Sll0147 IN SYNECHOCYSTIS SP. PCC 6803:

THE CruA LYCOPENE CYCLASE

A Thesis in
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by

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ABSTRACT

The sl0147 gene in the cyanobacterium Synechocystis sp. PCC 6803 was studied to determine its role in carotenoid biosynthesis. Through complementation of a cruA mutant of Synechococcus sp. PCC 7002, and by in vitro assays with purified protein, the sl0147 product was shown to be a lycopene cyclase, converting lycopene, 1-hydroxy-lycopene and γ-carotene into β-carotene. Sl0147 belongs to the newly identified family of lycopene cyclases, the CruA family, so Sl0147 has been re-designated as CruA of Synechocystis sp. PCC 6803.

The CruA of Synechococcus sp. PCC 7002 was previously and indirectly shown to be a lycopene cyclase in the carotenoid biosynthetic pathway of that organism. Sequence comparisons show that the sl0147 product of Synechocystis sp. PCC 6803 shares 65% amino acid sequence identity with the CruA of Synechococcus sp. PCC 7002. A cruA deletion mutant of Synechococcus sp. PCC 7002, designated as Synechococcus sp. PCC 7002 ΔcruA::aacC1, segregated fully. Unlike the wild-type strain, the ΔcruA::aacC1 mutant accumulated lycopene, 1-hydroxy-lycopene and γ-carotene. By using the heterologous gene expression system in Synechococcus sp. PCC 7002 based on its endogenous plasmid pAQ1, two complementation strains were obtained based on the Synechococcus sp. PCC 7002 ΔcruA::aacC1 strain, named 7002_pAQ1::6803cruA-N(His)$_{10}$ and 7002_pAQ1::6803cruA-C(His)$_{6}$ respectively. Pigment analysis of these two strains showed the same pigments profile as that of the wild-type, demonstrating the sl0147 product had lycopene cyclase activity in vivo. In contrast, expression of these same proteins in Escherichia coli strains producing lycopene showed no activity. These data strongly suggested that E. coli strains were unable to produce an essential cofactor for activity of the sl0147 gene product.

To obtain additional information about the Sl0147/CruA protein, CruA was purified from Synechococcus sp. PCC 7002 expression strains by metal chelation chromatography of
detergent solubilized membranes. The as-purified Sll0147 product was yellow-green in color due to the presence of non-covalently bound chlorophyll $a$ and pheophytin $a$. Small amounts of lycopene and $\beta$-carotene were also detected in the as-purified protein fractions. Gel electrophoresis, immunoblotting, and mass spectrometry identified the yellow-green protein as the product of $sll0147/cruA$.

The as-purified CruA protein was able to convert lycopene and $\gamma$-carotene to $\beta$-carotene \textit{in vitro}. These enzymatic data provide the first direct evidence that Sll0147 and by extension other CruA proteins are lycopene cyclases capable of synthesizing $\beta$-carotene from lycopene. The findings reported in this thesis fill a major gap in the biosynthetic pathway of carotenoids in \textit{Synechocystis} sp. PCC 6803 specifically and cyanobacteria in general.
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Chapter 1

Introduction

1.1 Cyanobacteria

Cyanobacteria are the only prokaryotes that perform oxygenic photosynthesis (1). They use the light energy to convert carbon dioxide into organic materials for growth. All but one currently described cyanobacterium are photoautotrophs (2). So far, there are only two described mechanisms of phototrophy, the collection of light energy and its conversion into chemical energy. The first one is the retinalophototrophy, which employs bacterio- or proteo-rhodopsins, retinal-binding proteins act as light-driven proton or chloride pumps that respond to light stimuli. Organisms that use this mechanism are phototrophs but have not yet been shown to be photosynthetic, because retinal-containing proteins do not mediate electron transfer reactions. The other mechanism is the called “chlorophototrophy,” which depends on photochemical reaction centers (RCs) that contain (bacterio)-chlorophyll [(B)Chl] (2). Cyanobacteria employ this chlorophototrophic mechanism for their photoautotrophic growth. They have Chl a-containing photosystem (PS) I and PS II and also light-harvesting phycobilisomes, which are associated mainly with PS II. They have both type-1 RCs and type-2 RCs, having [4Fe-4S] clusters and quinones as terminal electron acceptors respectively. Within these RCs, the harvested light energy initiates electron transfer through oxidation of a chlorophyll and reduction of an electron acceptor, and secondary electron transfer produces proton-motive force that can be coupled to ATP synthesis (2). Evidence has shown that cyanobacteria are the progenitor(s) of the plant
plastids (1), so cyanobacteria and their photosynthesis mechanisms are widely studied to shed light on the higher plant photosynthesis studies.

1.2 *Synechococcus* sp. PCC 7002

*Synechococcus* sp. PCC 7002 is a unicellular, euryhaline/marine cyanobacterium commonly used as a model organism for studies of cyanobacteria. Compared to other reported cyanobacteria, it has a faster growth rate with a doubling time of ~3 hours under optimal growth conditions (3). It is naturally transformable with high transformation efficiency (4). It can also grow photoheterotrophically with glycerol supplementation (5). Its complete genome has been sequenced and is comprised of a relatively small 3.0 mega-basepair (Mbp) chromosome and 6 endogenous plasmids, pAQ1, pAQ3, pAQ4, pAQ5 pAQ6 and pAQ7, which range in size from 4.8 to 176 kbp. The smallest plasmid, pAQ1, which has the highest copy number, has been used to construct an efficient gene expression system in *Synechococcus* 7002 (6).

1.3 *Synechocystis* sp. PCC 6803

In 1996 the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 became the first photosynthetic organism to have its 3,573,470 bp genome completely sequenced (7). This non-nitrogen (N₂)-fixing cyanobacterium and its close relatives are common soil and freshwater organisms (1). *Synechocystis* sp. PCC 6803 has been widely used as a model organism for genetic and physiological studies of oxygenic photosynthesis, due to its natural transformability by exogenous DNA (8) and its relatively rapid heterotrophic growth at the expense of glucose (9).
1.4 Carotenoids

Colored carotenoids are a major class of lipophilic terpenoids (10). They were first isolated from food materials in the early 19th century, and the first complete structures were elucidated in the 1930’s (11). These natural pigments can contain a linear array of up to 13 conjugated double bonds, which having the formal repeating unit of –CH=CH-CCH3=CH- (12). This conjugated bond system, i.e., the chromophore, gives carotenoids their characteristic colors, which range from light yellow to deep red (10).

Carotenoids are widely distributed in both BChl-containing anoxygenic photosynthetic and Chl-containing oxygenic photosynthetic bacteria, and also in a variety of non-photosynthetic bacteria (10). In both retinalophototrophs and chlorophototrophs, carotenoids are thought to play at least three diverse roles, including light harvesting, photoprotection, and structure stabilization (13). Carotenoids can function as accessory light-harvesting pigments, absorbing light in the blue-green and yellow regions of the spectrum, and then transferring that energy to neighboring (B)Chl molecules [e.g., see (14)]. Another example are the photosynthetic dinoflagellates, which use about equal amounts of carotenoids and chlorophylls to harvest light (15). Carotenoids with nine or more conjugated double bonds can also protect the photosynthetic apparatus by directly quenching both triplet excited-state BChls and singlet oxygen, due to their relatively low-lying triplet energy states (14) (16) (17). In many light-harvesting antenna complexes and reaction centers, carotenoids are surrounded by aromatic residues or Chls, and thus carotenoids play an important role in structural stabilization of such proteins (18). Other members of the carotenoids, the xanthophylls, have been shown to be essential structural components of the chlorophyll a/b light-harvesting complex in higher plants and algae (19).

Of the more than 700 chemically distinct carotenoids that have been described, the most commonly occurring ones are hydrophobic tetraterpenoids that contain a C40 methyl-branched
hydrocarbon backbone (10). In cyanobacteria, carotenoids are integral components of both PS I and PS II, and the dominant carotenoid associated with the photosystems is β-carotene (20) (21) (22). In most cases, these carotenoids exist within van der Waals contact with one or more Chl molecules (18). β-Carotene has also been identified as an intermediate involved in secondary electron transfer pathways in PS II core complexes, which are active when oxygen evolution is blocked or when the complexes are placed at low temperature (23) (24). Most cyanobacteria synthesize β-carotene (Appendix C, 25). In cyanobacterium *Synechocystis* 6803, β-carotene is required for the assembly of PS II (26).

### 1.5 Biosynthesis of carotenoids

Various modifications in carotenoids have led to their various specific functions. However, they all derive from the universal isoprenoid precursors isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAP) from one of two pathways, the mevalonate pathway and the non-mevalonate (2C-methyl-D-erythritol 4-phosphate) pathway (27). All cyanobacteria utilize the latter pathway through 2C-methyl-D-erythritol 4-phosphate as an intermediate to produce both IPP and DMAP (27), which are further used to produce the C<sub>20</sub> precursor, geranylgeranyl pyrophosphate (GGPP), as shown in Figure 1-1. One IPP and one DMAP are first condensed to produce geranyl pyrophosphate (GPP), and two additional IPP molecules are condensed with GPP into farnesyl pyrophosphate (FPP) and then GGPP (28).
Figure 1-1: Synthesis of geranylgeranyl pyrophosphate (GGPP), the C\textsubscript{20} precursor of the C\textsubscript{40} carotenoids (25).

In cyanobacteria, the biosynthesis of the C\textsubscript{40} carotenoids starts by the condensation of two molecules of the C\textsubscript{20} precursor GGPP into the first C\textsubscript{40} hydrocarbon, 15-\textit{cis} phytoene. 15-\textit{cis} phytoene is converted into 9,15,9'-\textit{tri-cis-}\textit{ζ}-carotene, and then into 9,9'-\textit{di-cis-}\textit{ζ}-carotene by a putative isomerase NnrU (Z-ISO), and finally into all-\textit{trans} lycopene (ψ,ψ-carotene) (29). This linear compound lycopene is considered to be the precursor of cyclic α-, β- or γ-carotenes (for example formulas, see Figure 1-2), and additionally the xanthophylls (30). The cyclizations of the ψ-ends of lycopene are isomerization reactions producing no net change in mass or redox state of the substrate. In the cyclization reactions, the C(2) carbon is attacked by H\textsuperscript{+} to form a carbocation at C(5), and subsequent loss of a proton at C(4), C(6) or C(18) forms the β, ε, or γ rings, respectively (31). The enzymes involved in these cyclization reactions of lycopene to produce
carotenoids with cyclic end-groups are called lycopene cyclases.

