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Identification and Characterization of Enzymes Involved in Post-translational Modifications of
Phycobiliproteins in the Cyanobacterium *Synechocystis* sp. PCC 6803

A Thesis

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

Master of Science
in
Biological Sciences

by

Crystal Miller

B.S. University of New Orleans, 2004

August, 2007

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ABSTRACT

The goal of this research is to identify and characterize enzymes responsible for post-translational modifications of phycobiliproteins (PBP) in the cyanobacterium *Synechocystis* sp. PCC 6803. Asparagine 72 is methylated to produce gamma-N-methylasparagine on beta subunits of PBP *in vivo*. A candidate for this methyl transferase is CpcM (sl10487). Methylase assays showed that CpcM is specific for the beta subunits of PBP with no methylation on the homologous alpha subunits. CpcM methylates PBP after chromophorylation but before the PBP assemble into trimers. Candidates for the lyases responsible for attachment of phycocyanobilin to phycocyanin in *Synechocystis* sp. PCC 6803 are two *cpeS*-like genes (*cpcS* and *cpcU*) and one *cpeT*-like gene (*cpcT*). Through absorbance and fluorescence spectra, it was determined that CpcS and CpcU together catalyze the addition of phycocyanobilin to Cys-82 on beta-phycocyanin *in vitro* and that CpcT is a lyase that adds phycocyanobilin to Cys-153 on beta-phycocyanin.

INTRODUCTION

1.1 Cyanobacteria

Cyanobacteria are photosynthetic, gram-negative prokaryotes belonging to the kingdom Monera. They have the distinction of being the world's oldest oxygen-evolving organisms and fossils have been found which are more than 3.5 billion years old (Schopf, 1983). Through their oxygenic photosynthetic capabilities during the Archaean and Proterozoic eras, they created the oxygen rich atmosphere that we breathe today (Bengston, 1994). Cyanobacteria also contributed to the origin of plants and other oxygen-evolving organisms such as red, green, and cryptophyte algae; through endosymbiosis, they became the chloroplasts of these eukaryotes (Bengston, 1994; Sidler, 1994). Plants also evolved from cyanobacterial genes that migrated to the host nucleus from the plastid, thus demonstrating an evolutionary relationship between plants and cyanobacteria (Hughes and Lamparter, 1999).

Cyanobacteria, like plants, require a light-harvesting complex that helps the organism perform photosynthesis. These complexes are responsible for capturing light and then efficiently transferring this energy to the photosynthetic reaction centers. In cyanobacteria this complex is the phycobilisome (PBS) which forms regular arrays that electrostatically interact with the stromal side of the thylakoid membrane transferring energy to photosystem II (PSII) (Figure 1) (Glazer *et al.*, 1983; Sidler, 1994). The PBS is a large, water-soluble, macromolecular antenna complex comprised mostly of light-absorbing phycobiliproteins (Glazer, 1989).

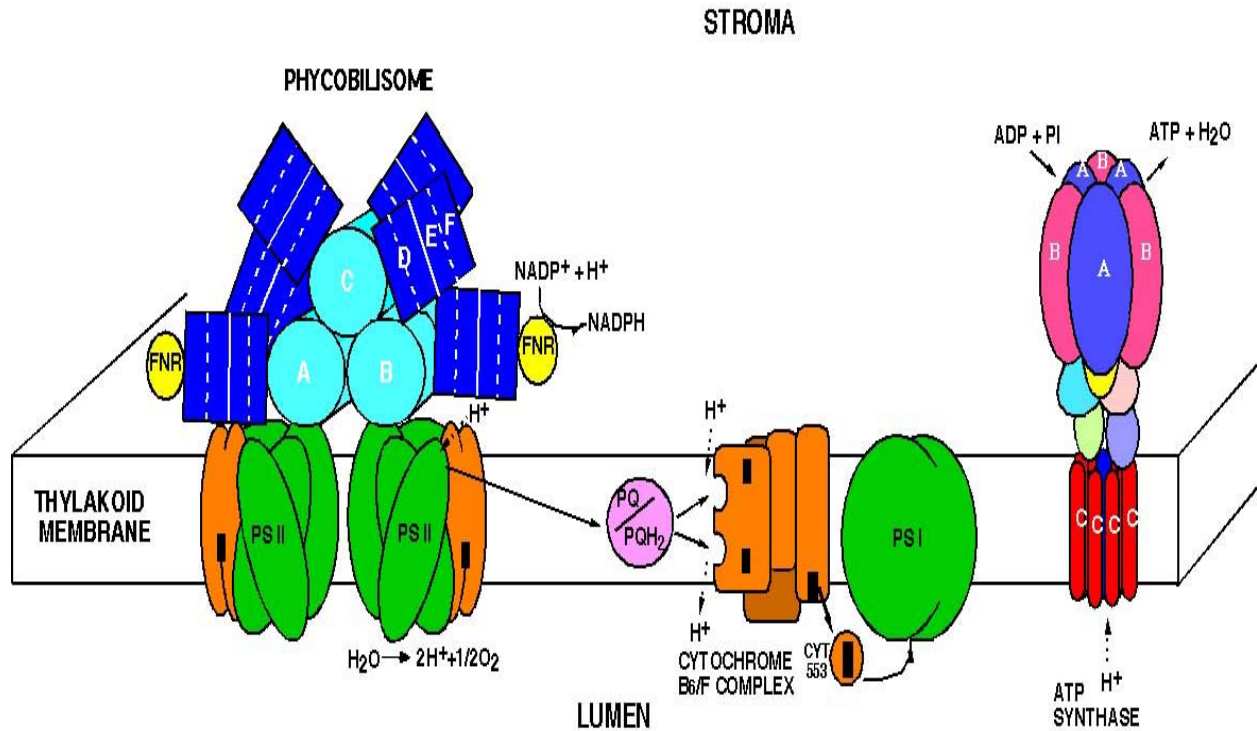


Figure 1: Phycobilisome structure and interaction with PSII and the thylakoid membrane The PBS consists of a core composed of stacked PBP trimeric discs and radiating rods composed of stacked PBP hexameric discs. A collection of polypeptides known as “linker” polypeptides mediate the assembly of the PBS and regulate the spectroscopic properties of the individual PBP within the PBS. The arrangement of the PBP in the PBS coincides to that of spontaneous excitation energy flow from higher to lower energy transitions with phycoerythrin (PE- λ_{max} 565 nm) at the periphery of the rods, phycocyanin (PC- λ_{max} 620 nm) on the interior of the rods proximal to the core, and allophycocyanin (AP- λ_{max} 650 nm) in the core (modified from Beale, 1994).

1.2 Phycobiliproteins and Bilin Chromophores

1.2.1 Phycobiliprotein Structure and Function

Phycobiliproteins serve as the light-harvesting antenna in many photosynthetic organisms such as prokaryotic cyanobacteria, eukaryotic red algae and cryptomonads (Glazer, 1989).

Phycobiliproteins can comprise up to 30-40% of total protein in a typical cyanobacterial cell (Arciero *et al.*, 1988), and absorb light in the visible range of 450-665 nm, which covers the absorption of light wavelengths between the two maxima covered by chlorophyll *a* (420 and 680 nm), during photosynthesis (Apt *et al.*, 1995). The four spectroscopic classes of phycobiliproteins are phycoerythrocyanin (PEC, λ_{\max} 575 nm), phycoerythrin (PE, λ_{\max} 565-575 nm), phycocyanin (PC, λ_{\max} 615-640 nm), and allophycocyanin (AP, λ_{\max} 650-655 nm) (Sidler, 1994). The cyanobacteria used in this study, *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002, contain only two major phycobiliproteins, AP and PC (Toole *et al.*, 1998).

Each phycobiliprotein consists of equimolar amounts of two dissimilar polypeptide chains, the α and β subunits, with molecular weights of approximately 17 and 18 kDa, respectively (Glazer and Fang, 1973; Glazer, 1989). Sequence alignment data of the α and β subunits have revealed that a large number of residues are highly conserved in all phycobiliprotein subunits (Schirmer *et al.*, 1986). Each phycobiliprotein α and β subunit, together, form a monomer. These monomers assemble into trimers $(\alpha\beta)_3$, and there is further stacking of two trimers in a face-to-face arrangement to form a hexamer $(\alpha\beta)_6$. These trimers and hexamers are disc-like structures possessing a central cavity where “linker” proteins bind to connect the trimers and hexamers together (Apt *et al.*, 1995).

Each α or β subunit bears one to three covalently attached bilin chromophores (Glazer, 1989). These heme-derived open-chain linear tetrapyrrole bilin prosthetic groups are covalently

attached to cysteinyl residues on the α or β subunit via a thioether linkage (Duerring *et al.*, 1990; Frankenburg *et al.*, 2001; Glazer, 1989). Most bilin chromophores are attached to the cysteine at the vinyl group ring A bilin (Sidler, 1994). The four bilins naturally occurring in cyanobacteria are: phycocyanobilin (PCB, blue), phycoviolobilin (PVB, purple), phycoerythrobilin (PEB, red) and phycourobilin (PUB, yellow) (Figure 2) (Glazer, 1989; Sidler, 1994). Each bilin has a different number of double bonds in conjugation, which gives it its own unique spectroscopic properties (Glazer, 1989). The only bilin attached to the phycobiliproteins in *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 is PCB.

1.2.2 Phycocyanobilin:Ferredoxin Oxidoreductase (PcyA)

The precursor molecule for all bilins is heme. Heme is a prosthetic group that consists of an iron atom contained in the center of a porphyrin, a large heterocyclic organic ring. Phycocyanobilin:Ferredoxin Oxidoreductase (PcyA) catalyzes the synthesis of phycocyanobilin from biliverdin (Frankenburg *et al.*, 2001). This enzymatic reaction is dependent on a four-electron reduction of biliverdin using ferredoxin as the electron donor (Figure 3). Recombinant PcyA forms a very stable complex with biliverdin which is the precursor of all bilins in the bilin biosynthetic pathway (Frankenburg *et al.*, 2001; Frankenburg and Lagarias, 2003).

1.3 Phycobilisome Structure and Function

The PBS consists of a core composed of stacked phycobiliprotein trimeric discs and radiating rods composed of stacked phycobiliprotein hexameric discs. The core is composed of three phycobiliprotein structures that run parallel to one another, and there are six or more rods of phycobiliproteins that branch out from the core. A collection of polypeptides known as

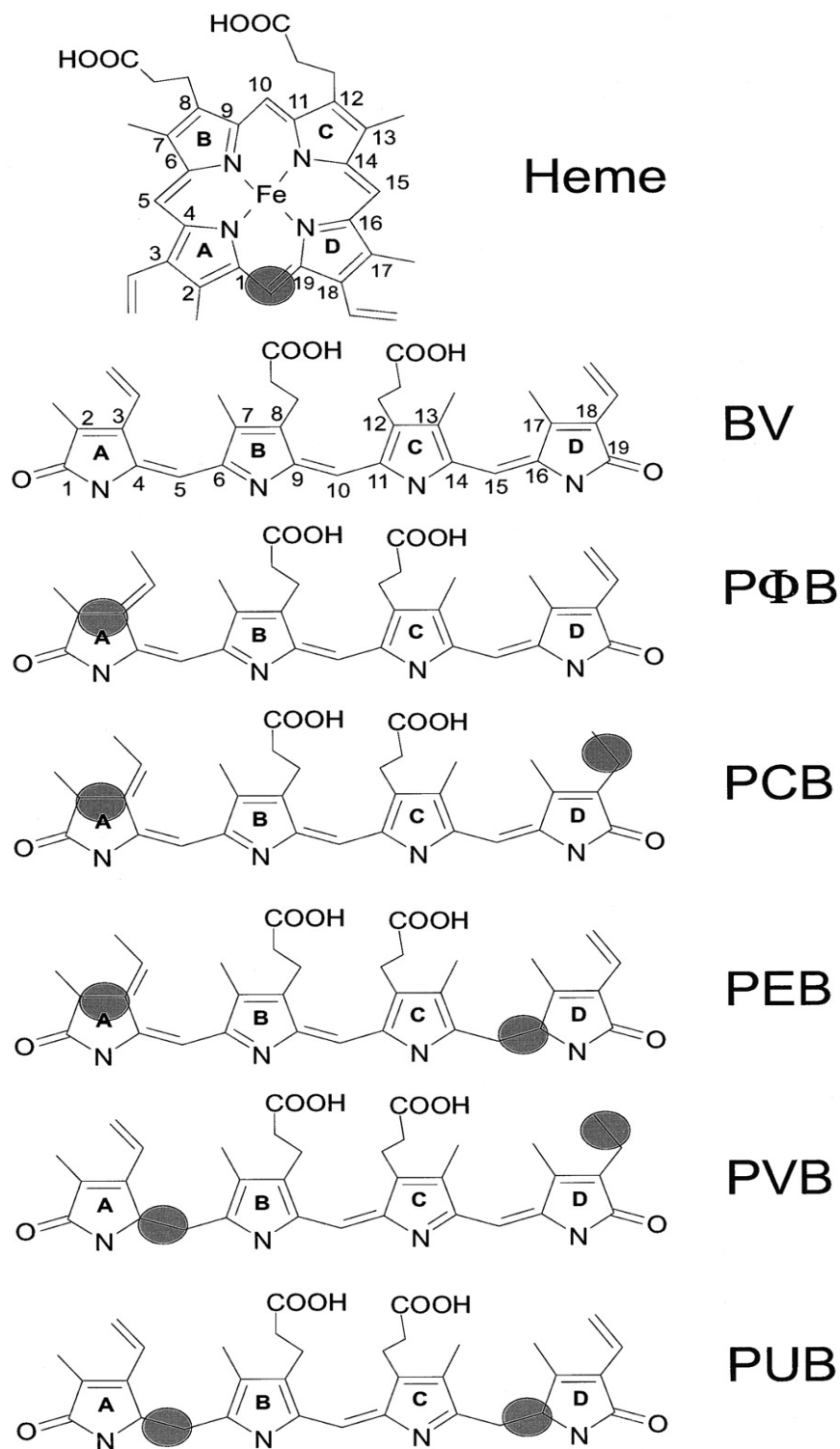


Figure 2: Phycobiliprotein Synthesis and Structure. Structure of heme and the natural bilins biliverdin (BV), phytochromobilin (found in phytochromes [PΦB]), phycocyanobilin (PCB), phycoerythrobilin (PEB), phycoviolobilin (PVB), and phycourobilin (PUB). Heme oxygenase converts heme to biliverdin by cleaving between rings A and D at the positions marked. Differences in the other bilins with respect to biliverdin are also indicated (Hughes and Lamparter, 1999).

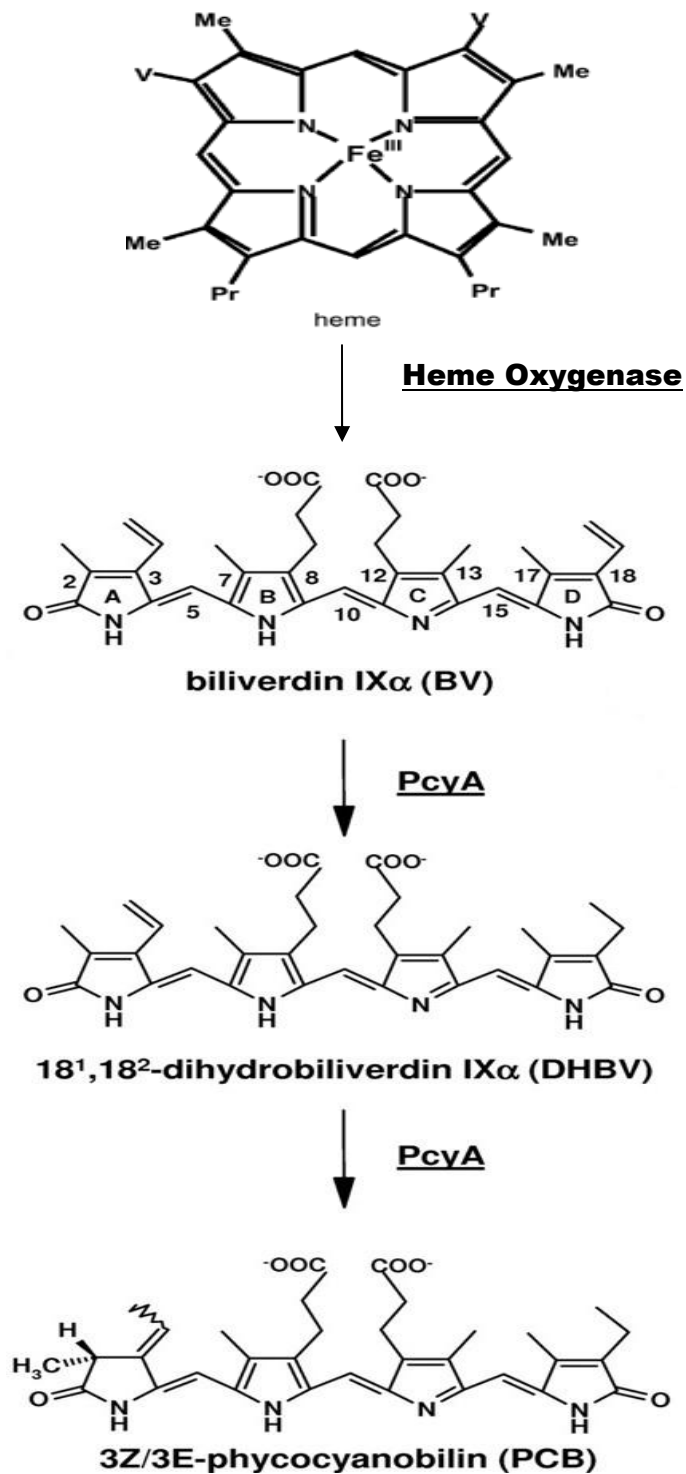


Figure 3: Biosynthesis of phycocyanobilin. Conversion of heme to phycocyanobilin (modified from Hagiwara *et al.*, 2006).

“linker” polypeptides mediate the assembly of the PBS and regulate the spectroscopic properties of the individual phycobiliproteins within the PBS by interacting with the bilins near the central cavity of the trimers (Glazer, 1989). The arrangement of the phycobiliproteins in the PBS coincides with spontaneous excitation energy flow from higher to lower energy transitions from PEC (λ_{\max} 575) or PE (λ_{\max} 565 nm) at the periphery of the rods, to PC (λ_{\max} 620 nm) on the interior of the rods proximal to the core, and then to AP (λ_{\max} 650 nm) in the core. Energy is then transferred to PSII (λ_{\max} 680 nm). In the organisms *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 the phycobiliprotein composition in the core is AP, and in the rods only PC is found (Figure 4).

Steady state fluorescence emission originates almost exclusively from “acceptor” bilins which both absorb excitation energy and fluoresce (Glazer, 1989). In C-phycoyanin and other phycobiliproteins, the bilin located at β -84 (β -82 in *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002) is the “acceptor”, and the bilins located at α -84 and β -155 (β -153) are “donors” that absorb light energy and transfer this excitation energy to another bilin (Schirmer and Vincent, 1987). The α -84 and β -82 bilins are found toward the center of the trimeric discs in regions that have structural similarity to the heme-binding pocket in the globin family of proteins, whereas the chromophore β -153 is located in a short loop unique to the β -subunit of phycoyanin and is close to the edge of the trimeric disc (Pastore and Lesk, 1990; Schirmer et al., 1985). Energy absorbed by any of the “donor” bilins in the PBS is quickly localized on the terminal energy “acceptor” bilins and the emission of these bilins overlaps precisely the absorption spectrum of the reaction center of PSII creating a radiationless directional transfer of energy (Glazer, 1989).

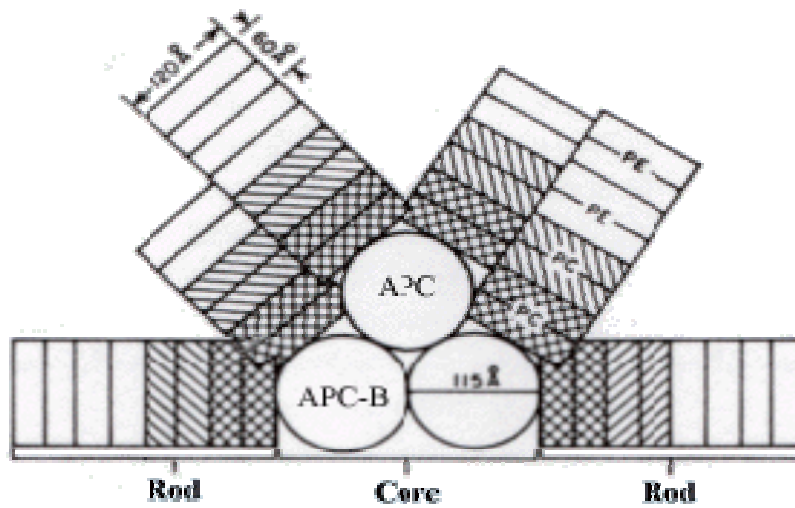
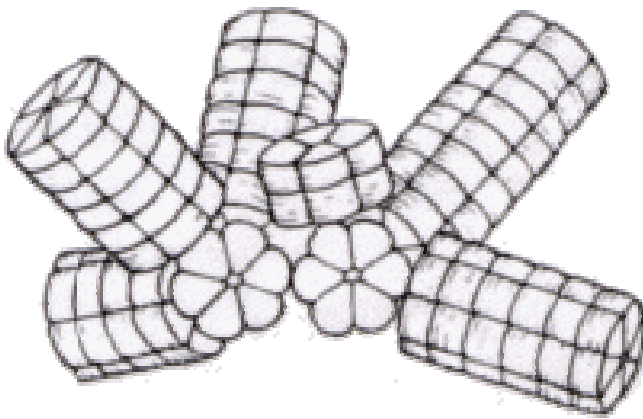
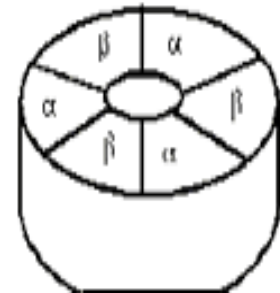
A**B****C**

Figure 4: Phycobilisome (PBS) Structure. Panel A) PBS phycoerythrin (PE) shown in the periphery of the rod structures, phycocyanin (PC) shown in the interior rods, and allophycocyanin (AP) shown inside the core of the PBS structure. Panel B) PBS structure is shown three dimensionally with core trimeric discs and rod hexameric discs. Panel C) A diagram of a phycobiliprotein disc with alternating α and β subunits (Glazer, 1989).

The structure and composition of the PBS corresponds to rapid energy absorption and transfer throughout the antenna complex and photosynthetic membrane to PSII with greater than 95% efficiency (Swanson and Glazer, 1990). The radiationless energy transfer occurs through a dipole-induced dipole resonance energy transfer called the Förster energy-transfer mechanism (Förster, 1965, 1967). The energy transfer to Chl *a* of PSII occurs 150 picoseconds after the excitation of peripheral phycoerythrins and 120 picoseconds after excitation of phycocyanins (Sidler, 1994).

The allophycocyanin core of the PBS contains some low abundance subunits that are important for energy transfer from the core to PSII. ApcF is a β -like allophycocyanin subunit that is present in two copies per phycobilisome and ApcD is an α -like allophycocyanin subunit, present in two copies per PBS. Ashby and Mullineaux (1999) investigated the role of the phycobilisome core components, ApcD and ApcF, in transferring energy from the phycobilisome to PS I and PS II in the cyanobacterium *Synechocystis* sp. PCC 6803. The genes encoding these proteins were disrupted in the genomes of wild type *Synechocystis* sp. PCC 6803. When both ApcF and ApcD are absent, the phycobilisomes are unable to transfer energy to either reaction center. The major route of energy transfer to both reaction centers appears to involve ApcF rather than ApcD (Ashby and Mullineaux, 1999).

1.4 Phycobiliprotein Post-Translational Modifications

1.4.1 Phycobiliprotein Methylation

The efficient radiationless transfer of energy throughout the PBS and photosynthetic membrane to PSII has been shown to be facilitated by a unique post-translationally modified residue, γ -*N*-methylasparagine. This modified residue has been found in all phycobiliproteins

isolated from cyanobacteria, red algae, and cryptomonads (Wilbanks *et al.*, 1989). This highly conserved γ -*N*-methylnasparagine residue is located at the β -72 position, and no such modification is present on the homologous α position (Klotz *et al.*, 1986; Klotz and Glazer, 1987). The methylation is also thought to be conserved in the allophycocyanin subunits, ApcB and ApcF (Swanson and Glazer, 1990). The conservation of asparagine at position 72 is demonstrated in a phycobiliprotein alignment in Figure 5.

Radiotracer experiments have shown that the methyl group of γ -*N*-methylnasparagine is derived from the *S*-methyl of methionine, implicating *S*-adenosylmethionine, which is found naturally in cells, as an intermediate methyl transfer agent (Klotz and Glazer, 1987). This means that the enzyme responsible for this methylation is an *S*-adenosylmethionine (AdoMet)-dependent methyltransferase. These enzymes have the very simple function of transferring a methyl group from the sulfonium atom of AdoMet to a variety of nucleophiles, including oxygen, sulfur, carbon, and in the case of γ -*N*-methylnasparagine, a nitrogen atom, on proteins, nucleic acids, carbohydrates, lipids, and small molecules in all organisms (Clarke, 2002). γ -*N*-methylnasparagine results from the methylation on the side-chain amide nitrogen on the asparagine residue located at β -72 of phycobiliproteins leading to *N*-4-methylnasparagine or γ -*N*-methylnasparagine (Figure 6) (Klotz *et al.*, 1986; Klotz and Glazer, 1987). Besides providing the efficient radiationless transfer of energy during photosynthesis, studies on synthetic peptides have suggested that methylation can slow the spontaneous deamidation of the side chain of asparagine residues by 45-fold (Klotz and Thomas, 1993). The methylation reaction adds bulk to the side-chain amide group and removes the possible participation of one of the two amide hydrogen atoms from participating in hydrogen bonding schemes (Klotz and Thomas, 1993).

Phycobiliprotein Alignment

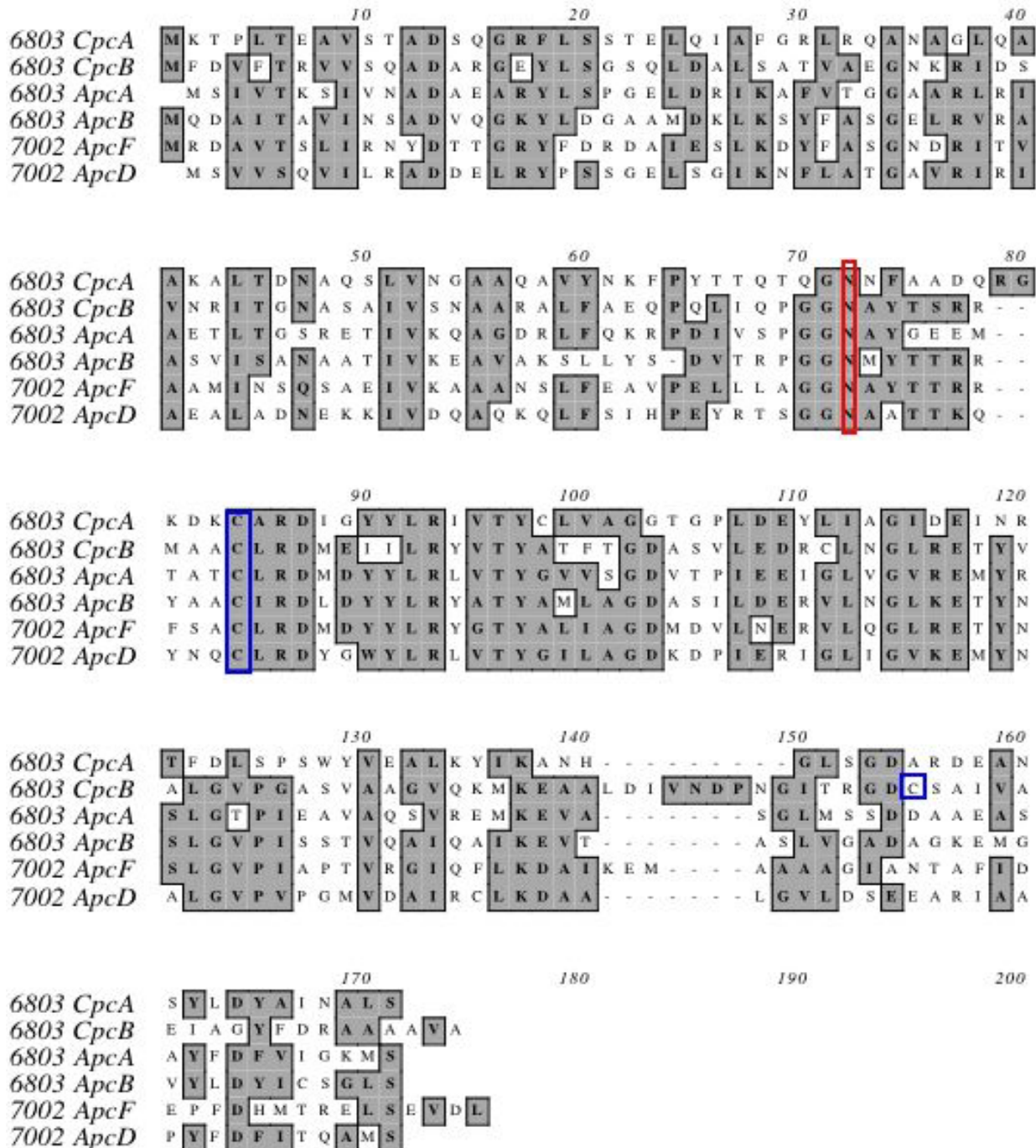


Figure 5: Alignment of phycobiliprotein sequences from *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002. Indicated in the red box is the conserved asparagine residue at position 72. Indicated in the blue boxes are the CpcB bilin attachment site cysteines at positions 82 and 153.

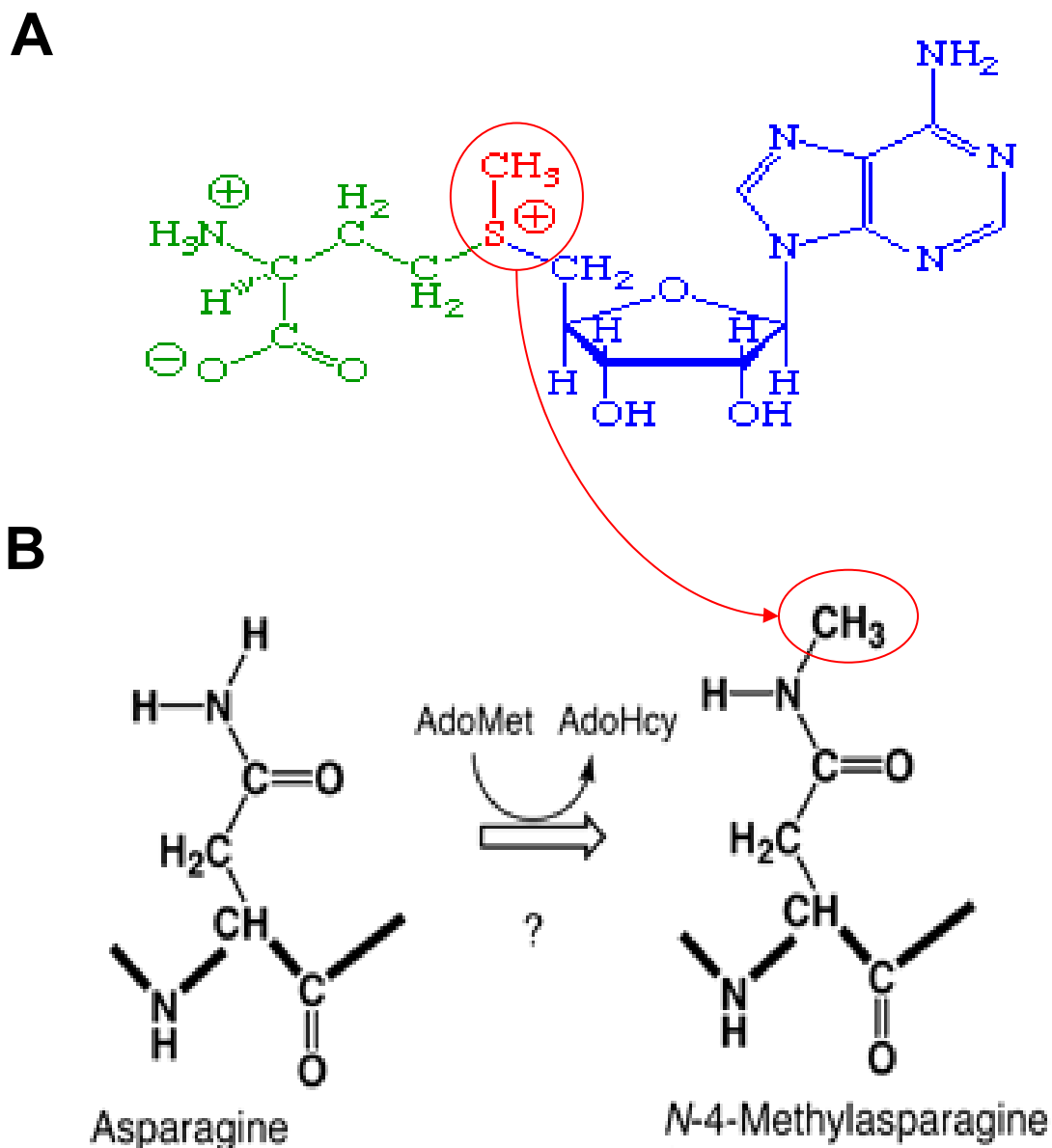


Figure 6: Methylation of β -72 asparagine. Panel A) Diagram of *S*-adenosylmethionine (SAM): In red is the very reactive sulfonium group with a methyl group attached, the amino group is homocysteine in green, and the nucleoside is adenosine in blue. Panel B) Diagram of the methylation of asparagine to *N*-4-Methylasparagine by an unknown enzyme transferring the methyl group of the sulfonium atom of SAM to the side-chain amide nitrogen (Clarke, 2002).

The fact that there is no modification on the homologous α position, the modification is highly evolutionarily conserved, and the modification is energetically costly implies the important role of this modification in photosynthesis. The side chain of γ -*N*-methylasparagine at β -72 is located in close proximity to the chromophore at β -82 (Figure 7) (Swanson and Glazer, 1990), which serves as the terminal energy acceptor in C-phycoyanin. This location suggested that this methylation might affect the spectroscopic properties of the chromophore at β -82.

Swanson and Glazer (1990) developed a protocol using [methyl- 3 H]S-adenosylmethionine and apo-phycoyanin (purified from *Escherichia coli* expressing the genes for the PC α and β subunits from *Synechococcus* sp. PCC 7002) as substrates to assay the phycobiliprotein asparagine methylase activity in extracts from *Synechococcus* sp. PCC 6301. This assay allowed the partial purification of the activity that completely methylates PC and AP at the β -72 residue. Swanson and Glazer isolated two mutants of the cyanobacterium *Synechococcus* sp. PCC 7942, pcm-1 and pcm-2, after incubation with the mutagen *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) which produces G:C to A:T transitions. The mutants, pcm-1 and pcm-2, produced PC and AP unmethylated at β -72 and were shown to lack the methylase activity (Swanson and Glazer, 1990). The phycobilisomes from these mutant strains displayed greater emission from PC and AP and lower fluorescence emission quantum yields (14%) compared to fully methylated phycobilisomes. These results showed that site-specific methylation of phycobiliproteins significantly contributes to the efficiency of directional energy transfer to the terminal energy acceptors in the PBS (Swanson and Glazer, 1990).

