The supernatant was loaded onto the column. The resin and the extract were incubated together for 15 minutes and then added to a column. The extract was allowed to flow through the column two times fast and one time slowly. The column was washed with 50 ml Buffer A1 (20 mM Tris-HCl, pH 8.0, 100 mM Na/KCl, 20 mM imidazole, 5% glycerol), Buffer B (20 mM Tris-HCl, pH 8.0, 1 M Na/KCl), and Buffer A2 (20 mM Tris-HCl, pH 8.0, 100 mM Na/KCl, 30 mM imidazole). CpcU was eluted from the Ni-NTA resin by the addition of 20 ml Buffer C (20 mM Tris-HCl, pH 8.0, 100 mM Na/KCl, 200 mM imidazole) followed by another 10 ml of Buffer C. The eluted fractions were dialyzed exhaustively against Buffer O. They were then concentrated in the same manner as for CpcS and stored in 2 ml aliquots at -20°C.

Recombinant CpcV (in pAED4) was purified only to the whole-cell extract stage. The cells were thawed and resuspended in Buffer O and homogenized and passed through a French

press as for CpcS. The extract was then centrifuged at 15,000 rpm (26,000 * g) for 25 minutes. The supernatant contained the whole cell extract and this was frozen in 2 ml aliquots at -20°C.

2.4.4 CpcB/CpcA Protein Purification

CpcB/CpcA was co-purified by adapting the procedure from Arciero *et al.,* (1988a). *CpcBA* expression cells were resuspended in 50 mM Na-phosphate, pH 7.0, 10 mM 2-mercaptoethanol (BME) homogenized and passed through a French Press as for like CpcS. Extracts were centrifuged at 15,000 rpm (26,000 * g) for 25 minutes. Ammonium sulfate was added to the supernatant to 38% (w/v) and left overnight at 4°C. After centrifugation at 15,000 rpm (26,000 * g) for 20 minutes, the pellet was resuspended in as small an amount of Na-phosphate buffer, pH 7.0, as possible and was loaded onto a DEAE (DE-52, Whatman; 2.5x 15 cm) column. The flow through of the column contained CpcBA and was collected. Ammonium sulfate was added to 50% (w/v) and left overnight at 4°C. The mixture was centrifuged as described above, and the pellet was collected. The pellet was resuspended in Na-phosphate buffer, pH 7.0, and dialyzed against the same buffer to remove the ammonium sulfate. A separate DEAE column was poured as described above for CpcS. This column was run using the BioLogic LP system at room temperature. 10-ml aliquots of CpcBA were loaded onto the column using the same program as described for CpcS. Fractions were collected and analyzed by SDS-PAGE to determine which fractions contained CpcBA. These were pooled and dialyzed against 50 mM Tris-HCl, pH 8.0, 1 mM β-ME and concentrated (Amicon, YM10) and stored at -20°C. Chromatograph and SDS-PAGE analysis of CpcBA purification can be seen in Results (Figures19 and 20).

2.5 Interaction Assays

Interaction assays were performed to determine if any of the recombinant proteins interacted with each other. Reactions were performed in the same manner as described below for the CpcU and CpcS interaction *in vitro*. Protein concentrations were estimated from SDS-PAGE analysis. 100 μg of CpcU (all his-tagged proteins used this concentration of protein) was added to 50 μg (non-his tagged products used this desired concentration of protein) of CpcS and the proteins were left to interact for 15 minutes on ice. The Ni-NTA resin (150 μl) was spun for 4 minutes as 4000 * g. The supernatant was discarded and 500 μl of Buffer O were added and mixed with the resin. The resin was centrifuged, and the supernatant was discarded. The interaction was then added to the washed resin and allowed to mix for 1 hour on a rotating wheel at room temperature. The mixture was centrifuged for 5 minutes at 3000 * g. The supernatant was discarded and 500 μl Buffer A1 was added followed by centrifugation for 5 minutes at 3000 * g. The same process was followed for Buffer B and Buffer A2. Elution of the protein occurred by the addition of 40 μl Buffer C. An equal amount of 2X SDS loading buffer was added, and the sample was boiled, centrifuged at 12,000 * g for 5 minutes, and loaded on a 15% Tris SDS-PAGE gel (12 minutes at 100 volts; 45 minutes at 200 volts).

2.5.1 Western Blot Analysis

Western Blots were performed to identify the non-His tagged proteins that copurified with the His tagged protein. Two 15% SDS-PAGE gels were run (one was loaded with prestained molecular weight standards). One gel was stained with Coomassie blue and the other one was transferred to a PVDF membrane. A piece of PVDF membrane was cut to the size of the gel. Whatman paper (3 mm) was cut into four pieces, each slightly larger than the gel. The

membrane was soaked in 100% methanol for one minute and then put into transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% (v/v) methanol, 0.1% SDS). The four sheets of Whatman paper were soaked in cold transfer buffer. The western blot transfer was set up as follows: two pieces of Whatman paper, the SDS gel, the PVDF membrane, and two pieces of Whatman paper. After each layer a glass rod was used to remove any air bubbles. The apparatus was put into the regular SDS gel box with a frozen cooling unit. Transfer buffer covered the sandwich, and the transfer performed at 100 volts for 1 hour. The blot was taken out and placed in blocking buffer (TTBS and 5% (w/v) non-fat dried milk) for 30 minutes at room temperature or overnight at 4°C. The blot was washed in TTBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) and then placed in 100 ml TTBS, and the primary antibodies were added (all antibodies made for these proteins worked best at 1:10,000 dilution). Primary antibodies were created in rabbits (W.M. Schluchter unpublished results). The primary antibodies were incubated with the blot for 1 hour at room temperature followed by a brief wash and three 5 minute washes of 100 ml TTBS each. The secondary antibodies (goat-anti rabbit; horse radish peroxidase conjugated antibodies purchased from BioRad) were added to 1:25,000 dilution and incubated with the membrane for 45 minutes at room temperature and washed the same as after the primary antibodies. The Immune-Star™ HRP luminal/enhancer and Immune-Star™ HRP peroxide buffer (Biorad) were mixed in a 1:1 ratio. The blot was incubated in the mixture for 1 minute, and chemiluminescence was detected by the Chemi-Doc System (Biorad). Blots were stored in TTBS in case they needed to be stripped and probed with other antibodies.

2.6 *In vitro* Bilin Addition Reactions with PCB

Bilin addition assays were performed using phycocyanobilin cleaved and purified from

Spirulina sp. These reactions contained 1 ml CpcBA (final concentration for reaction: 1 mg/ml) and 100 μl pAED4 for control reactions (final concentration for reaction: 1 mg/ml), 200 μl CpcS (final concentration for reaction: 1 mg/ml), 100 μl CpcU (final concentration for reaction: 1 mg/ml), 100 μl CpcT (final concentration for reaction: 1 mg/ml), 100 μl CpcV (final concentration for reaction: 1 mg/ml), or a combination of these. Differing amounts of the proteins were added because some of the proteins were more concentrated than others. The proteins were incubated on ice for 15 minutes before PCB was added to 10 μM concentration from a 2 M stock in DMSO. The reactions were then placed in a water bath at 30°C for 2 hours, in the dark. Fluorescence and absorbance spectra were taken; a sample of each reaction (20 μl) was taken for SDS-PAGE analysis.

2.6.1 Absorbance and Fluorescence Spectra

Fluorescence emission spectra were acquired with slits set at 3 nm (excitation and emission) and the excitation wavelength set at 590nm using a PTI international (model QM-1) fluorometer. This fluorometer is equipped with a 75-W continuous xenon arc lamp as a light source. Spectra were taken from 600-750 nm.

The absorbance spectra were gathered on a dual-beam Lambda 35 UV-Vis spectrophotometer (Perkin Elmer, Shelton, CT.). The blank for absorbance scans was 1 ml of Buffer O. Absorbance spectra were taken from 450-750 nm.

2.7 PcyA Purification

The *pcyA* expression plasmid was kindly provided by Dr. J. C. Lagarias. This enzyme

was overexpressed as a GST fusion and purified using glutathione agarose (from Sigma) and the following procedure (modified from Frankenburg *et al*., 2001; Kohchi *et al*., 2001: Frankenburg and Lagarias, 2003). The DNA was transformed and protein was overexpressed at 37°C. Cells were grown for 4 hours, induced with IPTG, and grown for another 4 hours. Protein was extracted in the same way as for CpcS. To swell the lyophilized glutathione agarose powder, 10 ml of ddH_20 was added for at least 2 hours. 5 ml of glutathione resin was washed with 2 column volumes of equilibration buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), and the GST-PcyA whole cell extract was added. The whole cell extract was allowed to flow through two times fast and one time slowly. The next step was to wash the column with two column volumes of equilibration buffer. The protein was eluted with two aliquots (10 ml each) of elution buffer (50 mM Tris-HCl, pH 8.0, 5 mM oxidized glutathione). The protein was dialyzed at 4°C against 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM β-mercaptoethanol.

2.8 *In vitro* Bilin Addition Reactions with PcyA

Biliverdin is reduced by PcyA in two sequential, 2-electron reductions using reduced ferredoxin to produce phycocyanobilin (Frankenburg *et al*., 2001; Frankenburg and Lagarias, 2003). Only a small amount of PCB is produced by PcyA (concentration of PCB is more like *in situ*), so almost no mesobiliverdin (MBV) is produced. Four reactions were set up containing the following: **1)** four ml of purified CpcB/CpcA (total of 1 mg/ml) and 200 μl of pAED4 control extract **2)** four ml of CpcB/CpcA and 200 μl purified CpcS (total of 1 mg/ml) **3)** four ml CpcB/CpcA and 600 μl purified HT-CpcU (total of 1 mg/ml) **4)** four ml CpcB/CpcA and 200 μl CpcS and 600 μl HT-CpcU. A separate series of reactions were set up with CpcT: **1)** four ml CpcB/CpcA (total of 1 mg/ml) and 200 μl pAED4 for a control reaction **2)** four ml CpcB/CpcA

and 200 μl CpcT (total of 1 mg/ml). The following were also added to each reaction mixture: Hepes pH 7.3, to 50 mM, $MgCl_2$ to 1 mM, 6.5 mM glucose-6-phosphate, 1.6 mM $NADP^+$, 1.1 u/ml glucose-6-phosphate dehydrogense, 4.6 μM recombinant *Synechococcus* sp. PCC 7002 ferredoxin (Schluchter, 1994) , 0.025 u/ml Spinach FNR, 10 μM BSA, 5 μM biliverdin (Porphyrin Products, Logan, UT), and 10 μM PcyA (Frankenburg *et al*., 2001; Frankenburg and Lagarias, 2003). Concentration of biliverdin was determined by using the extinction coefficient ε_{696}=30.8$mM^{-1}cm^{-1}$ (Lamparter, Esteban, and Hughes, 2001). Reactions were incubated in a 30°C water bath for two hours in the dark. Another aliquot of biliverdin was added (for a final concentration of 10 μM), and the reactions were allowed to continue for another two hours in the 30°C water bath. Reactions were centrifuged for 10 minutes at 10,000 rpm in centrifuge. Fluorescence and absorbance spectra were taken as described above.

To further clean up the reactions, small DEAE columns were poured and equilibrated with two column volumes of Na phosphate buffer, pH 7.0. Each reaction, plus 100 μl phycocyanin, was loaded onto separate DEAE columns and put though a gradient of Na phosphate buffers, pH 7.0. 5 mM Na phosphate buffer was added and a stepwise gradient was established using 10 mM, followed by 50 mM, 100 mM, and finally 150 mM Na phosphate buffer. The colored products were collected after the addition of 100 mM Na phosphate buffer, pH 7.0. The samples were then dialyzed exhaustively against 50 mM Na phosphate buffer, pH 7.0, with 1 mM β-mercaptoethanol at 4°C. The reactions were concentrated (Amicon YM10) five fold, and a sample was taken for SDS- PAGE and zinc staining (described above).

2.9 Tryptic Digestion of Phycocyanin

The procedure was taken from Arciero *et al*. (1988a). Concentrated samples, from PcyA

reactions, were diluted 1:4 with ddH_2O and were titrated to pH 2.0 with 1N HCl (pH was estimated using a pH strip). They were incubated 45 minutes in the dark at room temperature to allow protein unfolding. Trypsin (TPCK from Worthington) to 2% (w/w) was added from a stock solution in 1mM HCl. Ammonium bicarbonate was then added to 0.1M from a 1M stock. The reaction was then titrated to pH 7.5 with 1N NaOH and incubated two hours in a 30°C water bath. A second aliquot of trypsin was added and allowed to incubate for two hours at 30°C. The reaction was quenched by addition of glacial acetic acid to 30% (v/v). The peptides were then loaded onto C_{18} Sep-pak cartridges (Waters). The C_{18} cartridge was pre-wetted prior to sample loading with 100% Acetonitrile and washed with 100% 0.1% TFA (tri-fluoroacetic acid). The peptides were eluted with 1.5 ml 60% acetonitrile and 40% 0.1% TFA. The samples were dried down using a Speed Vac at 30°C in the organic mode. Samples were stored in the dark at –20 °C until HPLC analysis.

2.10 High Performance Liquid Chromotography (HPLC)

Tryptic peptides were separated on a C_{18} RP-HPLC column (5 mm x 10 mm x 250 mm; Waters Corporation) using a Waters HPLC equipped with a 600E pump and a photodiode array detector. Separation was carried out as described Arciero *et al.*, 1988a, using 0.1M Na phosphate, pH 2.1 (buffer D) and 100% acetonitrile (ACN) (buffer A). The program was as follows with a flow rate of 1.5 ml/min: starting conditions 20% buffer A and 80% buffer D, 3-31 minute gradient from 20% buffer A to 80% buffer A, 32-39 minute gradient from 80% buffer A back to starting conditions of 20% buffer A. Bilin-containing peptides were monitored at 600 nm and the retention time of each peak was determined.

RESULTS

3.1 Creation of *cpcBA, cpcS, cpcU, cpcV,* and *cpcT* Clones

After CpcE and CpcF were characterized as bilin lyases (Fairchild and Glazer, 1994; Swanson *et al*., 1992; Zhou *et al*., 1992), Dr. Schluchter's lab, along with her collaborators at Pennsylvania State University, decided to look at *Synechococcus* sp. PCC 7002 to determine if this organism also had bilin lyases. This was made easier after the genome of *Synechococcus* sp. PCC 7002 was completely sequenced (Li *et al*., unpublished). Cobley *et al*., (2002) found a gene cluster in *Fremyella diplosiphon* that contained *cpeS* and *cpeT*. The exact function of these genes was not known, but mutants lacking *cpeT* had no phycoerythrin. They believed that CpeT was involved in transcription of the phycoerythrin operon (J. Cobley, personal communication). It was found that *cpeS* and *cpeT* are present in most species of cyanobacteria, even those that do not produce phycoerythrin. Three genes were found to be paralogs of *cpeS* in *Synechococcus* sp. PCC 7002 (*cpcS, cpcU, cpcV*) and one gene was found to be a paralog of *cpeT (cpcT)*. The genes were amplified by PCR and cloned as described in Materials and Methods. To determine if the plasmids purified from transformants were correct, another restriction enzyme digest was performed using the same restriction enzymes. The transformants with both insert and vector were then sequenced to determine if the clones were correct.

