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Characterization of Enzymes Involved in Bilin Attachment to Allophycocyanin in the Cyanobacterium *Synechococcus* sp. PCC 7002

Shervonda Williams
University of New Orleans

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Characterization of Enzymes Involved in Bilin Attachment to Allophycocyanin in the
Cyanobacterium *Synechococcus* sp. PCC 7002

A Thesis

Submitted by 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

Shervonda Williams

B.S. University of New Orleans, 2002

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ABSTRACT

The goal of this research is to identify and characterize enzymes involved in bilin attachment to the phycobiliprotein allophycocyanin in the cyanobacterium *Synechococcus* sp. PCC 7002. Candidates for lyases responsible for attachment of phycocyanobilin to allophycocyanin are two *cpeS*-like genes termed *cpcS* and *cpcU*, and one *cpeT*-like gene termed *cpcT*. *In vitro* bilin attachment reactions were conducted in the presence of the recombinant substrate apo-allophycocyanin (HT-ApcAB). Size exclusion HPLC showed that CpcS and HT-CpcU form a 1:1 heterodimeric complex and that HT-ApcAB is present as a monomer ($\alpha\beta$). Absorbance and fluorescence spectroscopy illustrated that both CpcS and HT-CpcU were required to get holo-allophycocyanin with phycocyanobilin attached to the cysteine-81 residue. Absorbance of the product at 615 nm was consistent with holo-monomeric allophycocyanin. Experiments were performed with HT-ApcD ApcB and HT-ApcF ApcA, but size exclusion HPLC showed they were in aggregated form.

Keywords: Allophycocyanin, Bilins, Bilin lyase, Cyanobacteria, Phycobiliproteins, Phycobiliprotein biosynthesis, Phycocyanin, Phycocyanobilin.

INTRODUCTION

1.1 Phycobilisome origin:

Cyanobacteria are considered to be one of the oldest and largest bacterial groups on the earth and date back to 3.5 billion years. Cyanobacteria are able to perform oxygenic photosynthesis. This autotrophic strategy utilizes energy and electrons derived from the splitting of water, resulting in the release of oxygen. Evolution of the process of oxygenic photosynthesis resulted in the current oxygen rich atmosphere utilized by most organisms today. Chloroplasts from eukaryotic organisms such as red, green and cryptophan algae are derived from endosymbiotic events where a cyanobacterial ancestor was engulfed (Bengston, 1994). The evolutionary process included cyanobacterial genes migrating to the host nucleus during co-habitation (Hughes and Lamparter, 1999).

Photosynthesis is performed by capturing light using chlorophyll and transferring the energy to photosystem II within the thylakoid membrane of the organism (Glazer et al., 1983). In prokaryotic cyanobacteria and eukaryotic red algae, light harvesting is carried out by large water-soluble protein structures called phycobilisomes (PBS). PBS are found on the cytoplasmic surface of the thylakoid membrane and function to transfer their excitation energy to the membrane-intrinsic photosystem II complex (Figure 1) (Glazer 1985; Grossman et al., 2001). PBS consists of proteins termed phycobiliproteins which absorb light energy and linker proteins which are necessary for proper PBS assembly. Phycobiliproteins absorb light in the visible range between 450nm and 665nm which is where chlorophyll absorbs weakly. The absorbed excitation energy is transferred to the photosynthetic photosystems with high quantum efficiency (Zhao et al., 2006, Bald et al., 1996). PBSs comprise 40-50% of the total protein in a

cyanobacterial-photosynthetic cell when the organism is grown under low light conditions (Glazer 1989; Glazer 1985).

PBS of both cyanobacteria and red algae consists of two domains; the center core and the peripheral rods. The center core contains three allophycocyanin trimers which form 2 or 3 cylindrical complexes essentially held together by specific linker proteins that assist in energy transfer to the reaction centers (Figure 1) (Arciero, Bryant, and Glazer; 1988, Arciero et al., 1988a). The peripheral hexameric rods radiate from the core complexes and are composed of phycocyanin, in some organisms phycoerythrin or phycoerythrocyanin which may be present at the end of the rods (Grossman et al., 2001, Grossman et al., 1993). Linker polypeptides assist with assembly of phycobiliproteins, aid in stabilization of PBS complexes, and modify the absorption qualities of phycobiliproteins thereby facilitating unidirectional energy transfer from phycoerythrin (if present) to phycocyanin, to allophycocyanin, and finally to photosystem II (Glazer 1985). Thus, PBS function by absorbing light energy in the 500-650 nm range, converting it to 680 nm and transferring the energy to chlorophyll for photosynthesis with almost 100 percent efficiency (Anderson and Toole, 1998).

Phycobiliproteins are a family of water-soluble proteins that are similar to one another in amino acid sequence and three-dimensional structure (Glazer 1985; Grossman et al., 1993). They expand the range of light energy that can function in photosynthetic electron transfer with the aid of the spectral properties of the major phycobiliproteins (Anderson and Toole, 1998). There are four major classes of phycobiliproteins: allophycocyanin (APC $\lambda_{\text{max}} = 650\text{-}655\text{ nm}$); phycocyanins (PC, $\lambda_{\text{max}} = 615\text{-}640\text{ nm}$); phycoerythrins (PE, $\lambda_{\text{max}} = 565\text{-}575\text{ nm}$); and phycoerythrocyanin (PEC; $\lambda_{\text{max}} = 575\text{ nm}$) (Sidler, 1994; Glazer et al., 1992). Each

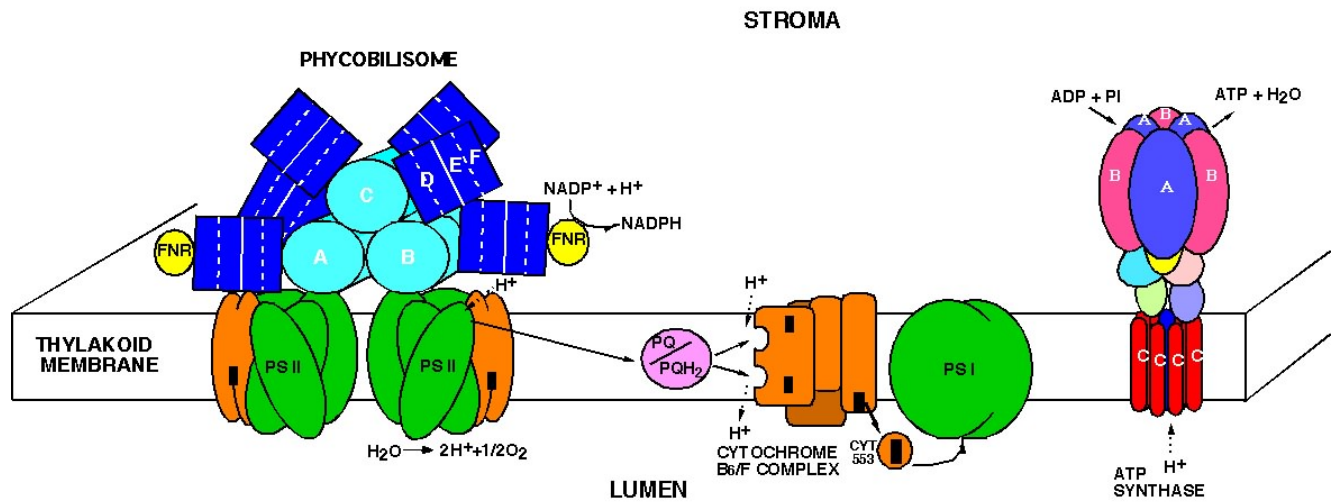


Figure 1: Illustration of Phycobilisome in conjunction with the photosystem complex found in the thylakoid membrane. The PBS consists of linker proteins and the phycobiliproteins phycocyanin and allophycocyanin. Phycocyanin is found within the radiating rods and is arranged in hexameric discs that assist in channeling excitation energy to the core. The core of the PBS consists of trimeric allophycocyanin which is involved in energy transfer to chlorophyll a. The phycobiliproteins are arranged in a manner that provides efficient energy transfer from the higher energy phycocyanin (PC- λ_{max} 620 nm) to lower energy allophycocyanin (AP- λ_{max} 650 nm) in the core. Figure taken from www.bmb.psu.edu

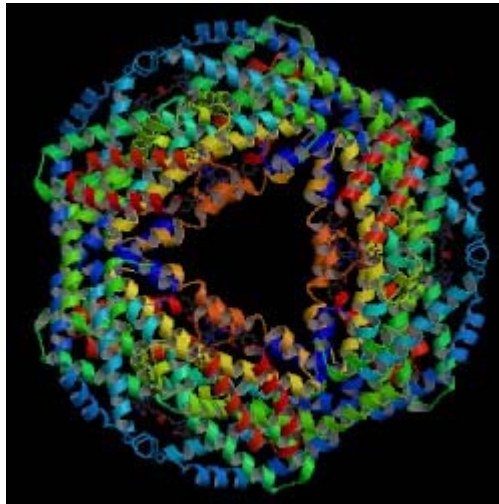
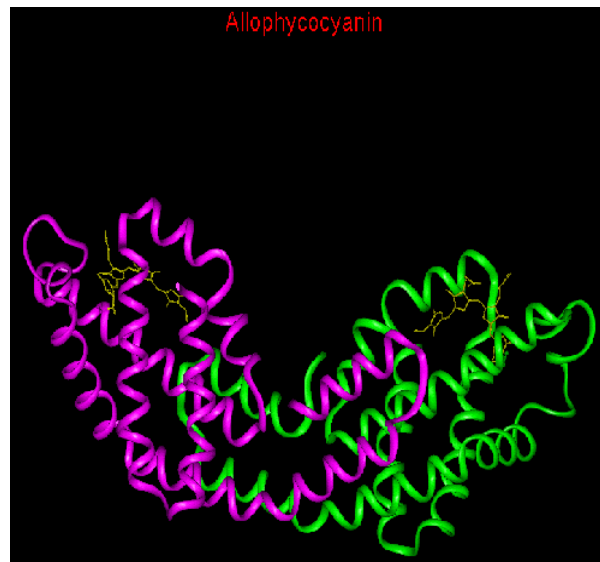
A**B**

Figure 2: Crystal structure of allophycocyanin. Panel A) Trimeric form of allophycocyanin. Panel B) Monomeric alpha and beta structure of allophycocyanin showing bilin chromophores in their stretched conformation attached at conserved cysteine residues. The green ribbons represent the alpha subunit and the purple ribbons represent the beta subunit. (www.rcsb.org-Protein data base)

phycobiliprotein consists of equimolar amounts of two polypeptides, alpha and beta, ranging in molecular weight from 15 to 22 kDaltons (Figure 2) (Glazer 1985; Arciero et al., 1988a, Arciero et al., 1988b). The alpha and beta subunits of phycobiliproteins are arranged in a stacked cylinder shape of trimers ($\alpha\beta$)₃ or hexmers ($\alpha\beta$)₆ (Swanson and Glazer, 1990; Arciero, Bryant, and Glazer, 1988) (Figure 3). The cylindrical shape allows for energy transfer through the central cavity from the radiating rods to the core (Figure 3, 4). Light energy is transferred from phycocyanin (620nm) to allophycocyanin (650nm) to photosystem II (680nm). (Sidler, 1994)

The trimers and hexamers are connected by linker proteins and have covalently attached prosthetic chromophores called bilins (Figure 4). Bilins or chromophores are heme-derived linear tetrapyrroles that attach to specific conserved cysteine residues via thioether linkages to the vinyl group on ring A on the bilin chromophore (Grossman et al., 2001; Frankenburg et al., 2001; Glazer 1989; Sidler, 1994) (Figure 5). Each alpha and beta subunit carries at least one and up to three bilin chromophores (Glazer 1989; Glazer 1985). The four bilins found in cyanobacteria vary in the position of specific conjugated double bonds allowing them to emit a unique range of color. When attached, each bilin possesses either an R or S stereochemistry at the C3' (Figure 5). The bilins are phycocyanobilin (PCB, blue), phycoerythrobilin (PEB, red), phycobiliviolin (PVB/PXB, purple), and phycourobilin (PUB, yellow) (Figure 6) (Grossman et al., 1993; Glazer 1989; Sidler, 1994).

1.2 Allophycocyanin subunits:

Cyanobacteria and red algae contain membrane bound peripheral antenna complexes that function to transfer energy to the photosystem complexes. These complexes are composed of 350-600 polypeptides and contain covalently attached bilin chromophores (Anderson and Toole,

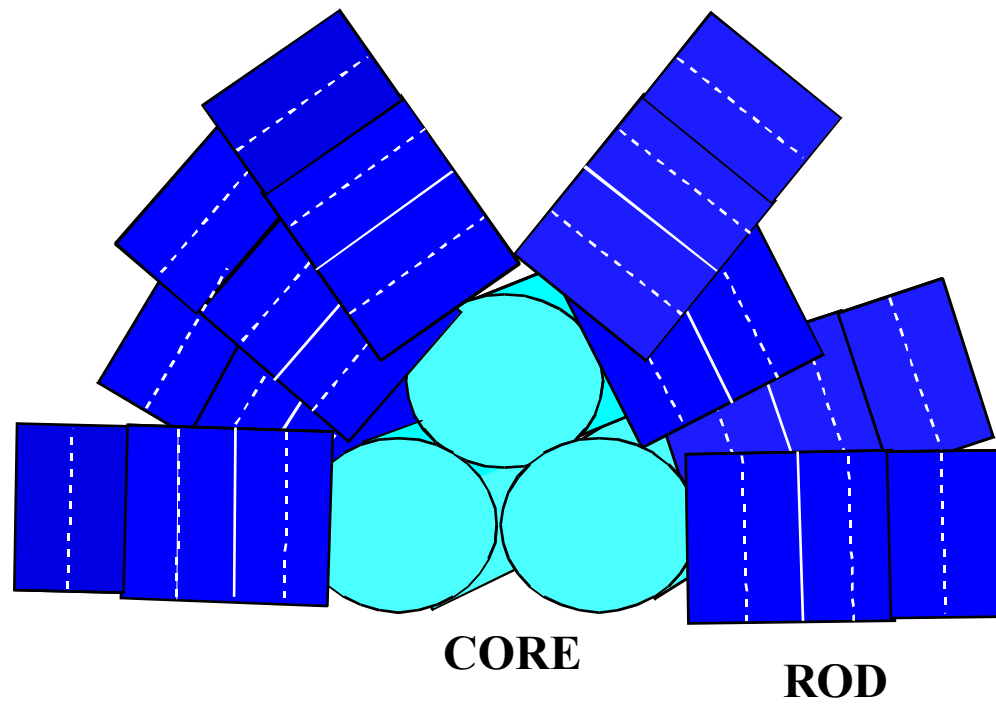
1998). Within the trimeric and hexameric aggregations of phycobiliproteins, linker polypeptides are present and function in holding the stacks of phycobiliprotein discs together and interact with bound chromophores to absorb energy at a specific wavelength (Holzwarth, 1991). PBS consists of both phycobiliproteins and linker polypeptides which aid in energy transfer or act as energy acceptors within the PBS (Capuano et al., 1991; Gindt et al., 1994). *Synechococcus* sp. PCC 7002 contains an *apcABC* operon that encodes for the alpha and beta subunits of allophycocyanin as well as a small rod core linker polypeptide involved in PBS assembly encoded by *apcC* (Maxson et al., 1989; Gindt et al., 1994). It was determined through inactivation of the *apcC* gene, that it was not replaced by another linker protein and that the *apcC* mutants contained incomplete PBS that were less stable and grew 25% slower than the wild type. These findings by Maxson et al determined that PBS core assembly and stability requires the presence of the ApcC protein (1989). Three allophycocyanin core substituents L_{CM} , α^{AP-B} , and β^{18} are present in two copies of each PBS. The L_{CM} and the α^{AP-B} , encoded by *apcE* and *apcD* respectively, are believed to be involved in binding of core trimers as well as transferring energy from the PBS core to chlorophyll *a* of the photosystems (Bald et al., 1996). β^{18} , encoded by *apcF*, appears to be involved in energy transfer to photosystem II (Gindt et al, 1994, Zhao et al, 2005). The gene that encodes for the high molecular weight anchor polypeptide, *apcE*, is expressed as a monocistronic unit separate from the *apcABC* operon. Table 1 shows the function and copy number of each allophycocyanin subunit found in *Synechococcus* sp. PCC 7002.

The L_{CM} is a large phycobiliprotein that tunnels light energy to the chlorophyll *a* of photosystem II and is involved in core assembly and the anchoring of the PBS to the photosystem II complex (Bald et al., 1996). The ApcE apoprotein or L_{CM} has a molecular weight

of 75-120 kDa, contains a chromophore binding site, and is believed to be the terminal emitter of the PBS (Bald et al., 1996; Zhao et al., 2005; Grossman et al., 1993).

The L_{CM} contains 1 major domain with about 40% homology to the phycobiliprotein subunits and the C-terminal which contains two to five repeated domains and is believed to play a role in disc assembly (Bald et al., 1996; Ajlani and Vernotte, 1998). Researchers identified a domain labeled the PB domain that is found within the N-terminus and contains a 50-65 amino acid insert labeled the PB-loop. They found that the PB domain is part of the core subunits and consists of this PB-loop that extends out from the PBS core toward the photosystem membrane. Ajlani and Vernotte hypothesized that the PB-loop is involved in the attachment of the PBS to the photosystem II or its membrane (1998) (Bald et al., 1996). In order to determine this, they removed the sequence consisting of 54 amino acids that encodes the PB-loop. They found that removal of this section yielded assembled PBS, a blue-green phenotypic color, and growth time comparable to the wild type. The researchers also inactivated the entire *apcE* gene that encodes the L_{CM} . Ajlani and Vernotte found an increase growth time of 36 hours compared to 7 hours found in the wild type, a greenish phenotypic color, and unassembled PBS. The researchers also found that the mutant had similar amounts of chlorophyll when compared to the wild type but contained less phycobiliprotein and more carotenoid than the wild type (Ajlani and Vernotte, 1998). Due to the lack of L_{CM} , the mutant exhibited a lower fluorescence due to the inability of the phycobiliproteins to attach to the photosystem II complex (Ajlani and Vernotte, 1998). The conclusion was that inactivation of the PB-loop did not have adverse affects on PBS assembly and that the mutant behaved like the wild type.

A



B

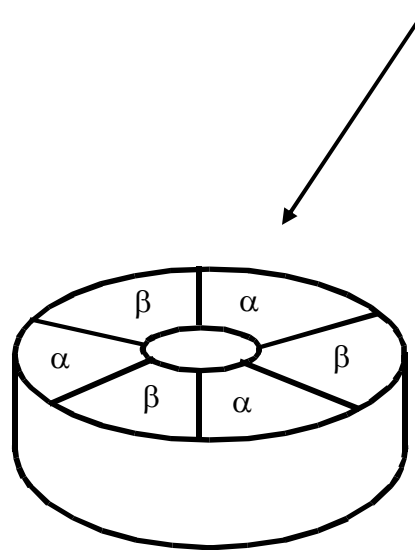


Figure 3: PBS structure. Panel A) Illustration of stacked phycobiliproteins. The phycobiliprotein phycocyanin is the major constituent of the rod. The core is composed of the phycobiliprotein allophycocyanin. Panel B) Diagram of alternating alpha and beta subunits in a hexameric conformation.

Table 1: **Allophycocyanin Subunits and Function.**

Gene	Protein Name	Function	Copies per PBS
apcA	ApcA	Encodes the alpha subunit of allophycocyanin.	32
apcB	ApcB	Encodes the beta subunit of allophycocyanin.	34
apcD	ApcD	Encodes the the alpha chain of allophycocyanin beta subunit.	2
apcE	ApcE	Encodes core-membrane Lcm. Anchors PBS to photosystems and transfers energy to chlorophylls.	2
apcF	ApcF	Encodes β 18 polypeptide. Transfers energy from allophycocyanin to linking protein apcE.	2

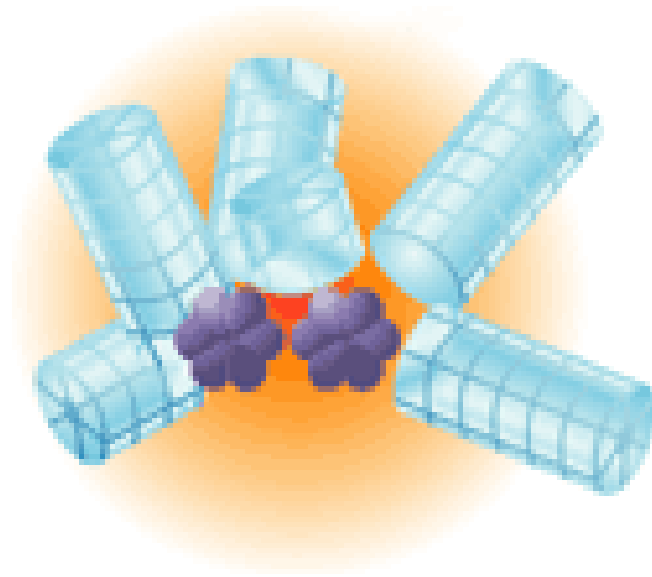


Figure 4: **Spacial diagram of phycobilisome.** Figure 4 illustrates the association of the phycobiliprotein subunits into stacked discs forming radiating rods. The core of the phycobilisome associates to form trimers and hexamers of alpha and beta subunits. www.htrf.com/products/labeling/ind_labeling_reagents/

Zhao et al determined that the anchor polypeptide was soluble in 4M urea and possessed a phycocyanobilin binding site at cysteine 195. They expressed the full length ApcE as well as the N-terminal portion of the polypeptide containing just the cysteine binding site 195 and incubated both in the presence of phycocyanobilin. After zinc stain analysis of the SDS-PAGE, Zhao et al found that ApcE had the ability to attach phycocyanobilin to itself without the aid of an enzyme (2005). The reaction products had an absorption max at 660 nm and a red shifted fluorescence emission at 675 nm. It was also determined that the N-terminal portion of ApcE, which is made up of 240 amino acids, was the only portion necessary and actively involved in attaching phycocyanobilin to itself (Gindt et al., 1994; Zhao et al., 2005).

The gene encoding the α^{AP-B} of the core, *apcD*, is not found near the *apcABC* operon in *Synechococcus* sp PCC 7002 and is transcribed as a monocistronic mRNA (Grossman et al, 1993). ApcD is found in two copies per PBS and its primary function is to act as a terminal energy transmitter and efficiently transfer this energy from chromophores attached to the PBS to the photosynthetic reaction centers attached to the membrane (Bald et al., 1996; Maxson et al., 1989; Zhao et al., 2005). Ashby and Mullineaux found that inactivation of *apcD* in *Synechocystis* sp. PCC 6803 caused the cells to have increased levels of phycobilisomes and photosystem I per cell but a decreased amount of phototsystem II (1999). They only found a small reduction in energy transfer from phycobilisome to the reaction centers (Ashby and Mullineaux, 1999).

Lastly, the β^{18} polypeptide, encoded by the monocistronic gene *apcF*, is also found in two copies per PBS and transfers energy from allophycocyanin to the linking protein (L_{CM}) and photosystem II (Gindt et al., 1994). They found that inactivation of both *apcF* and *apcD* in

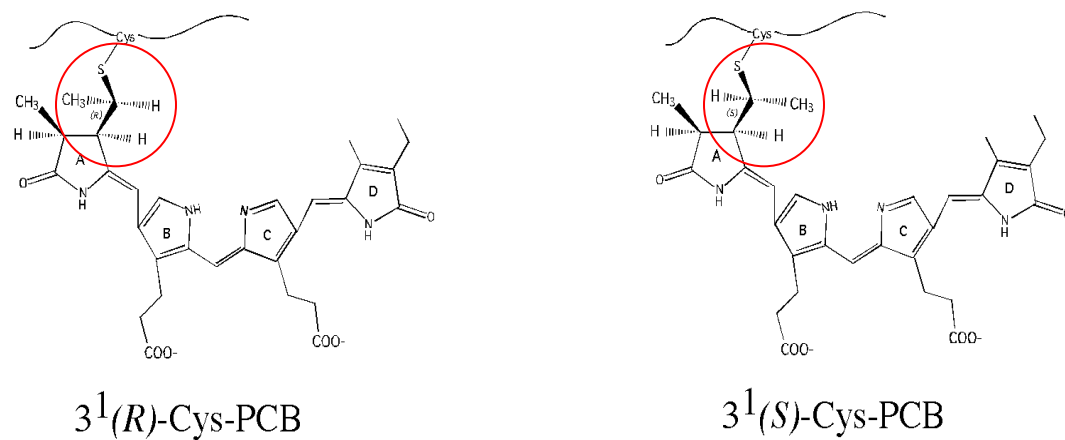
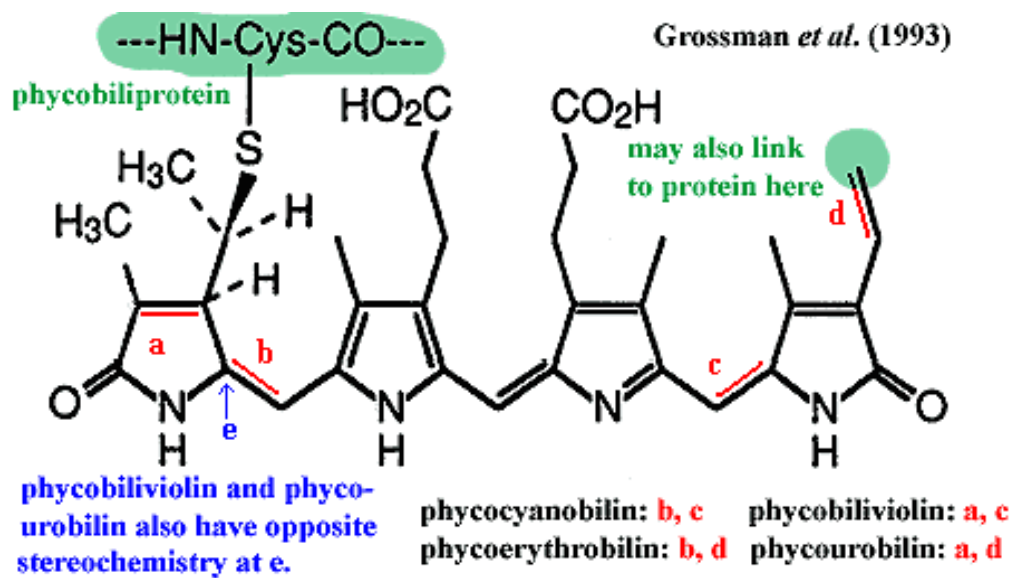


Figure 5: Bilin structures when attached to phycocyanin. Illustration of R and S stereochemistry of phycocyanobilin (Shen et al, 2006).

A



B

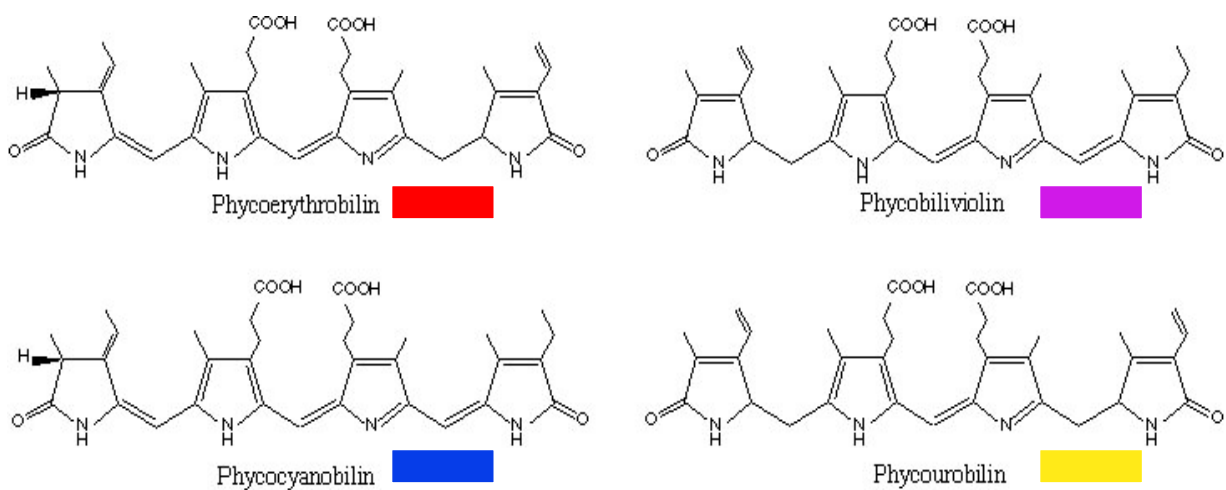


Figure 6: Bilin chromophores. Panel A) Diagram illustrates the C3' site on the bilin at the cysteine attachment site. Also shows the different variations of conjugated double bonds required to synthesize a particular bilin. Panel B) Illustration of bilins in stretched conformation which attach to conserved cysteine residues found on the phycobiliprotein. The bilins absorb at different wavelengths and emit various hues of the visible spectrum. Phycocyanobilin (PCB, blue), phycoerythrobilin (PEB, red), phycobiliviolin (PVB/PXB, purple), phycourobilin (PUB, yellow).

Synechocystis sp. PCC 6803 did not inhibit phycobilisome assembly but greatly reduced energy transfer from the phycobilisome to the reaction center and illustrated that the proteins may be involved in functional coupling of phycobilisomes to the reactions centers (Gindt et al., 1994, Ashby and Mullineaux, 1999). However, inactivation of *apcF* and *apcD* caused a decrease in cell growth, led to a lower level of photosystem I and photosystem II, and the mutant cells showed a decrease in the number of phycobilisomes per cell (Ashby and Mullineaux, 1999).