![Structures of selected carotenoids and their characteristic end groups. Linear end-groups are referred to as $\psi$ (psi)-ends. $\beta$ (beta)-rings have the double bond at the C5, C6 position while the $\varepsilon$ (epsilon)-rings have the double bond at the C4, C5 position.](image)

Figure 1-2: Structures of selected carotenoids and their characteristic end groups. Linear end-groups are referred to as $\psi$ (psi)-ends. $\beta$ (beta)-rings have the double bond at the C5, C6 position while the $\varepsilon$ (epsilon)-rings have the double bond at the C4, C5 position. (25).

Starting from $\beta$-carotene, or in some cases from lycopene or $\gamma$-carotene, various oxidations or further modifications produce the xanthophylls found in cyanobacteria. These reactions include the addition of hydroxy-, keto- or glycosyl- groups to the carotenoids, and also the introduction of additional double bonds (e.g., 3′,4′- double bond) in the case of myxoxanthophyll.

### 1.6 Lycopene Cyclases

Until now, four classes of lycopene cyclases have been identified in photosynthetic and nonphotosynthetic bacteria. The classical monomeric bacterial CrtY-type lycopene cyclase was
identified in *Erwinia uredovora*; it converts lycopene to β-carotene when heterologously expressed in *Escherichia coli* (30). This kind of lycopene cyclase is commonly found in carotenogenic proteobacteria [*e.g.*, see (30) (32)], *Streptomyces* spp. (33), and the *Chloroflexi* but does not occur in cyanobacteria. The first member of the CrtL family, CrtL from the cyanobacterium *Synechococcus* sp. PCC 7942, was expressed in *E. coli*, and it converted the acyclic hydrocarbon lycopene into the bicyclic β-carotene (34). Lycopene cyclases in this family include the β- and ε-cyclases, and are found in some cyanobacteria and higher plants (35). Lycopene cyclases in the third family, the heterodimeric cyclases of some Gram-positive bacteria [*e.g.*, see (36) (37)], are related to the lycopene cyclases of archaea (38) (39) and halophilic bacteria (40). The first member of the fourth family of lycopene cyclases, encoded by the *cruA* gene of the green sulfur bacterium *Chlorobaculum* (formerly *Chlorobium*) *tepidum*, was identified in a complementation assay in *E. coli* (41). Orthologs of *cruA* are found in all the cyanobacterial genomes lacking CrtL- or CrtY- homologs, and a paralogous gene, *cruP*, is found in the same species. The only exceptions are the *Synechococcus* spp. PCC 7942 and 6301, which have one CrtL lycopene cyclase and one CruP homolog. The CruA family proteins are predicted to be members of the FixC superfamily of dehydrogenases. Like the CrtL- and CrtY- type lycopene cyclases, the CruA family lycopene cyclases are predicted to be integral membrane proteins with a flavin-binding domain (41).

### 1.7 Lycopene Cyclases in *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803

The crude methanol extracts of both *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 contain seven carotenoid pigments. These are synechoxanthin, myxoxanthophyll (myxol dimethylfucoside for *Synechocystis* sp. PCC 6803 and myxol fucoside for *Synechococcus*
sp. PCC 7002, respectively), zeaxanthin, 3′-hydroxy-echinenone, cryptoxanthin, echinenone and β-carotene, as shown in Figure 1-3 (25). The fact that both *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 produce β-carotene indicates they both have lycopene cyclase(s) in their genomes.

Two homologs of CruA were found in the cyanobacterium *Synechococcus* sp. PCC 7002, which were named CruA and CruP, respectively. The lycopene cyclase activity of *Synechococcus* sp. PCC 7002 CruP (CruA Paralog) was demonstrated in *E. coli*: CruP converted lycopene into γ-carotene (β,ψ-carotene), but the pigment composition of a cruP disruption mutant was identical to that of wild-type *Synechococcus* sp. PCC 7002. The activity of CruA activity was inferred from the pigmentation of an insertional mutant, but the complementation assay for CruA activity in *E. coli* failed to show any conversion of lycopene into cyclic carotenoids (41). The data

Figure I-3: Pigment analysis of crude methanol extracts by HPLC (24). Pigments are 1, synechoxanthin; 2, myxoxanthophyll (myxol fucoside); 2′-myxoxanthophyll (myxol dimethylfucoside); 3, zeaxanthin; 4, 3′-hydroxy-echinenone; 5, cryptoxanthin; 6, echinenone; 7, β-carotene (25).
suggested that CruP might be a lycopene monocyclase and that it probably acted as an accessory cyclase to CruA (41).

One feature that makes cyanobacterial CruA unique within the lycopene cylase family is that cyanobacterial CruA homologs possess a large N-terminal extension of about 150 amino acids that is not observed in CruA of green sulfur bacteria. This domain is completely specific to CruA, and no sequences similar to this domain are found in GenBank. This unique domain may play an important role in the lycopene cyclase activity of CruA, and could perhaps bind an unidentified cofactor that is related to photosynthesis. Alternatively, this domain could be required for binding between CruA and another protein required for cyclase activity, as occurs in the heterodimeric lycopene cyclases (41) (42). These possibilities may well explain why the *Synechococcus* sp. PCC 7002 CruA activity was not detected in the complementation assay in *E. coli*.

Homologs of both *cruA* and *cruP* are also found in the genome of *Synechocystis* sp. PCC 6803, which is the source of genes studied in this thesis. Open reading frame (ORF) sll0147 (2007 bp) on the *Synechocystis* sp. PCC 6803 genome encodes a protein of 668 amino acids. The protein product of ORF sll0147 of *Synechocystis* sp. PCC 6803 Sll0147 shares 65% identity with the *Synechococcus* sp. PCC 7002 CruA and 35% identity to the *Chlorobaculum tepidum* CruA. Thus, it is highly probable that *Synechocystis* sp. PCC 6803 sll0147 encodes a lycopene cyclase activity. The purpose of this thesis study was to demonstrate that the sll0147 product has lycopene cyclase activity. Identification of the *Synechocystis* sp. PCC 6803 lycopene cyclase will fill a major gap in our understanding of β-carotene biosynthesis in cyanobacteria in general and *Synechocystis* sp. PCC 6803 in particular (see Figure 1-4).
Figure 1-4: Putative β-carotene biosynthetic pathway in *Synechocystis* sp. PCC 6803 (29) (41) (43) (this thesis).
References


Chapter 2

Materials and Methods

2.1 Cell Cultures and Growth Conditions

2.1.1 Synechococcus sp. PCC 7002

*Synechococcus* sp. PCC 7002 wild-type cells were generally grown in liquid or on solid medium A supplemented with 1 mg mL$^{-1}$ NaNO$_3$ (designated as medium A$^+$) (1). Liquid cultures were grown at 38 °C under constant cool white fluorescent illumination at the intensity of 250 µmol photons m$^{-2}$ s$^{-1}$, bubbled with air supplemented with 1% (v/v) CO$_2$ (designated as standard growth condition). Agar plates were prepared by solidifying the A$^+$ growth medium with 1.5% (w/v) Bacto™ agar from BD Difco (Sparks, MD) supplemented with 0.3% (w/v) sodium thiosulfate. For the cruA deletion mutant strain *Synechococcus* sp. PCC 7002 ΔcruA::aacC1, the medium was supplemented with 10 mM glycerol as carbon and energy source, 100 µg mL$^{-1}$ gentamycin as selective antibiotic, and the cells were grown at low light intensity of 30 µmol photons m$^{-2}$ s$^{-1}$. When the cruA deletion mutant strain was grown on solid medium, molecular biology grade agarose was used instead of the Difco Bacto™ agar. For the two complementation strains, 7002_pAQ1::6803cruA-N(His)$_{10}$ and 7002_pAQ1::6803cruA-C(His)$_6$, the growth conditions were the same as for the wild-type strain except for the addition of 100 µg mL$^{-1}$ gentamycin and 100 µg mL$^{-1}$ spectinomycin as selective antibiotics. For strain storage and maintenance purposes, the strains were frequently re-inoculated on solid A$^+$ medium supplemented with proper antibiotic(s) and kept at an ambient level of CO$_2$ at 30 °C under a light
intensity of 30 µmol photons m\(^{-2}\) s\(^{-1}\) for the cruA deletion mutant strain and 150 µmol photons m\(^{-2}\) s\(^{-1}\) for the wild-type strain and the complementation strains.

2.1.2 *Synechocystis* sp. PCC 6803

Wild-type *Synechocystis* sp. PCC 6803 strain was grown at 30 °C under a light intensity of 150 µmol photons m\(^{-2}\) s\(^{-1}\) bubbled with air supplemented with 1% (v/v) CO\(_2\) (designated as standard growth condition) in medium B-HEPES. This medium was prepared by supplementing BG-11 medium (2) with 4.6 mM HEPES-KOH and 18 mg L\(^{-1}\) ferric ammonium citrate. The attempts of selecting the cruA deletion mutants were carried out on solid B-HEPES medium under a light intensity of 30 µmol photons m\(^{-2}\) s\(^{-1}\), although no transformants were obtained. The solid B-HEPES medium was prepared by adding 1.5% (w/v) molecular biology grade agarose to the same medium.

2.1.3 *Escherichia coli* strains

Electro-competent *Escherichia coli* strains DH5α, DH10B, or BL21 (DE3) were used for routine recombinant DNA procedures. *E. coli* was grown at 37 °C in LB liquid or solid (15 g L\(^{-1}\) Bacto\(^{TM}\) agar) media supplemented with 100 µg mL\(^{-1}\) ampicillin or 100 µg mL\(^{-1}\) spectinomycin as required.

The lycopene-producing *E. coli* strain BL21(DE3) harboring plasmid pACLyc (see Figure 2-1) was grown at 30 °C for 24 h in liquid LB medium containing 34 µg mL\(^{-1}\) chloramphenicol before extraction of lycopene from the cells. The γ-carotene producing *E. coli* strain BL21(DE3) harboring plasmid pCPL1 (Figure 2-2) was grown at 30 °C for 24 h in liquid LB medium containing 34 µg mL\(^{-1}\) chloramphenicol and 100 µg mL\(^{-1}\) ampicillin.
Figure 2-1: Schematic representation of the pAC-LYC plasmid (3).

