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Identification and Characterization of a New Class of Bilin Lyase Responsible for Attachment of Phycocyanobilin to Cys-153 on the β-subunit of Phycocyanin in Synechococcus sp. PCC 7002

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Identification and Characterization of a New Class of Bilin Lyase

THE cpcT GENE ENCODES A BILIN LYASE RESPONSIBLE FOR ATTACHMENT OF PHYCOCYANOBILIN TO CYS-153 ON THE β-SUBUNIT OF PHYCOCYANIN IN SYNECHOCOCCUS SP. PCC 7002[*][S]

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***Synechococcus* sp. PCC 7002 and all other cyanobacteria that synthesize phycocyanin have a gene, *cpcT*, that is paralogous to *cpeT*, a gene of unknown function affecting phycoerythrin synthesis in *Fremyella diplosiphon*. A *cpcT* null mutant contains 40% less phycocyanin than wild type and produces smaller phycobilisomes with red-shifted absorbance and fluorescence emission maxima. Phycocyanin from the *cpcT* mutant has an absorbance maximum at 634 nm compared with 626 nm for the wild type. The phycocyanin β-subunit from the *cpcT* mutant has slightly smaller apparent molecular weight on SDS-PAGE. Purified phycocyanins from the *cpcT* mutant and wild type were cleaved with formic acid, and the products were analyzed by SDS-PAGE. No phycocyanobilin chromophore was bound to the peptide containing Cys-153 derived from the phycocyanin β-subunit of the *cpcT* mutant. Recombinant CpcT was used to perform *in vitro* bilin addition assays with apophycocyanin (CpcA/CpcB) and phycocyanobilin. Depending on the source of phycocyanobilin, reaction products with CpcT had absorbance maxima between 597 and 603 nm as compared with 638 nm for the control reactions, in which mesobiliverdin becomes covalently bound. After trypsin digestion and reverse phase high performance liquid chromatography, the CpcT reaction product produced one major phycocyanobilin-containing peptide. This peptide had a retention time identical to that of the tryptic peptide that includes phycocyanobilin-bound, cysteine 153 of wild-type phycocyanin. The results from characterization of the *cpcT* mutant as well as the *in vitro* biochemical assays demonstrate that CpcT is a new phycocyanobilin lyase that specifically attaches phycocyanobilin to Cys-153 of the phycocyanin β-subunit.**

Cyanobacteria and red algae contain peripheral, light-harvesting complexes called phycobilisomes (PBS).[2] These macromolecular complexes are composed of two types of proteins: colored phycobiliproteins (PBPs), which absorb and transmit light energy to the photosynthetic reaction centers, and non-pigmented linker proteins, which direct the assembly of PBS (1). In many cyanobacteria, including *Synechococcus* sp. PCC 7002, PBS are composed of two blue-colored PBPs: phycocyanin (PC) and allophycocyanin (AP). These PBPs are composed of two subunits, α and β (2), which are members of the globin superfamily (3). The α- and β-subunits of PC and AP carry at least one linear tetrapyrrole chromophore (bilin) called phycocyanobilin (PCB), which is covalently attached to specific cysteine residues via thioether linkages (see supplemental Fig. 1) (4). The bilin chromophores of PBPs are derived from heme and are related to the phytochromobilin (5), the photoisomerizable chromophore of the plant protein, phytochrome (6). X-ray crystallographic analyses of PC have shown that the chiral $C3^1$ carbons of the PCB chromophores attached at cysteines α84 and β82 are in the *R* configuration, whereas the $C3^1$ carbon of the PCB at cysteine β153 is in the *S* configuration (7) (see supplemental Fig. 1).

The N-terminal domains of phytochromes contain a bilin lyase activity that attaches phytochromobilin to a cysteine to produce holo-phytochrome (8). Many prokaryotic organisms contain phytochrome-like proteins, and several of these have also been shown to attach their bilin chromophores autocatalytically (8–15). However, when PCB is mixed with apo-PC, the major product is a more oxidized bilin, mesobiliverdin (MBV), which contains an extra double bond between C2 and C3 (see supplemental Fig. 1); MBV is covalently bound at cysteines α84 and β82, but no PCB is bound at cysteine β153 (16, 17). These observations led Arciero *et al.* (16) to propose that enzymes would be required for the attachment of bilin chromophores to PBPs. The first such enzyme to be identified and characterized was the α-PC PCB lyase, a heterodimer of the CpcE and CpcF proteins from *Synechococcus* sp. PCC 7002 (18–20). PecE and PecF are similar in sequence to CpcE and CpcF, respectively, and were shown to comprise the α-phycoerthrocyanin (PEC) lyase in *Anabaena* sp. PCC 7120 (21, 22) and *Mastidocladus laminosus* (23–25). The α-PEC subunit is very similar in sequence and structure to α-PC; the main difference is that PCB is attached to α-PC, whereas a different isomer phycobiliviolin is attached to α-PEC (26). The PecE/PecF lyase attaches PCB to the α-subunit of PEC and then isomerizes PCB to phycobiliviolin (23, 25, 27).

Three paralogous genes with sequence similarity to *cpcE* are found in the genome of *Synechocystis* sp. PCC 6803, and their products appear to be involved in PBP degradation or repair (28). One CpcE paralog, NblB, participates in PBP turnover during nutrient deprivation, presumably

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[2] The abbreviations used are: PBS, phycobilisome(s); AP, allophycocyanin; C-PE, cyanobacterial phycoerythrin; HT, His_6 tagged; HTApcA/ApcB, recombinant His_6-tagged apo-allophycocyanin; MBV, mesobiliverdin; PBP, phycobiliprotein; AP, allophycocyanin; PC, phycocyanin; PCB, phycocyanobilin; PE, phycoerythrin; PEC, phycoerythrocyanin; rCpcB/CpcA, recombinant apo-phycocyanin; HPLC, high pressure liquid chromatography.

by removing PCB from PBPs (29, 30). Because PBPs are still produced in mutants in which all four *cpcE* paralogs have been inactivated, it is likely that other enzymes are involved in bilin attachment to PBPs (31). However, Zhao *et al.* (32, 33) recently suggested that PBPs themselves might act as lyases at some chromophore attachment sites. For ApcE, a large core membrane linker protein that contains a PCB attached to a Cys within its N-terminal phycobiliprotein domain, these authors showed that ApcE can autocatalytically bind PCB (33) optimally in the presence of 4 M urea (34, 35). From studies with full-length and truncated forms of ApcE, it was shown that the PBP domain portion of ApcE contains an intrinsic lyase activity, and it was suggested the same might be true for other PBPs.

For the past several years we sought to identify additional PBP bilin lyases. In *Fremyella diplosiphon*, the *cpeCDESTR* operon encodes linker proteins for phycoerythrin (PE) assembly as well as CpeR, a regulator of the *cpeBA* operon that encodes the apoPE subunits (36). A *cpeT* transposon mutant does not produce detectable PE (37). Interestingly, *cpeT* orthologs and/or paralogs are present in the sequenced genomes of all cyanobacteria that synthesize PBPs except for *Prochlorococcus marinus* strain MED4 (see "Results" and "Discussion"). To explore the function of these *cpeT*-like gene products in an organism that does not synthesize PE, we characterized the *cpeT*-paralogous gene, denoted *cpcT*, from *Synechococcus* sp. PCC 7002 through reverse genetics and *in vitro* enzymatic analyses. Our results demonstrate that CpcT is a new bilin lyase that specifically attaches PCB to cysteine 153 of CpcB.

EXPERIMENTAL PROCEDURES

Growth of Cyanobacterial Cultures—Wild-type and mutant strains of *Synechococcus* sp. PCC 7002 were grown in medium A supplemented with 1 mg/ml of $NaNO_3$ (medium A^+) (38). The *cpcT*:*aacCI* mutant was grown in A^+ medium amended with 10 mM glycerol and 50 μg/ml of gentamicin. Liquid cultures were bubbled with 1% (v/v) CO_2 in air and continuously illuminated with cool-white fluorescent lamps. Growth rates were determined by monitoring the optical density at 730 nm as a function of time.

Construction of the cpcT Mutant—Total DNA from *Synechococcus* sp. PCC 7002 wild-type and mutant strains was isolated as described (39). Using DNA of *Synechococcus* sp. PCC 7002 as template, a 1.2-kb DNA fragment including *cpcT* was amplified by PCR using the *cpcT*-F forward primer (5′-CCTTTTTCCTAAGCTTAGACAATCAGC-3′) and the *cpcT*-R reverse primer (5′-TGATCGAGACGGATCCAAACACTGGGAG-3′) (see Fig. 2). The resulting product was digested with HindIII and BamHI, cloned into pUC19, and sequenced to verify the insert. For insertional inactivation of the *cpcT* gene, a 1-kb DNA fragment, which encodes the *aacCI* gene and confers resistance to gentamicin, was inserted into the unique StuI site within the *cpcT* coding sequence (see Fig. 3*A*). This construction was used to transform cells of *Synechococcus* sp. PCC 7002 (40). Transformants were selected on medium A^+ plates containing gentamicin and subjected to several rounds of streaking on selective medium. Segregation of the *cpcT* and *cpcT*::*aacCI* alleles was verified by PCR using the *cpcT*-F and *cpcT*-R primers (see above).