1.3 Biosynthetic pathway for bilins

The main precursor for all bilins in cyanobacteria is biliverdin IX-alpha (Frankenberg and Lagarias, 2003). To synthesize phycocyanobilin, the enzyme PcyA reduces biliverdin IX-alpha using electrons from monomeric phycocyanobilin ferredoxin oxidoreductase (PcyA). Phycocyanobilin is the bilin found attached to phycocyanin and allophycocyanin in *Synechococcus* sp. PCC 7002 (Glazer, 1989) (Figure 7).

1.4 Bilin Attachment of Phycobiliproteins

The alpha subunit of phycocyanin possesses a phycocyanobilin at conserved cysteine residue 84 while the beta subunit of phycocyanin contains a phycocyanobilin at cysteines 82 and cysteine-153 (Arciero, Bryant, Glazer 1988). There was speculation that bilins either attached spontaneously, like plant phytochromes, or utilized enzymes termed lyases.

Arciero et al. hypothesized that phycocyanobilin and phycoerythrobilin could spontaneously attach at the appropriate conserved cysteine residues on phycocyanin (1988a). They cloned and expressed the genes that encode the alpha and beta polypeptides of

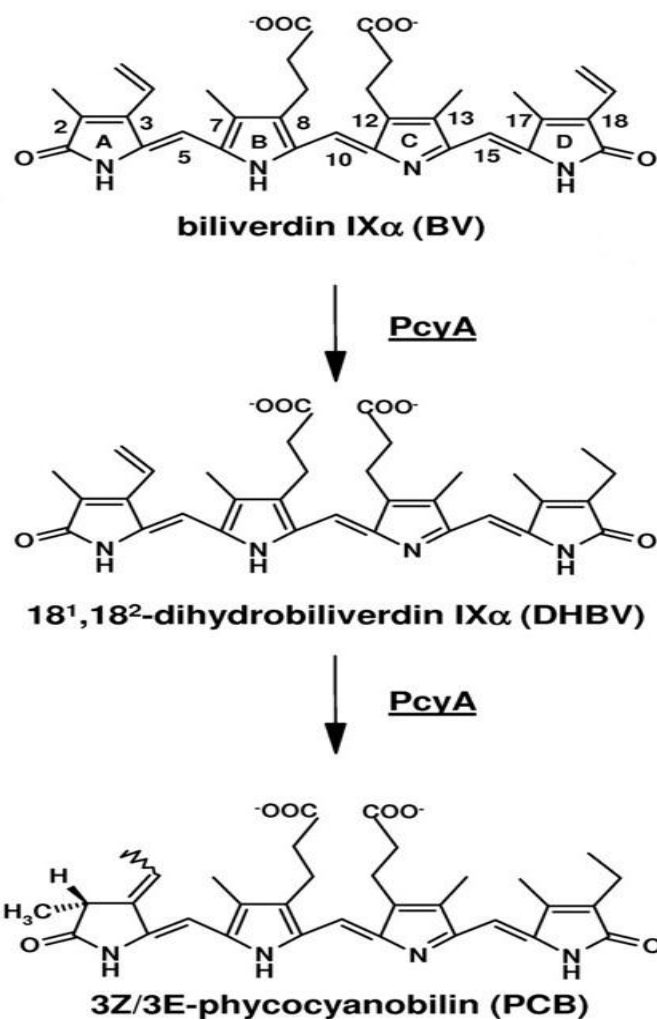
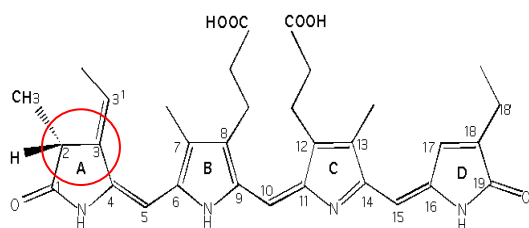


Figure 7: Biliverdin to phycocyanobilin. Biosynthetic pathway converting the bilin biliverdin to phycocyanobilin with the aid of the enzyme PcyA. (Frankenberg et al., 2001; Hagiwara *et al.*, 2006).

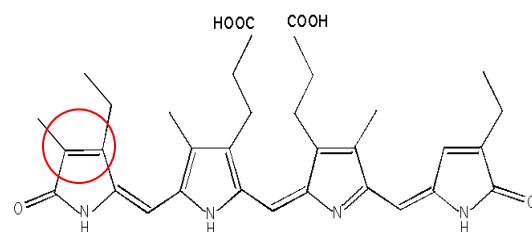
phycocyanin, denoted *cpcA* and *cpcB* respectively, from the organism *Synechococcus* sp. PCC 7002. The recombinant purified proteins were added to purified bilins to determine if spontaneous attachment of phycocyanobilin to the recombinant apo-phycocyanin would occur (Arciero et al 1988a). They observed that the product had a 24 nm red shift in absorption maximum compared to the native phycocyanin and found that a bilin was attached only to the alpha-84 and beta-82 sites, not to the beta-153 site (Arciero et al 1988a). The *in vitro* reaction of apo-phycocyanin with phycocyanobilin resulted in the formation of a bilin termed mesobiliverdin which possessed an extra double bond between carbon-2 and carbon-3 position of ring A when compared to phycocyanobilin (Figure 8) (Arciero et al., 1988a; Arciero et al., 1988b; Fairchild and Glazer, 1994a; Fairchild and Glazer, 1994b). Since the formation of mesobiliverdin occurred autocatalytically *in vitro*, it was suggested that *in vivo* bilin addition to the proper cysteine site required enzyme catalysis (Arciero et al 1988c).

1.5 Lyases for alpha phycocyanin:

The first phycobiliprotein bilin lyase to be identified was encoded by the *cpcE* and *cpcF* genes from *Synechococcus* sp. PCC 7002. These two genes, *cpcE* and *cpcF*, are encoded downstream of the genes *cpcA*, *cpcB*, *cpcC*, and *cpcD* which are PBS rod structural components (Zhou et al., 1992). Researchers found that insertional inactivation of either gene affected only phycocyanobilin addition to the alpha subunit in phycocyanin but not to any other phycocyanobilin addition site (Fairchild et al., 1992; Zhou et al., 1992). The mutants created by the inactivation of *cpcE* and *cpcF* produced less than 10% of the native phycocyanin, grew at half the rate of normal cells and were a different phenotypic color when compared to the wild



Phycocyanobilin



Mesobiliverdin

Figure 8: Phycocyanobilin vs. Mesobiliverdin. Illustration shows phycocyanobilin and mesobiliverdin in stretched conformation. The red circles indicate the different double bonds found between the two bilins at the C3 position.

type strains (Swanson et al, 1992, Zhou et al 1992). They also found that the phycocyanin alpha subunits of the mutants had no bilin attached, and that the phycocyanin beta subunits were chromophorylated normally. Zhou et al. suggested that *cpcE* and *cpcF* encode subunits of a lyase specifically involved in chromophorylation of the alpha subunit of phycocyanin (1992).

In 1992, Fairchild et al. used *in vitro* experiments to show that recombinant CpcE and CpcF produce a bilin lyase and act together as a heterodimer to add phycocyanobilin to the alpha-84 site of apo-alpha phycocyanin (Fairchild and Glazer, 1994a; Fairchild et al., 1992). Both *cpcE* and *cpcF* were expressed, purified, and tested in reactions containing apo-alpha-phycocyanin bound to resin. Reactions were set up using CpcE alone, CpcF alone, as well as the heterodimer CpcE/F to determine which protein(s) was actively involved in phycocyanobilin attachment. The two proteins together produced a product that was comparable to the absorption and fluorescence spectra of native alpha phycocyanin. The reaction product was placed on a Reverse Phase HPLC column to show that a single chromopeptide eluted in the same position as the bilin tripeptide derived from alpha phycocyanin and with the same absorbance (Fairchild et al., 1992).

The results confirmed that the *cpcE* and *cpcF* genes encode a lyase required for proper bilin addition of phycocyanobilin to cysteine-84 of the alpha subunit of phycocyanin. Fairchild also observed that CpcE and CpcF catalyzed the reverse reaction by removing phycocyanobilin from holo-alpha-phycocyanin and transferring it to resin-bound apo-alpha-phycocyanin.

In 2006, Zhou et al. focused on the amino acids and functional domains required for CpcE and CpcF to catalyze bilin addition properly. They used CpcE and CpcF of the cyanobacterium *Mastigocladus laminosus* PCC 7603. Functional domains were determined by site-directed mutagenesis and deletion mutations in conserved regions of the proteins. They

determined that if the N-terminus of CpcE was truncated by 41 amino acids and the C-terminus had a 39-amino acid deletion there was no activity (Zhou et al., 2006). This observation suggested that truncation prevented complex formation between CpcF and CpcE and interferes with proper refolding of CpcE. It was also observed that a truncation of 53 amino acids from the C-terminus and 20 amino acids from the N-terminus caused CpcF to become insoluble when expressed in *E. coli* cells, prevented proper folding in the presence of CpcE (Zhou et al., 2006). They also tested whether CpcE/CpcF was involved in transferring bilins to phycocyanin subunits. Phycocyanobilin was incubated with CpcE/CpcF and placed on an affinity column. They determined that phycocyanobilin could be bound by CpcE/CpcF and later transferred to an acceptor such as the alpha subunit of phycocyanin (Zhou et al., 2006). Zhou et al also found that when phycocyanobilin was incubated with apo-alpha phycocyanin and CpcE/CpcF simultaneously, there was formation of a non-enzymatic by-product. However, initial incubation of CpcE/CpcF with phycocyanobilin and later addition of the acceptor formed no by-product (Zhao et al., 2006).

In 1995, Jung et al determined that *Anabaena* sp. PCC 7120 contained a *pec* operon composed of 5 genes. The genes *pecB* and *pecA* encode for the beta and alpha subunits of phycoerytherin, *pecC* encodes a linker protein associated with the rods, and *pecE* and *pecF* had no known function. However, sequence analysis determined that *pecE* and *pecF* were highly similar to *cpcE* and *cpcF*. They created mutants deleting *pecE*, *pecF*, and portions of both. The resulting mutants grew much slower, possessed less phycocyanobilin, lacked holo-phycoerythrocyanin, and had a reduced amount of phycoerythrocyanin linker polypeptide present when compared to the wild type (Jung et al, 1995). They also observed that when portions of both *pecE* and *pecF* were absent, the alpha subunit of phycoerythrocyanin contained

phycocyanobilin as opposed to the normal phycobiliviolin. Based on these findings, it was suggested that PecE and PecF encode a lyase for the phycoerythrocyanin alpha subunit (Jung et al, 1995). Zhao et al. and Storf et al. further investigated the function of the lyase genes *pecE* and *pecF* found in *Mastigocladus laminosus*. They determined that PecE and PecF catalyzed the covalent addition of phycocyanobilin to the cysteine 84 residue on the alpha subunit of phycoerythrocyanin and the isomerization reaction of phycocyanobilin to 3¹-Cys-phycoviolobilin (2000, 2001).

1.6 Lyases for beta Phycocyanin:

A new class of phycobiliprotein lyase was identified in *Synechococcus* sp PCC 7002. The newly discovered genes termed *cpcS* and *cpcT* are present in at least one copy in every cyanobacterial genome. The genes were first found within the *cpeCDEST*R operon and were originally termed *cpeS* and *cpeT* in *Fremyella diplosiphon* (Cobley et al, 2002). In *Synechococcus* sp. PCC 7002, there are three open reading frames that show similarity to *cpeS*. The genes are now termed *cpcS*, *cpcU*, and *cpcV*. There is only one open reading frame similar to *cpeT* now called *cpcT*. Recently, results proved that only *cpcS*, *cpcU*, and *cpcT* produce functional enzymes required for bilin addition to the beta subunit (Saunée, 2006; Shen et al., 2006).

It was determined through BLAST analysis that orthologs of the *cpeT* gene were found in all cyanobacteria producing phycobiliproteins. A paralog of the *cpeT* gene was identified in *Synechococcus* sp. PCC 7002 and termed *cpcT* (Shen et al, 2006). The *cpcT* gene was cloned

into an expression vector and expressed in *E. coli* cells. The recombinant CpcT protein was used in bilin addition reactions with recombinant purified alpha and beta phycocyanin and phycocyanobilin. Shen et al. found that the reaction products containing the potential lyase CpcT and phycocyanin were very fluorescent with an emission maximum at 623nm and an absorbance maximum at 597nm compared to the control reactions which had a fluorescence emission at 657 nm and an absorbance maximum at 638nm (2006). Tryptic digestion and Reverse Phase HPLC analysis was performed on the CpcBA reaction products, and showed that the beta-phycocyanin attachment site was at the cysteine 153 residue (Figure 9) (Shen et al., 2006). Insertional inactivation of the *cpcT* gene in *Synechococcus* sp. PCC 7002 caused an almost two fold increase in the doubling time of the cells which were yellow in color when compared to the wild type. The fluorescence emission of the mutant cells was obtained at an excitation of 590 nm. Shen et al. found that the fluorescence emission peak at 645 nm was much lower than that of the wild type indicating decreased phycocyanin production (2006). A sucrose density gradient was conducted and determined that the PBS in the *cpcT* mutant were much smaller than the wild type and there was an aggregation of light-blue phycobiliproteins located in the upper most layer of the gradient signifying defects in PBS assembly (Shen et al., 2006)

Among the new family of lyases, CpcS and CpcU were found to be involved in bilin addition to the beta subunit of phycocyanin as well (Saunée 2006). Prior to performing bilin addition reactions, Saunée determined that recombinant CpcS and CpcU interacted with one another during Ni-NTA chromatography and formed a 1:1 stoichiometric complex during purification. Subsequent reactions utilizing recombinant alpha and beta phycocyanin and the bilin donor phycocyanobilin were performed. The reaction products containing the recombinant lyases CpcS and CpcU had an absorbance maximum at 621 nm and fluorescence maximum at

638 nm whereas the control reactions possessed an absorbance maximum at 629 nm and a fluorescence maximum at 647 nm (Saunée 2006). The absorbance and fluorescence reading were consistent with previous research conducted by Debreczeny et al (2003) which demonstrated that a fluorescence emission at 640 nm indicated chromophore addition to the cysteine-82 residue (Figure 9). Fluorescence emission was also conducted using SDS-PAGE-zinc stain analysis which confirmed that a bilin was being added to the beta subunit of phycocyanin (Figure 9) (Saunée, 2006).

In 2006, Zhao et al. focused on the *alr0617* open reading frame found in *Anabaena* sp. PCC 7120 which they found to be homologous to the *cpeS* genes found in *Fremyella diplosiphon*, *Gloeobacter violaceus* PCC 7421, and *Synechococcus* sp. WH8102. They expressed the *alr0671* gene (*cpcS*) with and without tags and incubated it in the presence of phycocyanobilin and the beta subunit of phycocyanin. The reaction products had an increased absorbance around 619 nm and a bright fluorescence at 643 nm. These results indicated that the beta subunit of phycocyanin contained phycocyanobilin at the cysteine-84 residue. The same experiment was conducted using the beta subunit of phycoerythrocyanin. The reaction products had an absorption max at 602 nm and a fluorescence emission at 629 nm which was similar to those found with a chromophore attached at the cysteine-84 residue on the beta subunit of phycoerythrocyanin from *Mastigocladus laminosus* (Zhao et al., 2006).

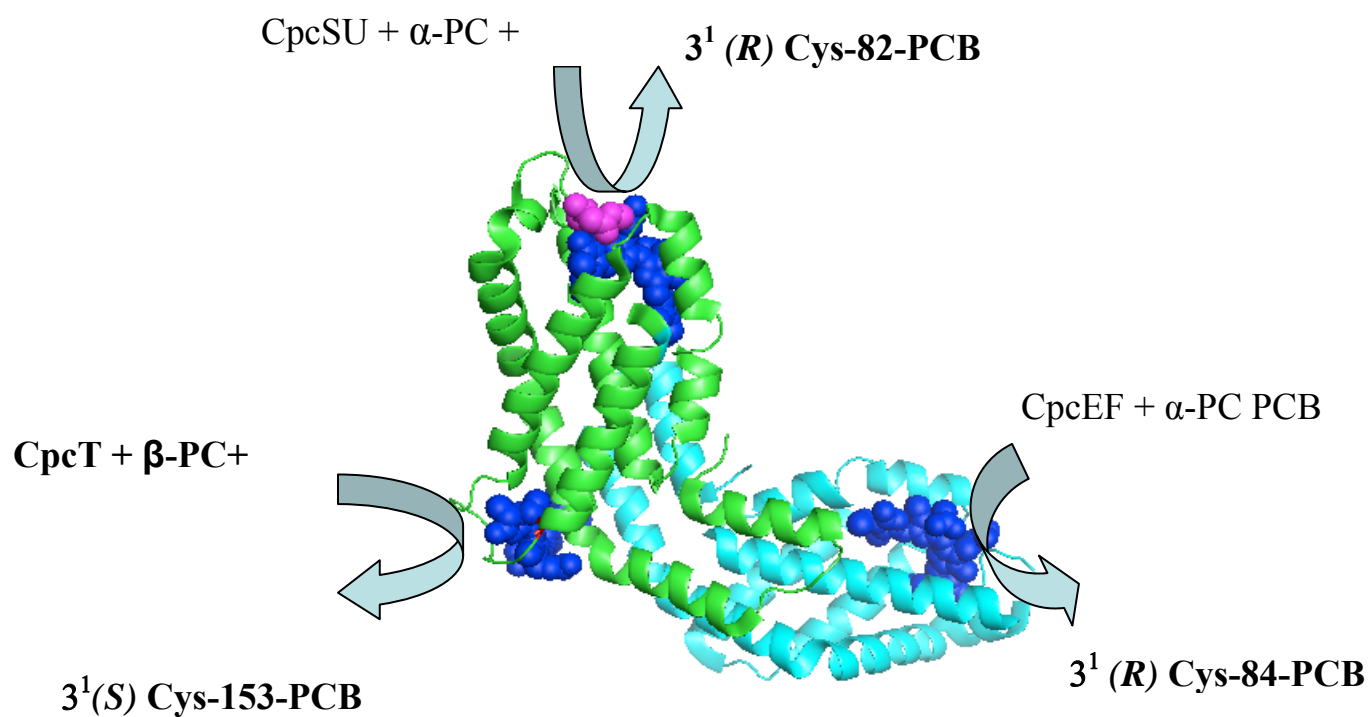


Figure 9: Lyases involved in bilin attachment of phycocyanin. Fairchild and Glazer determined that CpcE/F are required for phycocyanobilin attachment to the alpha subunit of phycocyanin at cysteine-84 (Fairchild and Glazer, 1994a, Fairchild et al, 1992). In 2006, Shen et al., and Saunée demonstrated that the new found lyases CpcSU and CpcT were involved in phycocyanobilin attachment to the beta subunits of phycocyanin at cysteines-82 and -153 respectively.

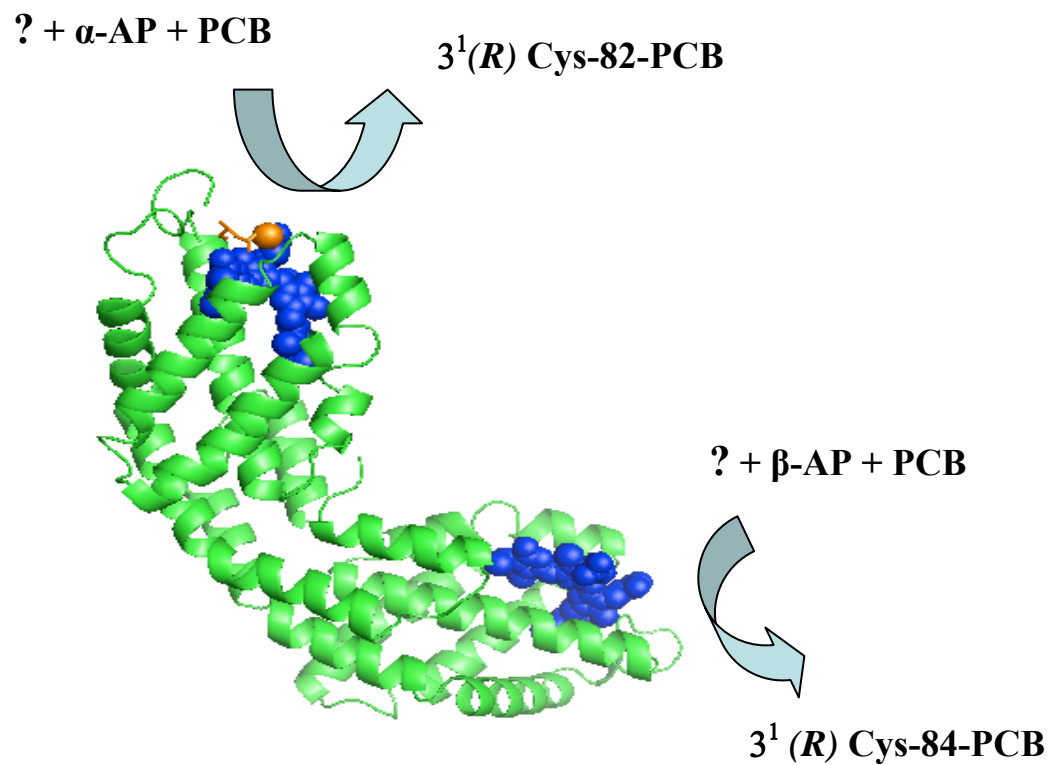


Figure 10: Unidentified lyases involved in bilin attachment of allophycocyanin. Illustration of allophycocyanin with unknown lyases involved in bilin attachment to the alpha and beta subunits at cysteine-81.

1.7 Purpose of Research

Most research on lyases has focused primarily on the binding properties and the lyases involved in bilin attachment to the alpha subunit of phycocyanin. Newly discovered lyases CpcS, CpcU, and CpcT, found in *Synechococcus* sp PCC 7002 have been shown to add bilins to the phycocyanin beta subunits at cysteine 82 and 153. However, little research has been conducted on the bilin addition sites of allophycocyanin and whether these specific lyases are involved in bilin addition to various subunits of allophycocyanin like ApcF and ApcD. The research presented here focuses on the enzymes involved in biosynthesis and whether the lyases CpcS, CpcU, and CpcT act as lyases in bilin addition to alpha subunit at cysteine 84, the beta subunit at cysteine 82, and other bilin addition sites found on the polypeptides ApcD and ApcF of allophycocyanin (Figure 10). The initial goal was to clone *apcA*, *apcB*, *apcD*, and *apcF* genes into plasmids. Once the correct construct was created and sequenced, the recombinant forms of the proteins were produced in *E. coli* cells and purified using chromatography techniques. The second goal of the research was to conduct bilin addition assays using apo-allophycocyanin to determine if CpcS, CpcT, CpcU, and CpcV act as lyases to attach bilins to allophycocyanin subunits. Lastly, complex formation among the subunits was probed using SEC HPLC.

MATERIALS AND METHODS

Materials:

Chemicals used in the laboratory were purchased from Fisher Chemical Co. (Houston, TX), and Sigma-Aldrich Chemical Co. (St. Louis, MO). Restriction enzymes used in cloning were purchased from New England Biolabs (Beverly, MA) and Promega Corp. (Madison, WI). PCR primers were produced and purchased from Qiagen (Alameda, CA) and Opedron Biotechnologies (Huntsville, AL). Gene Clean kits were purchased from Qbiogene (Carlsbad, CA). QIAquick PCR purification kits and QIAprep spin miniprep kits were purchased from Qiagen Incorporated (Valencia, CA). PCR Master Mix was purchased from Promega Corporation (Madison, WI). The cloning and expression cells DH5 α , TOP10, and BL21*DE3* were purchased from Invitrogen (Carlsbad, CA). BL21-T1^r cells were purchased from Sigma-Aldrich Incorporated (St. Louis MO). Plasmid midiprep kits and SDS-PAGE gels were purchased from Bio-Rad Laboratories (Hercules, CA). Big Dye kit with fluorescent ddNTPs for sequencing was purchased from Applied Biosystems (ABI) (Foster City, CA).

2.1 Construction of Recombinant Expression Plasmids

2.1.1 Polymerase Chain Reaction (PCR)

PCR reactions were conducted with 40 pmol of primer, 2-10ng of *Synechococcus* 7002 chromosomal DNA, and Platinum *Pfx* DNA polymerase using 1mM magnesium sulfate and 10nM dNTP mixture (Invitrogen Corporation Carlsbad, CA). The PCR reactions were performed in the W.M.Keck Foundation Conservation and Molecular Genetics Lab using a Thermocycler (i-Cycler, Bio-Rad). The amplification program was as follows: denaturation for 4 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 50°C, 1 minute at 68°C with a final extension for 7 minutes at 68°C. The QIA quick PCR purification kit was used to remove any unincorporated dNTPs and primers from the finished reaction. As per the manufacturer instructions, the PCR product was absorbed onto a silica membrane, centrifuged, and the final PCR product was eluted in water or low salt buffer.

2.1.2 Agarose Gel/DNA Separation

Purified PCR products, digestion and restriction reactions were separated on 0.8% agarose gels with TAE buffer (1X). Agarose gels were stained in 10µg/ml ethidium bromide solution for 10 minutes and placed under ultraviolet light. When necessary, the DNA bands were excised from the stained gel and purified using the GeneClean kit as per manufacturers instructions provided (Qbiogene Carlsbad, CA).

2.1.3 Creation of *apcA*, *apcB*, *apcAB*, *apcBD*, and *apcFA*

The *apcA* gene was amplified by polymerase chain reaction (PCR) using *Synechococcus* sp. PCC 7002 chromosomal DNA and primers 7002 *apcA*5' and 7002 *apcA*3' (Table 2). The amplified PCR product was purified to remove remaining and unincorporated dNTP's. The 0.5-kb purified fragments as well as the 5.7-kb pET100 vector were combined in a ligation reaction following manufacturer's instructions (Invitrogen, Carlsbad, CA.). The ligation was transformed into TOP10 competent cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA.). The transformants were plated on ampicillin LB agar plates and incubated overnight at 37°C. Individual colonies were selected and 2ml cultures started overnight. Plasmids were purified from cultures using Qiagen miniprep kits to identify correct constructs (Qiagen, Valencia, CA.) (Figure 11). The plasmids were cut with restriction enzymes *NdeI* and *EcoRI* and run on a 0.8% agarose gel for 1 hour at 100 volts.

The *apcB* gene was amplified by polymerase chain reaction (PCR) using *Synechococcus* sp. PCC 7002 chromosomal DNA and primers 7002 *apcB*5' and 7002 *apcB*3' (Table 2). The amplified PCR product was purified to remove remaining and unincorporated dNTP's. The 0.5-kb purified fragments as well as the 5.7-kb pET100 vector were combined in a ligation reaction as described (Carlsbad, CA.). The ligation was transformed into TOP10 competent cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA.). The transformants were plated on ampicillin LB agar plates and incubated overnight at 37°C. Individual colonies were selected and 2ml cultures started overnight. Plasmids were purified from cultures using minipreps to identify correct constructs (Qiagen, Valencia, CA.) (Figure 12). The plasmids were cut with the same restriction enzymes as stated above and run on an agarose gel.

Both the *apcA* and *apcB* genes were amplified by polymerase chain reaction (PCR) using *Synechococcus* sp. PCC 7002 chromosomal DNA and primers 7002 apcA5' and 7002 apcB3', respectively (Table 2). The amplified PCR product was purified to remove remaining and unincorporated dNTP's. The 1.1-kb purified fragments as well as the 5.7-kb pET100 vector were combined as described (Carlsbad, CA.). The ligation was transformed into TOP10 competent cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA.). The transformants were plated on ampicillin LB agar plates and incubated overnight at 37°C. 2ml overnight cultures were started to identify correct constructs (Qiagen, Valencia, CA.) (Figure 13). The plasmids were cut with restriction enzymes and analyzed on an agarose gel.

The *apcD* gene was amplified by PCR using *Synechococcus* sp. PCC 7002 chromosomal DNA and the primers 7002 apcD5' and 7002 ApcD3' (Table 2). The 0.6-kb purified gene was combined with the 5.7-kb histidine tagged pET100 vector. The ligation was transformed into TOP 10 competent cells. The transformants were plated on Ampicillin LB agar plates and incubated at 37°C overnight. The *apcB* genes were amplified by PCR with primers apcB5'-SacI and apcB3'-SacI. Both the ApcD-pET100 construct and the ApcB construct were digested with SacI and ligated together. The transformants were screened by digesting the plasmids with *HindIII* to check for orientation (Figure 14). The ligation was transformed into TOP10 competent cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA.). The transformants were plated on an ampicillin LB agar plates and incubated overnight at 37°C. Individual colonies were selected and 2ml cultures started overnight. Plasmids were purified from cultures using Qiagen miniprep kits to identify correct constructs (Qiagen, Valencia, CA.) (Harris and Schluchter).

The *apcF* genes were also amplified by PCR using *Synechococcus* sp. PCC 7002 chromosomal DNA and primers 7002 *apcF*5', and 7002 *apcF*3' (Table 2). The 0.6-kb purified gene was combined with the 5.7-kb histidine tagged pET100 vector. The ligation was transformed into TOP 10 competent cells. The transformants were plated on Ampicillin LB agar plates and incubated at 37°C overnight. The *apcA* gene was amplified by PCR with primers *apcA*5'-*SacI* and *apcA*3'-*SacI*. Both *apcF*-pET100 and the *ApcA* gene were digested with *SacI* and ligated together. The transformants were screened by digesting the plasmids with *HindIII* to check for orientation (Figure 15). The ligation was transformed into TOP10 competent cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA.). The transformants were plated on ampicillin LB agar plates and incubated overnight at 37°C. Individual colonies were selected, 2ml overnight cultures were started, and correct constructs identified (Turner and Schluchter).