Figure 2-2: Schematic representation of the pCPL1 plasmid (4).
2.2 Deletion of cruA gene in Synechococcus sp. PCC 7002

Left and right flanks of the cruA gene were amplified from genomic DNA of Synechococcus sp. PCC 7002 using the four primers described in Table 2-1. Two restriction sites HindIII and XbaI were designed at ends of the flanks. These primers were designed to delete the entire cruA coding sequence. The restriction sites were selected so as to avoid creating restriction sites that already existed in the fragments to be used in mutant construction. PCR products were separated on agarose gels by electrophoresis, cut from the gel, purified using the Eppendorf PerfectPrep Gel Clean-up Kit (Hamburg, Germany), and digested with HindIII and XbaI respectively. The digestion product was purified using the same methods. The aacC1 gene, encoding resistance to gentamicin was digested from plasmids pMS255 with enzymes HindIII and XbaI, to excise the antibiotic resistance cartridge. This gentamicin cartridge was separated by agarose gel electrophoresis, purified as described above, and ligated to the left and right flanks at a ratio of 3:1:3 (flank1:cassette:flank2) with T4 DNA ligase. The ligation product was then transformed to Synechococcus sp. PCC 7002 using an optimized transformation protocol based on the described one (5). After bubbling for 5 h, the 2-mL liquid transformation mixture was placed in reduced light (30 µmol photon m⁻² s⁻¹) without bubbling for 24 h. Cells were then plated directly onto solid medium containing glycerol as carbon source and gentamicin as the selective antibiotic. Because the gentamicin resistance cartridge has its own promoter, the correct transformants were selected from the pool with the cartridge inserted into the chromosome in both directions. Single colonies were picked from plates and repeatedly streaked on selective media until segregation was confirmed. Segregation was confirmed by PCR amplification of the gene loci using the upstream primer of the left flank and the downstream primer of the right flank.
2.3 Construction of the two complementation strains

The *sll0147* locus was amplified by PCR from the genome DNA of *Synechocystis* sp. PCC 6803 using primers described in Table 2-2. The PCR products of *sll0147* were digested with NdeI and BamHI for the N-terminal, deca-His-tagged construction and BspHI and BamHI for the C-terminal, hexa-His-tagged construction, respectively. The digestion products were cloned into expression plasmid pAQ1Ex-\(P_{cpcBA}\) (Figure2-3). This plasmid contains two flanking regions that enable the integration of foreign DNA cassettes with selectable markers into a neutral site within the endogenous plasmid pAQ1 of *Synechococcus* sp. PCC 7002. The N-terminal deca-His-tagged and the C-terminal hexa-His-tagged *sll0147* constructions were cloned into pAQ1Ex-\(P_{cpcBA}\) to construct the plasmids pAQ1::cruA-N(His)\(_{10}\) and pAQ1::cruA-C(His)\(_{6}\), respectively. To construct the *Synechococcus* sp. PCC 7002_pAQ1::6803cruA-C(His)\(_{6}\) strain, plasmid pAQ1::cruA-C(His)\(_{6}\) was linearized by restriction enzyme *ScaI* and subsequently transformed into the *Synechococcus* sp. PCC 7002 *cruA* deletion mutant strain using the same method described in Chapter 2.2. The C-terminal, His-tagged *sll0147* gene was introduced into endogenous pAQ1 by homologous recombination. For selection, 100 µg mL\(^{-1}\) spectinomycin was used as selective antibiotics and PCR verification was carried out using the appropriate cloning primers. To obtain the 7002_pAQ1::6803cruA-N(His)\(_{10}\) strain, the plasmid pAQ1::cruA-N(His)\(_{10}\) was linearized by restriction enzyme *ScaI* and subsequently transformed into wild-type *Synechococcus* sp. PCC 7002, creating the intermediate strain of wild-type *Synechococcus* sp. PCC 7002 with the N-terminus His-tagged *sll0147* gene on its endogenous plasmid pAQ1. This intermediate strain was named wt7002::6803cruA-N(His)\(_{10}\). Using the primer pair 7002F1 and 7002R2, a DNA sequence of gentamicin resistance cartridge with two flanking sequence on each end was amplified by PCR of the *Synechococcus* sp. PCC 7002 *cruA* deletion mutant genome. This PCR product was purified and transformed into the intermediate strain wt7002::6803cruA-N(His)\(_{10}\) as described
above. Segregations of 7002_pAQ1::6803cruA-N(His)\textsubscript{10} strain were carried out on selective solid A\textsuperscript{+} medium and verified by PCR of the cruA locus on the genome.

![Diagram of pAQ1Ex-P\textsubscript{cpcBA} plasmid](image)

Figure 2-3: Schematic representation of the pAQ1Ex-P\textsubscript{cpcBA} plasmid (6).

### 2.4 Deletion of sll0147 gene in Synechocystis sp. PCC 6803

The construction of sll0147 deletion mutant of *Synechocystis* sp. PCC 6803 was similar to that for *Synechococcus* sp. PCC 7002 except for two differences. Firstly, the kanamycin resistance cartridge was obtained by PCR from the plasmid pRL161 using primer pair KmF/KmR shown in Table 2-1. The PCR product obtained was digested with EcoRI and Xhol in order to ligate to the digested left and right flanking sequences, respectively. The growth conditions as mentioned in Chapter 2.1.2 were followed for wild-type strains. For the selection of an sll0147
deletion mutant, a lower light intensity of 15 µmol photons m$^{-2}$ s$^{-1}$ was employed together with growth on solid B-HEPES medium supplemented with 5 mM glucose and 30 µg ml$^{-1}$ kanamycin.

### 2.5 Pigment Extraction and Analysis

Pigments were analyzed by reversed-phase high-performance liquid chromatography (HPLC). *Synechococcus* sp. PCC 7002 cells were generally grown until mid-exponential phase (OD$_{730}$ nm = ~1.0) for pigment analyses. An aliquot of a liquid culture (1.0 ml) was harvested by centrifugation, and the pigments were extracted by sonication of the cell pellet in 500 µl acetone:methanol (7:2, v/v). Debris was removed by centrifugation (12,000 × g for 3 min) and the supernatant was filtered through a 0.2-µm teflon (polytetrafluoroethylene) syringe filter. Fresh extracts were prepared from freshly harvested fresh cells each time prior to analysis. Pigments were separated on a 25 cm × 4.6 mm analytical Discovery 5 µm C18 column (Supelco, Bellefonte, PA) using an HPLC system (Agilent Model 1100) equipped with a diode array detector (model G1315B) and controlled with Agilent ChemStation software (Agilent Technologies, Palo Alto, CA). The protocol method named “jegpsu.m” was used for the carotenoids separation consisting the gradient [time in min, %B]: [0, 20], [10, 70], [40, 100], [50, 100], and [60, 20] and a flow rate of 1 mL min$^{-1}$ on the analytical column. Solvent A of the “jegpsu.m” method consisted of water: methanol: acetonitrile (62.5:21:16.5, v/v/v) containing 10 mM ammonium acetate, and solvent B of the “jegpsu.m” method was methanol: ethyl acetate: acetonitrile (50:30:20, v/v/v). Pigments were identified according to their retention time, absorption spectra, and comparisons to established standards in the laboratory.
The substrates lycopene and γ-carotene were prepared by extracting the *E. coli* BL21 (DE3)::pACYC and the *E. coli* strain BL21(DE3)::pCPL1 cell pellets, respectively, using pure acetone. Extractions were repeated several times until the cell color disappeared. Sonication was employed to improve extraction efficiency. After centrifugation, the colored acetone phases containing lycopene or γ-carotene were collected in 20 ml vials; the extracts were concentrated by evaporation to ~10 ml under dim light under nitrogen gas. An aliquot (100 µl) was used for analysis and quantitation. The HPLC system was the same as described above, with the modified method for analysis of *E.coli* strains. The method “ecolicar.m” [time in min, %B]: [0, 70], [10, 90], [15, 100], [25, 100] and [26, 70] was used. Solvent A of “ecolicar.m” method consisted of water:methanol:acetonitrile (62.5:21:16.5, v/v/v), and solvent B of “ecolicar.m” method was methanol:ethyl acetate:acetonitrile (50:30:20, v/v/v). The lycopene or γ-carotene peak area of this injection was measured and recorded. The remaining lycopene or γ-carotene extraction was divided into aliquots in reaction vials, dried individually, and stored at -20 °C until needed.

### 2.6 Protein Purification

The complementation strain 7002_pAQ1::6803cruA-C(His)_6 was cultured in 3.0 L of A⁺ medium supplemented with 10 mM glycerol, 100 µg ml⁻¹ gentamycin and 100 µg mL⁻¹ spectinomycin, under standard growth conditions. The cells were harvested when the OD₇₅₀ nm of the culture reached 1.5, which is the late exponential phase of cell growth. From this step on, all additional purification steps were performed at 4 °C. The pellets were resuspended in 45 ml of binding buffer (50 mM Tris-HCl pH 6.8, 300 mM NaCl, 10 mM imidazole), supplemented with 10 µg mL⁻¹ DNase I and 0.5 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF). This resuspended cell solution was disrupted by three passages through a chilled French pressure cell
at 138 MPa. Cell debris was removed by centrifugation at 4,500 × g for 20 min in a Sorvall SS-34 rotor (Sorvall, Milford, MA). The supernatant was the cell lysate that contained both the membrane fraction and the soluble fraction. This supernatant was centrifuged at 133,200 × g for 45 min, to separate the membrane and soluble fraction. Both fractions were collected and analyzed for the presence of the target protein, Sll0147. The membrane fraction, which is the pellet of this ultracentrifugation step, was resuspended in 45 ml Binding Buffer supplied with 0.5 mM protease inhibitor PMSF and 1% (w/v, final concentration) lithium dodecyl sulfate (LDS). This resuspended membrane solution was incubated at 4 °C with gentle shaking for 1 h. The solubilized membranes were subjected again to ultracentrifugation at 133,200 × g for 20 min, and the supernatant fraction will be referred to below as “the solubilized membrane extract”.

Other detergents were also tested for their ability to solublize the membrane fraction. These detergents and their final concentrations in the solublization mixtures were 2% (w/v) n-dodecyl-β-D-maltoside (DM), 0.8% (w/v) octyl glucoside (OG) and 1% (w/v) Triton X-100.