Isolation of Phycobilisomes—Cyanobacterial cells (4–6 liters) grown to late-exponential growth phase ($OD_{730\,nm}$ = 1.5–2.0) were harvested by centrifugation. Cell pellets were washed once with 0.75 M potassium phosphate buffer, pH 7.0, and resuspended in 50 ml of 0.75 M potassium phosphate buffer, pH 7.0, 10 mM EDTA. Cells were broken by two passages through a chilled French pressure cell operated at 138 megapascals. The broken cell suspension was stirred at room temperature until homogenous after addition of 2% (w/v) Triton X-100 and centrifuged at 17,000 × *g* at 20 °C to remove unbroken cells and debris. PBS were isolated following a procedure described previously (41). The PBS fraction was dialyzed against 0.75 M potassium phosphate, pH 7.0, 10 mM EDTA and centrifuged at 27,000 × *g* for 15 min to pellet membrane fragments. Freshly prepared PBS samples were immediately used for spectroscopic measurements or stored at −70 °C until required.

For purification of PC, isolated PBS were dialyzed against 50 mM potassium phosphate buffer, pH 7.0, and applied onto a DEAE column (1.5 × 20 cm; DEAE-Sepharose (Sigma)). PBPs were eluted with a linear gradient of NaCl (10 to 400 mM NaCl) prepared with 50 mM potassium phosphate buffer, pH 7.0, 10 mM EDTA. Appropriate fractions were pooled, precipitated by addition of 0.18 g/ml of solid ammonium sulfate, and resuspended in 50 mM potassium phosphate buffer, pH 7.0, 10 mM EDTA.

Absorption and Fluorescence Spectroscopy of PBS and PBPs—The relative PBP contents of cells were determined by heat-bleaching liquid cell suspensions at 65 °C for 5 min as described previously (42, 43). Absorption spectra of whole cells, PBS, and PBPs were measured using a GENESYS 10 spectrophotometer (ThermoSpectronic, Rochester, NY). Data were analyzed using the IgoR-Pro (Wavemetrics, Inc., Lake Oswego, OR). Fluorescence excitation and emission spectra were measured using a SLM 8000C spectrofluorometer as described (39). For sample preparation, isolated PBS were adjusted to a concentration of 50 μg/ml of protein in 0.75 M potassium phosphate buffer, pH 7.0, and purified PBPs were adjusted to 20 μg/ml of protein in 50 mM potassium phosphate buffer, pH 7.0, 10 mM EDTA. To record low temperature fluorescence emission spectra of intact cells, cells in exponential growth phase ($OD_{730\,nm}$ = 0.6–0.7) were harvested by centrifugation and resuspended in 25 mM HEPES-NaOH buffer at pH 7.0 ($OD_{730\,nm}$ = 1.0 cm^{-1}). Glycerol was added to a final concentration of 60% (v/v), and the cell suspension was frozen in liquid nitrogen. The excitation wavelength was 440 nm for chlorophyll and 590 nm for PBS and PBPs. A long-pass filter (with transmission at >600 nm) was used at the inlet of the emission monochromator to minimize scattered light contributions.

SDS-PAGE Analysis—Protein fractions were analyzed by polyacrylamide gel electrophoresis in the presence of SDS as described (39). PBPs were resolved on 10–22% (w/v) acrylamide gradient gels, and the resolved proteins were visualized by staining with Coomassie Blue. Bilin-linked proteins were visualized by their UV-induced fluorescence after soaking the gel in 100 mM $ZnSO_4$ for 2 min (44). Recombinant proteins were analyzed on 15% (w/v) acrylamide gels as described (45).

Formic Acid Cleavage of Phycocyanin—For dilute acid cleavage of the Asp-Pro peptide bond in the β-PC subunit (46), purified PC (40 μg) was precipitated by the addition of cold acetone to 80% (v/v). After incubation at −20 °C for 30 min, the solution was centrifuged for 15 min at 14,000 × *g*. The pellet was resuspended in formic acid (70 μl), and then adjusted to 70% formic acid (v/v) by addition of distilled H_2O. After incubation at 37 °C for 10–12 h, the sample was dried under vacuum, resuspended in distilled water, and precipitated with cold 80% (v/v) acetone. The pellet was resuspended in SDS loading buffer and analyzed by SDS-PAGE.

Construction of Recombinant Expression Plasmids—The *cpcT* gene was amplified by PCR from total *Synechococcus* sp. PCC 7002 DNA as template by using the oligonucleotides cpcT1.6 (5′-AGCAATTTCATATGTCCCACTCTACCGATGCC-3′) and cpcT1.4 (5′-CTTTCTCGAGTTTAGCCGCCATAATTTTTGCTTCTCTCC-3′). The resulting PCR product was digested with NdeI and XhoI and cloned into T7 expression vector pAED4 that had been digested with the same enzymes. The *cpcB* and *cpcA* genes were amplified by PCR from total *Synechococcus* sp. PCC 7002 DNA as template using oligos CpcB.5 (5′-

GAGATAAACATATGTTTGATATTTTTACCCGGGTTG-3′) and SyncpcA162 (5′-ACTAAGCTTAATTAGCTGAGGGCG-3′). The resulting PCR product and pAED4 were digested with NdeI and HindIII and ligated. The *Synechococcus* sp. PCC 7002 *apcAB* genes were amplified by PCR from total DNA using oligos 7002apcA5 (5′-CACCATGAGTATTGTCACGAAATCCATCG-3′) and 7002 apcB3 (5′-CGAATTCTTAGCTTAAGCCAGAGCAGATG-3′). The *apcAB* operon was cloned into pET100 using the Champion™ pET Directional TOPO® Expression Kit (Invitrogen) by following the protocol suggested by the manufacturer. The resulting ApcA product carries a His_6 tag at its N terminus (HT-ApcA). All expression clones were verified by complete resequencing at the W. M. Keck Conservation and Molecular Genetics Lab (University of New Orleans).

Protein Overexpression and Purification—Each expression plasmid was transformed into *Escherichia coli* BL21(DE3) cells, and colonies were selected on LB medium in the presence of ampicillin (Ap; 100 mg/ml). For overexpression of pAED4::*cpcT* or pAED4::*apcAB*, a 50-ml overnight culture was inoculated into a flask containing 1 liter of LB medium supplemented with 100 mg/ml of ampicillin, and the cell suspension was shaken at 30 °C for 4 h. Protein expression was induced by the addition of 0.5 mM isopropyl β-D-thiogalactoside, and cells were grown for an additional 4 h. To produce a control *E. coli* extract, plasmid pAED4 was transformed into BL21(DE3) cells, and the cells were treated as described above for pAED4::*cpcT*. For overproduction of apo-PC (CpcA + CpcB), a 50-ml overnight culture of *E. coli* BL21(DE3) cells harboring plasmid pAED4::*cpcBA* was used to inoculate a flask containing 1 liter of LB medium at 30 °C, and the cell suspension was shaken for 11 h with no isopropyl β-D-thiogalactoside induction. The *E. coli* cells were harvested by centrifugation and stored at −20 °C until required.

To purify CpcT, *E. coli* overexpression cells were resuspended in 20 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM dithiothreitol (cells from a 1-liter culture were resuspended in 20 ml of buffer), and cells were disrupted by three passages through a chilled French pressure cell operated at 138 megapascals. The extract was clarified by centrifugation at 17,000 × *g* for 20 min, the supernatant was adjusted to 30% (w/v) ammonium sulfate, and the resulting solution was left to stand overnight at 4 °C. After centrifugation at 17,000 × *g* for 20 min, the CpcT-containing supernatant was collected. CpcT was precipitated by the addition of ammonium sulfate to a final concentration of 50% (w/v). Following centrifugation at 17,000 × *g*, the CpcT-containing pellet was resuspended in a small volume of 50 mM Tris-HCl, pH 8.0, and dialyzed against the same buffer. CpcT was further purified by anion-exchange chromatography (Whatman DE-52 DEAE-cellulose; 2.5 × 12.5 cm) that had been equilibrated with 50 mM Tris-HCl, pH 8.0, 1 mM NaN_3 (Buffer A). Aliquots (~10 ml) of the CpcT solution were loaded at room temperature onto the column using a BioLogic LP system (Bio-Rad). The column was developed using the following program at a flow rate of 2 ml/min: 0–30 min, 100% Buffer A; 30–120 min, 0 to 100% Buffer B (50 mM Tris-HCl, pH 8.0, 1.0 M NaCl, 1 mM NaN_3); 120–150 min, 100% Buffer B; 150–180 min, 0 to 100% Buffer A; 180–210 min, 100% Buffer A. Fractions with $A_{280\,nm} \geq 0.05$ were collected and analyzed by SDS-PAGE. Fractions containing CpcT were pooled, dialyzed against 20 mM Tris-HCl, pH 8.0, 50 mM NaCl and stored in aliquots at −20 °C until required.

