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ORIGINAL ARTICLE

Synechocystis sp. PCC 6803 CruA (sll0147) encodes lycopene cyclase and requires bound chlorophyll *a* for activity

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Abstract The genome of the model cyanobacterium, *Synechococcus* sp. PCC 7002, encodes two paralogs of CruA-type lycopene cyclases, SynPCC7002_A2153 and SynPCC7002_A0043, which are denoted *cruA* and *cruP*, respectively. Unlike the wild-type strain, a *cruA* deletion mutant is light-sensitive, grows slowly, and accumulates lycopene, γ-carotene, and 1-OH-lycopene; however, this strain still produces β-carotene and other carotenoids derived from it. Expression of *cruA* from *Synechocystis* sp. PCC 6803 ($cruA_{6803}$) in *Escherichia coli* strains that synthesize either lycopene or γ-carotene did not lead to the synthesis of either γ-carotene or β-carotene, respectively. However, expression of this orthologous $cruA_{6803}$ gene (sll0147) in the *Synechococcus* sp. PCC 7002 *cruA* deletion mutant produced strains with phenotypic properties identical to the wild type. $CruA_{6803}$ was purified from *Synechococcus* sp. PCC 7002 by affinity chromatography, and the purified protein was pale yellow-green due to the presence of bound chlorophyll (Chl) *a* and β-carotene. Native polyacrylamide gel electrophoresis of the partly purified protein in the presence of lithium dodecylsulfate at 4 °C confirmed that the protein was yellow-green in color. When purified $CruA_{6803}$ was assayed in vitro with either lycopene or γ-carotene as substrate, β-carotene was synthesized. These data establish that $CruA_{6803}$ is a lycopene cyclase and that it requires a bound Chl *a* molecule for activity. Possible binding sites for Chl *a* and the potential regulatory role of the Chl *a* in coordination of Chl and carotenoid biosynthesis are discussed.

Keywords Carotenoids · Carotenogenesis · Lycopene cyclase · β-Carotene · Chlorophyll · Photosynthesis

Abbreviations

BChl	Bacteriochlorophyll
Chl	Chlorophyll
DM	*n*-Dodecyl-β-D-maltoside
DMAP	Dimethylallyl pyrophosphate
FAD	Flavin adenine dinucleotide
FPP	Farnesyl pyrophosphate
GGP	Geranyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
IPP	Isopentenyl pyrophosphate
LDS	Lithium dodecylsulfate
OG	*n*-Octyl-β-D-glucopyranoside
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulfonylfluoride
PS	Photosystem
SDS	Sodium dodecylsulfate

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Introduction

Carotenoids are widely distributed in chlorophototrophic microorganisms, phototrophs dependent upon either chlorophyll (Chl) or bacteriochlorophyll (BChl) (Domonkos et al. 2013; Maresca et al. 2008a; Takaichi 2011). However, carotenoids are also synthesized in many heterotrophic bacteria, fungi and even some insects (Moran and Jarvik 2010). Carotenoids may play a role in membrane structure and stabilization, but carotenoids generally play three important roles in chlorophototrophs: light harvesting, photoprotection, and structural stabilization of proteins (Cogdell 1978; Frank and Cogdell 1996; Zakar et al. 2016). Carotenoids also occur in organisms that contain retinal-binding bacteriorhodopin, proteorhodopsin, or halorhodopsin (Bryant and Frigaard 2006). Retinal, an oxidative cleavage product derived from β-carotene, additionally plays a photochemical role in these organisms through a light-induced isomerization cycle that results in H^+, Cl^-, or Na^+ translocation and energy storage across the cytoplasmic membrane (Bryant and Frigaard 2006; Kandori 2015). A subfamily of retinal-binding proteins, xanthorhodopsins, also binds a molecule of salinixanthin, a xanthophyll carotenoid that acts as an antenna pigment (Balashov et al. 2005; Balashov and Lanyi 2007; Luecke et al. 2008).

Structural studies have shown that carotenoids are important components of both photosystems (PSs) I and II in cyanobacteria (Grotjohann and Fromme 2005; Shen 2015; Umena et al. 2011; Zakar et al. 2016), and the water-soluble, orange carotenoid-binding protein is an important regulatory molecule promoting non-photochemical quenching of phycobilisomes in these organisms (Kirilovsky and Kerfeld 2013). The predominant carotenoid associated with PS I and PS II is all-*trans* β-carotene, and in most cases, the β-carotene molecules occur within van der Waals contact of one or more Chl molecules. When oxygen evolution is blocked, or when PS II core complexes are placed at low temperature, β-carotene can also become an electron donor in secondary electron transfer pathways (Tracewell et al. 2001). One molecule of echinenone, (β,β-caroten-4-one) and one Chl *a* molecule are also important structural components of the cytochrome b_6f complex of cyanobacteria (Cramer et al. 2005; Kurisu et al. 2003). Because of the significant roles of β-carotene in the structure, function, and assembly of the major complexes of the cyanobacterial photosynthetic apparatus, all cyanobacteria synthesize β-carotene. *Prochlorococcus* spp. and *Acaryochloris marina* additionally synthesize α-carotene (β,ε-carotene) (Takaichi et al. 2012).

Cyanobacterial carotenoids are C_{40} molecules synthesized from the universal isoprenoid precursor isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DMAP), by the non-mevalonate (2*C*-methyl-D-erythritol 4-phosphate) pathway (Vranová et al. 2013). IPP and DMAP are condensed to produce the C_{10} precursor, geranyl pyrophosphate (GPP) (Suppl. Fig. S1A). Two additional IPP molecules are sequentially condensed with GPP to produce C_{15} farnesyl pyrophosphate (FPP) and then the C_{20} intermediate, geranylgeranyl pyrophosphate (GGPP). The head-to-head condensation of two C_{20} molecules of GGPP produces the initial C_{40} intermediate, 15-*cis*-phytoene (Suppl. Fig. S1B). The sequential actions of CrtP, Z-ISO, CrtQ, and CrtH convert phytoene into all-*trans*-lycopene (Chen et al. 2010; Domonkos et al. 2013). Lycopene is the precursor of cyclic α-, β-, γ-, δ-, and ε-carotenes as well as xanthophyll carotenoids, and lycopene cyclases catalyze the formation of the cyclic end-groups of these carotenoids (Domonkos et al. 2013; Maresca et al. 2008a). The primary cyclization reactions of the ψ-ends of lycopene to form β- or ε-rings are isomerization reactions that produce no net change in mass or redox state of the substrate.

Four types of lycopene cyclases have been identified in bacteria and archaea (Maresca et al. 2007, 2008a). The bacterial CrtY-type of lycopene cyclase was first identified in *Erwinia uredovora* (Misawa et al. 1990), and cyclases similar to CrtY are commonly found in carotenogenic *Proteobacteria*, *Streptomyces* spp., and members of the *Chloroflexi*, but CrtY has not been found in cyanobacteria (Maresca et al. 2008a). When heterologously expressed in *Escherichia coli*, CrtY introduces two β-rings into lycopene, producing β-carotene (Misawa et al. 1990; Schnurr et al. 1996). The first member of the CrtL subfamily was identified in the cyanobacterium, *Synechococcus* sp. PCC 7942, and when *crtL* was expressed in *E. coli*, it also converted lycopene into β-carotene (Cunningham et al. 1994). Lycopene cyclases in this family include the β- and ε-cyclases, which are found in some cyanobacteria and higher plants (Stickforth et al. 2003). The heterodimeric $CrtY_c/CrtY_d$ lycopene cyclases of some Gram-positive bacteria [e.g., see Krubasick and Sandmann (2000) and Viveiros et al. (2000)] are related to the lycopene cyclases of archaea (Peck et al. 2002; Hemmi et al. 2003) and halophilic bacteria (Mongodin et al. 2005). 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 by phylogenetic profiling and by a complementation assay in *E. coli* (Maresca et al. 2007). Orthologs of *cruA* are found in all cyanobacterial genomes that lack homologs of *crtL*, and a paralogous gene, *cruP*, is usually found in these same species. Exceptions include *Synechococcus* spp. PCC 7942 and PCC 6301, which each have

a CrtL-type lycopene cyclase and a homolog of CruP (Maresca et al. 2007). Like CrtL- and CrtY-type lycopene cyclases, CruA family members belong to the FixC superfamily of dehydrogenases; CruA-type lycopene cyclases are predicted to be atypical membrane proteins with flavin-binding domains (Maresca et al. 2007; Yu et al. 2010).