The Sll0147 protein was purified by nickel chelation chromatography. TALON™ metal affinity resin (Clontech, Mountain View, CA) was equilibrated with 10 bed volumes of binding buffer. The solubilized membrane extract was incubated with 2 ml of pretreated resin at 4 °C with gentle shaking for 2 h. The mixture was loaded onto a column by gravity flow, and the flow-through fraction was collected for analysis. The resin was washed with 10 bed volumes of wash buffer (50 mM Tris-HCl, pH 6.8, 300 mM NaCl, 20 mM imidazole, 0.1% (w/v) DM). Sll0147 was eluted from the column with 10 bed volumes of Elution Buffer (50 mM Tris-HCl pH 6.8, 300 mM NaCl, 250 mM imidazole, 0.1% DDM). Fractions (~20 ml) were collected, and aliquots were prepared from each fraction for analysis by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE).
Purified Sll0147 protein was dialyzed overnight against 1:200 (v/v) 50 mM Tris-HCl, pH 6.8, supplemented with 0.005% (w/v, final concentration) LDS at 4 °C using dialysis tubing (60-kDa cutoff). The harvested protein was concentrated using Microcon Ultracel YM-50 (Millipore, Billerica, MA) to a final concentration of ~2 mg protein ml\(^{-1}\). Protein concentrations were determined using the Coomassie Plus Protein Assay Kit from Thermo Scientific (Rockford, IL).

### 2.7 In vitro Enzyme Assays

Lycopene and \(\gamma\)-carotene were prepared from *E. coli* BL21 DE3 strains. These strains produced lycopene or \(\gamma\)-carotene as the only carotenoid. An overnight culture was usually harvested by centrifugation, and the carotenoid was extracted using acetone by sonication. The extract aliquots were dried under a stream of nitrogen in the reaction vials and stored in dark at -20 °C.

The concentration of the as-purified Sll0147 protein (CruA) was determined using the Bradford method before addition to the reaction mixture. The 72 µL reaction mixture included the following (final concentrations): 3 mM ATP, 6 mM MgCl\(_2\), 6 mM MnCl\(_2\), 50 mM Tris-HCl, pH 6.8, 0.03 µg µL\(^{-1}\) lycopene (0.056 mM) (powder, dried at the bottom of each reaction vial), 0.5 mM FMN, 0.5 mM FAD, 0.5 mM NAD\(^+\), 0.5 mM NADP\(^+\), 0.5 mM NADH, 0.5 mM NADPH, 0.1% (w/v) DM and 3.6-14.4 µg (0.625-2.5 µM) Native form as-purified Sll0147. This protocol was named “reaction condition 1.” A modified reaction mixture (100 µL) was used with the following additions (final concentrations): 3 mM ATP, 6 mM MgCl\(_2\), 6 mM MnCl\(_2\), 50 mM Tris-HCl pH 6.8, 0.022 µg µL\(^{-1}\) lycopene (0.041 mM) or 0.012 µg µL\(^{-1}\) \(\gamma\)-carotene (0.022 mM) (powder, dried at the bottom of each reaction vial), 0.5 mM NADH, 0.5 mM NADPH, 0.1% (w/v) DM and 4-10 µg (0.5-1.3 µM) Native form as-purified Sll0147. These reaction conditions
were named “reaction condition 2.” Parallel controls were also performed without adding the substrate. Reactions were incubated at 30 °C with gentle shaking for 16 h.

The reaction was stopped by adding 100 µL acetone to the reaction vial. This 200 µL mixture was centrifuged at 12,000 × g for 3 min, and the ~200 µL supernatant was collected in a new Eppendorf tube. Acetone (150 µL) was added to the pellet and the solution was sonicated for 1 min, centrifuged, and added to the supernatant from the previous ~200 µL extraction. This step was repeated once to make the whole extraction volume ~500 µL. An aliquot was injected and analyzed for pigment content by HPLC. The “ecolicar.m” method was used on the same HPLC system as described in Chapter 2.3.

2.8 Data Collection and Calculations

The Agilent ChemStation software can analyze and integrate each peak and output various peak values, including height and area. Calculations were based on the peak areas from each injection to calculate changes before and after each reaction. Internal standards were used to normalize the data collected from individual injections.
Reference


Table 2-1: Primers for construction of deletion mutants.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Synechococcus</em></td>
<td>7002F1</td>
<td>AAACCGATTACAGCCATAAAA</td>
<td></td>
</tr>
<tr>
<td>sp. PCC 7002</td>
<td>7002R1</td>
<td>TTAAGCTTGGAAATTACAGATGGTG</td>
<td>HindIII</td>
</tr>
<tr>
<td><em>Synechococcus</em></td>
<td>7002F2</td>
<td>GGCTCTAGAAATGTATTAGGACCTTGCAACGGAT</td>
<td>XbaI</td>
</tr>
<tr>
<td>sp. PCC 7002</td>
<td>7002R2</td>
<td>TTCCACGTTGGCGGTGCTGTCC</td>
<td></td>
</tr>
<tr>
<td><em>Synechocystis</em></td>
<td>6803F1</td>
<td>AATGCAGGGCAGGTGTGGGG</td>
<td></td>
</tr>
<tr>
<td>sp. PCC 6803</td>
<td>6803R1</td>
<td>CCGGAATTCCGGTGGAAAATGGACTAAAAAG</td>
<td>EcoRI</td>
</tr>
<tr>
<td><em>Synechocystis</em></td>
<td>6803F2</td>
<td>CCGCTCGAGATTAGGGCGACATTAGAAA</td>
<td>Xhol</td>
</tr>
<tr>
<td>sp. PCC 6803</td>
<td>6803R2</td>
<td>GCCCTCAAACCTCCACCC</td>
<td></td>
</tr>
<tr>
<td><em>Synechocystis</em></td>
<td>KmF</td>
<td>CCGGAATTCAAGCTTGCATGCGTCAGGT</td>
<td>EcoRI</td>
</tr>
<tr>
<td>sp. PCC 6803</td>
<td>KmR</td>
<td>CCGCTCGAGCGAAGACCTCCAGCATGAGT</td>
<td>Xhol</td>
</tr>
</tbody>
</table>
Table 2-2: Primers for expression of *Synechocystis* sp. PCC 6803 *sll0147* in pAQ1Ex-*P* _pcpBA*.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
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<td>NtermF</td>
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<td>NdeI</td>
</tr>
<tr>
<td>NtermR</td>
<td>CGCGGATCTTTACTTTGAGTTGAGCTAATT</td>
<td>BamHI</td>
</tr>
<tr>
<td>CtermF</td>
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<td>BspHI</td>
</tr>
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<td>CtermR</td>
<td>CGCGGATCTTTAATGATGATGATGATGATGATGATTTGGATT</td>
<td>BamHI</td>
</tr>
<tr>
<td></td>
<td>GGTGAGCTAATTTG</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3

Results

3.1 Sequence comparison

The protein product of *sll0147* of *Synechocystis* sp. PCC 6803 was compared with CruA of *Synechococcus* sp. PCC 7002 using bl2seq (www.ncbi.nlm.nih.gov/BLAST). The result is shown in Figure 3-1.
Figure 3-1. Amino acids alignment result of *Synechocystis* sp. PCC 6803 Sll0147, *Synechococcus* sp. PCC 7002 CruA, and *Chlorobaculum tepidum* CruA sequences.
Both the *Synechococcus* sp. PCC 7002 CruA (656 amino acids) and the *Chlorobaculum tepidum* CruA (512 amino acids) have been identified as lycopene cyclases that can convert lycopene to either γ-carotene or β-carotene or both (1). The 65% amino acid sequence identity shared by *Synechocystis* sp. PCC 6803 Sll0147 (668 amino acids) and *Synechococcus* sp. PCC 7002 CruA, and the 35% identity shared by *Synechocystis* sp. PCC 6803 Sll0147 and *Chlorobaculum tepidum* CruA, indicated a possible function of Sll0147 as lycopene cyclase in *Synechocystis* sp. PCC 6803. In addition, as mentioned in Chapter 1.7, the ~150 amino acids N-terminal extension common in cyanobacterial CruA sequences is present in the Sll0147.

### 3.2 Deletion mutagenesis of *Synechococcus* sp. PCC 7002 cruA

As described in Chapter 2.2, the entire *cruA* gene of *Synechococcus* sp. PCC 7002 was deleted and replaced by *aacC1*, which encodes gentamicin resistance, as shown in Figure 3-2. The segregation of the correct mutant was monitored until the PCR amplification of the *cruA* locus showed full deletion of *cruA*. The PCR result is shown in Figure 3-3.

![Figure 3-2](image.png)

Figure 3-2: Scheme of the deletion of *Synechococcus* sp. PCC 7002 *cruA* locus.

Segregation of the *cruA* deletion locus was verified by PCR amplification of genomic DNA using the primer pair 7002F1/7002R2. PCR verification of the presence of the gentamicin
resistance cartridge was done using the primer pair for the \textit{aacC1} gene. The result shows the complete replacement of \textit{cruA} (1971 bp) by the gentamycin cartridge (~800 bp).

![Image of gel electrophoresis](image)

**Figure 3-3:** Deletion of \textit{cruA} locus. Lane 1, wild-type \textit{Synechococcus} sp. PCC 7002. Lane 2, \textit{Synechococcus} sp. PCC 7002 \textit{ΔcruA::aacC1} mutant strain.

The pigments produced by both the \textit{Synechococcus} sp. PCC 7002 wild-type strain and the \textit{Synechococcus} sp. PCC 7002 \textit{ΔcruA::aacC1} mutant strain were analyzed by HPLC following the method described in chapter 2. Compared to the wild-type pigment profile, the deletion mutant accumulates lycopene, \(\gamma\)-carotene and 1-hydroxy-lycopene (see Figure 3-4). This result is consistent with the conclusion that CruA possesses lycopene cyclase activity, and it agrees with previous \textit{cruA} insertional inactivation data for \textit{Synechococcus} sp. PCC 7002 (1).
Figure 3-4: HPLC analysis (“jegpsu.m” method) of wild-type *Synechococcus* sp. PCC 7002 (lower line) and the cruA deletion mutant *Synechococcus* sp. PCC 7002 ΔcruA::aacC1 (upper line). 1. 1-hydroxy-lycopene; 2. lycopene; and 3. γ-carotene. Carotenoids: s, synechoxanthin; m, myxoxanthophyll; z, zeaxanthin; c, cryptoxanthin; e, echinenone; β, β-carotene.