The amino acid sequences were aligned using MacVector (Accelrys, San Diego, Ca.). Each of the four proteins from *Synechococcus* sp. PCC 7002 was aligned to translations of five different cyanobacterial genomes (Genbank and Cyanobase): *Synechocystis* sp. PCC 6803, *Fremyella diplosiphon, Gloeobacter violaceus* PCC 7421, *Prochlorococcus marinus* SS120, and *Nostoc* sp. PCC 7120. Using ClustalW (v1.4) multiple sequence alignment, four different

alignments were created (one for each protein) (Figures 10-13). CpcS in *Synechococcus* sp. PCC 7002 is very similar in amino acid sequence to slr2049 in *Synechocystis* sp. PCC 6803 with 76.9% similarity (Figure 10). Similarity scores for the CpcS alignment are as follows: *Gloeobacter violaceus* PCC 7421 with 68% (gene name *glr1191*), *Nostoc* sp. PCC 7120 with 64.1% (gene name *alr0617*), *Fremyella diplosiphon* with 44.7% (gene name *cpeS*), and *Prochlorococcus marinus* with 41.4% (gene name *Pro034*). It makes sense that CpcS from *Synechococcus* sp. PCC 7002 is most similar to slr2049 from *Synechocystis* sp. PCC 6803, as both organisms only contain phycocyanobilin and the phycobiliproteins phycocyanin and allophycocyanin.

CpcU has the lowest similarity scores among the three *cpeS*-like genes (Figure 11). The similarity scores for CpcU are as follows: *Nostoc* sp. PCC 7120 with 48.1% has the highest similarity (gene name *alr0617*), *Synechocystis* sp. PCC 6803 with 43.7% (gene name *sll0853*), *Prochlorococcus marinus* SS120 with 40.1% (gene name *Pro0343*), *Fremyella diplosiphon* with 35.2% (gene name *cpeS*), and *Gloeobacter violaceus* PCC 7421 with 34.7% (gene name *glr1614*).

The CpcV alignment (Figure 12) showed it has the most amino acid similarity to *Gloeobacter violaceus* PCC 7421 (gene name *gll1531*) with 60.6% similarity. Further similarity scores for CpcV were: *Nostoc* sp. PCC 7120 with 59.1% (gene name *all5292*), *Prochlorococcus marinus* SS120 with 36.4% (gene name *Pro0343*), *Synechocystis* sp. PCC 6803 with 35.1% (gene name *slr2049*), and *Fremyella diplosiphon* with 34.1% (gene name *cpeS*).

CpcT was aligned to the same five cyanobacterial species as the *cpeS*-like sequences (Figure 13). CpcT has the most amino acid similarity to slr1649 from *Synechocystis* sp. PCC

Alignment of CpcS

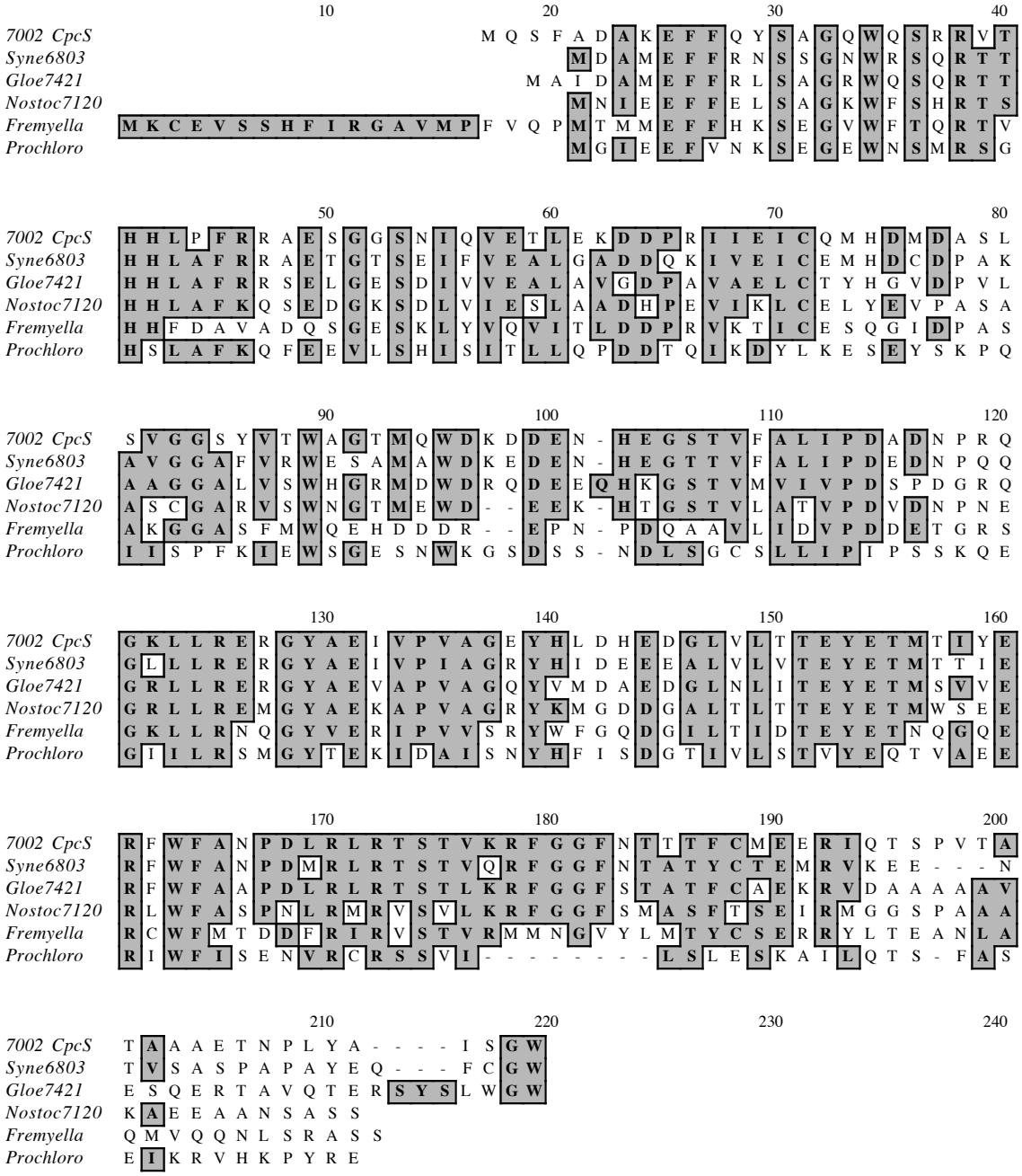


Figure 10: Alignment of amino acid sequences of CpcS presumed paralogs from different cyanobacteria. *Synechococcus* sp. PCC 7002 gene name *cpcS, Synechocystis* sp. PCC 6803 gene name *slr2049*, *Fremyella diplosiphon* gene name *cpeS, Gloeobacter violaceus* PCC 7421 gene name *glr1191*, *Prochlorococcus marinus* SS120 gene name *Pro0343*, and *Nostoc* sp. PCC 7120 gene name *alr0617*. Boxed in grey are the conserved amino acid residues.

Alignment of CpcU

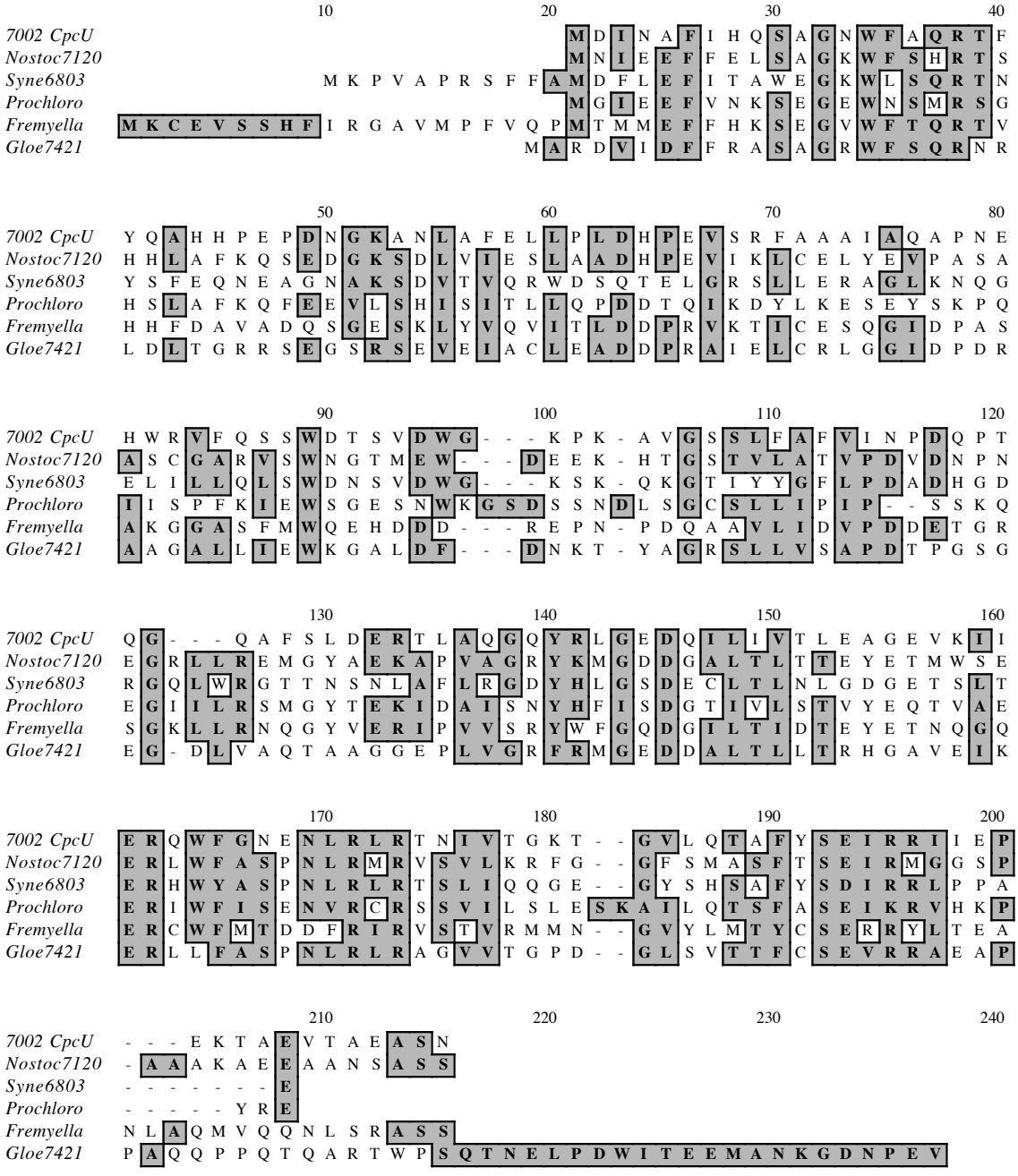


Figure 11: Alignment of amino acid sequences of CpcU presumed paralogs from different cyanobacteria. *Synechococcus* sp. PCC 7002 (gene name *cpcU*), *Synechocystis* sp. PCC 6803 (gene name *sll0853*), *Fremyella diplosiphon* (gene name *cpeS), Gloeobacter violaceus* PCC 7421 (gene name *glr1614*), *Prochlorococcus marinus* SS120 (gene name *Pro0343*), and *Nostoc* sp. PCC 7120 (gene name *alr0617*). Boxed in grey are the conserved amino acid residues.

Alignment of CpcV

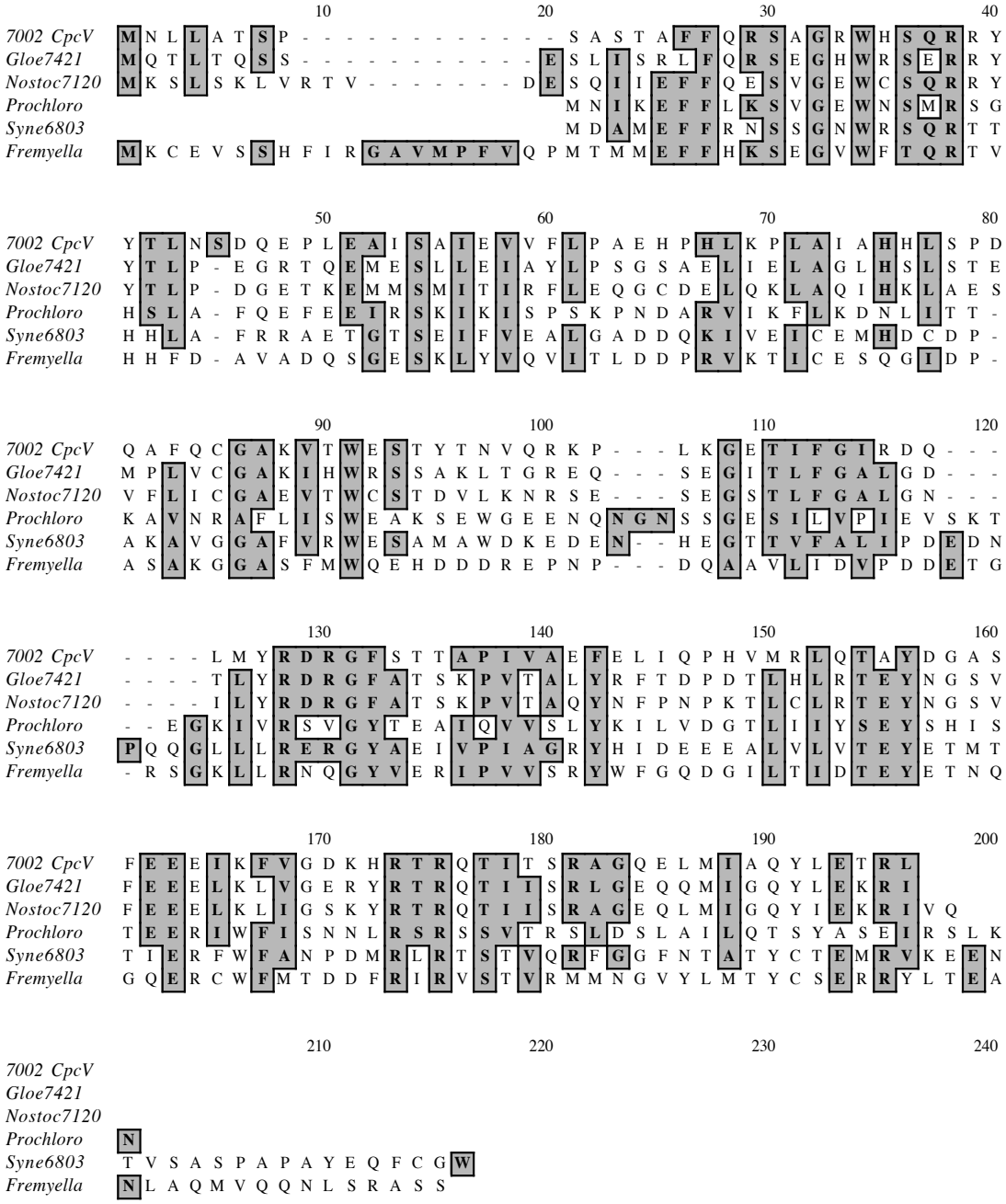


Figure 12: Alignment of amino acid sequences of CpcV presumed paralogs from different cyanobacteria. *Synechococcus* sp. PCC 7002 (gene name *cpcV)*, *Synechocystis* sp. PCC 6803 (gene name *slr2049)*, *Fremyella diplosiphon* (gene name *cpeS*)*, Gloeobacter violaceus* PCC 7421 (gene name *gll1531*), *Prochlorococcus marinus* SS120 (gene name *Pro0343*), and *Nostoc* sp. PCC 7120 (gene name *all5292*). Boxed in grey are the conserved amino acid residues.