Two homologs of *cruA,* which were named *cruA* and *cruP*, respectively, were identified in the genome of the cyanobacterium *Synechococcus* sp. PCC 7002 (Maresca et al. 2007). The lycopene cyclase activity of *Synechococcus* sp. PCC 7002 CruP (i.e., CruA Paralog) was demonstrated in *E. coli:* CruP converted lycopene into γ-carotene (i.e., β, ψ-carotene) but β-carotene was not produced (Suppl. Fig. S1B). The carotenoid composition of a *cruP* mutant was similar to that of wild-type *Synechococcus* sp. PCC 7002 and importantly still allowed the synthesis of β-carotene and allowed nearly wild-type growth (Maresca et al. 2007). Similar results have been observed for *Synechocystis* sp. PCC 6803 (Liang et al. 2008). The activity of CruA as a lycopene cyclase could be inferred from the altered carotenoid content of a *cruA* insertion mutant, but attempts to express *cruA* heterologously in *E. coli* failed to show any conversion of lycopene into γ-carotene or γ-carotene into β-carotene (Maresca et al. 2007). Collectively, the data suggested that CruP was a lycopene monocyclase when expressed in *E. coli*, but that this enzyme might produce some β-carotene in cyanobacteria. Maresca et al. (2007) concluded that CruP probably acts as an accessory lycopene cyclase to CruA, which phenotypically was inferred to be the major lycopene cyclase in most cyanobacterial cells. Surprisingly, Bradbury et al. (2012) detected no lycopene cyclase activity when they attempted to express the *Synechocystis* sp. PCC 6803 *cruP* gene in an *E. coli* strain that could synthesize lycopene. The reason for this discrepancy from the results of Maresca et al. (2007) is currently unknown.

Cyanobacterial CruA homologs possess a large, N-terminal extension of about 120 amino acids that is not observed in CruA of green sulfur bacteria (Maresca et al. 2007) (Suppl. Fig. S2). This domain is unique to cyanobacterial CruA, and no other sequences similar to this domain are found in GenBank. This unique domain might play an important role in the lycopene cyclase activity of cyanobacterial CruA. To explain the various conflicting observations described above, we hypothesized that this domain binds an unidentified cofactor related to photosynthesis that cannot be produced by *E. coli*. Alternatively, this domain might mediate binding of another protein to CruA that is required for cyclase activity, analogous to the situation for heterodimeric lycopene cyclases (Krubasick and Sandmann 2000; Viveiros et al. 2000). Either of these possibilities could explain why *Synechococcus* sp. PCC 7002 *cruA* is inactive when expressed in *E. coli*.

Orthologs of *Synechococcus* sp. PCC 7002 *cruA* and *cruP*, namely sll0147 and sll0659, respectively, are also found in the genome of *Synechocystis* sp. PCC 6803. *Synechocystis* sp. PCC 6803 CruA is predicted to be a protein of 668 amino acids, which shares 65 % sequence identity with CruA from *Synechococcus* sp. PCC 7002 and 35 % identity to CruA from *Chlorobaculum tepidum*. The purpose of this study was to demonstrate that $CruA_{6803}$ (sll0147) has lycopene cyclase activity and to understand why this protein is inactive when expressed in *E. coli*. A biochemical demonstration that sll0147 truly encodes lycopene cyclase would fill a significant gap in our understanding of carotenogenesis in cyanobacteria.

Materials and methods

Cell cultures and growth conditions

Synechococcus sp. PCC 7002 was obtained from the Pasteur Culture Collection (Rippka et al. 1979) and was grown in liquid or on solidified medium A supplemented with 1 mg $NaNO_3$ mL^{-1} (designated as medium A^+) (Stevens et al. 1973; Stevens and Porter 1980). Liquid cultures were grown at 38 °C under cool-white fluorescent illumination at an irradiance of 250 µmol photons m^{-2} s^{-1} and were sparged with air supplemented with 1 % (v/v) CO_2. These conditions have been defined as "standard growth conditions" for this cyanobacterium (see Ludwig and Bryant 2011). Agar plates were prepared by solidifying the A^+ medium with 1.5 % (w/v) Bacto™ Agar (BD Difco, Sparks, MD) supplemented with 0.3 % (w/v) sodium thiosulfate. Wild-type *Synechocystis* sp. PCC 6803 was grown in medium B-HEPES at 30 °C at an irradiance of 150 µmol photons m^{-2} s^{-1} with sparging with 1 % (v/v) CO_2 in air (designated as standard growth conditions for this organism). This medium was prepared by supplementing BG-11 medium (Dubbs and Bryant 1991) with 4.6 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)-KOH and 18 mg ferric ammonium citrate L^{-1}.

For construction of the *cruA* deletion mutant (*ΔcruA::aacC1*) of *Synechococcus* sp. PCC 7002, the medium was supplemented with 10 mM glycerol, a metabolizable carbon/energy source (Lambert and Stevens 1986), and 100 µg gentamycin mL^{-1}, and the cells were grown under 30 µmol photons m^{-2} s^{-1}. When the *cruA* mutant strain was grown on a solid medium, molecular-biology-grade agarose was used. For the two complemented strains carrying plasmids pAQ1Ex [$(His)_{10}$-$cruA_{6803}$] and pAQ1Ex [$cruA_{6803}$-$(His)_6$], the growth

conditions were the same as for wild type and the *ΔcruA::aacC1* mutant except for the addition of 100 μg gentamycin mL^{-1} and 100 μg spectinomycin mL^{-1}. For strain storage and maintenance purposes, the strains were frequently re-inoculated on solid A^+ medium supplemented with appropriate antibiotic(s) and kept at ambient CO_2 at 30 °C under an irradiance 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 and the complemented strains.

Electro-competent *E. coli* strains DH5α, DH10B, or BL21 (DE3) were used for routine recombinant DNA procedures. *E. coli* was grown at 37 °C in Luria–Bertani (LB) liquid medium or LB medium solidified with Bacto™ Agar (15 g L^{-1}), supplemented with 100 μg ampicillin mL^{-1} or 100 μg spectinomycin mL^{-1} as required. The lycopene-producing *E. coli* strain BL21(DE3) harboring plasmid pAC-LYC (Cunningham et al. 1994) was grown at 30 °C for 24 h in liquid LB medium containing 34 μg chloramphenicol mL^{-1} before extraction of lycopene from the cells. The γ-carotene producing *E. coli* strain BL21(DE3) harboring plasmids pAC-LYC and pCPL1 (Maresca et al. 2008b) was grown at 30 °C for 24 h in liquid LB medium containing 34 μg chloramphenicol mL^{-1} and 100 μg ampicillin mL^{-1}.