ApoPC (rCpcB/CpcA) was purified by modifying the procedure of Arciero *et al.* (16) as follows. *E. coli* BL21(DE3) cells harboring plasmid pAED4::*cpcBA* were resuspended in 50 mM sodium phosphate, pH 7.0, 10 mM 2-mercaptoethanol and disrupted by three passages through a chilled French pressure cell operated at 138 megapascals. Extracts were clarified by centrifugation at 17,000 × *g* for 20 min, and the cell extract was adjusted to 38% (w/v) with solid ammonium sulfate. After centrifugation at 17,000 × *g* for 20 min, the pellet was resuspended in 150 ml of 50 mM sodium phosphate, pH 7, and loaded onto a DEAE-cellulose anion-exchange column (Whatman DE-52; 2.5 × 15 cm). The flow-through containing apoPC (rCpcB/CpcA) was collected, and ammonium sulfate was added to 50% saturation (w/v). This mixture was clarified by centrifugation as described above, and the pellet containing apoPC was collected, resuspended in sodium phosphate buffer, and dialyzed exhaustively against the same buffer to remove the ammonium sulfate. ApoPC (the α:β ratio was ~1:1 as judged by SDS-PAGE) was purified further by anion exchange chromatography on a separate DEAE-cellulose column. After dialysis of the precipitated apoPC against Buffer A, this column was loaded with 10-ml aliquots of apoPC and developed with the same program described above for purification of CpcT. Pooled fractions containing apoPC were dialyzed against 50 mM sodium phosphate, pH 7.0, 1 mM 2-mercaptoethanol and concentrated by ultrafiltration (YM10 membrane; Millipore/Amicon, Billerica, MA) to ~1 mg/ml of protein as estimated by SDS-PAGE.

E. coli BL21(DE3) cells from overproduction of apoAP (HTApcA/ApcB) were resuspended in 20 mM Tris-HCl, pH 8.0, 10 mM 2-mercaptoethanol and disrupted by three passages through a chilled French pressure cell operated at 138 megapascals. Extracts were clarified by centrifugation at 17,000 × *g* for 20 min to pellet cell wall debris, inclusion bodies, and unbroken cells. HTApcA/ApcB was co-purified by metal affinity chromatography as described (47). The purified protein solution, which contained both subunits in a ~1:1 ratio as judged by SDS-PAGE, was dialyzed against 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM 2-mercaptoethanol, concentrated by ultrafiltration to ~1 mg/ml, and stored in aliquots at −20 °C until required.

In Vitro Bilin Addition Experiments—Phycocyanobilin from *Spirulina* sp. (purchased from a local health food store) was cleaved from PBPs as described (48). PCB was purified by reverse phase HPLC as described (16). Bilin addition reactions contained apoPC (1.0 ml, 1 mg/ml of protein) and either CpcT (100 μl, 1 mg/ml of protein) or a control pAED4 extract (100 μl, 1 mg/ml of protein). These mixtures were allowed to stand on ice for 15 min to allow CpcT to interact with apo-PC, after which time PCB was added to a final concentration of 10 μM PCB from a stock solution (2 mM in Me_2SO). The addition reactions were incubated for 2 h at 30 °C in the dark.

Alternatively, *in vitro* PCB addition reactions utilized PCB that was produced *in situ* from the biosynthetic enzyme PcyA from *Anabaena* sp. PCC 7120 (49–51). PcyA reduces biliverdin to phycocyanobilin in two sequential 2-electron reductions using reduced ferredoxin as electron donor. Because PCB is produced in very small amounts and is rapidly consumed, less MBV product results from the non-enzymic addition of bilins to the apoproteins. The *pcyA* expression plasmid was kindly provided by Dr. J. C. Lagarias (Department of Biochemistry, University of California, Davis, CA). The enzyme was overproduced as a glutathione *S*-transferase fusion and purified as described (49–51). Because the glutathione *S*-transferase tag did not interfere with the PcyA enzyme activity, it was not removed. Bilin addition reactions utilizing PcyA were conducted as follows. Solutions of rCpcB/CpcA or HTApcA/ApcB (1.0 ml at ~1 mg/ml of protein) and either CpcT or pAED4 control extract (100 μl at 1 mg/ml of protein) were prepared. The following were added to each reaction mixture: HEPES-NaOH buffer, pH 7.3, to 50 mM; $MgCl_2$ to 1 mM; glucose 6-phosphate to 6.5 mM; $NADP^+$ to 1.6 mM; 1.1 unit/ml of glucose-6-phosphate dehydrogenase; recombinant *Synechococcus* sp. PCC 7002 ferredoxin to 4.6 μM (52); 0.025 unit/ml of spinach ferredoxin:$NADP^+$ oxidoreductase;

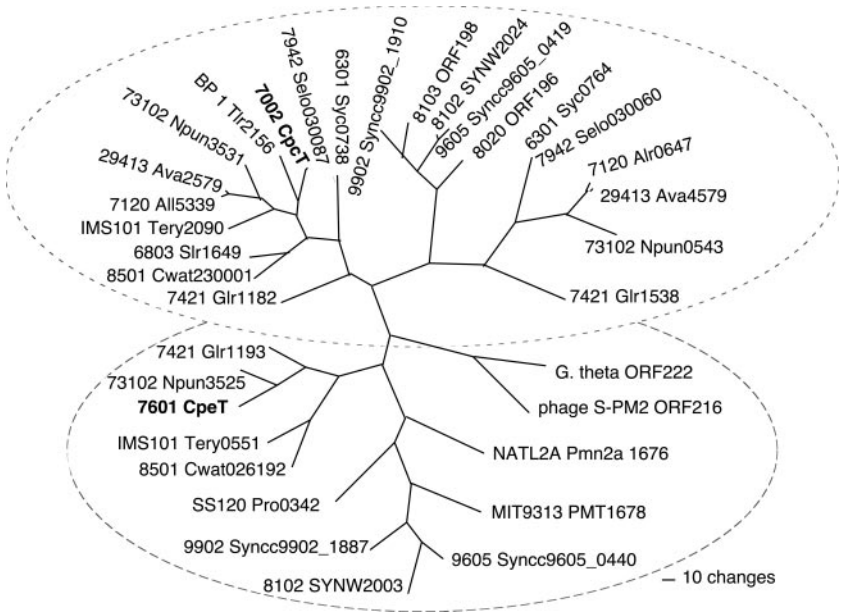

FIGURE 1. **Unrooted neighbor-joining tree for proteins paralogous to CpcT.** Amino acid sequences of CpcT and CpeT proteins were compared from *Synechococcus* sp. PCC 7002 (*7002*), *Synechocystis* sp. PCC 6803 (*6803*), *Anabaena* sp. PCC 7120 (*7120*), *T. elongatus* BP-1 (*BP-1*), *Gloeobacter violaceus* PCC 7421 (*7421*), *S. elongatus* PCC 7942 (*7942*), *Synechococcus* sp. PCC 6301 (*6301*), *Calothrix* PCC 7601 (*F. diplosiphon*) (*7601*), *P. marinus* SS120 (*SS120*), *P. marinus* MIT9313 (*MIT9313*), *Prochlorococcus* sp. CC9605 (*9605*), *Prochlorococcus* sp. CC9902 (*9902*), *P. marinus* MIT9313 (*MIT9313*), *P. marinus* SS120 (*CCMP1375*) (*SS120*), *P. marinus* strain NATL2A (*NATL2A*), *Synechococcus* sp. WH8102 (*8102*), *Synechococcus* sp. WH8103 (*8103*), *Crocosphaera watsonii* WH8501 (*8501*), *Nostoc punctiforme* PCC 73102 (*73102*), *Anabaena variabilis* ATCC 29413 (*29413*), *Trichodesmium erthraeum* IMS101 (*IMS101*), *G. theta* (*G. theta*), Bacteriophage S-PM2 (*phage S-PM2*). The phylogenetic tree was generated by PAUP Phylogenetic Analysis program, based on the aligned sequences produced from the ClustalW function within MacVector version 8.1.1 (Accelerys, San Diego, CA).

bovine serum albumin to 10 μM; biliverdin to 5 μM (from a stock solution at 3 mM in Me_2SO; Porphyrin Products, Logan, UT); and PcyA to 10 mM. Reactions were incubated in the dark at 30 °C for 1 h. A second aliquot of biliverdin was added (to produce a final concentration of 10 μM), and the reaction was incubated for an additional hour at 30 °C. The solutions were centrifuged for 10 min at 14,000 × *g* in a microcentrifuge. Fluorescence emission spectra were recorded with the excitation wavelength set at 590 nm and with the slits set at 3 nm (excitation and emission). Absorption spectra were recorded with a dual-beam Lambda 35 UV-visual spectrophotometer (PerkinElmer Life Sciences). The fluorescence emission spectra of bilin addition products were measured using a PTI International (model QM-1) fluorometer, equipped with a 75-watt continuous xenon arc lamp as light source.