6803 with 72.4% similarity; this is the same as with CpcS but not with CpcU and CpcV. The rest of the similarity scores for CpcT are as follows: *Nostoc* sp. PCC 7120 with 65.7% (gene name *all5339*), *Gloeobacter violaceus* PCC 7421 with 57.1% (gene name *glr1182*), *Fremyella diplosiphon* with 51.7% (gene name *cpeT*), and *Prochlorococcus marinus* SS120 with 46.8% (gene name *Pro0342*).

3.2 Overproduction of CpcS, CpcU, CpcV, CpcT, and CpcBA

For CpcS, CpcU, CpcV, and CpcT to be tested for their lyase activity (bilin attachment activity), the expression plasmids were transformed into BL21 or BL21*DE3* cells for overproduction as described in Materials and Methods. Expression cells were harvested and analyzed by SDS-PAGE (Figure 14). Figure 14 shows both the samples taken before induction (t=0) and samples taken after induction (t=4). Lanes 1 and 2 show the overexpression of CpcS at t=0 (protein induced) and t=4 (4 hours after induction. CpcV overexpression can be seen in lanes 12 and 13(t=0 and t=4). Lanes 4, 6, 9, and 11 show CpcU and CpcV (his-tagged) after induction. These lanes show that there is more protein being made at t=4, but it is not as much as the t=4 samples of proteins from proteins with pAED4 vectors. This may be because of the different promoter utilized (T^7 versus P^{Trc}). Figure 14 also shows the relative size of these proteins. These proteins are about the size that was calculated. Table 2 shows the calculated molecular weights and isoelectric points for each of the proteins. The cells were harvested and were purified as described in Materials and Methods.

CpcS was purified using DEAE anion exchange chromatography (described in Materials and Methods). A chromatograph of the CpcS purification (Figure 15) shows the absorbance and conductivity. Fractions containing protein were collected and SDS-PAGE analysis (Figure 16)

Alignment of CpcT

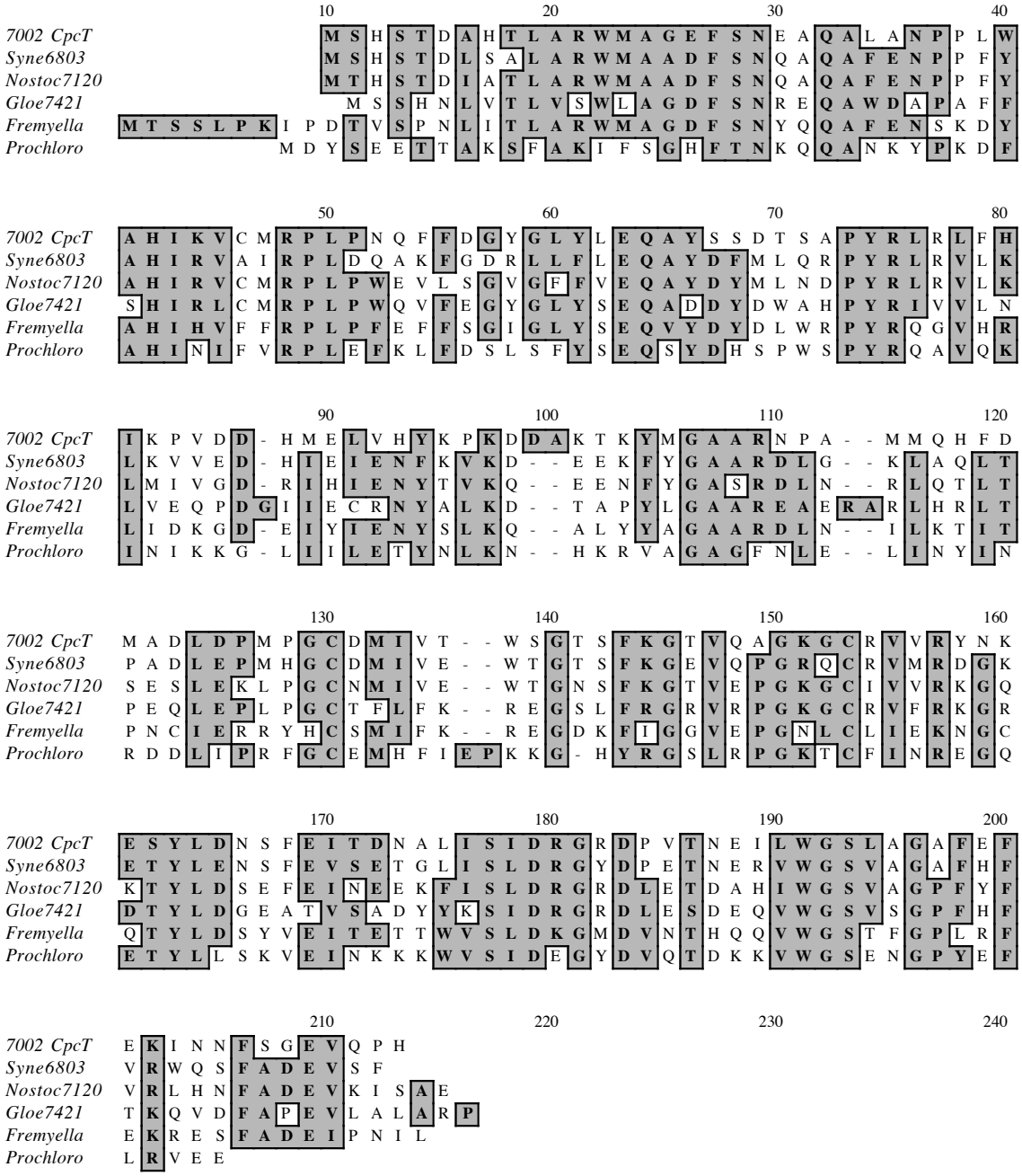


Figure 13: Alignment of amino acid sequences of CpcT presumed paralogs from different cyanobacteria. *Synechococcus* sp. PCC 7002 (gene name *cpcT*), *Synechocystis* sp. PCC 6803 (gene name *slr1649*), *Fremyella diplosiphon* (gene name *cpeT*), *Gloeobacter violaceus* PCC 7421 (gene name *glr1182*), *Prochlorococcus marinus* SS120 (gene name *Pro0342*), and *Nostoc* sp. PCC 7120 (gene name *all5339*). Boxed in grey are the conserved amino acid residues.

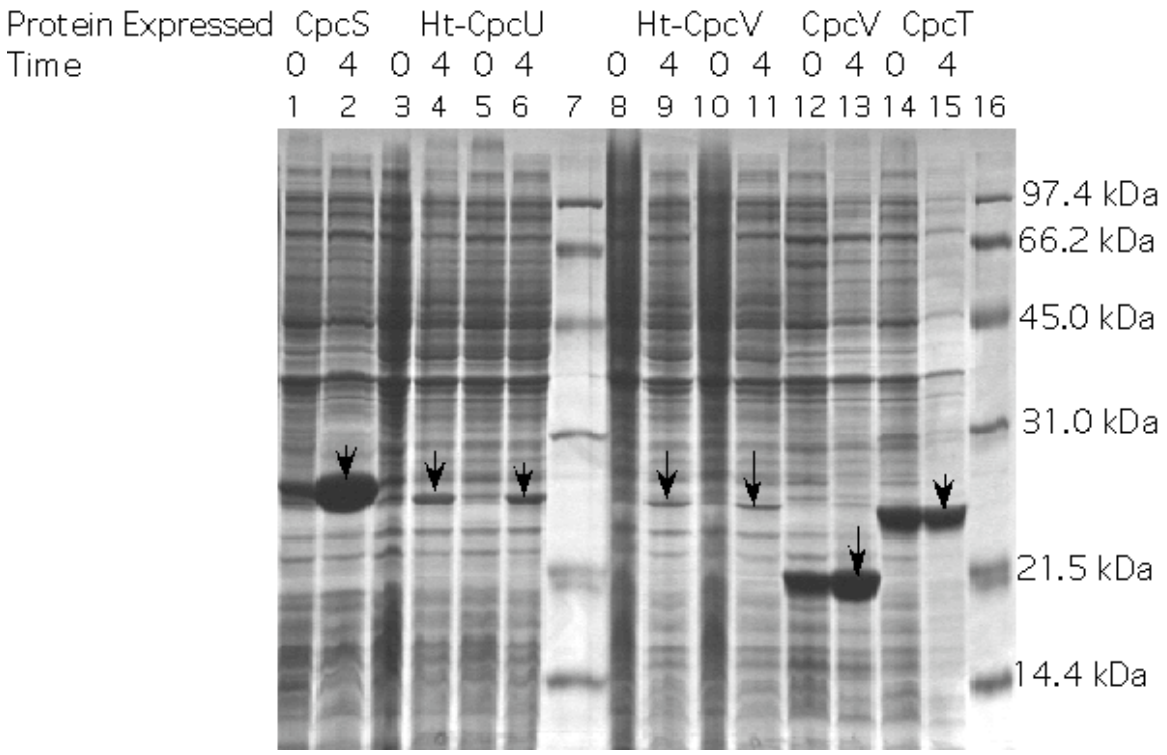


Figure 14: SDS-PAGE analysis of overexpression of CpcS, HT-CpcU, HT-CpcV, CpcV, and CpcT in *E. coli* 1 ml samples were taken at the time of induction (t=0) and 4 hours after induction with IPTG (t=4). The addition of IPTG inactivates a transcriptional repressor that controls the expression of the cloned genes. HT stands for histidine tag. Lane 1 contains CpcS t=0, lane 2 contains CpcS t=4, lane 3 and 5 contain HT-CpcU t=0, lane 4 and 6 contain HT-CpcU t=4, lane 7 is the low molecular weight standard (S), lane 8 and 10 contain HT-CpcV t=0, lane 9 and 11 contain HT-CpcV t=4, lane 12 contains CpcV t=0, lane 13 contains CpcV t=4, lane 14 contains CpcT t=0, lane 15 contains CpcT t=4, and lane 16 contains low molecular weight standard (S).

Table 2: Calculated molecular weights and Isoelectric points

Protein	Calculated Molecular Weight	Calculated Isoelectric Point
CpcS	22,526.3 kDa	4.79
CpcU (his-tagged)	23,471.71 kDa	5.53
CpcV	19,851.25 kDa	7.44
CpcV (his-tagged)	22,728.22 kDa	6.57
CpcT	22,515.91 kDa	5.68
CpcB	18,334.58 kDa	5.34
CpcA	17,620.5 kDa	5.68

was performed to determine which fractions had pure CpcS contained in them. Lanes 1 and 2 show fractions from the smaller peaks before the large peak between 120 and 150 minutes. One of the fractions from the large peak can be seen in Lane 3. This is the most pure fraction. Lanes 4, 5, and 6 show fractions taken after the peak and no pure CpcS was seen. Fractions included in the large peak were pooled together, dialyzed, and concentrated down for use later.

CpcT was also purified using DEAE anion exchange chromatography (described in Materials and Methods). The chromatograph of CpcT purification (Figure 17) shows absorbance and conductivity. Fractions containing protein were collected and SDS-PAGE analysis was performed. Figure 18 shows the SDS-PAGE gel of purified CpcT. Lanes 1 and 2 show fractions from some of the smaller peaks before the large peak (seen in Figure 17). Lane 3 shows the fraction from the one of smaller peak at about 110 minutes. Lane 4 shows the fraction from the major peak at 120-135 minutes. Lane 4 has more CpcT protein present. Lane 5 shows a fraction after the large peak. Lanes 3 and 4 both show pure fractions. These fractions were pooled together, dialyzed, and concentrated for later use.

CpcBA/pAED4 was transformed into Bl21*DE3* cells, and the CpcB and CpcA apoproteins were made by growing cultures at 30°C for 11 hours with no induction with IPTG, under ampicillin selection. Previous experiments were performed to optimize the production of protein, and it was found that the optimal amount of folded soluble protein was produced at 30°C with no induction. Previous research by Plank, Toole, and Anderson (1995) showed that when CpcB from *Synechocystis* sp. PCC 6803 was insertionally inactivated no CpcA was detected. Analysis of the mutants showed that by interrupting one of the phycocyanin subunits the other subunit was also absent (Plank, Toole, and Anderson, 1995). For this reason CpcB and CpcA were expressed together.

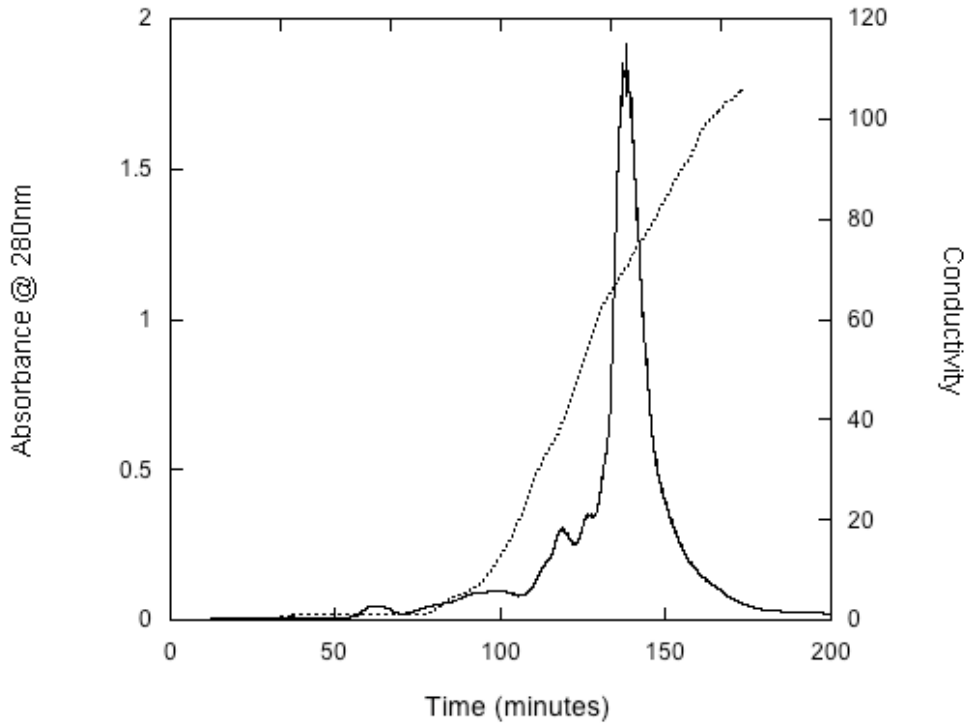


Figure 15: Purification on a DEAE column for CpcS. Absorbance at 280 nm is shown by the solid line while conductivity is shown by the dotted line. The most pure fraction of CpcS eluted at the major peak (between 120-135 minutes).