Construction of *cruA* mutants in cyanobacteria

The upstream and downstream flanking sequences of the *cruA* gene were amplified from genomic DNA of *Synechococcus* sp. PCC 7002 using the oligonucleotide primers described in Table S1 (also see Fig. 1). Restriction sites for HindIII and XbaI were included at 5′ and 3′ ends of the flanking sequences, respectively. These primers were designed to replace the entire *cruA* coding sequence with an antibiotic resistance cassette (see Fig. 1). PCR products were purified by electrophoresis on agarose gels, excised from the gel, purified using the PerfectPrep™ Gel Clean-up Kit (Eppendorf, Hauppauge, NY), and digested with HindIII and XbaI, respectively, and repurified. The *aacC1* gene, encoding resistance to gentamicin, was excised from plasmid pMS255 with enzymes HindIII and XbaI. This gentamicin cartridge was purified as described above and ligated to the left and right flanking sequences at a DNA fragment ratio of 3:1:3 (wt:wt:wt; flank 1:*aacCI* cassette:flank 2) with T4 DNA ligase. The ligation product was directly transformed into *Synechococcus* sp. PCC 7002 as described (Frigaard et al. 2004). After a 5-h incubation under normal growth conditions, the transformation mixture (~2.0 mL) was placed under reduced light (30 μmol photons m^{-2} s^{-1}) without aeration for 24 h. Cells were then plated directly onto solid medium containing 10 mM glycerol and gentamicin. Single colonies were picked from plates and repeatedly streaked on selective media until segregation was confirmed by analytical PCR using the upstream the downstream primers (see Fig. 1 and Table S1) used to produce the flanking DNA fragments. PCR amplicons were further confirmed by DNA sequencing.

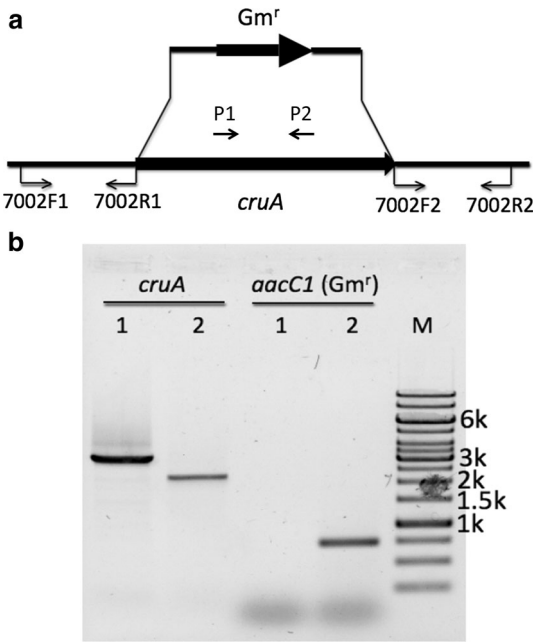

Fig. 1 Deletion of the *cruA* gene of *Synechococcus* sp. PCC 7002. **a** The scheme at the top shows the complete deletion of the *cruA* gene and its replacement by the *aacC1* gene encoding gentamicin (Gm^R) resistance. **b** PCR amplicons showing the complete segregation of the *cruA* and *ΔcruA::aacC1* alleles. The lanes on the left show PCR amplicons produced with primers 7002F1 and 7002R2 (see **a**) using genomic DNA from the wild type (*lane 1*) and the *ΔcruA::aacC1* mutant (*lane 2*) as templates. The PCR amplicon in *lane 2* is smaller because the *aacC1* gene is smaller than the *cruA* gene. *Lanes* on the *right* show products obtained from internal primers *aacCI*_P1 (P1) and *aacCI*_P2 (P2) (see **a** and Table S1) using genomic DNA from the wild type (*lane 1*) and the *ΔcruA::aacC1* mutant (*lane 2*) as templates. The *aacC1* gene is only present in the *ΔcruA::aacC1* mutant strain. M, DNA standards (k = kbp)

Pigment extraction and analysis

Synechococcus sp. PCC 7002 cells were typically grown until late exponential phase ($OD_{730\ nm}$ = ~0.7 to 1.5) for pigment analyses, which were performed by reversed-phase, high-performance liquid chromatography (HPLC). Cells from 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 syringe filter. Extracts were prepared from freshly harvested fresh cells each time prior to

analysis. Pigments were separated by reversed-phase chromatography on a 25 cm × 4.6 mm analytical Discovery 5-μm C_{18} column (Supelco, Bellefonte, PA) using an Agilent Model 1100 HPLC system equipped with a model G1315B diode array detector and controlled with Agilent ChemStation software (Agilent Technologies, Palo Alto, CA). The HPLC conditions for carotenoid separation used the following gradient conditions [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 this method was water–methanol–acetonitrile (62.5:21:16.5, v/v/v) containing 10 mM ammonium acetate; solvent B was methanol–ethyl acetate–acetonitrile (50:30:20, v/v/v). Pigments were identified according to their retention time and absorption spectra as well as by comparison with standards established in the laboratory (Graham and Bryant 2008, 2009; Maresca et al. 2007).

Lycopene and γ-carotene were purified by extracting the *E. coli* BL21 (DE3):pAC-LYC and the *E. coli* strain BL21(DE3) pAC-LYC/pCPL1 cell pellets, respectively, using 100 % acetone. Extractions were repeated with sonication until cells were colorless. After centrifugation the combined colored acetone extracts containing lycopene or γ-carotene were placed in 20-mL vials, and the extracts were concentrated by evaporation to ~10 mL under dim light under a stream of 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 pigments derived from *E. coli* strains. The HPLC gradient conditions were as follows [time in min, %B]: [0, 70], [10, 90], [15, 100], [25, 100], and [26, 70]. Solvent A consisted of water–methanol–acetonitrile (62.5:21:16.5, v/v/v), and solvent B was methanol–ethyl acetate–acetonitrile (50:30:20, v/v/v). The lycopene or γ-carotene was quantified, and the lycopene or γ-carotene was divided into aliquots in reaction vials, dried, and stored at −20 °C until needed.

Protein analyses and immunoblotting

Protein concentrations were determined using the Coomassie Plus protein assay kit from Thermo Scientific (Rockford, IL). Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate (SDS) and immunoblotting was performed as previously described (Shen and Bryant 1995; Shen et al. 2002). PAGE in the presence of lithium dodecylsulfate was performed at 4 °C by replacing SDS from electrophoresis buffers and stocks with lithium dodecylsulfate (LDS). Tryptic peptide mass fingerprinting was performed at the Proteomics and Mass Spectrometry Core Facility in the Huck Institutes for the Life Sciences at The Pennsylvania State University, University Park, PA.

Purification of CruA

The *cruA* mutant strain of *Synechococcus* sp. PCC 7002 harboring plasmid pAQ1Ex [$cruA_{6803}$-$(His)_6$] was cultured in 3.0 L of A^+ medium supplemented with 10 mM glycerol, 100 μg gentamycin mL^{-1}, and 100 μg spectinomycin mL^{-1} under standard growth conditions. The cells were harvested by centrifugation when $OD_{730\ nm} = 1.5$, and all additional purification steps were performed at 4 °C. The cell 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 DNase I mL^{-1}, and the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 0.5 mM. This cell suspension was disrupted by three passages through a chilled French pressure cell at 138 MPa. Unbroken cells and large cell debris were removed by centrifugation at 4500×*g* for 20 min. The supernatant contained both the membrane and the soluble cytosolic fraction, which were separated by centrifugation at 133,200×*g* for 45 min. Both fractions were collected and analyzed for the presence of the target protein, CruA (sll0147) (see below). The membrane fraction (i.e., the pellet) from ultracentrifugation was resuspended in binding buffer (45 mL) supplemented with 0.5 mM PMSF and 1 % (w/v, final concentration) LDS. The resuspended membrane solution was incubated at 4 °C with gentle shaking for 1 h. The solubilized membranes were subjected to ultracentrifugation at 133,200×*g* for 20 min; the supernatant from this centrifugation step will be referred to below as the "solubilized membrane extract." Other detergents were also tested for their ability to solubilize the membrane fraction. These detergents and their final concentrations in the solubilization mixtures were 2 % (w/v) *n*-dodecyl-β-D-dodecyl maltoside (DM), 0.8 % (w/v) *n*-octyl-β-D-glucoside (OG), and 1 % (w/v) Triton X-100.