Tryptic Digestion—Tryptic digestion of PC was performed as described (16). The peptides were bound to C_{18}-Sep-Pak cartridges, eluted with 60% acetonitrile and 40% 0.1% trifluoroacetic acid, and dried under vacuum (16). Samples were stored in the dark at −20 °C until analyzed by HPLC.

Tryptic peptides were separated on a C_{18} reverse phase HPLC column (5 × 10 × 250 mm) using a Waters HPLC equipped with a model 600E pump and a photodiode array detector. Peptide separation was carried out as described (16) using 0.1 M sodium phosphate buffer, pH 2.1, and 100% acetonitrile. Bilin-containing peptides were detected at 600 nm.

RESULTS

Phylogenetic Analysis of CpcT and CpeT-like Proteins—Since the original characterization of the CpcE/CpcF bilin lyase (18–20, 53), we have been interested in identifying the remaining phycocyanobilin lyases. We recently focused on genes paralogous to *cpeT* for two reasons. First, PBPs are "signature genes" for cyanobacteria (54), and logically, all genes required for PBP biogenesis should also be exclusively found in cyanobacteria and missing from the genomes of organisms that do not synthesize PBPs. Second, Cobley and co-workers (36) showed that a null mutation in *cpeT*, a gene found in an operon (*cpeCDESTR*) containing genes related to PE regulation (*cpeR*) and PBS biogenesis/structure (*cpeCDE*), caused PE levels to fall below detection limit (37). Blast analyses identified *cpeT* orthologs and/or paralogs in the sequenced genomes of all cyanobacteria except *P. marinus* MED4 (see "Discussion") (Cyanobase and JGI Microbial Genome data base), including the recently completed genome sequence of *Synechococcus* sp. PCC 7002.[3]

A multiple sequence alignment was conducted to compare these similar sequences (see supplemental Fig. 2). The result of a phylogenetic analysis of this family of proteins is shown in Fig. 1. The CpeT/CpcT family of proteins forms two main groups. The first group (*lower dashed circle*) contains CpeT from *Calothrix* sp. PCC 7601 (also known as *F. diplosiphon*), and this protein, denoted 7601 CpeT, groups most closely with apparent orthologs from organisms that synthesize PE. As suggested from the mutagenesis results (37), this distribution of sequences strongly suggests that this protein subgroup might have a role in C-phycoerythrin (C-PE) biosynthesis. Interestingly, a bacteriophage (phage S-PM2) also possesses a *cpeT* gene; this phage infects marine cyanobacteria that produce large amounts of PE (55). Finally, an apparent ortholog of *cpeT* is also found in the nucleomorph genome of the cryptomonad *Guillardia theta*, which also synthesizes PE (56).

The second major grouping (*upper dashed circle*) contains at least two subgroups. The grouping to the *upper left* in Fig. 1 contains the CpeT paralog from *Synechococcus* sp. PCC 7002 (denoted 7002 CpcT) along with other CpeT-like proteins from organisms that similarly cannot synthesize PE and only synthesize PC and AP (*e.g. Synechocystis* sp. PCC 6803, *Thermosynechococcus elongatus* BP-1, and *Synechococcus elongatus* PCC 7942). Other proteins in subgroup 7002 CpcT are found in organisms that produce PC as well as other PBPs. This distribution of sequences suggests a paralogous function, perhaps a role in PC biogenesis, which will be established below. These proteins have been designated CpcT (C-phycocyanin) to denote this essential difference. The remaining sequences found at the *upper right* of Fig. 1 appear to form two distinct clades. The first contains marine strains of cyanobacteria, whereas the other includes sequences that are more divergent and that are distributed among organisms with no obvious pattern regarding PBP synthesis.

Insertional Inactivation of the cpcT Gene in Synechococcus sp. PCC 7002—To investigate CpcT function in *Synechococcus* sp. PCC 7002, the *aacCI* gene was cloned into the unique StuI site within the *cpcT* gene

[3] T. Li, J. Marquardt, G. Shen, J. Zhao, and D. A. Bryant, unpublished results.

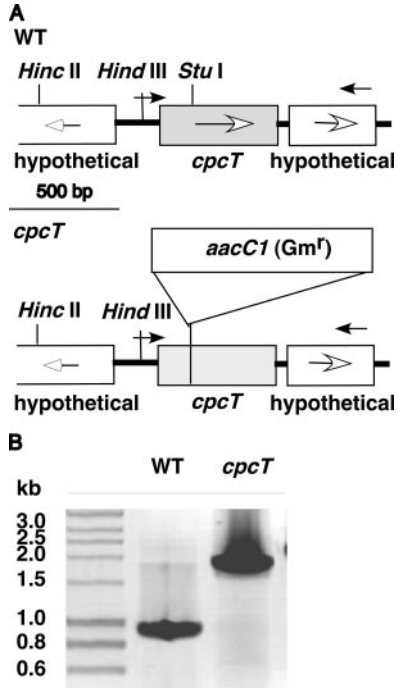

FIGURE 2. **Cloning and mutagenesis of the *cpcT* gene in *Synechococcus* sp. PCC 7002.** *A*, physical maps of the DNA fragments containing the *cpcT* gene (*shaded*) from wild-type (*above*) and the *cpcT* mutant (*below*) and showing the position of the inserted gentamycin-resistance cartridge in the unique StuI site within the *cpcT* gene. *Open arrows* in *boxes* indicate the direction of transcription. *Filled arrows* show the locations of primers used for PCR amplification. *B*, PCR analysis to verify the segregation of the *cpcT* mutant. PCR amplifying *cpcT* from wild type (*WT*) and the *cpcT*::*aacC1* mutant (*cpcT*) are shown after the amplicons were separated by agarose gel electrophoresis. The amplicons from the wild-type (0.9 kb) and *cpcT*::*aacC1* mutant (1.9 kb) have the expected sizes, and no product of 0.9 kb is observed in the mutant, which shows that the *cpcT* and *cpcT*::*aacC1* alleles are fully segregated in the mutant.

(Fig. 2*A*). The resulting construction was used to transform wild-type *Synechococcus* sp. PCC 7002 cells to gentamicin resistance. After repeated streaking on selective medium, DNA was isolated from selected transformants, and PCR analyses using primers *cpcT*-F and *cpcT*-R were performed to verify that the *cpcT* and *cpcT*::*aacCI* alleles had fully segregated. As shown in Fig. 2*B*, these primers amplify a 0.9-kb fragment when DNA from the wild-type strain was used as the template, but these same primers amplify a 1.9-kb fragment when transformant DNA was used as the template (*lane 2*). Because no 0.9-kb fragment was amplified from the transformant, the *cpcT* and *cpcT*::*aacCI* alleles had segregated fully, and the resulting *cpcT* mutant strain was homozygous at the *cpcT* locus. Although the *cpcT* gene is universally distributed among PBP-containing cyanobacteria that synthesize PC, these results establish that this gene is not essential.

Physiological Characterization of the cpcT Mutant—The *cpcT* mutant could grow photoautotrophically; however, the doubling time (7.8 ± 0.4 h) for the *cpcT* mutant was much longer than that for the wild type (4.2 ± 0.3 h) under standard growth conditions (1% (v/v) CO_2 in air; light intensity = 250 μE m^{-2} s^{-1}). The reason for this growth rate difference could immediately be seen from the coloration of the cells (see supplemental Fig. 3). The *cpcT* mutant cells were much more yellow-green in color than wild-type cells due to a significantly lower PC content. When the PC content was measured for both cell types, the wild type had a relative PC content of 380, whereas the relative PC content of the *cpcT* mutant was only 230, a reduction of ~40%. To examine PBS function *in vivo*, fluorescence emission spectra of whole cells were measured under conditions that excite primarily the PBP (λ_{ex} = 590 nm) (Fig. 3). Compared with the wild type, the 645-nm

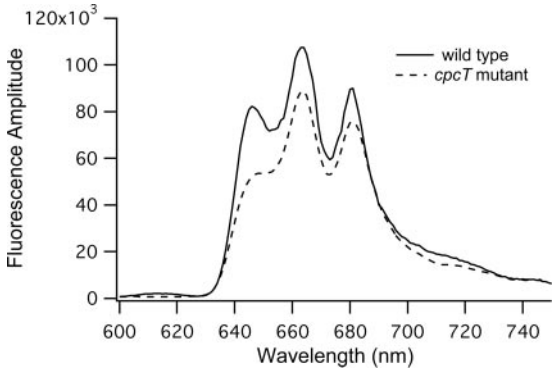

FIGURE 3. **Low temperature (77 K) fluorescence emission spectra.** Fluorescence emission spectra for wild-type cells (*solid line*) and *cpcT* mutant cells (*dashed line*) of *Synechococcus* sp. PCC 7002. Spectra were collected from solutions containing equal numbers of cells ($OD_{730\ nm}$ = 0.5), and the excitation wavelength was 590 nm. Each spectrum is the average of four spectra.

fluorescence emission peak of PC is significantly reduced in cells of the *cpcT* mutant. Fluorescence emission from AP at ~660 nM and the PBS terminal emitters, ApcD and ApcE, at ~680 nm was also somewhat reduced in the *cpcT* mutant cells relative to that for the wild-type cells. Because the PC of the *cpcT* mutant absorbs less light than wild-type PC and thus transfers less light energy to these acceptor proteins, these smaller differences at longer wavelength are probably secondary effects. Nevertheless, the results strongly suggest that inactivation of the *cpcT* gene causes a defect in PC synthesis.