2.1.4 Ligations

A Champion pET100 Directional TOPO expression cloning vector kit™ from Invitrogen Corporation (Carlsbad, CA) was used as per the manufacturer's instructions. The pET100 vector has an ampicillin resistance marker as well as a T7 promoter that controls gene expressions of exogenously added genes in *E. coli*. The T7 promoter is derived from bacteriophage T7 and, in addition contains a *lac* operator sequence. The T7 RNA polymerase is provided by BL21 (DE3) Star™ *E. coli* cells. When large amounts of T7 RNA polymerase are synthesized it binds to the T7 promoter and expresses the gene of interest. The pET100 vector also contains a sequence encoding 17 amino acids that includes six encoded histidines which aids in the purification of a

fusion protein using metal affinity chromatography (nickel-NTA resin). The ligations consisted of 5-10 ng of PCR product, 1µl of pET100 vector, 1µl of salt solution, and ddH₂O. In the TOPO[®] expression cloning vector kit, a four base pair overhang (CACC) is added to the forward primer and cloned into the PCR product. The vector also contains a four base pair overhang (GTGG) which anneals to the 5' end of the PCR product and aids in stabilizing the PCR product in the correct proper orientation. Ligations were incubated for 5 minutes at room temperature.

2.1.5 Transformations

Ligation reactions were transformed into One Shot TOP10 (Invitrogen, Carlsbad, CA) chemically competent *E. coli* cells using the guidelines provided. The transformation reaction called for 2-5 µl of ligation incubated with 50 µl of cells. The cell mixture was incubated on ice for 5 to 30 minutes, heat shocked for 30 seconds at 42°C, reintroduced to ice for 2 minutes then 300 µl of S.O.C (0.5% Bacto yeast extract (w/v), 2% Bacto-tryptone (w/v), 10mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 1mM NaOH, 20mM glucose) was added for cell recovery. The cell mixture was placed in a 37°C water bath for 1 hour without shaking prior to plating on Luria-Bertani (LB) (1% NaCl (w/v), 1% Bacto-tryptone (w/v), 0.5% Bacto yeast extract) plates containing ampicillin (100µg/ml) and incubated overnight at 37°C.

2.1.6 *cpcS*, *cpcU*, *cpcV*, *cpcT*

The *cpcS*, *cpcT*, and *cpcV* genes were cloned into the pAED4 vector as described (Saunée, 2006). These genes are not fused to a tag and are expressed from the T7 promoter. The *cpcU* and *cpcV* genes were cloned into pBS150v as described (Saunée, 2006). This vector

Table 2: Primers used for PCR amplification with Restriction enzyme sites underlined

Primer	Sequence 5' to 3'	Use
7002 apcA 5'	<u>CACCATGAGTATTGTCACGAAATCCATCG</u>	Amplification of apcA
7003 apcA 3'	<u>AGAATTCTGATTACTCCATTGCTCCGATGAC</u>	Amplification of apcA
7004 apcA 5'-Sac	<u>ATTTGAGCTCATATCTAGAGGAGGAATC</u>	Amplification of apcA
7005 apcA 3'-Sac	<u>AACGGAGCTCCAGAGGGTAAAAACCAC</u>	Amplification of apcA
7004 apcB 5'	<u>CACCATGCAAGACGCAATTACTTCTGTA</u>	Amplification of apcB
7005 apcB 3'	<u>CGAATTCTTAGCTTAAGCCAGAGCAGATG</u>	Amplification of apcB
7006 apcB 5'-Sac	<u>TTAAGAGCTCCTAAGAGAAGGAGTTATAACAATGCAAGA</u>	Amplification of apcB
7007 apcB 3'-Sac	<u>GATAGAGCTCGTAAAAGCTTATAAAGAGCTAGACAG</u>	Amplification of apcB
7006 apcD 5'	<u>CACCATGAGCGTCGTTAGTCAAGTTATC</u>	Amplification of apcD
7007 apcD 3'	<u>AACGATATCCTAGGACATTGCTTGGGTAATGAAG</u>	Amplification of apcD
7008 apcF 5'	<u>CACCATGCCGGACGCTGTTACAAGTC</u>	Amplification of apcF
7009 apcF 3'	<u>TCAGATATCTTAGAGATCCACTTCGCTCAGTTC</u>	Amplification of apcF

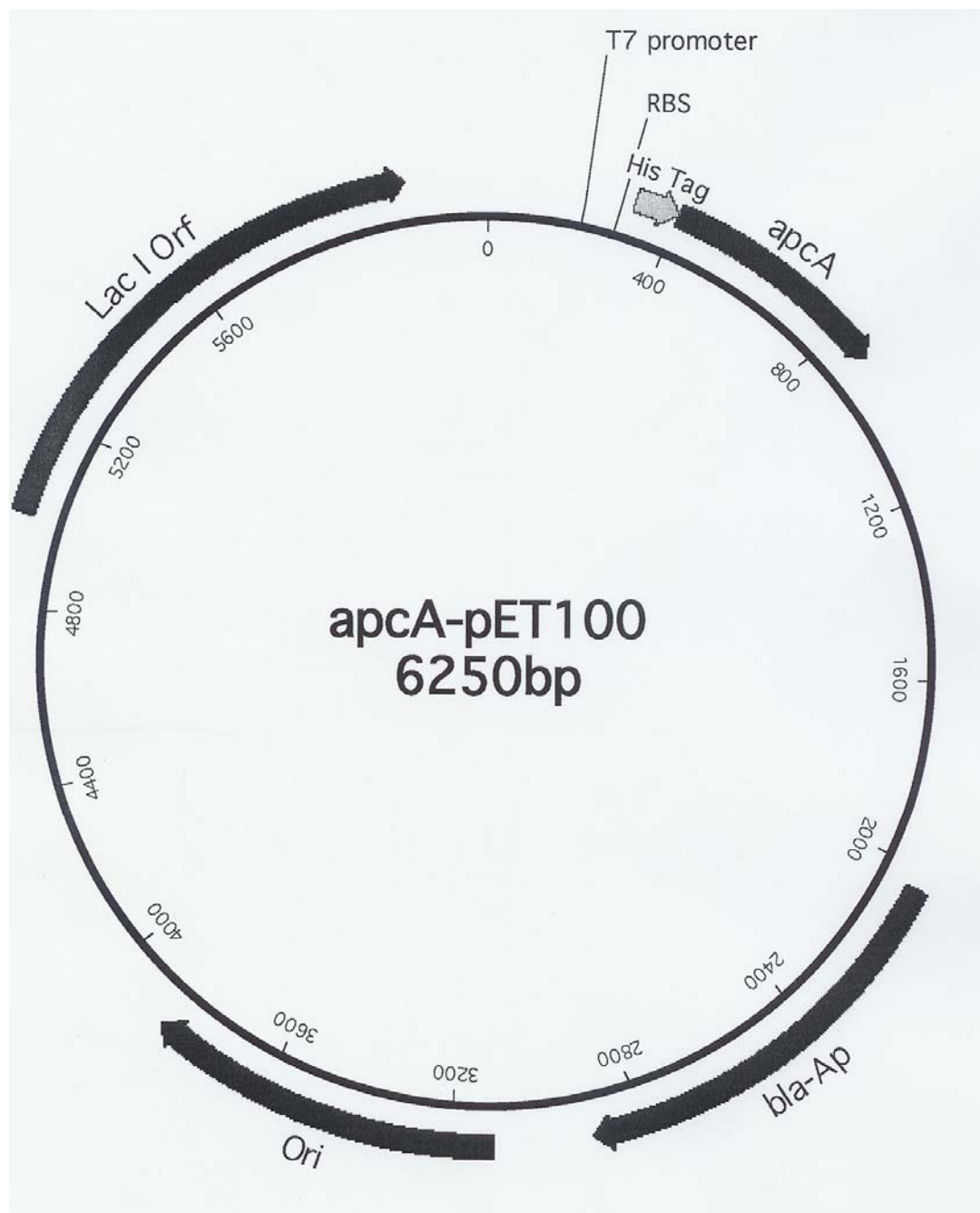


Figure 11: Plasmid map of clone *apcA*/pET100

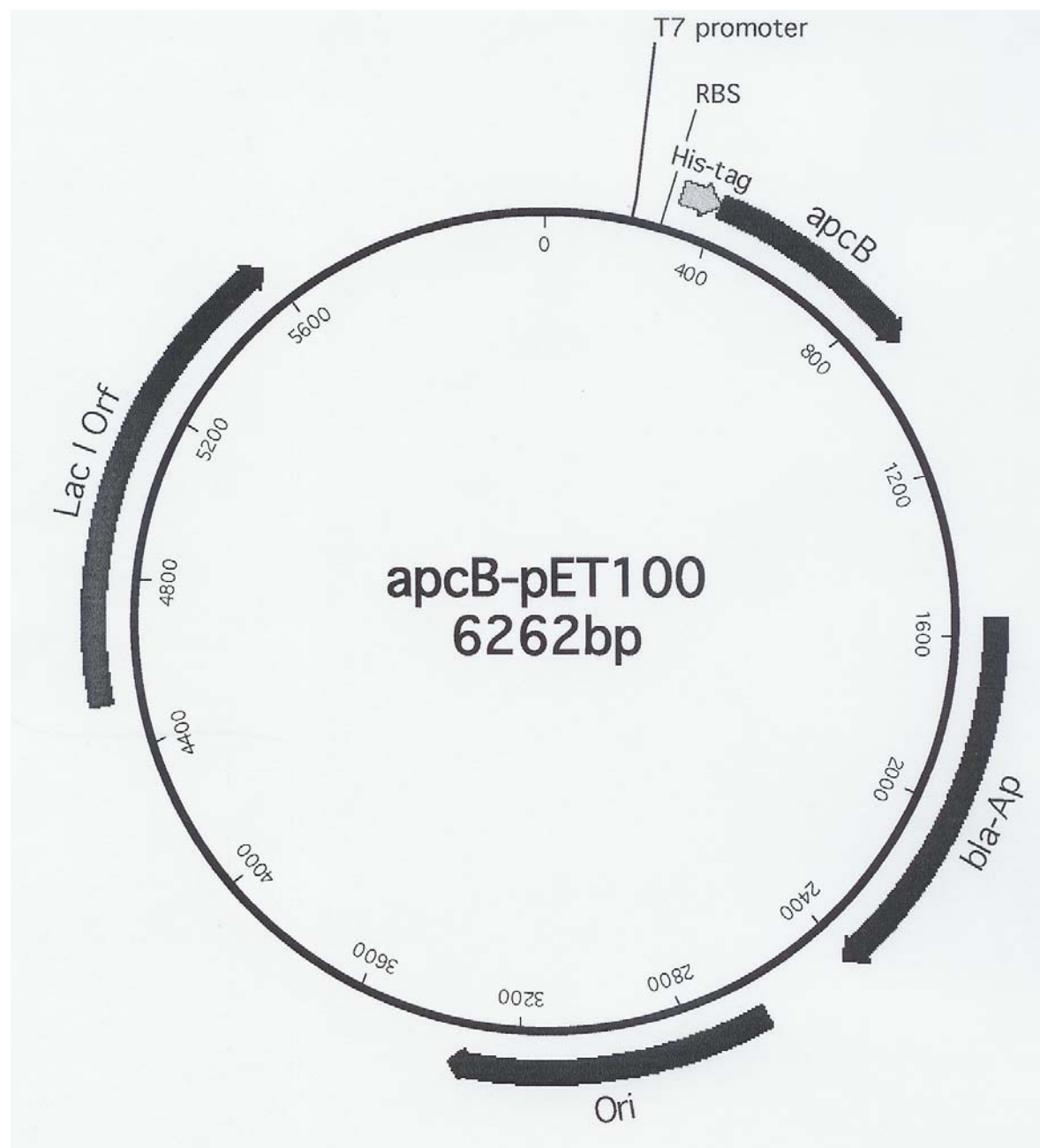


Figure 12: Plasmid map of clone *apcB/pET100*

fuses the genes to a histidine tag at the N-terminus and uses the pTrc promoter to control expression.

2.1.7 Sequencing

All clones were sequenced at the W. M. Keck Conservation and Molecular Genetics lab to confirm the lack of mutations. The sequencing reactions contained 2 µl of template (between 300-500ng), 1 µl of primer (0.8 pmol/µl) and 2 µl of Big Dye from ABI following kit directions. To remove any unincorporated dNTPs, the sequencing reaction was placed on Sephadex G-50 spin column. The Sephadex G-50 was removed from the refrigerator and allowed to equilibrate at room temperature for 15 minutes. 650µl of slurry was removed and placed in a sterilized spin column. The column containing the Sephadex G-50 slurry was centrifuged for 3 minutes at 14,000 x g. The column was placed into a new 1.5 ml eppendorf tube and the sequencing reaction was aliquoted onto the slurry and the tube was centrifuged again for 3 minutes at 14,000 x g. The purified sample was dried in a speed vac for 8 minutes. 20 ul of hi di (formamide) was added and the solution then loaded onto the ABI PRIZM 3100 Genetic Analyzer. The chromatogram generated by the sequencer was analyzed by the Macvector computer program (Accelrys, San Diego, Ca.) to determine if the construct was correct and to make sure there were not mutations.

2.2 Recombinant Protein Expression Analysis

2.2.1 SDS-PAGE Analysis

A 15% SDS-PAGE Tris-HCl was loaded with samples and run 12 minutes at 100 volts followed by 45 minutes at 200 volts (Laemilli, 1970). The completed gel was placed into Coomassie blue stain (10% methanol, 10% acetic acid) overnight and allowed to de-stain in 10% methanol combined with 10% acetic acid. For bilin-peptide visualization, gels were first incubated in 100mM ZnSO₄ for 10 minutes. Afterwards, bilins were visualized by fluorescence with excitation at 560nm the FX scanner.

2.2.2 Recombinant Production and Purification

The *apcA*/pET100, *apcB*/pET100, *apcAB*/pET100, *apcDB*/pET100, *apcFA*/pET100, or pAED4 cloning vectors were transformed individually into BL21 *DE3* and BL21 cells. A 50 ml overnight culture containing LB and 100 µg/ul of ampicillin was placed into a 37°C shaking incubator. The 50 ml culture was added to 1 liter of LB antibiotic and allowed to shake for 4 hours at 37°C. To induce T7 RNA polymerase production, 0.5 mM IPTG (isopropyl-β-D-thiogalactoside) was added to the 1 liter culture and incubation was continued.

The 1 liter culture was removed from the shaking incubator and cells were harvested by centrifugation for 20 minutes at 10,000 rpm (14,000 x g) and frozen at -20°C until later use.

2.2.3 Purification of HT-ApcA, HT-ApcB, HT-CpcU, HT-ApcAB, HT-ApcBD, and HT ApcFA

Proteins containing a Histidine tag were purified by metal affinity chromatography using Ni-NTA resin (Qiagen, Valencia, CA.). The frozen *E. coli* cell pellet was resuspended in Buffer 0 (20mM Tris-HCl, pH 8.0, 100 mM Na/KCl), homogenized, and passed through a French Pressure Cell Press at 20,000 psi 3 times. The whole cell extract was centrifuged and clarified by centrifugation at 10,000 rpm (14,000 x *g*) in a Sorvall® GSA rotor for 30 minutes. To prepare the resin, 10 ml of Ni-NTA resin was washed with 15ml of Buffer 0 and centrifuged for 8 minutes at 5,000x *g*. The supernatant was added to the resin and allowed to interact on ice for 30 minutes with occasional shaking. The supernatant along with the resin were added to a Glass Econo-column® (2.5 cm x 10 cm; BioRad, Richmond, CA) and allowed to filter through 2 times quickly and 1 time slowly. To move any unwanted contaminants, the agarose was washed with 50 ml of Buffer A1 (20mM Tris-HCl, pH 8.0, 100 mM Na/KCl, 20 mM imidazole, 5% glycerol), 0.5 M Buffer B (20 mM Tris-HCl, pH 8.0, 0.5 M Na/KCl), and Buffer A2 (20 mM Tris-HCl, pH 8.0, 100 mM Na/KCl, 30 mM imidazole). The histidine-tagged proteins were eluted from the column by adding 10 ml aliquots of Buffer C (20mM Tris-HCl, pH 8.0, 100 mM Na/KCl, 200 mM imidazole). Next, proteins were dialyzed against two changes of Buffer O containing 10 mM (1st change) and 1 mM (2nd change) 2-mercaptoethanol. After 8 hours, the dialyzed protein was removed, concentrated using Amicon YM100, and stored in -20°C in 2 ml aliquots.

2.2.4 Purification of CpcS, CpcT, CpcV,

Recombinant CpcS and CpcT were purified as described (Saunée, 2006). HT-CpcV was purified by Ni-NTA as described (Saunée, 2006). CpcS, CpcT, and HT-CpcV were transformed into BL21 cells and incubated overnight in a 30°C shaking incubator. Cells containing the CpcS expression plasmid were shaken for 8 hours with 4 hours of IPTG induction, whereas cultures expressing CpcV were incubated for 10 hours with no IPTG induction and the resulting protein purified using DEAE anion exchange chromatography.

All fractions were collected from the DEAE column analyzed by SDS-PAGE to determine concentration and purity (Data not shown). Those containing the most recombinant purified protein were pooled together and further concentrated. Recombinant CpcT underwent the same conditions as that of CpcS and was concentrated for later use (Data not shown).

2.3 Bilin Addition Reactions

2.3.1 Phycocyanobilin Addition Reactions

In vitro bilin addition reactions were conducted with phycocyanobilin extracted from *Spirulina sp.* (as described by Arciero et al., 1988a). The phycocyanobilin reactions contained 500µl-1 ml of HT-ApcA (0.73mg/ml), HT-ApcB (0.68mg/ml), HT-ApcAB (1.25mg/ml), HT-ApcBD (0.97mg/ml), or HT-ApcFA (0.82mg/ml). Reactions were set up that included various combinations of: 50-100 µl of *E.coli* whole cell extract containing pAED4 cloning vector as a control, 100 µl of purified or whole cell CpcS (1mg/ml), 250 µl of HT-CpcU (1mg/ml), 50µl of

purified CpcT (1mg/ml), and 100 μ l of purified HT-CpcV (1mg/ml)(as described in Saunée, 2006). 3 μ M of purified and concentrated phycocyanobilin was added from a 2mM stock in DMSO to each reaction and placed in a 30°C water bath for 1 hour in the dark. A second aliquot of phycocyanobilin (3 μ M) was added, and the reactions were incubated for an additional 1.5 hours in the dark at 30°C. Upon completion the reactions were centrifuged for 10 minutes at 15,000 rpm (26,000 $\times g$), and the fluorescence and absorbance spectra were taken. A 20 μ l aliquot of each reaction was separated by SDS-PAGE for zinc enhanced bilin fluorescence analysis followed by Coomassie staining protein.

2.3.2 Fluorescence and Absorbance Spectra

The fluorescence emission spectra were gathered on LS 55 Luminescence Spectrometer (Perkin Elmer, Shelton, CT). The Fluorometer has a xenon discharge lamp (7.3 W average power at 50 Hz) as a light source. The slits were set at 3 nm and the excitation wavelength set at 590nm. The fluorescence emission spectra were collected between 600-750 nm.

Absorbance spectra were acquired using a dual-beam Lambda 35 UV-Vis spectrophotometer (Perkin Elmer, Shelton, CT.). A blank 1 ml of Buffer 0 was used and the spectra were acquired from 450-750nm.

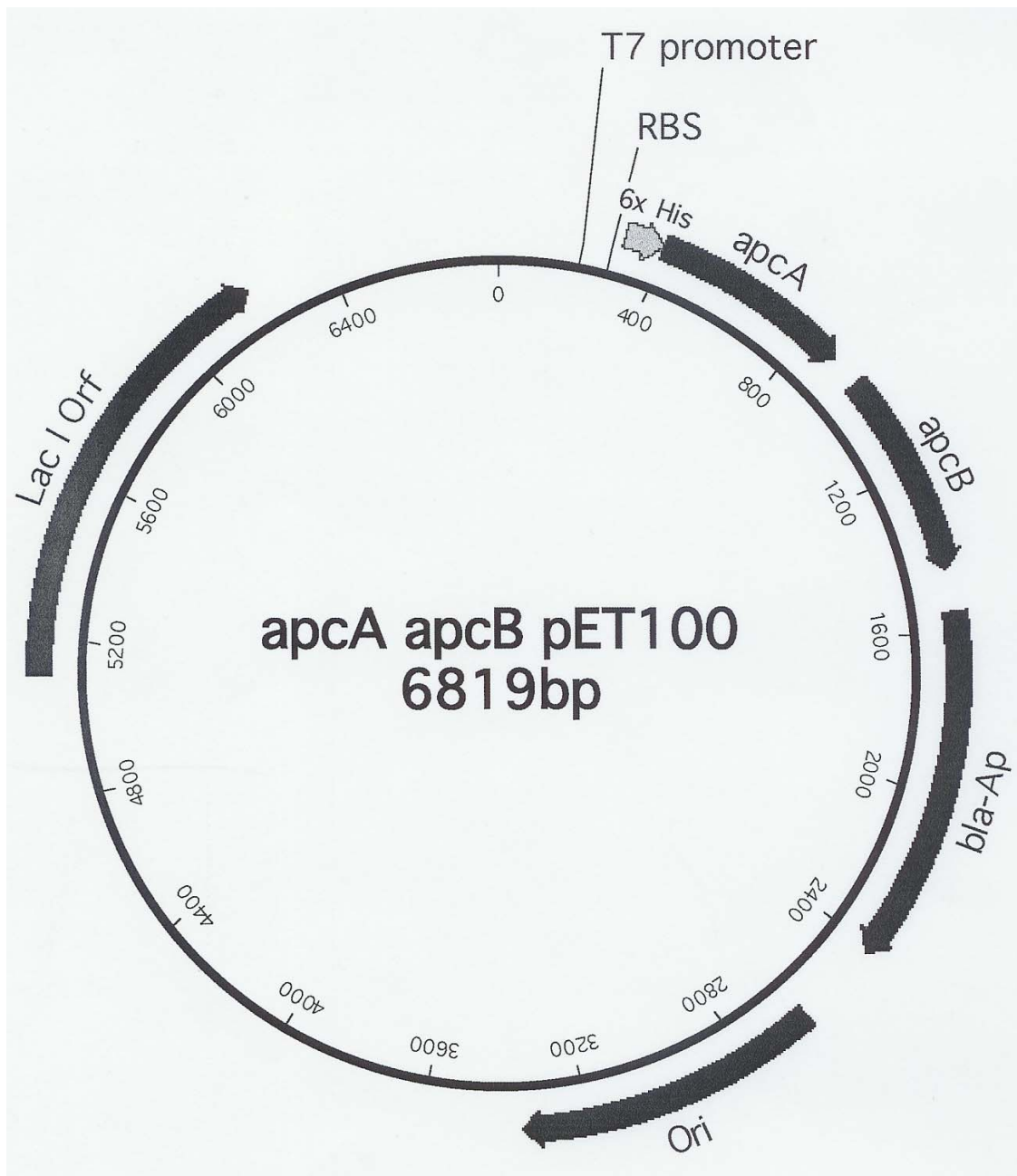


Figure 13: Plasmid map of clone *apcAapcB/pET100*.

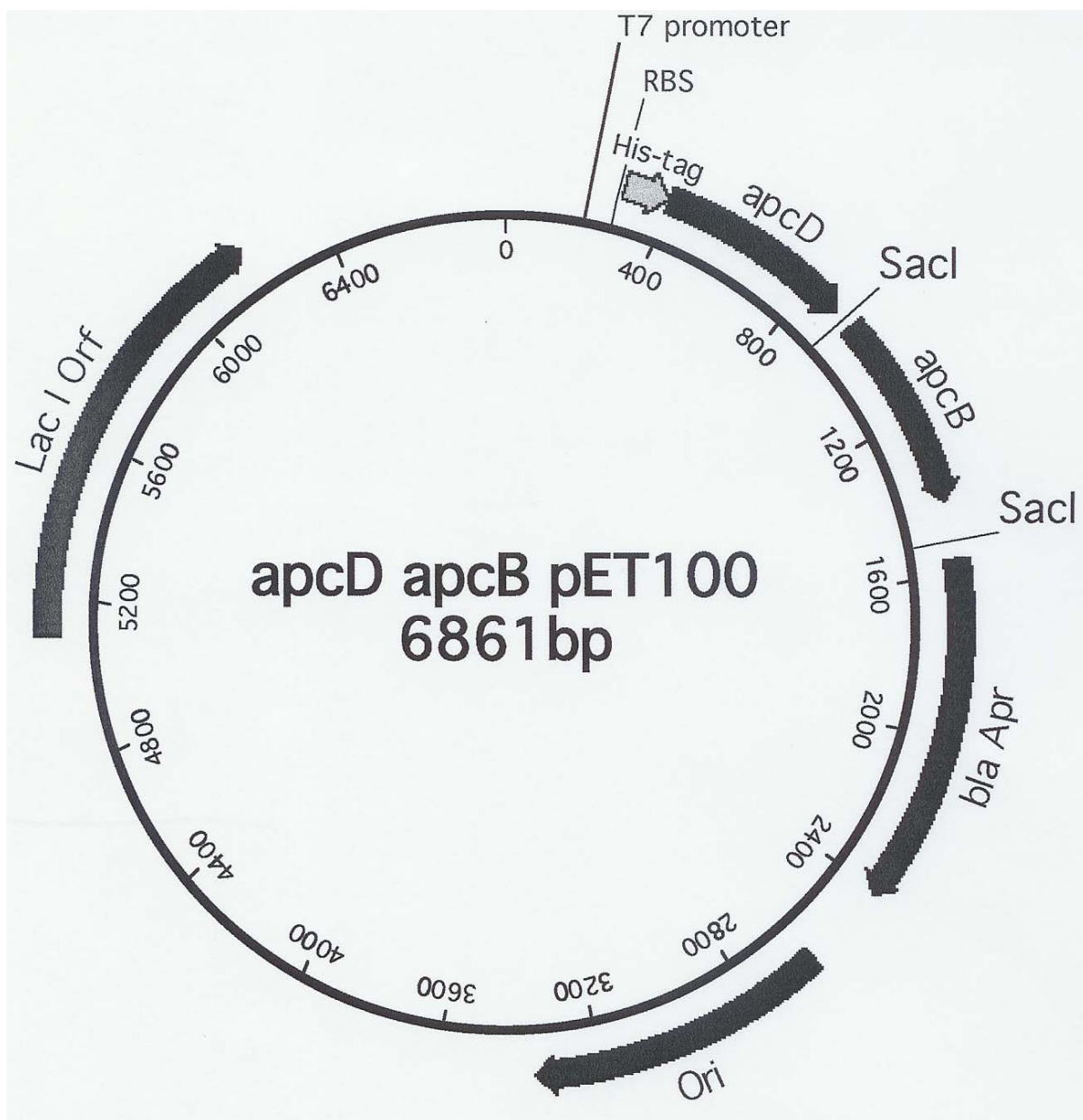


Figure 14: Plasmid map of clone *apcD apcB/pET100*

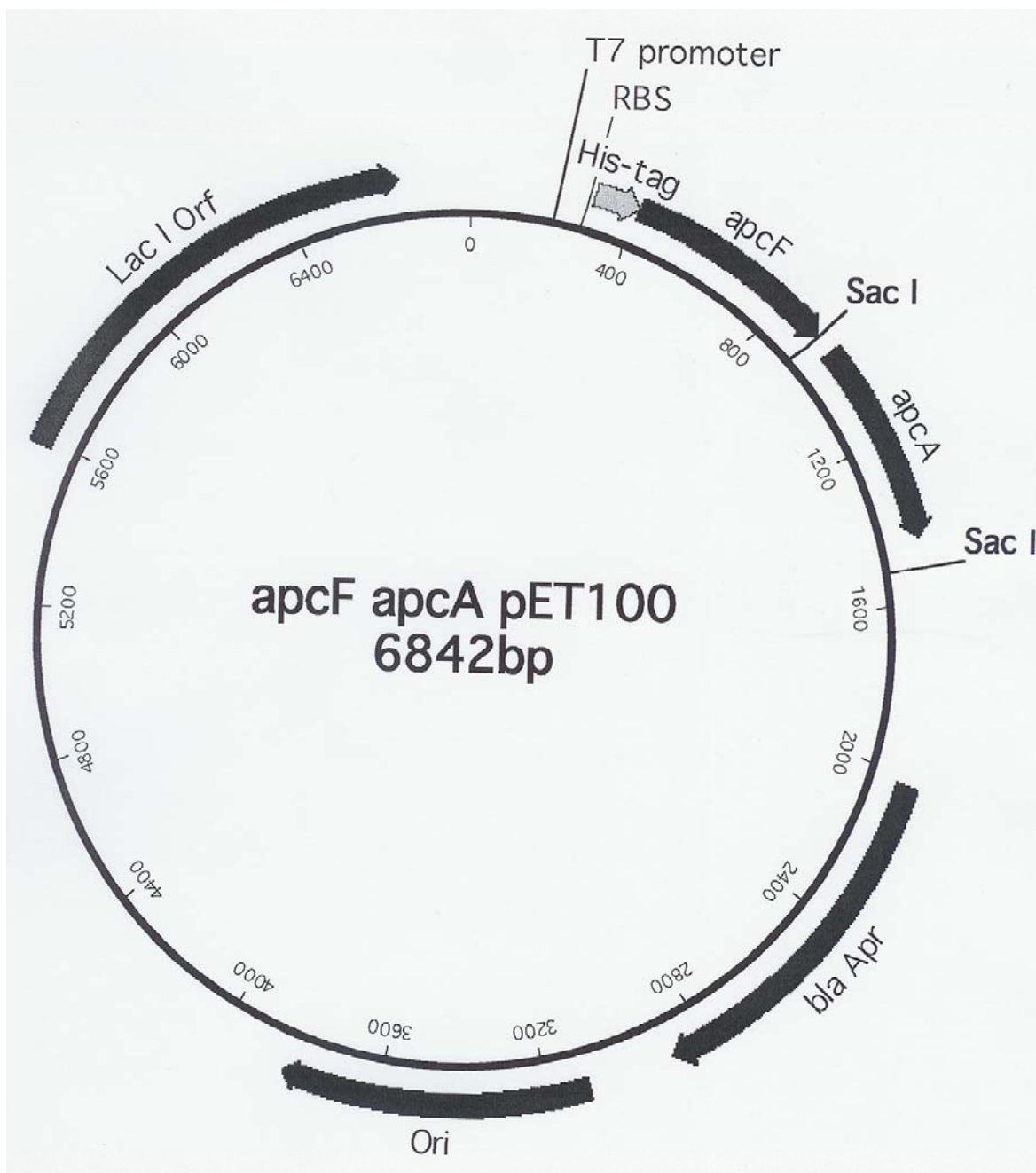


Figure 15: Plasmid map of *apcF apcA/pET100*

2.3.3 Recombinant GST-PcyA purification

The GST-PcyA plasmid was donated by Dr. J. C. Largaris (Frankenberg et al., 2003). The GST-PcyA gene from *Anabaena* sp. PCC 7120 was cloned into a vector fusing it to a GST tag. The BL21 cells containing the GST-PcyA construct was allowed to grow for 4 hours prior to IPTG induction (0.5mM) and then allowed to grow for another 4 hours post IPTG induction. The culture was removed and centrifuged for 20 minutes at 15,000 x g. The enzyme was purified on glutathione resin beads (Sigma, St. Louis, Mo). The glutathione agarose was stored in a lyophilized powder and prior to purification the agarose was swollen for 2 hours at room temperature by the addition of 10 ml of ddH₂O to 740 mg of powder. After adding approximately 7 ml of glutathione resin, the column (2.5 cm x 10 cm) was washed with 2 column volumes of equilibration buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl) prior to addition of the GST-PcyA whole cell extract. The column was washed again with 2 column volumes of equilibration buffer, and the PcyA/pGEX-2T protein was eluted with 10 ml of elution buffer (50mM Tris-HCl, pH 8.0, 5 mM oxidized glutathione). The protein was dialyzed against 50mM Tris-HCl, pH 8.0 at 4°C overnight.