$CruA_{6803}$ 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 pretreated resin (2 mL) 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 loaded 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). $CruA_{6803}$ 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 % DM). Fractions (~20 mL) were collected, and aliquots were prepared from each fraction

for analysis by SDS-PAGE. Purified $CruA_{6803}$ was dialyzed overnight against 1:200 (v/v) 50 mM Tris–HCl, pH 6.8, supplemented with 0.005 % (w/v) LDS at 4 °C at a concentration of ~2 mg $CruA_{6803}$ mL^{-1}.

In vitro enzyme assays

Lycopene and γ-carotene were prepared from *E. coli* BL21 DE3 strains harboring plasmids pAC-LYC or pAC-LYC and pCPL1 (Maresca et al. 2007), respectively. Cells from an overnight culture were harvested by centrifugation, and carotenoids were extracted by sonication in acetone. The extracts were dried under a stream of nitrogen in reaction vials and stored in the dark at −20 °C until required. The concentration of as-purified $CruA_{6803}$ was determined using the Bradford method adding the enzyme to the reaction mixture. Because of the low yield and the instability of $CruA_{6803}$, it was not possible to optimize the reaction conditions. The molar extinction coefficient (ε) at 473 nm for β-carotene is 110,100 M^{-1} cm^{-1}, and the molecular weight of β-carotene is 536.9 g mol^{-1} (Britton et al. 1994).

The complete reaction mixture (100 µL) for the in vitro assays contained the following: (reflecting final concentrations): 6 mM $MgCl_2$, 6 mM $MnCl_2$, 50 mM Tris–HCl, pH 6.8, 2.2 µg lycopene (4.1 µM) or 120 µg γ-carotene (223 µM), 0.5 mM NADH, 0.5 mM NADPH, 0.1 % (w/v) DM and 0.4–1.0 mg mL^{-1} (0.5-1.3 µM) of as-purified $CruA_{6803}$. Parallel control reactions in which no substrate was added were performed for each test condition. Reactions were incubated at 30 °C with gentle shaking for 16 h. Reactions were stopped by adding 100 µL acetone to the reaction vials. The 200-µL solutions were centrifuged at 12,000×*g* for 3 min, and the supernatants (~200 µL) were collected and transferred to a clean microcentrifuge 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 extraction. This step was repeated so that the final pigment solution was approximately 500 µL. The pigment content of an aliquot of this solution was analyzed by HPLC as described above.

Results

Construction and validation of a *cruA* deletion mutant of *Synechococcus* sp. PCC 7002

In previous studies, only a small part of the *cruA* gene of *Synechococcus* sp. PCC 7002 was deleted (Maresca et al. 2007), and the remaining sequences could have interfered by recombination with the complementing plasmids that were used in this study. To avoid this complication, the *cruA* gene was completely deleted as shown in Fig. 1A. The upstream and downstream flanking sequences of the *cruA* gene were ligated to the *aacC1* gene (conferring resistance to gentamycin), and after transformation, selection, and repeated streaking, transformants lacking the entire *cruA* gene were obtained. PCR analyses showed that the *cruA* and *ΔcruA::aacC1* alleles had fully segregated in the mutant strain (Fig. 1B). Figure 2 shows HPLC elution profiles monitored at 491 nm for carotenoid pigments extracted from the *Synechococcus* sp. PCC 7002 *cruA* mutant (top) and wild type (bottom). The *ΔcruA::aacC1* mutant strain grew very slowly and had about half the amount of β-carotene per cell as the wild type (data not shown). Furthermore, the *ΔcruA::aacC1* mutant accumulated 1′-OH-lycopene (peak 1), lycopene (peak 2), and γ-carotene (peak 3), which are never observed in the wild type (Fig. 2) (Graham and Bryant 2008, 2009; Maresca et al. 2007). We interpret this phenotype to indicate that CruP (or some other unknown lycopene cyclase) can slowly synthesize β-carotene in *Synechococcus* sp. PCC 7002, but we also infer from these as well as previously published results (Maresca et al. 2007; also see below) that CruA is the principal lycopene cyclase in this cyanobacterium. Inefficient conversion of lycopene and γ-carotene into β-carotene would account for the accumulation of

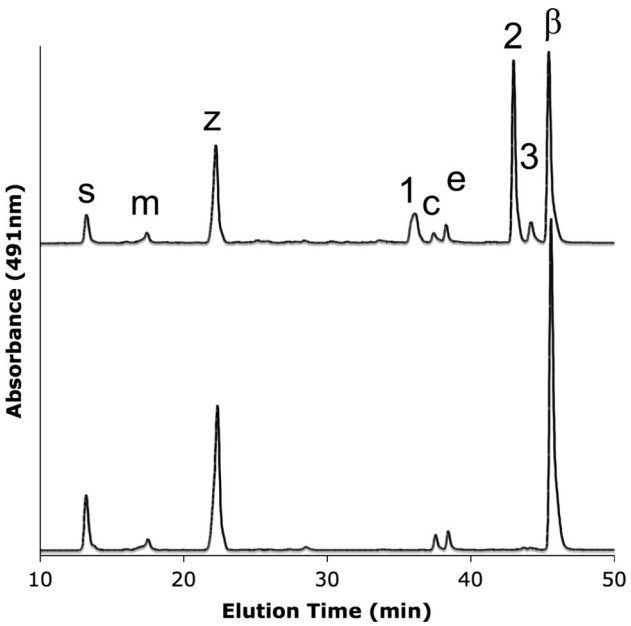

Fig. 2 HPLC analysis of carotenoids in wild type and the *ΔcruA::aacC1* mutant strains fo *Synechococus* sp. PCC 7002. HPLC analysis of *Synechococcus* sp. PCC 7002 wild type (*lower trace*) and the *ΔcruA::aacC1* deletion mutant (*upper trace*). Carotenoids exclusively produced in the mutant strain: *1* 1-OH-lycopene; *2* lycopene; and *3* γ-carotene. Carotenoids produced in the wild-type strain: *s* synechoxanthin; *m* myxoxanthophyll; *z* zeaxanthin; *c* cryptoxanthin; *e* echinenone; *β* β-carotene (see Graham and Bryant 2008, 2009 for identification of these carotenoids)

lycopene and the other carotenoids that are not observed in the wild type. The altered carotenoid composition and the slow, light-sensitive growth of the *cruA* mutant comprise an obvious phenotype that provides the basis for a complementation assay to assess the functionality of putative lycopene cyclases.

Construction and characterization of expression plasmids for *cruA* in *E. coli* and the *cruA* mutant of *Synechococcus* sp. PCC 7002

Expression plasmids for the *Synechocystis* sp. PCC 6803 *cruA* gene ($cruA_{6803}$) were constructed using the pAQ1Ex plasmid and the P_{cpcBA} promoter from *Synechocystis* sp. PCC 6803 (Xu et al. 2011). This promoter is constitutively and strongly expressed in *E. coli* as well as *Synechococcus* sp. PCC 7002 (Xu et al. 2011; Zhou et al. 2014). The full-length $cruA_{6803}$ gene in these expression constructs was modified to include a poly-histidine tag at either the N terminus or the C terminus (Xu et al. 2011). These expression strains were co-transformed into *E. coli* BL21 (DE3) cells together with plasmids pAC-LYC (Cm^R) or pAC-LYC and pCPL (Cm^R, Ap^R), which direct the synthesis of lycopene and γ-carotene, respectively. Expression of a immunoreactive protein of the ~77-kDa $CruA_{6803}$ polypeptide in the *E. coli* strains could readily be detected by immunoblotting with antibodies directed against the poly-histidine tag (Fig. 3). However, no conversion of pink-colored lycopene into orange-colored γ-carotene or β-carotene was observed in any of these strains (data not shown). Thus, while the *cruA* genes were clearly expressed in these *E. coli* strains, no lycopene cyclase activity was detected in *E. coli*. Because no activity was detected, these proteins were not further characterized.