Thomas *et al.* (1993) used these same mutants, pcm-1 and pcm-2, to study the relative rates of PSII electron transfer by observing steady state rates of oxygen evolution. Oxidation of

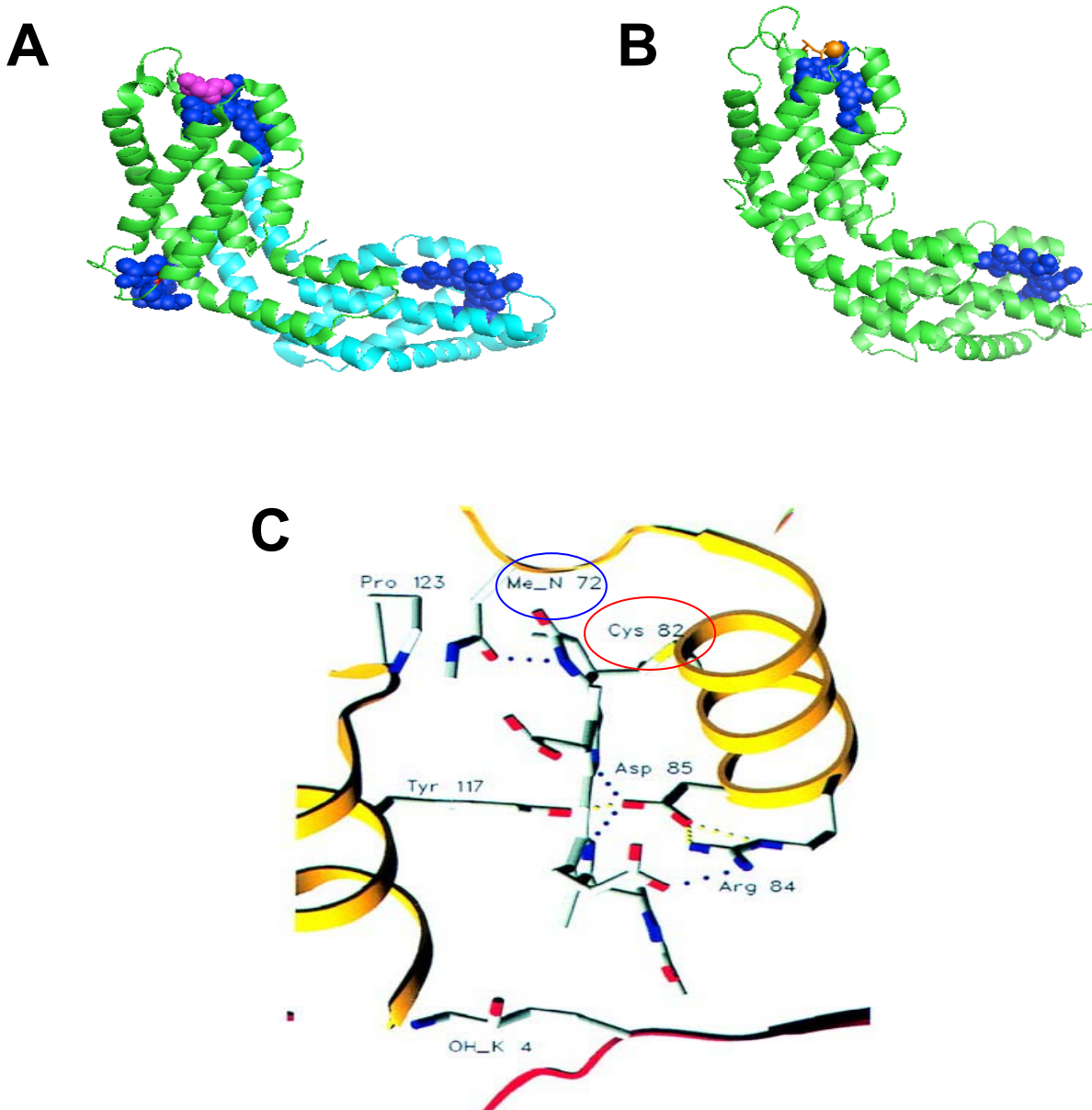


Figure 7: Figures of β -72 γ -N-Methylasparagine. Panel A) Crystal structure of phycocyanin. The γ -N-methylasparagine is highlighted in pink color. Panel B) Crystal structure of allophycocyanin. The γ -N-methylasparagine is highlighted in brown color. Panel C) A ribbon diagram of the β subunit of phycoerythrin. Circled in red is the cysteine β -82 chromophore and circled in blue is the area where the β -72 asparagine residue is located showing the β -82 and β -72 close proximity (Brejc *et al.*, 1995; Stec *et al.*, 1999; Wilk *et al.*, 1999).

water to O₂ requires abstraction of 4 electrons requiring the photochemistry of a 1 photon/1 electron event (Einstein's Law), this requires a mechanistic interface between the 1-electron photochemistry, and the 4-electron oxidation process. A schematic model explaining these observations, proposed by Kok and coworkers, has been widely accepted (Kok *et al.* 1970). This model for the photooxidation of water, called the S state mechanism, consists of a series of five states, known as S₀ to S₄, which represent successively more oxidized forms of the water-oxidizing enzyme system, or oxygen-evolving complex. The light flashes advance the system from one S state to the next, until state S₄ is reached. State S₄ produces O₂ without further light input and returns the system to S₀. After the steady state has been reached, a complex has the same probability of being in any of the states S₀ to S₃ (S₄ is unstable and occurs only transiently), and the yield of O₂ becomes constant (Kok *et al.*, 1970). Thomas *et al.* (1993) using xenon lamps for illumination and DCBQ and DMBQ as artificial electron acceptors, found that the mutants demonstrated lower rates of electron transfer through PSII, and it was shown that methylation at β-72 can improve the PSII quantum efficiency (the average number of electrons photoelectrically emitted per incident photon of a given wavelength) from 0.82 to 0.95 (observed in wild-type) which is consistent with the 14% lowering of PBS fluorescence quantum yield originally shown (Thomas *et al.*, 1993; Swanson and Glazer, 1990).

In 1995, these same researchers used two mutants of the cyanobacterium *Synechococcus* sp. PCC 7002 that contained either Asp or Gln in place of the Asn located at β-72. Steady-state spectroscopic measurements demonstrated that these substitutions affected both the ground (lowest-energy) to excited (any energy greater than ground) state transition and the excited-state characteristics of the β-82 chromophore. The lifetime of a system in an excited state is usually short. Spontaneous emission of a quantum of energy (such as a photon) usually occurs shortly

after the system is promoted to the excited state, returning the system to a state with lower energy (a less excited state or the ground state). Energy-transfer efficiency represented by steady-state fluorescence quantum yield decreased 7-10% in the mutants compared to wild-type. It was concluded that γ -*N*-methylnasparagine plays a role in establishing the environment surrounding β -82 thereby minimizing non-radiative energy loss (Thomas *et al.*, 1995).

The effect of γ -*N*-methylnasparagine is critical to the efficiency of photosynthesis and therefore the survival of many organisms. Now that this is known, the enzyme that performs this vital modification needs to be ascertained. Swanson and Glazer in 1990 developed an assay for the methylase activity using [methyl-³H]S-adenosylmethionine which allowed for the partial purification of the enzyme in cell extracts that methylates phycocyanin and allophycocyanin at β -72, but the gene that encoded this presumed enzyme was never elucidated.

1.4.2 Bilin Attachment to Phycobiliproteins

Phycocyanobilin chromophore is covalently attached to cysteinyl residues on phycocyanin, one on the α subunit (α -84) and two on the β subunit (β -82 and β -153) (reviewed in Glazer, 1989). Researchers first speculated that the addition of bilins to phycobiliproteins was a spontaneous occurrence, like it is in bilin addition to apo-phytochrome, but *in vitro* bilin addition experiments provided support for the requirement of enzymes called lyases (Arciero *et al.*, 1988). These lyases function to attach bilins through thioether linkages to specific cysteinyl residues of apophycobiliproteins (proteins without bilins attached) (Glazer, 1994).

Arciero *et al.*, (1988) performed *in vitro* addition experiments with phycocyanobilin and apo-phycocyanin. The α and β subunits of PC from *Synechococcus* sp. PCC 7002, CpcA and CpcB, respectively, were overexpressed in *Escherichia coli* cells. The copurified monomeric

($\alpha\beta$) apo-PC subunits were incubated with PCB at room temperature in the dark for 16 hours, which resulted in the formation of covalent bilin adducts. The reaction was assayed by the change of the visible absorption spectrum of the adducts with noticeable spectroscopic differences between the *in vitro* produced PC and the native PC. Amino acid analysis of the *in vitro* produced chromopeptides revealed that PCB reacted specifically only at the cysteinyl residues α -84 and β -82 leaving the β -153 site unchromophorylated. In addition, NMR spectra of these *in vitro* products showed that the actual covalent bilin product was more oxidized than PCB and that the bilin of the two adduct peptides, α -84 and β -82, was 3'cysteinylmesobiliverdin which has an extra double bond in the C2-C3 position of ring A (Figure 8). These results indicate that the major bilin products arising from an *in vitro* nonenzymatic reaction of PCB with apo-PC differ from the bilins present in wild-type PC. This supported strongly the hypothesis that the addition of PCB to apo-PC is not a spontaneous event, but rather that enzymes are required for chromophorylation (Arciero *et al.*, 1988).

1.5 Enzymes Involved In PBP Post-Translational Modifications

1.5.1 The *sl10487* Gene Denoted *cpcM*

Donald Bryant's lab (Pennsylvania State University) cloned the *apcE* gene, which encodes an allophycocyanin core linker protein from *Synechococcus* sp. PCC 7002 (J. Zhou, 1992), and upstream from this gene they found an unidentified partial ORF, which showed similarity to methyltransferases (J. Zhou, 1992). This homology to known methyltransferases suggests that the product of this ORF is likely to exhibit methylase activity. Since, at the beginning of this research project the genome of *Synechococcus* sp. PCC 7002 had not been fully sequenced, a similar cyanobacterium whose genome had been fully sequenced, *Synechocystis* sp.

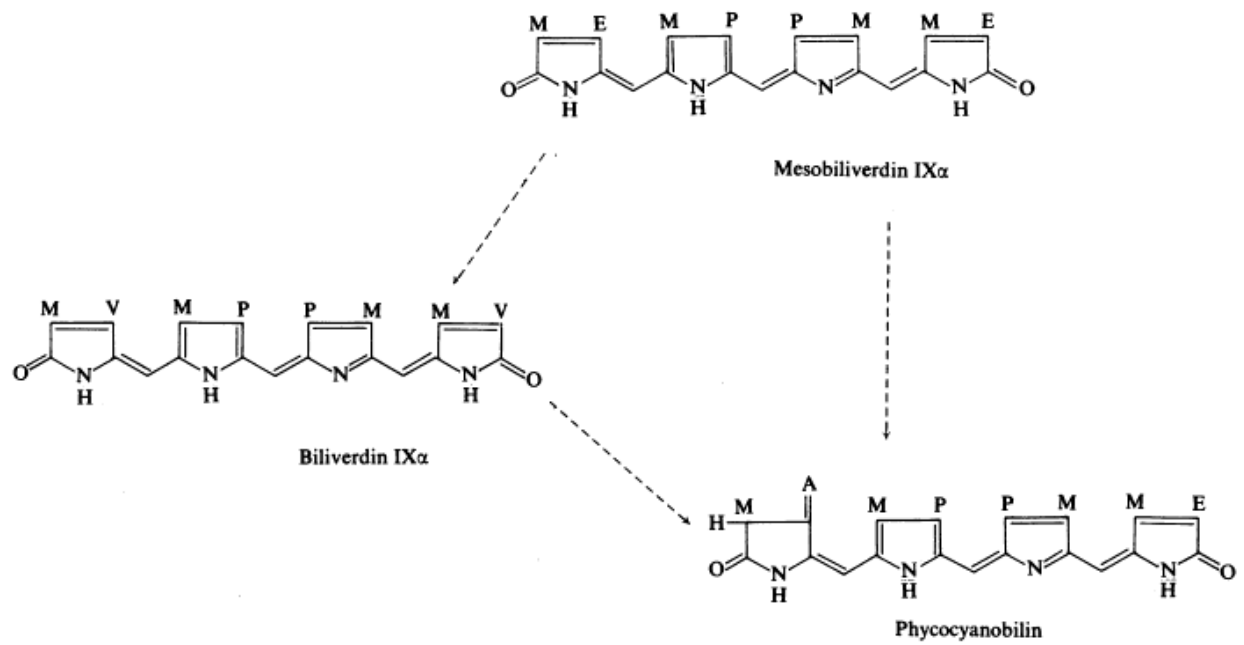


Figure 8: Structure of mesobiliverdin compared to phycocyanobilin. Abbreviations used: M, -CH₃; V, -CH=CH₂; P, -CH₂CH₂CO₂H; E, -CH₂CH₃; A, =CH-CH₃ (modified from Brown and Holroyd, 1984).

PCC 6803, was used to further investigate this ORF. The sequence analysis of the genome for *Synechocystis* sp. PCC 6803 assigned a potential protein-coding region, similar to the *Synechococcus* sp. PCC 7002 ORF found in Donald Bryant's lab, to the area of the genome *sll0487* (Accession No. X05302) (H.R Leonard, 2002). Genomic sequencing of several cyanobacterial strains, including *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002, has provided very useful information in identifying the candidate gene that functions to methylate PBP. Gene homologs of the *sll0487* gene encoding a hypothetical methyltransferase from *Synechocystis* sp. PCC 6803 have been found to be located upstream of the *apcE* gene in the genomes of *Synechococcus* sp. PCC 7002 and several other cyanobacteria. As shown in Figure 9, this gene exists in all of the sequenced genomes of cyanobacteria. Based on the bioinformatic analysis and results of characterization of mutants in two different cyanobacterial strains, the cyanobacterial gene *sll0487* from *Synechocystis* sp. PCC 6803 is designated as the *cpcM* gene.

When the *Synechocystis* sp. PCC 6803 *cpcM* gene was compared against the Genbank database the highest scored hit had a high sequence similarity (65%) to a SAM-dependent methyltransferase from the cyanobacterium *Nostoc punctiforme* PCC 73102, and the next highest scored hits were putative methyltransferases from other cyanobacteria. A conserved domain search resulted in significant similarities with several methylases including SAM-dependent methyltransferases (64.6%). This *cpcM* gene was also only found in genomes of organisms that contain phycobiliproteins. The proximity of this ORF to a phycobiliprotein structural gene and its similarity to other methylases made this gene product, CpcM, a candidate for the asparagine methylase.

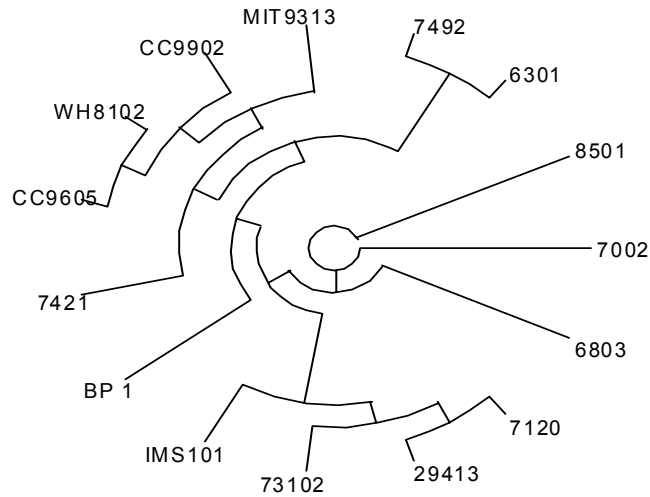


Figure 9: Phylogenetic tree of CpcM proteins from cyanobacteria. Amino acid sequences of CpcM proteins were compared from *Synechococcus* sp. PCC 7002 (7002), *Synechocystis* sp. PCC 6803 (6803), *Anabaena* sp. PCC 7120 (7120), *Nostoc punctiforme* PCC 73102 (73102), *Anabaena variabilis* ATCC 29413 (29413), *Thermosynechococcus elongatus* BP-1 (BP 1), *Synechococcus* sp. WH8102 (8102), *Crocospaera watsonii* WH8501 (8501), *Trichodesmium erthraeum* IMS101 (IMS101), *Gloeobacter violaceus* PCC 7421 (7421), *Synechococcus elongatus* PCC 7942 (7942), *Synechococcus* sp. PCC 6301 (6301), *Prochlorococcus marinus* MIT9313 (MIT9313), *Prochlorococcus* sp. CC9605 (CC9605), *Prochlorococcus* sp. CC9902 (CC9902), *Prochlorococcus marinus* MIT9313 (MIT9313) (G.Shen, W.M. Schluchter, and D.A. Bryant unpublished results).

Previously, the *cpcM* gene was inactivated by interposon mutagenesis (H.R. Leonard, 2002). The *cpcM* mutant as compared to the wild-type demonstrated a 32.5% and 13.7% decrease in doubling time when grown with glucose and without glucose, respectively (H.R. Leonard, unpublished results). State transition measurements using fluorescence emission spectra during the exponential phase and under green light resulted in no light-induced transition from state 2 to state 1 in the *cpcM* mutant and impaired excitation energy transfer from phycocyanin to allophycocyanin and from Photosystem I (G. Shen, H. Leonard, W. Schluchter, D.A. Bryant, unpublished results). This phenotype was consistent with CpcM being the asparagine methyl transferase.

1.5.2 α Subunit Lyases: CpcE/F and PecE/F

The first phycobiliprotein lyases discovered were CpcE and CpcF from the phycocyanin of the cyanobacterium *Synechococcus* sp. PCC 7002. The *cpcE* and *cpcF* genes were first identified because they were present in an operon including the PBP structural genes: *cpcA*, *cpcB*, *cpcC*, and *cpcD*. The function of the *cpcE* and *cpcF* genes were explored using knockout mutagenesis techniques resulting in mutants that were yellow-green in color, had increased doubling times, and low levels of phycocyanin (Zhou *et al.*, 1992). About 90% of the phycocyanin lacked a phycocyanobilin chromophore on the α subunit, however the β subunit of PC had the correct bilin incorporated at both cysteinyl residues (Fairchild *et al.*, 1992). Because knocking out the *cpcE* and *cpcF* genes resulted in cyanobacterial cells that could not attach PCB to the α subunit of PC (Swanson *et al.*, 1992), the authors concluded that these genes might encode the lyases for bilin attachment to the α subunit.

Fairchild *et al.* (1992) performed *in vitro* reactions with recombinant *cpcE* and *cpcF*, PCB, and apo- α PC attached to resin. The product of this reaction was holo- α PC (protein with bilins attached) which was determined by absorbance and fluorescence spectroscopy. Additional work by Fairchild and Glazer (1994) showed that CpcE and CpcF form a heterodimer that attaches PCB to PC. Tooley *et al.* (2001) recreated the pathway of α PC biosynthesis within *Escherichia coli*. They created two plasmids: one plasmid contained all the essential genes for bilin biosynthesis, including enzymes that convert heme to PCB and another plasmid that contained the genes *cpcA* (apo- α PC) and *cpcE* and *cpcF* (lyases). After induction of the products, one-third of the apo- α PC was converted to holo- α PC. Through spectroscopic and SDS-PAGE analysis, this holo- α PC demonstrated similar absorbance and fluorescent properties as compared to native PC found within cyanobacteria. No mesobiliverdin was found in *Escherichia coli* meaning that there was no non-enzymatic addition of bilin which would result in the unnatural addition of mesobiliverdin instead of the enzymatic addition of phycocyanobilin, the correct bilin.

Other α subunit lyases discovered are PecE and PecF from *Anabaena* sp. PCC 7120, a cyanobacterium that contains phycoerythrocyanin (PEC) in addition to phycocyanin in its rods. The genes *pecE* and *pecF* were found in the *pec* operon containing other PEC structural genes: *pecB*, *pecA*, and *pecC* (Jung *et al.*, 1995). The β subunits of PEC and PC carry two PCB chromophores at cysteinyl residues β -82 and β -153, but the PEC α subunit carries one phycoviolobilin (PVB) (see Figure 2) at cysteinyl residue α -84 (Tooley and Glazer, 2002). The *pecE* and *pecF* genes of *Anabaena* sp. PCC 7120 show 47% similarity to *cpcE* and *cpcF* genes of *Synechococcus* sp. PCC 7002. Jung *et al.* (1995) created *pecE* and *pecF* single and double

knockout mutants, and similar to the *cpcE* and *cpcF* mutants, these mutants produced less PEC, and the α subunit had the incorrect bilin attached.

Zhao *et al.* (2000) performed *in vitro* reactions by adding overexpressed *pecE* and *pecF* from *Escherichia coli* to apo- α PEC and PCB resulting in the product holo- α PEC that was identical to native holo- α PEC found inside cyanobacteria based on absorbance spectroscopy. When apo- α PEC was incubated with PCB without PecE and PecF, the bilin adduct formed was mesobiliverdin instead of PVB which suggests that PecE and PecF proteins are necessary for the addition of PCB and then its subsequent isomerization to PVB. Further research has concluded that PecE is responsible for attachment of PCB to PEC and PecF is responsible for the isomerization of PCB to PVB (Storf *et al.*, 2001; Zhao *et al.*, 2002). Tooley and Glazer (2002) recreated the pathway of α PEC biosynthesis within *Escherichia coli*. They created two plasmids: one plasmid contained all the essential genes for PCB biosynthesis and another plasmid that contained the genes *pecA* (apo- α PEC) and *pecE* and *pecF* (lyases). After induction of the products, two-thirds of the apo- α PEC was converted to holo- α PEC. This holo- α PEC demonstrated similar absorbance and fluorescent properties as compared to native PEC found within cyanobacteria (Tooley and Glazer, 2002).

1.5.3 β Subunit Lyases: CpcS and CpcT

Until recently, very little was known about the attachment of bilins to the two cysteine residues of the phycocyanin β subunit, β -82 and β -153. In 2002 Cobley *et al.*, discovered a gene required for the expression of the *cpe* operon in the cyanobacterium *Fremyella diplosiphon* called *cpeR*. One operon contains the genes encoding the α and β subunits of phycoerythrin (PE), *cpeA* and *cpeB*. The PE linker polypeptide operon contains the genes: *cpeC*, *cpeD*, *cpeE*, *cpeS*,

cpeT, and *cpeR*. The *cpeS* and *cpeT* genes were found in the genomes of other organisms containing phycobiliproteins, even in species lacking phycoerythrin. A *cpeT* transposon mutant was lacking in PE, and initially Cobley believed that the CpeT protein was involved in the regulation of transcription for the PE operon (J. Cobley, personal communication). However, there was no direct evidence that the *cpeS* and *cpeT* genes were transcriptional regulators as they had no sequence similarity to such DNA binding proteins.

Recently, a group of four genes: *cpcS*, *cpcT*, *cpcU*, and *cpcV* have been identified in *Synechococcus* sp. PCC 7002 that show similarity to these *cpeS* and *cpeT* genes. Three of the genes are similar to the *cpeS* gene found in *Fremyella diplosiphon*: *cpcS*, *cpcU*, and *cpcV*, and there is one gene that is an ortholog of the *Fremyella diplosiphon* gene *cpeT*, *cpcT*. Shen *et al.* (2006) created a *cpcT* mutant in *Synechococcus* sp. PCC 7002 which contained 40% less PC than wild-type and produced smaller PBS with red-shifted absorbance and fluorescence. Purified PC from the mutant was cleaved with formic acid and analyzed via SDS-PAGE. Results showed that there was no PCB bound to the peptide containing cysteine β -153. Recombinant CpcT was used to perform *in vitro* bilin addition assays with apo-PC (CpcB/CpcA) and PCB. The reaction products had an absorbance maxima between 597 and 603 nm as compared to 638 nm for control reactions (with mesobiliverdin attached). After trypsin digestion and Reverse-Phase HPLC, the CpcT reaction product had one major PCB-containing peptide. This peptide had a retention time identical to that of the tryptic peptide that includes PCB-bound, cysteine β -153 of wild-type PC. Shen *et al.* (2006) concluded, from characterization of the *cpcT* mutant and *in vitro* biochemical assays, CpcT is a new PCB lyase that specifically attaches PCB to cysteine β -153 of PC.

Zhao *et al.* (2006) expressed the gene *alr0617*, which is homologous to *cpeS* and *cpcS*, from the cyanobacterium *Anabaena* sp. PCC 7120 in *Escherichia coli*. The gene product (CpeS)

was then incubated with PCB and CpcB which resulted in a rapid increase of absorption around 619 nm and with a fluorescence emission around 643 nm. In both cases, PC and PEC, the binding reactions to cysteine β -82 catalyzed by CpeS are at least 10-fold increased compared to the spontaneous, non-enzymatic addition of PCB to the respective subunits. Zhao *et al.* (2006) concluded that CpeS catalyzes the site-selective attachment of PCB to cysteine β -82 in both CpcB and PecB.

Recently, it was shown that *Synechococcus* sp. PCC 7002 proteins CpcS and CpcU form a 1:1 complex and catalyze the addition of phycocyanobilin to the β -82 cysteinyl residue on phycocyanin *in vitro* (N. Saunée, 2006). Bilin addition reactions using the CpcSU complex and apo-CpcBA showed the addition of PCB to the β -82 cysteinyl residue. The PCB was generated *in situ* from PcyA using ferredoxin for the 4 electron reduction of biliverdin to PCB (Frankenburg *et al.*, 2001; Frankenburg and Lagarias, 2003). After incubation at 30°C for four hours a color change from blue-green to blue was visible. Absorbance and fluorescence spectra maxima of the reaction sample were 621 nm and 638 nm, respectively, which were different from the control reactions (no lyases added) and consistent with the presence of PCB at β -82 (absorbance and fluorescence maxima at 620 nm and 640 nm, respectively) (Debreczeny *et al.*, 1993). PcyA bilin addition reactions with apo-CpcBA and either CpcS or CpcU alone resulted in results similar to the control (N.Saunée, 2006).

To determine which cysteinyl residue the CpcSU lyase was adding to, tryptic digestion and Reverse-Phase HPLC was performed on a PcyA bilin addition reaction sample. The CpcSU-product and *Synechococcus* sp. PCC 7002 holo-phycocyanin were each digested with trypsin. The products of the trypsin cleavage were injected onto a C₁₈ HPLC reverse phase column and the elution profiles were analyzed. The holo-PC had three peaks: α -84 with a retention time of

20.1 minutes, β -82 with a retention time of 23.1 minutes, and β -153 with a retention time of 30.1 minutes. The CpcSU reaction sample only had one peak with a retention time of 23 minutes corresponding to that of the β -82 cysteinyl residue PCB site. This indicated that CpcSU is a lyase specific for bilin attachment at the Cys-82 on β phycocyanin of *Synechococcus* sp. PCC 7002 (N. Saunée, 2006).

Synechococcus sp. PCC 7002 PC β subunit has two PCB attachment sites, β -153 and β -82, and each requires two different lyases to perform this attachment, CpcT for the former and CpcSU for the latter. The reason that separate lyases are required for the two sites may have to do with the stereochemistry of the attachment sites. The PCB that attaches to the β -153 cysteine has S stereochemistry on the chiral carbon at C3¹, whereas the PCB that attaches to the β -82 cysteine has R stereochemistry on the chiral carbon at C3¹ (Figure 10) (Schirmer *et al.*, 1987; Shen *et al.*, 2006; Zhao *et al.*, 2006). The β -153 site has been shown to have S stereochemistry in all solved x-ray crystal structures of phycobiliproteins (Duerring *et al.*, 1990; Duerring *et al.*, 1991; Ritter *et al.*, 1999). With this information Shen *et al.*, (2006), hypothesized that it is probable that CpcT and its paralogs may also be responsible for attachment of any possible substrate to the β -153 or its corresponding site (Shen *et al.*, 2006). A compilation of all lyases used to chromophorylate *Synechococcus* sp. PCC 7002 phycocyanin and their locations are summarized in Figure 11.

1.6 Purpose of this Work

The first part of the research presented here is to further characterize the *sl10487* gene (*cpcM*) from the cyanobacterium *Synechocystis* sp. PCC 6803. Recombinant CpcM from *Synechocystis* sp. PCC 6803 will be assayed for methyl transferase activity to phycobiliprotein

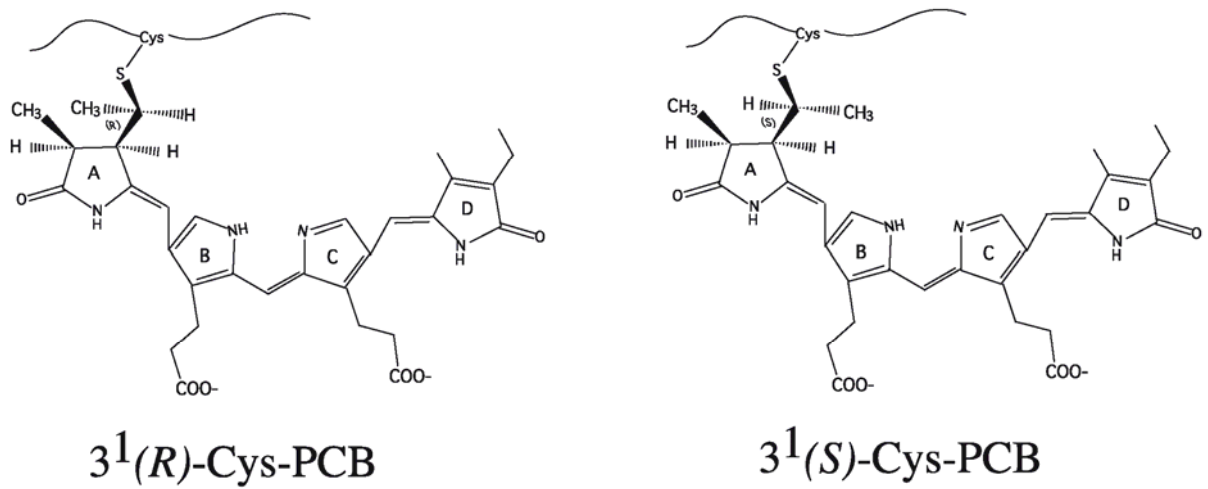


Figure 10: Structure of phycocyanobilin (PCB) in the R and S stereochemistry forms (modified from Shen *et al.*, 2006).

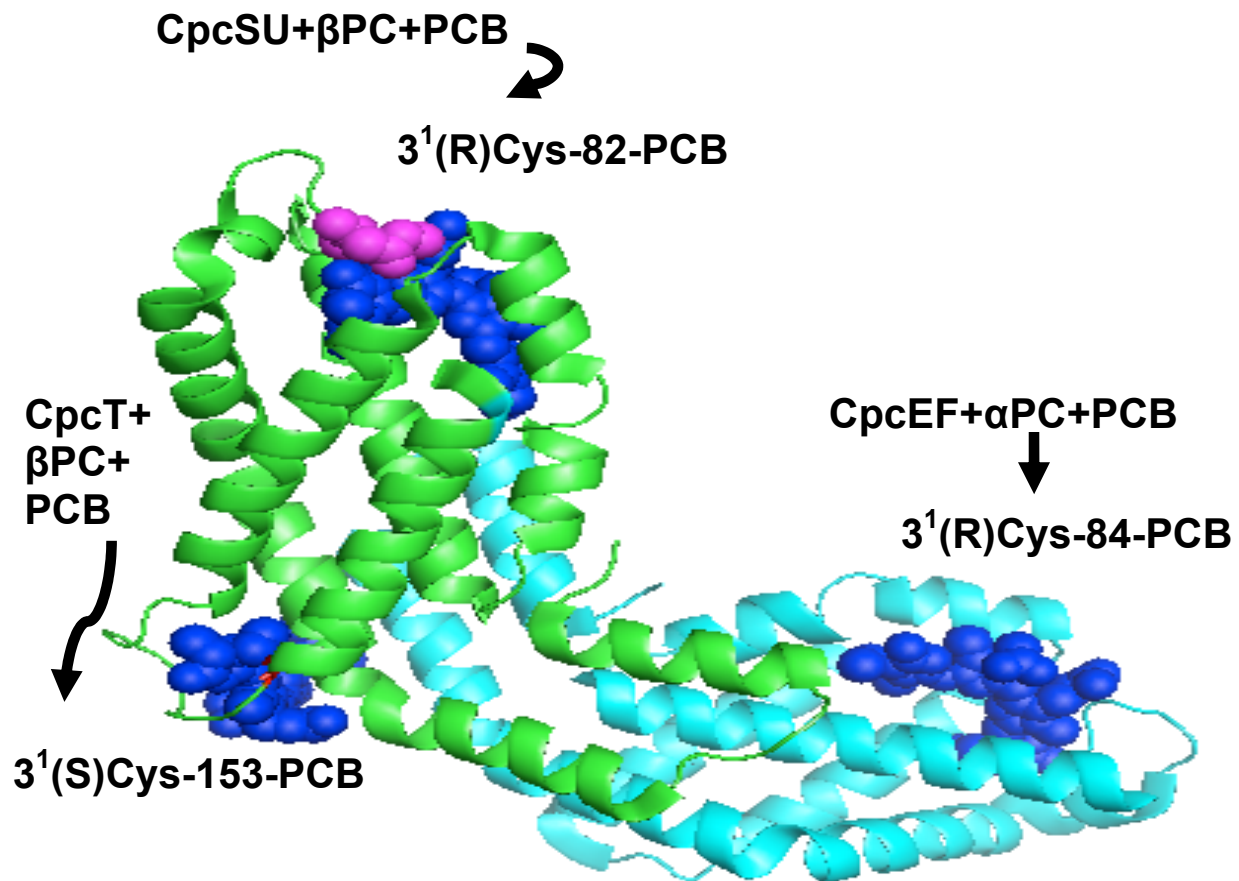


Figure 11: Crystal structure of phycocyanin. Summary of lyases required for addition of phycocyanobilin to phycocyanin in *Synechococcus* sp. PCC 7002. Stereochemistry is shown for α -84 (3^1 chiral carbon as R stereochemistry), β -82 (3^1 chiral carbon as R stereochemistry), and β -153 (3^1 chiral carbon as S stereochemistry)

subunits *in vitro* using [methyl-³H]S-adenosylmethionine. Through scintillation counting and autoradiograms enzymatic activity can be analyzed and quantitated.

The second part of this research focuses on phycobiliprotein biosynthesis in *Synechocystis* sp. PCC 6803. *Synechocystis* sp. PCC 6803 contains two genes that are *cpeS*-like genes, *slr2049* and *slr0853*, which are denoted *cpcS* and *cpcU*, respectively. This cyanobacterium also contains one *cpeT*-like gene, *slr1649*, denoted *cpcT*. The first goal of this part of the research is to clone these genes into plasmids and then purify the recombinant proteins from *Escherichia coli*. The second goal is to perform bilin addition reactions with apo-PC to determine if these proteins are in fact adding a bilin to either or both of the β subunit bilin attachment sites, β -82 and β -153. Lastly, biochemical analysis will be used to determine if the correct bilin addition product occurred in the presence of these lyases. These results can be compared to those achieved for *Anabaena* sp. PCC 7120 CpeS and for *Synechococcus* sp. PCC 7002 CpcSU (heteromer).

MATERIALS AND METHODS

Materials:

All chemicals were purchased from Fisher Chemical Company (Houston, TX) and Sigma Chemical Company (St. Louis, MO). Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and Promega Corporation (Madison, WI). The GeneClean kits were purchased from Qbiogene (Carlsbad, CA). PCR primers were purchased from Qiagen (Alameda, CA) and Operon Biotechnologies (Huntsville, AL). QIAquick PCR purification kit and QIAprep spin miniprep kit were purchased from Qiagen Incorporated (Valencia, CA). PCR Master Mix was purchased from Promega Corporation (Madison, WI). DH5 α , TOP10, 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). Big Dye was purchased from Applied Biosystems (ABI) (Foster City, CA). Amplify solution and [methyl-³H]S-adenosylmethionine was purchased from Amersham BioSciences (Piscataway, NJ). S-adenosylmethionine was purchased from Sigma Chemical Company (St. Louis, MO) and nitrocellulose filters were purchased from Fisher Chemical Company (Houston, TX). Kodak tritium sensitive film, developer and fixer solutions were purchased from Sigma-Aldrich Incorporated (St. Louis, MO). Sep-pak cartridges were purchased from Waters Corporation (Milford, MA).

2.1 Construction of Recombinant Expression Plasmids

2.1.1 Polymerase Chain Reaction (PCR)

All PCR amplification reactions were performed with 40 pmol of each primer, 10 ng of *Synechocystis* sp. PCC 6803 or *Synechococcus* sp. PCC 7002 chromosomal DNA, and 25 μ l of PCR Master Mix. 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 until retrieved. 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 without binding. The pure PCR product was eluted in water.

2.1.2 DNA Purification from Agarose Gels

Electrophoresis 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 ran for 60 minutes at 100 volts using an EC570-90 voltmeter (EC Apparatus Corporation, Holbrook, NY). After separation, gels were then stained in 10 mg/ml ethidium bromide for 15 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 Restriction Enzyme Digestion and Ligation

Four different vectors were used to create recombinant expression plasmids: pGEX-2T, pET100, pBS150V, and pAED4. The pGEX-2T vector contains an ampicillin resistance gene and contains a section of DNA that codes for an extra 232 amino acids at the N-terminus. This section encodes the protein glutathione-*S*-transferase (GST) fusion tag, which makes the recombinant protein easy to purify using glutathione agarose chromatography. The pGEX-2T vector also has a lac fusion promoter that allows the protein to be expressed when lactose (or IPTG) is present. The pET100 vector (Invitrogen) contains an ampicillin resistance gene, a lac fusion promoter, and contains a section of DNA that codes for an extra 37 amino acids at the N-terminus, which includes six histidines. This series 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 spectinomycin resistance gene, a lac fusion promoter, and also encodes a his-tag from the extra 23 amino acids at its N-terminus. The pAED4 vector contains an ampicillin resistance gene and a T7 promoter cloned into it, but contains no fusion tag. BL21*DE3* cells have the T7 RNA polymerase gene cloned behind the lac promoter, which is why pAED4 plasmids must be transformed into BL21*DE3* cells to promote protein expression.