### 3.3 Construction of plasmids pAQ1::cruA-N(His)$_{10}$ and pAQ1::cruA-C(His)$_{6}$

The expression plasmid pAQ1Ex-P$_{cpcBA}$ was used as the platform to develop expression constructions. This plasmid has the cpcBA promoter sequence from *Synechocystis* sp. PCC 6803, a spectinomycin resistant cartridge (encoded by aadA gene) and a deca-histidine sequence that can be fused to genes for tagging proteins of interest. For the plasmid pAQ1::cruA-N(His)$_{10}$ (Figure 3-5), PCR using the primer pair NtermF/NtermR was used to clone the slt0147 gene from
Synechocystis sp. PCC 6803 genome DNA. The sll0147 gene was introduced into the NdeI and BamHI cloning sites in the expression plasmid (see Figure 3-5). Similarly, the plasmid pAQ1::cruA-C(His)$_6$ (Figure 3-6) was constructed using the primer pair NtermF/NtermR followed by digestion with BmpI and BamHI, ligation and transformation. DNA sequencing results for both plasmids confirmed the correct insertion of sll0147.

Figure 3-5: Schematic representation of the constructed pAQ1::cruA-N(His)$_{10}$ plasmid.
**3.4 Assays of Sll0147 activity in E. coli**

Three plasmids, pAQ1Ex-P\(_{cpcBA}\), pAQ1::cruA-N(His)\(_{10}\) and pAQ1::cruA-C(His)\(_{6}\), all conferring resistance to spectinomycin, were transformed into the lycopene-producing *E. coli* strain BL21(DE3) pACLyc (Cm\(^r\)) and the γ-carotene-producing *E. coli* strain BL21(DE3) pCPL1 (Cm\(^r\), Amp\(^r\)), respectively. Colonies were cultured for 48 h in darkness at room temperature on LB plates with addition of corresponding antibiotics. Six *E. coli* strains were obtained and named *E. coli* (pACLyc, pAQ1Ex-P\(_{cpcBA}\)), *E. coli* (pACLyc, pAQ1Ex::cruA-N(His)\(_{10}\)), *E. coli* (pACLyc, pAQ1Ex::cruA-C(His)\(_{6}\)), *E. coli* (pCPL1, pAQ1Ex-P\(_{cpcBA}\)), *E. coli*
(pCPL1, pAQ1Ex::cruA-N(His)$_{10}$) and E. coli (pCPL1, pAQ1Ex::cruA-C(His)$_6$). Liquid cultures were prepared for pigment analysis to detect any possible changes in the carotenoids produced. Immunoblotting experiments, using a commercial antibody to the His-tag, showed that the product of the sll0147 open reading frame was expressed as the appropriately sized protein in each of the complementation strains but the protein was not observed in the negative control cells yet (Figure 3-7). This experiment showed that the putative lycopene cyclase was produced in each strain as expected.

Figure 3-7: Immunoblot analysis of E. coli whole cell lysate samples. The antibodies used are anti-6 × His epitope tag (rabbit) antibody peroxidase conjugated (ROCKLAND). The corresponding strains analyzed are: lane 1, E. coli (pACLYC, pAQ1Ex-P$_{pcpB}$); lane 2, E. coli (pACLYC, pAQ1Ex::cruA-N(His)$_{10}$); lane 3, E. coli (pACLYC, pAQ1Ex::cruA-C(His)$_6$) lane 4, E. coli (pCPL1, pAQ1Ex-P$_{pcpB}$); lane 5, E. coli (pCPL1, pAQ1Ex::cruA-N(His)$_{10}$); and lane 6, E. coli (pCPL1, pAQ1Ex::cruA-C(His)$_6$).

Lycopene-producing E. coli strains, such as BL21(DE3)/pACLYC, are pink-colored, while γ-carotene-producing E. coli strains, such as BL21(DE3)/pCPL1 are orange-colored. Therefore, production of an active lycopene cyclase should convert lycopene/γ-carotene into γ-/β-carotene, and would show a color change for strains that originally produced only lycopene. However, there no visible change in color occurred in any of the strains producing the sll0147 gene product (Figure 3-8). The pigments profiles of the complementation strains were the same as the negative controls, and moreover, HPLC analyses were identical to the controls as well (data not shown). These experiments convincingly demonstrate that, although the Sll0147 proteins
were produced as expected, these proteins were not active when expressed in E. coli strains producing lycopene or γ-carotene.

Figure 3-8-1: Colored colonies of the E. coli complementation strains E. coli (pACYC, pAQ1Ex-P_{pcpBA}), E. coli (pACYC, pAQ1Ex::cruA-N(His)$_{10}$) and E. coli (pACYC, pAQ1Ex::cruA-C(His)$_{6}$).

Figure 3-8-2: Colored colonies of the E. coli complementation strains E. coli (pCPL1, pAQ1Ex-P_{pcpBA}), E. coli (pCPL1, pAQ1Ex::cruA-N(His)$_{10}$) and E. coli (pCPL1, pAQ1Ex::cruA-C(His)$_{6}$).
3.5 Heterologous expression of Sll0147 in Synechococcus sp. PCC 7002

Using the heterologous pAQ1Ex-P_{pcBA} expression system in Synechococcus sp. PCC 7002, two expression strains, named the 7002_pAQ1::6803cruA-N(His)_{10} and 7002_pAQ1::6803cruA-C(His)_{6}, were constructed by transformation. PCR was used to verify that the Synechococcus sp. PCC 7002 cruA locus was mutated and that the sll0147 gene had been correctly inserted by transformation and recombination with endogenous plasmid pAQ1 (Figure 3-9).

Figure 3-9: PCR verification of the two complemented strains, 7002_pAQ1::6803cruA-N(His)_{10} and 7002_pAQ1::6803cruA-C(His)_{6}. Lane 1: verification of the cruA locus in wild-type Synechococcus sp. PCC 7002. Lane 2: verification of the cruA deletion locus in Synechococcus sp. PCC 7002::cruA. Lane 3: verification of the presence of sll0147 on recombinant pAQ1.

The pigment profiles of the two complementation strains were also obtained by HPLC and are shown in Figure 3-10. As previously observed, the wild-type strain produces β-carotene and other carotenoids but does not accumulate any lycopene or γ-carotene. When the cruA gene is inactivated, lycopene and γ-carotene accumulated to quite significant levels in cells (Figure 3-4). Strains complemented with the sll0147 gene restored the wild-type phenotype completely, and complementation was observed when the resulting protein had a His-tag at either the N-terminus or the C-terminus. This experiment convincingly demonstrates that the sll0147 product has
lycopene cyclase activity. Thus, *sll0147* encodes a lycopene cyclase and can be renamed *cruA*. It should be noted that these same constructs were inactive when expressed in *E. coli*. This observation is consistent with previous experiments, and it strongly suggests that *E. coli* lacks the ability to produce an active form of the CruA protein.

![Figure 3-10: HPLC analysis (“jegpsu.m” method) of the serial of Synechococcus sp. PCC 7002 strains, wild-type Synechococcus sp. PCC 7002 (trace 1), Synechococcus sp. PCC 7002 ΔcruA::aacC1 (trace 2), the two complementation strains, 7002_pAQ1::6803cruA-N(His)₁₀ (trace 3) and 7002_pAQ1::6803cruA-C(His)₆ (trace 4). 1, 1-hydroxy-lycopene; 2, lycopene; and 3, γ-carotene; s, synechoxanthin; m, myxoxanthophyll; z, zeaxanthin; c, cryptoxanthin; e, echinenone; β, β-carotene.](image)

### 3.6 CruA purification and identification

As described in chapter 2.6, C-terminal, hexa-Histidine tagged CruA of *Synechocystis* sp. PCC 6803 was purified from the complementation strain 7002_pAQ1::6803cruA-C(His)₆. The eluted protein from the metal chelation column had a yellow-green color. This yellow-green color remained even after dialysis and concentration (Figure 3-11).
Figure 3-11: As-purified CruA (~500 µL) in a 1.5-mL microcentrifuge tube showing the yellow-green color of the protein.

The absorption spectrum of the as-purified protein was taken from 250 nm to 800 nm and the results are shown in Figure 3-12. According to the absorption spectrum, the color appeared to come from the chlorophyll $a$, which was assumed to be bound to mature CruA. In addition, the shoulder on the spectrum at 450 nm to 520 nm indicated that carotenoids might also be present.

Figure 3-12: Spectrum of the as-purified CruA in 50 mM Tris-HCl, pH 6.8 buffer. The absorption maxima for the Qy and Soret peaks are 678nm and 438nm, respectively.
Colored products were obtained when the purification was performed using other detergents. However, the absorption spectra suggested that a smaller amount of chlorophyll was bound to the protein (Figure 3-13).

![Absorbance Spectra](image)

**Figure 3-13**: Absorbance spectra of the different detergent-treated, as-purified CruA preparations in 50 mM Tris-HCl, pH 6.8 buffer. The detergents used are: 1% (w/v) LDS (black line), 2% (w/v) DM (blue line), 0.8% (w/v) OG (red line) and 1% (w/v) Triton X-100 (grey line).

According to these spectral profiles, and assuming that a higher level of bound chlorophyll might be indicative of a more native protein, 1% (w/v) LDS appeared to be the best detergent and concentration among those tested. Thus, 1% (w/v) LDS was used for in subsequent experiments.

The N-terminal, deca-histidine tagged CruA of *Synechocystis* sp. PCC 6803 was also purified from the complementation strain 7002_pAQ1::6803cruA-N(His)10. Subcellular fractions, such as the whole-cell lysate, the cell debris, the solubilized membrane extract, the soluble fraction, the flow through fraction after binding with the TALON™ metal affinity resin, the
fraction after washing the resin with wash buffer, and the elution fractions were collected during
the purification process. Immunoblot analysis using anti-histidine antibodies was performed for
each of the fractions but CruA was not detected in any fractions (data not shown). This might
indicate that the N-terminal domain was being removed by proteolysis.

The calculated mass for the C-terminally His-tagged CruA protein is 77.1 kDa. Two
types of samples were prepared for gel electrophoresis: the not heated, as-purified CruA and the
same sample with an additional heat treatment at 65 °C for 7.5 min. The two samples contained
2% (w/v) SDS and 10% (v/v) glycerol brought in by the loading buffer. Two SDS-PAGE gels
were run at a low voltage of 120 V at 4°C for about 2.5 hours with the prepared samples. One
was processed as a “green gel” and the other one was stained with Coomassie Blue (Figure 3-14).