When these same two plasmids were transformed into the *ΔcruA::aacC1* mutant of *Synechococcus* sp. PCC 7002, however, clear evidence for complementation of the *cruA*

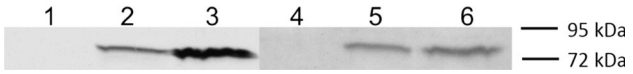

Fig. 3 Immunoblot analysis of *E. coli* whole-cell lysates from strains expressing poly-His-tagged $cruA_{6803}$. The antibodies are rabbit polyclonal antibodies directed against a poly-$(His)_6$ epitope tag and conjugated to horseradish peroxidase. The predicted mass of $CruA_{6803}$ with an N-terminal poly-$(His)_{10}$ tag is 77.7 kDa and with a C-terminal poly-$(His)_6$ tag is 77.1 kDa. The corresponding strains are: *lane 1 E. coli* BL21 (DE3) (pAC-LYC, pAQ1Ex-P_{cpcBA}); *lane 2 E. coli* BL21 (DE3) (pAC-LYC, pAQ1Ex [$(His)_{10}$-$cruA_{6803}$]; *lane 3 E. coli* BL21 (DE3) (pAC-LYC, pAQ1Ex [$cruA_{6803}$-$(His)_6$]); *lane 4 E. coli* BL21 (DE3) (pAC-LYC, pCPL1, pAQ1Ex-P_{cpcBA}); *lane 5 E. coli* BL21 (DE3) (pAC-LYC, pCPL1, pAQ1Ex [$(His)_{10}$-$cruA_{6803}$]); *lane 6 E. coli* BL21 (DE3) (pAC-LYC, pCPL1, pAQ1Ex [$cruA_{6803}$-$(His)_6$]). The control lanes (*1* and *4*) do not contain an immunoreactive protein at ~77 kDa, while all other strains produce an immunoreactive protein with the expected size

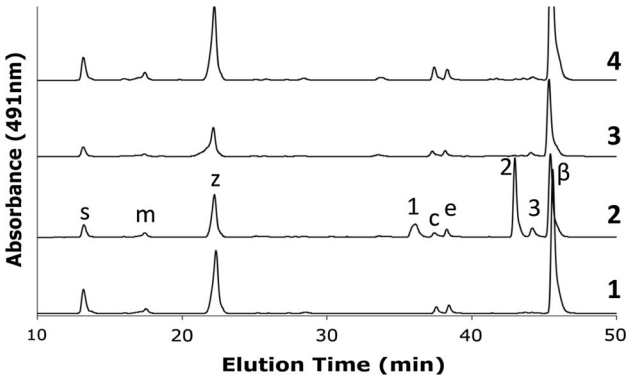

Fig. 4 HPLC analysis of *Synechococcus* sp. PCC 7002 strains. Elution profile 1, wild type; Elution profile 2, *ΔcruA::aacC1* mutant; Elution profile 3, *ΔcruA::aacC1* mutant strain complemented with plasmid pAQ1Ex [$(His)_{10}$-$cruA_{6803}$]; Elution profile 4, *ΔcruA::aacC1* mutant strain complemented with plasmid pAQ1Ex [$cruA_{6803}$-$(His)_6$]. Carotenoids exclusively produced in the mutant strain: *1* 1-OH-lycopene; *2* lycopene; and *3* γ-carotene. Carotenoids produced in the wild-type strain: *s* synechoxanthin; *m* myxoxanthophyll; *z* zeaxanthin; *c* cryptoxanthin; *e* echinenone; *β* β-carotene (see Graham and Bryant 2008, 2009 for details concerning the identification of these carotenoids)

mutant phenotype described above was observed (Fig. 4). The complemented strains were no longer light-sensitive and had doubling times similar to the wild type (data not shown). Moreover, these complemented strains no longer accumulated 1′-OH-lycopene, lycopene, and γ-carotene, but predominantly accumulated β-carotene and zeaxanthin like the wild type (Fig. 4). Collectively, these data support the hypothesis that $CruA_{6803}$ (sll0147) is a lycopene cyclase. However, the active protein produced in *Synechococcus* sp. PCC 7002 must clearly be fundamentally different from the inactive protein that is produced in *E. coli*.

Purification of *Synechocystis* sp. PCC 6803 CruA produced in *Synechococcus* sp. PCC 7002

To characterize the active form of $CruA_{6803}$, *Synechococcus* sp. PCC 7002 cells harboring plasmid pAQ1Ex [$cruA_{6803}$-$(His)_6$] were grown to late exponential phase, and $CruA_{6803}$ was purified by metal chelation chromatography by taking advantage of the C-terminal poly-$(His)_6$ tag. Preliminary experiments indicated that cells producing the C-terminally tagged protein accumulated more CruA protein than cells producing the N-terminally tagged protein. Preliminary experiments additionally showed that $CruA_{6803}$ was very tightly associated with the cellular membranes, so several detergents were tested for their ability to solubilize and release CruA from the membranes. These experiments indicated that the yield and Chl *a* content of the protein (see below) were highest with 1 % (w/v) lithium dodecylsulfate (LDS) (Fig. 5). Crude cell membranes were solubilized with 1 % (w/v) LDS at 4 °C, and

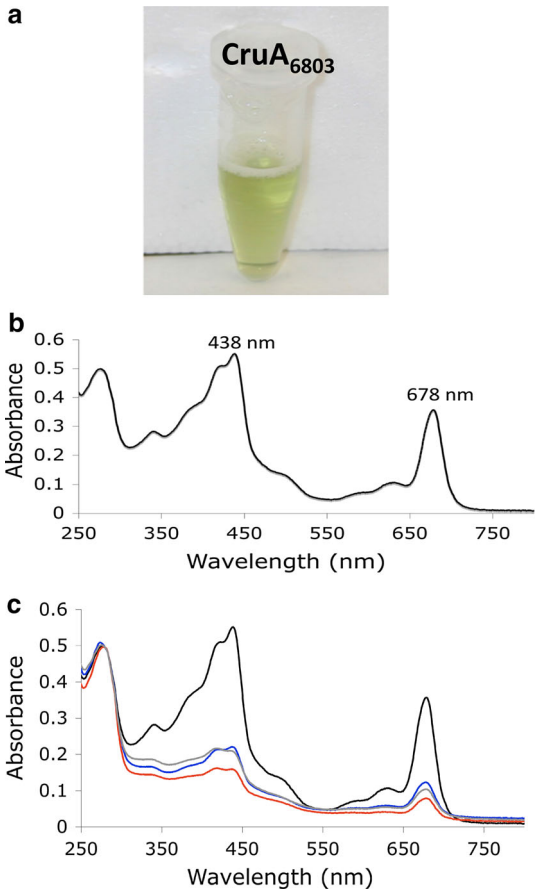

Fig. 5 Appearance of purified $CruA_{6803}$ and the absorbance spectrum of the purified protein. **a** As-purified $CruA_{6803}$ showing the yellow-green color of the purified protein obtained with 1 % (w/v) LDS. **b** Absorbance spectrum of the as-purified $CruA_{6803}$ protein in 50 mM Tris–HCl, pH 6.8 buffer. The absorption maxima for the Q_y (678 nm) and Soret (438 nm) peaks of Chl *a* are indicated. The shoulder at about 490 nm indicates the presence of some carotenoids in the sample. **c** Absorbance spectra of as-purified samples of $CruA_{6803}$ using different detergents in 50 mM Tris–HCl buffer, pH 6.8. 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 (*gray line*). The absorption spectra were normalized at 280 nm to compare the Chl *a* and carotenoid contents of the samples