The cleaned PCR amplification product of interest and its corresponding vector were digested with restriction enzymes. The digestions were composed of: at least 1 μg of DNA, 1 μl of restriction enzyme (10 units/ μl), 1 μl of 10X restriction enzyme buffer, and ddH₂O to give a total volume of 10 μl . The digestion reactions were allowed to incubate at 37°C for 1 hour then 1 μl of 10X DNA loading buffer was added to each digest. The digests were then separated on a 0.8% agarose gel and the bands were purified. The digested gene of interest and vector were added together and incubated at room temperature (16°C) overnight. The ligations were

composed of: 200 ng of digested PCR product, 50 ng of digested vector, 1 μ l of T4 DNA ligase, 1 μ l of 10X ligase buffer, and ddH₂O to give a total volume of 10 μ l.

2.1.4 Transformation

After overnight incubation, the ligations were transformed into either Top10 or DH5 α *Escherichia coli* cells using the method suggested by supplier. Transformation was obtained by adding 5 μ l of ligation to 50 μ l of cells. This mixture was put on ice for 5 minutes after a light tapping and then heat shocked at 42°C for 30 seconds. After the heat shock, the cells were placed on ice for 2 minutes and 250 μ l of S.O.C [0.5% Bacto-yeast extract (w/v), 2% Bacto-tryptone (w/v), 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 1 mM NaOH, 20 mM glucose] was added. Next, the cells were placed in a 37°C water bath for 1 hour.

Luria-Bertani [LB, 1% NaCl (w/v), 1% Bacto-tryptone (w/v), and 0.5% Bacto-yeast extract] plates with either ampicillin (100 μ g/ml) or spectinomycin (100 μ g/ml) were used to plate the transformations. The appropriate antibiotic was used to select for cells that contained vectors which hopefully contained inserts as well. The entire transformation sample was about 300 μ l; this allowed for 100 μ l and 200 μ l aliquots to be spread plated on two separate antibiotic LB plates.

2.1.5 Minipreps and Midipreps

Plasmid DNA was purified using the miniprep method from each colony grown in 5 ml LB with antibiotics using manufacturer's guidelines from the QIAprep spin miniprep kit. After a 0.8% agarose gel confirmed the correct size of the transformed molecule, another digest was performed on the ligated construct. The same enzymes used to originally digest both the gene of

interest and vector were used to digest the ligated vector using the same protocol. The digested DNA was then separated on a 0.8% agarose gel to confirm that bands of DNA representing the correct sizes of the insert and vector appear confirming a correct clone. A large scale midiprep (from a 50 ml LB with antibiotics culture) is then performed using the midiprep kit, again following manufacturer guidelines. Midiprep DNA was used for sequencing.

2.1.6 *cpcBA*, *apcAB*, *apcFA*, *cpcM*, *cpcS*, *cpcU*, and *cpcT*

The *cpcB* and *cpcA* genes were amplified together by PCR from wild-type *Synechocystis* sp. PCC 6803 chromosomal DNA using oligonucleotides *cpcB*.1 and *cpcA*3' (see Table 1) (W.M. Schluchter, unpublished results). All clones were made from 6803 chromosomal DNA unless otherwise specified. After PCR purification, the resulting 1.3-kb product was cloned into the 5.2-kb his-tagged vector pBS150V using restriction enzymes *Nde*I- and *Hind*III (Figure 12).

The *apcA* and *apcB* genes were amplified together by PCR using the primers *apcA*5' and *apcB*3' (Table 1). After PCR purification, the resulting 1.1 kb *apcAB* operon was cloned into the 5.7-kb his-tagged pET100 vector using Invitrogen's Champion™ pET Directional TOPO® Expression Kit (Carlsbad, CA), following manufacturer directions (Figure 13).

The *apcF* gene was PCR amplified from wild-type *Synechococcus* sp. PCC 7002 chromosomal DNA using the primers 7002ApcF5' and 7002ApcF3' (Table 1). After PCR purification, the resulting 0.6-kb product was cloned into the 5.7-kb his-tagged pET100 vector using Invitrogen's Champion™ pET Directional TOPO® Expression Kit, following manufacturer guidelines. Once the ligation was confirmed correct the *apcF*/pET100 plasmid was then used to construct another clone. The *apcA* gene was PCR amplified using the primers *apcA*5'Sac and *apcA*3'Sac (Table 1). The resulting 0.6-kb product was digested with *Sac*I and

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

Primer	Sequence (5' to 3')	Use
cpcB.1	GGAGATTAAT <u>CATATG</u> TTCGACGTATTCAC	Amplification of <i>cpcB</i>
cpcA3'	CCCAAGCTTCCAGGCCAGCTAGAAT	Amplification of <i>cpcA</i>
apcA5'	CACCATGAGTATCGTCACGAAATCAATCTG	Amplification of <i>apcA</i>
apcB3'	GGAATTCAGGACTAGCTCAAGCCAGA	Amplification of <i>apcB</i>
7002ApcF5'	CACCATGCGGGACGCTGTTACAAGTC	Amplification of <i>apcF</i>
7002ApcF3'	TCAGATATCTTAGAGATCCACTTCGCTCAGTTC	Amplification of <i>apcF</i>
7002apcA5-Sac	ATTTGAGCTCATATCTAGAGGAGGAATC	Amplification of <i>apcA</i>
7002apcA3-Sac	AACGGAGCTCCAGAGGGTAAAAACCAC	Amplification of <i>apcA</i>
slI0487.5.2	TTCGGATCCATGTTGTCCAACCTCCGAC	Amplification of <i>cpcM</i>
slI0487.3.2	AATTCCCGGGCATCGAGAAGTCG	Amplification of <i>cpcM</i>
slr2049cpeS.5	GAGTTAATCCATATGGATGCAATGGAATTCTTTCGC	Amplification of <i>cpcS</i>
slr2049cpeS.3	CTGGAAGCTTACGGGGCCTACCAGCCACA	Amplification of <i>cpcS</i>
slI0853cpeS2.5	CAATAGCCTCATATGAAGCCTGTTGCCCAAGAA	Amplification of <i>cpcU</i>
slI0853cpeS2.3	GTA ^{CT} CGAGA ^{ACT} GTTACTCTGCCGGAGG	Amplification of <i>cpcU</i>
slr1649cpeT1.5	CAATAACTACATATGTCCCATTCCACTGATCTTT	Amplification of <i>cpcT</i>
slr1649cpeT1.3	GTCAAGCTTGCTAAAACTAACTTCATC	Amplification of <i>cpcT</i>
slr1649GST5	TTGGATCCATGTCCCATTCCACTGATCTTTCC	Amplification of <i>cpcT</i>
slr1649GST3	GTGAATTCTGGCTAAAACTAACTTCATCGGCG	Amplification of <i>cpcT</i>
pBS150NcoI	CAGACCATGGGTCATCATCATCATCACG	Sequencing of <i>cpcBA</i>
pBS150V.3	AAATGCAGGGCCAGTGCCAAGCTT	Sequencing of <i>cpcBA</i>
T7 Promoter Primer	TAATACGACTCACTATAGGG	Sequencing of <i>apcAB</i> , <i>apcFA</i>
T7 Reverse Seq. Primer	TAGTTATTGCTCAGCGGTGG	Sequencing of <i>apcAB</i> , <i>apcFA</i>
pGEX2T5	GCCTTTGCAGGGCTGGCAAG	Sequencing of <i>cpcM</i> , <i>cpcT</i>
pGEX2T3	CCGTCATCACCGAAACGCG	Sequencing of <i>cpcM</i> , <i>cpcT</i>
pAED45.2	AGCAGCCGGATCTGATATCATCG	Sequencing of <i>cpcS</i> , <i>cpcU</i>
pAED43.2	TAACGGATCCAAGGAGATATACATATG	Sequencing of <i>cpcS</i> , <i>cpcU</i>

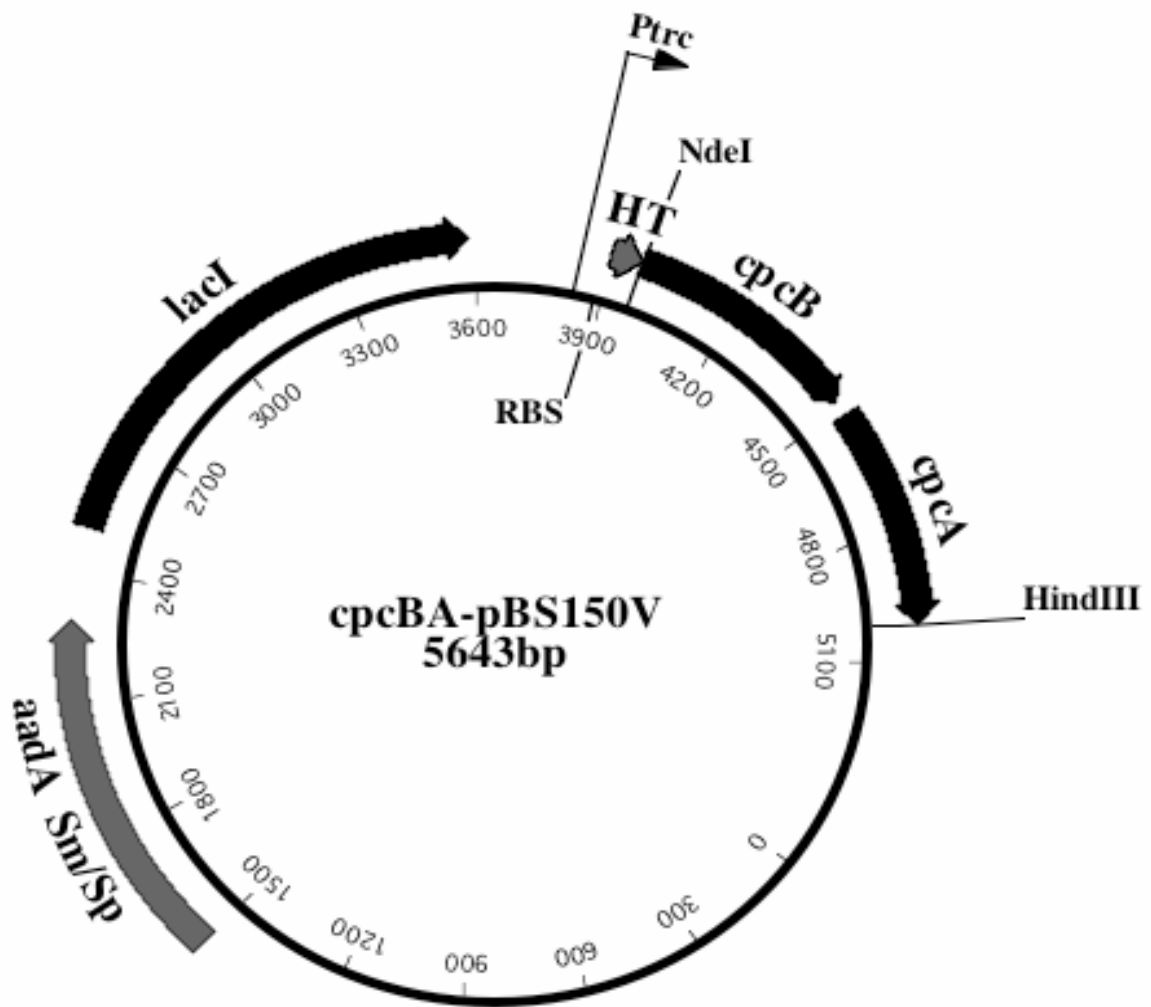


Figure 12: Plasmid map for *Synechocystis* sp. PCC 6803 clone *cpcBA*/pBS150V.

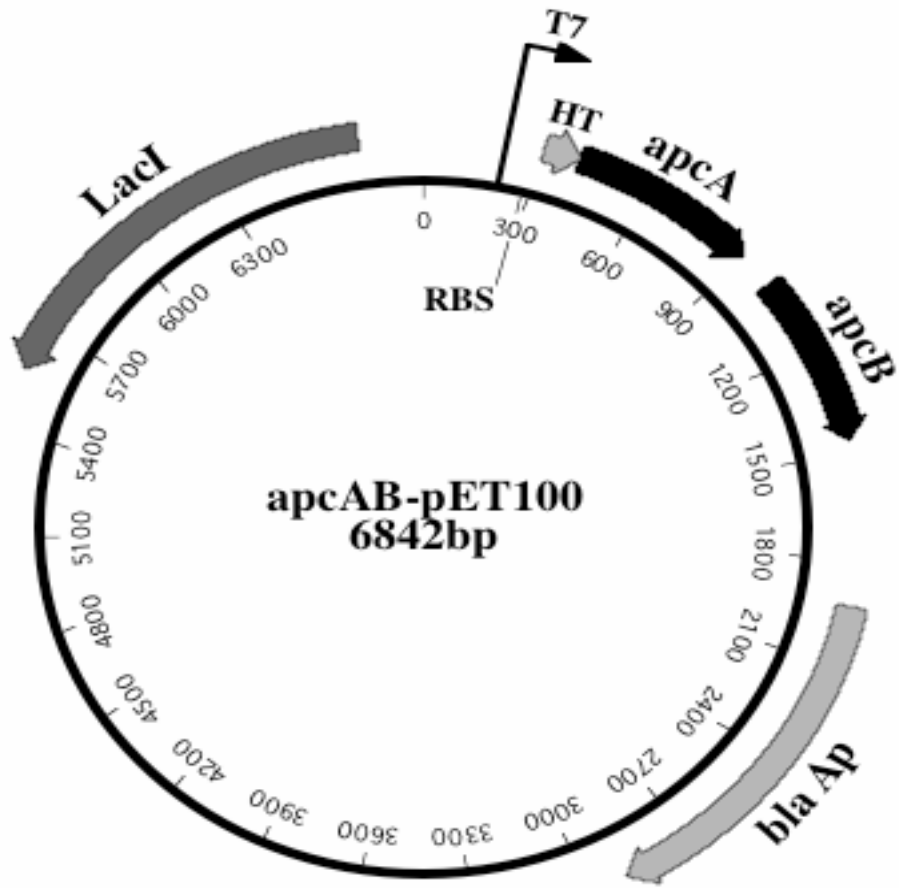


Figure 13: Plasmid map for *Synechocystis* sp. PCC 6803 clone *apcAB/pET100*.

cloned into *SacI*-digested *apcF*/pET100 plasmid of 6.3-kb such that *apcA* will be cotranscribed with *apcF*. The plasmid was digested with *HindIII* to confirm the correct orientation of the *apcA* gene within the plasmid. A plasmid construct of *apcFA*/pET100 (HT-*apcF*) was made and a midiprep was performed (Figure 14) (A. Fletcher, B. Turner, and W.M. Schluchter, unpublished results). Primers used to PCR amplify the *cpcM* gene were sll0487.5.2 and sll0487.3.2 (Table 1). The resulting 1.3-kb product was digested with *BamHI* and *SmaI* and cloned into *BamHI*- and *SmaI*-digested GST-tagged cloning vector pGEX-2T of 4.9-kb (Figure 15).

The *cpcS* gene was amplified by PCR using the oligonucleotides slr2049cpeS.5 and slr2049cpeS.3 (Table 1). After PCR purification, the resulting 0.6-kb *cpcS* gene was cloned into the 3.3-kb vector pAED4 using the restriction enzymes *NdeI* and *HindIII* (Figure 16). The *cpcU* gene was amplified by PCR using the primers sll0853cpeS2.5 and sll0853cpeS2.3 (Table 1). After PCR purification, the resulting 0.6-kb *cpcU* gene was cloned into the 3.3-kb vector pAED4 using the restriction enzymes *NdeI* and *XhoI* (Figure 17).

The *cpcT* gene was the only gene ligated into two different vectors. The gene was amplified by PCR using the primers slr1649cpeT1.5 and slr1649cpeT1.3 (Table 1). After PCR purification, the resulting 0.6-kb *cpcT* gene was cloned into the 3.3-kb vector pAED4 using the restriction enzymes *NdeI* and *HindIII* (Figure 18). The *cpcT* gene was also PCR amplified using the primers slr1649GST5 and slr1649GST3 (Table 1). After PCR purification, the resulting 0.6-kb gene was cloned into the 4.9-kb GST-tagged vector pGEX-2T using the restriction enzymes *BamHI* and *EcoRI* (Figure 19). Only the GST-tagged *cpcT* plasmid was used for further study.

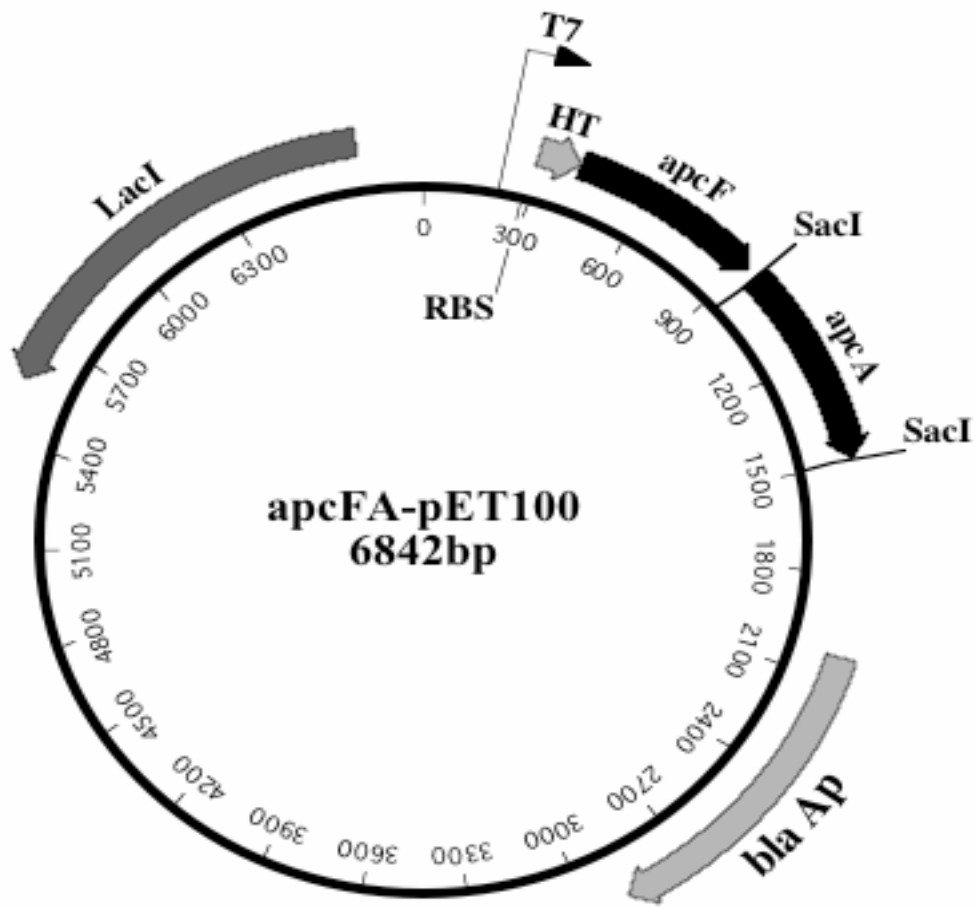


Figure 14: Plasmid map for *Synechococcus* sp. PCC 7002 clone *apcFA/pET100*.

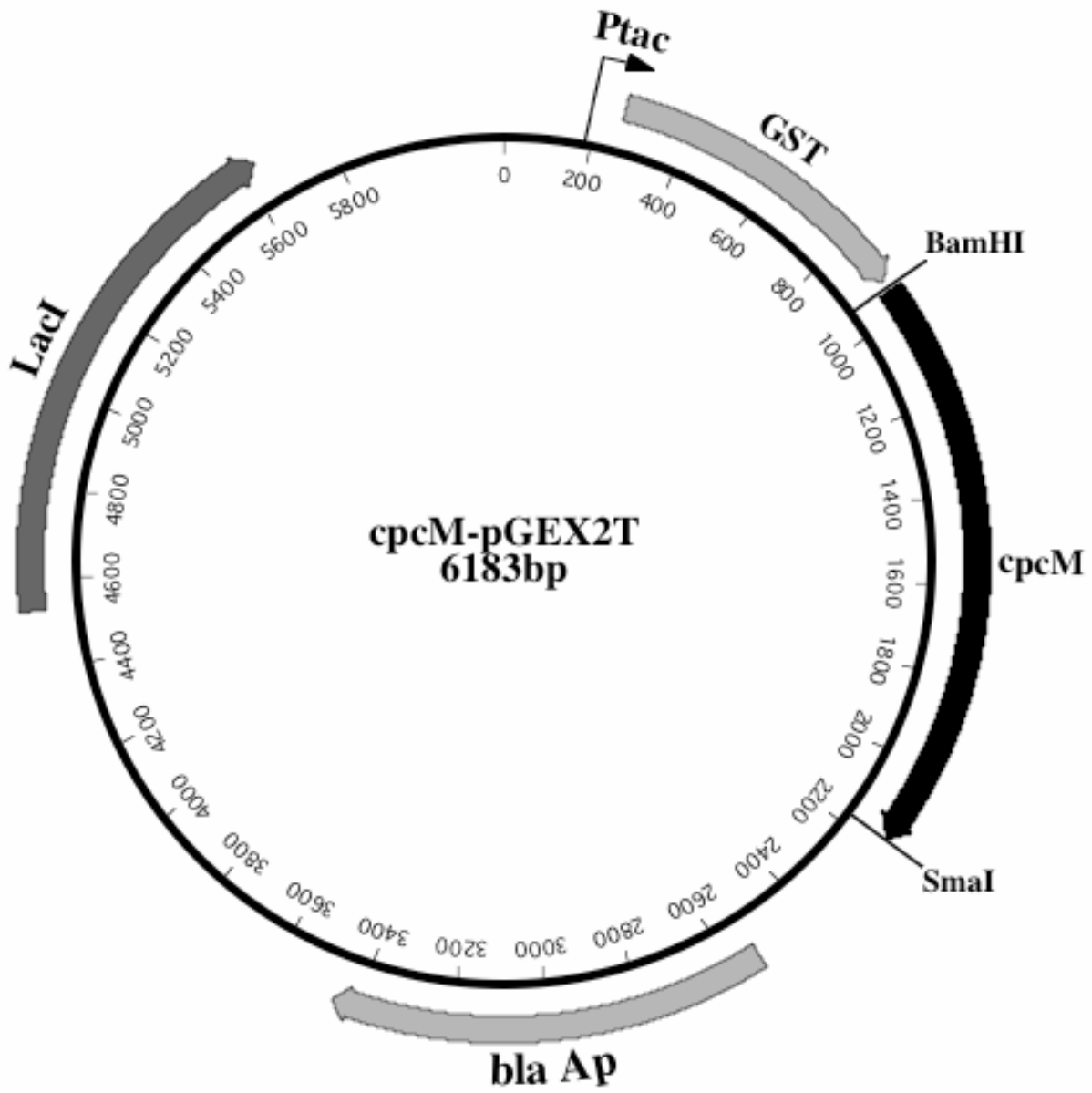


Figure 15: Plasmid map for *Synechocystis* sp. PCC 6803 clone *cpcM/pGET-2T*.

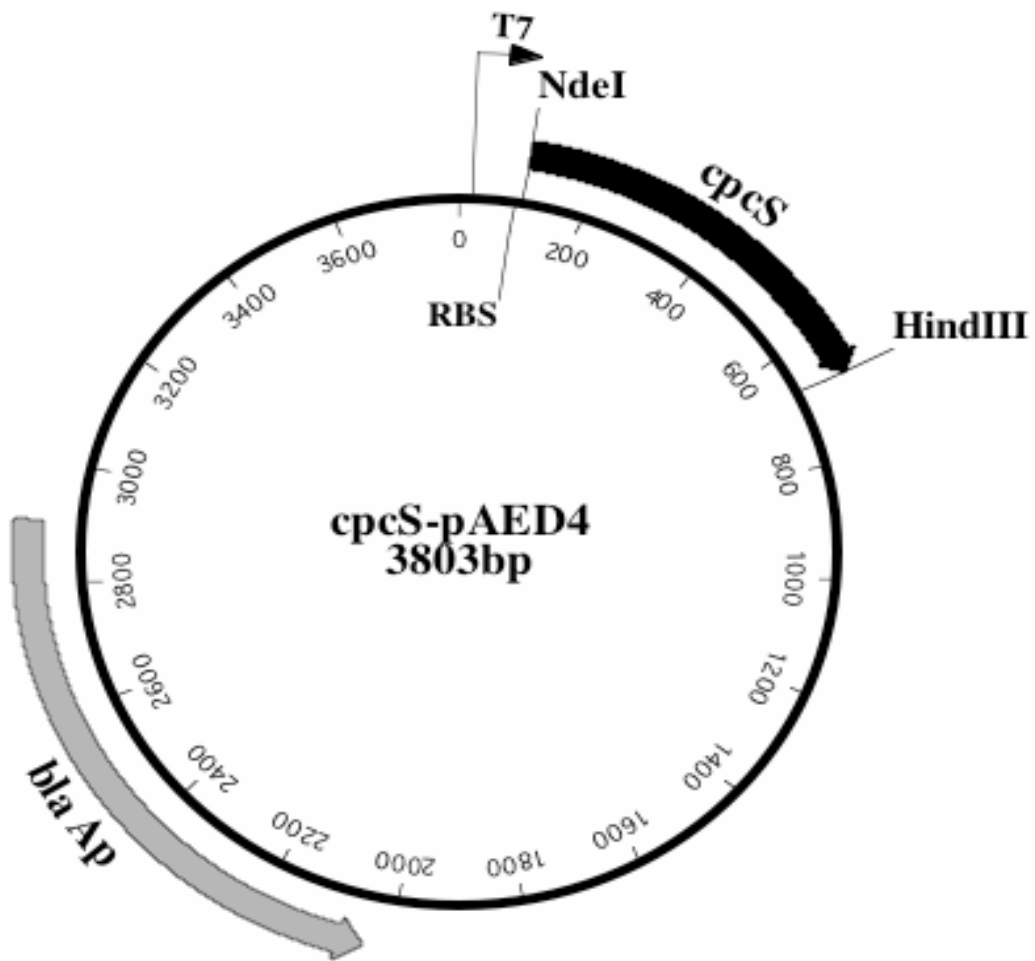


Figure 16: Plasmid map for *Synechocystis* sp. PCC 6803 clone *cpcS/pAED4*.

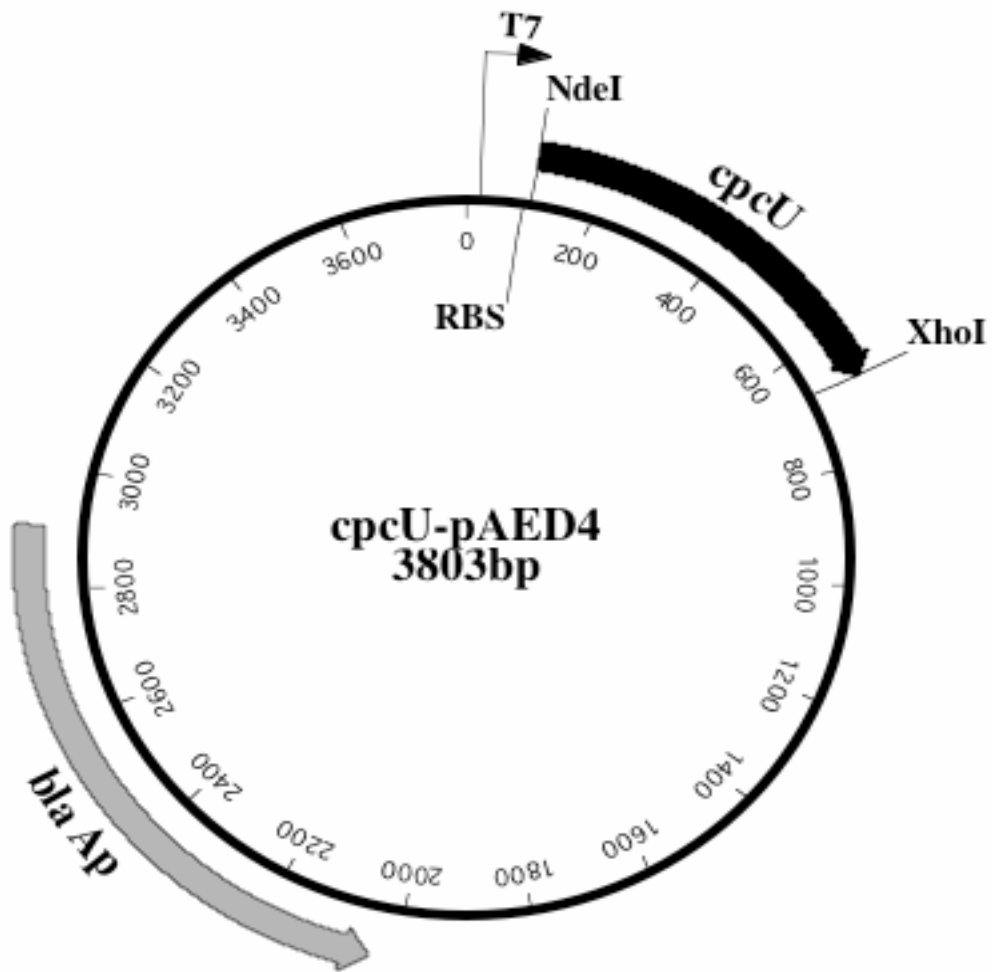


Figure 17: Plasmid map for *Synechocystis* sp. PCC 6803 clone *cpcU*/pAED4.

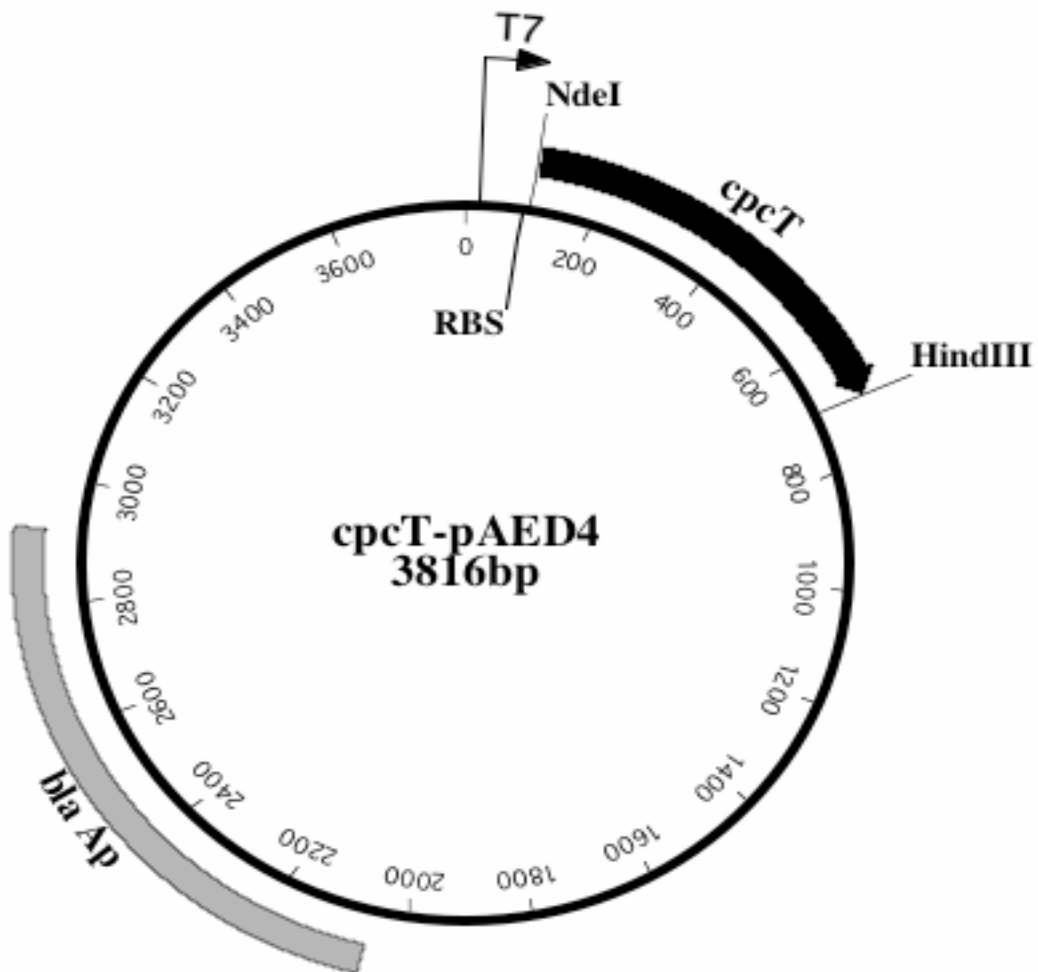


Figure 18: Plasmid map for *Synechocystis* sp. PCC 6803 clone *cpcT/pAED4*.

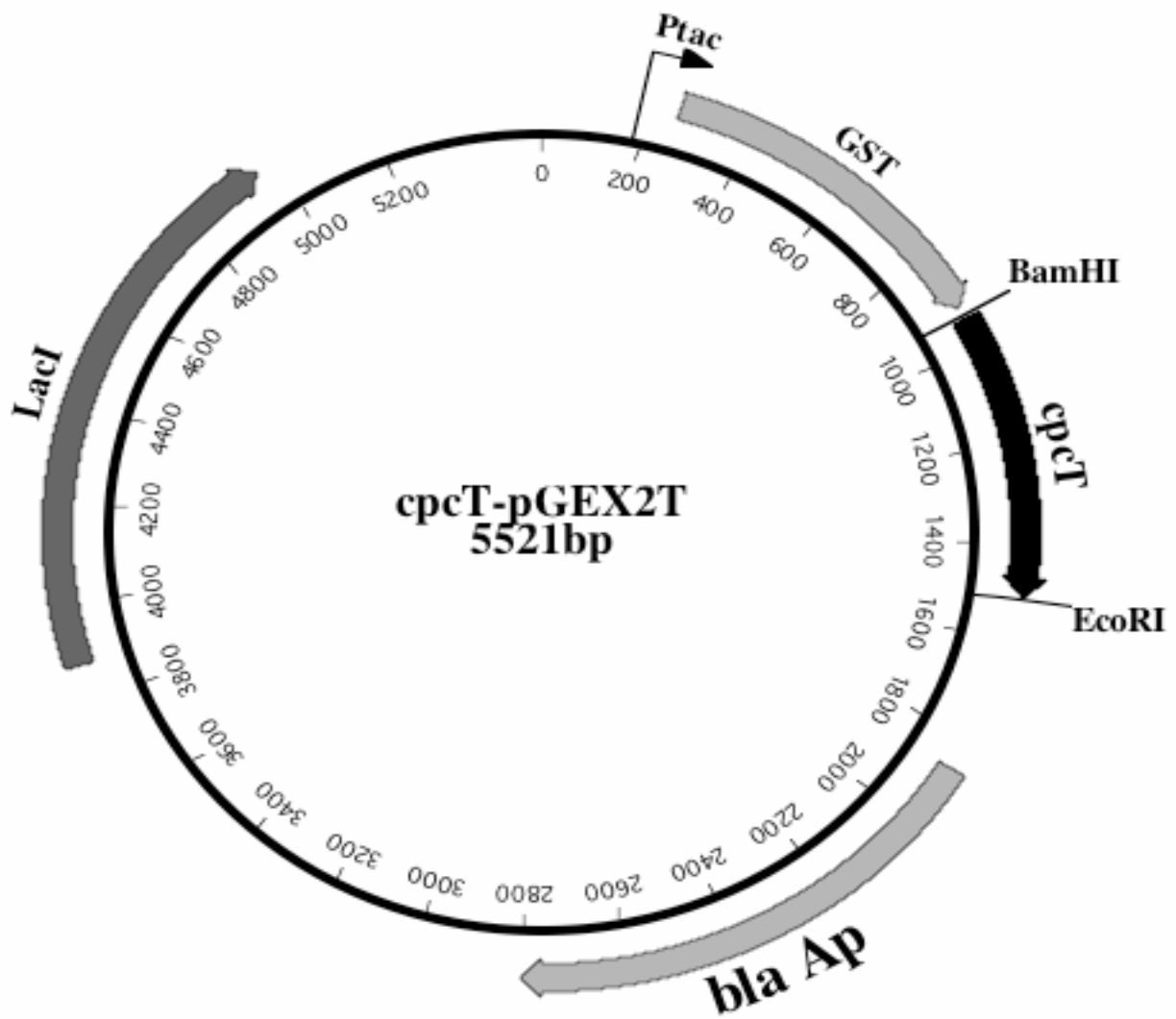


Figure 19: Plasmid map for *Synechocystis* sp. PCC 6803 clone *cpcT*/pGEX-2T.