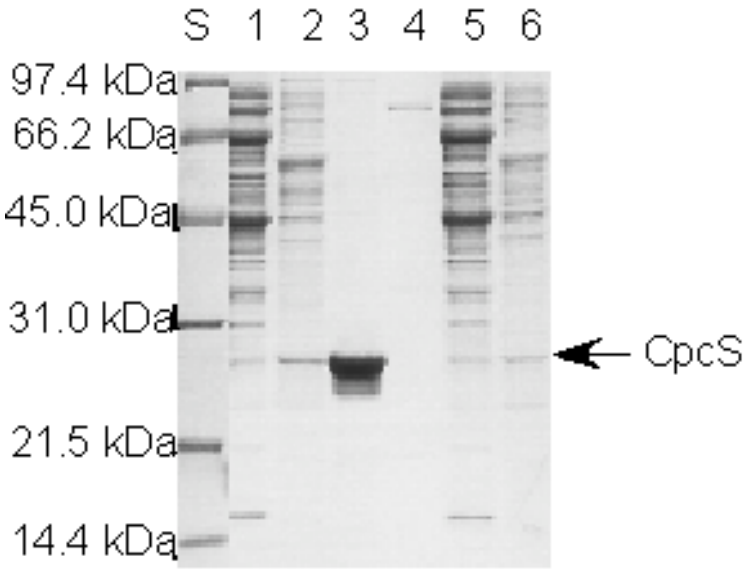


Figure 16: SDS-PAGE Analysis of CpcS DEAE purification. Lanes 1 and 2 show fractions from the smaller peaks before the large peak (seen in Figure 15). Fraction from the large peak can be seen in Lane 3. This is the most pure fraction. Lanes 4, 5, and 6 show fractions taken after the large peak- no pure CpcS.

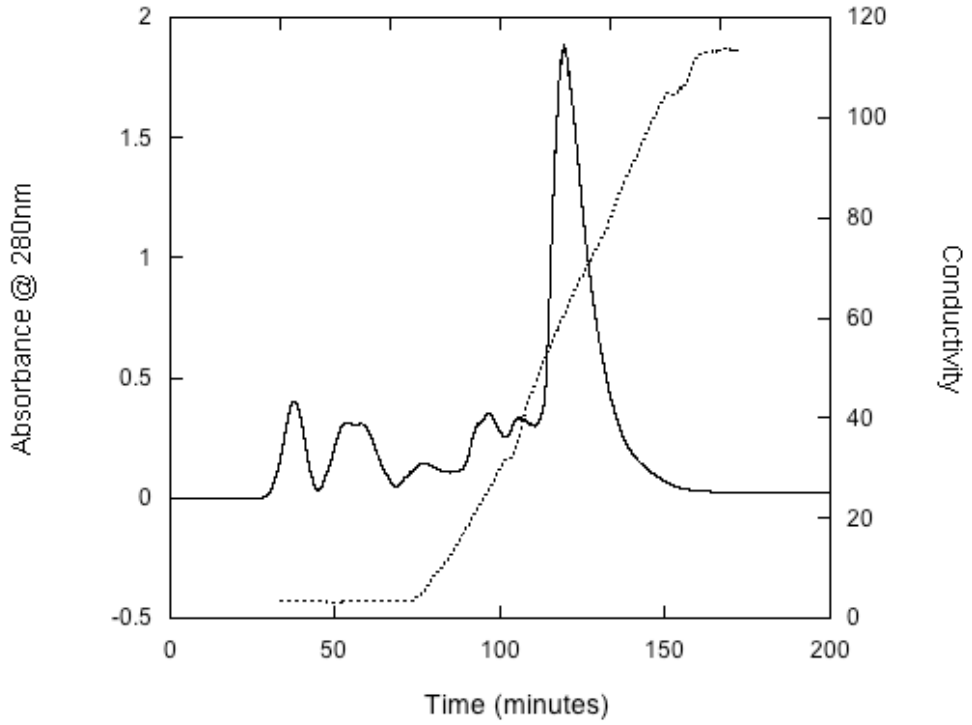


Figure 17: Purification on a DEAE column for CpcT. Absorbance at 280 nm is shown by the solid line while conductivity is shown by the dotted line. The most pure fraction of CpcT eluted at the major peak (between 120-135 minutes).

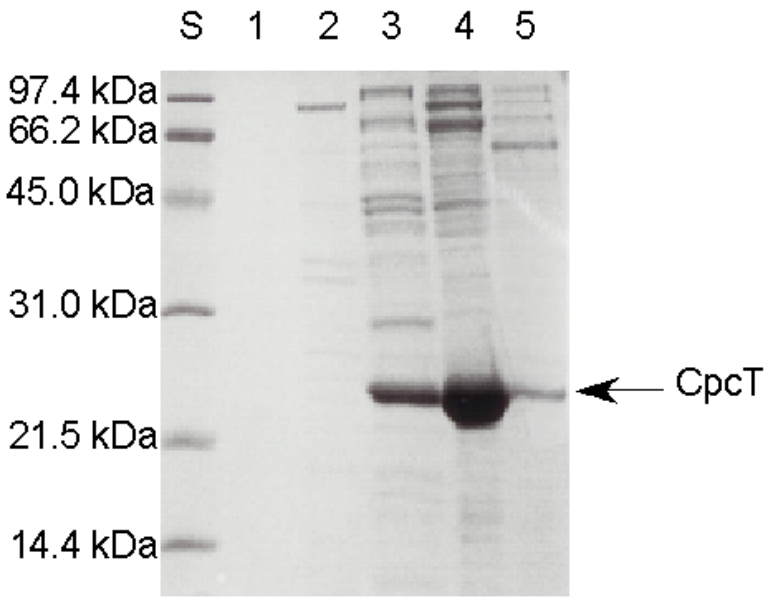


Figure 18. SDS-PAGE analysis of CpcT DEAE purification. Lanes 1 and 2 show fractions from some of the smaller peaks before the large peak (seen in Figure 17). Fraction from the large peak can be seen in Lane 3 and 4. Lane 3 shows the fraction from the smaller peak at about 110 minutes. Lane 4 shows the fraction from the major peak at 120-135 minutes. Lanes 3 and 4 are the most pure fractions. Lane 5 shows a fraction after the large peak.

Cells were harvested as described in Materials and Methods. Whole cell extract of CpcBA before any purification steps were taken can be seen in Figure 19. Purification steps were taken to reduce any interference in further experiments by *E. coli* proteins. The CpcBA DEAE chromatograph can be seen in Figure 20. SDS-PAGE analysis of DEAE anion exchange chromatography can be seen in Figure 21. SDS-PAGE of CpcBA from whole cell extract (Figure 21) shows different fractions from the purification. The purest fraction, and most concentrated, was found in lane 8 and corresponds to the large peak seen in figure 19 eluting around 90-100 minutes. No CpcBA was seen in Lanes 1-3. Lanes 5-7 show fractions from the smaller peaks around 50 and 75 minutes. Pure fractions (lanes 5-7 and 8) were pooled, dialyzed, and concentrated. Purified protein was used in further experiments.

3.3 Protein/Protein Interactions: CpcS/CpcU

Some enzymes are composed of two subunits, like CpcE/CpcF. It was important to find out if any of the proteins interacted with each other to form complexes. This was done by adding Ht-CpcU, Ht-CpcV, or Ht-CpcB with each different protein as well as different combination of CpcS, CpcV, and CpcT. In each reaction, one of the HT proteins was used as bait. Other, non-HT subunits were added as prey and were expected to be purified only if they could bind tightly to the HT bait. The only combination that interacted strongly and reliably was HT-CpcU and CpcS. HT-CpcU was added to purify CpcS, and this combination was allowed to interact for 30 minutes. HT-CpcU was then purified on a small amount of nickel resin (see Materials and Methods) and eluted with 40µl of buffer C. The results of this experiment can be seen in Figure 22, panel A. Lane 1 contains CpcS and HT-CpcU after the purification steps. Lane 3 and lane 4 show the purified CpcS and purified HT-CpcU, respectively before they were added together for

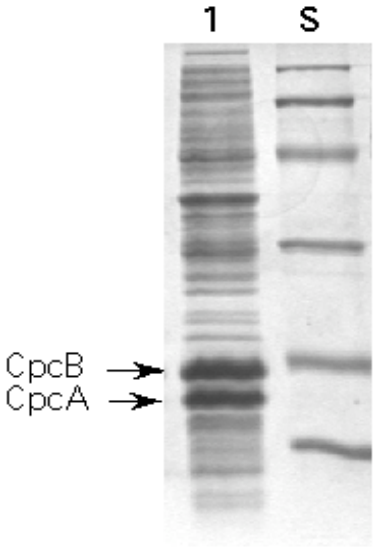


Figure 19: SDS-PAGE of E. coli whole cell extract containing CpcBA. CpcBA before DEAE and FPLC purification. The standard is marked S and the whole cell extract of CpcBA is in lane 1. This extract in lane 1 was used later for DEAE purification seen in figure 19.

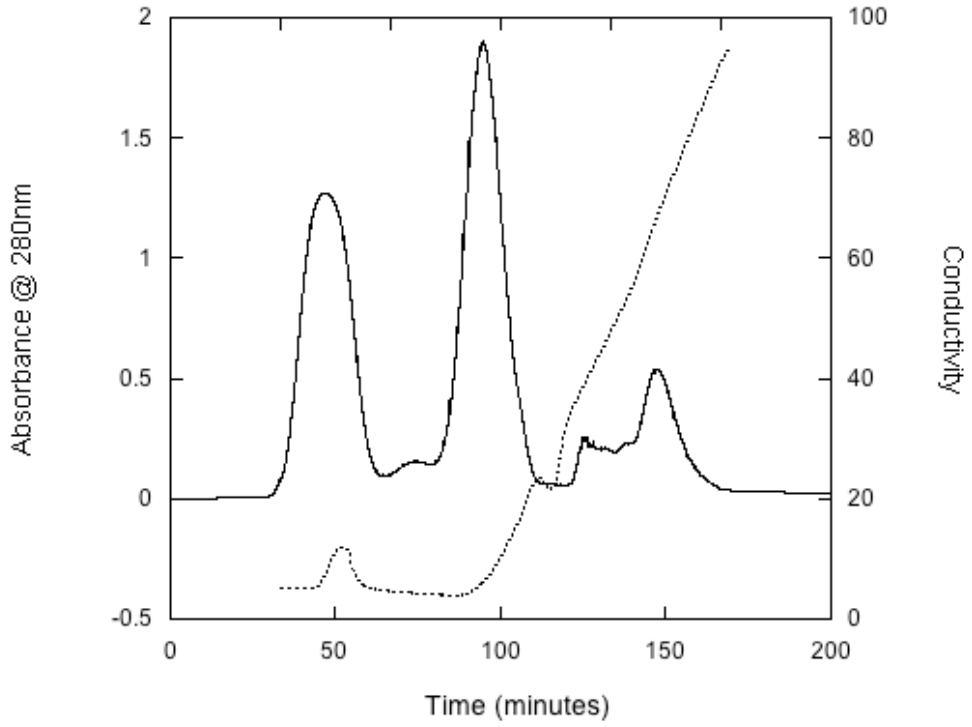


Figure 20: Purification of CpcBA on a DEAE cellulose column. Absorbance at 280 nm is shown by the solid line and conductivity is shown by the dashed line. The most pure fractions of CpcBA eluted at the second peak (about 90-102 minutes).

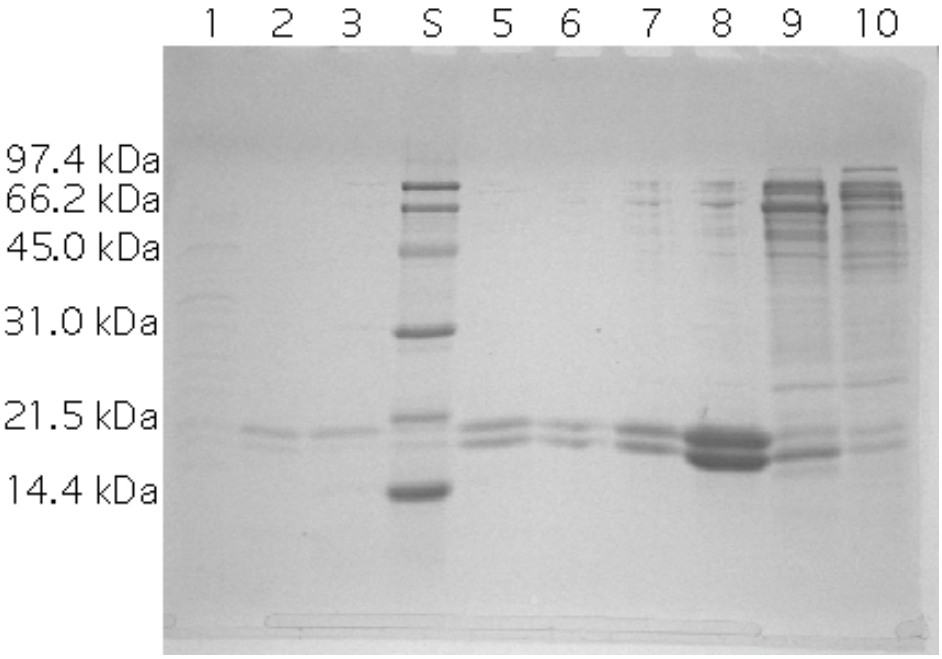


Figure 21: SDS-PAGE of CpcBA fractions after FPLC. SDS-PAGE of CpcBA from the whole cell extract shown in Figure 18. CpcBA was purified by DEAE, the fractions were run on a 15% Tris-HCl SDS polyacrylamide gel. The purest fraction was found in lane 8 and corresponds to the large peak seen in figure 19 eluting around 90-100 minutes. Lane 5-7 were also kept and pooled together for an addition pure sample. Both the products from lane 8 and lane5-7 were dialyzed and concentrated.

the reaction. In order to verify that CpcS was the protein that copurified with HT-CpcU, a western blot was performed shown in panel B. Primary antibodies were generated against recombinant CpcS in rabbit. Lane 1 is purified CpcS, lane 3 is CpcU, and lane 4 is the CpcS and HT-CpcU pull-down interaction assay eluate. Lane 2 is again the low molecular standard. Only lane 1 and lane 4 show bands because these are the only two places that CpcS is found on the gel. The antibodies only bind to CpcS and thus one band is seen in each lane where CpcS is present. This shows that indeed HT-CpcU is interacting with CpcS to form a complex that copurifies during Ni-NTA chromatography. A further experiment was performed to see if CpcS and CpcU for a 1:1 complex. Equal amounts of whole cell extracts of CpcS and HT-CpcU were added together and allowed to interact and were purified by Ni-NTA chromatography. The product was run on a 15% SDS-PAGE gel, and the results can be seen in Figure 23. Lane 1 shows that there are equal amounts of CpcU and CpcS in the interaction complex. This shows that there is 1.1 stoichiometry in the CpcSU complex.