Phycobiliprotein Characterization of the cpcT Mutant—PBS were isolated from the wild-type and *cpcT* mutant to analyze further the effects of CpcT deficiency on PC synthesis and PBS assembly. After sucrose density gradient ultracentrifugation of cell extracts, several differences were noted between gradients for the wild-type and *cpcT* mutant. First, the PBS of the mutant strain did not migrate as far as the PBS of the wild type (data not shown). This observation suggests that the PBS of the *cpcT* mutant are smaller in size than those of the wild type. Second, the gradients loaded with extracts from the *cpcT* mutant had an intense, blue-colored band near the top of the gradient that was not observed for gradients loaded with extracts from wild type. Because this fraction typically contains PBPs from dissociated PBS and becomes enriched when there is a defective or missing PBP or linker polypeptide (18, 21, 57–62), the enrichment of PBPs in this fraction in the *cpcT* mutant indicates that there is a defect in the assembly or stability of a PBP or PBS component.

Blue-colored fractions were collected from the sucrose gradients, and these fractions were analyzed by SDS-PAGE and $ZnSO_4$ staining to enhance bilin fluorescence. As shown in Fig. 4*A*, *lane 1*, wild-type PBS contain four major bilin-containing polypeptides: β-PC (two PCBs), α-AP (one PCB), α-PC (one PCB), and β-AP (one PCB). When the PBPs from the PBS complexes of the *cpcT* mutant are visualized by zinc-enhanced bilin fluorescence (Fig. 4*A*, *lane 3*), two principal differences can be observed. First, the β-PC subunit in the *cpcT* mutant (designated β^*) migrates slightly faster than the corresponding protein in the wild type. Immunoblot analysis with antibodies specific for β-PC verified the identity of this protein (data not shown). Second, the fluorescence intensity of the β^*-subunit was similar to or less than that for the α-PC subunit. The fluorescence intensity of the β-PC subunit is normally greater than that of the α-PC subunit because β-PC has two PCB chromophores, whereas α-PC has only one.

The PBPs present in the fraction from the top of the sucrose gradient for the *cpcT* mutant are shown in Fig. 4*A*, *lane 2*. The major PBP in the upper fraction from sucrose gradients for the *cpcT* mutant migrates

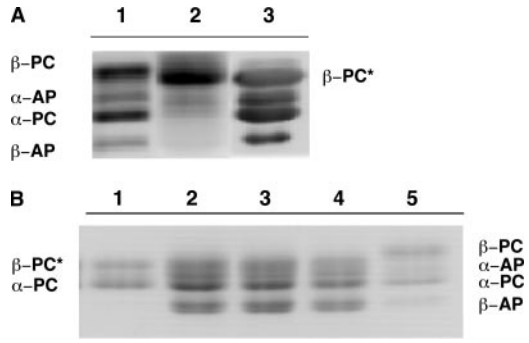

FIGURE 4. **SDS-PAGE analysis of isolated PBS (*A*) and PBP-containing fractions (*B*) from the wild-type and *cpcT* mutant of *Synechococcus* sp. PCC 7002.** *A*, PBPs (10 μg of protein) isolated from sucrose gradients were separated by SDS-PAGE and visualized by bilin fluorescence after $ZnSO_4$ staining. Phycobiliproteins in wild-type PBS (*lane 1*) were compared with phycobiliproteins isolated from the top of the sucrose gradients (*lane 2*) or from the lower PBS-containing fraction from the *cpcT* mutant (*lane 3*). *B*, assessment of the purification of phycobiliproteins from the *cpcT* mutant after DEAE ion-exchange chromatography by SDS-PAGE. Phycobiliproteins from fractions 1 (*lane 1*), 3 (*lane 2*), 5 (*lane 3*), and 7 (*lane 4*) from the DEAE column were compared with phycobiliproteins of the wild-type (*lane 5*). Bilin fluorescence was visualized after $ZnSO_4$ staining. Polypeptides are identified at the *left* and *right*.

slightly faster than the β-PC subunit and appears to be identical to the β*-PC subunit observed in the PBS. Only minor amounts of α-PC are found in this fraction, which also contains small amounts of α-AP and β-AP.

A potential explanation for all of these observations is that the β*-PC subunit from the *cpcT* mutant is missing one of its PCB chromophores (PCB has a mass of 587 Da). The absence of this PCB chromophore could affect the binding of β-PC* to α-PC, and this could account for the reduced amount of PC in the PBS and the non-stoichiometric amount of α-PC in the upper fraction of the sucrose gradient. A corollary hypothesis is that CpcT is a PCB lyase that is specifically involved in the chromophorylation of CpcB/β-PC.

Analysis of $\alpha\beta$-PC from the cpcT Mutant*—To characterize the modified PC (denoted $\alpha\beta$*-PC) in the *cpcT* mutant further, $\alpha\beta$*-PC was purified from dissociated PBS by anion-exchange chromatography on DEAE-Sephacryl. Fractions were collected, and proteins were analyzed by SDS-PAGE and $ZnSO_4$ staining to observe bilin fluorescence (Fig. 4*B*). The earliest eluting fraction (Fig. 4*B*, *lane 1*) contained the most pure PC and was used for further analyses. For comparison, $\alpha\beta$-PC was also purified in a similar manner from dissociated PBS from the wild type. The absorption spectrum of $\alpha\beta$*-PC shows a prominent shoulder at about 580 nm and has an absorption maximum at 634 nm that is significantly red-shifted relative to the λ_{max} of purified, wild-type PC at 626 nm (Fig. 5*A*). Fig. 5*B* shows a difference spectrum that was generated by normalizing the absorption spectra at 650 nm and subtracting the absorption spectrum of $\alpha\beta$*-PC from the *cpcT* mutant from the spectrum of wild-type PC. This difference spectrum had a maximum at 603 nm. The fluorescence emission spectra of PC and $\alpha\beta$*-PC are shown in Fig. 5*C*. Wild-type PC has a minor fluorescence emission maximum at 620 nm and its principal emission maximum occurs at 646 nm, but the $\alpha\beta$*-PC from the *cpcT* mutant has an obvious reduction in emission at 620 nm and a slightly red-shifted, principal emission maximum at 648 nm.

The absorption properties of the three PCB chromophores of native PC are well characterized (63–65). The α-84 and β-82 PCB chromophores of PC absorb maximally between 617 and 628 nm; the fluorescence emission maximum of the β-82 PCB, the terminal fluorescence emitter in PC, lies between 644 and 648 nm (63–65). The absorbance maximum of the PCB chromophore bound to cysteine β153 of PC occurs in the range 594–600 nm and has a fluorescence emission