2.2 Sequencing

All recombinant expression plasmid clones were sequenced in the W.M. Keck Conservation and Molecular Genetics laboratory to confirm that the clones were correct and no mutations had occurred. To sequence the genes the following was combined: 2 μ l of template DNA (300-500 ng), 1 μ l of primer (0.8 pmol/ μ l), and 2 μ l of Big Dye. The cycle sequence on the Thermocycler was as follows: an initial denaturing step performed only once, a second denaturing step at 96°C for 2 seconds, an annealing step at 50°C for 15 seconds, and an extension step at 60°C for 15 seconds. The three steps were repeated 25 times. To remove unincorporated dNTPs and primer, a slurry of Sephadex G-50 was used with a 2.0 ml eppendorf tube and a mini-column (USA Scientific). To the tube/column combination 650 μ l of Sephadex was added and centrifuged for 3 minutes at 14,000 x g. The column was placed in a new 1.5 ml eppendorf tube and the DNA sample was added to the column and centrifuged for 3 minutes at 14,000 x g. The clean samples were then dried in a speed vac for 8 minutes. After drying, 20 μ l of hi di (formamide) was added to each sample and then loaded into the ABI PRIZM 3100 Genetic Analyzer. The resulting sequences were then analyzed using the MacVector program (Accelrys, San Diego, CA) to compare sequence alignment to actual gene sequence. This confirmed if the constructs were correct and if mutations had been introduced.

2.3 SDS-PAGE Analysis

The protein samples were boiled for 15 minutes and separated by SDS-PAGE using a 15% Tris-HCl 8.6 x 6.8 cm resolving gel or a 10-20% Tris-HCl 13.3 x 8.7 cm resolving gel. 15% Tris-HCl SDS-PAGE gels were subjected to electrophoresis at 100 volts for 12 minutes followed by 45 minutes at 200 volts and 10-20% Tris-HCl gels were electrophoresed for 60

minutes at 200 volts. Gels were stained overnight in Coomassie Blue and then destained (10% methanol, 10% acetic acid). To assay for the presence of covalent bilins, zinc ions were added to bind to bilin for detection by 550 nm Vis light. 100 mM ZnSO₄ was added to SDS-PAGE gel and incubated for 2-3 minutes. The gel was then scanned for bilin fluorescence at 550 nm and images were acquired.

2.4 Recombinant Protein Production and Purification

2.4.1 Protein Production

BL21 cells were transformed with the plasmids containing *cpcBA*, *apcAB*, *apcFA*, *cpcM*, and *cpcT* and streaked onto a LB plate containing the appropriate antibiotic. Plasmids containing *cpcS* and *cpcU* were transformed into BL21DE3 cells like the plasmids mentioned above. To produce proteins encoded from plasmids transformed into BL21DE3 cells, a 50-ml overnight starter culture was added to each 1-L LB with the appropriate antibiotic and grown for 4 hours at 37°C. Gene expression was induced by the addition of 0.5 mM isopropyl β-D-thiogalactoside (IPTG), and cells were then allowed to grow for an additional 4 hours. To express genes from plasmids transformed into BL21DE3 cells, a 25-ml overnight starter culture was added to each 1-L LB with the appropriate antibiotic and grown for 6 hours at 30°C. No IPTG was added during growth period. All expression cells were harvested by centrifugation at 3273 x g and frozen at -20°C until later.

2.4.2 CpcBA, ApcAB, ApcFA, and CpcA Purification

CpcBA, ApcAB, and ApcFA each contain a subunit that is a his-tagged protein. In the case of CpcBA, CpcB is his-tagged and CpcA co-purify with HT-CpcB. In ApcAB, ApcA is his-

tagged, and ApcB will co-purify with it, and in ApcFA, ApcF is his-tagged, and ApcA will co-purify with it. The *cpcA* gene was expressed using the pBS414V vector which encodes HT-CpcA and the lyases CpcE and CpcF (Tooley *et al.*, 2001). All his-tagged proteins were purified from cells as follows: cell pellets were thawed on ice for around 30 minutes and resuspended in 20 ml of cold (0-4°C) buffer 0 [20mM Tris·HCl (pH 8.0), 100 mM Na/KCl]. The suspensions were vortexed, homogenized, and then the cells were broken by passage through a French Pressure Cell three times at 18,000 psi. Five ml of nickel-nitrilotriacetic acid (Qiagen Incorporated, Valencia, CA), or Ni-NTA, was incubated with 10 ml of buffer 0 on ice for 20 minutes then centrifuged for 5 minutes at 5,000 x g. After centrifugation of the cell extract at 15,000 x g in a Sorvall® GSA rotor for 30 min, to pellet unbroken cells and inclusion bodies, the supernatant was applied to a Ni-NTA Glass Econo-Column® (2.5 cm x 10 cm; BioRad, Richmond, CA) containing 5 mls of resin. The supernatant was slowly filtered through the resin three times. The agarose was washed with one column full each of buffer A1 [20 mM Tris·HCl (pH 8.0), 100 mM Na/KCl, 20 mM imidazole, 5% glycerol] buffer B [20mM 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]. His-tagged proteins were eluted from the agarose with 20 ml buffer C [20 mM Tris·HCl (pH 8.0), 100 mM Na/KCl, 200 mM imidazole] and the proteins were dialyzed against 2-L buffer 0 and 1 mM β-mercaptoethanol overnight at 4°C on a magnetic stirrer and against another 2-L for 4 hours longer.

2.4.3 CpcM, CpcT, GST, and PcyA Purification

GST-tagged proteins CpcM, CpcT, PcyA and GST itself (from the pGEX-2T vector) were all purified using Glutathione affinity chromatography. The *pcyA*/pGEX-2T plasmid was

kindly provided by Dr. J. C. Lagarias (U.C. Davis). The *pcyA*/pGEX-2T plasmid and the pGEX-2T vector alone were transformed and gene expressed in the same manner as CpcM and CpcT. Cell pellets were thawed on ice for around 30 minutes and resuspended in 20 ml of cold (0-4°C) buffer 0. The suspensions were vortexed, homogenized, and the cells were then broken by passage through a French Pressure Cell three times at 18,000 psi. After centrifugation of the cell extract at 15,000 x g for 20 minutes, the supernatant was applied to a glutathione-agarose (Sigma Chemical Company, St. Louis, MO) column (2.5 cm x 10 cm) with a 5 ml bed volume that was previously washed with 2 column volumes of equilibration buffer [20 mM Tris·HCl (pH 7.5), 150 mM NaCl]. The lyophilized glutathione agarose powder was also previously swollen by adding 10 ml of ddH₂O to 740 mg of powder (74 mg/ml) and allowed to swell for at least 2 hours. The supernatant was filtered through the agarose three times slowly. Another two columns full of equilibration buffer was applied to the column, and the protein was eluted using 20 ml of elution buffer [50 mM Tris·HCl (pH 8.0), 5 mM glutathione]. Proteins were dialyzed against 2-L 50 mM Tris·HCl (pH 8.0), 1 mM EDTA, and 1 mM β-mercaptoethanol overnight at 4°C on a magnetic stirrer and then in another 2-L for 4 more hours.

2.4.4 CpcS and CpcU Purification

To purify CpcS and CpcU, expression cells were resuspended in 50 mM Tris-HCl (pH 8.0), vortexed, homogenized, and passed through a French Pressure Cell at 18,000 psi three times. Whole cell extracts were centrifuged at 15,000 x g for 20 minutes. The supernatant was brought to 45% (w/v) ammonium sulfate and was left at 4°C overnight. The ammonium sulfate precipitation was centrifuged at 15,000 x g for 20 minutes. Most of the protein was in the pellet so the supernatant was discarded. The pellet was resuspended in 50 mM Tris-HCl (pH 8.0) and

dialyzed at 4°C against the same buffer overnight and then another 4 hours in fresh buffer to remove the ammonium sulfate. To purify the proteins, anion exchange chromatography was performed using a Diethylaminoethyl cellulose (DEAE) column. To make the DEAE column 200 g of DEAE powder (DE52; Whatman, Maidstone, England) was added to 400 ml of 50 mM Tris-HCl (pH 8.0) and stirred on a stir plate for 2 hours. The solution then sat overnight at room temperature to let the DEAE swell and allow any fines rise to the top. The fines at the top of the DEAE slurry were poured off the next day. Then a DEAE column (2.5 cm x 20 cm) was poured and equilibrated with 2 column volumes of buffer containing 50 mM Tris-HCl (pH 8.0) and 1 mM NaN₃ (Buffer A). Using 10 ml aliquots of the protein solution, the DEAE column was loaded 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, up to 100% Buffer B [50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1mM NaN₃]; 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 protein were pooled and dialyzed against 50 mM Tris-HCl (pH 8.0) overnight at 4°C and then another 4 hours in fresh buffer. CpcS and CpcU were concentrated using Amicon Ultra-15 concentrators (Millipore Corporation, Billerica, MA) and stored in 2 ml aliquots at -20°C until needed.

2.5 Production of Recombinant HT-Holo- α -PC

Chromophorylation of recombinant *Synechocystis* sp. PCC 6803 phycocyanin α subunit was achieved by using two expression vectors containing genes from *Synechocystis* sp. PCC 6803, pBS414V and pAT101, which were designed by Tooley *et al.*, (2001). Both plasmids were

kindly provided by Dr. A. N. Glazer (U.C. Berkeley). The cassette in pBS414V vector contains a histidine tag and *cpcA* along with *cpcE* and *cpcF*, and provides all of the components (apo-HT-CpcA, CpcE, and CpcF) known to be both necessary and sufficient for the correct addition of PCB to apo-CpcA. The cassette in pAT101 contains *hox1* and *pcyA*, which provides the enzymes required for the conversion of heme to PCB, the proximal precursor to the polypeptide-bound bilin. By introducing them together into *Escherichia coli* cells, they produced all of the components and catalytic functions required for the formation of his-tagged holo- α -phycocyanin *in vivo*. Transformation, protein overexpression, and purification protocols were followed as addressed above for the other his-tagged proteins.

2.6 Purification of PC from the *Synechococcus* sp. PCC 7002 *cpcM* Mutant

The mutant was created by our collaborators G. Shen and D.A. Bryant at the Pennsylvania State University. For insertional inactivation of the *cpcM* gene, a 1 kb DNA fragment, which encodes the *aacCI* gene, which confers resistance to gentamicin, was inserted into the unique *Bgl*II site within the *cpcM* coding sequence. This construction was used to transform *Synechococcus* sp. PCC 7002 cells. Transformants were selected on medium A⁺ plates containing gentamicin and were subjected to several rounds of streaking on selective media. Segregation of the *cpcM* and *cpcM::aacCI* alleles were verified by PCR analysis (G. Shen and D.A. Bryant, unpublished results).

Synechococcus sp. PCC 7002 *cpcM* mutant cells were grown by our collaborator G. Shen at the Pennsylvania State University then shipped to us frozen. To purify PC (following the protocol of Arciero *et al.*, [1988a]), mutant cells were passed through a French Pressure Cell at 18,000 psi three times. Whole cell extracts were centrifuged at 15,000 x g for 20 minutes. The

supernatant was brought to 45% (w/v) ammonium sulfate and was left at 4°C overnight. The ammonium sulfate precipitation was centrifuged at 15,000 x g for 20 minutes. The pellet was resuspended in 10 mM phosphate buffer (pH 7.0) and dialyzed at 4°C against the same buffer overnight and then another 4 hours in fresh buffer to remove the ammonium sulfate. To purify the proteins, anion exchange chromatography was performed using a Diethylaminoethyl cellulose (DEAE) column. To make the DEAE column 200 g of DEAE powder (DE52; Whatman, Maidstone, England) was added to 400 ml of 50 mM Phosphate Buffer (pH 7.0) and stirred on a stir plate for 2 hours. The solution then sat overnight at room temperature to let the DEAE swell and allow any fines rise to the top. The fines at the top of the DEAE slurry were poured off the next day. Then a DEAE column (2.5 cm x 20 cm) was poured and equilibrated with 2 column volumes of buffer containing 50 mM Phosphate Buffer (pH 7.0). Using 10 ml aliquots of the protein solution, the DEAE column was loaded using the BioLogic LP system at room temperature (BioRad, Richmond, CA).

The column program (flow rate of 1.5 ml/min) is as follows: 0-30 minutes, 100% Buffer A [200 mM Phosphate Buffer (pH 7.0)]; 30-150 minutes, up to 100% Buffer B [10 mM Phosphate Buffer (pH 7.0)]; 150-180 minutes, 100% Buffer B; 180-210 minutes, 100% Buffer A. Fractions with ≥ 0.01 absorbance at 280 nm were collected, and blue-colored fractions were examined for purity by SDS-PAGE. Fractions containing protein were pooled and dialyzed against 50 mM Phosphate Buffer (pH 7.0) overnight at 4°C and then another 4 hours in fresh buffer. The *cpcM* mutant phycocyanin α and β subunits were concentrated using Amicon Ultra-15 concentrators (Millipore Corporation, Billerica, MA) and stored in 2 ml aliquots at -20°C until needed.

2.7 Demonstrating CpcM Methylase Activity

2.7.1 Asparagine Methyltransferase Assay

Methylase activity was assayed using the protocol of Swanson and Glazer, 1990. Either 14.5 μM apo-ApcAB, apo-CpcBA, or apo-ApcFA was combined with 1.25 μM -GST-CpcM and a reaction mixture containing 50 mM-Tris-HCl (pH 8.0), 10 mM-EDTA, 10 mM-dithiothreitol, 1.6 μM -S-adenosylmethionine, and 0.4 μM -[methyl- ^3H]S-adenosylmethionine in a total volume of 200 μl . Controls were set up with all the above (one for each substrate) except instead of the 1.25 μM -GST-CpcM, 1.25 μM -GST protein was used. All concentrations were determined by using Extinction Coefficients which are listed in Table 2. Reaction mixtures incubated at 30°C for up to 3 hrs and were terminated by addition of an equal volume of cold (0-4°C) 10% (v/v) trichloroacetic acid (TCA) and incubated overnight at 4°C. Methylase activity was analyzed by washing the precipitates onto 25 mm nitrocellulose filters and washing with 30 ml of 5% TCA followed by 20 ml of 95% ethanol. Filters were dried at 37°C for 15 min then placed in a scintillation tube with 4 ml of scintillation fluid and assayed by scintillation counting for ^3H in counts per minute (cpm). Time-controlled assays were also set up using 6803 apo-HT-CpcA versus 6803 holo-HT-CpcA and *Synechococcus* sp. PCC 7002 apo-CpcBA versus Cys-82-PCB-CpcB/CpcA in a total volume of 78.125 μl and precipitates were separated by SDS-PAGE. Multiple assays using 14.5 μM 7002 apo-CpcBA versus PC from *cpcM* mutant were performed in a total volume of 85 μl and were terminated every 2.5 minutes by TCA precipitation prior to loading onto SDS-PAGE.

Table 2: Molar Extinction Coefficients of proteins used in CpcM methyltransferase assays.

Protein	ϵ_{280}
6803 HT-ApcA/ApcB	$19,440 \pm 5\% \text{ M}^{-1}\text{cm}^{-1}$
6803 HT-CpcB/CpcA	$29,330 \pm 5\% \text{ M}^{-1}\text{cm}^{-1}$
7002 HT-ApcF/ApcA	$22,000 \pm 5\% \text{ M}^{-1}\text{cm}^{-1}$
6803 HT-CpcA	$21,290 \pm 5\% \text{ M}^{-1}\text{cm}^{-1}$
7002 CpcBA	$26,770 \pm 5\% \text{ M}^{-1}\text{cm}^{-1}$
6803 GST-CpcM	$106,360 \pm 5\% \text{ M}^{-1}\text{cm}^{-1}$
GST	$41,160 \pm 5\% \text{ M}^{-1}\text{cm}^{-1}$

2.7.2 SDS Polyacrylamide Gel Electrophoresis

To determine which subunits, if any, were methylated, assays were performed using the above reaction procedures. After the TCA precipitates of reaction mixtures incubated overnight, the samples were microcentrifuged at 14,000 x g for 15 min. The supernatant was removed, and the pellet was resuspended in 20 µl of 50 mM Tris·HCl (pH 8.0) and an equal volume of 2 X SDS loading buffer. Pre-stained molecular weight markers (BioRad) were used for protein molecular weight determination. The samples were then boiled for 15 minutes and separated by SDS-PAGE using either a 15% or 10-20% Tris-HCl resolving gel following SDS-PAGE protocol mentioned above. The gel was then soaked in a fixing solution (Isopropanol:Water:Acetic Acid in the ratio 25:65:10) and then in Amplify solution (Amersham BioSciences) for 30 minutes each, on a rocker. The gel was transferred to whatman paper and then dried down at 80°C under vacuum for 30 minutes (8.6 x 6.8 cm) or 60 minutes (13.3 x 8.7 cm) using a Model 583 Gel Dryer (BioRad, Richmond, CA). The dried down gel was then exposed to ³H-sensitive Kodak film at -80°C from 1-3 days. After exposure period, films were developed in the dark using Kodak GBX developer and replenisher and Kodak GBX fixer and replenisher solutions. Proteins with incorporated ³H were then visualized and images acquired using a BioRad densitometer (BioRad, Richmond, CA).

2.8 Demonstrating CpcS, CpcU, and CpcT Lyase Activity

2.8.1 *In Vitro* Bilin Addition Reactions with PCB

Bilin addition assays with *Synechocystis* sp. PCC 6803 proteins were performed using phycocyanobilin cleaved and purified from *Spirulina* sp. The reactions contained (1 mg/ml final concentration of all components): 1 ml CpcBA, 200 µl CpcT, and/or 100 µl pAED4 *E. coli*

extract containing vector alone. The reactions also contained 75 mM MgCl₂ and 75 mM Hepes (pH 7.3). The proteins were incubated on ice for 15 minutes before PCB was added up to a 10 μM concentration from a 2 M stock in DMSO. PCB (2 μM) was added in 15 minute intervals during the first hour of incubation. The reactions incubated at 30°C for a total of two hours in the dark. After the assays reacted, fluorescence and absorbance spectra were taken and a sample of each reaction was taken for SDS-PAGE analysis.

2.8.2 *In vitro* Bilin Addition Reactions with PcyA

Biliverdin is reduced by PcyA in two sequential, 2-electron reductions using reduced ferredoxin to produce PCB (Frankenberg et al., 2001; Frankenburg and Lagarias, 2003). Only a small amount of PCB is produced by PcyA, therefore almost no mesobiliverdin is produced (the non-enzymatic bilin addition). Five reactions were set up containing the following: 1) 2 ml of purified CpcB/CpcA (1mg/ml) and 200 μl pAED4 control extract 2) 2 ml of CpcB/CpcA and 200 μl CpcS (1 mg/ml) 3) 2 ml of CpcB/CpcA and 200 μl CpcU (1mg/ml) 4) 2 ml of CpcB/CpcA and 200 μl CpcS and 200 μl CpcU 5) 2 ml of CpcB/CpcA and 200 μl CpcT (1mg/ml). The following were also added to each reaction mixture: Hepes (pH 7.3) to 75 mM, MgCl₂ to 75 mM, 6.5 mM glucose-6-phosphate, 1.6 mM NADP⁺, 1.1 u/ml glucose-6-phosphate dehydrogenase, 4.6 μM recombinant *Synechococcus* sp. PCC 7002 ferredoxin (Gomez-Lojero *et al.*, 2004; Schluchter, 1994), 0.025 u/ml Spinach FNR, 10 μM BSA, 5 μM biliverdin (Porphyrin Products, Logan, UT), and 10 μM PcyA (Frankenberg et al., 2001; Frankenburg and Lagarias, 2003). The concentration of biliverdin was determined using the extinction coefficient $\epsilon_{696} = 30.8 \text{ mM}^{-1}\text{cm}^{-1}$ (Schluchter and Glazer, 1997). Reactions incubated in the dark at 30°C for two hours then a second aliquot of biliverdin was added (for a final concentration of 10 μM). The reactions

were allowed to incubate at 30°C for another two hours. Fluorescence and absorbance spectra were taken as described above.

2.8.3 Absorbance and Fluorescence Spectra

Absorbance spectra were gathered using a dual-beam Lambda 35 UV-Vis spectrophotometer (Perkin Elmer, Shelton, CT). The blank for absorbance scans was 1 ml of buffer 0. Absorbance spectra were acquired from 450-750 nm.

Fluorescence emission spectra were acquired with slits set a 3 nm (excitation and emission) and the excitation wavelength set at 590 nm using a LS 55 Luminescence Spectrometer (Perkin Elmer, Shelton, CT). The fluorometer is equipped with a xenon discharge lamp (7.3 W average power at 50 Hz) as a light source. Spectra were taken from 600-750 nm.

RESULTS

3.1 CpcM: The PBP γ -N-Methylasparagine Methyltransferase

3.1.1 Creation of *cpcBA*, *apcAB*, *apcFA*, and *cpcM* Clones

Working in collaboration with Donald Bryant's lab at the Pennsylvania State University, a gene was identified that showed high probability of being responsible for the methylation of the β -72 asparagine residue. After Donald Bryant's lab cloned the *apcE* gene, which encodes an allophycocyanin core linker protein from *Synechococcus* sp. PCC 7002, they found a partial ORF upstream from this gene, denoted *cpcM*, which showed similarity to methyltransferases (J. Zhou, 1992). Since at the time the genome of *Synechococcus* sp. PCC 7002 had not yet been fully sequenced, the ortholog of the ORF in *Synechococcus* sp. PCC 7002 was found in *Synechocystis* sp. PCC 6803 (H.R. Leonard, 2002).

The genes used in this research project were amplified by PCR and cloned as described in Materials and Methods. CpcM amino acid sequences from several cyanobacteria were aligned using ClustalW and Mac Vector software (Accelrys, San Diego, CA) (Figure 20). CpcM in *Synechocystis* sp. PCC 6803 is very similar in amino acid sequence to *all0012* in *Nostoc* sp. PCC 7120 cyanobacterium with 71% similarity. Other similarity scores for the CpcM alignment are as follows: *Nostoc punctiforme* PCC 73102 with 71% (unidentified gene name), *Anabaena variabilis* ATCC 29413 with 70% similarity (gene name *Ava_2618*), *Nodularia spumigena* CCY 9414 with 70% similarity (gene name *N9414_05394*), and *Synechococcus* sp. PCC 7002 with 45% similarity (gene name *cpcM*). All of these ORFs that showed high similarity to CpcM were similar to SAM-dependent methyltransferases or similar to other methylases.

Alignment of CpcM

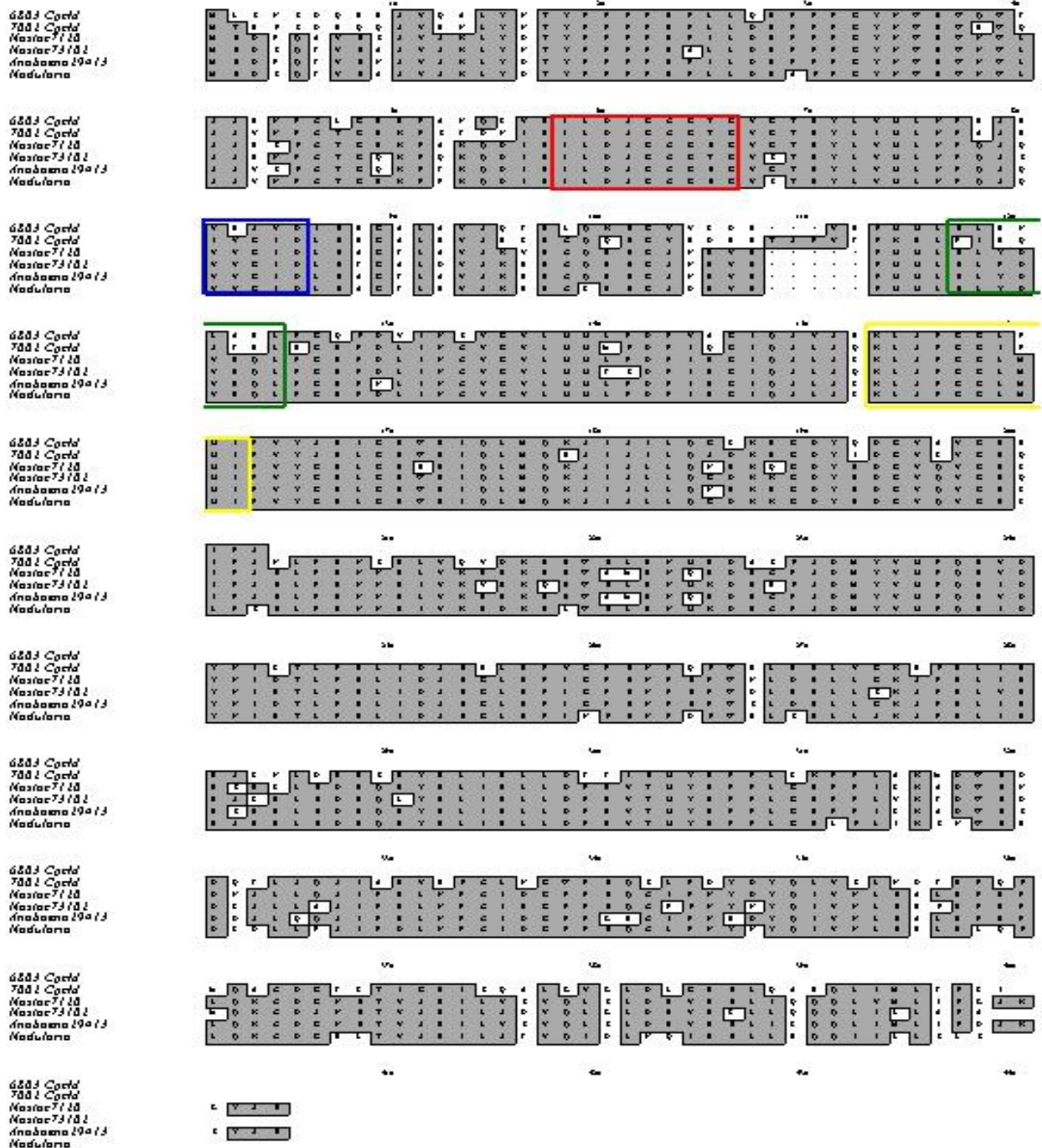


Figure 20: Alignment of amino acid sequences of *Synechocystis* sp. PCC 6803 CpcM presumed paralogs from different cyanobacteria. *Synechocystis* sp. PCC 6803 gene name *cpcM*, *Synechococcus* sp. PCC 7002 gene name *cpcM*, *Nostoc* sp. PCC 7120 gene name *all0012*, *Nostoc punctiforme* PCC 73102 gene name *SAM-dependent methyltransferase*, *Anabaena variabilis* ATCC 29413 gene name *Ava_2618*, and *Nodularia spumigena* CCY 9414 gene name *N9414_05394*. Boxed in grey are the exact matches of amino acid residues. Boxed conserved domains of SAM-dependent methyltransferases: red = motif I, blue = post I, green = motif II, and yellow = motif III.

3.1.2 Production and Purification of Recombinant Tagged CpcBA, ApcAB, ApcFA, and CpcM

Synechocystis sp. PCC 6803 his-tagged proteins: CpcB and ApcA, and *Synechococcus* sp. PCC 7002 his-tagged protein ApcF were purified using Ni-NTA resin after harvesting overproducing cells as described in Materials and Methods. 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 *et al.*, 1995). For this reason CpcB and CpcA, ApcA and ApcB, and ApcF and ApcA were expressed together. This copurification also allows for a higher yield of soluble protein, and when used in the asparagine methyltransferase assays there is an internal control protein with the presence of the alpha subunit. *Synechocystis* sp. PCC 6803 GST-tagged protein CpcM and the GST alone were purified using glutathione agarose resin after harvesting expression cells as described in Materials and Methods. Purified, tagged samples were analyzed by SDS- PAGE (Figure 21). Table 3 shows the calculated molecular weights and isoelectric points for each of the proteins used in this project. *Synechocystis* sp. PCC 6803 HT-CpcB has a calculated molecular weight of 21 kDa and CpcA has a calculated molecular weight of 17.6 kDa. These copurified proteins normally purified in a 1:1 ratio as visualized by SDS-PAGE, but not always. CpcBA was sometimes difficult to purify in a 1:1 ratio, but when achieved, those samples were used for reactions. These samples were fairly pure with very little other purified proteins (Figure 21A, lane 1), except for a ~25 kDa protein commonly seen in most Ni-NTA purifications from *E. coli*. The *Synechocystis* sp. PCC 6803 HT-ApcA has a calculated molecular weight of 21.5 kDa and ApcB has a calculated molecular weight of 17.2 kDa. These proteins, visualized via SDS-PAGE, always purified in a 1:1 ratio and the samples were always really pure with only small amounts

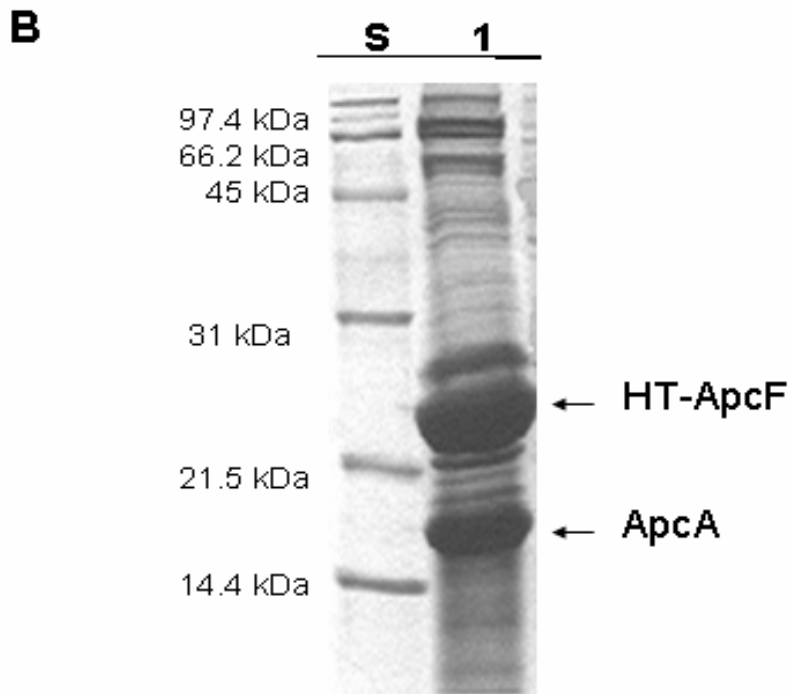
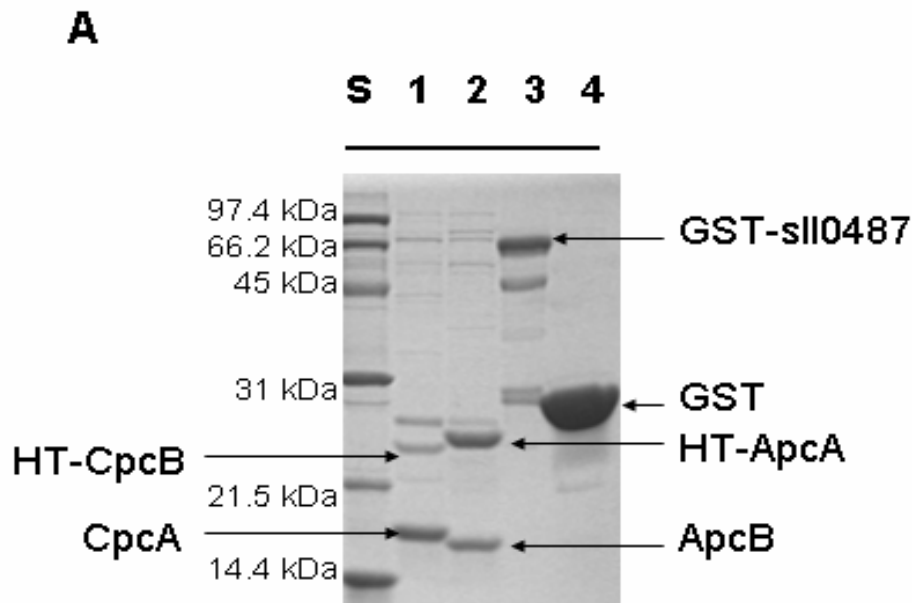


Figure 21: SDS-PAGE analysis of purification of *Synechocystis* sp. PCC 6803 tagged proteins CpcBA, ApcAB, CpcM, and CpcT and *Synechococcus* sp. PCC 7002 tagged proteins ApcFA; and the GST protein. Panel A) Samples were loaded as follows: phycobiliprotein subunits HT-CpcB/CpcA (lane 1) HT-ApcA/ApcB (lane 2) GST-CpcM (lane 3) glutathione-*S*-transferase (lane 4) Panel B) HT-ApcF/ApcA (lane 1). Molecular weight standards were loaded into the lane labeled “S”; the size of the bands is indicated on the left.

Table 3: Calculated molecular weights and Isoelectric points.

Protein*	Calculated Molecular Weight	Calculated Isoelectric Point
CpcB (his-tagged)	21,005.50 Da	5.75
CpcA	17,588.10 Da	5.27
ApcA (his-tagged)	21,539.20 Da	5.36
ApcB	17,217.20 Da	5.30
7002 ApcF (his-tagged)	22,824.00 Da	4.52
7002 ApcA	17,297.47 Da	4.48
CpcM (GST-tagged)	72,824.50 Da	5.52
CpcS	21,945.16 Da	4.45
CpcU	21,212.20 Da	5.49
CpcT (GST-tagged)	49,566.34 Da	5.73
GST	26,989.30 Da	6.45
Holo-CpcA (his-tagged)	21,052.80 Da	6.12
CpcA (his-tagged)	20,465.80 Da	6.12

*All recombinant proteins are from *Synechocystis* sp. PCC 6803 unless indicated otherwise.

of the 25 kDa *E. coli* protein (Figure 21A, lane 2). *Synechocystis* sp. PCC 6803 GST-CpcM has a calculated molecular weight of 72.8 kDa and when visualized by SDS-PAGE had a protein migrating at the size expected for GST-CpcM (Figure 21A, lane 3). Two other bands were visualized in lane 3 and are likely proteolytic degradation products of GST-CpcM (CpcM at 45 kDa and GST at 27 kDa). Purified GST is shown in Figure 21A lane 4 and has a calculated molecular weight of 27 kDa. *Synechococcus* sp. PCC 7002 HT-ApcF has a calculated molecular weight of 22.8 kDa, and ApcA has a calculated molecular weight of 17.3 kDa. ApcFA separated by SDS-PAGE in nearly a 1:1 ratio, but was not as pure as an ideal sample with other proteins appearing on the gel (Figure 21B, lane 1).

3.1.3 Recombinant Holo- α -PC

Chromophorylation of recombinant *Synechocystis* sp. PCC 6803 phycocyanin α subunit was achieved by using two expression vectors containing genes from *Synechocystis* sp. PCC 6803, pBS414V (containing *cpcA* along with *cpcE* and *cpcF*) and pAT101 (containing *hoxI* and *pcyA*), which were designed by Tooley *et al.*, (2001). By introducing them together into *Escherichia coli* cells, they produced all of the components and catalytic functions required for the formation of his-tagged holo- α -phycocyanin *in vivo*. The protein was purified using Ni-NTA (as described in Materials and Methods) and a sample was analyzed by SDS-PAGE (Figure 22). The gel was incubated with ZnSO₄ followed by Coomassie staining. ZnSO₄ allows bilin-containing polypeptides to be easily detected when the Zn⁺ ions bind to the bilin in the polypeptide, and enhance the fluorescence of the cyclic bilin in the presence of UV light (Raps, 1990). The band fluorescing on the zinc stained gel corresponds to the holo-HT-CpcA in the Coomassie stained gel which has a molecular weight of 21.1 kDa with one PCB attached.