3.4 Bilin Addition Reactions with CpcS/CpcU and CpcBA using PcyA Reactions

After CpcS and CpcU were seen shown to interact, assays were performed to determine if one or both subunits acted as a lyase for phycocyanobilin attachment to one of the two β phycocyanin cysteinyl residues. *In vitro* assays for chromophorylation were performed by adding CpcBA as a substrate and one or both subunits (CpcS, HT-CpcU, or CpcSU) to each assay. The synthesis of the chromophore precursor to phycocyanobilin is catalyzed by a enzyme called phycocyanobilin:ferredoxin oxidoreductase (PcyA). PcyA from *Nostoc* sp. PCC 7120 was used to produce phycocyanobilin *in situ*. This enzyme is dependent on ferredoxin and is responsible for the reduction of biliverdin to phycocyanobilin, (Frankenburg *et al*., 2001;

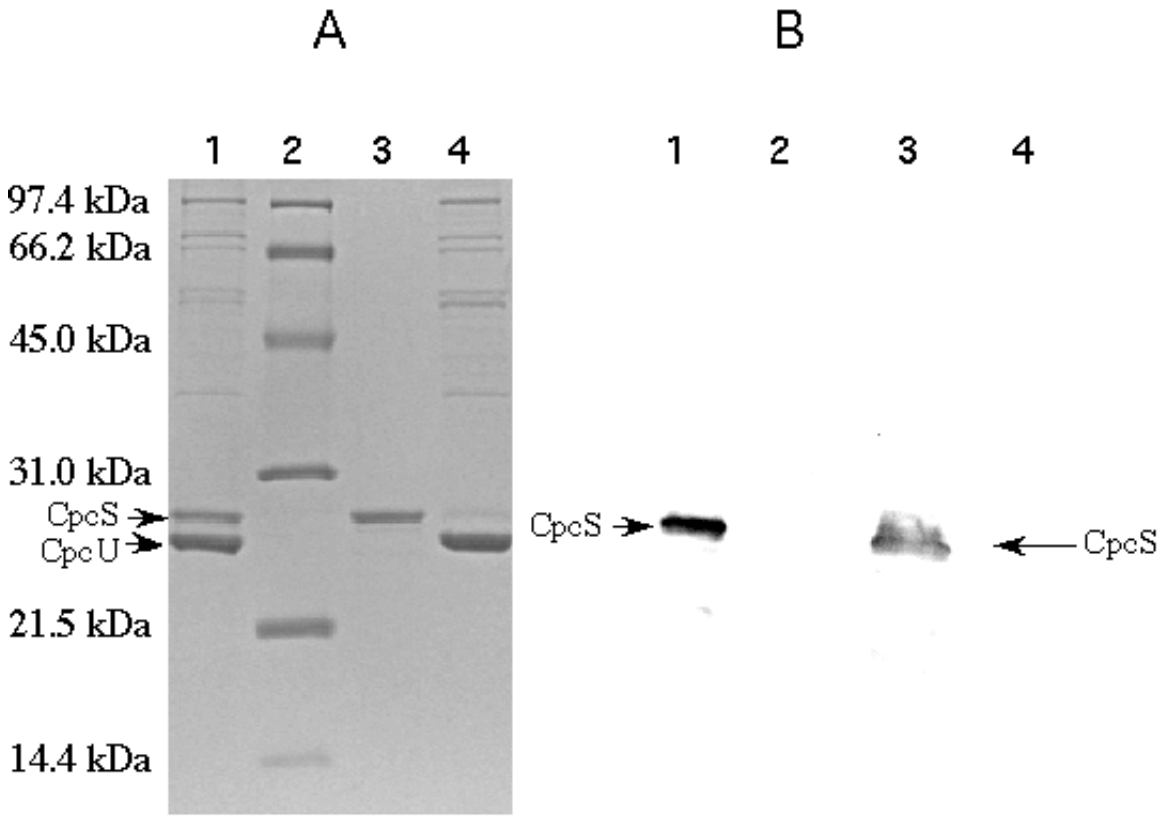


Figure 22: Protein/Protein interaction and western blot of CpcS/HT-CpcU. Panel A shows an SDS-PAGE gel of the interaction between HT-CpcU and CpcS. Lane 1 shows product of the interaction after purification has taken place. Lane 2 contains the low molecular standard (sizes of standard bands are shown to the left). Lane 3 is the CpcS purified extract that was used in the reaction. Lane 4 contains the HT-CpcU purified extract used. Panel B shows a western Blot using anti-CpcS antibodies were used detect CpcS. Lane 1 contains CpcS alone. Lane 2 is the low molecular standard. Lane 3 contains CpcU alone and lane 4 contains CpcS and CpcU

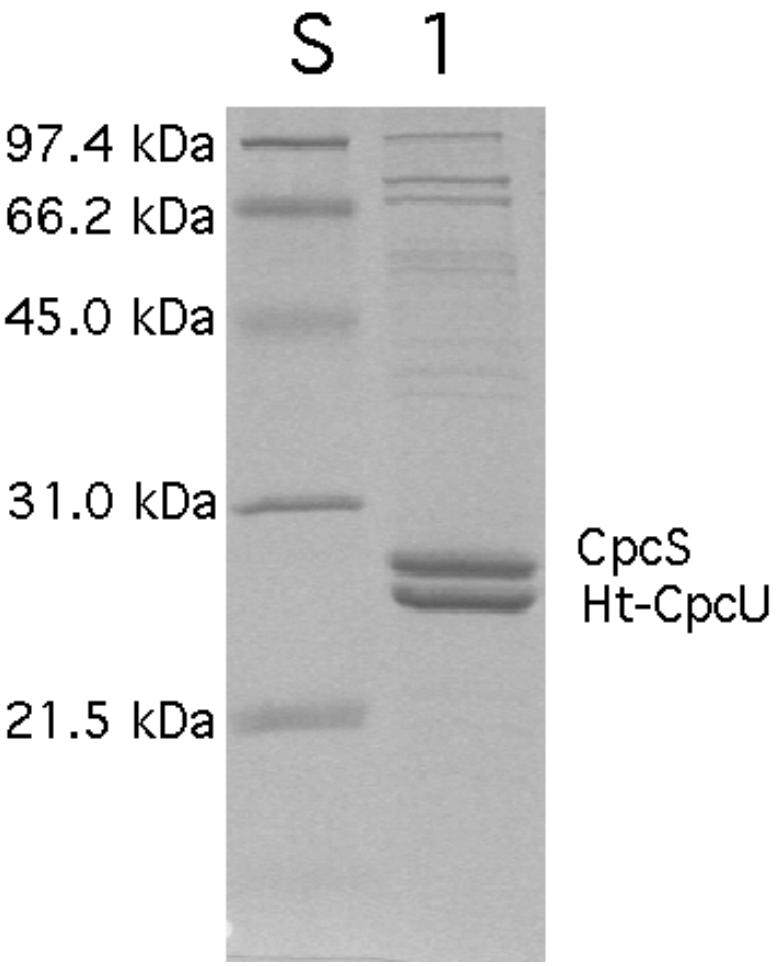


Figure 23: 1:1 Stoichiometry between CpcS and HT-CpcU. Low molecular weight standard marked as S (sizes of bands are shown to the left). Lane 1 shows equal amounts of CpcS and CpcU in the complex giving the complex a 1:1 stoichiometry between CpcS and CpcU.

Frankenburg and Lagarias, 2003). PcyA is a novel enzyme in that it requires no cofactors or metal, and it catalyzes a four electron reduction. It reduces the vinyl group on the D ring of biliverdin (see Figure 5 and 6) first followed by the reduction of the A ring. PcyA uses electron from ferredoxin to reduce biliverdin to phycocyanobilin (Hagiwara *et al*., 2006). Reactions were performed at 30°C with CpcBA alone (as control), CpcBA and CpcS alone, CpcBA and CpcU alone, and CpcBA with CpcS and CpcU together. Reactions were performed at 30°C because the PcyA enzyme from *Nostoc* is active at this temperature. After the four-hour reaction time, a clear color change from blue-green to blue could be seen for the reaction containing both CpcS and CpcU (CpcSU). No color difference was observed between the control reactions and either CpcS or CpcU alone. The absorbance and fluorescence spectra of these reactions can be seen in Figure 24 and 25 respectively. Figure 24 shows the product of the reaction with both CpcS and CpcU had a absorption maxima at 621 nm, while the control reaction and the single reactions with CpcS or CpcU had absorbance maxima of 629 nm and 631 nm. If the lyase is adding phycocyanobilin at the β-82 site than a peak at about 620 nm would be expected (Debreczeny *et al*., 1993), which is what is seen for CpcSU. The control reaction had an absorbance spectrum that is similar to the absorbance spectra of mesobiliverdin (the non-enzymatic unnatural product); (Arciero *et al*., 1988a&b).

The product of the CpcSU reaction was extremely fluorescent (Figure 25) and had a fluorescence emission maxima of 638 nm while the control reaction and the CpcS- and CpcU-single reactions were almost non-fluorescent with emission maxima of 647 nm. Energy absorbed by chromophores must either be transferred to another chromophore that is in close proximity or be released as fluorescence. The β-82 phycocyanobilin chromophore should fluoresce at approximately 640 nm (Debreczeny *et al*., 1993). Both the absorbance and

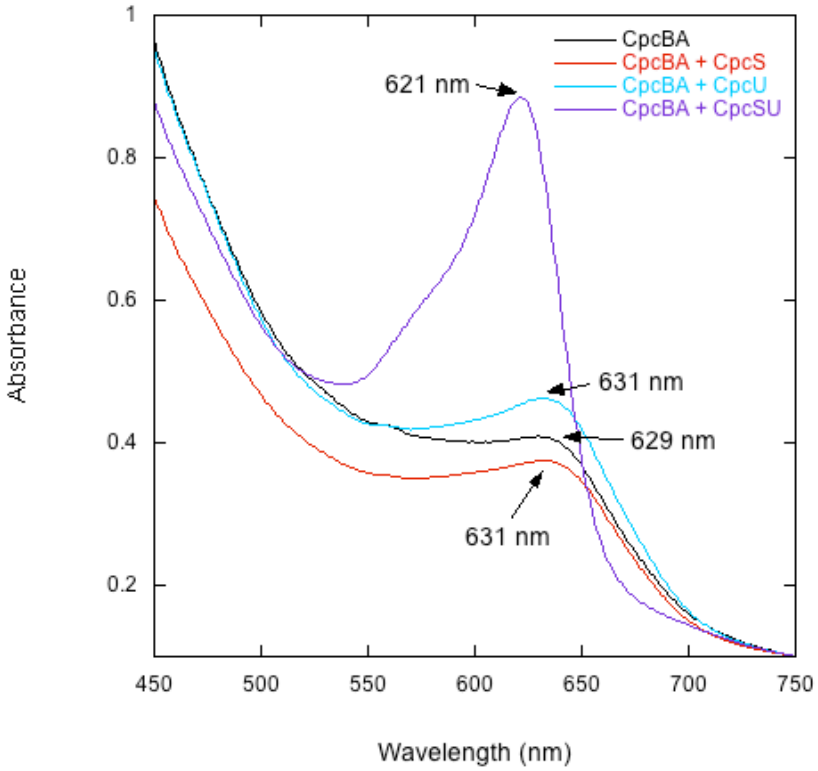


Figure 24: Absorbance emission spectra of *in vitro* PcyA bilin addition reactions with CpcS and CpcU and CpcBA. Absorbance spectra of reactions with CpcBA alone (control reaction=black line) and CpcBA with CpcS alone (red line), CpcU alone (light blue line), and CpcS and CpcU (purple line). Absorbance maximums are shown for the reactions.

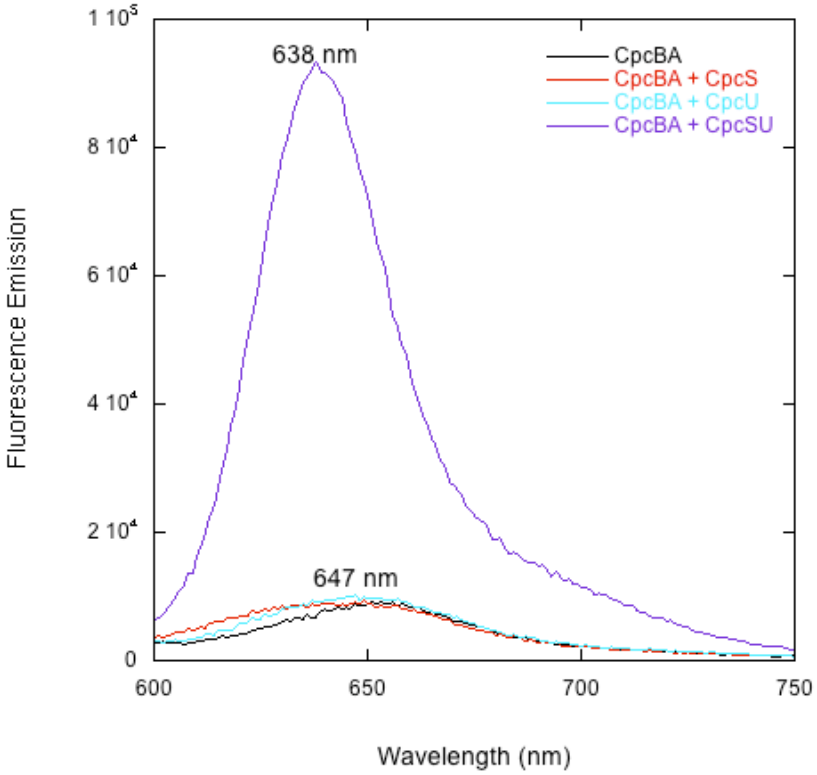


Figure 25: Fluorescence emission spectra of *in vitro* PcyA bilin addition reactions with CpcS and CpcU and CpcBA. Fluorescence spectra of reactions with CpcBA alone (control reaction=black line) and CpcBA with CpcS alone (red line), CpcU alone (light blue line), and CpcS and CpcU (purple line). Fluorescence was taken at 590 nm with 10 nm slits. The maximum for CpcBA with CpcS/CpcU was 638 nm, while all other maximums were 647 nm.

fluorescence spectra are consistent with addition of phycocyanobilin taking place at the β-82 cysteine site. However, because both CpcA (one addition site) and CpcB (two addition sites) are in the reaction mixture, it was necessary to determine which site contains a bilin added on to it. A 20μl aliquot of each reaction was run on an SDS-PAGE gel and stained with $ZnSO_4$ and later with Coomassie blue as described in materials and methods. $ZnSO_4$ allows bilin-containing polypeptides to be easily detected when the Zn^+ ions bind to the bilin in the polypeptide and become fluorescence in the presence of UV light (Raps, 1990). Figure 26 shows the zinc-stained and the Coomassie stained gel for PcyA reactions with CpcSU. Panel A shows phycocyanin from *Synechococcus* sp. PCC 7002, and the CpcBA product of CpcSU PcyA reaction. Lane 1 shows phycocyanin and it has 2 bands that correspond to the α and β subunits. Lane 2 shows the CpcBA reaction product of CpcSU, and it has only one fluorescent band corresponding to the β phycocyanin. Panel B of figure 26 shows the same gel after Coomassie blue staining.

3.5 Tryptic Digestion and Reverse-Phase HPLC: CpcS/CpcU

β phycocyanin has two phycocyanobilin addition sites; one at Cys-82 and one at Cys-153. In order to determine which site CpcSU was attaching phycocyanobilin to, tryptic digestion of CpcBA reaction products were performed. Reactions were carried out for 4 hours total at 30°C. The products of these reactions, as well as purified holo-phycocyanin from *Synechococcus* sp. PCC 7002, were digested with trypsin. Trypsin cleaves after the bond on the carboxyl terminus of positively charged arginine and lysine if they are not next to proline, so calculations of the size of the peptides can be made. This helps us to approximate when the peptides containing the bilin may elute from the HPLC column. The products of the trypsin cleavage were injected onto a C_{18} HPLC reverse phase column. The elution profile of the bilin-

peptides (taken at 600nm) can be seen in Figure 27. The top section of the figure shows the elution profile of tryptic digestion of phycocyanin. The lower half of the figure shows the elution profile of the tryptic peptides of the CpcSU-dependent CpcBA reaction product. There are three main peaks in the phycocyanin profile corresponding to the α-84, β-82, and β-153 cysteinyl residues were bilin addition can occur. The peptide containing the α-84 cysteine site eluted first at 20.1 minutes followed by the β-82 peptide at 23.1 minutes and the β-153 peptide at 30.1 minutes. This order of peptides is consistent with previously reported results from Arciero *et al*. (1988a). It is also consistent with the calculated mass of the tryptic peptides which are: α-84=1251 Da, β-82=1323 Da, and β-153=4075 Da (Shen *et al.*, 2006). The bottom half of the figure shows the elution profile for the CpcSU reaction. There is only one bilin-containing peak in this reaction product which has a retention time of 23.0 minutes. This corresponds to the retention time of the β-82 peptide, indicating that CpcSU is a lyase specific for bilin attachment at the Cys-82 on β phycocyanin.