![Figure 3-14: Green gel and denaturing SDS-PAGE gel of the as-purified CruA. The protein
amount loaded in each lane is 30 µg, and the calculated chlorophyll/pheophytin amount per lane
is 49.4 ng. Lane 1: native, as-purified CruA sample. Lane 2: as-purified CruA sample heated at 65
°C for 7.5 min.](image)
On the green gel, the native, as-purified CruA showed a visible green band while the heat-treated sample did not. This result indicated that the “green color” was bound to a protein molecule(s), which was denatured after heat treatment. The electrophoretic migration of this green band was much slower than the largest marker band (170 kDa) in the standards used. This suggested that a larger complex of chlorophyll and protein occurred prior to denaturation. The Coomassie Blue (CB) stain gel showed 3 bands for each sample, at ~300 kDa, ~72 kDa and ~43 kDa. The green band on the green gel (MS Sample #1) and the ~72 kDa band (MS Sample #2) corresponded to the calculated size of CruA on the SDS-PAGE gel were excised with a sterile blade. Tryptic peptide mass fingerprinting (PMF) was performed to confirm the identity of the as-purified protein at the Proteomics and Mass Spectrometry Core Facility of The Pennsylvania State University, University Park. The PMF results are shown in Figure 3-15 and Figure 3-16.

<table>
<thead>
<tr>
<th>1</th>
<th>MRELLYCEVP</th>
<th>TPATAEVVAV</th>
<th>LQGNWSPLVG</th>
<th>QKLPEQGLR</th>
<th>LRGSSQSSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>LSIFVWSLQR</td>
<td>TTYLKCVRWG</td>
<td>DTSFPQETAI</td>
<td>AKELRRALQG</td>
<td>KFPFTYPVPP</td>
</tr>
<tr>
<td>101</td>
<td>AIDLDRQNIF</td>
<td>QALQDRAWPET</td>
<td>VKFQQKFPQG</td>
<td>EYDLKRVVYW</td>
<td>EQRRNDRVAN</td>
</tr>
<tr>
<td>151</td>
<td>PQTPRQVVSQ</td>
<td>TSTTATAIPA</td>
<td>TFEDLILYLG</td>
<td>GALGSVHAAV</td>
<td>MARLVYRVL</td>
</tr>
<tr>
<td>201</td>
<td>IERLPFGRMN</td>
<td>REWNISRDREF</td>
<td>QRLIDILNFT</td>
<td>PEEFESVIAR</td>
<td>EYRGDFNKS</td>
</tr>
<tr>
<td>251</td>
<td>DAYNPPHLKA</td>
<td>NVLHTPTVLN</td>
<td>IALDSAKVLA</td>
<td>ICGHKLQQAG</td>
<td>GIWDETEFI</td>
</tr>
<tr>
<td>301</td>
<td>NATIDPNKVV</td>
<td>VHCINLENNQ</td>
<td>TKLQARLLV</td>
<td>DAMGTASPIA</td>
<td>WQLNGKTFN</td>
</tr>
<tr>
<td>351</td>
<td>SVCPTVGAVV</td>
<td>EGLDPVVWDN</td>
<td>AGYDVLNSHG</td>
<td>DISRGRQLW</td>
<td>ELFPGAGDEM</td>
</tr>
<tr>
<td>401</td>
<td>TIYLFHYHQV</td>
<td>NRENPGSLLSLE</td>
<td>MYEDFSSLIP</td>
<td>EYRCNLEKL</td>
<td>TWFKATFGYI</td>
</tr>
<tr>
<td>451</td>
<td>PGHFSTSAQD</td>
<td>RTIALDRILMA</td>
<td>IGDAASILQP</td>
<td>LVFTGFSGLV</td>
<td>RNLAKLDDL</td>
</tr>
<tr>
<td>501</td>
<td>HTAIQYDLLQ</td>
<td>AKLQINIRAY</td>
<td>QSNIAVTVWVF</td>
<td>SKGWVMVTGK</td>
<td>QLFPQVVMN</td>
</tr>
<tr>
<td>551</td>
<td>LNTFPGLLLAD</td>
<td>SAPTVAETF</td>
<td>KDRTTWLLFS</td>
<td>RLAKKAASKN</td>
<td>PQLFLWIFQM</td>
</tr>
<tr>
<td>601</td>
<td>AGTEDDLKNL</td>
<td>LVYFDFSRQA</td>
<td>LLNLAFRFWEY</td>
<td>PQWLRNSRNW</td>
<td>LKLSPQLW</td>
</tr>
<tr>
<td>651</td>
<td>SLLCFIQIQQ</td>
<td>PKLQASYK</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3-15: PMF result of the ~72 kDa band of the as-purified CruA sample.
The identification reports from the PMF experiment indicated that the protein sequence of the ~72 kDa band of the as-purified CruA sample matched the CruA predicted sequence with a high score of 134.19 and a coverage of 7.9%. For the green band, the score was even higher, 5751.463 and 48.2% coverage. Thus, the green band was shown to be a complex of CruA and chlorophyll a, while the ~72 kDa band derived from the heat-treated sample represented CruA with no cofactor bound, as the CruA monomer with no obvious cofactor bound. Because the 72 kDa polypeptide was detected before and after heat treatment, it is likely that some of the CruA in solution does not have the chlorophyll a cofactor bound to it.

To analyze the purified CruA further, an immunoblot experiment was performed using the same heat-treated sample that was used in the experiment shown in Figure 3-14. Anti-polyhistidine antibody was used in this immunoblot analysis. The immunoblotting result (Figure 3-17) showed a major band at ~72 kDa in the denatured sample, indicating the presence of the His-tagged CruA monomer. A minor band was also observed at ~43 kDa. It suggested that proteolysis of CruA was occurring. Because the protein was isolated from the C-terminally

Figure 3-16: PMF result of the green band of the as-purified CruA sample on the green gel.
tagged CruA, this degradation product must contain the C-terminus of the CruA polypeptide. Additional degradation products were also detected.

![Immunoblot analysis](image)

Figure 3-17: Immunoblot analysis of the as-purified CruA. Protein amount loaded in the well was 3 µg. The protein sample was heated at 95 °C for 1 min in loading buffer (2% SDS, 10% glycerol, final concentration) before loading and electrophoresis.

In summary, considering all the evidence, the as-purified, C-terminally hexa-His-tagged CruA occurred in solution as both the monomeric apo-CruA and as a CruA/Chl a complex. So, this preparation was used for in vitro enzyme assays.

### 3.7 In vitro enzyme assay

Using the method described in Chapter 2.7, the lycopene cyclase activity of the purified CruA was tested in an in vitro enzyme assay with lycopene or γ-carotene as the substrate. All of the HPLC analyses of the in vitro assays were performed by the “ecolicar.m” method. The elution profiles of lycopene and γ-carotene produced by *E. coli* strains BL21(DE3) pACLYC and BL21(DE3) pCPL1 are shown in Figure 3-18.
Figure 3-18: HPLC analysis ("ecolicar.m" method) of substrate lycopene (15.1 min) and substrate γ-carotene (16.4 min). The in-line absorption spectra of the two substrates are shown on the right.

All the controls and assays were done according to the composition of the reaction mixture in Chapter 2.7. A negative control was made for each reaction, for which no substrate was added to the reaction mixture. The negative controls all showed two peaks identified as lycopene and β-carotene in their HPLC 473 nm elution profiles (Figure 3-19). Two other peaks were identified as chlorophyll a (Chl a) and pheophytin a (Pheo a) in the 667 nm elution profile (Figure 3-20). This is in agreement with the absorption spectrum taken for the as-purified CruA solution, which showed the presence of Chl a. So, the as-purified CruA enzyme also had both its substrate lycopene and its product β-carotene bound to it. In addition, the presence of Chl a/Pheo a confirmed the assumption that the bound cofactor was Chl a. It was assumed that the Pheo a was a degradation product of the bound Chl a.
Figure 3-19: HPLC analysis ("ecolcar.m" method) of negative control at 473 nm. The absorption spectra of the two peaks are shown on the top, as lycopene (16.3 min) and β-carotene (18.3 min) characteristic spectra.

According to the peak areas of lycopene and β-carotene in Figure 3-19, the ratio of lycopene to β-carotene is calculated to be 1:15.1.

Figure 3-20: HPLC analysis ("ecolcar.m" method) of negative control at 667 nm. The absorption spectra of the two peaks are shown on the right. The peak at 11.9 min was identified as chlorophyll a and that at 16.8 minutes was identified as pheophytin a from their characteristic absorption spectra and co-elution with standards.
Data of parallel negative controls were also collected and similar results were obtained. Although there are minor peak area ratio changes, the peak area of chlorophyll \(a\) was consistently smaller than that of pheophytin \(a\). The extinction coefficient of chlorophyll \(a\) and pheophytin \(a\) at 667nm are \(89.8 \times 10^3\) M\(^{-1}\) cm\(^{-1}\) and \(52.6 \times 10^3\) M\(^{-1}\) cm\(^{-1}\) respectively, so, the amount of chlorophyll \(a\) was consistently lower than pheophytin \(a\) (see below).

### 3.7.1 Reactions using the reaction condition 1

As described in Chapter 2.7, the reaction condition 1 was initially used in the \textit{in vitro} enzyme assays performed. Different amounts of CruA were used in the 72 µL reaction mixtures. After incubation, the reactions were stopped, and the pigments were extracted and analyzed by HPLC. Peak areas were integrated by the Agilent ChemStation software and recorded in Table 3-1. To eliminate peak differences caused by differences in the amounts injected, the Chl \(a\)/Pheo \(a\) contents were used as an internal standard to normalize the data.