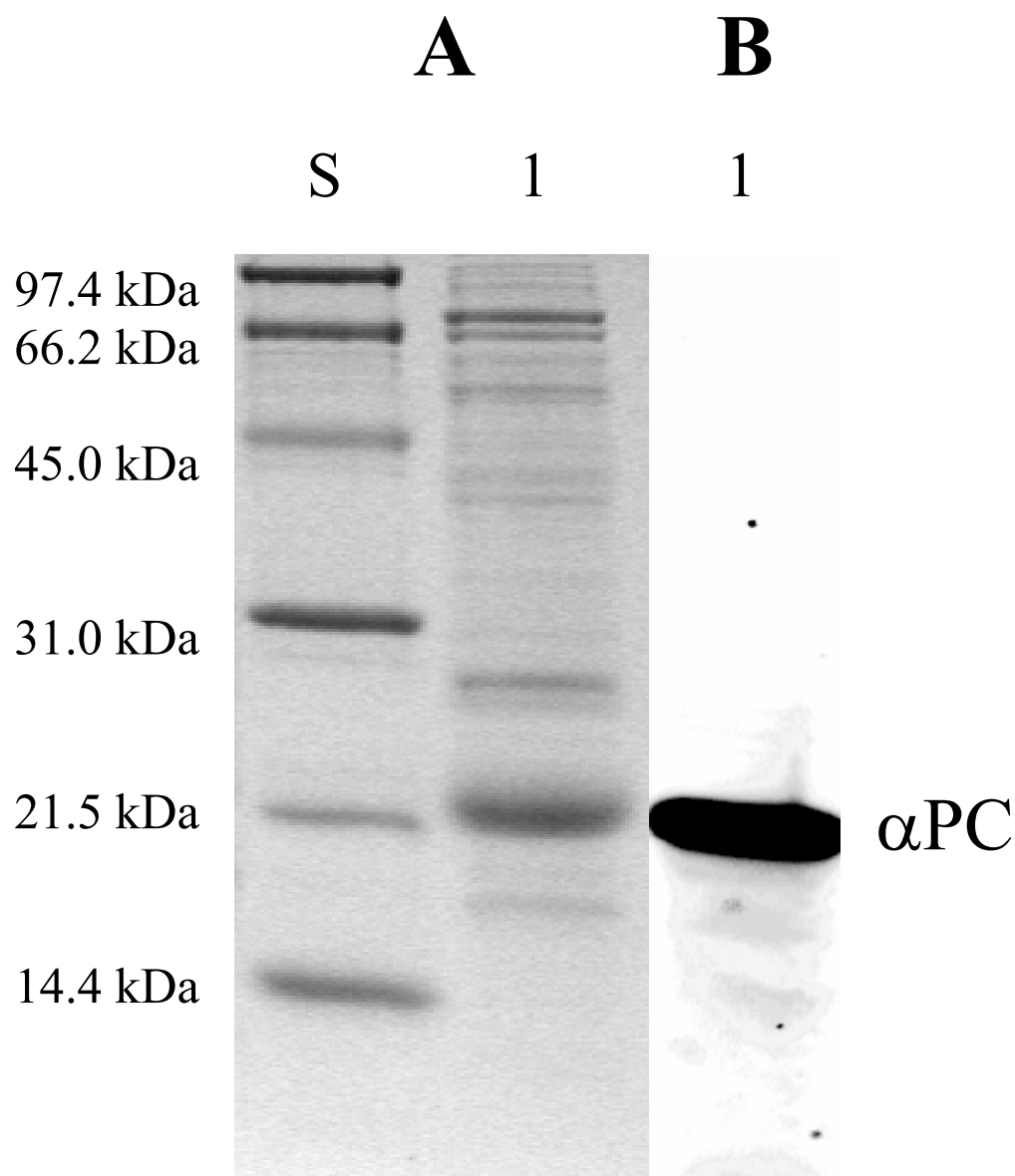


Figure 22: SDS-PAGE analysis of the expression of *Synechocystis* sp. PCC 6803 holo-CpcA and of *in vivo* chromophorylation of CpcA. Panel A shows the Coomassie stained SDS-PAGE gel of the purification of HT-CpcA in lane 1. Low molecular weight standard is marked as S and the sizes of the bands are indicated on the left. Panel B shows the zinc-enhanced fluorescence of the purified HT-holo-CpcA.

Absorbance and fluorescence spectra were taken of the purified holo-HT-CpcA and maxima were at 626 nm and 648.5 nm, respectively (Figures 23,24). This corresponds to the absorbance and fluorescence of PCB at the α -84 cysteinyl residue which has maxima at 625 nm and 645 nm, respectively (Tooley *et al.*, 2001). This holo-HT-CpcA was used in CpcM methylation assays.

3.1.4 Purification of PC from the *Synechococcus* sp. PCC 7002 *cpcM* Mutant

To generate the *Synechococcus* sp. PCC 7002 *cpcM* mutant, G. Shen (Pennsylvania State University) insertionally inactivated the *cpcM* gene with a gentamicin resistance cartridge and then transformed the construct into *Synechococcus* sp. PCC 7002 wild-type cells. The cells were then grown under optimal conditions and complete segregation was confirmed by PCR analysis (G. Shen, unpublished results). Through anion exchange chromatography the α and β phycocyanin subunits were purified from the *Synechococcus* sp. PCC 7002 *cpcM* mutant cells using a DEAE column. These phycobiliproteins are completely chromophorylated at all bilin attachment sites (α and β) but lack the methylated asparagine located at β -72. A chromatograph of the PC purification is shown in figure 25. Several blue fractions collected were separated via SDS-PAGE (Figure 26). Fractions from time points 125-200 minutes were separated via SDS-PAGE even though there was only one major peak between 125-145 minutes which is where all the protein was located. The most concentrated fractions were in lanes 3-6 which were time points: 133, 137, 141, and 144 minutes. Those fractions were pooled together, dialyzed, and concentrated.

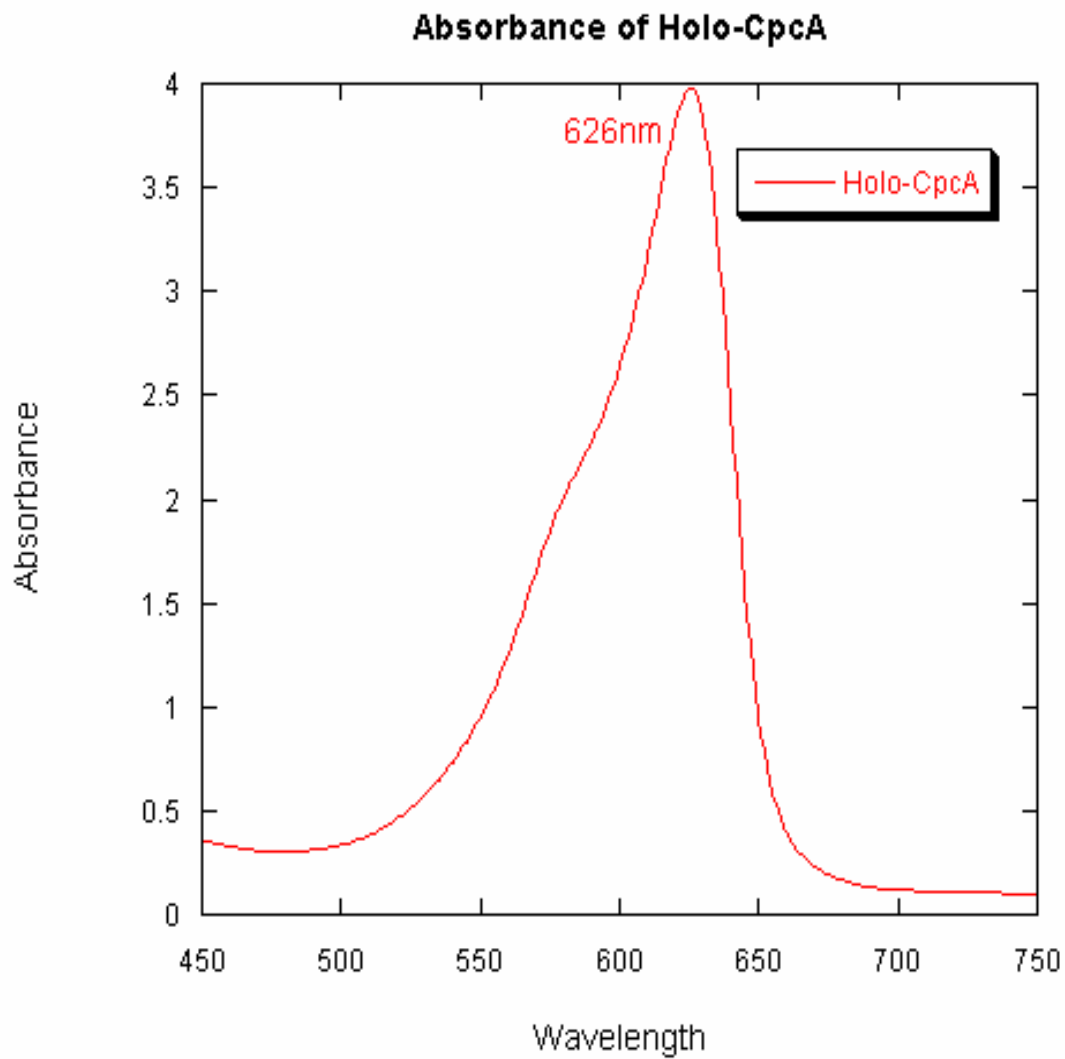


Figure 23: Absorbance spectrum of recombinant *Synechocystis* sp. PCC 6803 holo-HT-CpcA. Absorbance maximum is indicated for the protein at 626 nm.

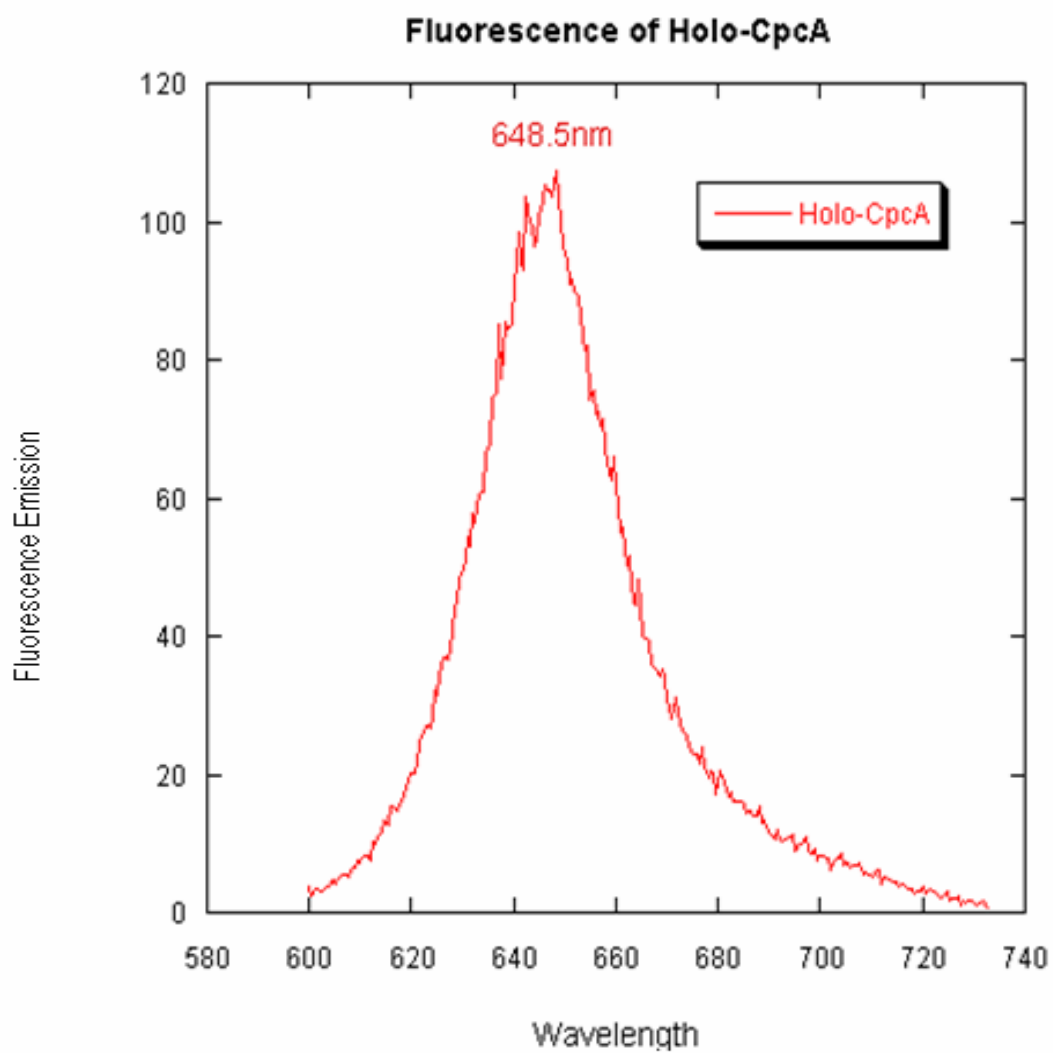


Figure 24: Fluorescence emission spectrum of recombinant *Synechocystis* sp. PCC 6803 holo-HT-CpcA. Excitation was at 590 nm with 3 nm slit widths. The emission maximum was at 648.5 nm.

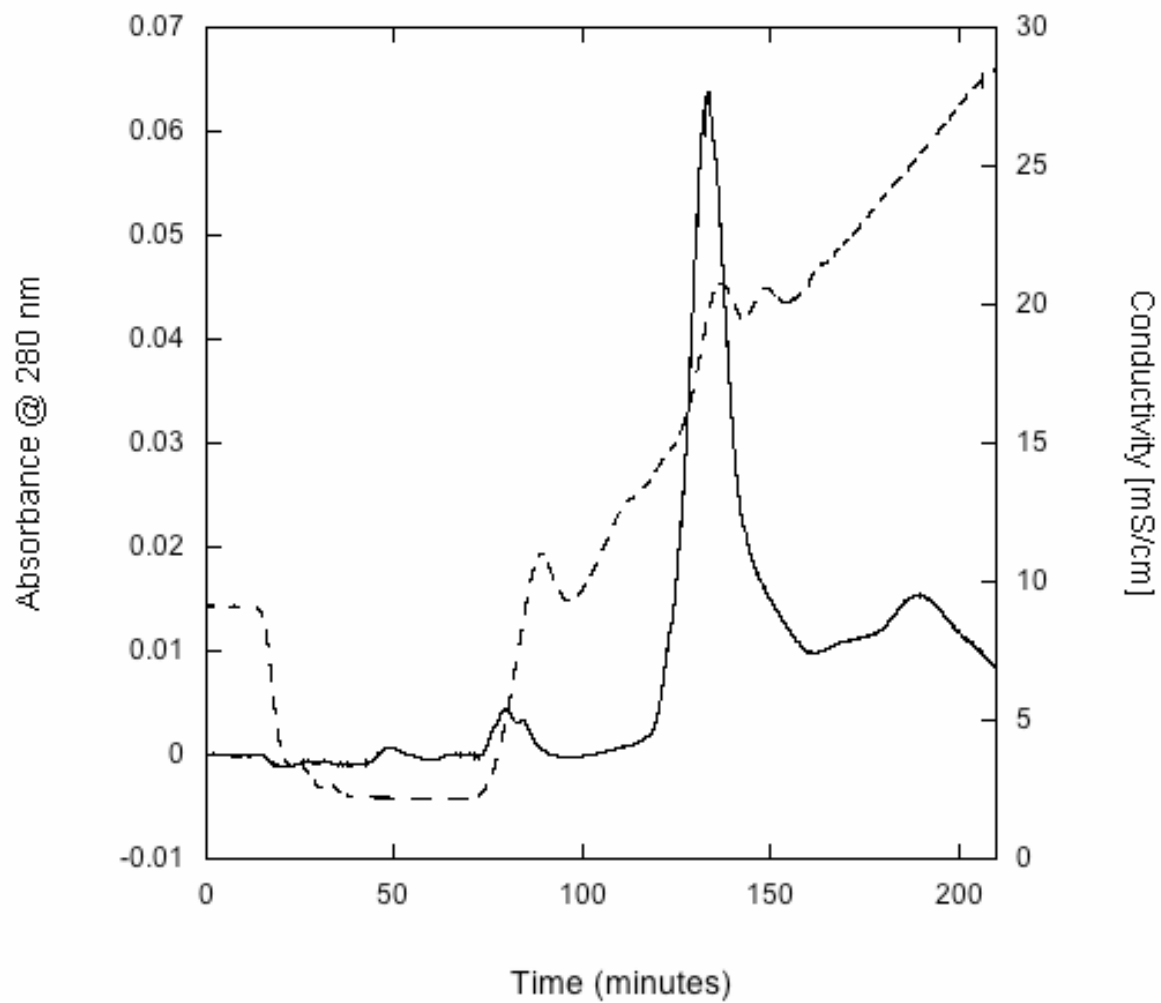


Figure 25: Purification of PC from *Synechococcus* sp. PCC 7002 *cpcM* mutant on a DEAE column. Absorbance at 280 nm is shown by the solid line while conductivity is shown by the dotted line. The most pure fraction of *cpcM* mutant CpcBA eluted at the largest peak (between 125-145 minutes).

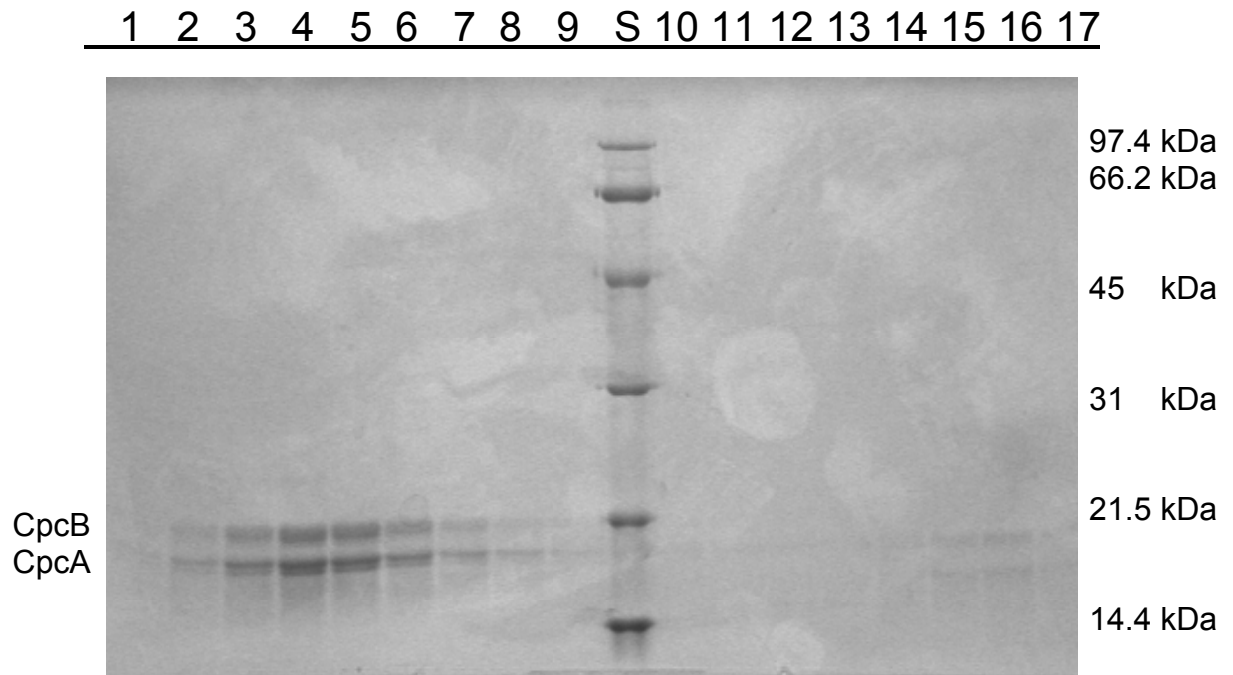


Figure 26: SDS-PAGE analysis of *cpcM* mutant CpcBA DEAE fractions. Lanes 1-9 show fractions of the largest peak (124-157 minutes), lanes 10-17 show fractions further down the largest peak and into the next smaller peak (160-205 minutes), and lane “S” is the molecular weight standard. Lanes 3-6 contained the most concentrated fractions (133-144 minutes). The size of the molecular weight bands are indicated on the right.

3.1.5 CpcM Methyltransferase Activity Assay: Time-controlled assay

Recombinant *Synechocystis* sp. PCC 6803 apo-phycoyanin and apo-allophycoyanin were used as substrates for the phycobiliprotein methyltransferase assay. Recombinant apo-phycoyanin and apo-allophycoyanin lack phycocyanobilin chromophores and are unmethylated at β -72. With [methyl- ^3H]-S-adenosylmethionine as the methyl donor, CpcM (the suspect methyltransferase) was assayed for its ability to incorporate tritium into TCA-precipitable material. Scintillation counting revealed that GST-CpcM was active with both apo-PC and apo-APC as substrates, but GST alone did not incorporate any tritium into the phycobiliprotein substrates. The scintillation counting results showed that for a 2 hour assay, apo-phycoyanin contained 23 times more ^3H counts per minute than its control counterpart containing no CpcM (just GST) and apo-allophycoyanin contained 33 times more ^3H counts per minute than its control counterpart with no CpcM (just GST). A time-controlled assay terminating the reactions every hour up to three hours showed maximum activity at two hours (figure 27). However, looking at the overall results, it was concluded that performing a one hour assay for further study would allow for enough tritium incorporation for sufficient detection.

3.1.6 β -Subunit Specificity Assay

Another assay was performed with the same components for only one hour and the scintillation counting revealed similar results as compared to the time-controlled assay (Figure 28). Not only was scintillation counting performed on the TCA precipitates, but the samples were also separated by SDS-PAGE. Since both α and β subunits were present, it was necessary to determine which subunit was being methylated. In cyanobacteria only the β subunit is methylated. The autoradiogram of the SDS-PAGE separation of the phycobiliprotein subunits

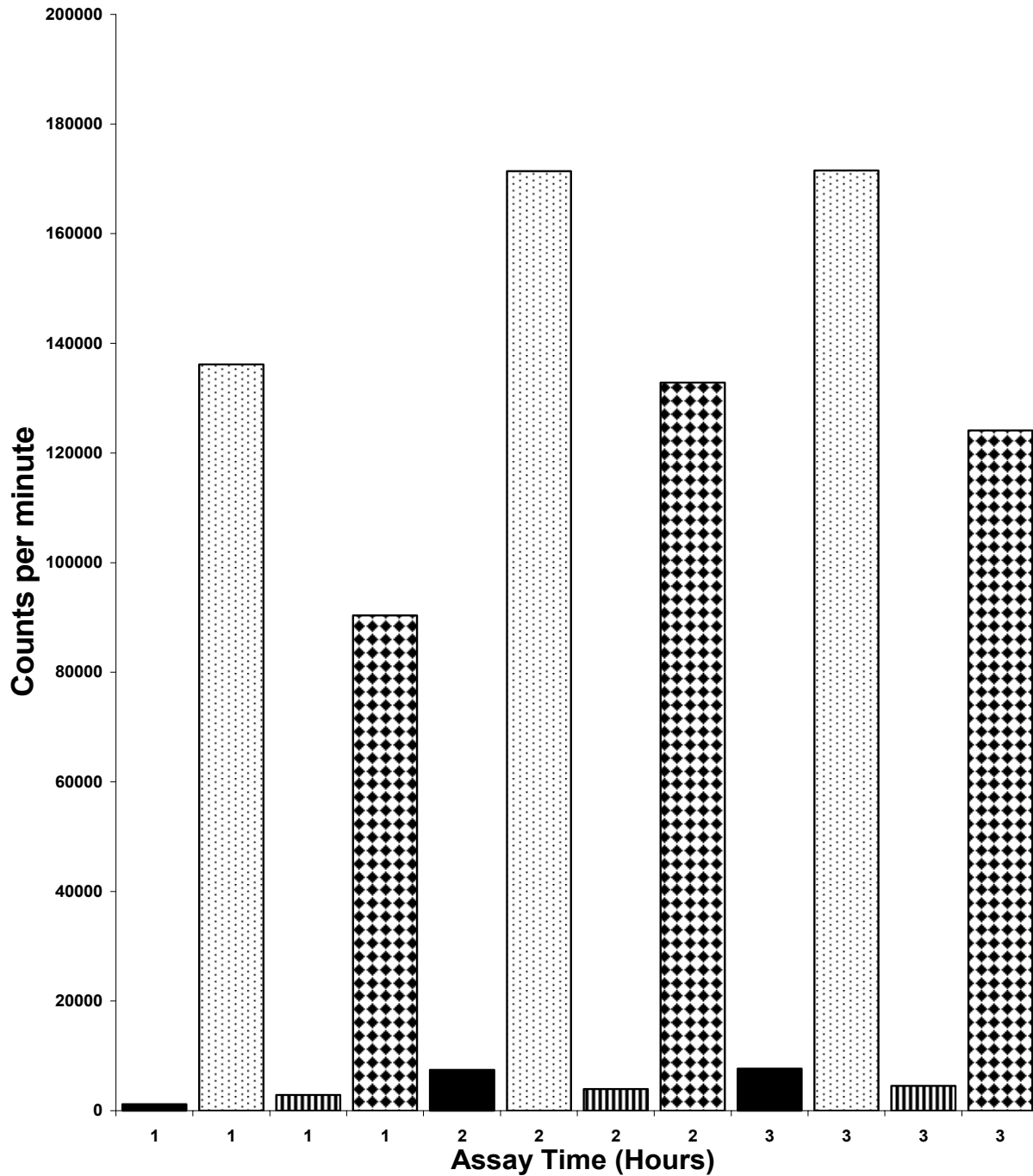


Figure 27: A time course of methylation of phycobiliproteins by CpcM. ^3H incorporation [into TCA-precipitable material] from [methyl- ^3H]-S-adenosylmethionine was measured by scintillation counting. Asparagine methylation in acid hydrolyzates of purified phycobiliproteins was quantitated radiochemically by determining the amount of ^3H methyl groups bound to protein in counts per minute. Time-controlled assays were quenched after 1, 2, or 3 hours to determine time of maximum enzyme incorporation.

revealed that the proteins in the control reactions contained no incorporated ^3H (Figure 29). However in the reaction containing GST-CpcM, ^3H was only incorporated into the HT- β subunit of both phycocyanin and allophycocyanin with no α subunits containing ^3H (Figure 29). The migration of the HT-CpcB subunit in Panel A, lane 1 (Figure 29) of the Coomassie stained gel is consistent with the position of the tritiated protein in Panel B, lanes 3 and 7 (Figure 29). The position of ApcB in the SDS-PAGE gel (Figure 29A, lane 2) is also consistent with the identity of the tritiated protein in Figure 29B, lanes 1 and 5 being ApcB. This is congruent with the fact that there is no methylated asparagine residue on the homologous α subunit in either phycocyanin or allophycocyanin purified from cyanobacteria (Klotz *et al.*, 1986; Klotz and Glazer, 1987). Since assays containing CpcM contained 8-21 times the number of counts per minute incorporated into phycobiliprotein as compared to controls, reactions containing GST, and CpcM reactions had ^3H incorporated into the β subunits only, it is concluded that CpcM is the phycobiliprotein β -72 methyltransferase.

3.1.7 CpcM Specificity: Apo Versus Holo

To determine where along the path of assembly of the phycobilisome that this methylation of the β -72 asparagine residue might occur, assays were performed using apo and holo phycobiliproteins. If CpcM was to show a preference to methylate one versus the other then we may know if the asparagine methylation occurs before or after chromophorylation of the PBP. First, CpcA was used in assays to verify that neither apo- nor holo-CpcA is a substrate. In a one-hour assay *Synechocystis* sp. PCC 6803 apo and holo-HT-CpcA was used as a substrate. The apo-HT-CpcA was purified from *E. coli* cells lacking PCB production (Figure 30A, lane 1) and holo- HT-CpcA was purified from *E. coli* cells containing PCB and the α -PC lyases (Figure 30B, lane 1). This was used to check whether CpcM shows any methylation of a chromophorylated α

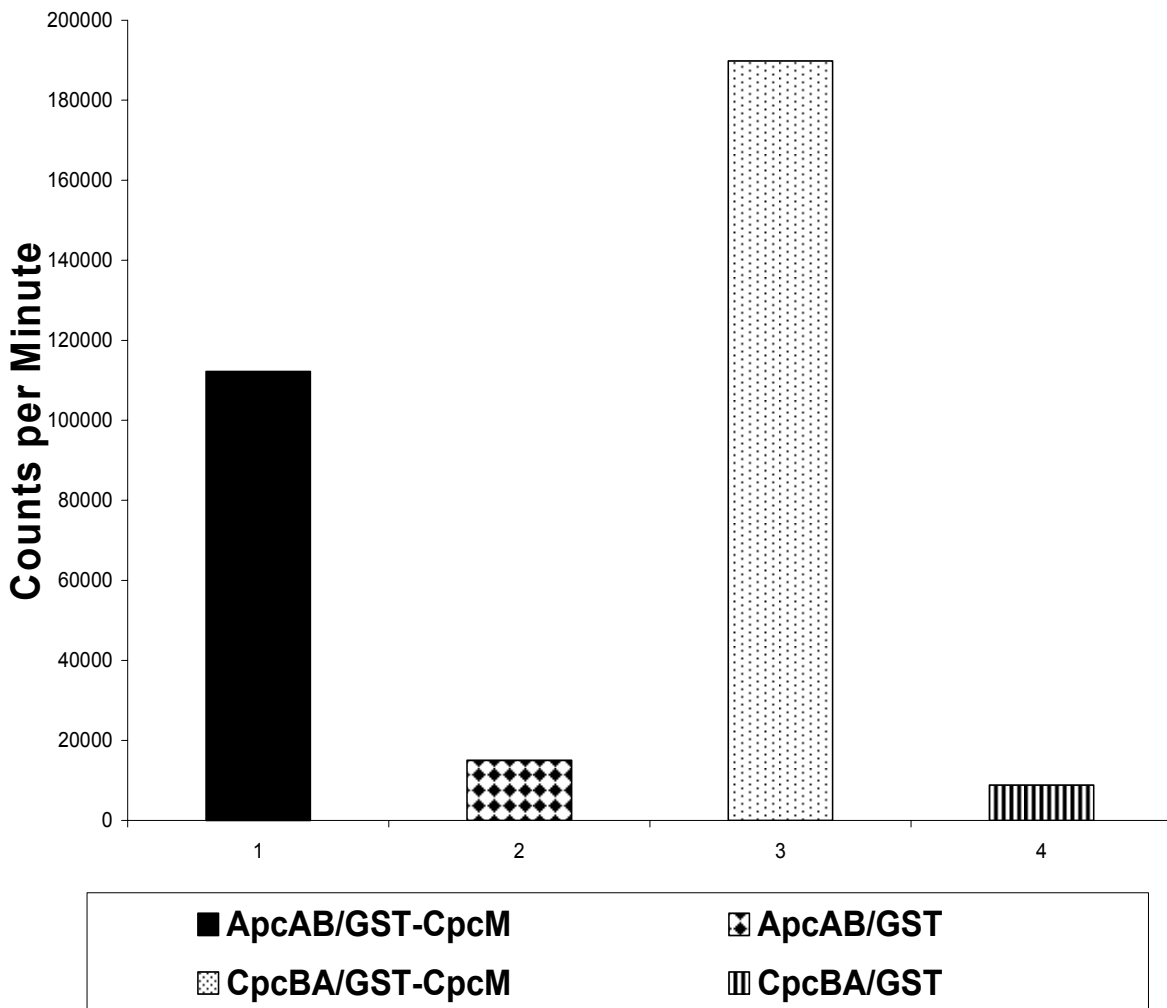


Figure 28: Graph of ^3H incorporation in recombinant *Synechocystis* sp. PCC 6803 HT-ApcAB and HT-CpcBA by CpcM. Asparagine methylation in acid hydrolyzates of purified phycobiliproteins was quantitated radiochemically by determining the amount of ^3H methyl groups bound to protein in counts per minute. Assay was quenched after one hour.

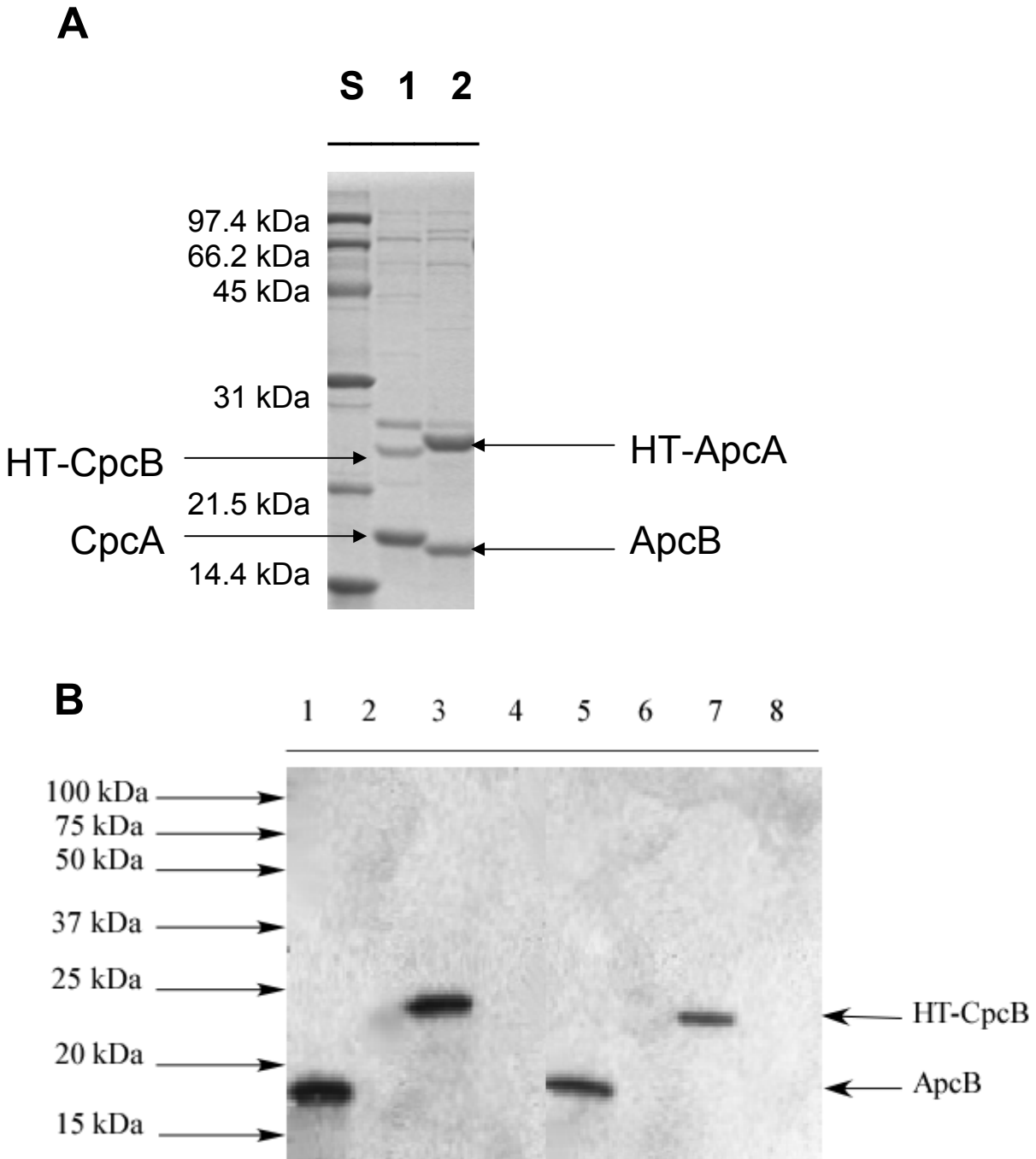


Figure 29: β -subunit specificity Assay. Panel A) Coomassie stained SDS-PAGE of purified substrates used in the assay. Lane 1 contains HT-CpcB/CpcA and lane 2 contains HT-ApcA/ApcB. Molecular weight standard was loaded into the lane labeled “S”. Weights are indicated on the left. Panel B) Autoradiogram of asparagine methyltransferase assay using HT-CpcB/CpcA and HT-ApcA/ApcB and CpcM. Samples were loaded as follows: ApcAB (GST-CpcM) 30 μ l (lane 1) and 10 μ l (lane 5), ApcAB control (GST only) 30 μ l (lane 2) and 10 μ l (lane 6), CpcBA (GST-CpcM) 30 μ l (lane 3) and 10 μ l (lane 7), and CpcBA control (GST only) 30 μ l (lane 4) and 10 μ l (lane 8). Prestained molecular weight standards were loaded on the gel and their sizes are shown at left. The positions of polypeptides are indicated to the right.