2.3.4 *In vitro* Bilin Addition Reactions using GST-PcyA

Multiple reactions were set up containing 500 µl to 1ml of recombinant HT-ApcA (0.73mg/ml), HT-ApcB (0.68mg/ml), HT-ApcAB (1.25mg/ml), HT-ApcBD (0.97mg/ml), or HT-ApcFA(0.82mg/ml) substrate and : 100 µl of *E. coli* whole cell extract containing pAED4 vector as a control, 100 µl of purified and whole cell CpcS, 250 µl of HT-CpcU (1mg/ml), 250 µl

of HT-CpcU/CpcS copurified complex (1mg/ml) , and/or 100 µl of CpcT (1mg/ml). In addition to the enzymes, the following were also added to each reaction mixture: 10 µM of GST-PcyA (Frankenburg et al., 2001; Frankenburg and Lagarias, 2003), 4.6 µM recombinant *Synechococcus* sp. PCC 7002 ferredoxin or from spinach (Sigma-Aldrich Chemical Co. St. Louis MO.) (Gomez-Lojero et al., 2003; Schluchter, 1994), 0.025 u/ml spinach FNR (Sigma-Aldrich Chemical Co. St. MO.), 6.5 mM glucose-6-phosphate, 1.1 U/ml glucose-6-phosphate dehydrogenase (from *Torula* Yeast, Sigma) , 1M Hepes pH 7.3, 50 to 75 mM MgCl₂ , 1.6 mM NADP⁺, 5µM biliverdin (Porphyrin Products, Logan, UT). Reactions were placed in a 30°C water bath for 1 hour in the dark. A second aliquot of biliverdin (5 µM) was added, and the reactions were incubated for an additional 1.5 hours in the dark at 30°C. Upon completion, the reactions were placed in a microcentrifuge for 10 minutes at 14,000 rpm (8000 x g). The absorbance/fluorescence emission spectra were obtained and an aliquot was separated by SDS-PAGE for zinc enhanced bilin fluorescence analysis followed by Coomassie staining for protein.

2.4 High Performance Liquid Chromatography (SEC-HPLC).

Size exclusion standards, recombinant and purified substrates, and individual lyase subunits were separated on a Bio-Sil[®] 250 SEC HPLC column (7.8 x 300mm)(Bio Rad, Hercules, Ca). The size exclusion standard was resuspended in 500 µl of ddH₂O and placed on ice for 2 minutes. 200µl of standard or recombinant protein was centrifuged in a microcentrifuge at 14,000 rpm (8,000 x g) for 5 minutes and the supernatant injected onto a Waters HPLC with a 600E pump and a photodiode array detector (Figure 16). Prior to each run, the SEC standards were injected,

analyzed, and used to calibrate the HPLC program. The program used a flow rate of 0.8ml/min in the presence of 50mM NaPO₄, pH 7.0 and ran for 55 minutes. The wavelengths of the samples were monitored at 280 nm (protein) and 360 nm (bilin). The components, retention time, and molecular weights of the standard are given in Table 3. Figure 17 shows the standard curve of the elution time against the log of the molecular weights. The standard curve generated an equation that was utilized to calculate the apparent molecular weight based on retention times of unknowns.

2.5 Urea Denaturation of Phycocyanobilin Reaction products.

Bilin addition reactions utilizing phycocyanobilin as the bilin donor were introduced to 8M Urea pH 2.0 and absorbance spectra acquired to determine the spectrum of the protonated bilin attached. The absorbance spectrum was compared to the mesobiliverdin spectrum found in the article by Arciero et al. describing in vitro bilin attachment to apophycocyanin (1988a). The bilin reactions were completed as stated and concentrated 3 fold using an Amicon YM10 micro concentrator. A 1:5 dilution of the concentrated bilin product in 8 M urea pH 2.0 was set up and analyzed by UV-Vis spectroscopy from 200 to 750 nm.

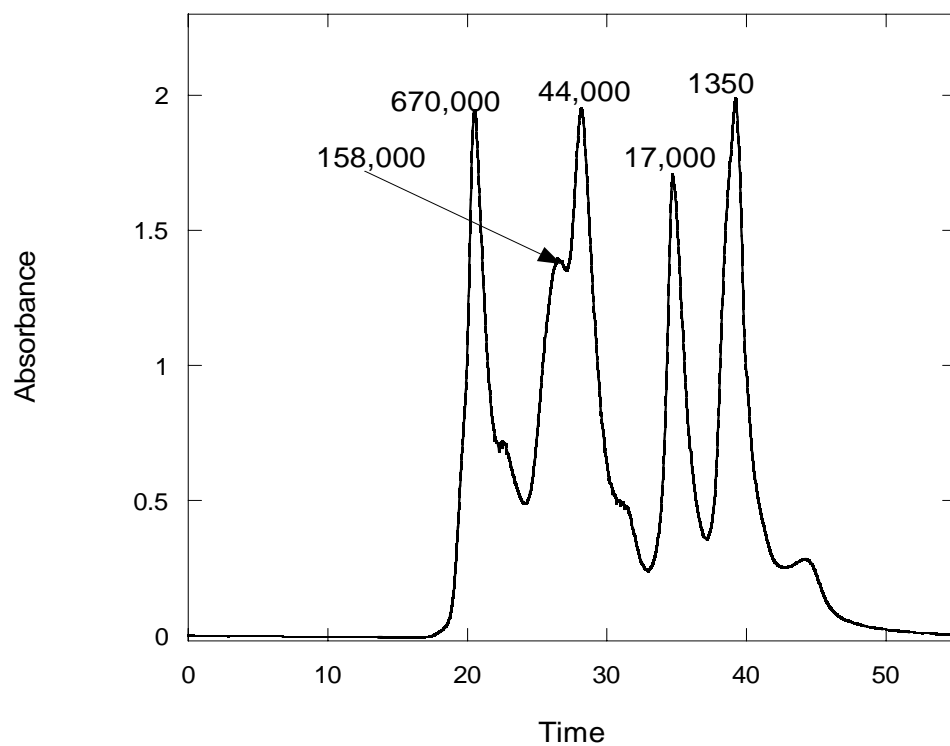
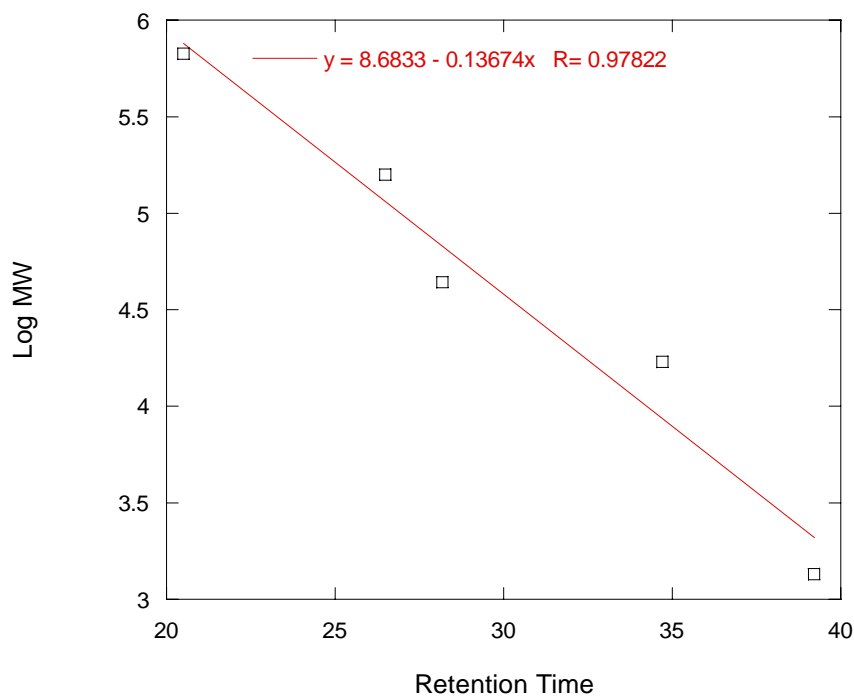


Figure 16: SEC Chromatogram of molecular weight standards. Image shows the molecular weights of each peak. Peaks are identified as thyroglobulin, γ -globulin, myoglobin, and Vitamin B12.

A



B

Table 3: SEC-HPLC Standards with Retention time and Molecular Weights.

Name	Retention Time	Log MW	Actual Molecular Weight
Thyroglobulin	18.883	5.8261	670000
γ -Globulin	24.383	5.1987	158000
Ovalbumin	25.767	4.6435	44000
Myoglobin	31.383	4.2304	17000
Vitamin B12	36.55	3.1303	1350

Figure 17: Equation of the regression line and table of retention times of SEC molecular weight standards. Panel A shows the regression line based up the observed retention times of components for SEC standards. It also shows the equation used to calculate the log and calculated molecular weights of recombinant lyases. Panel B is table 3 with retention time, log molecular weight, and actual molecular weight of each component found in the SEC standard.

2.6 Transfer reactions using holo-phycoyanin, HT-ApcAB, CpcS, and CpcU.

Transfer reactions were performed to determine if CpcS, CpcU, and/or CpcT are able to transfer bilins from a holo-phycobiliprotein to an apo-protein. 250 µl HT-ApcAB (1.25mg/ml), 15 µl of purified and whole cell CpcS, 30 µl of HT-CpcU (1mg/ml) together, 15 µl CpcT (1mg/ml) alone, 1mM MgCl₂, and 20mM Tris pH 8.0 were incubated on ice together for 30 minutes in the presence of 25 µl of purified phycocyanin (4.5 µg/µl). The reactions were placed on 500 µl of Ni-NTA resin and allowed to interact with the resin for 1 hour on ice and 2 hours at room temperature on a rotating wheel at room temperature. Prior to addition of the reaction, the Ni-NTA resin was spun for 4 minutes at 3000 rpm (4000 x g) and the supernatant was discarded. 500 µl of Buffer O was added to the resin and centrifuged again for 4 minutes at 4000 x g. The supernatant was removed, and the reaction components added. Upon completion of the transfer reactions, the products were centrifuged for 2 minutes at 3000 x g. The supernatant was retained and 600 µl of 0.5 M Buffer B was added and the solution centrifuged for 2 minutes at 3000 x g. Again, the supernatant was retained and 600 µl of Buffer A2 was added and the solution centrifuged for 2 minutes at 3000 x g to remove insoluble material. The supernatant was removed and retained and 200 µl of Buffer C was added and the mixture allowed to incubate at room temperature for 10 minutes. The reaction was centrifuged for 2 minutes at 3000 x g and the supernatant loaded on a 15% Tris-HCl SDS-PAGE gel for analysis. After electrophoresis, the gel was placed in 100mM ZnSO₄ for 10 minutes and analyzed for bilin addition by fluorescence emission before Coomassie staining.

RESULTS

3.1 Protein Alignment of Phycobiliprotein Subunits.

An alignment was performed to compare the allophycocyanin subunits to the alpha and beta subunit of phycocyanin using Clustal W (v1.4) multiple sequence alignment (MacVector, Accelrys) (Figure 18). The alpha subunit of both phycocyanin and allophycocyanin had 51.8% similarity; and the beta subunit of both phycobiliproteins had 55.8% similarity in their protein sequence. The alpha subunit of allophycocyanin found in *Synechococcus* sp. PCC 7002 has 1 conserved cysteine residues located at the 81 site while the beta subunit has 2 cysteine residue located at the 81 and 153 sites. The ApcD contains two cysteine residues located at the 81 and 132 sites and ApcF possesses only 1 cysteine residue at the 81 site (Figure 18). All allophycocyanin subunits contain only one phycocyanobilin attached at cysteine 81.

3.2 Recombinant protein expression of HT-ApcA, HT-ApcB, HT-ApcAB, HT-ApcBD, HT ApcFA, and HT-CpcU

The expression plasmids were transformed into BL21 DE3 cells for production of recombinant proteins in *E. coli* as stated in the Materials and Methods. After growth, the cells were collected, and their protein expression profile was analyzed by SDS-PAGE. Both HT-ApcA and HT-ApcB were both expressed independently in *E. coli* cells. The cells containing the recombinant proteins were harvested and analyzed by SDS-PAGE. The gel showed little to no expression of soluble protein (Data not shown).

It was determined that expression of HT-ApcA or HT-ApcB independently produced insoluble protein which aggregated into inclusion bodies (Data not shown). Next the *apcA* and *apcB* genes were expressed together in hopes of increasing soluble protein expression.

Figure 19 shows the *E. coli* whole cell extract containing HT-ApcAB after 4 hours of IPTG induction (lane 1), *E. coli* clarified extract containing HT-ApcAB (lane 2), the Ni-NTA flow through (lane 3), and the eluate containing purified HT-ApcAB (lane 4) (Figure 19). Lane 4 illustrates that both subunits are abundant and fairly pure, copurify in roughly equimolar amounts (sometimes less ApcB than HT-ApcA), and migrate on SDS-PAGE near the calculated molecular weights (HT-ApcA at 21.3 kDa. and ApcB at 17.2 kDa) (Figure 19, Table 4). Figure 19, lane 2 shows the *E.coli* whole cell containing recombinant HT-ApcA and ApcB subunits being expressed in a 1: 1 ratio; however, after purification HT-ApcA and ApcB are present in a 1.5: 1 ratio (Figure 19). To prevent the premature release of the ApcB subunit, a wash solution (Buffer B) containing 0.5 M salt (0.25 M KCl, 0.25 M NaCl) was used in place of the normal 1 M salt solution. It seems that the higher salt concentration greatly reduced the interaction between HT-ApcA and ApcB thereby reducing the amount of co-purified ApcB. Lane 3 shows an aliquot of the Ni-NTA flow-through during the purification process, and it appears that a small amount of HT-ApcA and ApcB are present, suggesting the column capacity had been reached (Figure 19).

After production of HT-ApcBD, the cells were harvested, and the protein expression profile was analyzed by SDS-PAGE (Figure 20). Figure 20 shows the whole cell extract in pellet form of the *E. coli* cells containing HT-ApcBD after 4 hours of induction with IPTG (lane 1), the Ni-NTA flow through (lane 2), and the eluate containing the purified HT-ApcBD (lane 4).

Formatted Alignments

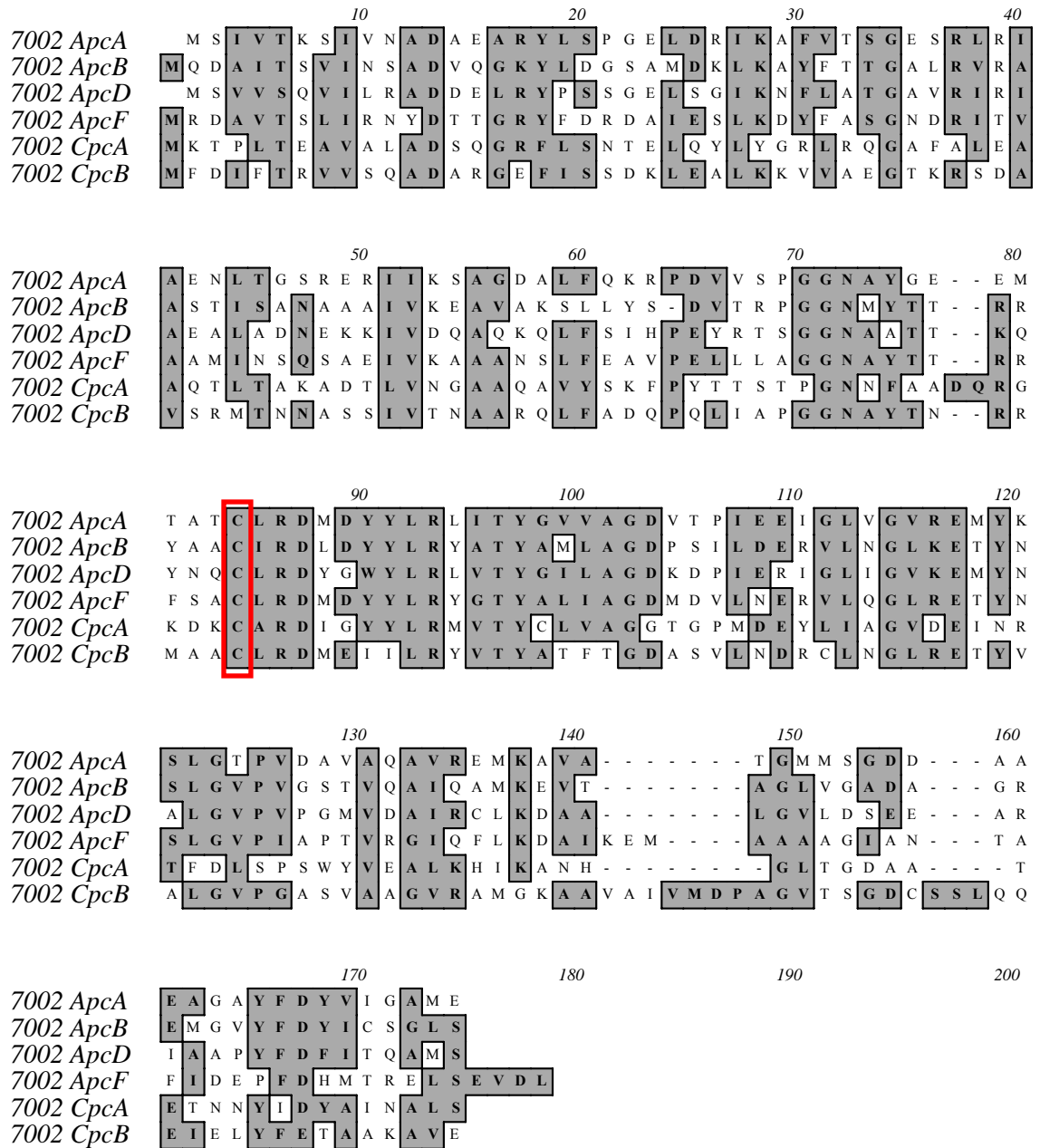


Figure 18: Alignment of protein sequence of allophycocyanin and phycocyanin subunits from *Synechococcus* sp. PCC 7002. Illustrates alignment of conserved cysteine residues found on ApcA and ApcB subunits as well as the linker proteins ApcD and ApcF. The allophycocyanin subunits were compared to the alpha and beta subunits of phycocyanin. The conserved cysteine residues where bilins are attached are outlined in red.

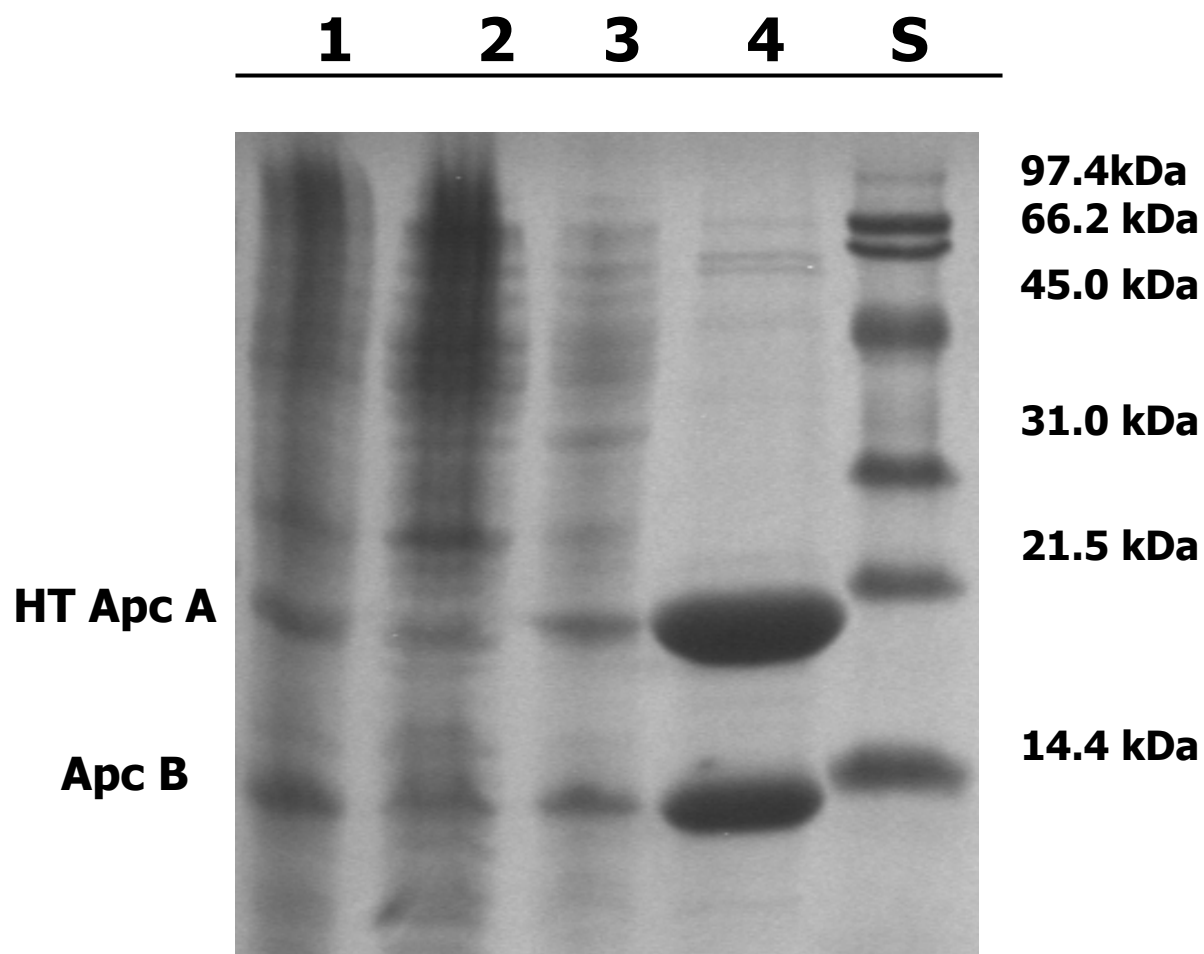


Figure 19: SDS-PAGE of *E. coli* whole cell extract containing HT-ApcAB. Lane 1 contains cells after 4 hours of IPTG induction. Lane 2 and 3 contain whole cell extract after clarification and Ni-NTA flow through from the purification procedure, respectively. Lane 4 contains purified recombinant HT-ApcAB and the S indicates the molecular weight markers whose sizes are shown at right.

Table 4: Calculated Molecular Weights and Isoelectric Points

Protein	Molecular Weight	p.I.
HT-ApcA	21,398.10 Da	5.04
HT-ApcB	21,321.40 Da	6.43
HT-ApcD	21,826.23 Da	5.86
HT-ApcF	22,848.16 Da	5.02
HT-CpcU	23,474.60 Da	5.77
ApcA	17,297.47 Da	4.48
ApcB	17,220.36 Da	6.58
CpcS	22,526.30 Da	4.79
CpcT	22,515.91 Da	5.68
CpcV	19,853.50 Da	7.54

Like HT-ApcAB, HT-ApcD and ApcB were produced together because expression of each gene independently resulted in production of little soluble protein.

Lane 4 of Figure 20 illustrates that both subunits are expressed in an abundant amount and are both fairly pure. During purification, 0.5 M Buffer B was also used to prevent the high salt concentration from weakening the interaction between HT-ApcD and ApcB. In the presence of a high salt buffer, the eluate contains less ApcB than HT-ApcD causing the recombinant protein to be in a 1.5:1 ratio. Lane 4 shows how both subunits copurify close to a 1:1 ratio and migrate near their expected molecular weights (HT-ApcD at 21.8 kDa. and ApcB at 17.2 kDa) (Figure 20). Expression levels of recombinant HT-ApcBD were much lower than those achieved for HT-ApcAB. The *E.coli* cell extract containing HT-ApcBD in lanes 2 and 3 shows very little unbound recombinant protein indicating adequate binding of the histidine tagged subunit to the Ni-NTA resin (Figure 20).

E. coli cells containing the recombinant protein HT-ApcFA were harvested and analyzed by SDS-PAGE as well (Figure 21). Both HT-ApcF and ApcA subunits were expressed together to ensure the production of soluble protein because independent expression of either resulted in the production of very little soluble protein. The recombinant HT-ApcFA is shown in Figure 21 where the *E. coli* whole cell extract containing the recombinant protein (lane 1), the Ni-NTA flow through (lane 2), and the eluate containing the purified HT-ApcFA (lane 3) (Figure 21). Figure 21, lane 2 shows the presence of HT-ApcFA in the flow-through indicating overloading of the Ni-NTA resin of the HT-ApcF protein as well as the copurifying ApcA subunit. Both subunits are expressed in relatively abundant amounts with a close to 1:1 ratio between HT-ApcF and ApcA present upon elution from the resin as shown in lane 3 (Figure 21). To retain both subunits in equal amounts during purification, a 0.5 M Buffer B solution was used, and each

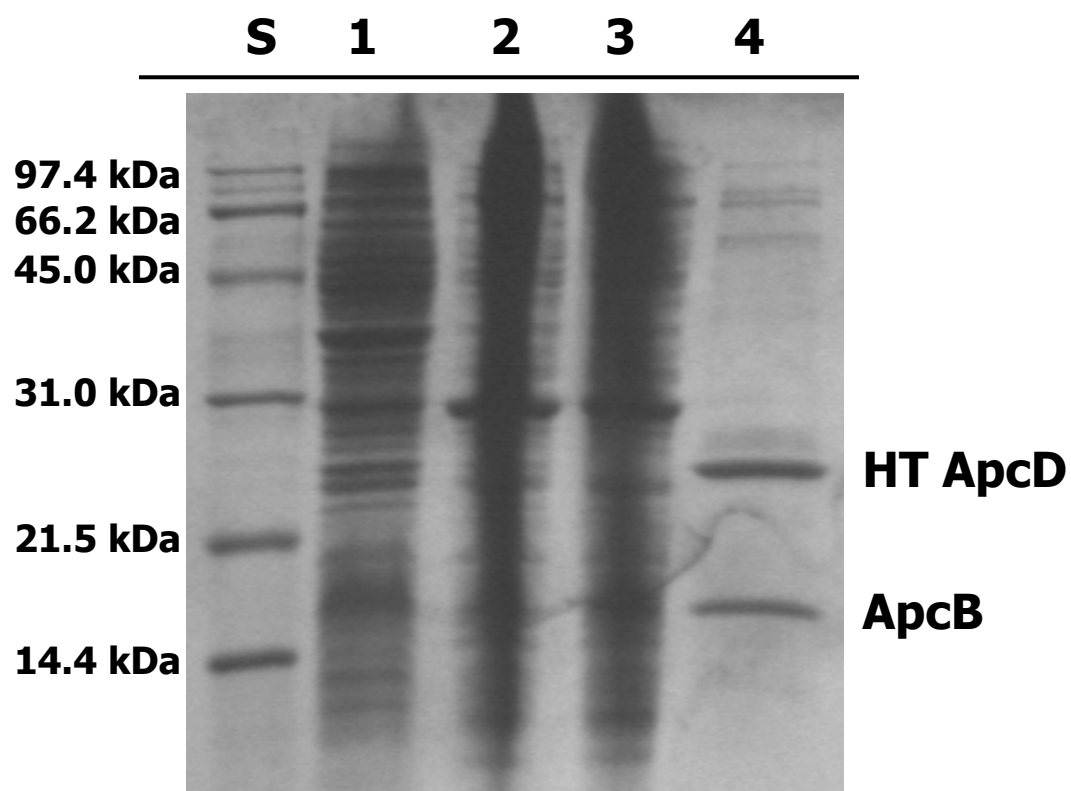


Figure 20: SDS-PAGE of fractions during purification of recombinant HT-ApcBD. S indicates the molecular weight markers whose sizes are shown at left; lane 1 contains cells after 4 hours of IPTG induction. Lanes 2 and 3 contain aliquots of clarified whole cell extract and Ni-NTA flow through taken from the purification procedure, respectively. Lane 4 contains purified recombinant HT-ApcBD.