after centrifugation, the clarified protein solution was subjected to metal chelation chromatography. Figure 5A shows that the purified $CruA_{6803}$ protein solution obtained with 1 % (w/v) LDS was pale yellow-green in color (The N-terminally tagged $CruA_{6803}$ protein solution was similarly yellow-green in color, but this protein was not characterized further). The absorption spectrum of this solution, as shown in Fig. 5B, suggested that Chl *a* and carotenoids absorbing at about 490 nm were present, a result confirmed by HPLC analyses (data not shown but see below). Figure 5C shows that attempts to use non-ionic detergents to purify the protein produced inferior results. The Chl *a* contents of CruA preparations made with Triton X-100, DM, or OG were consistently lower than that obtained with LDS (note that it is known that Triton X-100 can cause displacement of Chls from PS I (e.g., Yang et al. 1998). HPLC analyses showed that the ratio of pheophytin (Pheo) *a*:Chl *a* was generally larger when LDS was used (data not shown). Absorption spectra recorded as a function of time after isolation showed that all of the $CruA_{6803}$ preparations were unstable and that the absorption spectra of the isolated proteins changed over time due to the formation of Pheo *a* (data not shown).

Demonstration that $CruA_{6803}$ is a Chl *a*-binding protein

To verify that CruA was a Chl *a*-binding protein, purified and concentrated $CruA_{6803}$ was subjected to PAGE in the presence of 0.1 % (w/v) LDS at 4 °C. As shown in Fig. 6, a slowly migrating green band was clearly observed under these conditions, and the mobility of this band suggested that CruA formed oligomers, perhaps tetramers, with a molecular mass estimated to be ~300 kDa. When the CruA solution was heated at 65 °C for 7.5 min prior to electrophoresis, the green-colored band was no longer observed (Fig. 6a, b). These experiments further suggested that some of the CruA was dissociating into ~77-kDa

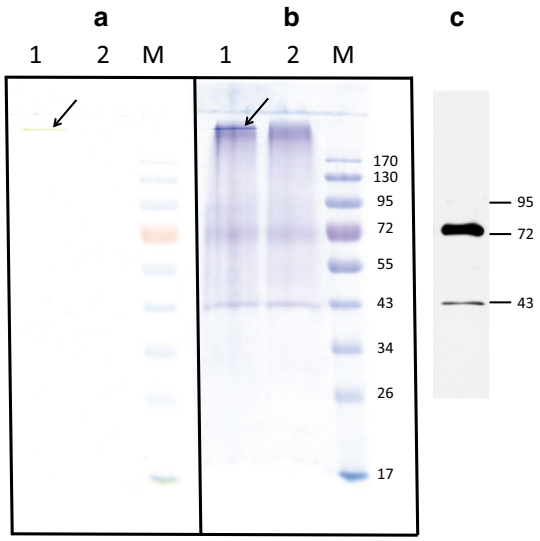

Fig. 6 Analysis of $CruA_{6803}$ by LDS-PAGE at 4 °C by native PAGE (**a**) and after (**b**) moderate heat denaturation, electrophoresis, and staining with Coomassie-blue R-250. The concentrated, as-purified CruA protein (*lanes 1*) and samples of $CruA_{6803}$ treated for 7.5 min at 65 °C in LDS dissociation buffer (*lanes 2*) were analyzed. The arrows indicate the green-colored $CruA_{6803}$ oligomer with a molecular mass of ~300 kDa. M, molecular mass markers (kDa). **c** Immunoblot after SDS-PAGE electrophoresis of heat-treated, as-purified $CruA_{6803}$ using antibodies against the poly-$(His)_6$ tag at the C terminus of the protein. The predicted mass of $CruA_{6803}$ with a C-terminal poly-$(His)_6$ tag is 77.1 kDa. The 43-kDa protein presumably is a degradation product derived from the C-terminal region of $CruA_{6803}$

monomers even in the absence of a heat treatment (Fig. 6b, c) and that a proteolysis product of about 43-kDa was also being formed (see below). Immunoblotting analysis with antibodies against the poly-$(His)_6$ epitope showed that the 77-kDa polypeptide was CruA and that the 43-kDa polypeptide was a degradation product derived from the C-terminus of the protein (Fig. 6c).

The green-colored band and the 77-kDa polypeptide produced after moderate heat treatment were excised from a gel similar to that shown in Fig. 6, and the extracted proteins were subjected to tryptic peptide mass fingerprinting (Fig. 7). This analysis unequivocally established that the green-pigmented protein was $CruA_{6803}$. Tryptic peptides covering 48 % of $CruA_{6803}$ were identified by mass spectrometry, and no other protein was identified by this analysis. Although the coverage was lower (7.9 % coverage), the 77-kDa colorless polypeptide was similarly identified as $CruA_{6803}$. Again, no other protein was identified; moreover, all of the peptides detected in the 77-kDa polypeptide were also identified in the green-pigmented complex. These results demonstrate that $CruA_{6803}$ binds Chl *a*, some carotenoids, and forms oligomers (possibly tetramers) that are sensitive to denaturation and proteolysis during storage and electrophoresis in the presence of LDS.

On the basis of the results from the LDS-PAGE, we estimated that at maximum only 20 % of the CruA was "native" and carried bound pigments. We further estimated that ~50 % of the total protein in our preparations was CruA, although this could be an underestimate. Based on these two assumptions, we calculated the ratio of Chl *a* and β-carotene to CruA polypeptide for three different CruA samples, which were determined in triplicate from three independent CruA preparations. The Chl *a*-to-protein ratio ranged from 0.7 to 1.44 for individual determinations, and the average value was calculated to be 1.01 ± 0.25. The β-carotene contents of the three preparations of CruA were more variable than the Chl *a* contents. Values ranged from 0.14 to 0.55 β-carotenes per CruA polypeptide, and the average β-carotene to CruA ratio was calculated to be 0.32 ± 0.19. These observations suggest that CruA binds at least 1.0 Chl *a* molecule per polypeptide, although this number could increase significantly if the assumptions underestimate the purity of the sample and proportion of the protein that is "native." The substoichiometric amount of bound β-carotene might be due to the dissociation of the carotenoid or could alternatively be due to adventitious binding of β-carotene to the enzyme. β-Carotene is the product (see below) and obviously might bind to the active site.

a

1	MRELLYCEVP	TPATAEVVAW	LQGNWSPLVG	QK**LPTEQGLR**	LRGSSGQSSE
51	LSIFVWSLQR	TTYLKCVRWG	DTSFPQETAI	AKELRRALGQ	KFPPTYPVPP
101	AIDLDR**QNIF**	**QALQDR**YPET	VKFFQKFPQG	EYDLKRVYWW	EQRWRDNVAN
151	PQTPRQVVSQ	TSTVTAIAPA	TPEFDLIYLG	GALGSVHAAV	MARLGYRVLL
201	IERLPFGRMN	REWNISRDEF	QRLIDLNLFT	PEEFESVIAR	EYRDGFNKFF
251	DAYNPPHLK**A**	**NVLHTPTVLN**	**IALDSAK**FLA	ICGHKLQQAG	GIIWDETEFI
301	NATIDPNKVV	VHCINLENNQ	TKLAQARLLV	DAMGTASPIA	WQLNGGKTFN
351	SVCPTVGAVV	EGLDPVVWDN	AYGDVLNSHG	DISRGRQLIW	ELFPGAGDEM
401	TIYLFHYHQV	NRENPGSLLE	MYEDFFSILP	EYRRCNLEKL	TWKK**ATFGYI**
451	**PGHFSTSAQD**	**R**TIALDRLMA	IGDAASLQSP	LVFTGFGSLV	RNLAKLTDLL
501	HTALQYDLLQ	AKQLNQIRAY	QSNIAVTWMF	SKGMMVPTGK	QLPPQQVNAM
551	LNTFFGLLAD	SAPTVAETFI	KDRTTWLLFS	RLALKAASKN	PQLLFWIWQM
601	AGTEDLLKWL	LVYFDFSRQA	LLNALFRFWY	PQWLINSRNW	LEKLSPQLWF
651	SLLCFGYQIQ	PKLAQQSK			