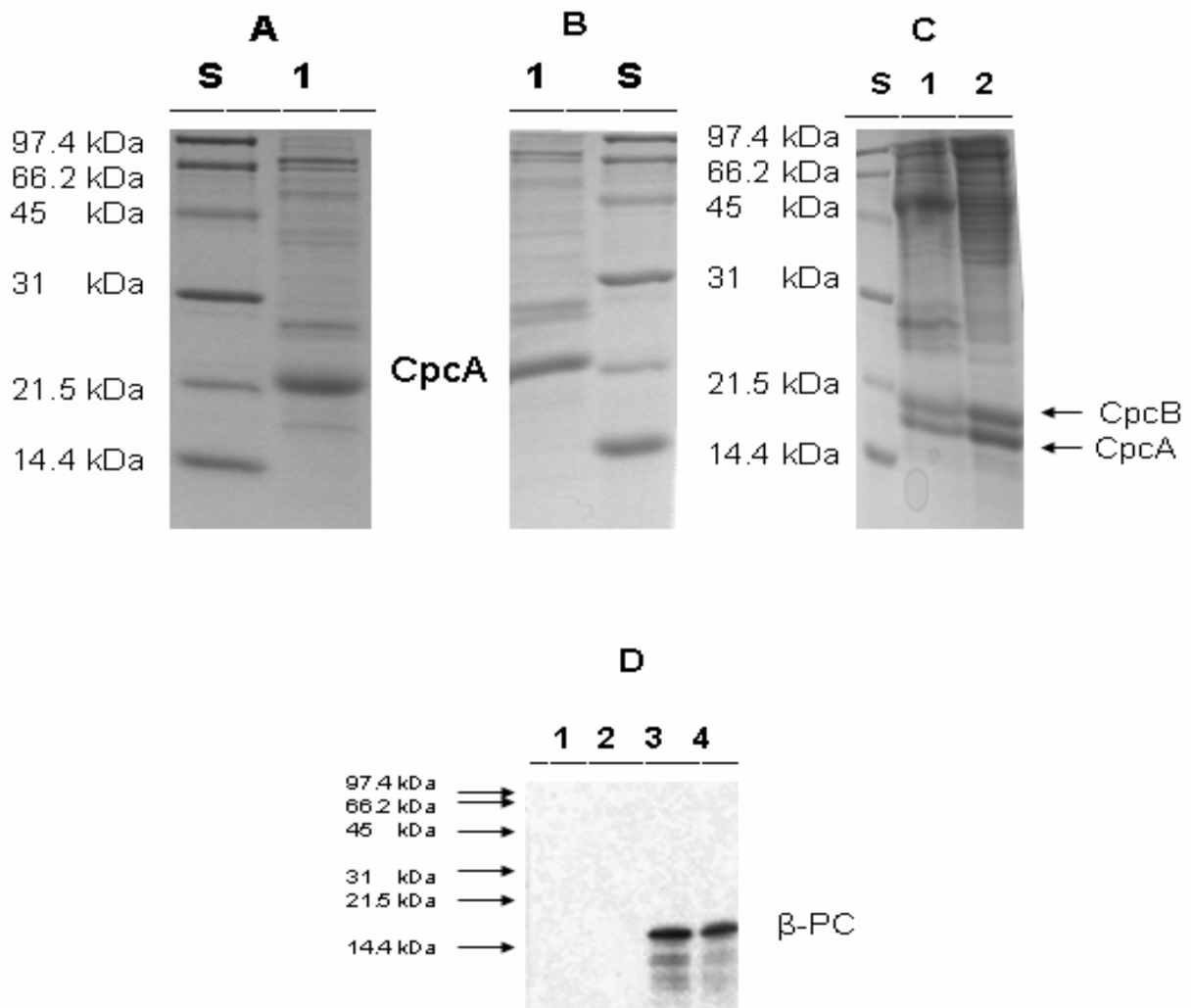
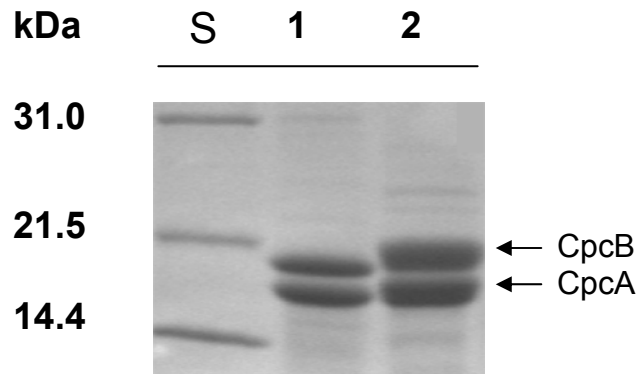


Figure 30: SDS-PAGE of CpcA, CpcBA, and of methylase assays using apo vs holo CpcA and CpcB (at Cys-82). Panel A) Coomassie stained SDS-PAGE of the *Synechocystis* sp. PCC 6803 α phycocyanin subunit. Lane 1 contains holo-HT-CpcA and lane S is the molecular weight marker. Panel B) Coomassie stained SDS-PAGE of *Synechocystis* sp. PCC 6803 α phycocyanin subunit. Lane 1 apo-HT-CpcA and lane S is the molecular weight marker. Panel C) Coomassie stained SDS-PAGE of the *Synechococcus* sp. PCC 7002 β phycocyanin subunits. Lane 1 contains (Cys-82-PCB)-CpcB, lane 2 contains apo-CpcB, and lane “S” is the molecular weight marker. Panel D) Autoradiogram of CpcM assay using the α and β proteins. Lane 1 contains the apo-HT-CpcA, lane 2 holo-HT-CpcA, lane 3 apo-CpcB, and lane 4 is (Cys-82-PCB)-CpcB. Prestained molecular weight standards were loaded on the gel and their sizes are shown at left. The position of the polypeptide is indicated to the right

subunit. After a one-hour assay, an autoradiogram was produced from the TCA precipitates. In lanes 1 and 2 of the autoradiogram (Figure 30, Panel D) no bands of α subunits, apo or holo, are visible, consistent with the expected specificity of CpcM. Also tested in an assay were *Synechococcus* sp. PCC 7002 apo-CpcBA and (Cys-82-PCB)-CpcB/CpcA which were used as substrates. These β phycocyanin proteins were kindly provided to me by Nicolle Saunée. She chromophorylated CpcB using the *Synechococcus* sp. PCC 7002 lyases CpcS and CpcU, which attach PCB only at Cys-82 on CpcB. The absorbance and fluorescence spectra of the (Cys-82-PCB)-CpcB were extremely high and at the correct wavelengths (N. Saunée, 2006). A Coomassie stained SDS-PAGE of the separated proteins used in the assay is also presented to show the size of the proteins (Figure 30, Panels A-C). After a one-hour assay both the apo and (Cys-82-PCB)-CpcB tritiated bands are visible in the autoradiogram, lanes 3 and 4, and appear to be equally methylated (Figure 30, Panel D).

To further investigate CpcM's activity towards unmethylated holo-PC from *cpcM* mutant and recombinant apo-PC, a time-controlled assay was performed using *Synechococcus* sp. PCC 7002 apo- β -phycocyanin and holo- β -phycocyanin both with CpcA co-purified. The holo-PC is purified from the *Synechococcus* sp. PCC 7002 *cpcM* mutant and is fully chromophorylated at all bilin addition sites (α and β) and lacks the methylation at β -72. Analysis of the PC purified from *Synechococcus* sp. PCC 7002 *cpcM* mutant showed that the unmethylated phycocyanin is in the form of a trimer instead of the monomeric forms that are created from recombinant protein (S.R. Williams and W.M. Schluchter, unpublished results). Methylase assays were performed (as described in Materials and Methods) but were terminated about every 2.5 minutes from 1.0 minute to 17.5 minutes. The TCA precipitates from all the reactions were separated by SDS-PAGE and an autoradiogram of the gel is shown in Figure 31B. A Coomassie stained gel of the

A



B



Figure 31: Apo Vs Holo Asparagine methyltransferase assay. Panel A) Coomassie stained SDS-PAGE analysis of the purified component used in the asparagine methyltransferase assays. *Synechococcus* sp. PCC 7002 phycobiliprotein samples were loaded as follows: apo-CpcB/CpcA (lane 1) *cpcM* mutant holo-PC (lane 2). Molecular weight standard is loaded in the lane labeled “S”; the size of each protein is indicated on the left side of the gel. Panel B) an autoradiogram of components after assays terminated in 2.5 minute increments. 10 μ l samples were loaded as follows: from 1.0 min to 17.5 minutes apo-CpcB/CpcA (lanes A) and holo-PC (lanes H). Prestained molecular weight standards were loaded on the gel and their sizes are shown at left. The position of the methylated polypeptide is indicated to the right.

substrates is presented in Figure 31A. On the autoradiogram there are other tritiated bands that appear that are not the size of CpcB. This is a result of proteolytic degradation of CpcB and is not CpcA; this was verified by Western blot analysis (not pictured). Results of the assay showed that the first tritiated CpcB to appear on the autoradiogram was in the reaction containing apo-CpcB at 5.0 minutes, and then the PBP methylation of apo-CpcB consecutively darkened in intensity through 17.5 minutes. The methylation of holo-CpcB was detected after 15.0 minutes. This indicates that CpcM may methylate the β -72 asparagine before chromophorylation of the phycobiliproteins since CpcM appears to have a greater affinity for apo-CpcB than for holo- β -PC in the trimeric form or it may just be methylating the apo-CpcB because it is a monomer. CpcM may methylate after chromophorylation of monomeric PBP but before they assemble into trimers.

To test this hypothesis, further assays were performed using *Synechococcus* sp. PCC 7002 apo-CpcBA recombinant protein and *Synechococcus* sp. PCC 7002 (Cys-82-PCB)-CpcB/CpcA provided by N. Saunée used in a previous assay. A coomassie stained SDS-PAGE of the proteins is pictured in Figure 30 Panel C. Both of these substrates are monomers but one is completely unchromophorylated and one has a bilin attached at the β -82 residue. Two assays were acid-quenched in 2.5 minute increments (Figure 32A&B). Again on the autoradiogram there are other tritiated bands that appear that are not the size of CpcB. This is a result of proteolytic degradation of CpcB and is not CpcA which was verified by Western blot analysis (not pictured). In Panel A the first tritiated band to appear on the autoradiogram was in the reaction containing (Cys-82-PCB)-CpcB at 2.5 minutes in lane 4. In Panel B the first tritiated band to appear on the autoradiogram, though faint, was in the reaction containing (Cys-82-PCB)-CpcB at 1.0 minute in lane 2. After 7.5 minutes both β subunits demonstrated equal methylation.

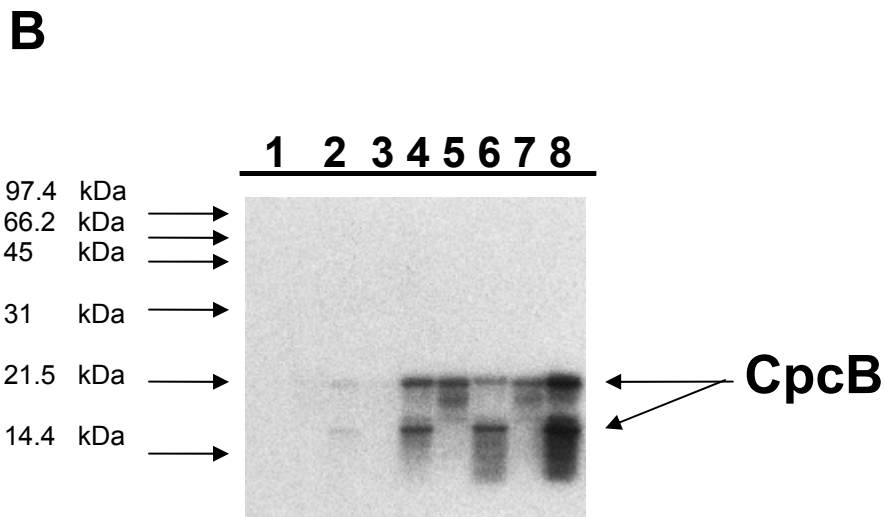
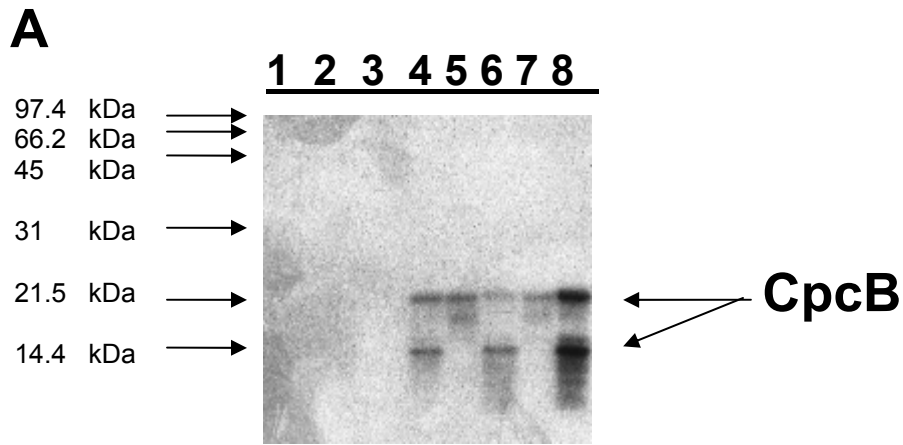


Figure 32: *Synechococcus* sp. PCC 7002 apo-CpcB vs (Cys-82-PCB)-CpcB asparagine methyltransferase assay. Panel A and B) two different autoradiograms of components after assays terminated in 2.5 minute increments. 10 μ l samples were loaded as follows: 1.0 min apo-CpcB/CpcA (lane 1), 1.0 min (Cys-82-PCB)-CpcB/CpcA (lane 2), 2.5 min apo-CpcB/CpcA (lane 3), 2.5 min (Cys-82-PCB)-CpcB/CpcA (lane 4) 5.0 min apo-CpcB/CpcA (lane 5), 5.0 min (Cys-82-PCB)-CpcB/CpcA (lane 6) 7.5 min apo-CpcB/CpcA (lane 7), and 7.5 (Cys-82-PCB)-CpcB/CpcA (lane 8). Prestained molecular weight standards were loaded on the gel and their sizes are shown at left. The position of the methylated polypeptide is indicated to the right.

The β subunit was not fully chromophorylated, lacking the PCB at Cys-153, but CpcM still showed a preference for one bilin attached on the (Cys-82-PCB)-CpcB versus the apo-CpcB monomeric subunits. These assays revealed that CpcM has a greater affinity for CpcB chromophorylated at Cys-82 which suggests that asparagine methylation may occur after the attachment of phycobiliprotein bilins but before holo-PC is trimerized.

3.1.8 CpcM Methylation of ApcF

ApcF is a β -like allophycocyanin subunit that is present in two copies per phycobilisome. ApcF is important for energy transfer from the phycobilisome to photosynthetic reaction centers, and it also contains a methylated asparagine residue like the phycocyanin and allophycocaynin β -72 γ -*N*-methylasparagine residue. Recombinant ApcF was produced with ApcA and purified from *E. Coli* for use in a CpcM assay (Figure 33 Panel A) (A. Fletcher, B. Turner, W. Schluchter, unpublished results). A one-hour assay was performed using purified ApcFA (his-tagged ApcF/ApcA) as the substrate to determine if CpcM also methylates ApcF. TCA precipitates were separated by SDS-PAGE, and the autoradiogram is shown in Figure 33B. As one can see in lane 1 of panel B there is one radioactive band that appears on the autoradiogram, and it is the size of the HT-ApcF protein. The co-purified ApcA shows no tritium incorporation, therefore was not methylated by CpcM. The autoradiogram results show that CpcM not only methylates the β -subunits of phycocyanin and allophycocyanin, but also methylates the allophycocyanin core component, ApcF.

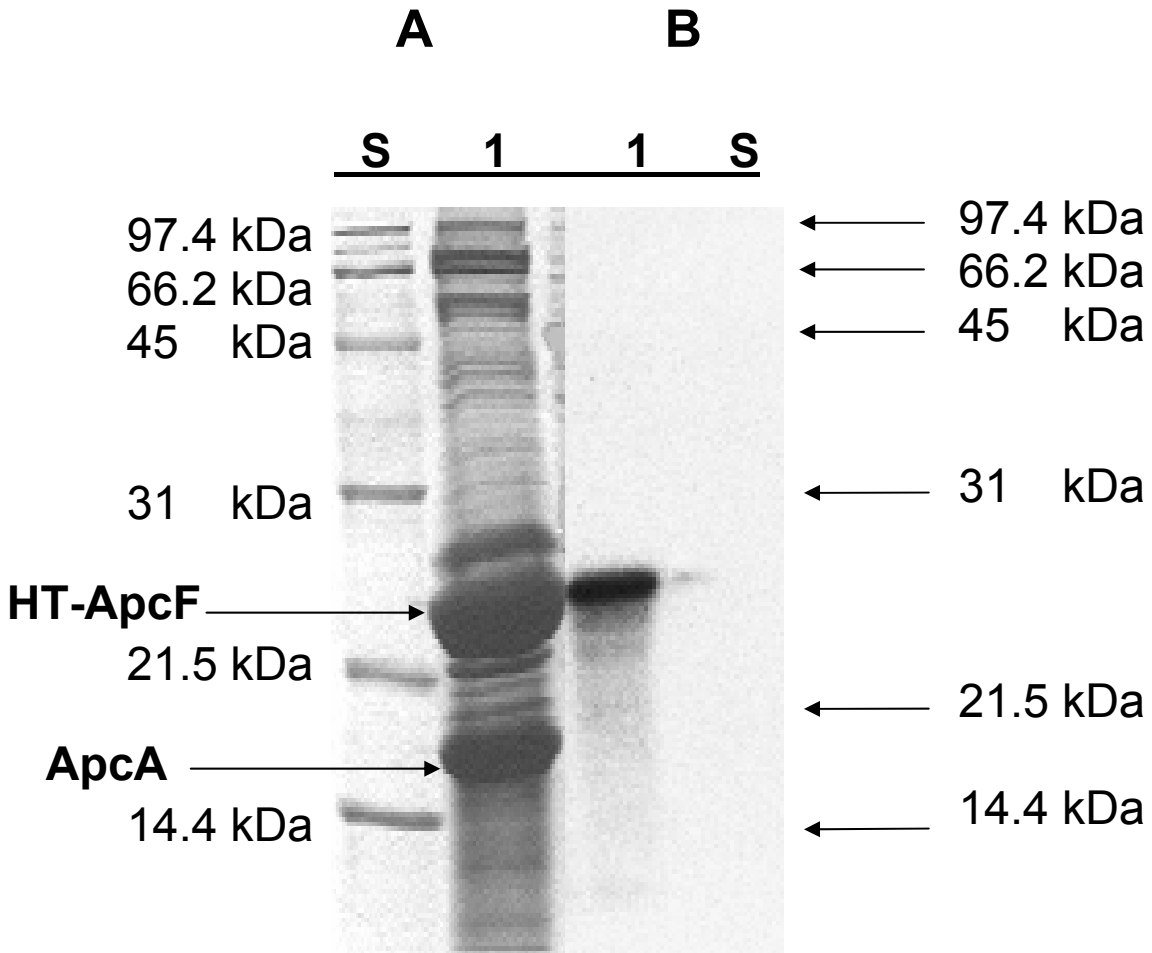


Figure 33: ApcF/CpcM methylase assay. Panel A) Coomassie stained SDS-PAGE of purified HT-ApcF/ApcA used in the methylase assay. Lane “S” contains the molecular weight marker and Lane 1 contains the HT-ApcF/ApcA proteins. Panel B) Autoradiogram of TCA precipitates from the methylase assay using CpcM. Lane 1 contains only a tritiated band for HT-ApcF and no ApcA shows radioactivity. Lane “S” contains the prestained molecular weight marker with weights indicated on the right.

3.2 CpcS, CpcU, and CpcT: PBP β -Subunit lyases

3.2.1 Creation of *cpcS*, *cpcU*, and *cpcT* Clones

Other genes of interest in this project are the PBP lyases of *Synechocystis* sp. PCC 6803. The *cpcS*, *cpcU*, and *cpcT* genes from *Synechocystis* sp. PCC 6803 were amplified by PCR and cloned as described in Materials and Methods. The transformants with both insert and vector were then sequenced to determine if the clones were correct. The amino acid sequences were aligned using Mac Vector (Accelrys, San Diego, CA). Each of the three proteins from *Synechocystis* sp. PCC 6803: CpcS, CpcU, and CpcT, was aligned to translations of two different cyanobacterial genomes (Genbank and Cyanobase): *Synechococcus* sp. PCC 7002 and *Fremyella diplosiphon* PCC 7601 and three other ORFs that showed the highest similarity against Genbank when using BLAST. Using ClustalW (v1.4) multiple sequence alignment, three different alignments were created (one for each protein) (Figures 34-36).

CpcS in *Synechocystis* sp. PCC 6803 showed the highest similarity to its *Synechococcus* sp. PCC 7002 ortholog, *cpcS*, with 61% similarity out of the three lyase-like genes, but other translations from Genbank scored higher (Figure 34). Similarity scores for the CpcS alignment are as follows: *Crocospaera watsonii* WH 8501 with 69% similarity (*hypothetical protein*), *Cyanothece* sp. CCY 0110 with 68% similarity (gene name *CY0110_04081*), *Lyngbya* sp. PCC 8106 with 64% similarity (gene name *L8106_24665*), *Synechococcus* sp. PCC 7002 with 61% similarity (gene name *cpcS*), and *Fremyella diplosiphon* with 31% similarity (gene name *cpeS*). Using Cyanobase (www.bacteria.kazusa.or.jp/cyanobase/) *Synechocystis* sp. PCC 6803 sequenced genome map, it was found that CpcS is located directly upstream (no other ORF between) of the phycobilisome rod-core linker polypeptide, CpcG (slr2051) (Kaneko *et al.*,

Alignment of CpcS



Figure 34: Alignment of amino acid sequences of *Synechocystis sp. PCC 6803 CpcS* presumed orthologs from different cyanobacteria. *Synechocystis sp. PCC 6803* gene name *cpcS*, *Crocosphaera watsonii* WH 8501 hypothetical protein, *Cyanothece* sp. CCY0110 gene name *CY0110_04081*, *Lyngbya* sp. PCC 8106 gene name *L8106_24665*, *Synechococcus sp. PCC 7002* gene name *cpcS*, and *Fremyella diplosiphon* gene name *cpeS*. Boxed in grey are the conserved amino acid residues.

1996). It is possible that they are co-transcribed because they are in the same direction and right next to each other.

CpcU in *Synechocystis* sp. PCC 6803 has the lowest similarity scores among the three lyase-like genes (Figure 35). The similarity scores for CpcU are as follows: *Cyanothece* sp. CCY0110 with 40% similarity (gene name *CY0110_18677*), *Crocospaera watsonii* WH 8501 with 37% similarity (gene name *CwatDRAFT_4215*), *Trichodesmium erythraeum* IMS101 with 31% similarity (gene name *Tery_3198*), *Synechococcus* sp. PCC 7002 with 29% similarity (gene name *cpcU*), and *Fremyella diplosiphon* with 18% similarity (gene name *cpeS*). Using Cyanobase BLAST *Synechocystis* sp. PCC 6803 similarity search, CpcU was the most similar ORF to CpcS in the entire *Synechocystis* sp. PCC 6803 genome with 27% identities, 50% positives, and only 2% gaps. Using Cyanobase *Synechocystis* sp. PCC 6803 sequenced genome map, it was found that CpcU is located approximately 115 kbp downstream of the phycocyanin alpha-subunit phycocyanobilin lyase, CpcE (slr1878) (Fairchild *et al.*, 1992; Zhou *et al.*, 1992; Swanson *et al.*, 1992).

CpcT in *Synechocystis* sp. PCC 6803 was 42% similar to its *Fremyella diplosiphon* paralog, *cpeT*, but there are other higher scoring alignments (Figure 36). The similarity scores for CpcT are as follows: *Cyanothece* sp. CCY0110 with 67% similarity (gene name *CY0110_13236*), *Crocospaera watsonii* WH 8501 with 67% similarity (gene name *DUF1001*), *Nostoc punctiforme* PCC 73102 with 64% similarity (gene name *Npun02004130*), *Synechococcus* sp. PCC 7002 with 54% similarity (gene name *cpcT*), and *Fremyella diplosiphon* with 42% similarity (gene name *cpeT*). Using Cyanobase *Synechocystis* sp. PCC 6803 sequenced genome map, it was found that CpcT is located approximately 216 kpb upstream of the

Alignment of CpcU

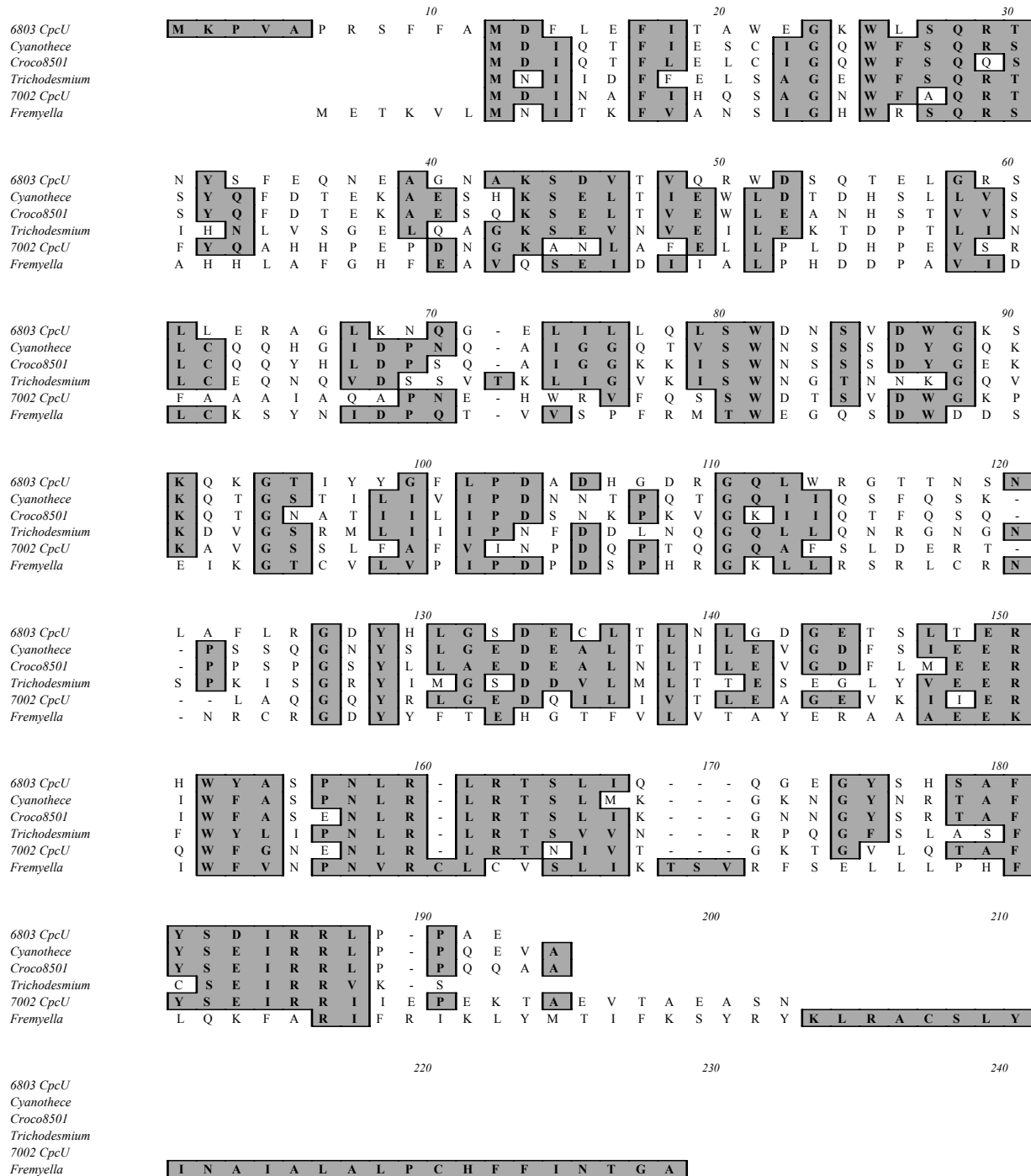


Figure 35: Alignment of amino acid sequences of *Synechocystis* sp. PCC 6803 CpcU presumed orthologs from different cyanobacteria. *Synechocystis* sp. PCC 680.3 gene name *cpcU*, *Cyanothece* sp. CCY0110 gene name *CY0110_18677*, *Crocospaera watsonii* WH 8501 gene name *CwatDRAFT_4215*, *Trichodesmium erythraeum* IMS101 gene name *Tery_3198*, *Synechococcus* sp. PCC 7002 gene name *cpcU*, and *Fremyella diplosiphon* gene name *cpeS*. Boxed in grey are the conserved amino acid residues.

Alignment of CpcT

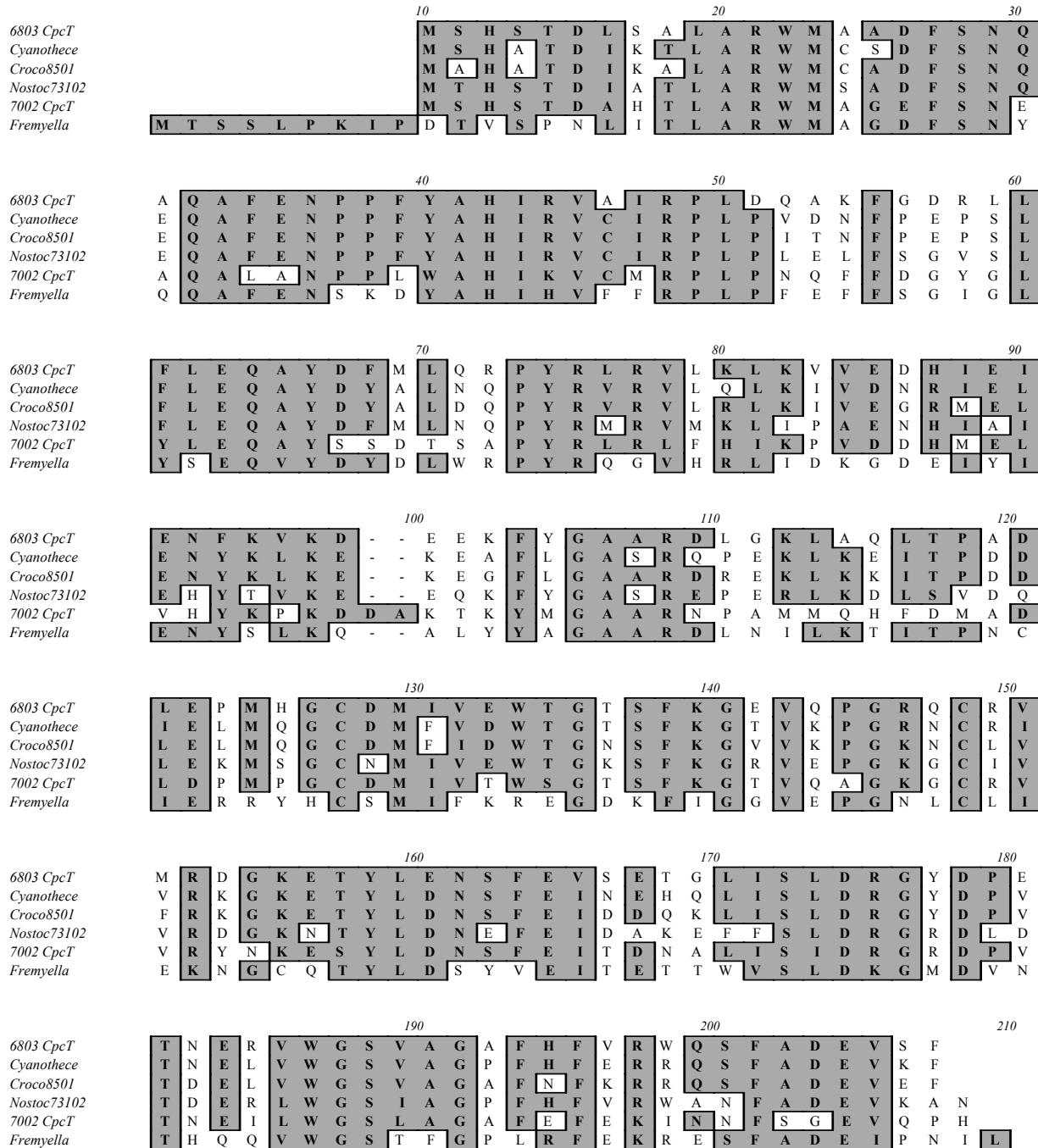


Figure 36: Alignment of amino acid sequences of *Synechocystis* sp. PCC 6803 CpcT presumed orthologs from different cyanobacteria. *Synechocystis* sp. PCC 6803 gene name *cpcT*, *Cyanothece* sp. CCY0110 gene name *CY0110_13236*, *Crocospaera watsonii* WH 8501 gene name *DUF1001*, *Nostoc punctiforme* PCC 73102 gene name *Npun02004130*, *Synechococcus* sp. PCC 7002 gene name *cpcT*, and *Fremyella diplosiphon* gene name *cpeT*. Boxed in grey are the conserved amino acid residues.

phycobilisome core-membrane linker polypeptide, ApcE (slr0335) (DiMagno and Haselkorn, 1993).

3.2.2 Production and Purification of Recombinant CpcS, CpcU, and CpcT

Recombinant *Synechocystis* sp. PCC 6803 CpcS has a calculated molecular weight of 21.9 kDa (Table 3) and was very difficult to purify. Numerous expression attempts ended in CpcS being present mostly in insoluble form (inclusion bodies). Recombinant CpcS was purified using DEAE anion exchange chromatography (described in Materials and Methods). A chromatograph of the CpcS purification is shown in Figure 37. Fractions containing protein were collected and SDS-PAGE analysis (Figure 38) was performed to determine which fractions contained CpcS. Recombinant CpcS ran near the size expected on the gel and lanes 1 and 2 show fractions from the smaller peaks before the large peak between 170 and 185 minutes. Lanes 3, 4, and 5 show fractions from the large peak (150-190 minutes) and the fraction in lane 4 is the most pure fraction. Fractions included in the large peak were pooled together, dialyzed, and concentrated.

Recombinant *Synechocystis* sp. PCC 6803 CpcU has a calculated molecular weight of 21.2 kDa (Table 3) and was also very difficult to express. Numerous expression attempts ended in insoluble protein. Eventually, expression conditions were optimized to get some soluble CpcU. Recombinant CpcU was purified using DEAE anion exchange chromatography (described in Materials and Methods). The chromatograph of the CpcU purification is shown in Figure 39. Fractions containing protein were collected, and SDS-PAGE analysis was performed. Figure 40 shows the SDS-PAGE gel of purified CpcU. Recombinant CpcU ran near the size expected on the gel and lanes 1 and 2 show the most pure fractions from the first peak between 75 and 90 minutes (seen in figure 40). Lanes 3 and 4 show fractions collected from the largest

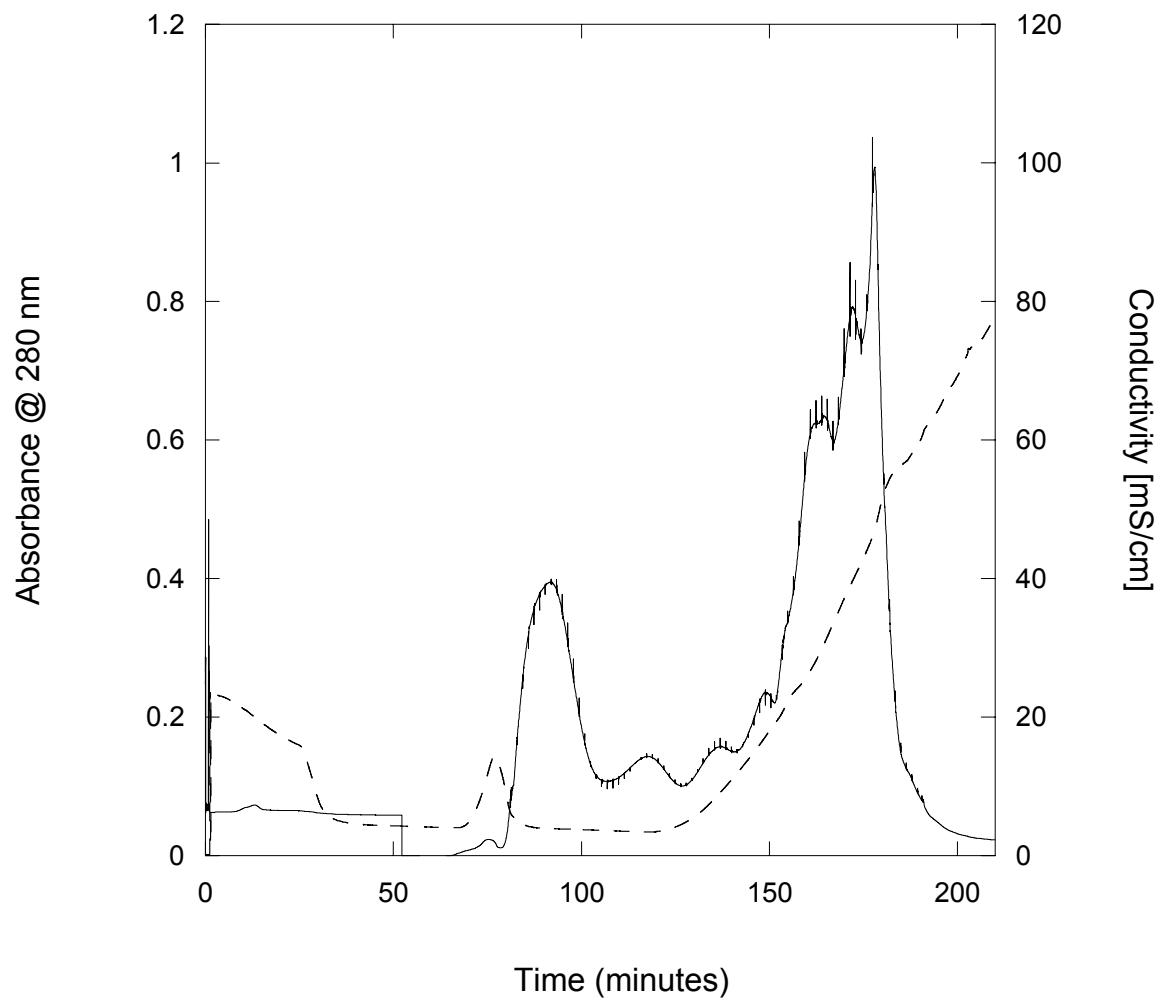


Figure 37: Purification on a DEAE column for *Synechocystis* sp. PCC 6803 CpcS. Absorbance at 280 nm is shown by the solid line while conductivity is shown by the dotted line. The most pure fraction of CpcS eluted at the major peak (between 170-185 minutes).