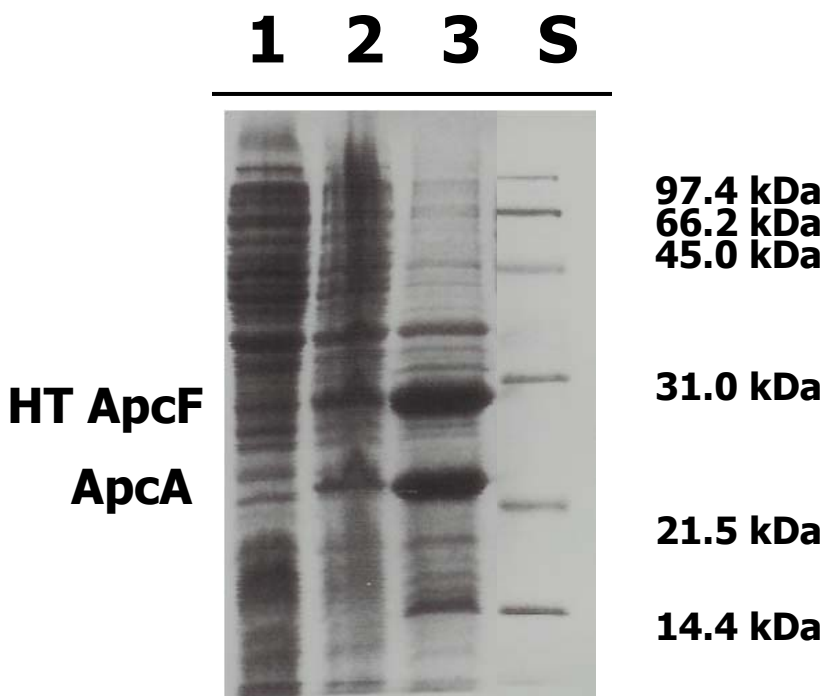


Figure 21: SDS-PAGE of purification fractions containing recombinant HT-ApcFA. Lanes 1 and 2 contains whole cell extract with no IPTG induction, whole cell extract after 4 hours with IPTG induction, respectively. Lane 3 is purified recombinant HT-ApcFA with relatively high expression levels. S indicates the molecular weight markers whose sizes are shown at right.

subunit migrated with the expected molecular weight (HT-ApcF at 22.8 kDa and ApcA at 17.2 kDa.) (Table 4). For the recombinant HT-CpcU, Figure 22 shows the *E. coli* whole cell extracts containing HT-CpcU before and after IPTG induction. Lanes 2 and 4 illustrates that HT-CpcU is produced (arrows) and migrates on the SDS-PAGE with an apparent molecular mass close to the calculated mass of 23.4 kDa. (Table 4).

3.3 Recombinant protein expression of CpcS, CpcT, and CpcV

Recombinant CpcS and CpcT were purified using DEAE anion exchange chromatography. Fractions containing CpcS were collected and analyzed by SDS-PAGE, shown in Figure 23. An aliquot of the *E. coli* cell culture containing the recombinant CpcS protein was taken prior to IPTG induction and is shown in lane 1. Post IPTG induction, an aliquot was removed from the *E. coli* cell culture and run on an SDS-PAGE as shown in lane 2 of Figure 23. Lane 2 illustrates increased protein expression after the addition of IPTG and confirms that expressed recombinant CpcS protein migrates according to the expected molecular weight of 22.5 kDa. (Table 4).

Aliquots of the *E. coli* cells containing CpcT were removed before and after IPTG induction and also run on an SDS-PAGE to confirm the calculated molecular weight of 22.5 kDa. (Data not shown, Table 4). Fractions containing CpcT were collected from the anion column and analyzed by SDS-PAGE as well (Data not shown).

Recombinant CpcV was also purified by DEAE anion exchange chromatography as described in Materials and Methods. A chromatograph of the CpcV purification illustrates the absorbance and conductivity of the protein during purification from the anion column (Figure

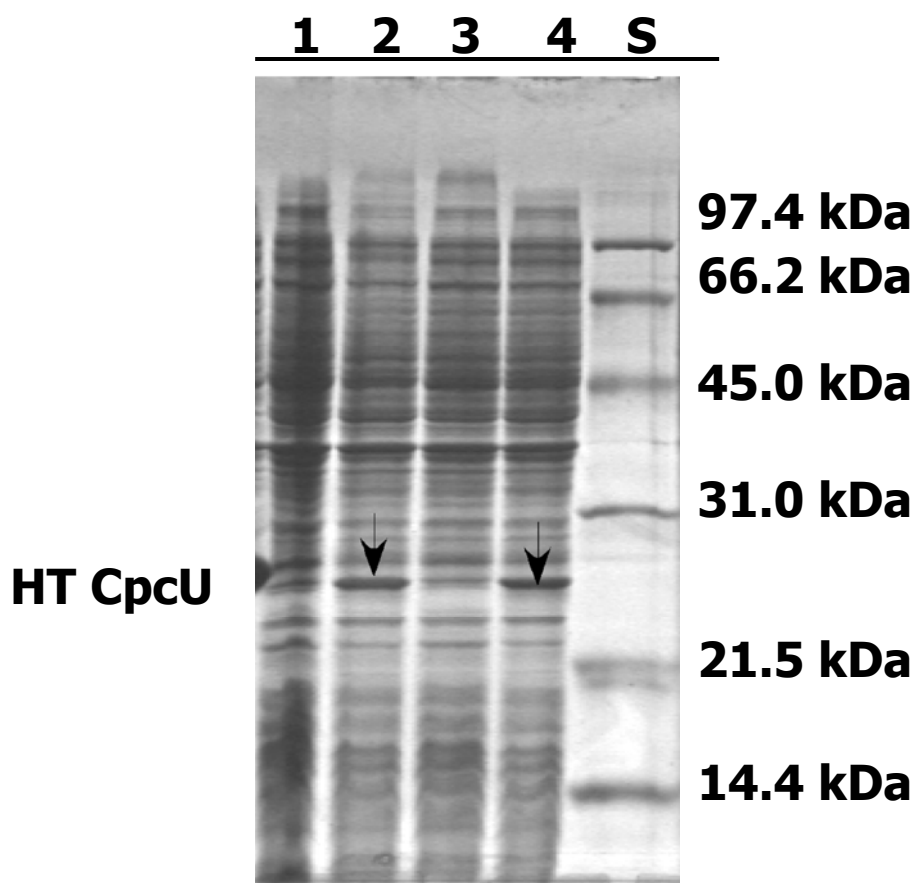


Figure 22: SDS-PAGE of *E.coli* whole cell extract containing HT-CpcU. Lanes 1 and 3 contain cell extract after 4 hours of growth. Lanes 2 and 4 contain cell extract taken from cells after 4 hours of IPTG induction and S is the molecular weight markers whose size are shown at right. The arrows show position of recombinant HT-CpcU after 4 hours of IPTG induction.

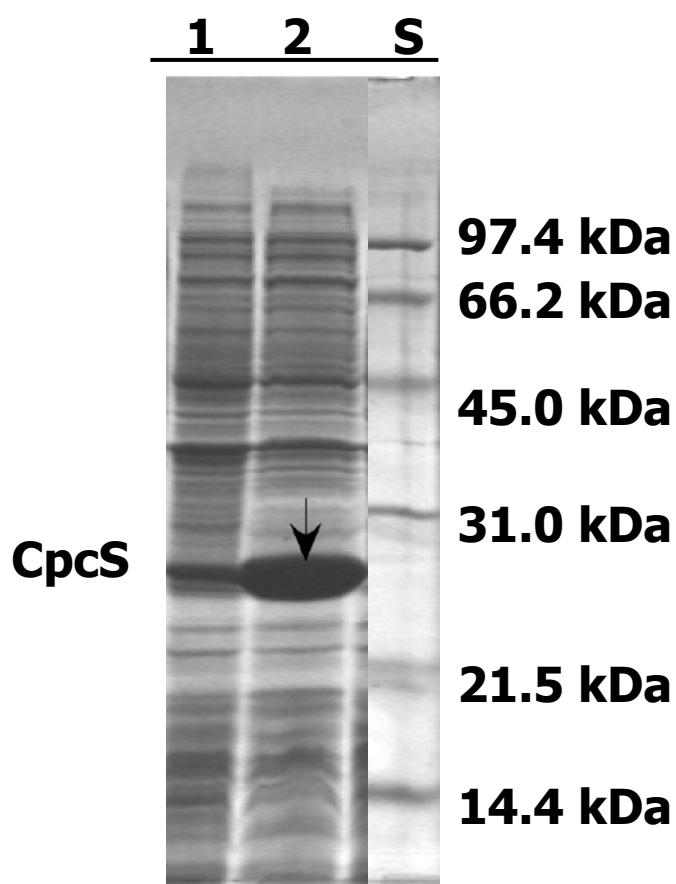


Figure 23: SDS-PAGE of *E. coli* whole cell extract containing CpcS.

Lane 1 contains cells before IPTG induction and lane 2 contains cells after 4 hours of IPTG induction of cells containing CpcS. S indicates the molecular weight markers whose sizes are shown at right. The arrow shows position of recombinant CpcS protein.

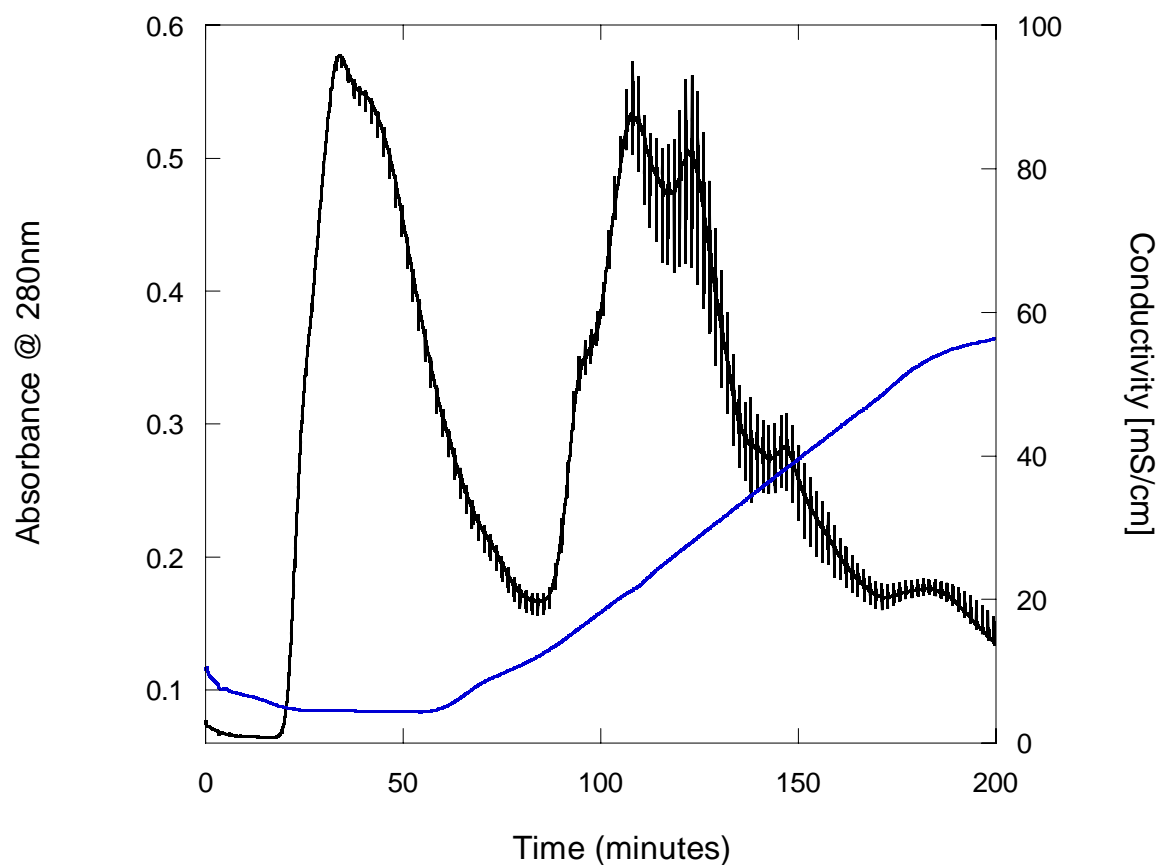


Figure 24: Chromatograph of purification on a DEAE column for CpcV. Absorbance at 280 nm is depicted by the black line while conductivity is depicted by the blue line. The purest fractions were found between 100-150 minutes with hash marks showing fractions collected.

24). Figure 25 panel A is the SDS-PAGE of the protein expression profile of *E. coli* extracts containing CpcV taken during different time intervals of expression at 4, 8, and 10 hours respectively. Fractions containing the largest amount of recombinant protein were collected between 100-150 minutes, pooled, and analyzed by SDS-PAGE (Figure 25, panel B). Panel B of Figure 25 shows purified aliquots containing an abundant amount of recombinant CpcV corresponding to the calculated molecular weight of 19.8 kDa.(Table 4).

3.4 HT-ApcAB SEC and Bilin Addition assays with PcyA bilin generation system.

Allophycocyanin is present and stable in two different forms. In cyanobacteria, it can be isolated and purified in the trimeric form consisting of 3 alpha and beta holo-subunits each. It can also be purified in a form consisting of only 1 alpha and beta subunit each under certain conditions. MacColl et al., determined that the absorption spectrum of the allophycocyanin trimer differs from that of the monomer (1981). The monomer has an absorption spectrum with a maximum at 615 nm; however, the allophycocyanin trimer has a red-shifted absorption spectrum with a broad shoulder at 632 nm and a sharp peak at 650 nm (MacColl et al., 1981).

It was important to determine the quaternary structure of the recombinant and purified substrates used in the bilin addition reactions. Size exclusion assays were conducted using a Bio-Sil® 250 SEC HPLC column (7.8 x 300mm). 200µl of recombinant protein as well as aliquots taken from bilin addition reaction products were injected onto the column in the presence of 50mM NaPO₄ buffer pH 7.0. Molecular weights of each sample were calculated as described in materials and methods. After the retention times of the standards were identified, recombinant substrate and individual lyases were also run on the SEC column to determine their calculated molecular weight based on their retention times.

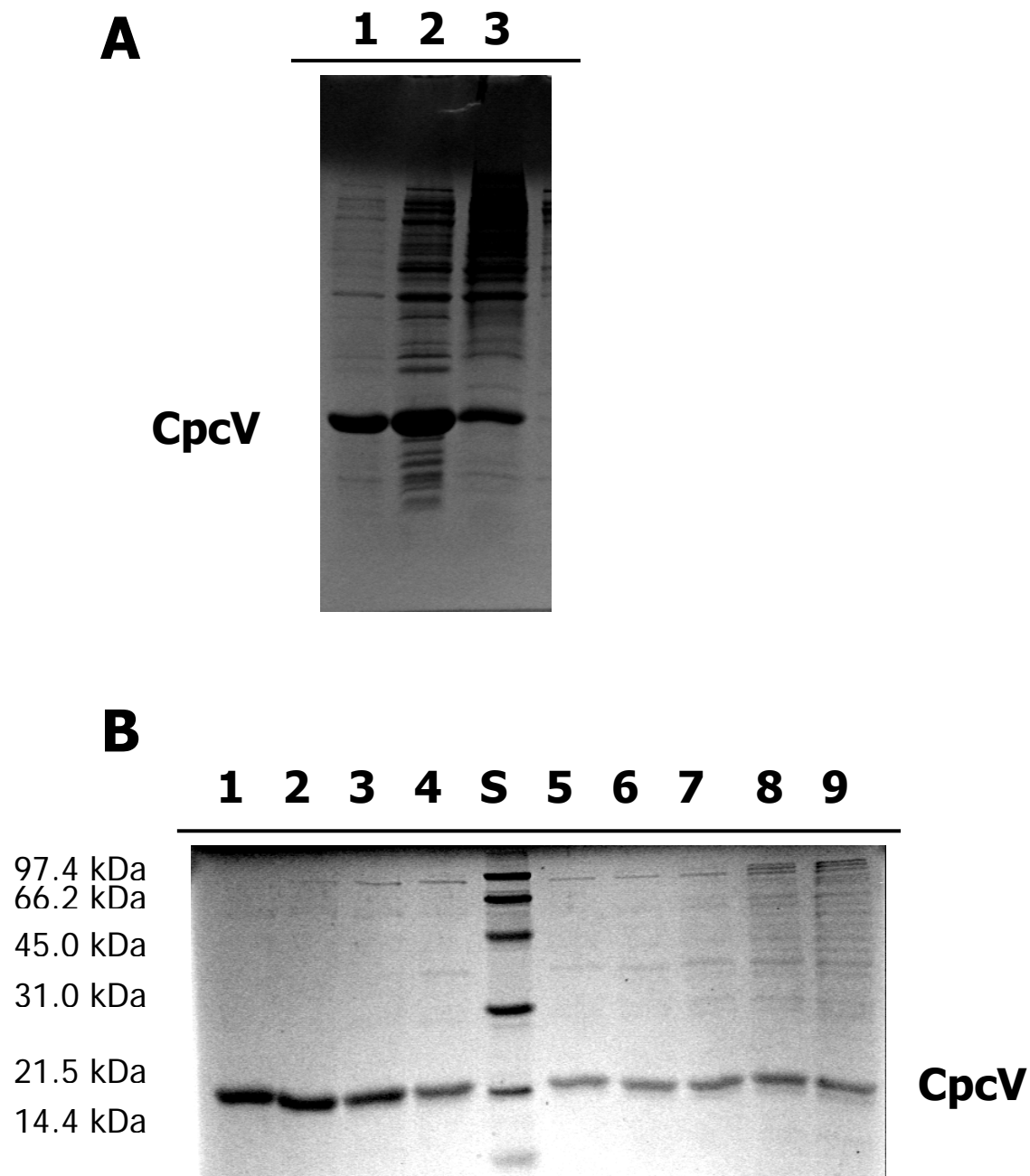


Figure 25: SDS-PAGE of *E.coli* whole cell extract containing CpcV. Panel A shows cells taken at different times during the expression. Lanes 1, 2, and 3 contain cells taken after 4, 8, 10 hours of expression with no IPTG induction. Panel B shows the SDS-PAGE analysis of fractions taken from the CpcV DEAE purification. Lanes 1-9 contain aliquots of fractions taken from the fractions collected between 100-150 minutes.

Figure 26 shows the chromatogram of HT-ApcAB with a sharp peak at 28.58 minutes and a second peak at 41.21 minutes with a calculated molecular weight of 26.87 kDa. and 435 Da., respectively. The second smaller peak most likely corresponds to traces of imidazole, with a formula weight of 68.08 grams/mole, which is used to elute the protein from the Ni-NTA column. Therefore, the HT-ApcAB substrate is behaving as a compact monomer because the calculated molecular mass is 39 Da.

Bilin addition assays were conducted to determine if CpcS, CpcT, or HT-CpcU catalyzed phycocyanobilin addition to the conserved cysteine residue found on the alpha or beta subunit of allophycocyanin. *In vitro* bilin attachment assays were performed using HT-ApcAB, HT-ApcBD, and HT-ApcFA as the substrate and a combination of CpcS, CpcT, and HT-CpcU in each assay. Phycocyanobilin is synthesized with the aid of the enzyme phycocyanobilin ferredoxin oxidoreductase (PcyA) and the bilin precursor biliverdin. PcyA is a monomeric protein from *Anabaena* 7120 and is able to produce phycocyanobilin *in situ* in limiting amounts. The enzyme requires the presence of ferredoxin and catalyzes a four electron reduction in the vinyl group of ring D of biliverdin followed by the reduction of ring A (see Figure 6-7). The electrons obtained from ferredoxin are used by PcyA to convert biliverdin to phycocyanobilin (Frankenburg and Lagarias, 2003; Hagiwara et al., 2006).

Bilin addition reactions were conducted in the dark in a 30°C water bath with the purified recombinant substrate HT-ApcAB in the presence of *E.coli* proteins (control), and HT-ApcAB with variations of CpcS, CpcT, and HT-CpcU, together and independently. The reactions were incubated for 3 hours in the dark and upon completion the change in color was observed.

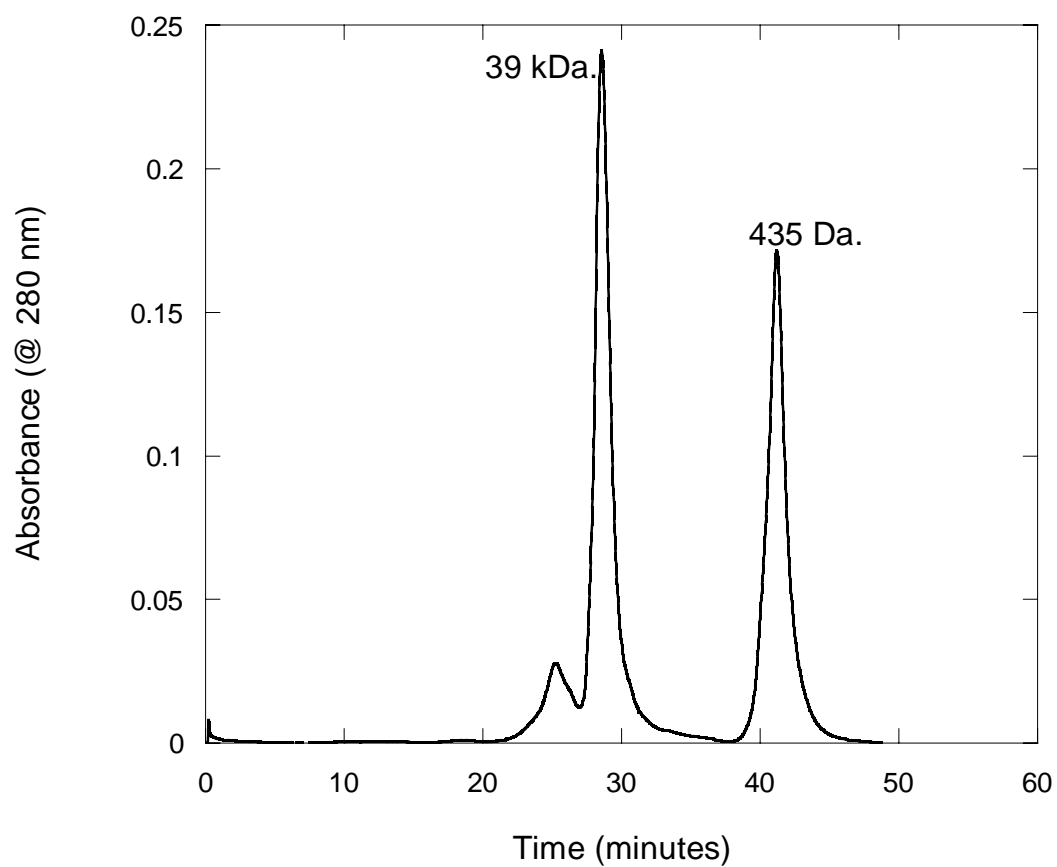


Figure 26: Size Exclusion chromatogram of recombinant HT-ApcAB protein. Peak 1 has a retention time of 28.58 and peak two has a retention time of 41.21 which is most likely imidazole. The elution was monitored at 280 nm and the apparent calculated masses are shown above the peaks.

In the presence of CpcS and HT-CpcU together, there was a color change from blue-green to blue; however, no significant color change was observed in other variations of the lyase reactions when compared to the control reactions. The reactions were removed from the 30°C water bath and centrifuged to move any insoluble matter, and the absorbance and fluorescence spectra of the reaction products were obtained. Figure 27 shows the absorbance spectra of HT-ApcAB reaction products containing CpcS and HT-CpcU had an absorbance maximum at 614 nm, whereas the control reaction and the single subunit reactions had absorbance maxima around 645 nm. These results are consistent with the finding by Beck and Sauer who determined that monomeric holophycocyanin possessed an absorbance maximum at 615 nm (1992). The control reactions as well as the single lyase reactions possessed an absorbance maximum similar to that of the non-enzymatic product mesobiliverdin.

One way to prove whether there was phycocyanobilin or mesobiliverdin production was to denature the HT-ApcAB control reaction product in acid urea. The fully protonated spectrum of the bilin would assist in this determination. Concentrated bilin addition reactions using phycocyanobilin as the bilin donor were denatured in 2.25 M Urea pH 2.0. An absorbance maximum around 660 nm indicates the covalent product is phycocyanobilin whereas an absorbance maximum around 680 nm would indicate the presence of mesobiliverdin (Arciero et al., 1988a). Figure 28 shows an absorbance maximum at 664 nm, indicating that phycocyanobilin is attached to the HT-ApcAB reaction product not mesobiliverdin. There was something distinct about the phycocyanobilin attached in the control reactions compared to the HT-ApcAB and CpcSU reactions therefore, the fluorescence emission spectra were obtained using a fluorimeter to probe how phycocyanobilin is interacting with the amino acid residues in the binding pockets. The control reaction product and the single lyase reaction products were

almost non-fluorescent, indicating that the phycocyanobilin attached to the protein is not interacting with the protein in the stretched conformation. Bilins can interact with the amino acids in the binding pocket and these interactions hold the bilin in a native state. If the bilin is not held in the stretched conformation, it can adopt a different absorbance and fluorescence. The absorbance at 645 nm and lack of fluorescence indicates that phycocyanobilin attached has not adopted the stretched conformation. The reaction products containing CpcS and HT-CpcU had an emission spectrum at 638 nm similar to that of holo-allophycocyanin monomers (Figure 29). Both the alpha and beta subunits possess a phycocyanobilin addition site on a conserved cysteine at position 81. To assay for the presence of bilins on each subunit, a 20µl aliquot from each reaction product was separated on an polyacrylamide gel and incubated with 100mM ZnSO_4 . Staining the SDS-PAGE gel with ZnSO_4 allows bilins attached to subunits to bind the Zn^{+} ions in the cyclic form and fluoresce at 540 nm allowing for easy detection. Figure 30 shows the zinc stained (panel B) and Coomassie stained SDS-PAGE gel (panel A) from the PcyA reactions with the HT-ApcAB substrate. Lanes 1, 2, and 3 of panels A and B contain the reaction products of the control, CpcS, and HT-CpcU reactions, respectively. The image shows that in the control, CpcS, and HT-CpcU reactions only the ApcB subunit is fluorescent, indicating phycocyanobilin attachment. However, in lane 4, where both CpcS and HT-CpcU are present, both the alpha and beta subunits contain phycocyanobilin. Lane 5 contains the reaction product from CpcS and CpcT and shows almost no bilin addition to either alpha or beta subunit. In the presence of both CpcS and HT-CpcU there is phycocyanobilin addition to both allophycocyanin subunits (lane 4) with an increased amount of bilin addition to the beta subunit here when compared to the control reaction (lane 1).

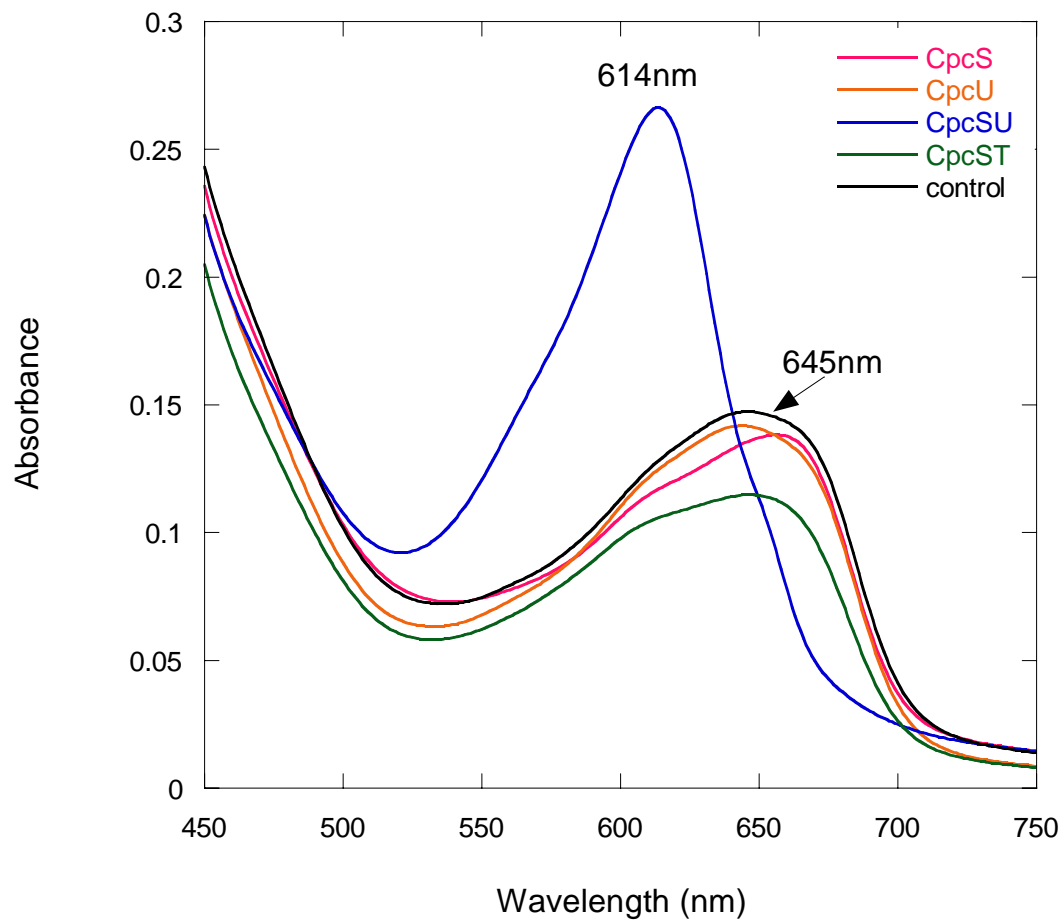


Figure 27: Absorbance spectra of PcyA bilin addition reactions with and HT-ApcAB and various lyase subunits. Absorbance spectra of reactions with HT-ApcAB and CpcS alone (red line), CpcU alone (orange line), CpcS and CpcU together (blue line), CpcS and CpcT together (green line), and HT-ApcAB alone (black line).