<table>
<thead>
<tr>
<th>CruA final concentration (mg mL(^{-1}))</th>
<th>(\beta)-carotene peak area at 473 nm (mAU)</th>
<th>Chlorophyll peak area at 667 nm (mAU)</th>
<th>Pheophytin peak area at 667 nm (mAU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>Control: 39.6</td>
<td>18.2</td>
<td>89.7</td>
</tr>
<tr>
<td></td>
<td>Assay: 60.1</td>
<td>12.1</td>
<td>96.9</td>
</tr>
<tr>
<td>1</td>
<td>Control: 101</td>
<td>7.6</td>
<td>220.8</td>
</tr>
<tr>
<td></td>
<td>Assay: 128.4</td>
<td>18.8</td>
<td>202</td>
</tr>
<tr>
<td>2</td>
<td>Control: 214.4</td>
<td>48.5</td>
<td>395.3</td>
</tr>
<tr>
<td></td>
<td>Assay: 278.9</td>
<td>23.4</td>
<td>410.4</td>
</tr>
</tbody>
</table>
To calculate the conversion of the reaction, the internal standard Chl \( a \)/Pheo \( a \) amount was first calculated for each injection. The extinction coefficients \( (\varepsilon) \) for Chl \( a \) and Pheo \( a \) at 667 nm are \( 89.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \) and \( 52.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \) respectively. The molecular weight is 893.5 g mol\(^{-1}\) for chlorophyll \( a \) and 871.2 g mol\(^{-1}\) for pheophytin \( a \). The concentrations of Chl \( a \) and Pheo \( a \) can be calculated using equation 3-1. It was assumed that the sum of Chl \( a \) and Pheo \( a \) for a certain amount of enzyme should be the same. Thus, the data in Table 3-1 can be normalized to produce the data in Table 3-2.

\[
\text{Chlorophyll } a \ (\mu\text{mol}) = \frac{\text{peak area (mAU)}}{\varepsilon_{\text{Chl}} (\text{M}^{-1} \text{ cm}^{-1})} / 60
\]

\[
\text{Pheophytin } a \ (\mu\text{mol}) = \frac{\text{peak area (mAU)}}{\varepsilon_{\text{Pheo}} (\text{M}^{-1} \text{ cm}^{-1})} / 60
\]

Equation 3-1: Calculation of the amounts of Chl \( a \) and Pheo \( a \).

<table>
<thead>
<tr>
<th>CruA final concentration (mg mL(^{-1}))</th>
<th>Sum of the Chl ( a )/Pheo ( a ) (nmol)</th>
<th>( \beta )-carotene peak area at 473 nm (mAU)</th>
<th>( \Delta \beta )-carotene peak area at 473 nm (mAU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.0318 Control 39.6</td>
<td>18.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0329 Assay 58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.0714 Control 101</td>
<td>34.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0675 Assay 135.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.1343 Control 214.4</td>
<td>64.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1344 Assay 278.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3-2: Normalized data from Table 3-1 for the enzyme assay (72 \( \mu \)L).

To convert the peak area into moles requires the use of the equation \( A = \varepsilon n l \). For each absorbent compound, the factor \( \varepsilon \) is the extinction coefficient factor for a specific wavelength.
The n and l in the equation are the concentration and the path length of the detection cuvette. For β-carotene, the molar extinction coefficient (ε) at 473 nm and 491 nm are $110.1 \times 10^3$ M$^{-1}$ cm$^{-1}$ and $71 \times 10^3$ M$^{-1}$ cm$^{-1}$. The cuvette length of the HPLC used in this assay is 1 cm. The molecular weight (MW) of β-carotene is 536.9 g mol$^{-1}$. And, the flow rate for the “ecolicar.m” is 1 mL min$^{-1}$. So, the concentration of the β-carotene can be calculated by equation 3-2:

$$\text{β-carotene (µg)} = \frac{\text{peak area (mAU) \times MW}_{\text{β-carotene (g mol}^{-1})/\varepsilon_{\text{β-carotene (M}^{-1}\text{cm}^{-1})}}}{60}$$

Equation 3-2: Calculation of the β-carotene amount.

So, a peak area of 1 mAU for β-carotene at 473 nm corresponds to 0.08 ng of β-carotene. Therefore, for the reactions above, at enzyme concentrations of 0.5 mg mL$^{-1}$, 1 mg mL$^{-1}$, and 2 mg mL$^{-1}$, the amount of β-carotene produced by the reaction was 1.47 ng, 2.78 ng and 5.14 ng, respectively.

This result showed that the in vitro lycopene cyclase activity of the as-purified CruA, conversion of the substrate lycopene to the product β-carotene was achieved. The activity of the CruA in the reaction conditions employed (30 °C with 0.1% detergent DM) may significantly decrease along with the incubation time due to the degradation of the enzyme. Thus, the lycopene cyclase activity (i.e., the conversion of lycopene into β-carotene) of Synechocystis sp. PCC 6803 CruA has been verified in vitro for the first time.

### 3.7.2 Reactions using the reaction condition 2

Another batch of as-purified CruA was obtained and a series of assays were carried out using the reaction conditions (Chapter 2.7, only omitting the FAD, FMN, NAD$^+$, NADP$^+$ from
the reaction condition 1, and keeping all the other additions the same). Two different enzyme concentrations were used in serial assays, and the data were collected in table 3-3.

<table>
<thead>
<tr>
<th>CruA final concentration (mg mL(^{-1}))</th>
<th>(\beta)-carotene peak area at 473 nm (mAU)</th>
<th>Chlorophyll (a) peak area at 667 nm (mAU)</th>
<th>Pheophytin (a) peak area at 667 nm (mAU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 0.4</td>
<td>145</td>
<td>12.2</td>
<td>172.6</td>
</tr>
<tr>
<td>Assay 0.4</td>
<td>135.4</td>
<td>12.5</td>
<td>140.7</td>
</tr>
<tr>
<td>Control 0.8</td>
<td>284.8</td>
<td>115.7</td>
<td>298.7</td>
</tr>
<tr>
<td>Assay 0.8</td>
<td>309.2</td>
<td>137.7</td>
<td>261.7</td>
</tr>
</tbody>
</table>

Using the same calculation method, the raw data can be normalized into table 3-4.

<table>
<thead>
<tr>
<th>CruA final concentration (mg mL(^{-1}))</th>
<th>Sum of the Chl (a)/Pheo (a) (nmol)</th>
<th>(\beta)-carotene peak area at 473 nm (mAU)</th>
<th>(\Delta) (\beta)-carotene peak area at 473 nm (mAU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 0.4</td>
<td>0.0570</td>
<td>Control 145</td>
<td>19.4</td>
</tr>
<tr>
<td></td>
<td>0.0469</td>
<td>Assay 164.4</td>
<td></td>
</tr>
<tr>
<td>Control 0.8</td>
<td>0.1161</td>
<td>Control 284.8</td>
<td>46.2</td>
</tr>
<tr>
<td></td>
<td>0.1085</td>
<td>Assay 331</td>
<td></td>
</tr>
</tbody>
</table>

Therefore, for the reactions above, at CruA final concentrations of 0.4 mg mL\(^{-1}\) and 0.8 mg mL\(^{-1}\), the amount of \(\beta\)-carotene produced by the reaction was 1.55 ng and 3.7 ng, respectively. Table 3-5 compares the amount of \(\beta\)-carotene product for the reaction condition 1 and the reaction condition 2.
Table 3-5: Comparison of Δβ-carotene amount between the two conditions.

| Reaction condition 1 |  | Reaction condition 2 |
|----------------------|---------------------------|
| CruA final concentration (mg mL⁻¹) | Δ β-carotene (ng) | CruA final concentration (mg mL⁻¹) | Δ β-carotene (ng) |
| 0.5 | 1.47 | 0.4 | 1.55 |
| 1 | 2.78 | 0.8 | 3.7 |
| 2 | 5.14 | | |

The results in table 3-5 show that, to achieve the same amount of product β-carotene, less CruA was needed in the modified protocol. Another finding was that the amount Chl a and Pheo a bound to the purified CruA differed among the different preparations. Slightly different handlings and different levels of CruA degradation might cause this difference. Differences in the Chl a/Pheo a:CruA ratio will probably affect the CruA enzyme activity. The CruA with a comparatively larger Chl a/Pheo a:CruA ratio may have better enzymatic activity.

### 3.7.3 Reactions using γ-carotene as substrate

Another possible substrate, γ-carotene, was also used in reaction assays. The as-purified CruA was the same batch as used in the data set presented in Table 3-3. Reaction mixtures (100 µL) were prepared, incubated and analyzed according to the reaction condition 2. The HPLC profile was recorded at 491 nm to have better resolution. Raw data for these γ-carotene assays were collected in Table 3-6.
Table 3-6: Raw data from HPLC peak profiles for the γ-carotene assays (100 µL).

<table>
<thead>
<tr>
<th>CruA final concentration (mg mL⁻¹)</th>
<th>β-carotene peak area at 491 nm (mAU)</th>
<th>Chlorophyll peak area at 667 nm (mAU)</th>
<th>Pheophytin peak area at 667 nm (mAU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control</td>
<td>305.8</td>
<td>172</td>
<td>332.4</td>
</tr>
<tr>
<td>1 Assay</td>
<td>514.5</td>
<td>53.6</td>
<td>346.8</td>
</tr>
<tr>
<td>1 Control</td>
<td>307.6</td>
<td>268.2</td>
<td>267.4</td>
</tr>
<tr>
<td>1 Assay</td>
<td>558.1</td>
<td>52.3</td>
<td>365.6</td>
</tr>
</tbody>
</table>

Using the same calculation method, these raw data were normalized into those in Table 3-7.

Table 3-7: Normalized data for the γ-carotene assays data in Table 3-6.

<table>
<thead>
<tr>
<th>CruA final concentration (mg mL⁻¹)</th>
<th>Sum of the Chl a/Pheo a (nmol)</th>
<th>β-carotene peak area at 491 nm (mAU)</th>
<th>Δ β-carotene peak area at 491 nm (mAU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1372</td>
<td>Control 305.8</td>
<td>283.5</td>
</tr>
<tr>
<td></td>
<td>0.1198</td>
<td>Assay 589.3</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.1345</td>
<td>Control 307.6</td>
<td>290.3</td>
</tr>
<tr>
<td></td>
<td>0.1255</td>
<td>Assay 597.9</td>
<td></td>
</tr>
</tbody>
</table>

The results of the two parallel cyclization reactions of γ-carotene were similar. The increase of the product β-carotene was 35.2 ng and 36.0 ng. These results confirmed a second cyclase activity for CruA, the conversion of γ-carotene to β-carotene.
3.8 The ratio of CruA to Chl a/Pheo a and β-carotene

The as-purified CruA had cofactor Chl a and Pheo a bound to it as well as its reaction product β-carotene (and smaller amounts of substrate lycopene). It was interesting to know the ratio of the CruA to Chl a/Pheo a and β-carotene.

Different batches of the as-purified CruA were analyzed and slight differences in the bound cofactors were observed between two batches, as shown in Table 3-8. For the calculations below, it was assumed that CruA contributed 50% of the protein in the as-purified CruA solution, and only 20% of the CruA in the solution is Native CruA.

Table 3-8: Observed bound cofactors amount of different assay injections.