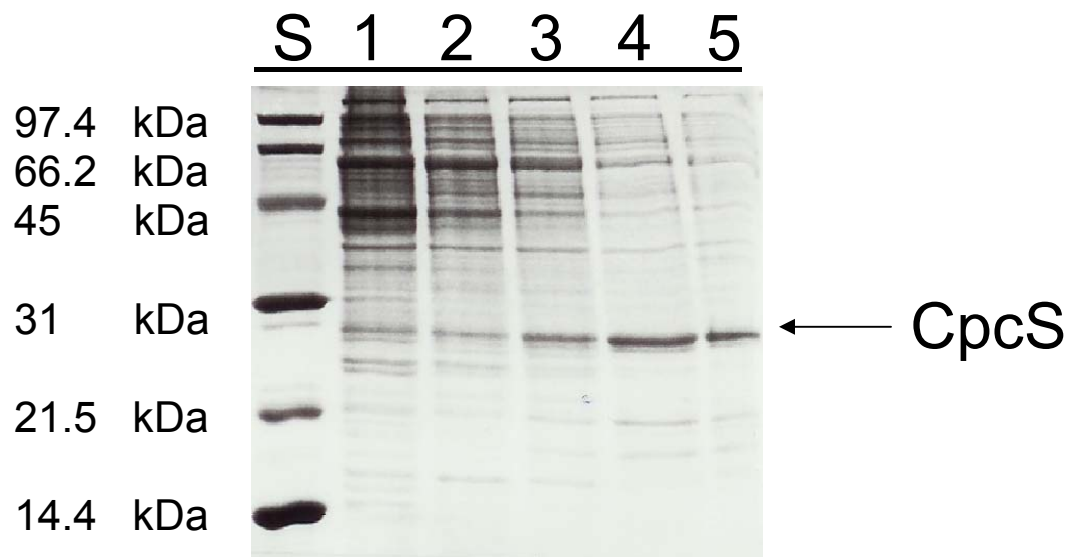


Figure 38: SDS-PAGE analysis of CpcS DEAE fractions. Lane 1 and 2 show fractions from the smaller peaks (110-130 min and 130-145 min, respectively) before the large peak (150-190 min) (seen in figure 23). Fractions from the large peak can be seen in lanes 3-5. Lane 4 is the most pure fraction. Molecular weight standard was loaded into the lane labeled “S”; the size of the bands is indicated on the left.

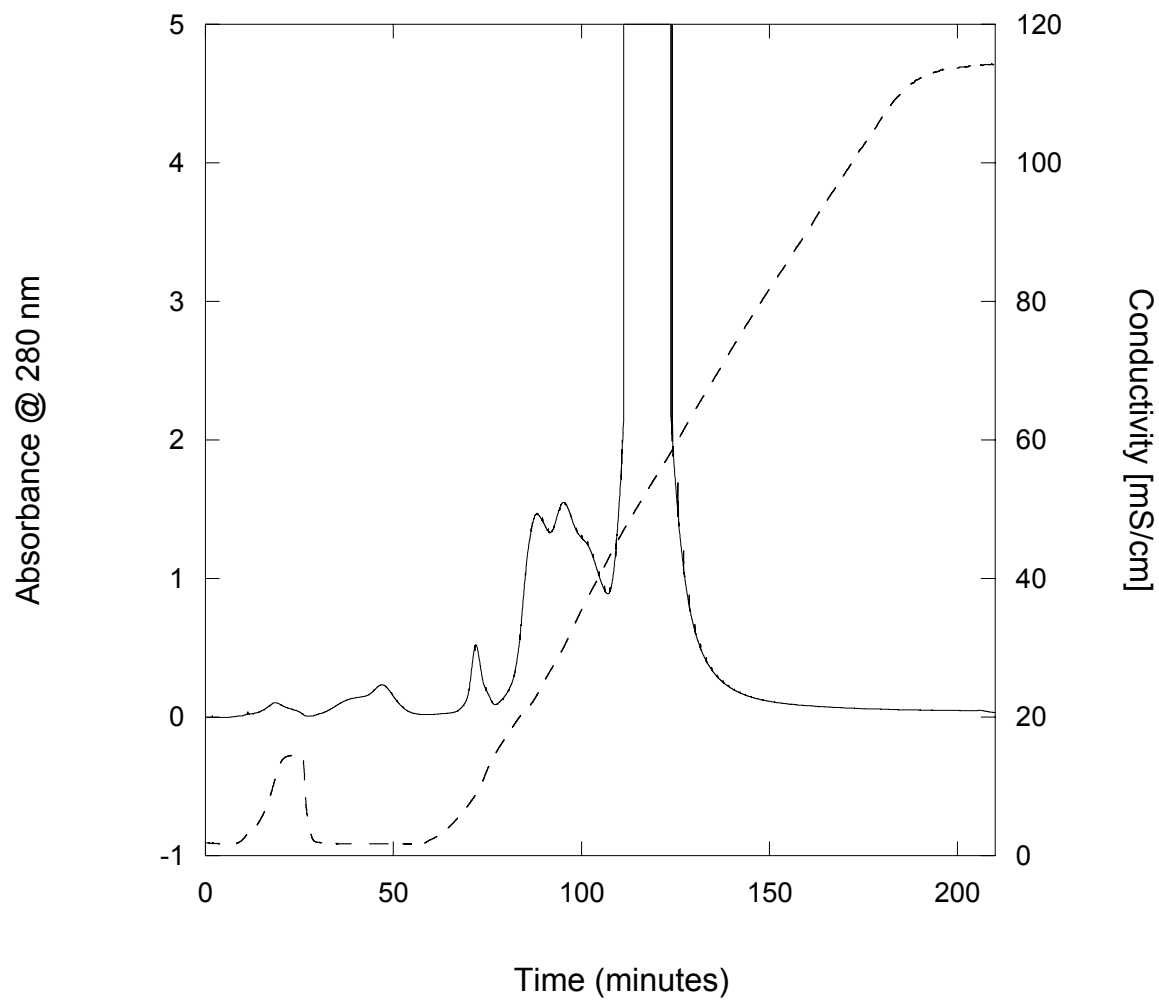


Figure 39: Purification on a DEAE column for *Synechocystis* sp. PCC 6803 CpcU. Absorbance at 280 nm is shown by the solid line while conductivity is shown by the dotted line. The most pure fractions of CpcU eluted at the first peak (between 75-90 minutes).

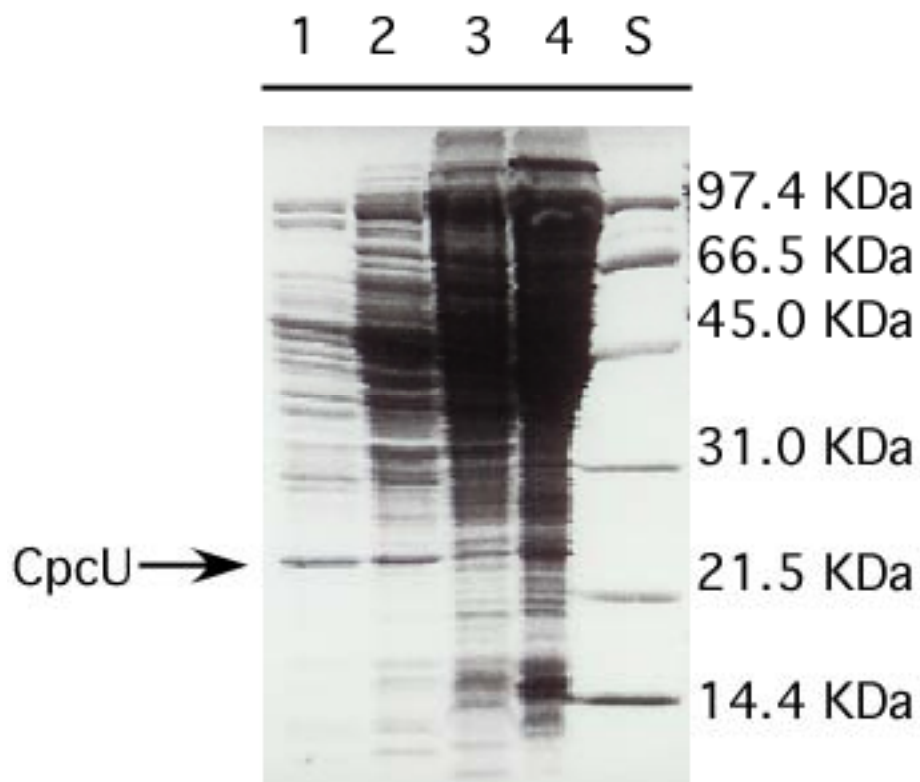


Figure 40: SDS-PAGE analysis of CpcU DEAE fractions. Lanes 1 and 2 contain the most pure fractions of CpcU from the first peak (75-90 min and 90-100 min, respectively) (seen in figure 25). Lanes 3 and 4 show fractions taken after the first peak which contain non-pure CpcU (110-130 min and 130-140 min, respectively). Molecular weight standard was loaded into the lane labeled “S”; the size of the bands is indicated on the right.

peak between 110 and 140 minutes which were not very pure. The pure fractions were pooled together, dialyzed, and concentrated.

Synechocystis sp. PCC 6803 GST-tagged protein CpcT from the pGEX-2T vector was purified using glutathione agarose resin after harvesting overproduced cells as described in Materials and Methods. A purified tagged sample was analyzed by SDS-PAGE (Figure 41 lane 1). Table 3 shows the calculated molecular weight and isoelectric point for the protein. GST-CpcT has a calculated molecular weight of 49.6 kDa which corresponds to the apparent molecular weight of the GST-CpcT band in lane 1 of Figure 41. The other two main polypeptides visible on the gel probably correspond to proteolytic products of GST as they are consistent with the molecular weight of GST (26.9 kDa).

3.2.3 Phycocyanobilin Addition with CpcT and CpcBA: PCB Reactions

Previous research has shown that *Synechococcus* sp. PCC 7002 CpcT does not interact or form complexes with any of the other lyase proteins in any significant way and was shown to be the lyase that adds PCB to the β -153 cysteinyl residue in *Synechococcus* sp. PCC 7002 (Shen et al., 2006; N. Saunée, 2006). *Synechocystis* sp. PCC 6803 CpcT was a candidate for the β -153 phycocyanobilin lyase in *Synechocystis* sp. PCC 6803, and chromophorylation reactions were performed to test this hypothesis. Recombinant *Synechocystis* sp. PCC 6803 HT-CpcB/CpcA and GST-CpcT were purified (Figure 21A lane 1 and Figure 41 lane 1, respectively). Bilin addition reactions were performed using HT-CpcB/CpcA alone (with *E. coli* extract containing empty vector pAED4) as a control and HT-CpcB/CpcA with GST-CpcT. For these reactions, phycocyanobilin (10 μ M) purified from *Spirulina* sp. was the source of phycocyanobilin.

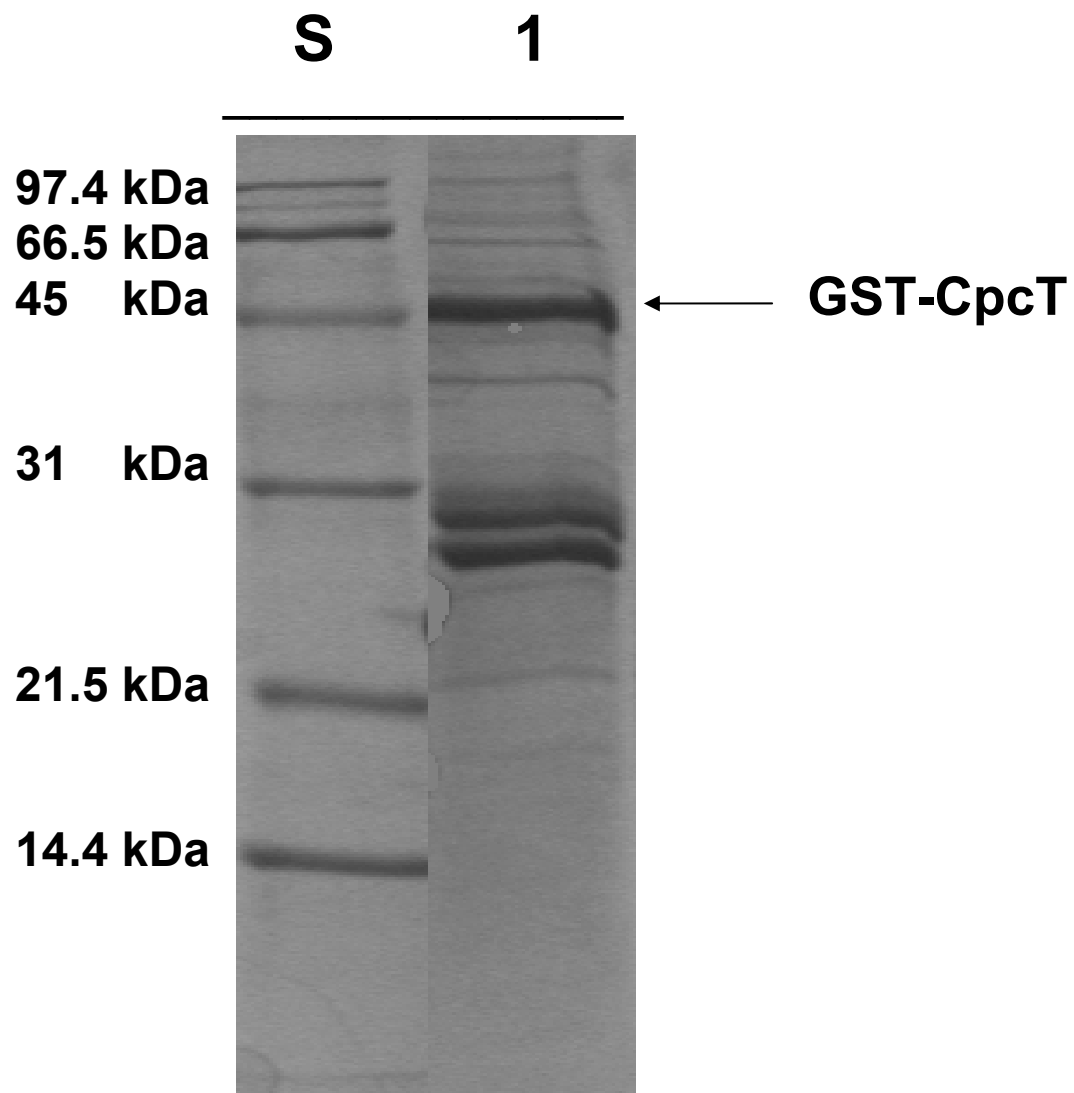


Figure 41: SDS-PAGE analysis of GST-CpcT purified sample. Lane 1 contains GST-CpcT and lane “S” is the molecular weight marker. Weights are indicated on the left.

After the addition of PCB to the CpcT reaction, a color change was noted within an hour. The color changed from blue-green to bluish/purple. The control reaction did not change color as significantly. Absorbance and fluorescence spectra can be seen in Figures 42 and 43, respectively. The absorption maximum of the CpcT-dependent product reaction is 601 nm with a second maximum at 640 nm while that of the control reaction is 640 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 640 where mesobiliverdin has its absorbance maximum, which is the more oxidized product seen after combining CpcBA with PCB in the absence of enzymes (Arciero *et al.*, 1988 a&b). In the CpcT reaction there is a significant shoulder at 640 nm which is likely due to some non-enzyme mediated addition at the Cys-82 on β -PC and on Cys-84 on α -PC. Figure 43 shows the fluorescence emission spectra for the same PCB reactions. The control reaction had a fluorescence maximum of 655 nm. The CpcT reaction has a peak at 629 nm which is consistent with PCB being attached at the β -153 site, and it has a strong second peak at 655 nm due to energy being absorbed by PCB at Cys-153 on β -PC and then being transferred to mesobiliverdin attaching to Cys-82 on β -PC. This mesobiliverdin fluoresces at 655 nm. Energy absorbed by chromophores must either be transferred to another chromophore that is in close proximity or be released as fluorescence. The CpcT reaction was overall more fluorescent than the control reaction which had almost double the fluorescence emission. This is consistent with the control product being mesobiliverdin; mesobiliverdin has only 5-10% of the fluorescence that phycocyanobilin has (Arciero *et al.*, 1988 a&b; Fairchild and Glazer, 1994).

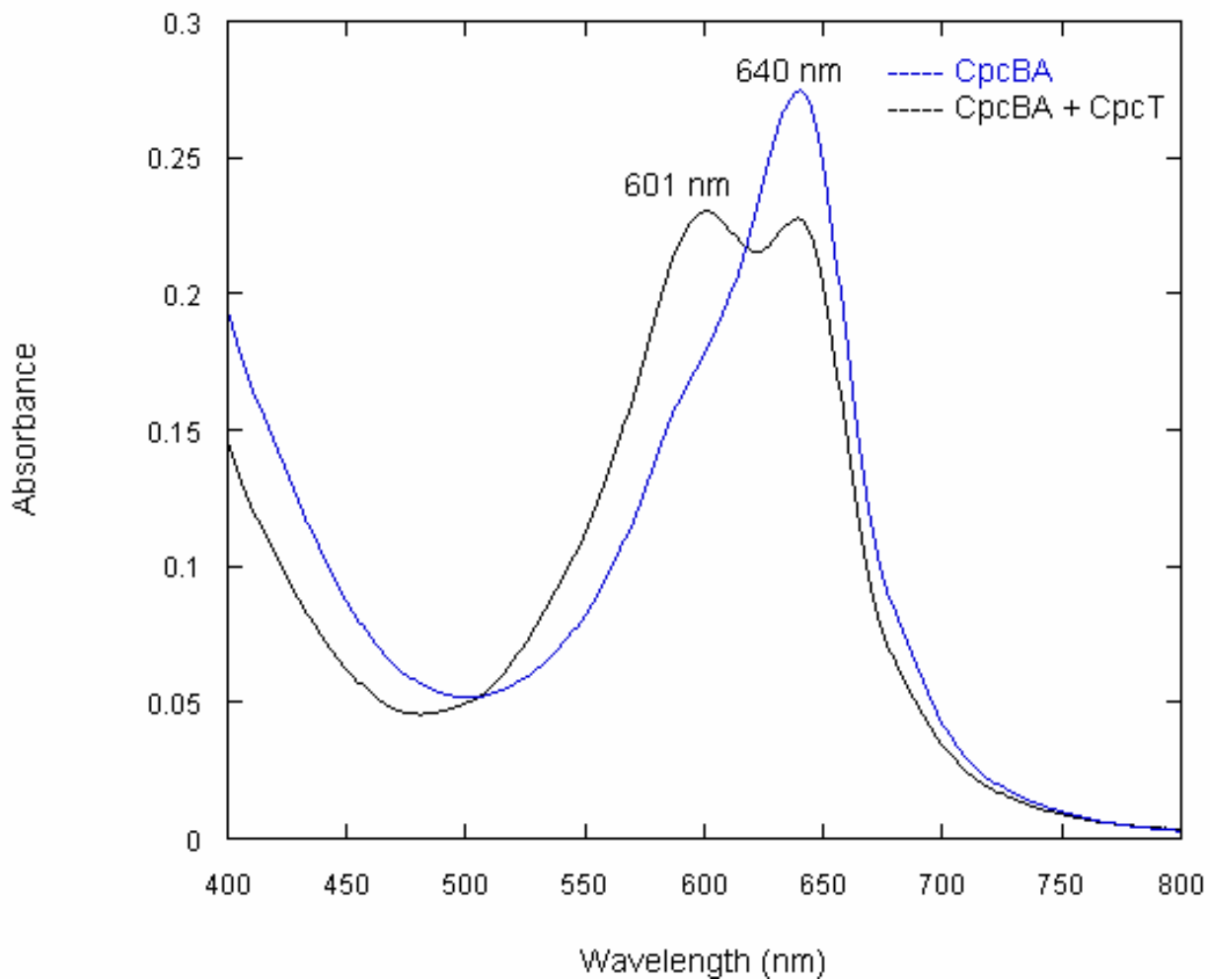


Figure 42: Absorbance spectra of *in vitro* PCB assays. Absorbance spectra of PCB reaction products with CpcBA and CpcBA with CpcT. The control reaction (CpcBA) is shown in blue, while the CpcT reaction is shown in black. The control reaction has a maximum at 640 nm while the CpcT reaction has a maximum at 601 nm.

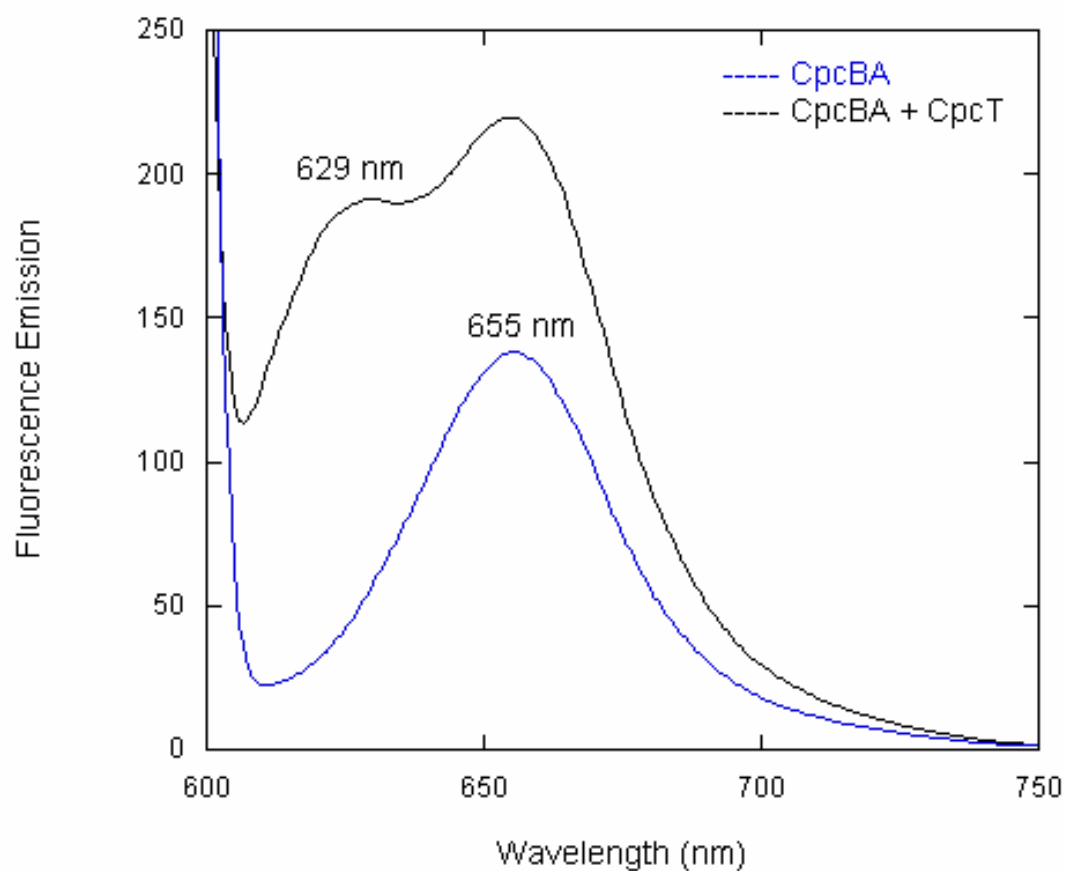


Figure 43: Fluorescence spectra of *in vitro* PCB assays. Fluorescence spectra of PCB reaction products with CpcBA and CpcBA with CpcT. The control reaction (CpcBA) is shown in blue, while the CpcT reaction is shown in black. The control reaction has a maximum at 655 nm while the CpcT reaction has a peak at 655 nm, as well, but a strong peak at 629 nm, also.

3.2.4 PcyA Reactions with CpcT

Because of the non-enzymatic mesobiliverdin product made during the PCB reactions, PcyA was used to generate PCB. Phycocyanobilin:ferredoxin oxidoreductase (PcyA) from *Nostoc* sp. PCC 7120 was used to produce PCB *in situ*. PcyA is a novel enzyme requiring no cofactors or metal, and it catalyzes a four electron reduction of biliverdin using ferredoxin to produce PCB. In these reactions there is little mesobiliverdin produced because only small amounts of phycocyanobilin are produced *in situ* (Frankenburg *et al.*, 2001; Frankenburg and Lagarias, 2003). Reactions were performed at 30°C because the PcyA enzyme from *Nostoc* is active at this temperature. PcyA along with other factors (ferredoxin, biliverdin, etc.) were added to reactions containing just CpcBA or CpcBA with CpcT. After the four hour incubation period at 30°C, a color change from yellow-green to blue could be seen. The control reaction again had little to no color change. The absorbance and fluorescence spectra of the reactions can be seen in Figure 44 and 45, respectively. Figure 44 shows the product of the reaction with CpcT had an absorbance maximum at 599 nm, while the control reaction had an absorbance maximum of 655 nm. If the CpcT lyase is adding at the β -153 site we expect a maximum around 600 nm (Debreczeny *et al.*, 1993; Shen *et al.*, 2006), consistent with the result in Figure 44 for CpcT. The control reaction had an absorbance spectra similar to that of mesobiliverdin (Arciero *et al.*, 1988 a&b), the non-enzymatic product, but it is not as significant as it is in the PCB reactions. The absorbance of the control product could also be a more oxidized version of mesobiliverdin as its maximum is red-shifted ~15 nm from that reported for mesobiliverdin (compare to Figure 42) The CpcT reaction product was fluorescent (Figure 45) with a fluorescence emission maximum of 621 nm, while the control reaction was much less fluorescent and with a maximum of 669 nm. Both the absorbance and fluorescence spectra of the CpcT-dependent CpcBA product

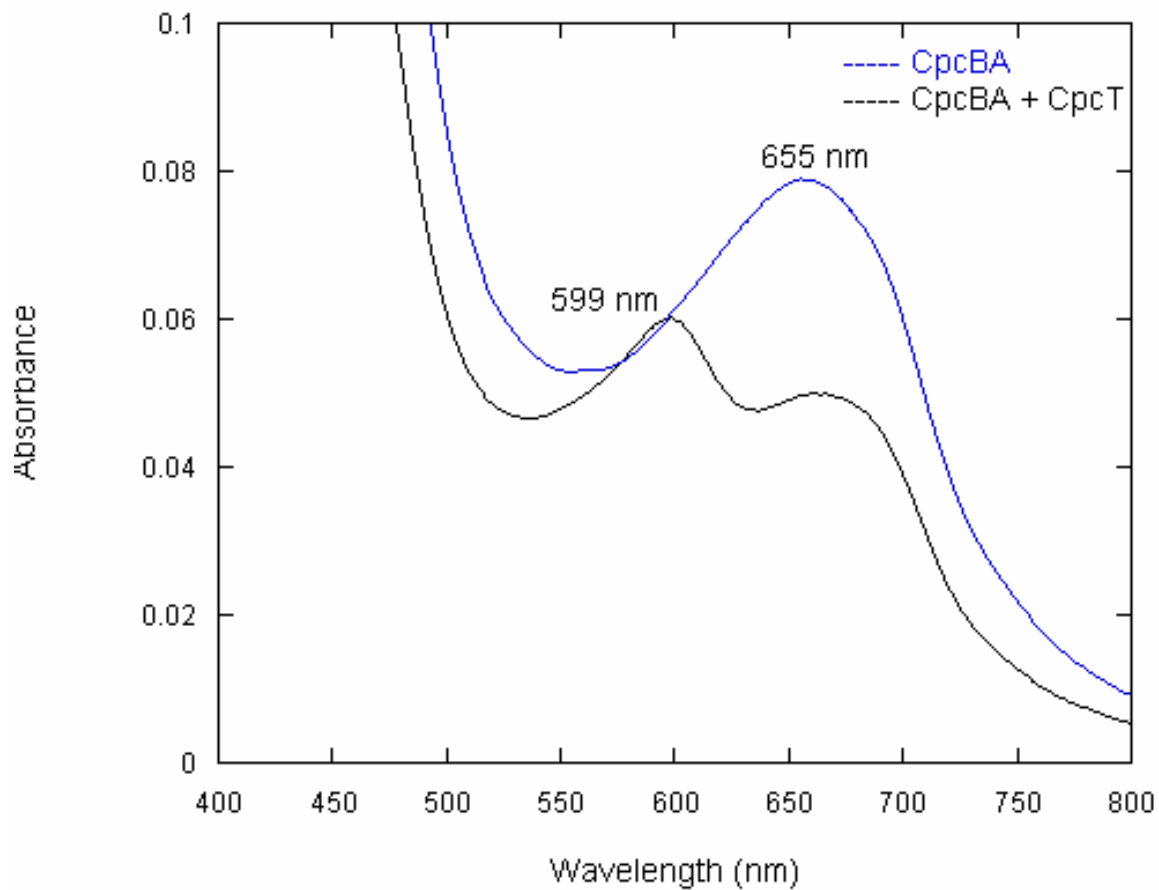


Figure 44: Absorbance spectra of *in vitro* bilin addition reactions with CpcBA using CpcT and PcyA. Absorbance spectra of reactions with CpcBA alone (control reaction=blue line) and CpcBA with CpcT (black line). Absorbance maxima are shown for the reactions. The control reaction has a maximum of 655 nm while the CpcT reaction has an absorbance maximum of 599 nm.

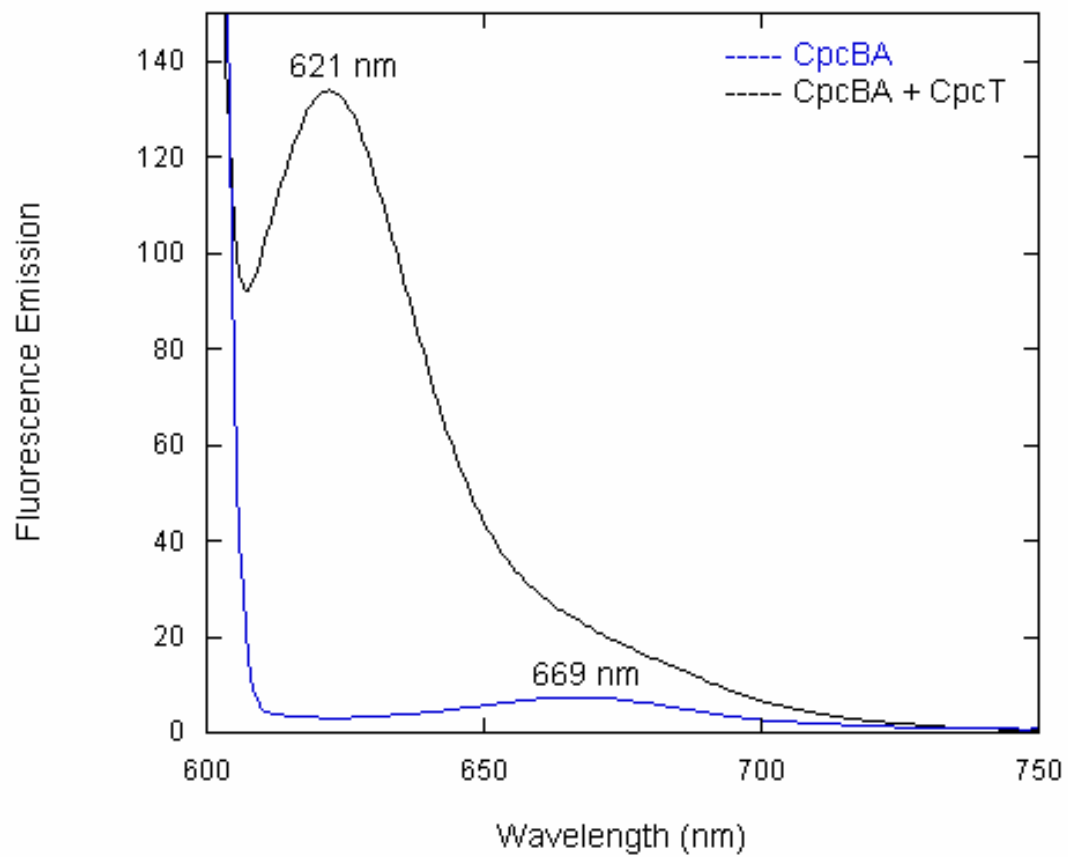


Figure 45: Fluorescence emission spectra of *in vitro* bilin addition reactions with CpcBA using CpcT and PcyA. Fluorescence spectra of reactions with CpcBA alone (control reaction=blue line) and CpcBA with CpcT (black line). Fluorescence maxima are shown for the reactions. The control reaction has a maximum of 669 nm while the CpcT reaction has a maximum of 621 nm.

have maxima that are consistent with addition of phycocyanobilin taking place at the β -153 cysteine and are consistent with the results obtained with CpcT from *Synechococcus* sp. PCC 7002 (Debreczeny *et al.*, 1993; Shen *et al.*, 2006).

3.2.5 Analysis of Bilin Addition by SDS-PAGE

Because CpcA has one bilin addition site and CpcB has two bilin addition sites and both proteins are present in the reaction mixture, it was important to determine which site has a bilin attached to it. Aliquots of chromophorylation reactions were separated by SDS-PAGE and incubated with ZnSO₄ and later with Coomassie blue (as described in Materials and Methods). Figure 46 shows zinc-enhanced fluorescence of bilins attached to proteins (Panel A) and the Coomassie stained gel (Panel B) for the PcyA reactions with CpcT (seen in Figures 44 and 45). The left side (Panel A) shows the products of the control (lane 1) and the CpcT PcyA reaction (lane 2) after the gel was incubated with zinc. The control reaction product contained a small amount of bilin (probably mesobiliverdin) attached to HT-CpcB, but the CpcT product clearly had more bilin attachment to HT-CpcB. There was not enough product to analyze which cysteine (β -82 or β -153) contained a PCB, but the absorbance and fluorescence properties of the CpcT product are consistent with addition at Cys-153 (Shen *et al.*, 2006).

3.2.6 Phycocyanobilin addition with CpcS/CpcU and CpcBA: PcyA Reactions

Previous research has shown that *Synechococcus* sp. PCC 7002 CpcS and CpcU form a complex with each other and comprise the lyase that adds PCB to the β -82 cysteinyl residue in *Synechococcus* sp. PCC 7002 (N. Suanée, 2006). However, recently CpeS from *Anabaena* sp.