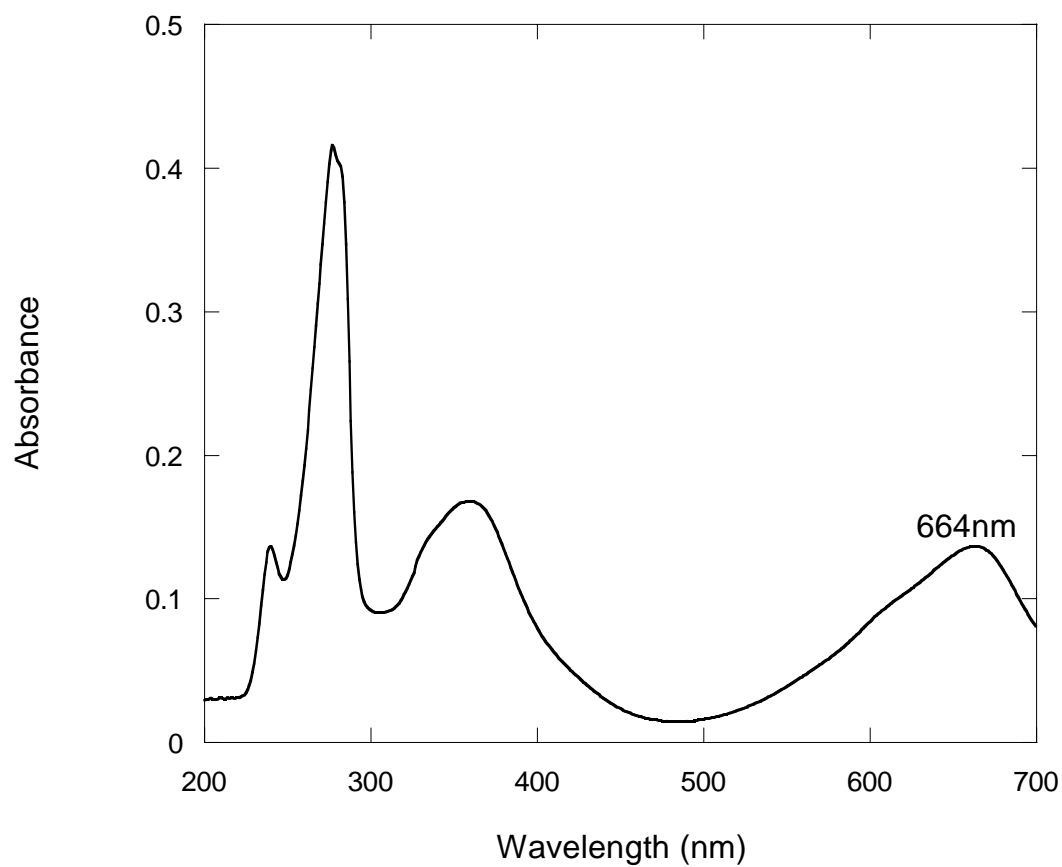


Figure 28: Absorbance spectrum of HT-ApcAB control phycocyanobilin bilin addition reaction in 8 M urea, pH 2. Figure illustrates a peak at 664 nm which is indicative of phycocyanobilin rather than mesobiliverdin at 680 nm.

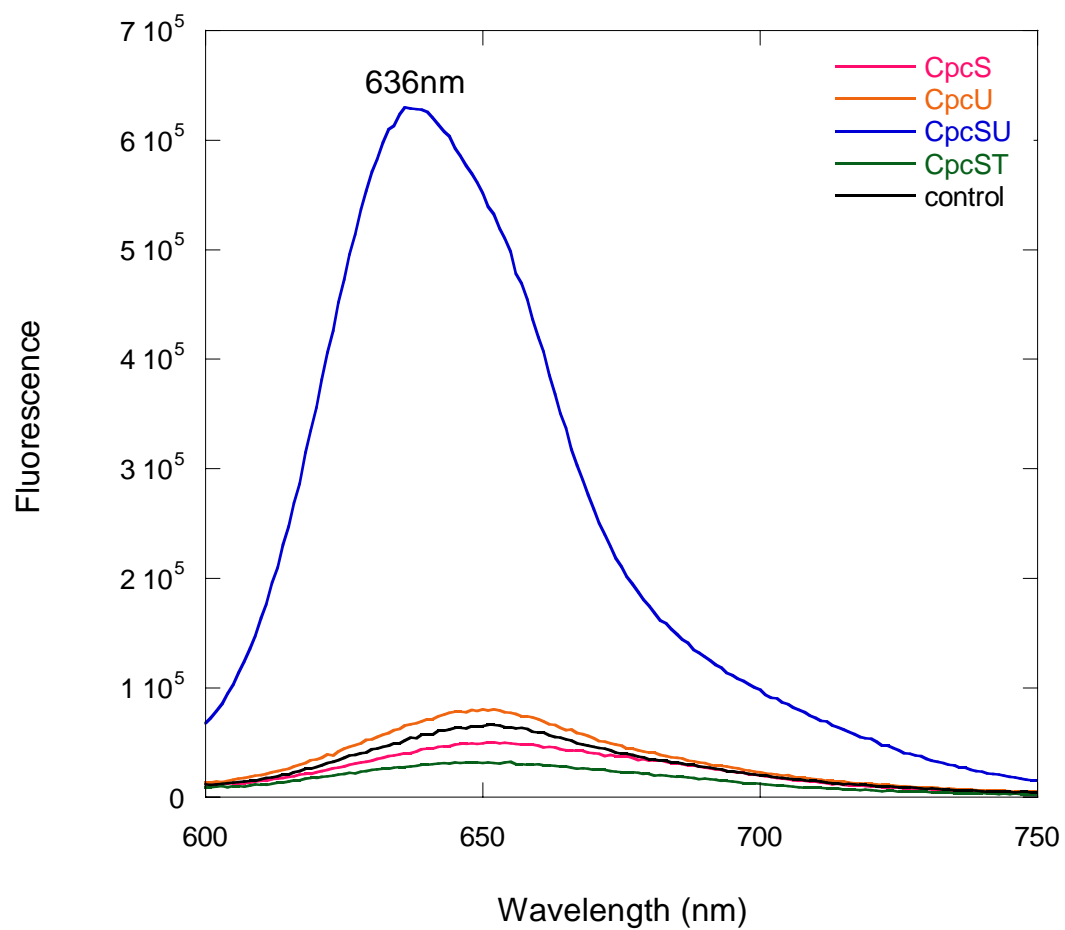


Figure 29: Fluorescence emission spectra bilin addition reactions with lyases to HT-ApcAB using PcyA. Fluorescence emission spectra of reactions with HT-ApcAB and CpcS alone (red line), CpcU alone (orange line), CpcS and CpcU together (blue line), CpcST (green line), and HT-ApcAB alone (black line).

Therefore, it was confirmed that CpcSU are required to attach phycocyanobilin to both the alpha and beta subunits of allophycocyanin and the product's spectroscopic properties mirror those of monomeric holo-allophycocyanin.

3.5 HT-ApcBD SEC and Bilin Addition Assays with PcyA Bilin Generation System.

Bilin addition assays containing PcyA and HT-ApcBD as the substrate were performed. The bilin addition reactions contained purified recombinant HT-ApcBD in the presence of CpcS, HT-CpcU, CpcT, and *E. coli* proteins for the control (pAED4 extract) and were incubated in the dark at 30°C for 3 hours. A color change from blue-green to blue was observed in the reaction product containing CpcS and HT-CpcU in comparison to no color change found in the control or the reaction containing CpcT. The absorbance and fluorescence spectra of the reaction products are shown in Figures 31 and 32. Figure 31 shows the reaction product containing both CpcS and HT-CpcU had an absorption maximum at 614 nm whereas the control and reaction containing CpcT had an absorption maximum at 645 nm and 647 nm respectively. According to Lundell and Glazer, the expected absorbance of ApcD/ApcB is 642 nm and ApcB alone is 615 nm (1981). The HT-ApcBD product from only the CpcSU reactions (not the control or single lyase reactions) had an absorbance of 614 nm. This is consistent with phycocyanobilin addition to ApcB. Figure 32 illustrates the fluorescence emission spectra of the reactions. The reaction product containing HT-ApcBD, CpcS, and HT-CpcU had an emission maximum of 640.5 nm, while the control and the CpcT reactions were non-fluorescent with an emission 655 nm.

S	-	+	-	+	+
U	-	-	+	+	-
T	-	-	-	-	+

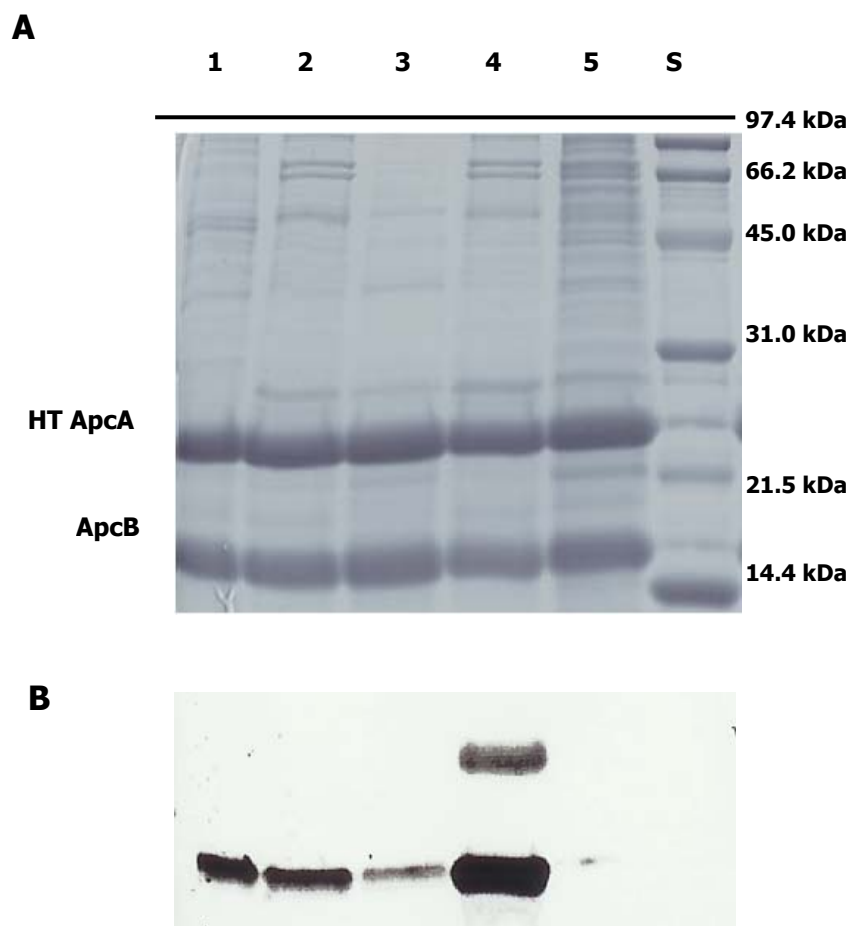


Figure 30: SDS-PAGE analysis of bilin addition reactions to HT-ApcAB. Panel A shows the Coomassie stained SDS-PAGE gel of PcyA bilin addition reaction products. Lane 1 contains HT-ApcAB (control), lane 2 contains CpcS alone, and lane 3 contains CpcU alone. Lane 4 shows the substrate in the presence of both CpcS and CpcU. Lane 5 has CpcS and CpcT. Panel B shows the zinc stained gel before Coomassie staining. As illustrated in lane 4, both CpcS and CpcU are required for covalent attachment of bilins to both the alpha and beta subunits of allophycocyanin.

Therefore, it appears that only in the CpcSU reaction is the phycocyanobilin addition product fluorescent, indicating a stretched conformation. A 20 μ l aliquot of each reaction product was analyzed on a SDS polyacrylamide gel and stained in 100mM ZnSO₄ before Coomassie staining (Figure 33). Lanes 1, 2, and 3 of both panel A and B contain the reaction products of the control, CpcS and HT-CpcU, and CpcT, respectively (Figure 33). The zinc-stained gel (panel B) shows bilin addition to solely the beta subunit in the control, CpcS and HT-CpcU reactions but no bilin addition to the ApcD subunit. The reaction product containing CpcT had a weak fluorescence emission in the zinc stained SDS-PAGE indicating little to no bilin addition by CpcT. However, the reaction products containing CpcS and HT-CpcU had the highest absorbance at 614 nm, and had the highest fluorescence compared to the control and CpcT reactions (Figures 31-32). The absorbance at 614 nm corresponds to the expected absorbance of holo-ApcB at 615 nm as noted by Lundell and Glazer (1981). Figure 33 shows that only the beta subunit is being chromophorylated and corresponding to the observed absorbance. The fluorescence indicates that phycocyanobilin is being held in the proper stretched conformation on the ApcB subunit causing it to emit fluorescence. In this assay system, ApcD was not a good substrate for bilin addition. ApcD is usually expressed by itself and attempting to express it alongside ApcB could potentially interfere with the folding mechanism of the protein. However, independent synthesis of ApcD resulted in low protein expression. Even though the clone had been sequenced to ensure no mutations were present, there was a concern that a mutation had developed where the cysteine-81 had been changed and therefore no bilin addition could take place. This clone had been subjected to extensive thaw after hurricane Katrina. The HT-ApcBD substrate was also subjected to size exclusion analysis to identify its quaternary structure.

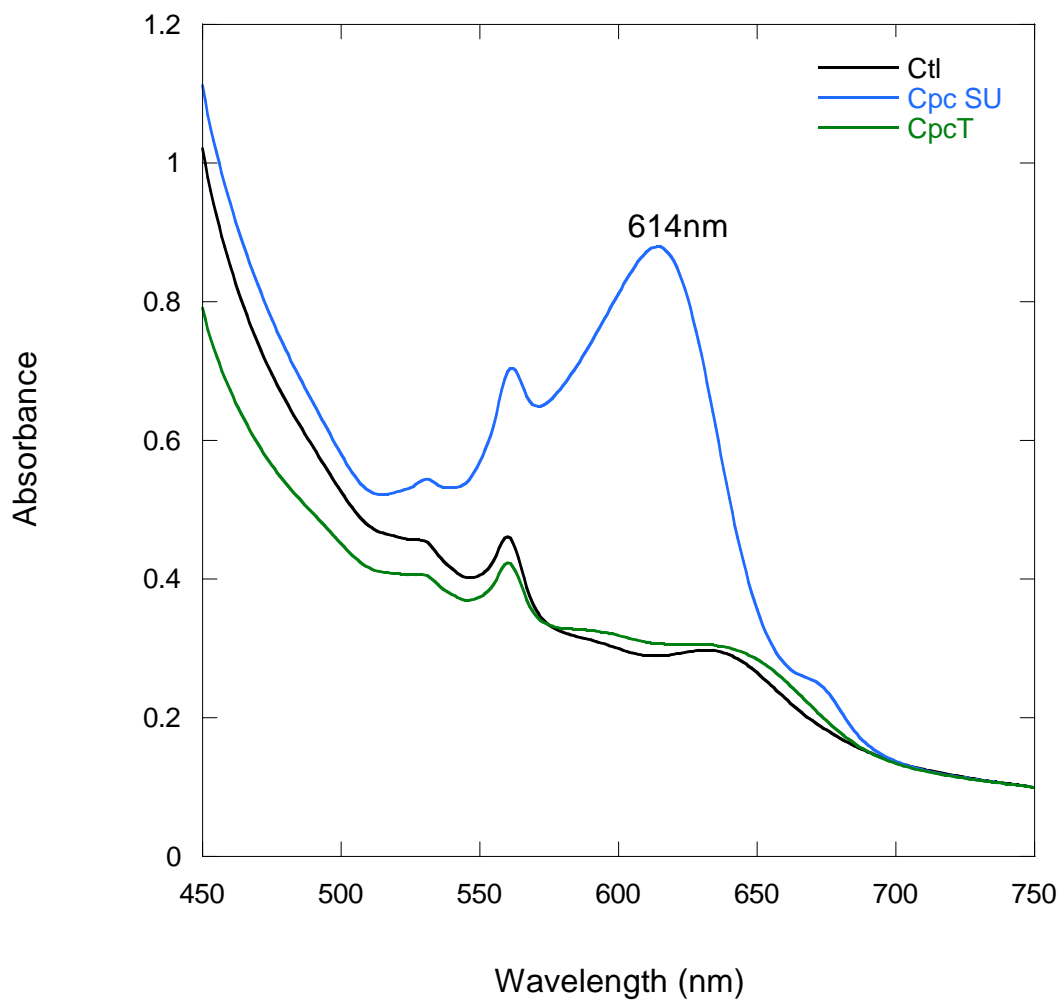


Figure 31: Absorbance spectra of bilin addition reactions to HT-ApcBD in the presence of various lyases. Absorbance emission spectra of HT-ApcBA, CpcS, and CpcU (blue line), CpcT alone (green line), and HT-ApcBD alone (black line).

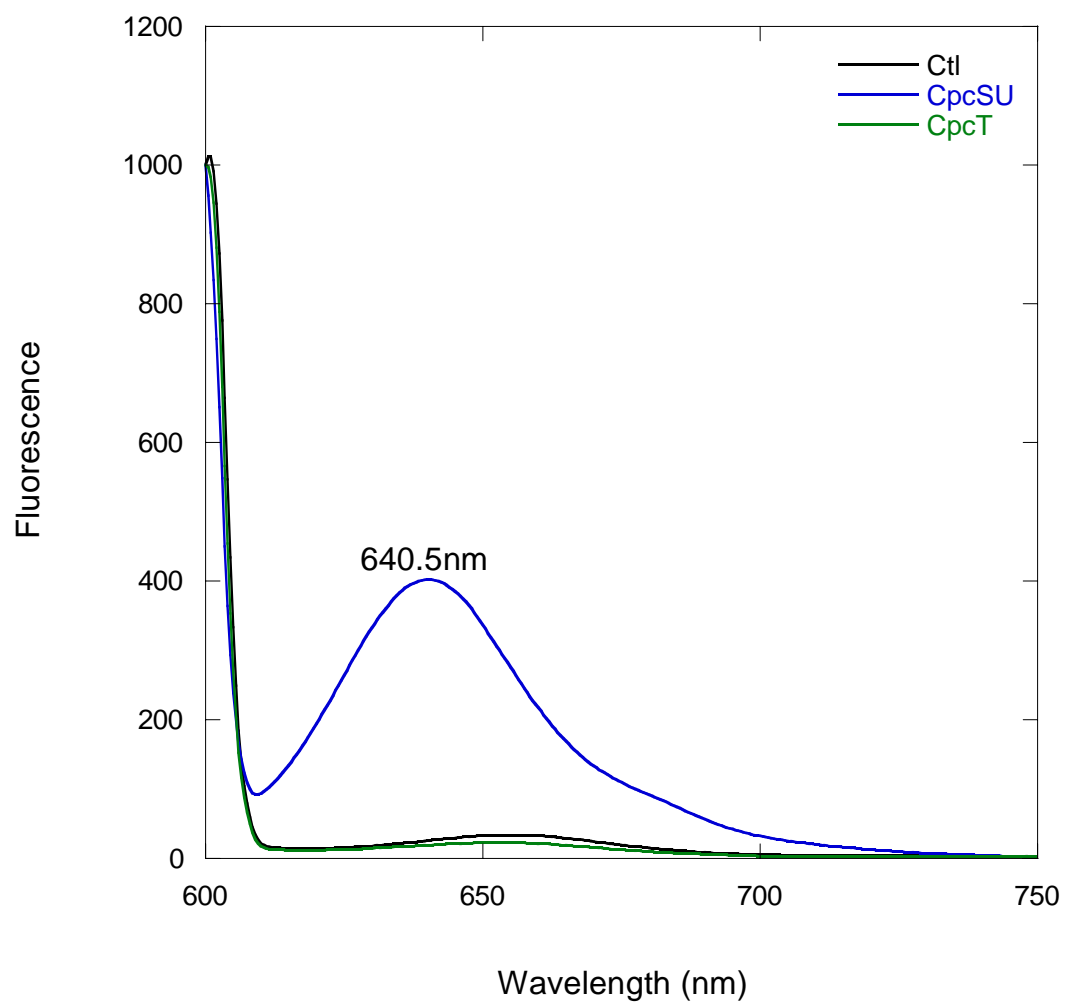
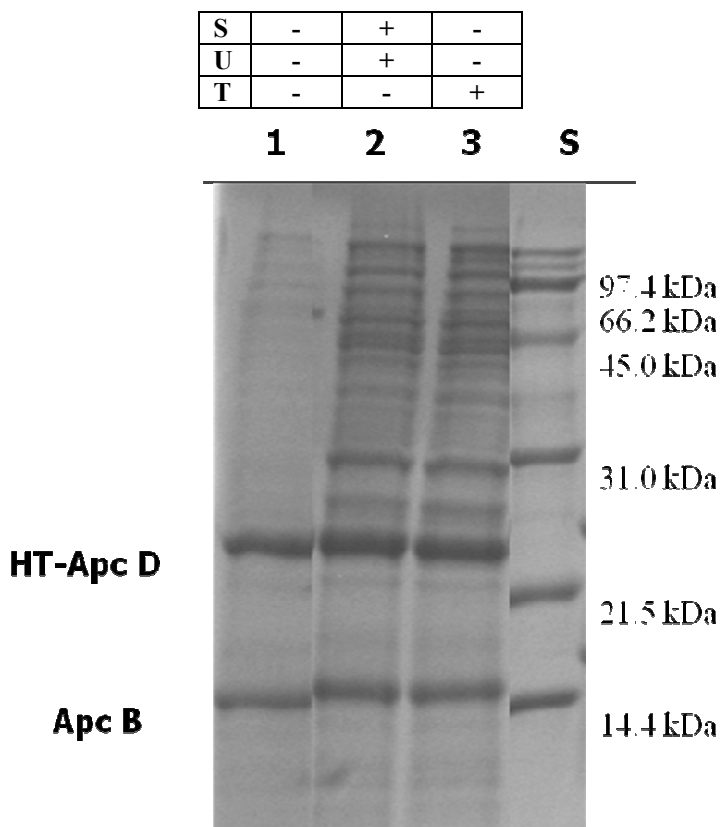


Figure 32: Fluorescence emission spectra of bilin addition reactions to HT-ApcBD and various lyases. Fluorescence emission of HT-ApcBD alone (black line), CpcT (green line), and CpcS and CpcU (blue line). Excitation was at 590 nm.

A



B

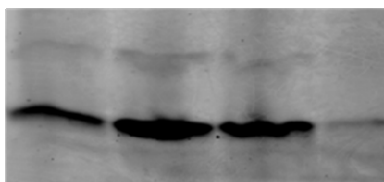


Figure 33: SDS-PAGE of bilin addition reactions to HT-ApcBD. Panel B shows the zinc stained SDS-PAGE gel of the PcyA reaction products. Lane 1 shows the control reaction, lane 2 shows the reaction containing both CpcS and CpcU, and lane 3 shows reaction containing CpcT. As illustrated, only the beta subunit contains a bilin and not the ApcD subunit. Panel A is the coomassie stained gel of the reaction products.

The recombinant substrate was injected onto the size exclusion column as described in the Materials and Methods. Figure 34 shows the chromatograph of HT-ApcBD with a broad peak and retention times of 24.5, 26.4, and 29.48 minutes, respectively. The observed retention times assisted in calculating the molecular weight of the substrate between 223 kDa, 212 kDa., and 44.8 kDa., respectively. The calculated molecular weight of HT-ApcBD is around 39 kDa. The large calculated molecular weights as well as the observed low absorbance and fluorescence illustrate that the majority of the substrate was an aggregated large complex and the majority of HT-ApcBD was not purified in a monomeric form like the HT-ApcAB substrate. In the future, purification techniques may need to be altered to attempt monomeric or trimeric isolation of the HT-ApcBD substrate in order to achieve optimal bilin addition to the cysteine residue.

3.6 Phycocyanobilin addition reactions with HT-ApcBD and HT-ApcFA

In the presence of the PcyA proteins, results showed bilin addition to both the alpha and beta subunits of allophycocyanin in the presence of CpcS and CpcU; however, only the beta subunit was chromophorylated on the substrate HT-ApcBD. Therefore, it was important to determine whether phycocyanobilin addition to the ApcD subunit was possible, whether the cysteine on the protein was present, reduced, and to determine whether the ApcD subunit was properly folded for phycocyanobilin addition. Bilin addition reactions were conducted again in the presence of the substrates HT-ApcBD using purified phycocyanobilin from *Spirulina* sp. as the bilin donor. Figure 35 shows the absorbance spectra of the reaction products of the substrate

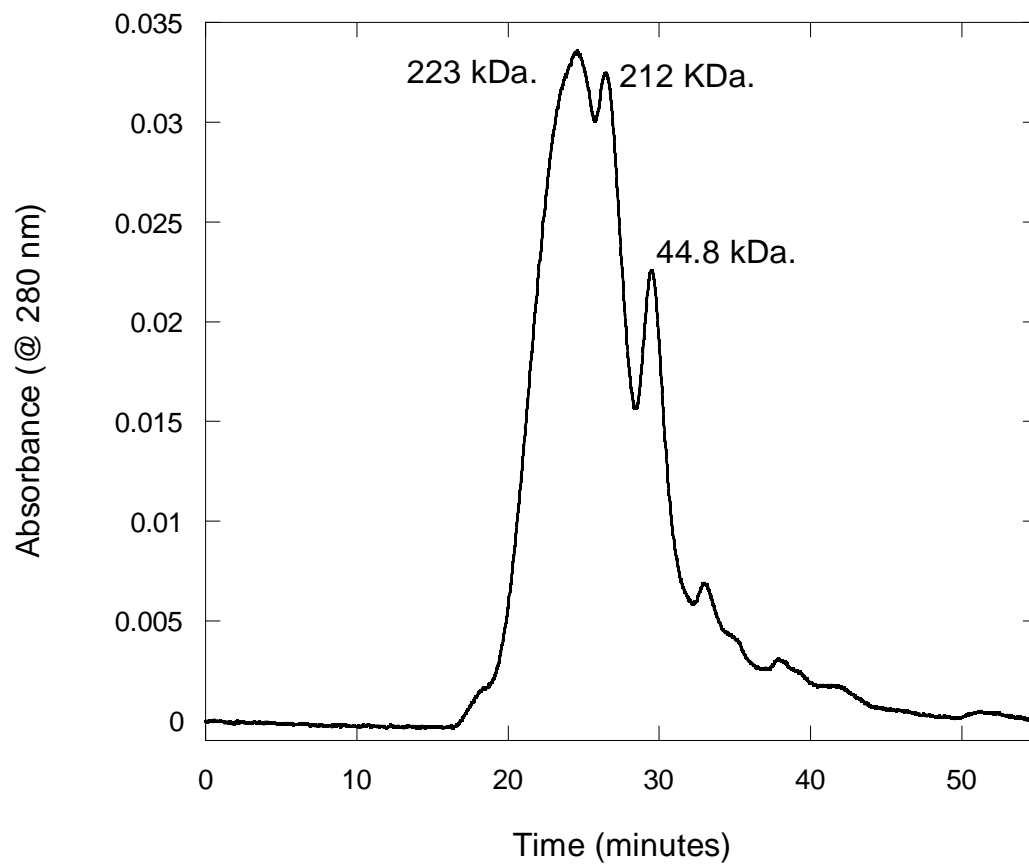


Figure 34: Size Exclusion chromatograph of HT-ApcBD. Figure shows a broad peak at 24.5, 26.4, and 29.483 minutes and the calculated molecular weight of the recombinant protein peaks are 223 kDa., 212 kDa., and 44.8 kDa. respectively.

HT-ApcBD in the presence of phycocyanobilin. A slight change in color from blue-green to blue was observed after 1 hour of incubation in both the CpcT, CpcS, and HT-CpcU reactions. Figure 35 shows the absorbance spectra of both CpcT and CpcSU reaction products with maxima at 639 nm and 637 nm, respectively. The absorbance was much higher in the reaction product containing CpcT in comparison to that of the control, CpcS, and HT-CpcU. The fluorescence emission of the bilin addition reactions containing phycocyanobilin was also taken. Figure 36 shows the fluorescence emission spectra of all the reaction products. All the reaction products had a fluorescence maximum at 655 nm illustrating that none of the reaction products were fluorescent, in comparison to other emission spectra (e.g. See Figure 29). The absorbance and fluorescence emission maxima expected for holo-ApcDB are at ~640 nm and 680 nm, respectively. If both subunits were properly folded and chromophorylated, then the proteins would be extremely fluorescent. To determine which of the subunits contained phycocyanobilin, SDS-PAGE and zinc staining were performed. Figure 37 shows the images of both the zinc stained SDS-PAGE gel and the Coomassie stained SDS-PAGE of the reaction products. Panel B shows zinc-enhanced fluorescence in lanes 1, 2, and 3 containing the control, CpcS and HT-CpcU, and CpcT, respectively. Based upon Figure 37, there is adequate bilin addition to both the ApcB and ApcD subunits of allophycocyanin. This confirms that there is an exposed and reduced cysteine on the ApcD subunit that is capable of bilin attachment. Panel A of Figure 37 shows the Coomassie stained gel of the same phycocyanobilin reaction products. It may be that the recombinant ApcD protein is not properly folded in *E. coli* or that it is more aggregated. Next, it was necessary to determine what lyases were involved in bilin addition to ApcF subunit of allophycocyanin.