<table>
<thead>
<tr>
<th>protein final concentration (mg mL⁻¹)</th>
<th>Volume of reaction mixture (µL)</th>
<th>Actual Native CruA molecule (nmol)</th>
<th>Chl a/Pheo a molecule (nmol)</th>
<th>β-carotene molecule (nmol)</th>
<th>Native CruA: Chl a/Pheo a: β-carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5b</td>
<td>72</td>
<td>0.045</td>
<td>0.0318</td>
<td>0.006</td>
<td>1: 0.7: 0.14</td>
</tr>
<tr>
<td>1a</td>
<td>72</td>
<td>0.09</td>
<td>0.0714</td>
<td>0.015</td>
<td>1: 0.8: 0.16</td>
</tr>
<tr>
<td>2a</td>
<td>72</td>
<td>0.18</td>
<td>0.1343</td>
<td>0.032</td>
<td>1: 0.74: 0.18</td>
</tr>
<tr>
<td>0.4b</td>
<td>100</td>
<td>0.05</td>
<td>0.0570</td>
<td>0.022</td>
<td>1: 1.14: 0.44</td>
</tr>
<tr>
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<td>0.1</td>
<td>0.1161</td>
<td>0.042</td>
<td>1: 1.16: 0.42</td>
</tr>
<tr>
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<td>0.13</td>
<td>0.1372</td>
<td>0.071</td>
<td>1: 1.06: 0.55</td>
</tr>
<tr>
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<td>0.1345</td>
<td>0.071</td>
<td>1: 1.04: 0.55</td>
</tr>
<tr>
<td>1c</td>
<td>100</td>
<td>0.13</td>
<td>0.1875</td>
<td>0.017</td>
<td>1: 1.44: 0.14</td>
</tr>
</tbody>
</table>

Note: In the above table 3-8, the footnote a, b and c in the first column indicates three different preparations of as-purified CruA used in assays.

So, the ratio of CruA: Chl a/Pheo a differs for different enzyme batches, from 1:0.7 to 1:1.44. By comparison, the amount of the bound product β-carotene was much smaller with a
ratio of CruA:β-carotene from 1:0.14 to 1:0.55. As shown above, a number of factors, including the choice of detergent and proteolytic degradation of the purified CruA might influence these ratios. Considering that a significant proportion of the as-purified CruA did not carry bound cofactors, these numbers are undoubtedly lower than would be expected for a completely native enzyme preparation.
Reference

Chapter 4

Discussion

4.1 The Synechocystis sp. PCC 6803 CruA and its cofactor chlorophyll a/pheophytin a

The function of the product of open reading frame sll0147 in the cyanobacterium Synechocystis sp. PCC 6803 was studied in this work. Through in vivo and in vitro enzyme activity assays, this protein has been demonstrated to have lycopene cyclase activity. Of the four families of lycopene cyclases identified so far, ORF sll0147 belongs to the newly identified family of lycopene cyclases, the CruA family, so it is re-designated as the cruA gene of Synechocystis sp. PCC 6803.

The C-terminal, hexa-His-tagged as-purified CruA was yellow-green in color, due to the presence of non-covalently bound chlorophyll a/pheophytin a and a relatively small amount of β-carotene. When Synechococcus sp. PCC 7002 cruA was expressed in an E. coli strain producing lycopene, no lycopene cyclase activity was observed (1). Expression of Synechocystis sp. PCC 6803 ORF sll0147 (cruA) in E. coli led to similar results (this study). The finding that CruA carries bound Chl a/Pheo a, and obvious fact that E. coli cannot synthesize Chls, suggests that the bound Chl a cofactor is essential for lycopene cyclase activity. Expression of the same genes that produced no activity in E. coli led to lycopene cyclase activity in the cyanobacterium Synechococcus sp. PCC 7002. Both the complementation assays with a cruA deletion mutant as well as purification and in vitro enzymes assays showed that CruA could be produced in an active form in cyanobacteria. It is important to note that CruP, a protein paralogous to CruA, exhibited
lycopene monocyclase activity when expressed in *E. coli* (1). Importantly, CruP lacks the N-terminal extension that is suspected to bind Chl *a* in CruA (alignment data not shown).

A recent study on the *Synechocystis* sp. PCC 6803 CP43 and CP47 complexes revealed that both purified His-tagged CP47 and His-tagged CP43 bind chlorophyll *a* and β-carotene (2). Previous studies also indicate that site-direct mutations of conserved histidine residues of *Synechocystis* sp. PCC 6803 CP47 lead to decrease amount of chlorophyll bound (3). So, it is possible that conserved histidine residue(s) in CruA sequences are responsible for binding the cofactor Chl *a*/Pheo *a*.

In addition, after identification of the CruA family lycopene cyclase, its orthologs are found in all available green sulfur bacterial genomes and in all cyanobacterial genomes that lack genes encoding CrtL- or CrtY-type lycopene cyclases. In addition, the cyanobacterial CruA differs from other members of the CruA family by possessing an N-terminal extension. With the evidence collected in this study, the N-terminal extension of cyanobacterial CruA might be responsible to bind the Chl *a*/Pheo *a* to form mature CruA *in vivo*. So, a comparison of 31 cyanobacterial CruA and the *Chlorobaculum tepidum* CruA was made. The alignment result is shown in the Appendix. Within the ~150 amino acids N-terminal extension sequences, no obviously conserved histidine residue was found as expected. However, an absolutely conserved glutamic acid (Glu) residue at position 35 exists among all of the cyanobacterial CruA compared. Serial structure studies on the light-harvesting chlorophyll *a/b*-protein complex associated with photosystem II (LHC-II) in green plants were previously carried out (4) (5) (6). They showed that the chlorophylls are attached to the LHC-II polypeptide by coordination of the central magnesium atom to polar amino-acid side chains or to main-chain carbonyls in the hydrophobic interior of the complex (4). Of the nine chlorophyll ligands they identified, three are charge compensated glutamates, forming ion pairs with adjacent arginines (4) (5) (6). In this CruA case, there are 4 partially conserved arginine residues in the extension sequences at positions 69, 100, 108 and
59

129, respectively. It is proposed here that one or more of these arginines could form Glu-Arg salt bridge(s), to serve as the chlorophyll a ligand(s) in CruA.

4.2 The possible catalytic mechanism of the cyclization reaction

CruA is member of the FixC superfamily of dehydrogenases, and as is the case in all other FixC dehydrogenases, CruA is predicted to have a flavin-binding domain. The cyclization reactions that convert the ψ-ends of lycopene or γ-carotene into rings of various configurations (β or ε rings) are isomerization reactions that produce no net change in mass or redox state of the substrate. Some of the studied flavoenzymes utilize the redox properties of flavin directly in catalysis and some do not (7). A recent study has showed that the CrtY-type lycopene cyclase (8) and the carotene cis-trans isomerase CrtISO (9) carry FAD cofactors, which must be in the reduced state for catalysis to occur. The mechanisms of their non-redox reactions were demonstrated with FAD_red residing in the stabilization of a transition state carrying a (partial) positive charge or of a positively charged intermediate via a charge transfer interaction.

In the *in vitro* assays carried out in this work, two reaction conditions were tested. The reaction condition 2 has redox factors NADH and NADPH, while the reaction condition 1 had NADH, NADPH, and additional FMN, FAD, NAD⁺ and NADP⁺. To convert the same amount of lycopene to β-carotene, reaction condition 1 required more as-purified CruA. This suggests that at least one of the following compounds was inhibitory to the lycopene cyclase activity: NAD⁺, NADP⁺, FMN and FAD. The oxidizing agents in the reaction mixture could oxidize the FAD, causing a decrease in the CruA activity. The results obtained showed the activity, but not much about the mechanism (e.g., whether the presence of FAD_red could increase the activity). Another improved reaction condition, namely reaction condition 3, containing only cofactors NADH,
NADPH and FAD, should be tested to see if these conditions might improve the catalytic efficiency of CruA.

*Synechocystis* sp. PCC 6803 CruA can be pre-classified as a non-redox flavoprotein with Chl *a*/Pheo *a* as the cofactor. This catalytic mechanism probably applies to all cyanobacterial CruA proteins, which have a flavin-binding domain and the unique N-terminal extension. Our lab has also produced His-tagged *Synechococcus* sp. PCC 7002 CruA in the *Synechococcus* sp. PCC 7002 Δ*cruA::aacC1* strain. Purification and characterization of the *Synechococcus* sp. PCC 7002 CruA will provide more information about the activity, the bound cofactor, and the cyclization mechanism as well.

### 4.3 The ratio of CruA to Chl *a*/Pheo *a* and to carotenoids

Calculations shown in the Results suggest that the molar ratio of CruA protein to chlorophyll *a*/pheophytin *a* was within the range 1:0.7~1.44. Factors such as the type of detergent used, the degree of purity of the enzyme, proteolysis/degradation of the native CruA, and the extraction of pigments before HPLC analysis could affect this ratio, causing a lower chlorophyll *a*/pheophytin *a* abundance. Thus, the studies conducted here lead to the conclusion that the chlorophyll *a* to CruA ratio is likely to be 1:1. The substrate lycopene and the product β-carotene were also be detected in as-purified CruA, albeit in comparatively lower amounts relative to chlorophyll *a*.

### 4.4 The two substrates, lycopene and γ-carotene

The linear substrate lycopene and the monocyclic substrate γ-carotene could be cyclized by CruA into β-carotene, with γ-carotene being the preferred substrate *in vitro*. In most
cyanobacteria, the primary carotenoid is β-carotene, but cyanobacteria, including *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 also produce the γ-carotene derivatives myxol and various myxoxanthophylls. It has been speculated that these two pigments are produced by separate biosynthetic branches, and these branches are independently regulated (10). This leads to the assumption that CruA recognizes both ends of the substrate *in vivo*, and once one β-ring is formed at one ψ-end, the CruA will cyclize the other ψ-end immediately to form the dicyclic β-carotene, for production of the downstream pigments such as synechoxanthin and zeaxanthin. The branch point is the modification of all-tans-lycopene, either into the production of β-carotene via CruA/CruP, or into 1-hydroxy-lycopene via CruF. In this case, it will be interesting to carry out *in vitro* CruA enzyme assays using the 1-hydroxy-lycopene as the substrate. This would help to resolve this aspect of carotenoid biosynthesis and could lead to insights into how the branches leading to β-carotene and myxoxanthophylls are regulated.
Reference


Appendix

Sequence comparison of 31 cyanobacterial CruA and *Chlorobaculum tepidum* CruA