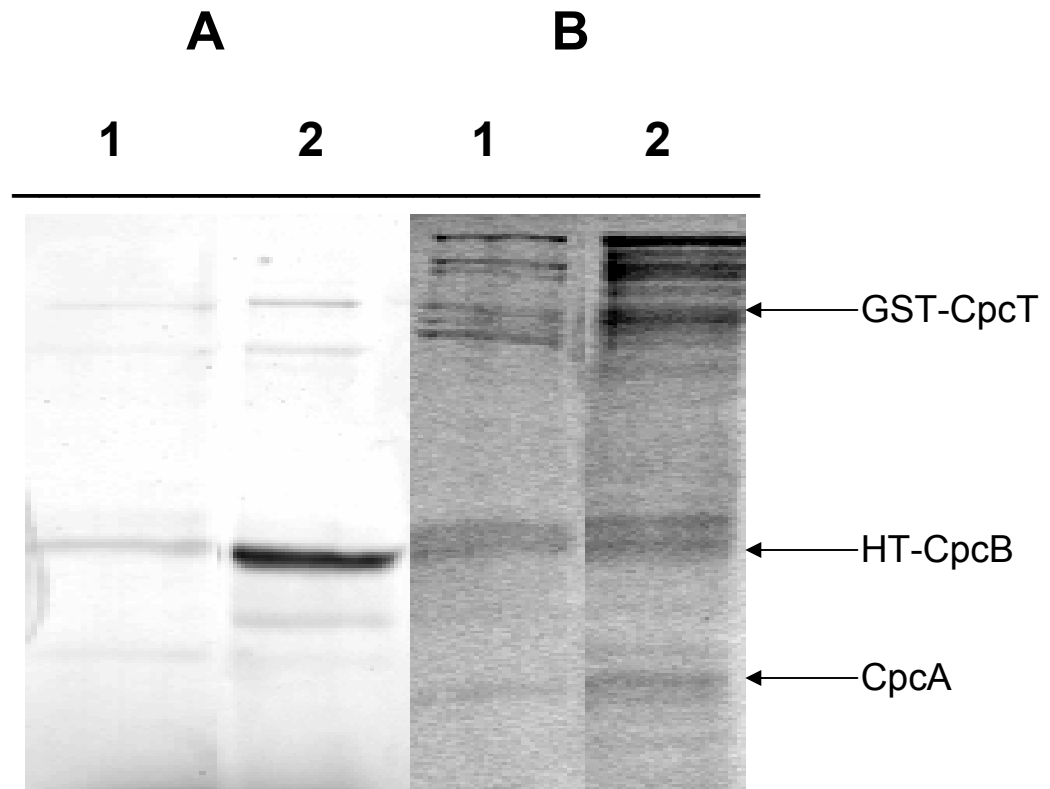


Figure 46: SDS-PAGE analysis of PcyA reactions containing CpcT. Panel A) Zinc-enhanced fluorescence of bilins attached to proteins present in the control reaction (lane 1) and the CpcT reaction (lane 2). Lane 1 contains the control reaction and lane 2 contains the CpcT reaction (fluorescence on the β -subunit). Panel B) Shows the same gel after staining in Coomassie blue. Lane 1 is the control reaction and lane 2 is the CpcT reaction. The protein identities are shown.

PCC 7120 was shown to act alone to attach PCB to Cys-82 (Zhao *et al.*, 2006). *Synechocystis* sp. PCC 6803 CpcS and CpcU were candidates for the β -82 phycocyanobilin lyase in *Synechocystis* sp. PCC 6803. *In vitro* assays for chromophorylation were performed by adding CpcBA as a substrate and one or both subunits (CpcS, CpcU, or CpcSU) to each assay. For these reactions, PcyA from *Nostoc* sp. PCC 7120 was used to produce phycocyanobilin *in situ*. After incubation the color changes were noted for the different sets of reactions. The control, CpcS alone, and CpcU alone reactions changed from yellow to a light green, but the reaction containing both CpcS and CpcU changed to a slightly bluish-green color.

The absorbance and fluorescence spectra of these reactions can be seen in Figures 47 and 48, respectively. Figure 47 shows the product of the reaction with both CpcS and CpcU had an absorbance maximum at 629 nm, while the control reaction and the single reactions with CpcS or CpcU had absorbance maxima between 655 nm and 672 nm. If phycocyanobilin is attached at the β -82 site then a peak around 620-625 nm would be expected (Debreczeny *et al.*, 1993), similar to the peak for CpcSU-dependent reaction product. The single reactions had an absorbance spectrum that is similar to the absorbance spectrum of mesobiliverdin (the non-enzymatic unnatural product) (Arciero *et al.*, 1988 a&b).

The product of the CpcSU reactions was fluorescent with an emission maximum of 640 nm while the control reaction and the CpcS and CpcU single reactions were barely fluorescent with an emission maxima of 649 (Figure 48). The β -82 phycocyanobilin chromophore should fluoresce at approximately 640 nm (Debreczeny *et al.*, 1993). Both the absorbance and fluorescence spectra are consistent with addition of phycocyanobilin taking place at the β -82 cysteine site, and both CpcS and CpcU were required to get the product. This is also consistent with the results obtained with CpcSU from *Synechococcus* sp. PCC 7002 (N. Saunée, 2006).

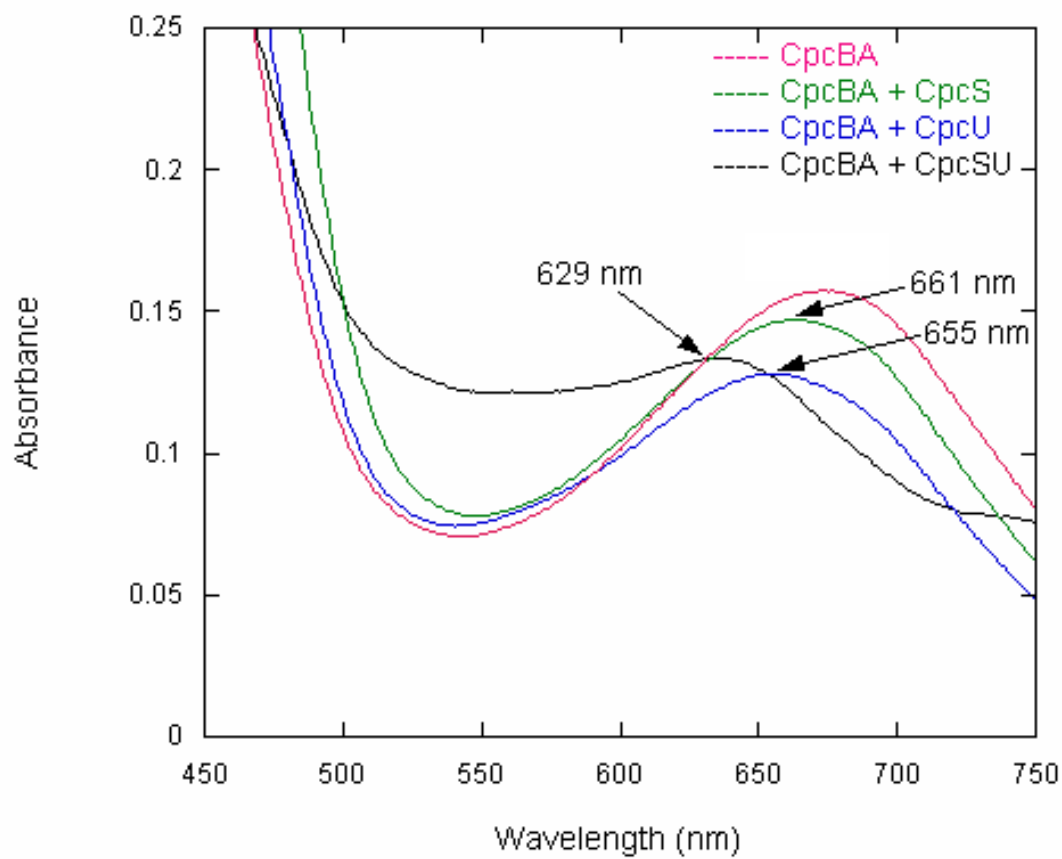


Figure 47: Absorbance spectra of *in vitro* bilin addition reactions with CpcBA using CpcS and CpcU with PcyA. Absorbance spectra of reactions with CpcBA alone (control reaction=red line) and CpcBA with CpcS alone (green line), CpcU alone (blue line), and CpcS and CpcU (black line). Absorbance maxima are shown for the reactions.

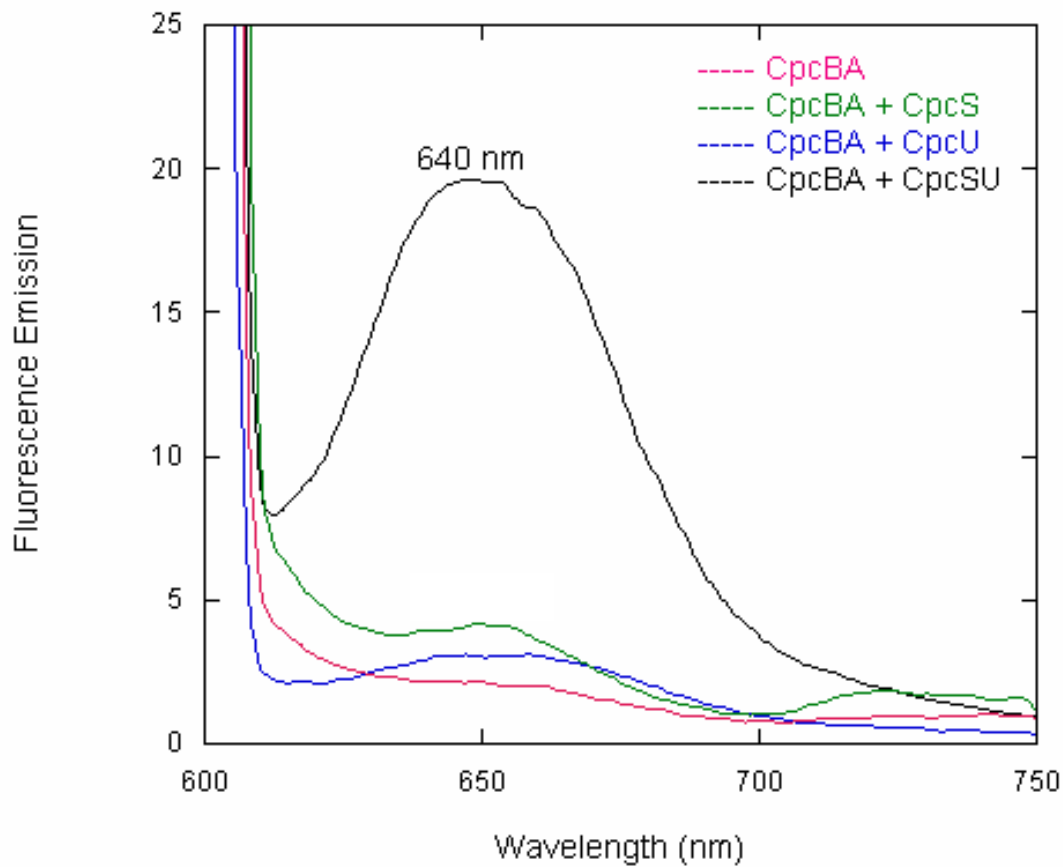


Figure 48: Fluorescence emission spectra of *in vitro* bilin addition reactions with CpcBA using CpcS and CpcU with PcyA. Fluorescence spectra of reactions with CpcBA alone (control reaction=red line) and CpcBA with CpcS alone (green line), CpcU alone (blue line), and CpcS and CpcU (black line). Fluorescence was taken at 590 nm with 10 nm slits. The maximum for CpcBA with CpcS and CpcU was 640 nm, while all other maxima were around 649 nm.

3.2.7 Analysis of Bilin Addition by SDS-PAGE

Both HT-CpcB and CpcA were included in the *in vitro* assay reactions. To determine which subunit the CpcS/CpcU lyases were adding to, the CpcS/CpcU reaction sample was separated via SDS-PAGE. 20 μ l aliquots from each reaction were separated on SDS-PAGE, incubated in ZnSO₄, followed by staining in Coomassie Blue. Figure 49 shows the bilin fluorescence (Panel A) and the Coomassie stained gel (Panel B) for reactions with CpcS and/or CpcU. The control showed very little bilin attachment (Panel A, lane 1), but all three reactions: CpcS (lane 3), CpcU (lane 4), and CpcSU (lane 2) displayed bilin attachment to both the α and β subunits. These attachments are due to mesobiliverdin in the control, CpcS, and CpcU reactions. There was an equivalent amount of bilin attached to CpcA as judged by fluorescence intensity in reactions containing CpcSU, CpcS, or CpcU (Figure 49A, lanes 2-4). The intensity of CpcB fluorescence in the CpcSU reaction (lane 2) was at least 2-fold higher than in the CpcS or CpcU-alone reactions (lanes 3 and 4, respectively). Panel B of Figure 43 shows the same gel after Coomassie staining. Proteins are identified on the gel. There was not enough product to analyze which cysteine contained a PCB, but the absorbance and fluorescence properties of the CpcSU product are consistent with addition of PCB to β -Cys-82.

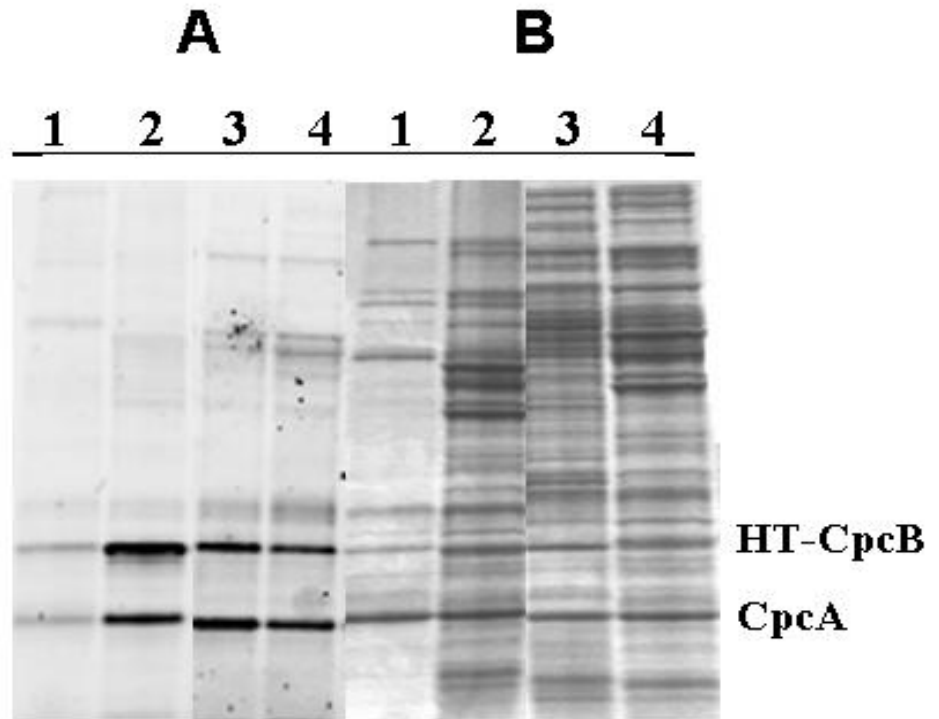


Figure 49: SDS-PAGE analysis of PcyA reactions containing CpcS and/or CpcU. Panel A) Zinc-enhanced fluorescence of bilins attached to proteins present in the control reaction (lane 1), the CpcSU reaction (lane 2), the CpcS reaction (lane 3), and the CpcU reaction (lane 4). Panel B) Shows the same gel after staining in Coomassie Blue.

DISCUSSION

4.1 CpcM Methyltransferase

Many observations have noted an unusual post-translational modification of γ -N-methylasparagine at the β -72 residue of many phycobiliproteins in cyanobacteria, red algae, and cryptomonads (Klotz *et al.*, 1986; Klotz and Glazer, 1987; Wilbanks *et al.*, 1989). Researchers have examined the consequences of this methylation for the structure and function of phycobiliproteins and of phycobilisomes (Swanson and Glazer, 1990). Results of many studies have shown that this site-specific methylation of a phycobiliprotein contributes significantly to the efficiency of energy transfer in phycobilisomes (Swanson and Glazer, 1990; Thomas *et al.*, 1993). Specifically, this methylation increases the energy transfer efficiency from phycobilisome components to the terminal energy acceptors in PSII and minimizes non-radiative energy losses (Thomas *et al.*, 1993; Thomas *et al.*, 1995). This prevention of nonradiative energy loss occurs by “fine-tuning” the spectroscopic properties of the neighboring β -82 chromophore (Thomas *et al.*, 1995). This protection of quantum energy yield presumably provides a selective advantage to cells growing in aquatic niches that are light-limiting (Thomas *et al.*, 1993). We have shown here that CpcM is responsible for methylation of the β -72 residues within phycobiliproteins of *Synechocystis* sp. PCC 6803.

We have shown that CpcM only methylates β subunits (CpcB, ApcF, and ApcB) and not α subunits (CpcA, ApcA) and these results match very well with previous researchers' and collaborators' work with the *cpcM* gene. The *cpcM* gene from *Synechocystis* sp. PCC 6803, was cloned and insertionally inactivated by interposon mutagenesis (H.R. Leonard, 2002). The mutant was analyzed to determine its effect on the assembly of the phycobilisome and

photosystem complexes. To assess the photosystem reaction centers, cells were frozen down to 77K, and the fluorescence emission of the chlorophylls associated with PSI and PSII were measured. No difference was observed in fluorescence amplitude from PSII and PSI associated chlorophylls between wild-type and the mutant. There was also no difference in the measured phycobiliprotein to chlorophyll ratio in mutant cells compared to wild-type (H.R. Leonard, 2002; G. Shen and D.A. Bryant, unpublished results).

To test whether PBS assembly was affected, PBS were purified from the *cpcM* mutant and wild-type cells. Spectral analysis of isolated phycobilisomes showed that there was no difference in the chromophore content of the phycobilisomes isolated from the *cpcM* mutant and wild-type. SDS-PAGE analysis showed that there was no difference in the composition of isolated phycobilisomes from wild-type and the *cpcM* mutant. These results imply that the *cpcM* mutation does not affect phycobilisome biogenesis and assembly (H.R. Leonard, 2002; G. Shen, W.M. Schluchter, and D.A. Bryant, unpublished results). In examining the growth curves, when the *cpcM* mutant and wild-type *Synechocystis* sp. PCC 6803 were grown under high-intensity white light, the *cpcM* mutant and wild-type displayed no differences in growth rates. When grown under low-intensity white light and green light, the *cpcM* mutant exhibited a much slower growth rate compared to wild-type indicating that this mutation has affected energy transfer from phycobilisomes to PSI and PSII (H.R. Leonard, 2002; G. Shen, W.M. Schluchter, and D.A. Bryant, unpublished results).

This is consistent with experiments performed by Swanson and Glazer on *Synechococcus* sp. PCC 7942 *pcm-1* and *pcm-2* mutants lacking methylation at the β -72 residue. Their mutant was still able to produce complete phycobiliproteins and phycobilisomes (Swanson and Glazer, 1990). However, Swanson and Glazer also noted that the mutants of *Synechococcus* sp. PCC

7942 deficient in PBP methylation had an increase in fluorescence emission between 630 and 660 nm indicating less efficient energy transfer was occurring between phycocyanin and allophycocyanin.

Because the original *cpcM* mutant was lost, *cpcM* mutant strains were generated through interposon mutagenesis in *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803. To investigate the possibility that the interruption of the *cpcM* gene affects energy transfer efficiency from phycobilisome to photosystems, state transition measurements were compared between wild-type and *sll0487::Km* cells. State transition measurements were done using fluorescence emission on exponential-phase cells. Fluorescence emission spectra of the purified phycobilisomes from *cpcM* mutant and wild-type revealed that the mutant exhibited obvious inefficiency of energy transfer from phycocyanin to allophycocyanin. No light-induced transition from state 2 to state 1 was observed in the *cpcM* mutant cells (G. Shen and D.A. Bryant, unpublished results). The decrease in excitation energy transfer would be consistent with the idea that the *cpcM* mutant's phycobilisomes are missing the methylation at the β -72 residue, and that this methylation is important in energy transfer within the PBS and from the PBS to PSI. These results are consistent with CpcM functioning in phycobiliprotein methylation. Since methylation of phycobiliproteins affects the cell's ability to respond to short-term changes in light-conditions, the *cpcM* mutant's inability to respond to short-term changes in light-conditions provides more evidence that the β -72 residue was not methylated.

LC-MS analyses of isolated PBS from the *cpcM* mutants confirmed that mutants lacking CpcM synthesize CpcB, ApcB, and ApcF that are not methylated. Trypsin digestion and further MS analyses of peptides showed that the peptide (Q L F A D Q P Q L I A P G G N A Y T N R)

including Asn72 (in bold) was 14 daltons (the weight of CH₃ replacing H) lighter than the equivalent peptide from the wild-type strains (G. Shen and D.A. Bryant, unpublished results). This provides evidence that CpcM is specific for Asn 72.

The timing of the post-translational methylation modification during the building of the phycobilisome was investigated by performing assays on chromophorylated and unchromophorylated phycobiliproteins. If CpcM were to show specificity towards apo versus holo protein, this would give us a clue to when the methylation occurs, either before or after the bilins were attached to the phycobiliproteins. When apo-CpcB and that same protein containing a bilin at Cys-82 (Cys-82-PCB)-CpcB were compared in assays, the presence of the bilin at Cys-82 on β -PC seemed to increase the affinity of the CpcM enzyme for the substrate.

When trimeric PC isolated from the *cpcM* mutant was compared with apo-PC (monomeric), the CpcM enzyme showed higher affinity towards the apo-PC. One explanation is that CpcM methylates the β -subunits early in the protein biogenesis either before, or just after bilins get added. Once the phycobiliprotein associates into trimers with linker proteins, the CpcM enzyme showed less affinity for this form. In examining the structure and position of the Asn72 with the PC trimers, Duerring *et al.*, noted that the Asn72 residue was readily accessible on the exterior of the trimer, and they speculated that the methyltransferase may recognize this as a substrate. Further research performing CpcM assays using CpcSU and CpcT-dependent CpcB reaction product versus apo-CpcB may allow us to examine the importance of bilins at all positions in the affinity of CpcM for its substrates. The conclusion from this research is that the methylation occurs after monomeric PBP are chromophorylated but before they assemble into trimers.

How does specificity for the β subunit and not the α subunit of PBP arise given the extremely similar primary and tertiary structures of these proteins (see Figure 5)? Comparing sequence alignments around the Asn72 there is a strong conservation of a short sequence among the β subunits that is not present in the α subunits. This signature sequence is P-G-G-N(-)-Y-T (Klotz *et al.*, 1986). This implies that the recognition domain for CpcM is within this region. This hypothesis could be tested by fusing this sequence to a protein like GST or by changing CpcA to contain this sequence.

Though there are only very slight structural differences between the α and β subunits of PBP, only the β -subunit carries the terminal energy acceptor bilin chromophore at Cys-82. It has been shown that the methylation of Asn72 provides for optimal energy transfer by decreasing the nonradiative energy loss of the β -82 chromophore, the terminal energy acceptor. This is proposed to happen by the methyl group on the Asn72 restricting conformational flexibility of the bilin. By keeping the chromophore rigid processes such as excited-state proton-transfer reactions, intersystem crossing, or photoisomerization are decreased (Thomas *et al.*, 1995). The methylation is specific for β subunits due to its purpose in altering the environment surrounding the terminal energy “acceptor” bilin located on the β subunit.

How does this post-translational modification affect Asn72 within the phycocyanin? When the Asn72 residue is methylated, the methylation reaction adds bulk to the side-chain amide group and also removes the possible participation of one of the two amide hydrogen atoms in hydrogen bonding schemes. Studies on synthetic peptides have suggested that methylation can slow the spontaneous deamidation of the side chain of asparagine residues by 45-fold (Klotz and Thomas, 1993). This is very important if Asn72 is supposed to hold the β -82 chromophore rigid

and in place for efficient photosynthesis. If the chromophore were to become flexible it would lead to nonradiative relaxation decreasing the efficiency of photosynthesis in the organism.

AdoMet-dependent methyltransferases, like CpcM, share a conserved catalytic domain structure due to the interaction of the enzymes with a common cofactor, *S*-adenosylmethionine (Schluckebier *et al.*, 1995). AdoMet-dependent methyltransferases show a similar folding pattern with a central parallel β -sheet surrounded by α -helices. The common three-dimensional structure of these enzymes is demonstrated in sequence motifs that are conserved among a large number of AdoMet-dependent methyltransferases (Ingrosso *et al.*, 1989; Lauster *et al.*, 1989; Posfai *et al.*, 1989; Kagan and Clarke, 1994; Malone *et al.*, 1995; Hamahata *et al.*, 1996). The conserved regions, which are designated as motif I, post-I, motif II, and motif III, are always found in the same order on the polypeptide chain and are separated by comparable intervals. The three-dimensional structures of AdoMet-dependent methyltransferases have revealed that motif I and post-I interact directly with AdoMet, whereas motifs II and III interact with each other and with a portion of motif I to form the central portion of the β -sheet (Figure 50). The enzymes lacking motifs may have three-dimensional structures distinct from the family of methyltransferases or may have diverged sufficiently for the motifs to become unrecognizable (Niewmierzycka and Clarke, 1999).

Is CpcM similar to any other methyltransferases? Another methyltransferase involved in helping its organism perform optimally is CheR. Sensory adaptation to persisting stimulation permits cells to respond with greater sensitivity to temporal changes in stimuli. In *Escherichia coli* and *Salmonella enterica*, adaptation during bacterial chemotaxis is in part mediated by reversible covalent modifications of transmembrane chemoreceptors, also referred to as methyl-accepting chemotaxis proteins, in which specific glutamate residues within the cytoplasmic

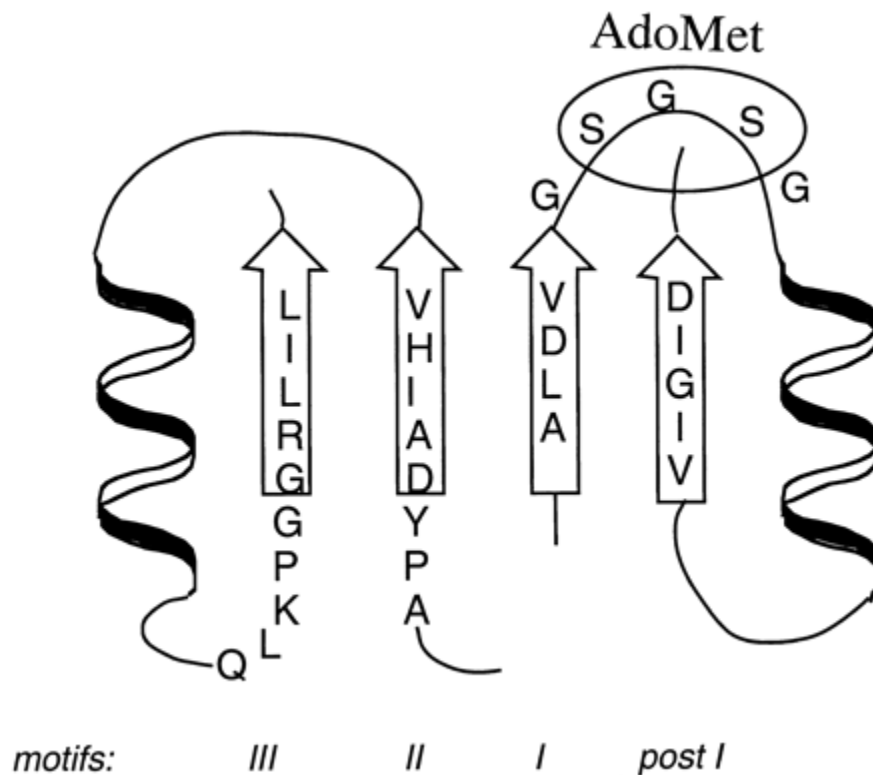


Figure 50: Schematic representation of the conserved methyltransferase motifs in relation to S-adenosylmethionine. The conserved motifs I, post-I, II, and III are shown with the sequence of the human protein L-isoaspartate (D-aspartate) O-methyltransferase. The arrangement of motifs is predicted based on known three-dimensional structures (Niewmierzycka and Clarke, 1999).

domains are methylated by methyltransferase CheR and demethylated by methylesterase/deamidase CheB (Kehry and Dahlquist, 1982). Though both CpcM and CheR's methylation may help their respective organisms to be more efficient, they are not similar in that one is reversible and one is not. Asparagine 72 methylation performed by CpcM is a covalent permanent post-translational modification. CheR/CheB's methylation/demethylation is regulated by negative feedback from receptors. CpcM methylation of Asn72 is probably more like other permanent modifications seen in ribosomal proteins.

Ribosomal protein methylation such as the δ -N-methylglutamine, is a permanent modification that makes ribosomes more cold hardy. Lhoest and Colson (1981) observed the effects on low-temperature assembly of *E. coli* ribosome L3 lacking the δ -N-methylglutamine modification. They suggested that the methylase represents a ribosomal assembly factor, as unmodified ribosomes, once assembled, appear to function normally. The gene encoding the methyltransferase was designated *prmB* (Colson *et al.*, 1979). Mutants in the *prmB* gene displayed a cold-sensitive phenotype and accumulated unstable and abnormal ribosomal particles (Lhoest and Colson, 1981). The PrmB methyltransferase is probably a distinct enzyme from CpcM. There is no sequence similarity in the methyl-accepting site of L3 and the PBP. Another permanent ribosomal protein methylation is N-5-methylglutamine. This is performed by the enzyme HemK and modulates the termination activity of release factors in ribosomal protein synthesis. A *hemK* knockout strain of *E. coli* not only suffered severe growth defects, but also showed a global shift in gene expression to anaerobic respiration (Nakahigashi *et al.*, 2002). The asparagine residue methylated by CpcM in phycobiliproteins is located in a similar sequence context to that of the glutamine residues in the ribosomal protein synthesis release factors

methylated by the HemK methyltransferase, where the amide residue in both cases is preceded by a pair of glycine residues (Clarke, 2002).

Several other amino acids have been found to be methylated by methyltransferases such as arginine, glutamate, and glutamine. What about the possibility of methylation of the side chain of glutamine residues by CpcM, since they only have one more methylene group in the side-chain than asparagine residues? This may be possible due to research performed by Thomas *et al.*, (1995) which showed when the asparagine residue at 72 was replaced with a glutamine residue in PBP, significant amounts of methylation were still detected. This suggests that CpcM might recognize either an asparagine or glutamine residue after the two glycine residues in the β -subunit signature sequence surrounding the 72 residue (Thomas *et al.*, 1995). There is also the fact that the asparagine residue methylated in phycobiliproteins is located in a similar sequence to that of the glutamine residues in the ribosomal protein methylated by the HemK methyltransferase, where the amide residue in both cases is preceded by a pair of glycine residues (Clarke, 2002). Research on γ -*N*-methylasparagine and δ -*N*-methylglutamine was performed by Klotz *et al.*, (1990). Both modified residues are similar in chemistry, and some methylation was detected by CpcM on glutamine residues. It seems probable that CpcM would methylate a glutamine residue if found in the same signature sequence as found in phycobiliproteins.

In conclusion for the CpcM experiments, we have deduced that CpcM is responsible for not only methylating CpcB and ApcB, but also ApcF. We demonstrated CpcM does not methylate the homologous α subunit in either phycocyanin or allophycocyanin. Through various assays we have concluded where along the path of phycobilisome assembly the asparagine methylation occurs. The methylation probably occurs after chromophorylation of the phycobiliproteins, but before formation of trimeric phycobiliproteins, but all forms of β -subunits

tested could be substrates for CpcM. We have also shown that CpcM is methylating Asn72 through the analysis of the *cpcM* mutant. In addition, we demonstrated that CpcM methylates β -PC in PC isolated from the *cpcM* mutant.

4.2 CpcS, CpcU, and CpcT Lyases

The second part of this research project demonstrated that *Synechocystis* sp. PCC 6803 CpcS, CpcU, and CpcT act as lyases to chromophorylate the β -subunit of phycocyanin. CpcT is a lyase that attaches phycocyanobilin to β -153 cysteine site of phycocyanin as demonstrated by absorbance, fluorescence, and zinc-enhanced fluorescence of SDS-PAGE. These results are consistent with the CpcT characterization from *Synechococcus* sp. PCC 7002, a similar species to that of *Synechocystis* sp. PCC 6803 (Shen *et al.*, 2006). Absorbance and fluorescence spectra of the CpcSU-dependent product show peaks where we would expect if bilin addition is taking place on the β -82 site of phycocyanin. These results are consistent with the CpcSU characterization in *Synechococcus* sp. PCC 7002 (Saunée, 2006). Therefore, it appears in these two cyanobacteria, both CpcS and CpcU are required to get bilin addition to β -82 PC.

Zhao *et al.* (2006) characterized the CpeS protein from *Anabaena* sp. PCC 7120 that is an ortholog to CpcS, called CpeS. They concluded that CpeS works alone to catalyze a site-specific attachment of phycocyanobilin to the β -84 (β -82, equivalent) cysteine in both CpcB and PecB (phycoerythrocyanin β subunit). Assays showed that CpeS does not require any other partner proteins to attach phycocyanobilin (Zhao *et al.*, 2006). This is in contrast to the results presented here that CpcS and CpcU are both required for bilin addition to β -82. One explanation for these differing results may be that CpeS acts as a homo-dimer while CpcSU has been shown to form a

1:1 stoichiometric complex in *Synechococcus* sp. PCC 7002 (N. Saunée, 2006), and this may be the case in *Synechocystis* sp. PCC 6803.

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 is part of a new class of bilin lyase found in *Synechococcus* sp. PCC 7002 (Shen et al., 2006). *cpcT* and *cpeT*-like genes are represented in all sequenced genomes of cyanobacteria, except *P. marinus* MED4 (which lacks phycobiliprotein). These genomes contain one copy of the *cpcT* or *cpeT*-like gene. Because of this sequence similarity, CpcT orthologs most probably function in attachment of the bilin at β -153, or the equivalent position (Shen et al., 2006). *cpcS* or *cpeS*-like genes and *cpcT* or *cpeT*-like genes can be found in all phycobiliprotein-containing cyanobacterial genomes and can be considered cyanobacterial signature genes.

Why are different lyases needed for the addition of PCB to the Cys-84 and Cys-82 position on α and β subunit since the spatial and stereochemical position of the bilins is the same? The lyases responsible for attachment to the α subunits, CpcE/F and PecE/F, act as heterodimers, can also act as bilin transferases (perform repairs), and in some cases isomerize the bilin (Fairchild et al., 1992; Fairchild and Glazer, 1994; Jung et al., 1995; Zhao et al., 2006). In contrast to the α -subunit lyases, the CpeS/CpcSU family attaches phycocyanobilin to β -82 and are not similar in sequence to CpcE/F or PecE/F. The CpcEF-type lyases appear to have more involvement in bilin removal/repair than the CpeS/CpcSU family does.

It is not clear why cyanobacteria have separate lyases for attachment on the α and β phycocyanin subunits. The locations of the attachment sites may be a reason for different lyases for the subunits. Bilins attached at the β -153 site are ‘peripheral bilins’ and are exposed because they do not have helices or other structures surrounding them and are at a location in the

biliprotein structure that bends and is exposed. 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 (Toole *et al.*, 1998). One reason that there are two different lyases for the two sites on the β phycocyanin subunit may have to do with the stereochemistry of the attachment sites. The PCB that attaches to the β -153 cysteine has an *S* stereochemistry on the chiral carbon at C3¹. The PCB that attaches to the β -82 cysteine has an *R* stereochemistry on the chiral carbon at C3¹ (Figure 10) (Schirmer *et al.*, 1987; Shen *et al.*, 2006; Zhao *et al.*, 2006). The PCB at β -153 has been shown to have *S* stereochemistry in all solved x-ray crystal structure phycobiliproteins (Duerring *et al.*, 1990; Duerring *et al.*, 1991; Ritter *et al.*, 1999). With this information Shen *et al.*, (2006), hypothesized that it is probable that CpcT and its paralogs may also be responsible for attachment of any possible substrate to the β -153 or its corresponding site (Shen *et al.*, 2006).

Future Work

Future experiments would be to achieve conclusive evidence of when CpcM methylation of PBP occurs during phycobilisome assembly. Bilin addition reactions using recombinant apo-CpcBA and PcyA using CpcSU and CpcT to fully chromophorylate the CpcB should be performed. This holo-CpcB should then be used in assays compared against apo-CpcB. These assays should give conclusive evidence as to when the CpcM methylation of β -72 occurs. Also, experiments to show that CpcM specifically methylates at β -72 should be performed. This can be achieved by narrowing down the recognition site for CpcM on β -subunits by making truncations. Or, one could subject the CpcB assay product to mass spectroscopy to confirm it is heavier at the peptide containing Asn72.

Futher lyase experiments would be to first achieve enough CpcSU-dependent product and CpcT-dependent product to perform tyrptic digestion and Reverse Phase-HPLC to determine precisely which cysteine bilin attachment sites these lyases are adding to. Next, we should determine the subunit structure of the CpcSU complex. More work also needs to be done to determine if cofactors can affect the lyase activity. Increasing the $MgCl_2$ concentration was shown to slightly increase the activity of CpcSU and CpcT in bilin addition reactions. Future work needs to determine if this or other metal ions can inhibit or help activate the lyases. Also, work on the *Synechocystis* sp. PCC 6803 allophycocyanin subunits using CpcS, CpcU, and CpcT to determine which lyases chromophorylate them could be performed. Allophycocyanin does not contain the β -153 bilin attachment site but does contain α -84 and β -82.

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