3.6 Phycocyanobilin Addition with CpcT and CpcBA

3.6.1 PCB Reactions

CpcT was not shown to interact (form complexes) with any of the other proteins in any significant way. CpcT was a candidate for the β-153 phycocyanobilin lyase. Chromophorylation reactions were performed to test this hypothesis. CpcBA and CpcT were overexpressed in BL21*DE3* cells and purified as described in Materials and Methods. Bilin addition reactions were performed using CpcBA alone or CpcBA with CpcT. For these reactions, phycocyanobilin (10 mM total) purified from *Spirulina* sp. was the source of bilin.

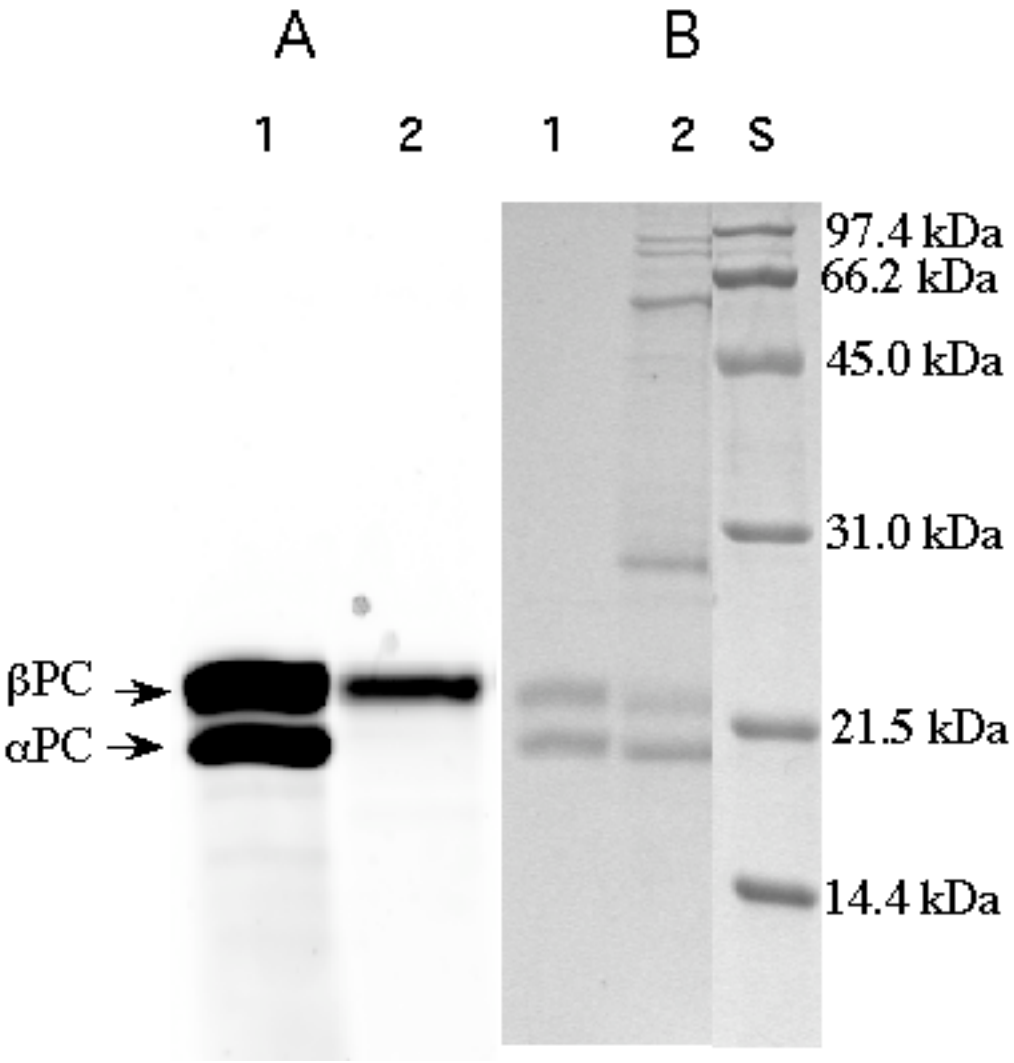


Figure 26: SDS-PAGE analysis of bilin addition reaction of PcyA products from CpcS and CpcU reactions. Panel A shows the zinc stain of wild type phycocyanin from *Synechococcus* sp. PCC 7002 and CpcSU PcyA reaction. For the phycocyanin, the top band corresponds to the β sites while the lower band corresponds to the α site where covalently bound bilins are found. Lane 2 shows CpcSU only has one band fluorescing and it corresponds to the β site. So CpcSU helps catalyze a covalent attachment of a bilin to one of the β sites. Panel B shows the same gel after Coomassie staining.

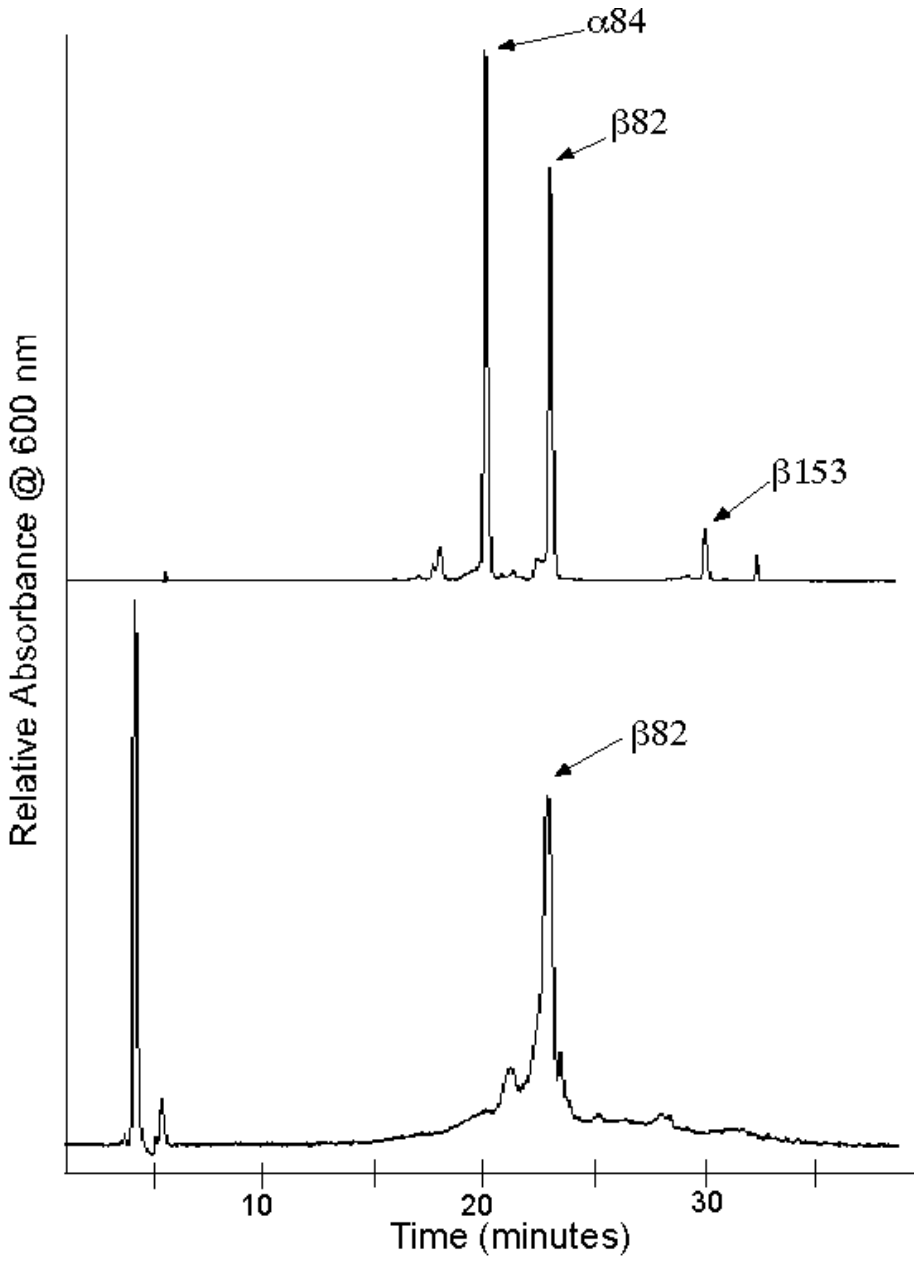


Figure 27: RP-HPLC separation of tryptic peptides from *in vitro* bilin addition assays (PcyA products) with CpcBA and CpcSU. Tryptic digestion elution pattern from RP-HPLC. The top half shows the elution pattern from wild type *Synechococcus* sp. PCC 7002 phycocyanin. The three bilin containing peptides are identified by arrows and show the cysteinyl residue that the peak corresponds to. The bottom half shows the elution pattern for PcyA reaction with CpcBA and CpcSU. This reaction only gives one peak at 23.0 minutes corresponding to the β-82 site.

After the addition on phycocyanobilin to the CpcT reaction, a color change was noted within 30 minutes. The color changed from blue-green to purple. The control reaction did not change color significantly. Absorbance and fluorescence spectra can be seen in figures 28 and 29 respectively. The absorption maximum of the CpcT reaction is 603 nm while that of the control reaction maximum is 638 nm. We would expect the maximum to be around 600 nm if the lyase is adding at the β-153 site (Debreczeny *et al*., 1993). The control reaction maximum is at 638 nm where mesobiliverdin has its absorbance maximum (Arciero *et al*., 1988a&b). In the CpcT reaction there is a slight shoulder at 638 nm which most probably from a competitive non-enzymatic product being created. Only a small amount of this competing reaction product is made. Figure 29 shows the fluorescence emission spectra for the same PCB reactions. The control reaction had a fluorescence maximum of 657 nm while the CpcT reaction had a fluorescence maximum of 624 nm. The CpcT reaction was extremely fluorescent while the control reaction had virtually no fluorescence. This is consistent with the control product being mesobiliverdin; mesobiliverdin has only 5-10% of the fluorescence that phycocyanobilin has (Arciero *et al*., 1988a&b; Fairchild and Glazer, 1994).

3.6.2 PcyA Reactions with CpcT

Because of the competitive non-enzymatic product made during the PCB reactions, PcyA reactions were performed. In these reactions there is almost no mesobiliverdin produced because only small amounts of phycocyanobilin are produced *in situ* (Frankenburg *et al*., 2001; Frankenburg and Lagarias, 2003). PcyA along with other cofactors (ferredoxin, biliverdin, etc.) were added to reactions containing just CpcBA or CpcBA with CpcT. After the 4-hour incubation period at 30°C, a color change from blue-green to purple could be seen. This color

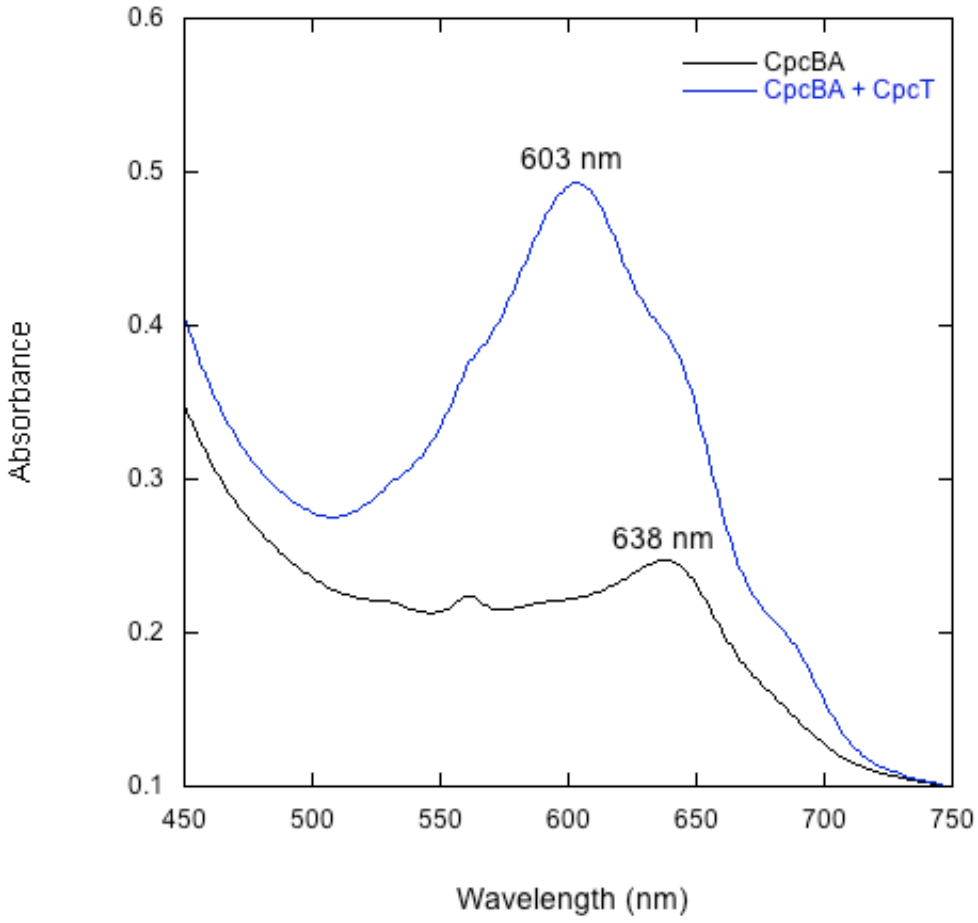


Figure 28: Absorbance spectra of *in vitro* PCB assays. Absorbance spectra of PCB reaction products with CpcBA and CpcBA with CpcT. The control reaction (CpcBA) is shown in black, while the CpcT reaction is shown in blue. The control reaction has a maxima at 638 nm while the CpcT reaction has a maxima at 603 nm.