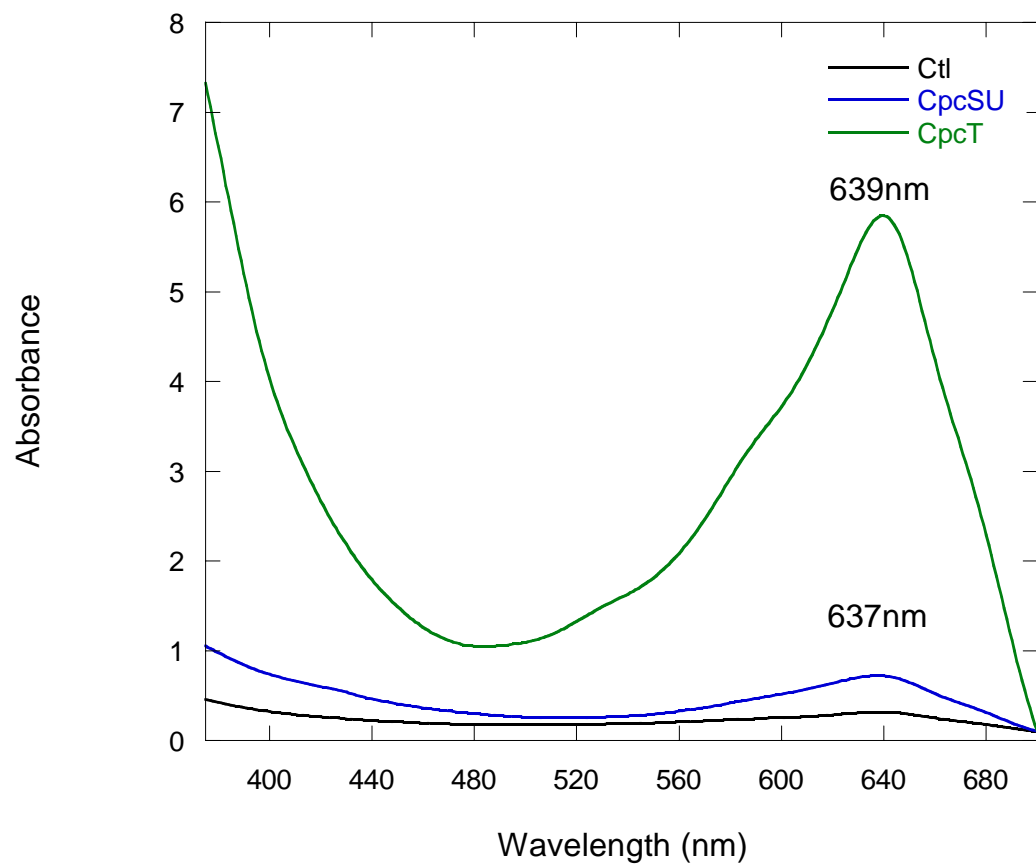


Figure 35: Absorbance spectra of phycocyanobilin bilin addition reactions of HT-ApcBD containing various lyase subunits. Absorbance spectra of reaction products containing CpcT alone (green line), CpcSU (blue line), and the HT-ApcBD control alone (black line).

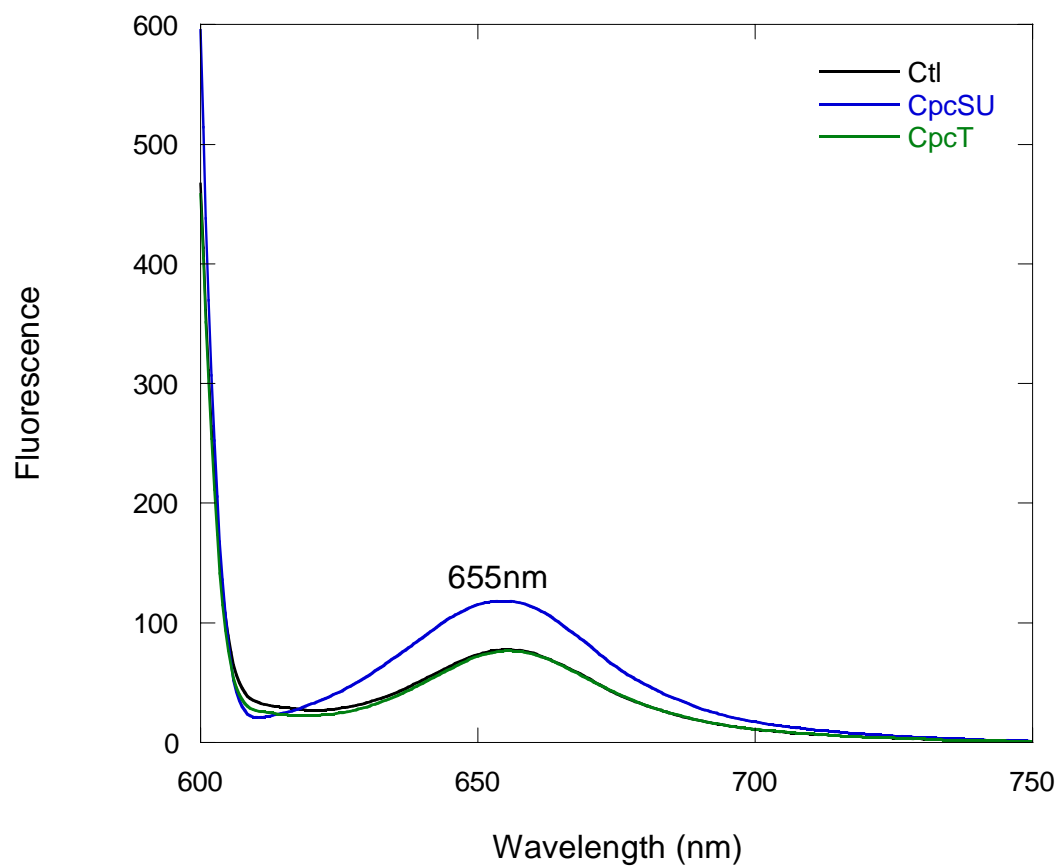
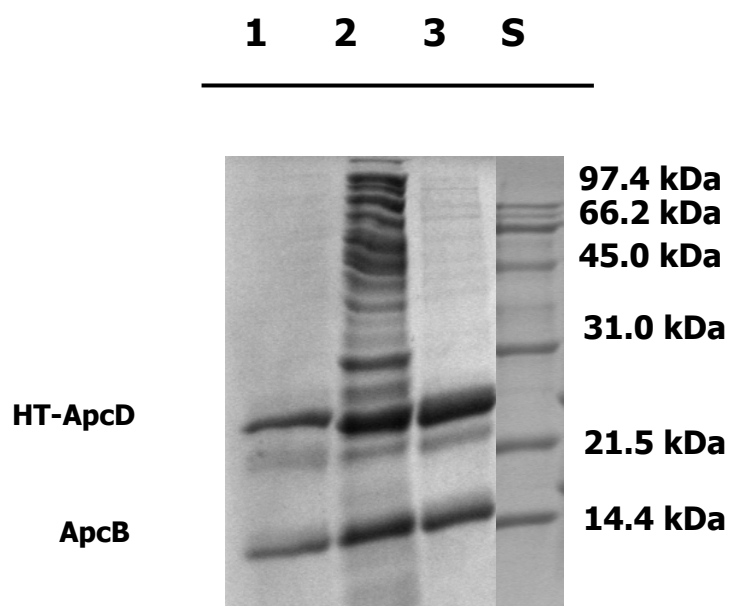


Figure 36: Fluorescence emission of phycocyanobilin bilin addition reactions to HT-ApcBD containing various lyase subunits. Fluorescence emission of reaction products containing CpcSU (blue line), CpcT alone (green line), and HT-ApcBD control alone (black line).

A



B

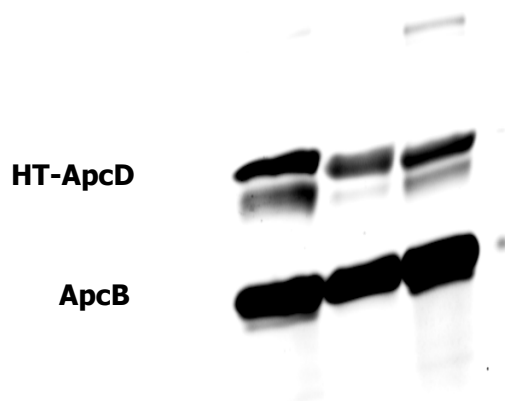


Figure 37: SDS-PAGE of phycocyanobilin addition reactions with HT-ApcBD and various lyase subunits. Panel A shows the Coomassie stained gel of the phycocyanobilin reaction products. Lane 1 shows the control reaction, lane 2 shows the reaction containing both CpcS and CpcU, and lane 3 shows products containing CpcT. Lane 4 is the molecular weight markers with the sizes to the right. The gel in panel A shows bilin addition to both the HT-ApcD and ApcB in the presence of CpcS and CpcU. Panel B shows the zinc stained SDS-PAGE gel.

Bilin addition reaction was conducted using the substrate HT-ApcFA in the presence of purified phycocyanobilin. The reactions were allowed to incubate at 30°C for 3 hours in the dark and a color change was observed after 1 hour of incubation. The absorbance and fluorescence spectra were taken after incubation and centrifugation. Figure 38 shows the absorbance spectra of the reaction products in the presence of the substrate HT-ApcFA. The Figure shows the reaction product containing two small peaks with CpcT present with a maxima at 591 nm and 627 nm while the control reaction had an absorbance of 575 nm and 624 nm. Both reactions had a very low absorbance. The reaction product containing CpcS and HT-CpcU had very little absorbance. The fluorescence emission of each reaction was also taken and was found to be very low as well. All the reaction products were non fluorescent indicating the potential of a bound bilin but in the more cyclic conformation resulting in low fluorescence (Figure 39). The expected absorbance of ApcF is 615 nm and the fluorescence is 642 nm (Ashby and Mullineaux, 1999). Neither the absorbance nor the fluorescence of the phycocyanobilin reaction products possessed these spectroscopic properties. Figure 40 shows the zinc stained and coomassie stained SDS-PAGE of the HT-ApcFA reaction products. Panel B of Figure 40 shows the zinc stained gel where lanes 2, 3, and 4 contain the control reactions, CpcS and HT-CpcU, and CpcT dependent reactions, respectively. The zinc-stained gel shows bilin addition to both the HT-ApcF and ApcA subunits in the presence of the lyase CpcT. However, little bilin addition occurred with CpcS and HT-CpcU or in the control. Although both subunits possessed bilins in the presence of CpcT, the resulting absorbance and fluorescence properties were not what one would expect for holo-ApcF; and it maybe that the phycocyanobilin is not in the proper conformation. The reaction product containing CpcT suggests that CpcT was capable of bringing phycocyanobilin in close proximity to the attachment site but did not assist in the covalent

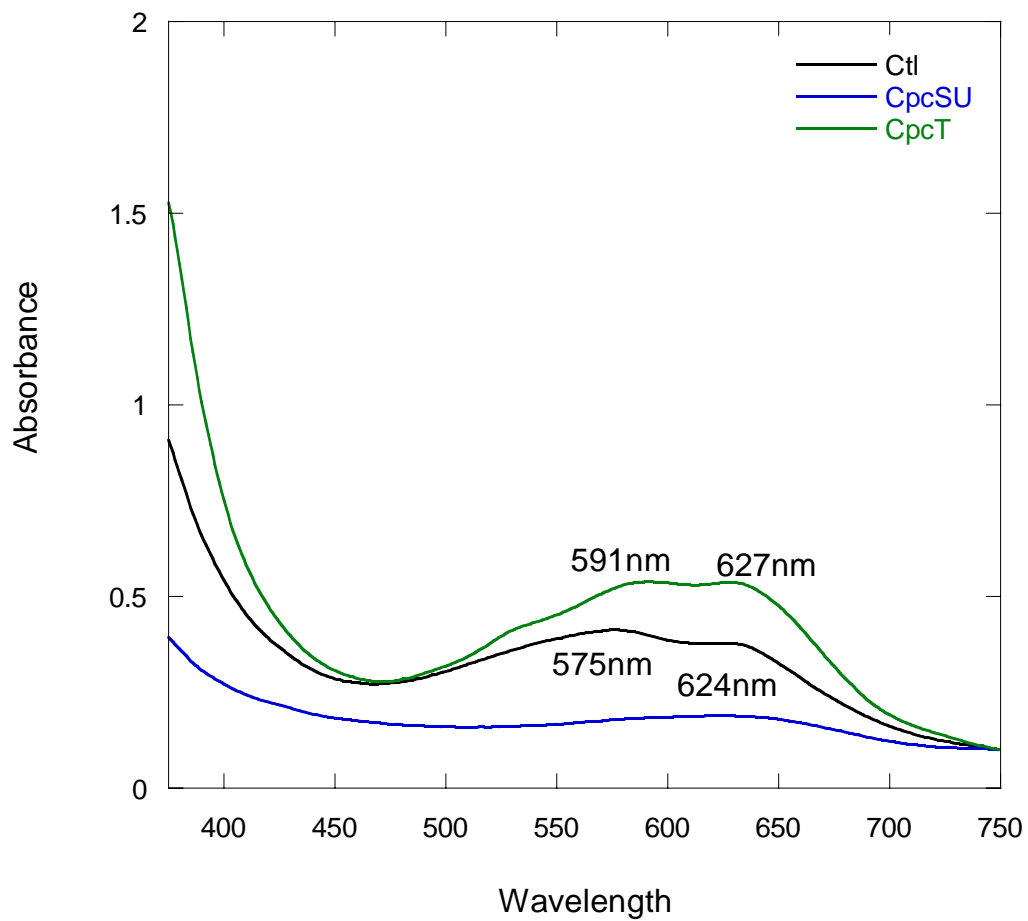


Figure 38: Absorbance spectra of phycocyanobilin bilin addition reactions containing HT-ApcFA, CpcSU, and CpcT. Absorbance spectra of CpcT alone (green line), the control containing only HT-ApcFA (black line), and CpcSU (blue line).

attachment or maintaining the bilin in the stretched conformation. Panel A of figure 40 shows the Coomassie stained gel of the reaction products.

Next, the HT-ApcFA was injected onto the size exclusion column to determine its quaternary structure. Figure 41 shows the chromatograph of the substrate with a retention times of 18.417, 24.06, 24.10, and 40.46 minutes showing that most of the protein was aggregated into a large complex and not purified in small globular format (void volume was 17.45). The calculated mass of monomeric HT-ApcFA substrate is around 245 kDa. Therefore, all of the substrate was aggregated and probably not in the appropriate conformation for bilin addition thereby causing bilin addition to be low (as judged by absorbance and fluorescence). The expected absorbance of ApcF is 615 nm and the fluorescence is 642 nm and neither of these spectral properties was observed during absorbance and fluorescence analysis. As stated earlier, a different purification process may need to be implemented to achieve monomeric or trimeric substrate isolation. Since so much of HT-ApcFA was aggregated (see SEC Figure 41) it is unlikely that it was an appropriate substrate for bilin addition here.

3.7 Transfer reactions containing phycocyanin, HT-ApcAB, CpcS, and CpcU.

Researchers used *in vitro* experiments to show that CpcE and CpcF are a bilin lyase and act together as a heterodimer to add phycocyanobilin to the alpha-84 site of apo-alpha phycocyanin (Fairchild and Glazer, 1994a, Fairchild et al., 1992). In 1992, Fairchild et al. showed that CpcE and CpcF catalyze the reverse reaction of removing phycocyanobilin from

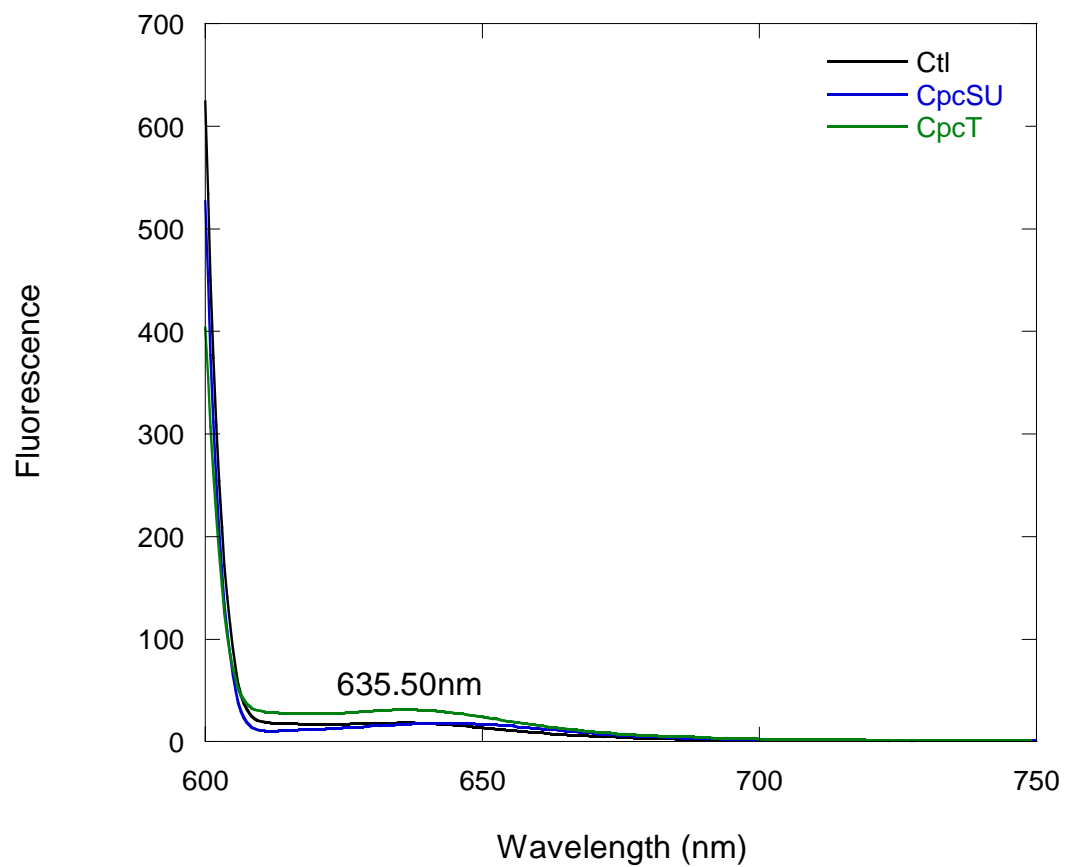
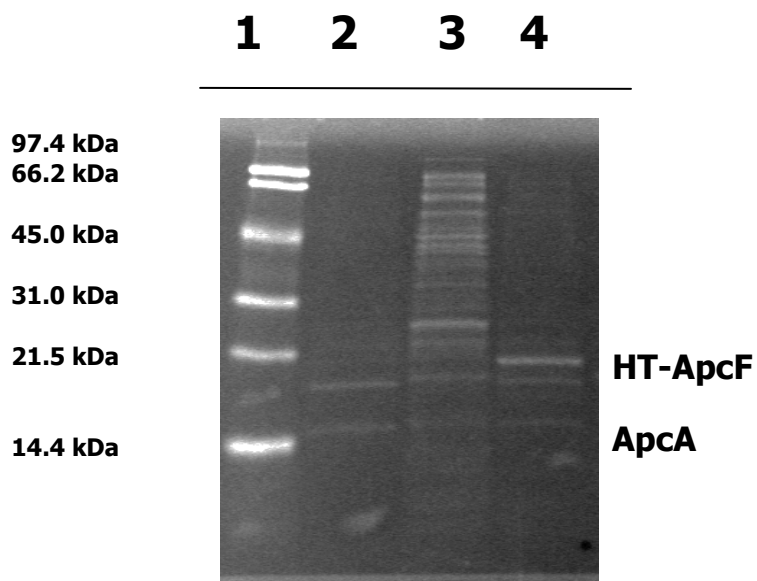


Figure 39: Fluorescence emission of phycocyanobilin bilin addition reactions of HT-ApcFA containing various lyase subunits. Fluorescence emission of reactions containing CpcT alone (green line), CpcSU (blue line), and the control with HT-ApcFA alone (black line).

A



B

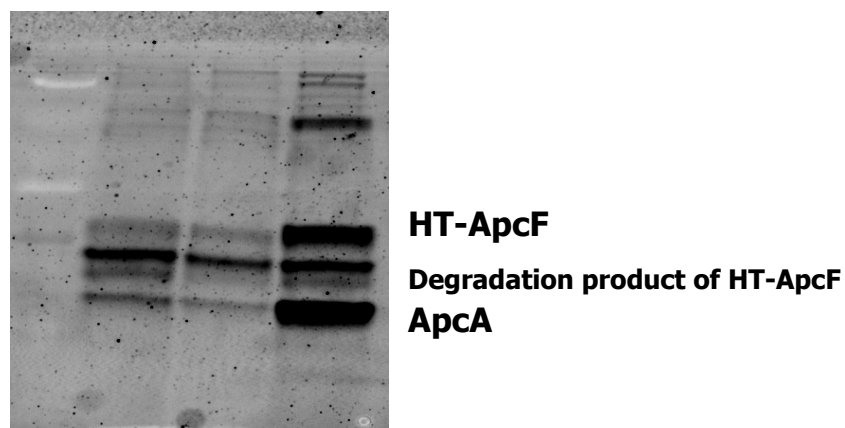


Figure 40: SDS-PAGE analysis of phycocyanobilin bilin addition reactions of HT-ApcFA with different lyase subunits. Panel A shows Coomassie stained gel of the HT-ApcFA phycocyanobilin reaction products. Lane 1 is the molecular weight marker whose sizes are to the left. Lanes 2, 3, and 4 contain the control, CpcS and CpcU, and CpcT respectively. Panel B shows the zinc stained gel prior to Coomassie staining.

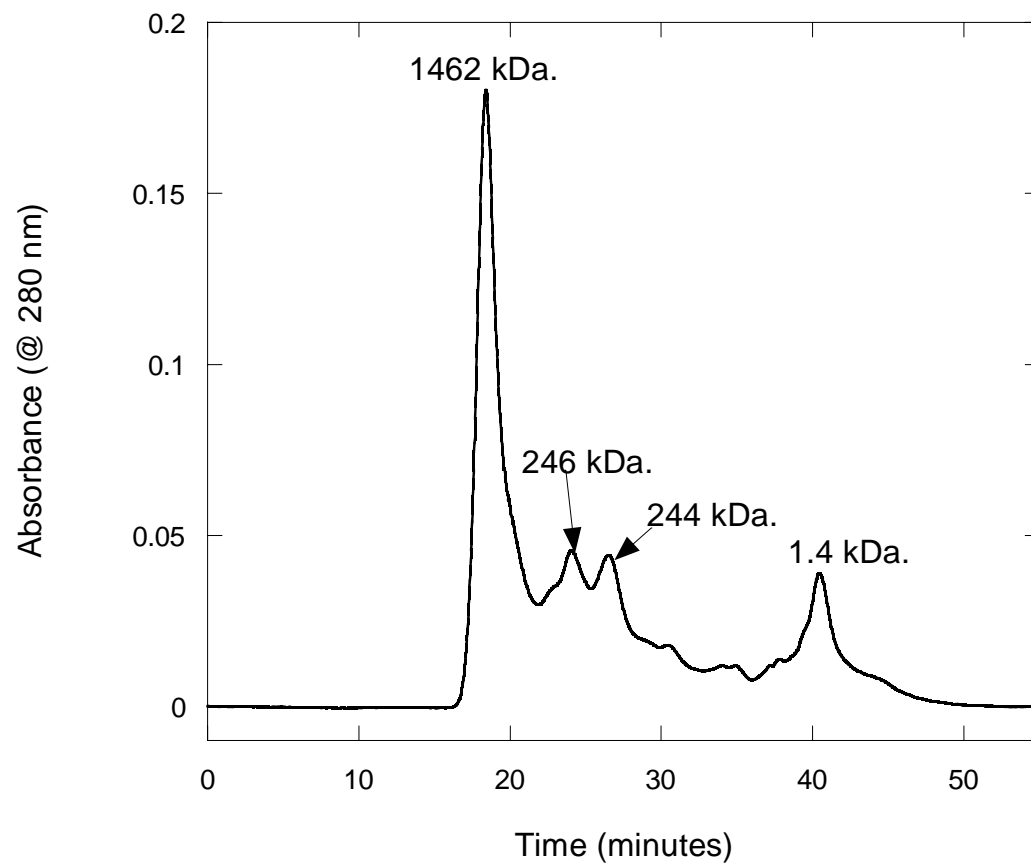


Figure 41: Size Exclusion chromatogram of recombinant HT-ApcFA. Figure show a sharp peak at 18.41; and smaller peaks at 24.06, 24.1, and 40.46 minutes and a calculated molecular weight of 240 kDa.

holo-alpha-phyocyanin and transferring it to resin-bound apo-alpha-phyocyanin. Later, it was determined that phyocyanobilin could be bound by CpcE/CpcF and later transferred to an acceptor such as the alpha subunit of phyocyanin (Zhou et al., 2006). Saunée et al. confirmed that CpcS and CpcU form a 1:1 heterodimeric complex involved in bilin addition to the cysteine-82 residue found on the beta subunit of phyocyanin (2006). The question was raised as to whether the CpcS/U complex like the CpcE/F complex could transfer bilins from holo-protein to apo-protein. The bilin transfer reactions were setup using holo-phyocyanin in the presence of apo-HT-ApcAB, CpcS, HT-CpcU, and/or CpcT and allowed to incubate together on ice. The reactions were introduced to Ni-NTA resin, allowed to interact on ice for 1 hour, at room temperature for 1.5 hours, purified, and analyzed by SDS-PAGE. Figure 42 panel A shows the Coomassie stained SDS-polyacrylamide gel of the transfer reactions. Lane 1 and 4 contain 5 µl and 20 µl of holo-phyocyanin respectively. Lane 2 contains the reaction products from the incubation of holo-phyocyanin, HT-ApcAB, CpcS and HT-CpcU. Lane 3 contains the reaction products from incubation of holo-phyocyanin, HT-ApcAB, and CpcT. Panel B shows the zinc stained SDS-PAGE before Coomassie staining and shows that CpcS, HT-CpcU, and CpcT are not involved in bilin transfer (Figure 42). Only lanes 1 and 4 are fluorescent and show bilins present due to holo-phyocyanin. Lanes 2 and 3 have no bilins attached to HT-ApcA or ApcB and confirms that CpcS, HT-CpcU, and CpcT can not transfer bilins from holo-protein to apo-protein (Figure 42).

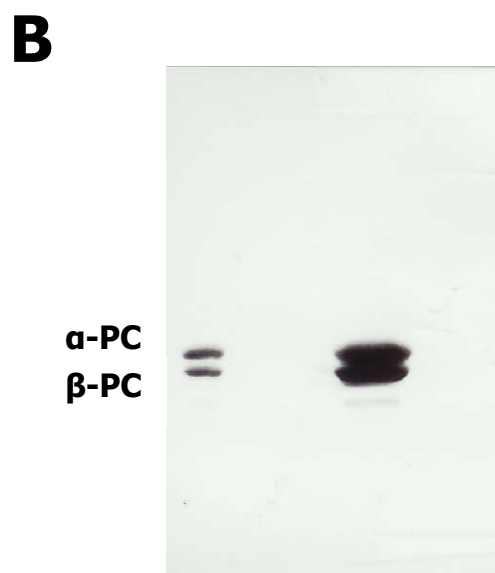
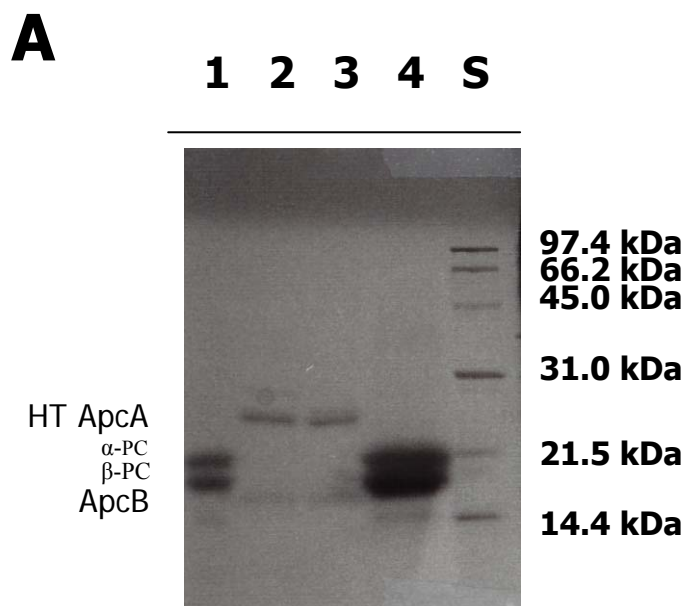


Figure 42: SDS-PAGE analysis of bilin transfer reaction products of HT-ApcAB containing phycocyanin, CpcS, CpcU, and CpcT. Lanes 1 and 4 contain 3.36 mM holo-phycocyanin. Lane 2 contains the reaction products from the incubation of holo-phycocyanin, HT-ApcAB, CpcS and CpcU. Lane 3 contains the reaction product of holo-phycocyanin, HT-ApcAB, and CpcT. Lane S indicates the molecular weight marker.