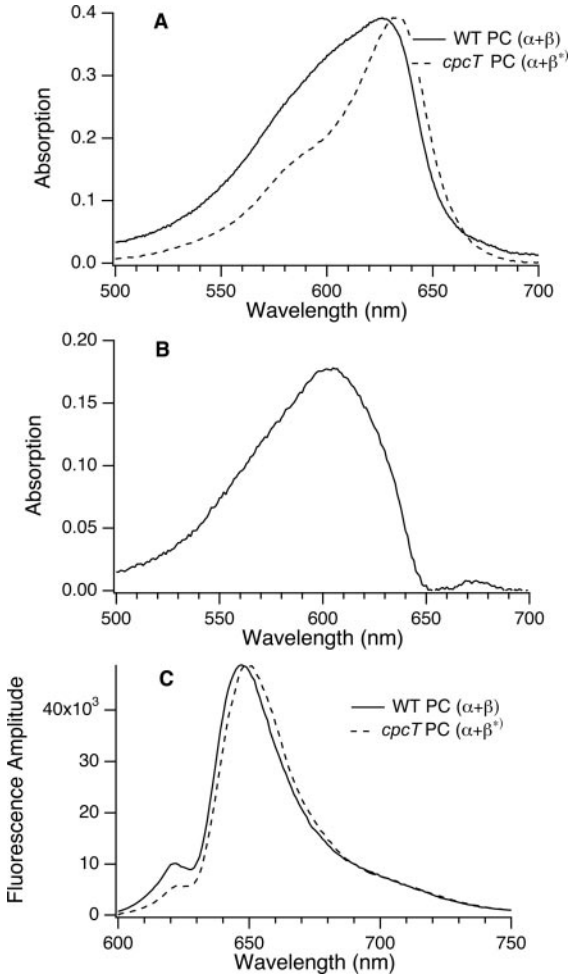

FIGURE 5. **Spectroscopic characterization of $\alpha\beta$*-PC purified from the *cpcT* mutant.** *A*, comparison of absorption spectra of the purified PC from wild-type (*solid line*) and $\alpha\beta$*-PC from the *cpcT* mutant (*dashed line*). The spectra were normalized at their maxima for comparison. *B*, absorption difference spectrum of the $\alpha\beta$-PC of wild-type and $\alpha\beta$*-PC of the *cpcT* mutant. This difference spectrum was calculated by subtracting the spectrum for PC from the *cpcT* mutant from the spectrum of wild-type PC after the spectra were normalized at 653 nm. *C*, fluorescence emission spectra of PC purified from *Synechococcus* sp. PCC 7002 wild-type (*solid line*) and the *cpcT* mutant (*dashed line*). The excitation wavelength was 590 nm, and the spectra were normalized at their maxima to facilitate their comparison.

maximum of 623–629 nm. The red-shifted absorbance spectrum and maximum of $\alpha\beta$*-PC, the absorption properties of the calculated difference spectrum, and the reduced fluorescence emission peak at 620 nm for $\alpha\beta$*-PC are consistent with the hypothesis that $\alpha\beta$*-PC lacks the PCB bound to the Cys-β153 residue.

To verify biochemically that cysteine β153 of $\alpha\beta$*-PC lacks its PCB chromophore, aliquots of $\alpha\beta$*-PC and wild-type PC were subjected to formic acid treatment as described under "Experimental Procedures." Formic acid does not affect the α-PC subunit but cleaves CpcB between Asp-144 and Pro-145 to yield two peptides. The larger peptide has a calculated mass of 15,996 Da, including the mass of one PCB chromophore (587 Da) attached at Cys-82; the smaller peptide has a calculated mass of 3532 Da, including the mass of one PCB bound to Cys-153. The formic acid-treated proteins were analyzed by SDS-PAGE, and bilin-carrying peptides were detected by their zinc-enhanced fluorescence. Treatment of wild-type PC with formic acid produces two fluorescent peptides as expected (Fig. 6*A*, *lane 1*). However, only the larger, 16-kDa fluorescent peptide is detected after formic acid treatment of $\alpha\beta$*-PC. This result conclusively demonstrates that the modified β*-PC

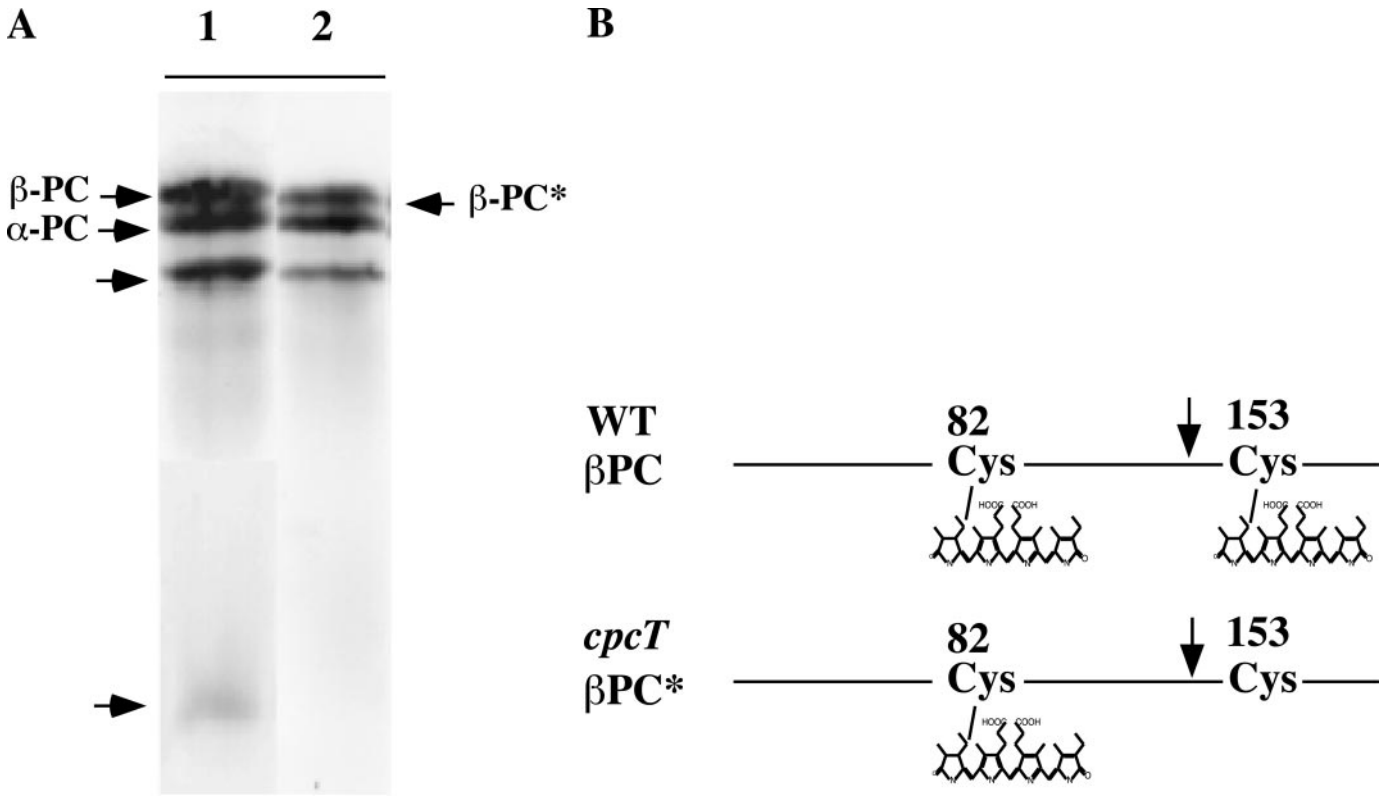

FIGURE 6. **Formic acid cleavage of PC purified from *Synechococcus* sp. PCC 7002 wild-type and the *cpcT* mutant.** *A*, PC proteins from wild-type (*αPC + βPC*) (*lane 1*) and PC proteins from the *cpcT* mutant (*α-PC + β*-PC*) (*lane 2*) were treated with formic acid and analyzed by SDS-PAGE. The gel was stained with $ZnSO_4$ to allow visualization of the chromopeptides by UV-induced fluorescence. *Arrows* at the *left* in *panel A* indicate the peptides that arise from the dilute acid cleavage of wild-type *β*-PC as indicated in the scheme shown in *panel B*. The *vertical arrows* in *panel B* show the position of the acid-sensitive Pro-Asp peptide bond.

subunit of the *cpcT* mutant lacks a PCB chromophore at Cys-153 as illustrated in the diagram Fig. 6*B*.

PCB Addition Assays with CpcT and rCpcB/CpcA—To verify the role of CpcT as a lyase for PCB attachment to Cys-153 of CpcB, *in vitro* chromophorylation assays were performed. Recombinant CpcT and rCpcB/CpcA were overproduced in *E. coli* BL21(DE3) cells, and the proteins were partly purified as described under "Experimental Procedures" (see supplemental Fig. 4). Bilin addition assays were carried out with purified rCpcB/CpcA with or without the addition of recombinant CpcT. In some experiments, PCB (10 mM) from *Spirulina* sp. PC was added, whereas in other experiments PCB was produced *in situ* by using PcyA from *Nostoc* sp. PCC 7120 (kind gift of J. Clark Lagarias) (49, 50) in the presence of ferredoxin, biliverdin (10 mM), and a ferredoxin regeneration system (see "Experimental Procedures"). When purified PCB was added to start the reactions, a color change from blue to purple could be seen in the reactions containing CpcT after 15 min, whereas the control reactions containing an equal volume of control *E. coli* extract (harboring empty vector pAED4) remained very faintly blue after incubation for 2 h at 30 °C. The absorbance spectra of these PCB reactions are compared in Fig. 7*A*. The reaction product produced in the presence of CpcT had λ_{max} at 603 nm, whereas the control reaction product had λ_{max} of 638 nm and had a spectrum similar to that expected for MBV (16, 17). The shoulder at 638 nm in the product spectrum of the reaction including CpcT probably arises from a small amount of competing, non-enzymatic PCB addition that produces MBV at cysteines *α*84 and *β*82 (see below). As shown in Fig. 7*B*, the product generated in the presence of CpcT was highly fluorescent and had an emission maximum at 624 nm, but the control reaction product was almost non-fluorescent by comparison and had an emission maximum at 657 nm. The properties of the control reaction product are consistent with the behavior of MBV adducts (16).