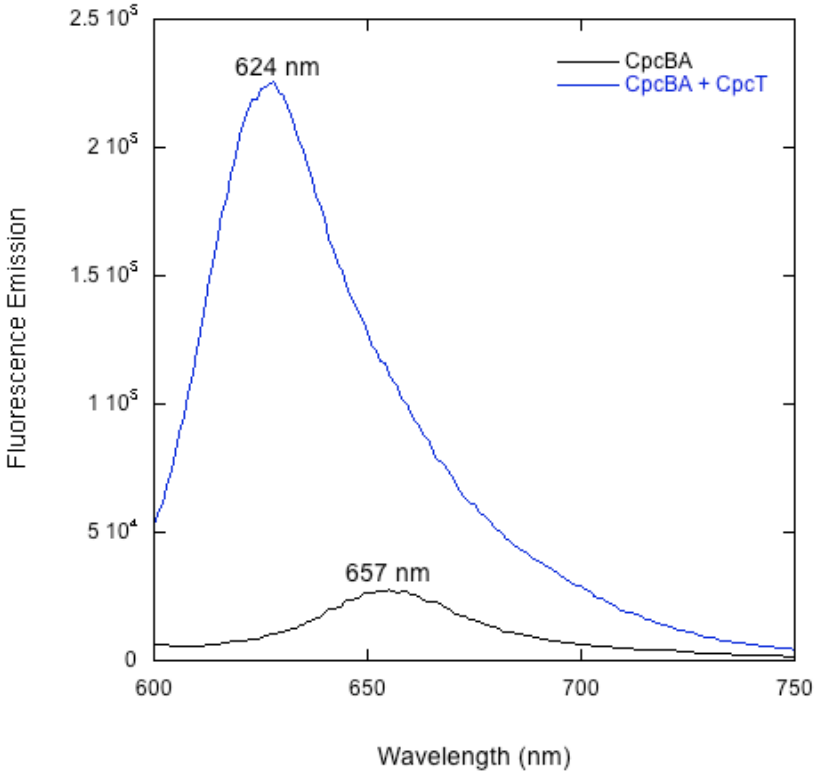


Figure 29: Fluorescence emission spectra of *in vitro* PCB assays. Fluorescence emission spectra of PCB reaction products with CpcBA and CpcBA with CpcT. The control reaction (CpcBA) is shown in black, while the CpcT reaction is shown in blue. Control reaction has a maxima at 657 nm while the CpcT reaction has a maxima at 624 nm.

change was slightly more pronounced than it was with the PCB reactions. The control reaction again had little to no color change. The absorbance and fluorescence spectra of the reactions can be seen in Figure 30 and 31 respectively. Figure 30 shows the product of the reaction with CpcT had an absorbance maximum at 597 nm, while the control reaction absorbance maximum of 638 nm. If the CpcT lyase is adding at the β-153 site we expect a maximum about 600 nm (Debreczeny *et al*., 1993), which is what we see for CpcT. The control reaction again had an absorbance spectra similar to that of mesobiliverdin (Arciero *et al*., 1988a&b). The CpcT reaction product was extremely fluorescent (Figure 31) and had a fluorescence emission maximum of 623 nm, while the control reaction had a maximum that could to be detected. Both the absorbance and fluorescence spectra give maxima that are consistent with addition of phycocyanobilin taking place at the β-153 cysteine.

Because CpcA (one addition site) and CpcB (two addition sites) are in the reaction mixture, we need to determine which site has a bilin attached to it. Aliquots were run on an SDS-PAGE gel and stained with $ZnSO_4$ and later with Coomassie blue as described in Materials and Methods. Figure 32 shows the zinc-stained and the Coomassie stained gel for PcyA reactions with CpcT. The left side shows the product of CpcT PcyA reaction. A bilin was attached only on the β site of CpcBA in the CpcT reaction. No attachment was seen on the control reaction (data not shown). The right side of figure 32 shows the same gel after Coomassie staining. CpcB, CpcA, and CpcT are identified on the gel.

3.7 Tryptic Digestion and Reverse-Phase HPLC: CpcT

For verification that CpcT catalyzes the attachment of phycocyanobilin to the β-153 cysteine, *in vitro* PCB reactions were performed as described above. The reaction contained

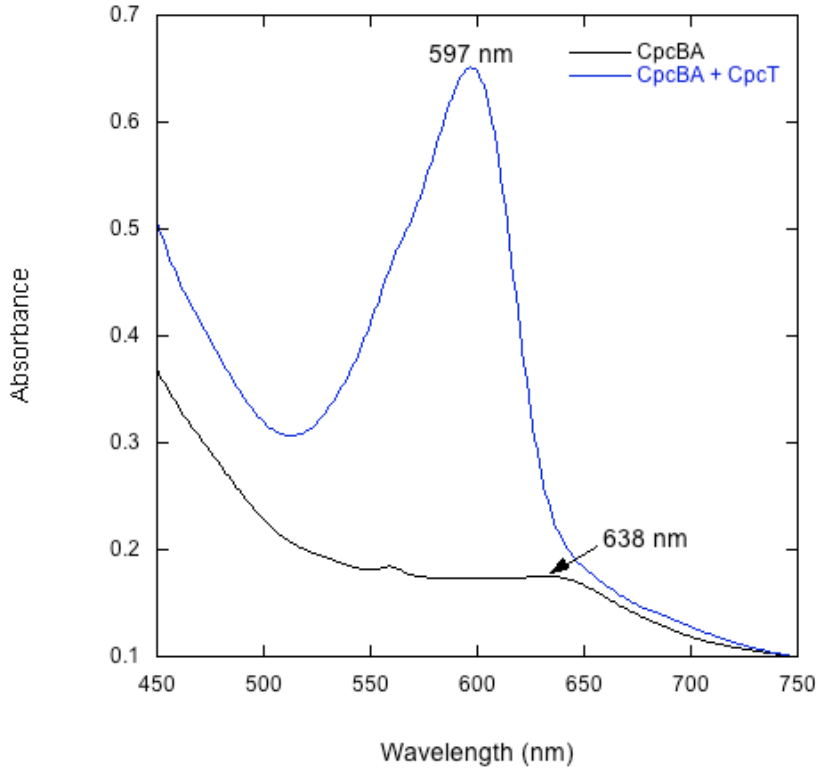


Figure 30: Absorbance spectra of *in vitro* PcyA bilin addition reactions with CpcT and CpcBA.
Absorbance spectra of reactions with CpcBA alone (control reaction=black line) and CpcBA with CpcT (blue line). Absorbance maximums are shown for the reactions. The control reaction has a maximum of 638 nm while the CpcT reaction has an absorbance maximum of 597 nm.

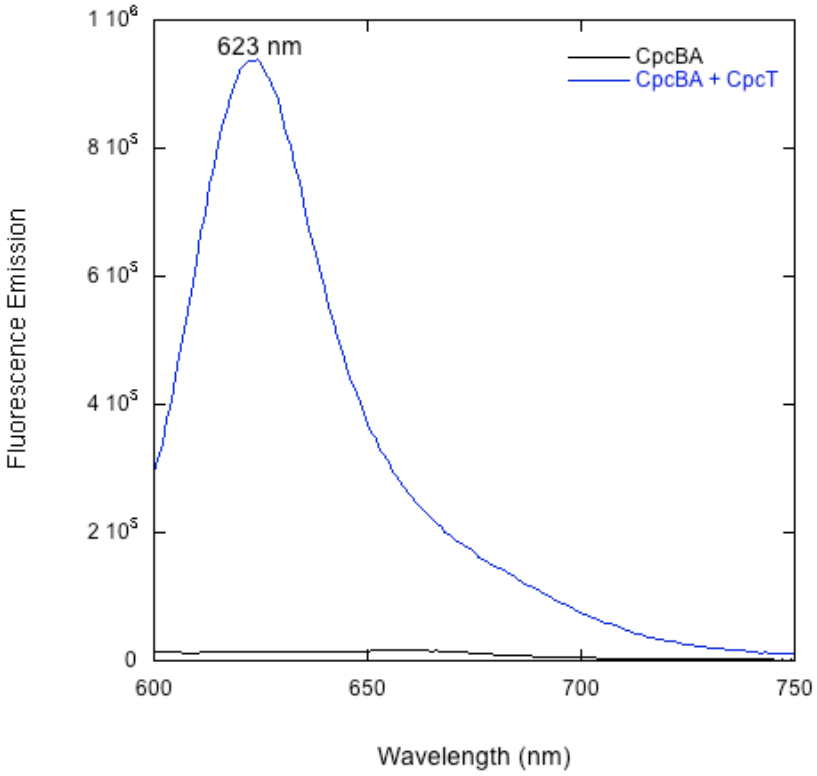


Figure 31: Fluorescence emission spectra of *in vitro* PcyA bilin addition reactions with CpcT and CpcBA. Fluorescence spectra of reactions with CpcBA alone (control reaction=black line) and CpcBA with CpcT (blue line). Fluorescence maximums are shown for the reactions. The CpcT fluorescence emission is so high that the little fluorescence seen by the control reaction can not be plotted. The maximum for CpcT is 623 nm.

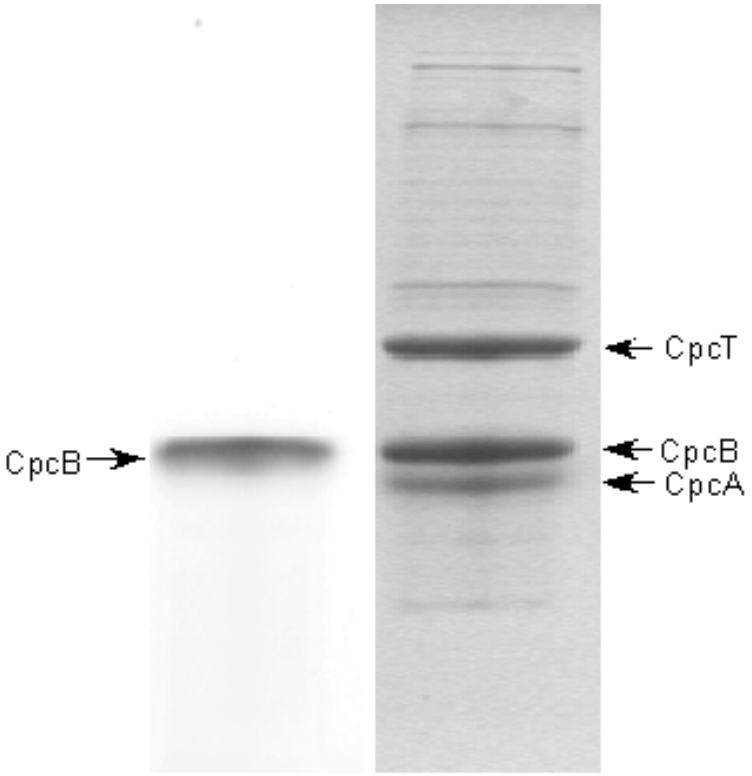


Figure 32: SDS-PAGE analysis of PcyA reactions containing CpcT. The left side of the gel shows CpcT PcyA reaction after Zinc staining. Only covalently bound bilin carried on CpcB. The right side of the gel shows the same gel after Coomassie staining. The protein identities are shown.

both CpcBA and CpcT. Reactions were carried out for 4 hours total at 30°C. The reaction products as well as purified holo-phycocyanin from *Synechococcus* sp. PCC 7002 were digested with trypsin. The product of the tryptic digestion was injected onto a C_{18} HPLC reverse phase column. The elution profile of the peptides (taken at 600nm) can be seen in Figure 33. The top half of the figure shows the elution profile of wild-type phycocyanin. The three main peaks in the phycocyanin profile correspond to the α-84, β-82, and β-153 cysteinyl residues. The peptide containing the α-84 cysteine site has a retention time of 20.4 minutes followed by the β-82 peptide at 23.2 minutes and the β-153 peptide at 30.3 minutes. The lower half of the figure shows the elution profile for the CpcT reaction. There is one major bilin-containing peak in this reaction product which has a retention time of 30.3 minutes. This corresponds to the retention time of the β-153 peptide. The two peaks with retention times slightly shorter than α-84 or β-82 site most probably have mesobiliverdin attached at the cysteinyl residue which would account for the shorter retention time. This shorter retention time for mesobiliverdin was previously reported by Arciero *et al*. (1988a). These results confirm that CpcT is the Cys-153 β phycocyanin phycocyanobilin lyase.

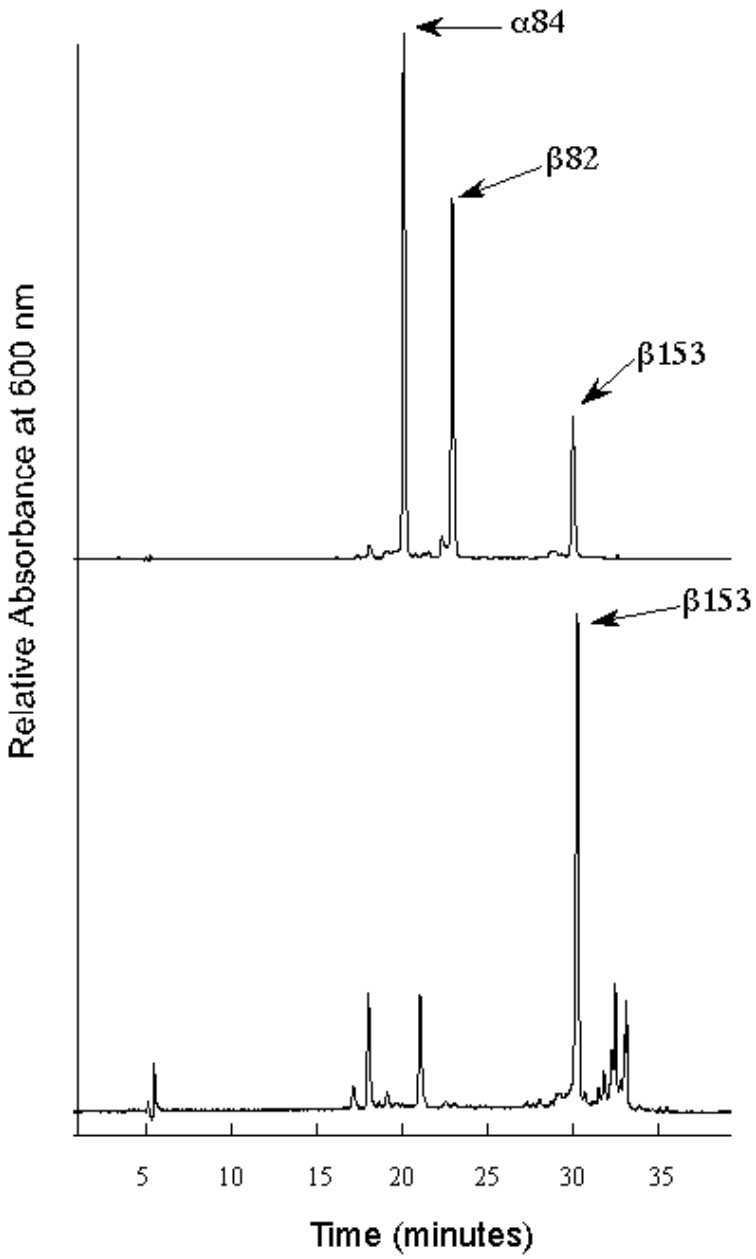


Figure 33: RP-HPLC separation of tryptic peptides from *in vitro* bilin addition assays (PCB products) with CpcBA and CpcT. Tryptic digestion elution pattern from RP-HPLC. The top half shows the elution pattern from wild type *Synechococcus* sp. PCC 7002 phycocyanin. The three bilin containing peptides are identified by arrows and show the cysteinyl residue that the peak corresponds to. The bottom half shows the elution pattern for PcyA reaction with CpcBA and CpcT. This reaction only gives one peak at 30.3 minutes corresponding to the β-153 site.