b

1	MRELLYCEVP	TPATAEVVAW	LQGNWSPLVG	QK**LPTEQGLR**	LRGSSGQSSE
51	LSIFVWSLQR	**TTYLK**CVR**WG**	**DTSFPQETAI**	**AK**ELRRALGQ	KFPPTYPVPP
101	AIDLDR**QNIF**	**QALQDRYPET**	**VK**FFQK**FPQG**	**EYDLK**RVY**WW**	**EQRWRDNVAN**
151	**PQTPR**QVVSQ	TSTVTAIAPA	TPEFDLIYLG	GALGSVHAAV	MARLGYR**VLL**
201	**IERLPFGR**MN	R**EWNISR**DEF	QR**LIDLNLFT**	**PEEFESVIAR**	EYR**DGFNK**FF
251	**DAYNPPHLK A**	**NVLHTPTVLN**	**IALDSAK**FLA	ICGHK**LQQAG**	**GIIWDETEFI**
301	**NATIDPNK VV**	**VHCINLENNQ**	**TKLAQAR**LLV	DAMGTASPIA	WQLNGGK**TFN**
351	**SVCPTVGAVV**	**EGLDPVVWDN**	**AYGDVLNSHG**	**DISR**GRQLIW	ELFPGAGDEM
401	TIYLFHYHQV	NR**ENPGSLLE**	**MYEDFFSILP**	**EYR**R**CNLEK**L	TWKK**ATFGYI**
451	**PGHFSTSAQD**	**R**TIALDR**LMA**	**IGDAASLQSP**	**LVFTGFGSLV**	**R**NLAKLTDLL
501	HTALQYDLLQ	AKQLNQIRAY	QSNIAVTWMF	SK**GMMVPTGK**	QLPPQQVNAM
551	LNTFFGLLAD	SAPTVAETFI	KDR**TTWLLFS**	**R**LALKAASKN	PQLLFWIWQM
601	AGTEDLLKWL	LVYFDFSR**QA**	**LLNALFR**FWY	PQWLINSR**NW**	**LEK**LSPQLWF
651	SLLCFGYQIQ	PK**LAQQSK**			

Fig. 7 Tryptic peptides identified by mass spectrometry for as-purified $CruA_{6803}$. **a** Peptides identified for the 77.1-kDa polypeptide in heat-denatured $CruA_{6803}$. **b** Peptides identified for the ~300-kDa green protein complex obtained from LDS-PAGE analysis of $CruA_{6803}$. Only $CruA_{6803}$ was identified by these analyses

In vitro assays with lycopene and γ-carotene show $CruA_{6803}$ is a lycopene cyclase

Substrates for in vitro enzyme assays were purified from *E. coli* strains harboring plasmids pAC-LYC or pAC-LYC and pCPL1 as described in the Materials and Methods. Because of the instability of the isolated CruA protein in the presence of LDS and DM, it was difficult to optimize the reaction conditions to assess the enzymatic activity in vivo. Nevertheless, it was possible to demonstrate cyclase activity with both lycopene and γ-carotene as substrates. Because CruA contained bound Chl *a*, lycopene, and β-carotene (lycopene–β-carotene = 1:15), it was necessary to include a control reaction with no added substrate for all conditions tested. Under the reaction conditions employed, much of the Chl *a* degraded to Pheo *a* during the time course of the 16-h incubation with substrate at room temperature (data not shown). Although the amount of Pheo *a* formed was variable, it was assumed that the sum of the Chl *a* and Pheo *a* values should represent the total amount of Chl *a* originally associated with the enzyme and that this number could be used to compare and normalize the recovery of β-carotene for each reaction. Consistent with this assumption, the recoveries of Chl *a* and Pheo *a* for the control and complete reaction mixes were similar and were directly proportional to the amount of enzyme added to the reaction mixture (data not shown). The ratio of lycopene to β-carotene for the enzyme preparation used for the assays summarized in Table 1 was about 1:15.

Table 1 shows the results obtained with as-purified $CruA_{6803}$ with either lycopene or γ-carotene as substrate. The data presented in Table 1 show that $CruA_{6803}$ could convert both lycopene and γ-carotene into β-carotene. The total amount of β-carotene in the reaction mixture increased substantially when $CruA_{6803}$ was included in reaction mixtures with either substrate, and the magnitude of the increase was roughly proportional to the amount of enzyme added to the reaction. Thus, $CruA_{6803}$ exhibited both mono- and bi-cyclase activity with lycopene (no γ-carotene intermediate was observed). Because the γ-carotene concentration (223 μM) tested was much greater than that for lycopene (4.1 μM), the increase in β-carotene content of the reaction mixture after 16 h was much greater. No γ-carotene intermediate was detected when lycopene was provided as the substrate; this observation also suggests that $CruA_{6803}$ efficiently converts this intermediate to β-carotene.

Discussion

Colored carotenoids are a major class of lipophilic C_{40} terpenoid compounds that were first isolated from foods, plants, and other sources in the early nineteenth century (Armstrong 1997; Maresca et al. 2008a). The first chemical structures of carotenoids were determined in the 1930s (Eugester 1995), and since that time more than 700 different carotenoids have been identified (Britton et al. 1994). The chromophores of carotenoids usually comprise nine to thirteen conjugated double bonds, and the precise chemical properties of the chromophores are responsible for the characteristic colors of carotenoids, which range from pale yellow to orange to deep purplish-red. In contrast to the complexity of carotenoids produced in nature, chlorophototrophic bacteria only synthesize 14 major classes of Chls (Chl *a, b, d, f*, divinyl-Chl *a*, divinyl-Chl *b*, 8-OH-Chl *a*) and BChls (BChl *a, b, c, d, e, f, g*) (Bryant and Liu 2013).

Table 1 In vitro assays with lycopene and γ-carotene as substrates for $CruA_{6803}$

CruA (mg mL^{-1})	Reaction	β-Carotene mAU at 473 nm	Δβ-Carotene[a] mAU at 473 nm	Δβ-Carotene (ng)
Assays with lycopene as substrate				
0.4	Control	145	19	1.55
	Assay	164		
0.8	Control	285	38	3.08
	Assay	323		
Assays with γ-carotene as substrate				
1.0	Control	306	283	35.2
	Assay	589		
1.0	Control	308	290	36.0
	Assay	598		

mAU milli-absorbance units

[a] Δ β-Carotene, difference in β-carotene between the assay reaction and the control

One of the factors that contributes to the diversity of carotenoid structures are enzymes that can cyclize the ψ-ends of linear carotenoids like lycopene, forming various carotenoids with cyclic (β-, ε-, ϕ-, and χ-) end-groups. In this study, we isolated and characterized the product of open reading frame sll0147 of the cyanobacterium, *Synechocystis* sp. PCC 6803. Previous studies had suggested that the ortholog of this protein in *Synechococcus* sp. PCC 7002 was a lycopene cyclase, but conclusive evidence was lacking because no β-carotene was produced when $cruA_{7002}$ was expressed in an *E. coli* strain that synthesizes lycopene (Maresca et al. 2007). Through in vivo and in vitro activity assays, we show here that sll0147 functionally belongs to the fourth family of lycopene cyclases, the CruA family, and accordingly we confirm the previous naming of this open reading frame as *cruA* (Maresca et al. 2007).