When PcyA was used to produce PCB, even more striking and clear-cut results were obtained (Fig. 7, *C* and *D*). The reaction product generated in the presence of CpcT had λ_{max} at 597 nm, whereas the control reaction in the absence of CpcT produced very little covalent product (Fig. 7*C*) and had λ_{max} at 638 nm. The reaction product generated in the presence of CpcT was highly fluorescent and had an emission maximum at 623 nm, whereas the product generated in the absence of CpcT had no detectable fluorescence emission.

Because both CpcB and CpcA were present in the reaction mixture, it was necessary to determine which subunit(s) was being chromophorylated by CpcT. An aliquot of the reaction mixture from the experiment in Fig. 7*C*, created using PcyA and CpcT, was analyzed by SDS-PAGE and subsequent $ZnSO_4$ staining (Fig. 8, *lane A*) and finally post-staining with Coomassie Blue (Fig. 8, *lane B*). As shown in Fig. 8*A*, only the *β*-PC subunit (CpcB) is fluorescent due to the presence of a covalently attached bilin. CpcT, CpcB, and CpcA were visualized after staining with Coomassie Blue, and their positions are indicated to the *right* (Fig. 8*B*), but neither of these proteins showed any zinc-enhanced fluorescence. When the control reaction products were subjected to the same analysis, no bilin fluorescence was detected on any protein (data not shown). Therefore, CpcT attaches PCB specifically to CpcB, the *β*-PC subunit.

To investigate whether CpcT plays any role in chromophore attachment to AP subunits, PCB addition reactions were also carried out using PcyA, HTApcA/ApcB, and CpcT. Following a 1-h incubation at 30 °C, products of PCB addition reactions (using PcyA) were characterized by absorption and fluorescence emission spectroscopy. No evidence of

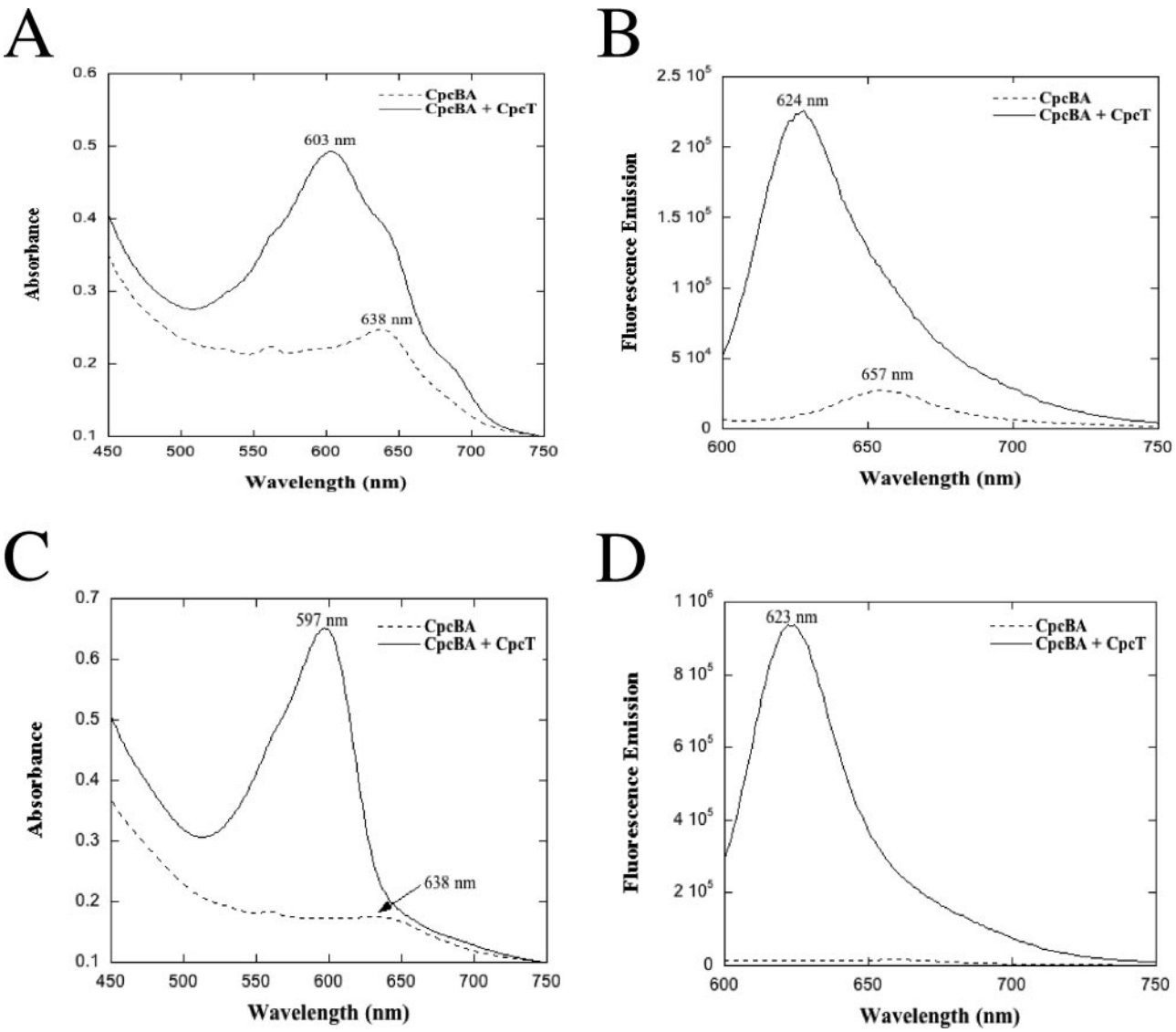

FIGURE 7. **Absorbance and fluorescence emission spectra of *in vitro* bilin addition assays with recombinant CpcB and CpcA (rCpcB/CpcA).** *A*, absorbance spectra of PCB addition products with apo-CpcB/CpcA as substrate were taken for reactions in which a control *E. coli* extract was added (*dashed line*) or CpcT was added (*solid line*). *B*, fluorescence emission spectra (λ_{ex} = 590 nm) of reactions described for *panel A*. *C*, absorbance spectra of addition reactions with rCpcB/CpcA in which PcyA was used to generate PCB as described under "Experimental Procedures." The *dashed line* shows the absorbance spectrum of a control reaction with no addition of CpcT, and the *solid line* shows the spectrum of the product produced in the presence of CpcT. *D*, fluorescence emission spectra (λ_{ex} = 590 nm) of the reactions described for *panel C*. The absorbance and fluorescence emission maxima of all spectra are indicated.

CpcT-catalyzed attachment of PCB to the HT-ApcA/ApcB proteins by CpcT was observed, and only non-enzymatic reaction products were detected (see supplemental Fig. 5). These results demonstrate that CpcT is specifically involved in chromophore attachment to CpcB and that CpcA, ApcA, and ApcB are not substrates for this lyase.

Tryptic Digestion of PC Followed by Reverse Phase HPLC Analyses—To verify that CpcT catalyzes the attachment of PCB to Cys-153 of *in vitro*, PCB addition reactions similar to those described in Fig. 7*A* were set up and allowed to proceed for 2 h at 30 °C. The products of this reaction, together with purified holo-PC from wild-type *Synechococcus* sp. PCC 7002 were subjected to digestion with trypsin. The trypsin cleavage products were resolved by HPLC on a C-18 reverse phase column. The elution profiles of the bilin-containing peptides, which absorb at 600 nm, are shown in Fig. 9. Trypsin digestion of holo-PC produced three major, bilin-bearing, tryptic peptides, which are identified by the position of the PCB-bearing cysteine in the polypeptide. The α84 peptide eluted first at 20.4 min, the β82 peptide eluted second at 23.2 min, and the β153 peptide eluted last at 30.3 min. This elution order corresponds to that reported previously (16) and also corresponds to the predicted masses of the tryptic peptides including PCB (α84 peptide = 1251 Da, β82 peptide = 1323 Da, and β153 = 4075 Da). When the *in vitro* CpcT/PCB reaction products with rCpcA/CpcB were similarly treated, only a single, major bilin-containing peptide was observed with a retention time of 30.3 min, matching the retention time of the β153 peptide obtained from digestion of holo-PC. Two minor peaks eluting slightly earlier in each case than the α84 and β82 peptides were also observed. Because the product analyzed in Fig. 9*B* was obtained from an incubation with free PCB and CpcT, it is likely that these peptides carry MBV attached to the α84 and β82 cysteine residues, which should shift their retention times slightly as reported previously (16). Consistent with this explanation, the product prior to trypsin digestion had a shoulder at ~638 nm indicative of the presence of MBV, and the absorbance spectra of the two minor peaks were significantly red-shifted when compared with the 30.3-min peak carrying PCB