3.8 Size Exclusion Chromatography of CpcSU and CpcT

Size Exclusion Chromatography (SEC) was used to determine the quaternary structure of the lyases involved in bilin attachment. In 1992, researchers determined that CpcE and CpcF synthesized a lyase involved in bilin addition of phycocyanobilin to cystein-84 of the alpha subunit of phycocyanin. They used *in vitro* reactions to show that CpcE and CpcF acted together as a heterodimer to add bilins to alpha subunit (Swanson et al., 1992; Zhou et al., 1992; Fairchild et al., 1992; Fairchild and Glazer, 1994a). Saunée determined that CpcS and CpcU form a 1: 1 complex, and it was presumed that the lyase complex was a heterodimeric structure like CpcE/F (2006). Recombinant and co-purified CpcS and CpcU were injected onto the size exclusion column and the results are shown in Figure 43. There were two small peaks and one sharp peak with a retention times of 18.76, 30.50, and 32.18 minutes. The first small peak may correspond to substrate eluting in aggregate form thereby signifying a large molecular weight complex. The smaller peaks eluting at 32.18 minutes could be individual subunits of CpcS and HT-CpcU that did not properly associate into a heterodimer. The calculated molecular weight of the complex was determined to be 32.5 kDa. based on the behavior in the SEC analysis (Figure 43). Based upon the results, the lyase complex CpcSU was determined to be a heterodimer as the complex co-purified in a 1:1 complex with a molecular weight near that expected for a heterodimer. The calculated molecular weight of CpcS and HT-CpcU is 45.9 kDa. (Table 4).

Shen et al determined that CpcT was involved in bilin attachment to the beta subunit of phycocyanin at the 153 site (2006). The CpcT lyase was injected onto the column to determine their quaternary structure. Figure 44 shows the chromatograph with a peak that had retention times of 18.53, 29.86, and 30.83 minutes and a calculated weight of the largest peaks were 39

kDa. and 29 kDa. (Figure 44). The results were again compared to the size exclusion standards and their retention times and it was determined that the CpcT lyase had a quaternary structure of a homotrimer based upon the large calculated molecular weight (Figure 44). The peak corresponding to 18.53 minutes is the substrate in a larger aggregate form. The smaller latter peaks eluting from the column at 29.86 and 30.83 minutes may be the substrate in a smaller dimeric and trimeric form, respectively. The actual molecular weight of CpcT is 22.5 kDa.

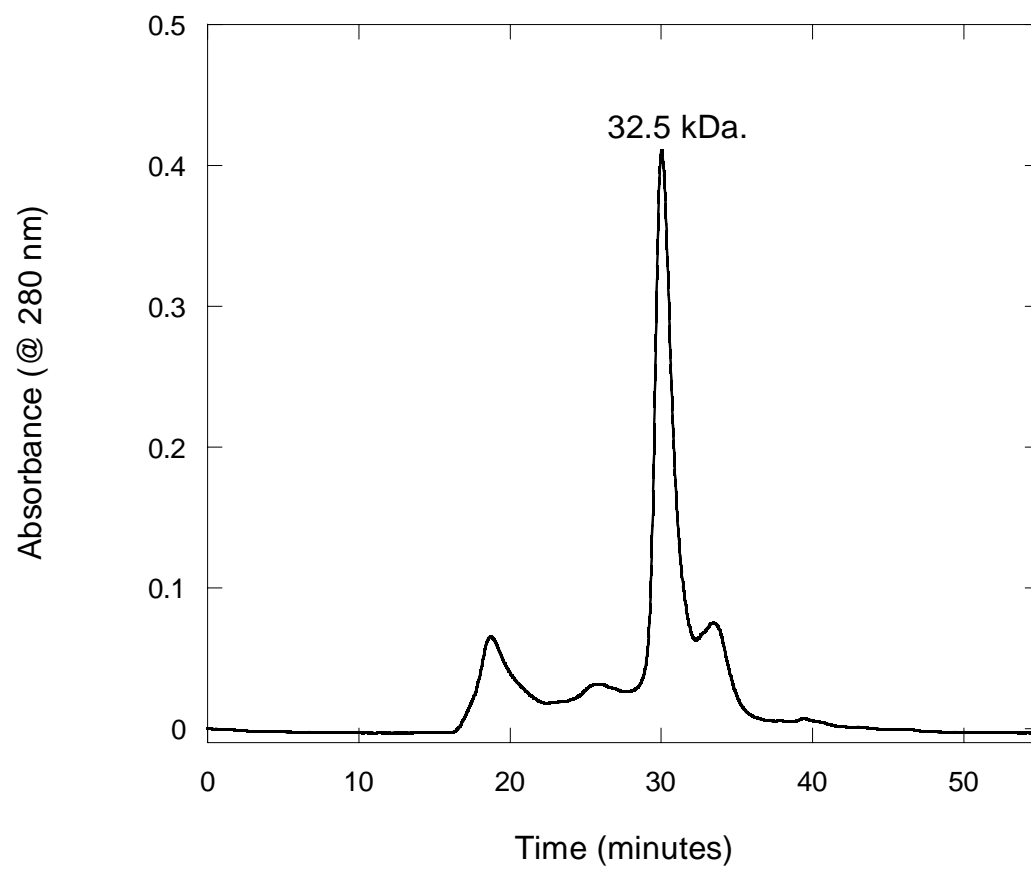


Figure 43: Size Exclusion chromatogram of recombinant CpcS/HT-CpcU copurified. The sharp peak at 30 minutes had a molecular mass of 32.5 kDa. protein was monitored at 280 nm.

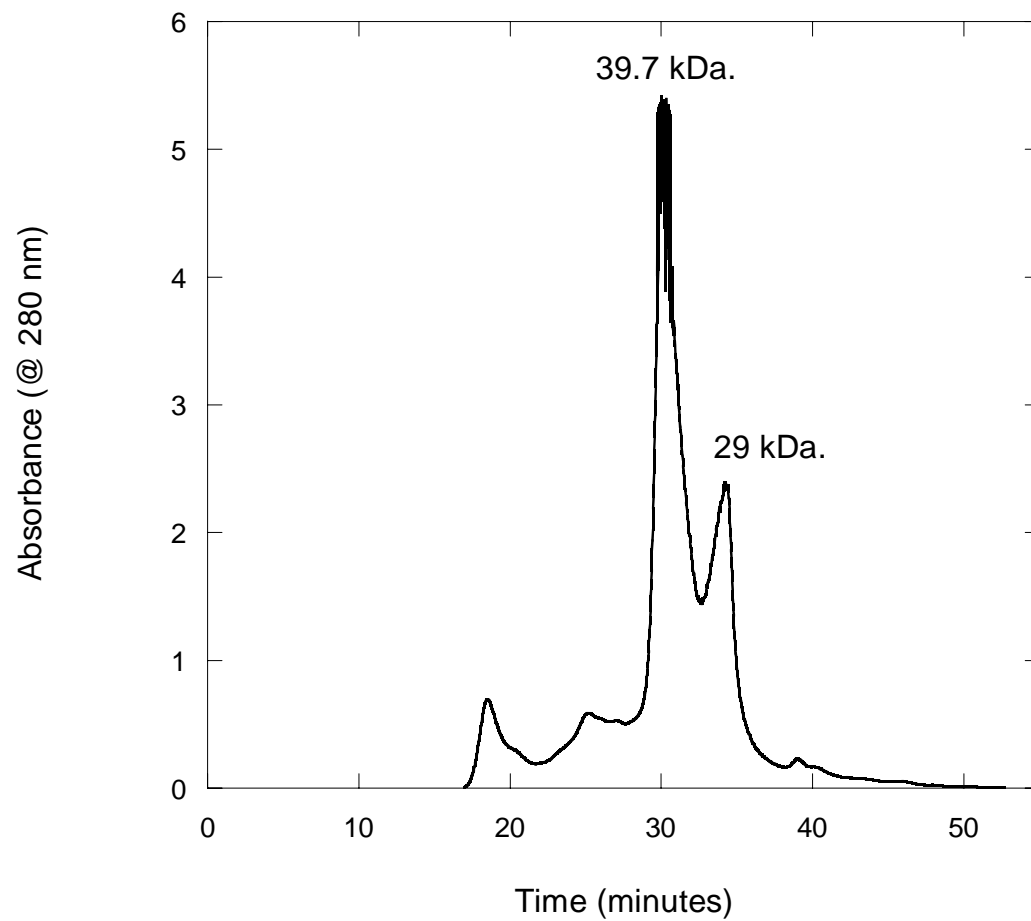


Figure 44: Size Exclusion chromatogram of recombinant CpcT. The peak at 33 minutes had a calculated molecular weight of 39.7 kDa. and 29 kDa. The protein was monitored at 280 nm.

DISCUSSION

The results presented here suggest the CpcS and CpcU proteins are required for allophycocyanin biogenesis. These results are supported by mutagenesis studies illustrating various phenotypic differences based on inactivation of specific genes. Single, double, and triple mutants inactivating *cpcS*, *cpcU*, and *cpcT* were created by Shen et al. in the species *Synechococcus* sp. PCC 7002 (Shen et al., 2006; Shen, Schluchter, and Bryant, manuscript under submission). The mutants exhibited obvious phenotypic differences compared to the wild type. The *cpcS*, *cpcU*, and *cpcT* mutants were yellowish in color as opposed to the normal blue-green color, had an increased doubling time, and produced far less phycocyanin than the wild type. An absorption spectra taken of the *cpcS* and *cpcU* mutants cells showed a reduction in absorption at 620 nm due to the diminished synthesis of phycobiliproteins. *CpcS*, *cpcU*, *cpcS cpcU*, and *cpcS cpcU cpcT* mutants also possessed lower chlorophyll content (~30%) but higher carotenoid content (46-93%) than the wild type. Carotenoids have an absorption maximum that is usually found in the visible region of the spectrum around 400 nm, and their primary function is to screen against violet and blue portions of the spectrum. Their cellular content increases when the genes necessary to produce chlorophyll *a* or phycobiliproteins are inactivated (Wilson et al, 2006). An analysis of the mRNA transcript levels and found no decrease in the *cpcBA* or *apcAB* mRNA transcript levels of the *cpcS* mutant. The analysis determined that the lack of phycobiliprotein synthesis was due to post-transcriptional malfunctions in either the translation or in the chromophorylation of the phycobiliprotein (Shen, Schluchter, and Bryant, manuscript under submission).

The phycobilisomes extracted from wild type *Synechococcus* sp. PCC 7002 were analyzed by sucrose gradient and were found to migrate further through the sucrose than the phycobilisomes from the *cpcS* and *cpcU* mutants, indicating the mutants made smaller phycobilisomes. A large amount of the phycobiliproteins purified from the mutant cells were found in the uppermost fraction of the sucrose gradient indicating disassembled phycobilisomes and free biliproteins. The inactivation of either *cpcS* and *cpcU* significantly impaired phycobilisome assembly. The sucrose gradient from the *cpcS cpcU cpcT* mutant also showed that all phycobiliproteins were found and recovered from the upper portion of the gradient (Shen, Schluchter, and Bryant, manuscript under submission). Fluorescence emission of whole cells of the *cpcS* and *cpcU* mutants showed a decreased amount of phycocyanin (emission at 640 nm) and a decreased amount of allophycocyanin cores (emission at 665 nm). The triple mutant showed even less emission and almost no energy transfer to photosystem II was occurring (Shen, Schluchter, and Bryant, manuscript under submission).

They also found that the *cpcS* and *cpcU* mutants possessed an altered beta phycocyanin subunit that was missing a phycocyanobilin chromophore. Through mass spectroscopy, researchers determined that the *cpcS* mutant possessed some beta subunits of phycocyanin containing two bilins that were covalently bound while other beta subunits from phycocyanin missing a bilin were also detected (Shen, Schluchter, and Bryant, manuscript under submission). A peak corresponding to the mass of one phycocyanobilin (588 Da.) was also detected suggesting some beta-phycocyanin had a bilin in the pocket that was not yet covalently attached. They further verified that a bilin was missing from cysteine-82 on the beta subunit of phycocyanin due to an increase in the fluorescence emission at 620 nm. It was concluded that the energy from the donor bilin found at cysteine-153 was not transferred with 100% efficiency

within the trimer causing this emission increase at 620 nm (Shen, Schluchter, and Bryant, manuscript under submission).

The mutants created by Shen et al. showed defects in the phycobiliprotein biosynthesis, bilin attachment, and phycocyanin production, however, it was important to determine how the mutation affected allophycocyanin biosynthesis. The *cpcS* and *cpcU* mutant whole cells showed a decrease in allophycocyanin fluorescence compared to the wild type whole cells. They also determined through immunoblot analysis with antibodies against ApcB that the *cpcSU* mutants contained only 27%, the *cpcSUV* mutant contained only 13%, and the *cpcSUT* mutant contained only 7% of the wild type ApcB levels (Shen, Schluchter, and Bryant, manuscript in preparation). A *cpcBAC* mutant that makes no phycocyanin contained nearly 70% of wild type levels of the beta-allophycocyanin subunit. These results support the hypothesis that CpcS and CpcU are required for allophycocyanin biosynthesis and that they act together to attach phycocyanobilin to the beta cysteine-82 subunit of phycocyanin and both the alpha and beta subunits of allophycocyanin (Shen, Schluchter, and Bryant, manuscript under submission).

Based upon the results presented here in this thesis and from the above summarized mutant analysis, it can be concluded that heterodimeric lyase CpcSU acts together to covalently attach bilins to the alpha and beta subunits of allophycocyanin (Figure 45). The *in vitro* bilin addition results clearly showed that the alpha subunit of allophycocyanin requires CpcS and CpcU for bilin addition. The beta subunit of allophycocyanin did contain phycocyanobilin without the aid of enzymes, but its fluorescence and absorbance properties were not those of native holo-ApcB (Beck and Sauer, 1992). Therefore, the phycocyanobilin attached to ApcB in the absence of enzymes is probably not interacting with the amino acid residues to maintain a

CpcSU + β -AP + PCB

$3^1(R)$ Cys-81-PCB

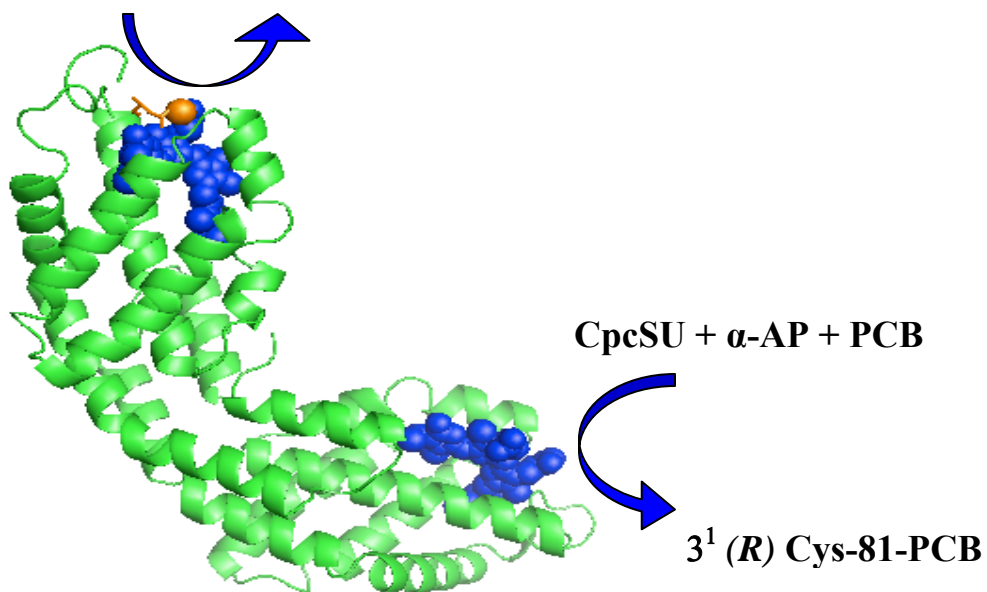


Figure 45: Lyases for alpha and beta subunits of Allophycocyanin.

Illustration of allophycocyanin with CpcSU lyases involved in bilin addition to both the alpha and beta subunits at cysteine-81 residues.

stretched conformation. This has been seen before with recombinant ApcA and ApcB subunits from *Anabaena* sp. PCC 7120 (P. Fung, W.M. Schluchter, A.N. Glazer, unpublished). It has also been observed that bilin addition can occur in the absence of enzymes when the detergent triton X 100 was present; however, the phycocyanobilin also did not adopt the normal stretched conformation and had altered spectroscopic properties (Zhao et al., 2004).

It was also demonstrated for the first time in this thesis that CpcS and CpcU form a 1:1 heterodimer. These results differ from those presented by Zhao et al. who showed that only the CpeS subunit from *Anabaena* sp. PCC 7120 was needed for bilin attachment to the beta subunit of phycocyanin and phycoerthrocyanin at the cysteine-82 position (2006). It appears that many organisms contain a one-subunit type lyase (CpcS-III type) while other organisms use a heterodimeric type. *Synechocystis* sp. PCC 6803 was shown to require both CpcS and CpcU for bilin attachment to the beta subunit of phycocyanin (Miller, 2007). Phylogenetic analysis shows that there are 5 major groups of CpeS type protein in organisms containing phycobiliproteins (Groups A-E)(Figure 46). The organisms in Group A contain phycoerytherin. Organisms that contain a CpcS protein in the C group form 3 clades. *Synechococcus* sp. PCC 7002 CpcS protein clusters with the CpcS-I clade. With one exception, every organism who contains a CpcS-I type lyase also contains a CpcU-type protein in group D. *Anabaena* sp. PCC 7120 CpeS clusters in the CpcS-III clade of group C. Shen et al. hypothesized that perhaps *Synechococcus* evolved a heterodimeric CpcSU lyase by a gene duplication event. An alternative hypothesis is that organism with a CpcS-III like lyase found in *Anabaena* sp. PCC 7120 had a gene deletion event. It is unknown whether the CpeS protein found in *Anabaena* sp. PCC 7120 is a homodimer.

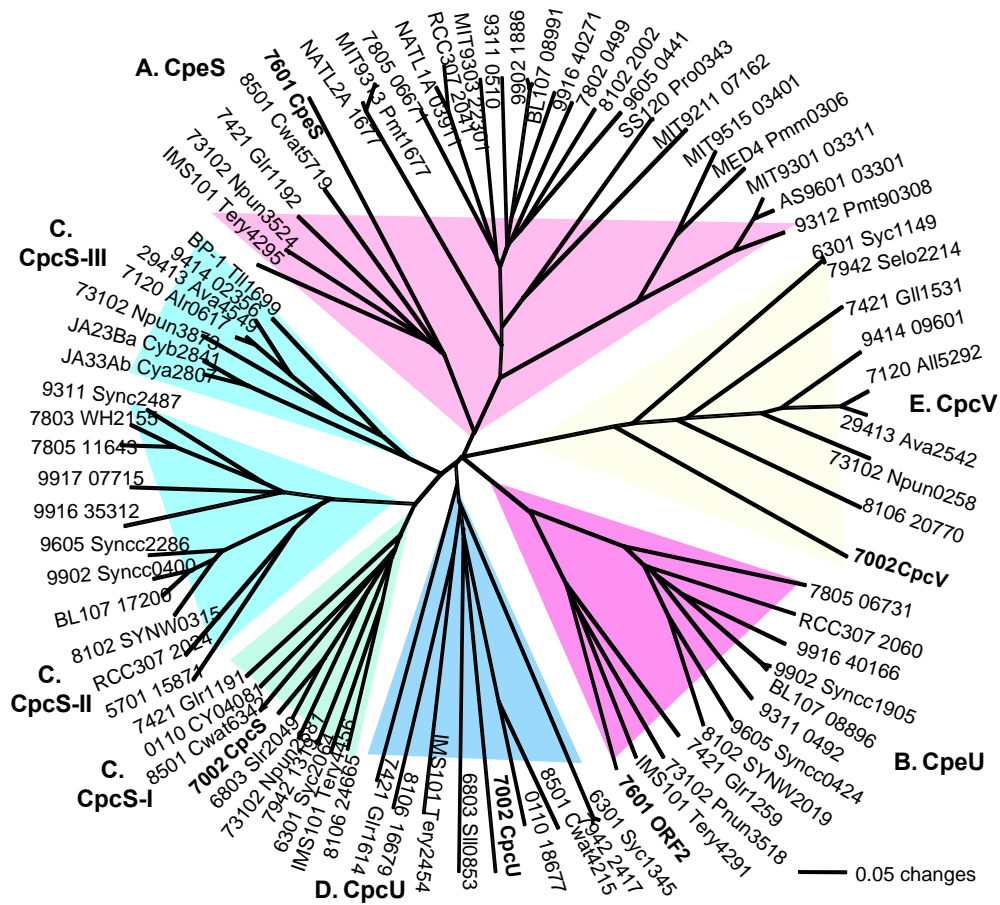


Figure 46: Phylogenetic analysis of the CpcS-I, CpcS-II, CpcS-III, CpcU, CpcV, CpeU and CpeS proteins from different cyanobacteria. Sequence alignments and comparisons were made on CpeS-like homologs from *Synechococcus* sp. PCC 7002 (7002), *Synechocystis* sp. PCC 6803 (6803), *Thermosynechococcus elongatus* BP-1 (BP-1), *Gloeobacter violaceus* PCC 7421 (7421), *Synechococcus elongatus* PCC 7942 (7942), *Synechococcus* sp. PCC 6301 (6301), *Synechococcus* sp. WH8102 (8102), *Synechococcus* sp. WH8103 (8103), *Synechococcus* sp. BL107 (BL107), *Synechococcus* sp. CC9311 (9311), *Synechococcus* sp. CC9902 (9902), *Synechococcus* sp. CC9605 (9605), *Synechococcus* sp. JA-3-3Ab (JA33Ab), *Synechococcus* sp. JA-2-3Ba (JA23Ba), *Synechococcus* sp. WH7803 (7803), *Synechococcus* sp. WH7805 (7805), *Synechococcus* sp. RS9916 (9916), *Synechococcus* sp. RCC307 (RCC307), *Synechococcus* sp. RS9917 (9917), *Synechococcus* sp. WH5701 (5701), *Crocospaera watsonii* WH8501 (8501), *Nostoc* sp. PCC 7120 (7120), *Nostoc punctiforme* PCC 73102 (73102), *Anabaena variabilis* ATCC 29413 (29413), *Trichodesmium erithraeum* IMS101 (IMS101), *Lyngbya* sp. PCC 8106 (8106), *Cyanothece* sp. CCY 0110 (0110), *Nodularia spumigena* CCY9414 (9414), *Calothrix* PCC 7601 [*Fremyella diplosiphon*] (7601), *Prochlorococcus marinus* SS120 (SS120), *Prochlorococcus marinus* MIT9312 (9312), *Prochlorococcus marinus* MIT9313 (MIT9313), *Prochlorococcus marinus* MIT9301 (MIT9301), *Prochlorococcus marinus* MIT9303 (MIT9303), *Prochlorococcus* sp. AS9601 (AS9601), *Prochlorococcus marinus* MIT9515 (MIT9515), *Prochlorococcus marinus* MIT9211 (MIT9211), *Prochlorococcus marinus* SS120 [CCMP1375] (SS120), *Prochlorococcus marinus* NATL1A (NATL1A), *Prochlorococcus marinus* NATL2A (NATL2A). The sequence alignment was made using the ClustalW feature of MacVector version 9.0 (MacVector, Inc., Cary, NC). The phylogenetic tree was generated using the PAUP phylogenetic analysis program. (Shen, Schluchter, and Bryant, manuscript under submission).

In 2007, Zhao et al categorized and tested two *cpeS*-like genes termed *cpeS1* (alr0617) and *cpeS2* (all5292); and two *cpeT*-like genes termed *cpeT1* (all5339) and *cpeT2* (alr0647) in *Anabaena* sp. PCC 7120 (Figure 46). He showed that only CpeS1 (alr0617) was capable of catalyzing the attachment of phycocyanobilin to all allophycocyanin subunits and that the remaining three proteins were incapable of phycocyanobilin addition to allophycocyanin subunits. Fluorescence emission via zinc stained SDS-PAGE and equivalent mass spectroscopy of tryptic peptides confirmed the attachment of bilins to the cysteine-81 site of ApcA, ApcB, ApcD, and ApcF. Therefore, it appears lyases are required for phycocyanobilin addition to ApcD and ApcF. It is likely that CpcSU will be required for this process in *Synechococcus* sp. PCC 7002 ApcD and ApcF also.

Based upon the results presented in this research, it is also clear that CpcS and CpcU are unable to transfer bilins from phycocyanin to apo-allophycocyanin. The transfer reactions presented in this research show that when phycocyanin is incubated with the apo-protein HT-ApcAB in the presence of CpcS and CpcU there is no bilin transfer (Figure 42). This illustrates that CpcS and CpcU are a different type of lyase from that of CpcE/F which can actively catalyze the reverse reaction by removing phycocyanobilin from holo-alpha-phycocyanin and transferring it to resin bound apo-alpha-phycocyanin (Fairchild et al., 1992). This result was also confirmed with CpeS from *Anabaena* sp. 7120 (Zhao et al, 2007).

The results presented here were unable to positively confirm that CpcT was involved in bilin addition to allophycocyanin and or its biosynthesis. Reactions consisting of phycocyanobilin, the substrates HT-ApcBD and HT-ApcFA, and the lyase CpcT show some bilin attachment to the alpha and beta subunits as well as the ApcD and ApcF subunits based upon the zinc stained SDS-PAGE. The bilin addition reactions were conducted with *E. coli*

whole cell extracts containing HT-ApcBD and HT-ApcFA. The hypothesis is that the presence of a chaperone protein found in *E. coli* may be involved in channeling the bilin in close proximity to the cysteine binding site. Another hypothesis is that these proteins are too aggregated to associate properly with lyases and that any phycocyanobilin that attaches is not appropriately interacting with the phycobiliprotein subunit as evidenced by the lack of fluorescence. It has been confirmed that CpcT is the lyase necessary for bilin attachment to the cysteine-153 on the beta subunit of phycocyanin; however the absorbance and fluorescence spectra of the allophycocyanin reaction products containing allophycocyanin in the presence of CpcT are inconclusive (Shen et al., 2006).

The question is why two different lyases are necessary for bilin attachment to the alpha and beta subunits of phycocyanin and allophycocyanin? One possibility is that the bilin attachment sites on the beta subunits are buried or situated toward the cavity of the protein as opposed to being openly exposed on the outside of the protein. Extensive research has been conducted on the protein structure of each major phycobiliprotein and results confirm that bilin attachment sites have different stereochemistry. The C₃¹ carbon of phycocyanobilin that attaches at the cysteine-82 site on beta-phycocyanin has a R stereochemistry; whereas the C₃¹ carbon that attaches at the cysteine-153 on beta-phycocyanin site has an S stereochemistry on phycocyanin (Figure 9; Shen et al., 2006; Zhao et al., 2006). The phycocyanobilin at the cysteine-82 and -81 site on all phycobiliproteins has R stereochemistry.

Unfortunately, past experiments have been unable to determine the exact function of CpcV. Results show that expression of the recombinant protein is possible; however, PcyA and phycocyanobilin *in vitro* bilin addition reaction assays were unable to confirm whether CpcV is a lyase involved in bilin attachment or phycobiliprotein biosynthesis. Shen et al. found that Group

E of CpcS-III (CpcV containing) clade includes divergent species and organisms that do not exhibit various pigmentation patterns (Figure 45). Therefore, the function of CpcV could not be inferred. Shen et al. determined that the CpcV protein of *Synechococcus* sp. PCC 7002 was a part of this clade (Shen, Schluchter, and Bryant, manuscript under submission). Results of the *cpcV* mutant found no obvious phenotypic differences and possessed spectroscopic properties equivalent to the wild type. However, the *cpcSUV* mutant had only half the level of ApcB when compared to the *cpcSU* mutant; suggesting CpcV may be involved in allophycocyanin biosynthesis when *cpcS* and *cpcU* are inactivated. Sequence analysis confirmed that CpcV was highly homologous to CpcS and CpcU but possessed a 22 amino acid truncation at the C-terminus and a 7 amino acid truncation in the middle of the protein perhaps causing structural and functional differences between CpcS and CpcU. Further experiments need to be conducted to ascertain whether CpcV in fact has a function and what that primary function is.

FUTURE WORK

A lot of information is still unknown about the enzyme activity of each new found lyase. Enzyme kinetic and activity assays should be conducted and quantitated. Also determination of how much substrate and concentrated enzyme is needed to achieve similar conditions as those found *in vivo* should be conducted. Another area of interest is the use of the multi-plasmid system pAT101 (containing *pcyA* and *hol*) with the expression plasmid HT-*apcD/apcB*-pET100 and HT-*apcF/apcA*-pET100 to determine *in vivo* bilin addition capabilities in the presence of the lyase complex CpcSU. Also, within the *in vitro* bilin addition reactions, to identify any necessary cofactors, optimal condition for maximum protein expression of both subunits of the substrate, as well as *in vivo* bilin addition requirements. Other important future experiments include determining the function of CpcV and whether it is involved in bilin addition. As stated earlier, some bilin addition assays were conducted in the presence of CpcV but results were inconclusive. Future work on cloning, expression, and purification of the anchor polypeptide ApcE will be important to determine if lyases are required to attach a bilin to this protein. It will be necessary to determine the appropriate absorbance and fluorescence spectra and compare this to previous research conducted on the subunit.

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Vita

Shervonda Williams was born in Houston, Texas. She received her B.S. in Biology from University of New Orleans in New Orleans, Louisiana. She lives in Kenner, Louisiana.