Attempts to express *cruA* heterologously in *E. coli* consistently failed to produce the lycopene cyclase activity that was hypothesized for this protein (Maresca et al. 2007; this study), although as shown here, $CruA_{6803}$ was synthesized and accumulated in the *E. coli* cells (Fig. 3). However, complementation studies with a *cruA* mutant, as well as in vitro reactions with purified $CruA_{6803}$, showed that CruA was expressed in an active form in the cyanobacterium *Synechococcus* sp. PCC 7002. Furthermore, Chl *a* and some β-carotene were bound to purified CruA. These results strongly imply that CruA is inactive as synthesized in *E. coli*, because *E. coli* is unable to synthesize Chl *a* that evidently is essential for the activity of the enzyme.

CruA is not predicted by structure-prediction algorithms to have any hydrophobic, trans-membrane α-helices. However, $CruA_{6803}$ was tightly associated with membranes and could only be solubilized with strong detergents. Based on these observations, it is possible that $CruA_{6803}$ might be structurally analogous to sulfide–quinone oxidoreductase, which is thought to be only partly embedded in, but does not extend across, the cytoplasmic membrane of *Aquifex aeolicus* (Marcia et al. 2009). This protein is believed to extend about 12 Å into the lipid bilayer by way of an amphipathic helix-turn-helix tripodal motif. A similar structural organization could allow CruA to have access to its very hydrophobic substrates, lycopene and γ-carotene, as well as to Chl *a*.

These observations suggest a mechanism by which the Chl *a* molecule could coordinate the synthesis of Chl *a* and carotenoids in cells. Assuming that Chl *a* can dissociate from CruA when (unbound) Chl *a* levels in cells are low, then the major lycopene cyclase activity in cells would be inactivated, and less lycopene would be converted to β-carotene, zeaxanthin, echinenone, and synechoxanthin (Maresca et al. 2008a). Based upon previous studies of *cruA* and *cruP* mutants (Maresca et al. 2007), CruP (or some other yet unidentified lycopene cyclase) could still produce some β-carotene, but it is perhaps more likely that lycopene would be diverted to the myxoxanthophyll branch of carotenoid synthesis (see Graham and Bryant 2009; Maresca et al. 2008a). Physiological studies of *cruA* and *cruP* mutants in *Synechococcus* sp. PCC 7002 suggest that CruP (or some other lycopene cyclase) can produce sufficient β-carotene to maintain cell viability, but that the residual activity in the *cruA* mutant cells cannot support wild-type growth rates. Growth is probably impaired because of a limitation in carotenoid biosynthesis that restricts the synthesis of PS I and PS II (Domonkos et al. 2013; Zakar et al. 2016). Finally, because CruP apparently lacks the N-terminal domain that putatively binds Chl *a*, CruP might be unable to coordinate carotenoid and Chl biosynthesis as hypothesized above.

Orthologs of CruA are found in all green sulfur bacteria and cyanobacteria that lack genes for CrtL-type lycopene cyclases (Maresca et al. 2007). Unlike CruP from cyanobacteria and the CruA family members produced by green sulfur bacteria (e.g., *Chlorobaculum tepidum*; Maresca et al. 2007), cyanobacterial CruA has a large (∼120 amino acids) N-terminal domain that does not occur in CruA proteins of green sulfur bacteria (see sequence alignment in Suppl. Fig. S2) or in CruP, and this domain is unrelated to any other protein domain in databases. Previous attempts to delete this domain to obtain an active form of the *Synechococcus* sp. PCC 7002 protein were unsuccessful (Maresca et al. 2007). This result further strengthens the hypothesis that the N-terminal domain of cyanobacterial CruA, together with bound Chl *a*, is essential for the lycopene cyclase activity of cyanobacterial CruA. Although histidine residues frequently serve as ligands to Chls in proteins, the N-terminal domain of cyanobacterial CruA does not contain a conserved histidine residue that could ligate a Chl *a* molecule. However, glutamate-arginine ion pairs can sometimes act as ligands to Chls (Kühlbrandt et al. 1994; Rögl and Kühlbrandt 1999; Yang et al. 1999), and an absolutely conserved glutamate residue occurs at position 35 in the sequence alignment shown in Suppl. Fig. S2. There are several mostly conserved arginine (positions 69, 100, 108, and 129) and lysine residues (positions 59 and 105) in the N-terminal domain that could form an ion pair with the conserved glutamate/aspartate residues (positions 30, 35, 90, and 143). Because relatively few amino acid residues are absolutely conserved in the N-terminal domain of CruA (Suppl. Fig. S2), this glutamate residue is an excellent candidate for ligating a Chl *a* molecule. Obviously, this hypothesis can be tested in future studies by site-directed mutagenesis.

CruA is a member of the FixC superfamily of dehydrogenases, and like all other FixC family members, CruA

is predicted to contain a flavin-binding domain (Maresca et al. 2007). The cyclization reactions that convert the ψ-ends of lycopene or γ-carotene into β- or ε-rings are isomerization reactions that produce no net change in the mass or redox state of the substrate. Some of the studied flavoenzymes of this family use the redox properties of the flavin directly in catalysis, but others do not (Bornemann 2002). A recent study showed that a CrtY-type lycopene cyclase (Yu et al. 2010) and a carotene *cis–trans* isomerase CRTISO (CrtH) (Yu et al. 2011) carry FAD cofactors, which must be in the reduced form for catalysis to occur. The mechanisms of their non-redox reactions were demonstrated with FAD_{red} participating in the stabilization of a transition state carrying a (partial) positive charge or of a positively charged intermediate via a charge-transfer interaction. $CruA_{6803}$ can be predicted to be a non-redox flavoprotein like CrtY but with Chl *a* as an additional cofactor required for activity. The catalytic mechanism described for CrtY could apply to other cyanobacterial CruA proteins, which also have a flavin-binding domain and the unique N-terminal extension. Future studies with improved CruA preparations will be required to study the enzyme mechanism. Although it is impossible to be sure at present, we suggest that the Chl *a* molecule(s) is required for structural (i.e., allosteric) reasons and is not directly required for catalysis.

A linear substrate, all-*trans*-lycopene, and a monocyclic substrate, γ-carotene, were both cyclized by CruA to produce β-carotene (Suppl. Fig. S1B). β-carotene and zeaxanthin are the predominant carotenoids produced in most cyanobacteria, including *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803. However, many cyanobacteria including these two model organisms also synthesize γ-carotene derivatives including myxol and other myxoxanthophylls (Graham and Bryant 2009; Lagarde and Vermaas 1999; Maresca et al. 2008a; Takaichi et al. 2001, 2005). Maresca et al. (2008a) proposed that separate biosynthetic pathways produce carotene derivatives and myxoxanthophylls and that these branches are independently regulated. CruA must recognize both lycopene and γ-carotene as substrates in vivo, as shown here in vitro, and once a β-ring is formed at one ψ-end of lycopene, CruA can cyclize the other ψ-end to form the dicyclic compound, β-carotene, which can further lead to the production of zeaxanthin and synechoxanthin (Graham and Bryant 2008, 2009; Maresca et al. 2008a). However, this is clearly not always the case, because the production of β-carotene from lycopene in brown-colored green sulfur bacteria requires two enzymes: a lycopene cyclase and a γ-carotene cyclase (Maresca et al. 2008b). The branch point is the modification of all-*trans*-lycopene, either into the production of β-carotene via CruA or CruP, or into 1-OH-lycopene via CruF (Graham and Bryant 2009; Maresca et al. 2008a). In this regard, it will be interesting in future studies to determine whether CruA can use 1-OH-lycopene as a substrate. Knowledge on this point would help to resolve this detail concerning carotenoid biosynthesis and could provide insights into how the branches leading to carotenes and myxoxanthophylls are regulated in cyanobacteria.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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