DISCUSSION

We have discovered a new family of genes in *Synechococcus* sp. PCC 7002, of which three encode phycobiliprotein lyases. These genes are not similar at the amino acid level to CpcE/CpcF, therefore these genes are considered a new family. Absorbance and fluorescence spectra of the CpcSU-dependent product show a maximum peak where we would expect a maximum peak if bilin addition is taking place on the β-82 site of phycocyanin. Zinc-enhanced fluorescence shows that there is addition only on the β subunit. Tryptic digestion confirms that CpcSU catalyzes bilin addition at the β-82 site. These results match very well with the mutant results from our collaborators.

The *Synechococcus* sp. PCC 7002 *cpcS* and *cpcU* mutants, created by G. Shen at Penn State University, show an obvious phenotypic difference from the wild-type cells. Both *cpcS* and *cpcU* mutants were yellowish in color, instead of blue-green, and had a severe reduction in phycocyanin produced. The *cpcS* mutant had 35% of wild-type levels of phycocyanin, and the *cpcU* mutant had only 30% phycocyanin. Loss of *cpcS* and *cpcU* also affected the doubling time of the mutants by severely slowing it. Wild-type doubling time was 3.8 hours while the *cpcS* mutant was 14.4 hours and *cpcU* mutant was 17.6 hours. Some phycocyanin in these mutants is missing the bilin at β-82 cysteine. Mass spectroscopic data showed that some β-phycocyanin did have two bilins attached, but an equivalent amount of β-phycocyanin was missing a bilin. The hypothesis is that phycocyanobilin is sitting in the β-82 pocket and that some of this bilin can slowly attach without enzymes. Consistent with this is the observation of a 587 Da peak in the mass spectroscopic data, the exact molecular weight of phycocyanobilin. Some phycocyanin

contains covalent phycocyanobilin at both β-sites because maybe there is very slow covalent addition (Shen *et al*., manuscript in preparation).

Further studies were performed using formic acid cleavage of phycocyanin purified from the CpcS and CpcU mutants (β*phycocyanin). This showed the β*-phycocyanin was missing a bilin at β-82 site, matching very well with the demonstrated recombinant activity of CpcSU (Shen *et al*., manuscript in preparation). Both the *in vitro* biochemical analysis presented here and the characteristics of the mutants described above support the conclusion that CpcS and CpcU act together as the Cys-82 β-phycocyanin phycocyanobilin lyase. Mutation results showed decrease in phycocyanin production but what about allophycocyanin production? *CpcS* and *cpcU* mutants also had a decrease in allophycocyanin production. This result could mean that CpcSU may have a role in allophycocyanin biosynthesis.

Zhao *et al.* (2006) characterized a protein from *Anabaena* sp. PCC 7120 that is a ortholog to CpcS, called CpeS. They concluded that CpeS works to catalyze a site-specific attachment of phycocyanobilin to the β-84 cysteine in both CpcB and PecB (phycoerythrocyanin β subunit). Assays showed that CpeS does not require any other proteins in the absence of phycocyanobilin (Zhao *et al*., 2006). This is in contrast to the results presented here that CpcS and CpcU interact to form a complex. One possibility for this is that CpeS acts as a homo-dimer.

There are similarities between the α-84 and β-84 binding sites. They are both spatially and stereochemically the same, but there are distinct differences between these two types of lyases. Lyases responsible for attachment on the α subunit act as heterodimers (CpcE/F and PecE/F) and are usually found encoded by genes in operons that include the gene for the substrate (apoprotein) (Fairchild *et al*., 1992; Fairchild and Glazer, 1994; Jung, Chang, and Glazer, 1995; Zhao *et al*., 2006). In contrast to the other α subunit lyases, CpeS can correctly

attach phycocyanobilin to β-84 (β-82 respectively) cysteinyl residue but its kinetic constants are like those of α-84 lyases (Zhao *et al*., 2006). CpeS in *Anabaena* sp. PCC 7120, which is encoded by a gene named *alr0617*, is not found near either the *cpc* or *pec* operon (Zhao *et al*., 2006). Even with the differences between CpeS and CpcSU, they both perform the same attachment in their respective organisms. The results that CpeS attaches phycocyanobilin to β-84 (equivalent of β-82) confirm the results found in this thesis about CpcSU.

CpcT is a lyase that attaches phycocyanobilin to β-153 cysteine site on phycocyanin as demonstrated by absorbance, fluorescence, and tryptic digestion results. These results match well with the *cpcT* mutant characterization performed by G. Shen at Penn State (Shen *et al*., 2006). The *cpcT* mutant was yellow green in color, compared to the blue-green color of wild-type. This color change can be accounted for because the *cpcT* mutant only had 60% phycocyanin content compared to wild-type. Doubling time for the mutant was also extended to almost twice that of the wild-type (wild-type-4.2 hours and *cpcT* mutant-7.8 hours). By examining phycobilisome function, Shen *et al*. (2006) also concluded that because fluorescence emission of phycocyanin was reduced, the *cpcT* mutant absorbed less light and suggests that the *cpcT* gene could function in phycocyanin synthesis. Other spectroscopic comparisons for the *cpcT* mutant showed that the difference spectra between phycocyanin isolated from the mutant and holo-phycocyanin (mutant absorbance spectrum minus the phycocyanin spectrum) matched very well with the CpcT-dependent phycocyanobilin reaction product from *in vitro* reactions, with a peak at ~600 nm. Formic acid cleavage results confirmed that phycocyanin from the *cpcT* mutant was missing phycocyanobilin at the Cys-153 β-phycocyanin site (Shen *et al*., 2006).

With *cpcT* mutant results confirming that CpcT catalyzes the attachment of phycocyanin at the β-153 site and CpcS and CpcU mutant results confirming attachment at the β-82 site, why

do cyanobacteria need two different lyases for the β subunit? It is also not clear why cyanobacteria have separate lyases for attachment on the α and β subunits of phycocyanin. The α-84 and β-82 cysteines are buried inside a crevice created by the α helices in the phycocyanin structure. Bilins attached at these sites are called 'central bilins' because of their location inside the biliprotein structure. Bilins attached at the β-153 site are 'peripheral bilins' and are more exposed because of the location of the attachment site (Toole *et al*., 1998). This location of attachment sites may be a reason for different lyases for the α and β subunits. One reason that there are two different lyases for the two sites on the β subunit of phycocyanin may be because of the stereochemistry of the attachment sites. The chiral carbon at $C3^1$ of phycocyanobilin, which attaches to cysteine β-82, has R stereochemistry. The chiral carbon at $C3^1$ of phycocyanobilin, which attaches to cysteine β-153, has S stereochemistry (Figures 4 and 34) (Schirmer *et al*., 1987; Shen *et al*., 2006; Zhao *et al*., 2006). All phycobiliproteins for which X-ray crystal structures have been solved contain S stereochemistry at β-153 (or the corresponding site) instead of R stereochemistry (Duerring *et al*., 1990; Duerring *et al*., 1991; Ritter *et al*., 1999). Because of this, it is probable that CpcT paralogs may also be responsible for attachment of any possible substrates to the β-153 corresponding site (Shen *et al*., 2006).

Which subunits, and therefore which lyases, evolved first? There is evidence that the β subunit evolved first. Origins of phycobiliproteins are still debated, but there is a consensus that the α and β subunits arose from a gene duplication event of a single ancestral gene. This makes sense when you look at the high degree of homology between the two subunits (Glazer and Wedemayer, 1995). Further evidence that the β subunit evolved first is that *Rhodella reticulata* (a unicellular red alga) has no α subunit. To date there has not been a photosynthetic bacterium that does not have a protein similar to the β subunit (Thomas and Passaquet, 1999). Glazer and

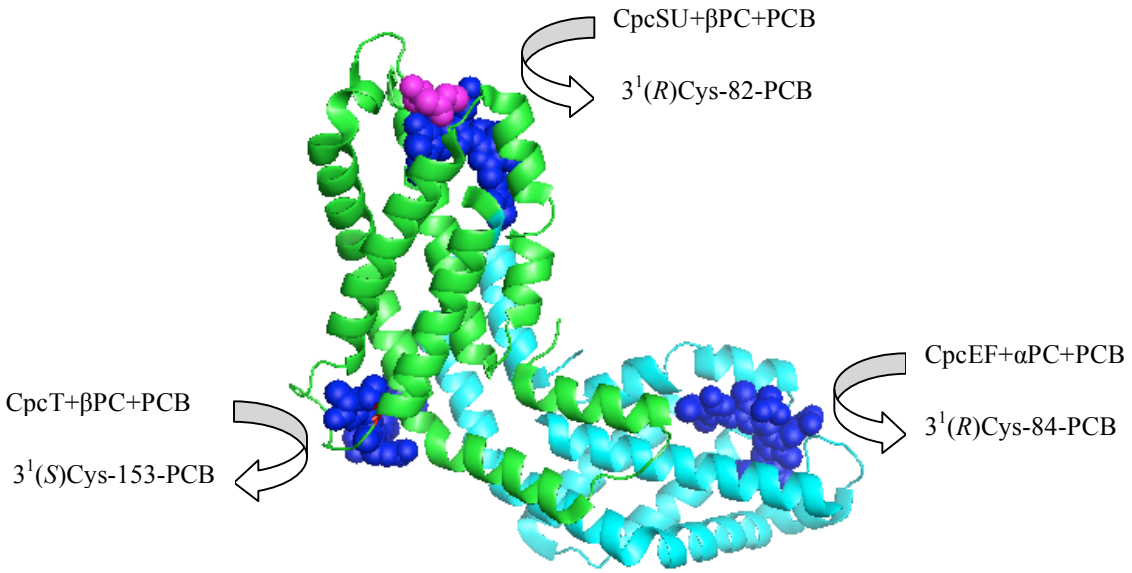


Figure 34: Crystal structure of phycocyanin. Summary of lyases required for addition of phycocyanobilin to phycocyanin. Stereochemistry is shown for α-84 (3^1 chiral carbon as R stereochemistry), β-82 (3^1 chiral carbon has R stereochemistry), β-153 (3^1 chiral carbon has S stereochemistry).

Wedemayer (1995) believe that phycobiliprotein evolution took place with allophycocyanin evolving first, then phycocyanin, and finally phycoerythrin. This scheme makes sense on different levels: 1) phycobiliproteins that act as acceptors (allophycocyanin) would have to appear first before the energy donor phycobiliprotein 2) the series is consistent with the complexity of chromophore binding sites on subunits-allophycocyanin has one on α and one on β subunit site, phycocyanin has one on α and two on β subunit sites, and phycoerythrin has two on α and three on β subunit sites. If the β subunit evolved first, than maybe the α subunit evolved through a gene duplication event taking place further along the evolutionary time line (Glazer and Wedemayer, 1995).

If the α subunit evolved later, why were these the CpcEF-type lyases necessary? In many phycobiliproteins, the α subunit can often have different bilins attached than the β subunit. Some of these lyases must also isomerize the bilin before attachment. The lyase/isomerase from *Mastigocladus laminosus* (*pecE/pecF*) is one such example. PecE/PecF catalyzed two separate reactions. The first reaction was the addition of phycocyanobilin to α-84 of phycoerythrocyanin α subunit. The second reaction was the isomerization of phycocyanobilin to phycoviolobilin. PecE/PecF works together to attach and isomerizes phycocyanobilin to phycoviolobilin (Storf *et al*., 2001; Zhou *et al*., 2002)). α subunit lyases may have evolved two different genes to attach and isomerize the bilin.

CpcT has no relation to α phycocyanin lyases CpcE/F or the lyase/isomerase PecE/F. Because CpcT is not related in sequence to CpcE/F or PecE/F, it comprises a new class of bilin lyase. *CpcT* and *cpeT*-like genes are represented in all sequenced genomes of cyanobacteria, all genomes except *P. marinus* MED4 (which does not contain phycoerythrin). These genomes contain one copy of the *cpcT* or *cpeT*-like gene. Because of the sequence similarity to CpcT,

CpeT paralogs most probably function in attachment of the bilin (chromophore) at β-153, or equivalent position (Shen *et al*., 2006).

CpcS, or *cpeS*-like, genes can be found in all phycobiliprotein-containing cyanobacterial genomes and can be considered a cyanobacterial signature gene. These different organisms have different copy numbers of *cpcS*-like genes. Table 3 shows the name of some of the cyanobacteria that have *cpcS*-like genes contained in their genome. *Synechococcus* sp. PCC 7002 has three copies of the *cpcS*-like gene (*cpcS, cpcU, cpcV*), while *Synechocystis* sp. PCC 6803 only has two copies of the *cpcS*-like gene (*slr2049* and *sll0853*). *Synechococcus* sp. PCC 7002 has one more copy of the *cpcS*-like gene than *Synechocystis* sp. PCC 6803, corresponding to *cpcV*. The *cpeS*-like gene is found not only in cyanobacteria that contain phycocyanin and allophycocyanin, but in cyanobacteria that contain other phycobiliproteins, such as phycoerythrin.

In theory, both bilins have the same stereochemistry at α-84 and β-82 sites so attachment by CpcSU might be possible. We were unable to demonstrate that CpcSU attaches phycocyanobilin to the α phycocyanin subunit. Swanson *et al*. (1992) characterized a *cpcE* revertant that had regained its ability to make phycocyanin again. The reverted mutants showed much higher phycocyanin levels than the mutants did. Upon further study of the reverted *cpcE* mutant, a single amino acid substitution within the α subunit of phycocyanin (α-129 Tyr to Cys) was found. This substitution may be enough to suppress the deficiency in bilin addition. The authors hypothesized that this substitution allowed another unknown lyase to recognize the α phycocyanin substrate and attach phycocyanobilin (Swanson *et al*., 1992). CpcSU is a good candidate for this lyase.

Table 3: List of some cyanobacteria with *cpcS*-like genes and the number of copies found in the genome.

Organism	Copy #	Gene name
Synechococcus sp. PCC 7002	3	*cpcS, cpcU, cpcV*
Synechocystis sp. PCC 6803	2	*slr2049, sll0853*
Synechococcus sp. WH 8120	3	*SYNW0315, SYNW2002, SYNW2019*
Anabaena sp. PCC 7120	2	*alr0617,all5292*
Synechococcus elongates PCC 7942	3	*Selo021319,Selo209501, Selo229501*
Gloeobacter violaceus PCC 7421	6	*glr1191, glr1192, glr1259, glr1614, gll1531, gll2024*
Synechococcus sp. WH 8020	1	*ORF200*
Fremyella diplosiphon	2	*cpeS, cpeS*-like
Anabaena variabilis ATCC 29413	2	*Avar020425, avar026076*
Prochlorococcus marinus SS120	1	*Pro0343*

Future Work

Future experiments would be to determine the enzyme kinetics of these lyases, CpcSU and CpcT. Experiments need to be performed to look at the function of CpcV. Some experiments were done with CpcV but no conclusive results were found. It is most likely that CpcV is an extra copy of the *cpeS*-like gene and may have no function. *Synechococcus* sp. PCC 7002 has the most copies of *cpeS*-like genes, with three copies. It may be that CpcV is an evolutionary dead end gene that has no real function. It could also be involved in bilin removal during N_2 starvation. But experiments need to be done to prove this point. More work needs to be done to determine if cofactors can affect the lyase activity. $MgCl_2$ (1 mM concentration) was shown to slightly increase the activity of CpcSU and CpcT in PcyA reactions. Future work needs to determine if this or other metal ions can inhibit or help activate the lyases. Experiments to show whether CpcSU can attach phycocyanobilin to the α and β subunits of allophycocyanin should also be performed. Since the stereochemistry is the same at both sites on allophycocyanin, CpcSU may attach bilin to each subunit.

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