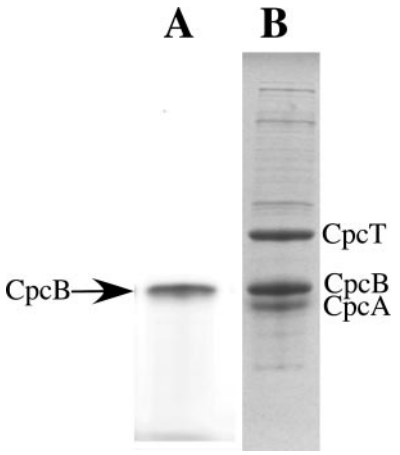

FIGURE 8. **SDS-PAGE analysis of the CpcT-dependent reaction product from PCB (generated by PcyA) addition with rCpcB/CpcA proteins.** An aliquot of the reaction product produced in the presence of CpcT was separated by SDS-PAGE. *A*, bilin fluorescence was visualized under UV light after the gel was stained with $ZnSO_4$. *B*, after staining with $ZnSO_4$, the same gel was stained with Coomassie Blue. The identity of each product is indicated. Only CpcB carries a covalently bound bilin.

bound to Cys-153 of CpcB. No tryptic peptides with 600-nm absorbance, not even peptides carrying MBV, were detected when CpcT was omitted from the lyase reaction with rCpcB/CpcA (data not shown). This is probably because the yield of peptides carrying MBV was below the level of detection when CpcT was omitted from the reaction. This is consistent with the results shown in Fig. 8*A*, which indicate that the MBV addition products (638 nm) to rCpcB/CpcA are ~3-fold lower in yield in the absence of CpcT than when CpcT is present.

DISCUSSION

Physiological and biochemical analyses of the *cpcT* mutant of *Synechococcus* sp. PCC 7002, and *in vitro* biochemical characterization of recombinant CpcT, clearly demonstrate that this protein is a lyase that attaches PCB to Cys-153 of CpcB. PC from the *cpcT* mutant was missing a PCB chromophore only at cysteine β153, and the difference spectrum (Fig. 5*B*) between wild-type PC and PC from the *cpcT* mutant closely matches the spectrum of the CpcT-dependent rCpcB/CpcA bilin attachment product (Fig. 7, *A* and *C*). The spectrum of PC from the *cpcT* mutant (Fig. 5*A*) also matches that of a PC mutant in which Cys-153 of CpcB was mutated to Ser (66). Recombinant CpcT specifically attaches PCB to Cys-β153 *in vitro*. Although some MBV addition products were detected when PCB was added at a high (10 μM) and probably non-physiological concentration, (see Figs. 7*A* and 9), as shown under "Results," these products appear to be the result of non-enzymatic addition reactions and were not seen when PcyA was used to generate PCB *in situ* (see Fig. 7*C*).

The phenotype of the *cpcT* mutant mirrors that described for other bilin lyase mutants. The upper region of the sucrose gradients used to isolate PBS from the *cpcT* mutant was enriched in β-PC subunits with faster electrophoretic mobility than that of wild-type β-PC. This apparently lower molecular mass was consistent with the absence of a single PCB (587 Da) (18). In *cpcE* and *cpcF* mutants, an excess of holo-β-PC was present in the top fraction of the sucrose gradient (18). It is unclear why we did not observe β^*-PC paired with holo-α-PC in the upper regions of the sucrose gradients. One explanation may be that the absence of a bilin at Cys-β153 reduces the binding interaction between the α- and β-subunits, as has been shown with sedimentation equilibrium studies (62), and that dimers of β^*-PC may be more stable and less susceptible to degradation than dimers of holo-α-PC. Absence of the β153 chromophore does not appear to impair PC assembly as much as

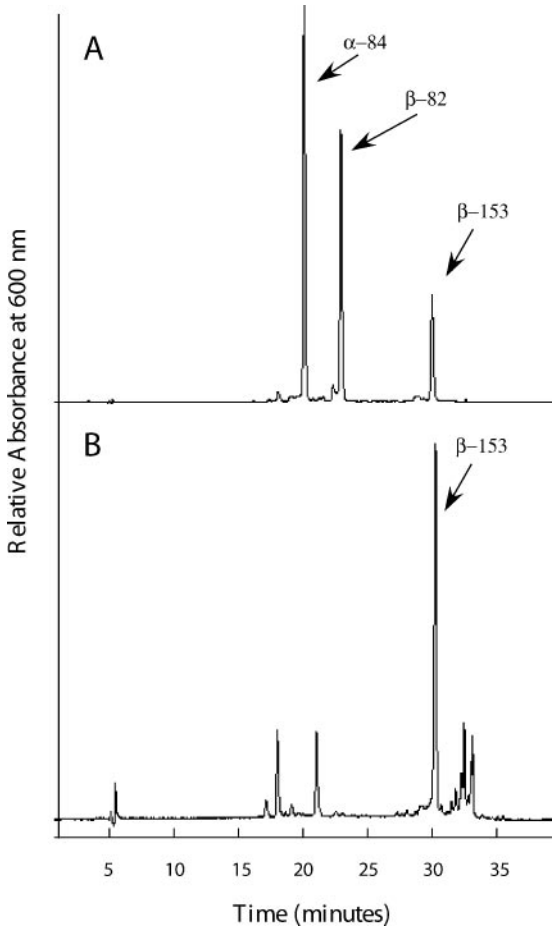

FIGURE 9. **Reverse phase HPLC separation of tryptic peptides of *in vitro* bilin addition products with rCpcB/CpcA in the presence and absence of CpcT.** *A*, PCB-containing tryptic peptides derived from holo-PC. Three bilin-containing peptides are indicated by *arrows* and identified by the cysteine residue to which the chromophore is attached. *B*, bilin-containing tryptic peptides derived from digestion of the rCpcB/CpcA after PCB addition in the presence of CpcT. The main peak at 30.3 min corresponds to the PCB-containing peptide containing the chromophor phosphorylation site at cysteine β153.

absence of the β82 chromophore (59, 62, 66, 67), however. The β153 chromophore binding site is located on the outer edge of the trimer, and its principal role is to harvest light energy and transfer that energy to the terminal acceptor chromophore at Cys-β82 (1).

We have recently identified and characterized another lyase (CpcS/CpcU) that attaches PCB to cysteine α82 of PC as well as the α82 and β82 cysteines of AP (68).[4] Another group has recently characterized a paralogous protein called CpeS, which alone attaches PCB to cystein α84 of PC and PEC of *Anabaena* sp. PCC 7120 (69). Why did nature evolve separate enzymes for each attachment site on phycocyanin? For the β-subunit, two different lyases are probably necessary because the stereochemistry of bilin attachment at the β82 and β153 attachment sites is different; the chiral carbon at $C3^1$ carbon of PCB attached to cysteine β82 has *R* stereochemistry, whereas that of PCB attached to cysteine β153 has *S* stereochemistry (see supplemental Fig. 1). For PBPs for which there are x-ray crystal structures, the stereochemistry of the site equivalent to β153 is always *S* rather than *R* (70–72). Therefore, it is likely that all CpcT paralogs will be responsible for attachment to the β153 equivalent sites of all potential substrates, although it remains possible that these proteins could also play a role in chromophore

[4] G. Shen, N. Saunée, S. R. Williams, W. M. Schluchter, and D. A. Bryant, manuscript in preparation.

attachment to γ-subunit linker proteins. This difference in attachment stereochemistry may arise from a required difference in the binding conformation of the substrate bilin or to an intrinsic difference in the enzyme mechanism of attachment when compared with lyases that attach chromophores to the α84 and β82 sites. In this regard, it should be noted that the enzyme for PCB attachment at the β82 cysteine of PC can chromophorylate the α84 cysteine to some extent, because some chromophorylation of CpcA occurs in *cpcE*/*cpcF* mutant strains, and this activity can be enhanced by suppressor mutations in *cpcA* (53). In support of this, we have demonstrated that the β82 lyase of *Synechococcus* sp. PCC 7002 (CpcS/CpcU) can also attach PCB to α84 of CpcA.[5] Zhao *et al.* (69) also noted that CpeS can attach PCB to cysteine α84 of both PEC and PC.

One of the main functions of the CpcT lyase may be to bind the PCB chromophore in the proper orientation during the attachment reaction such that the *S* isomer at C3′ is formed. A continuing question regarding bilin lyases is whether the function of the enzyme is to bind the bilin in the appropriate conformation as it approaches the apo-protein or whether the function of the enzyme is to act as a "chaperone" to bind the apoPBP subunit in the appropriate conformation to allow PCB attachment to the appropriate cysteine residue. Supporting the first hypothesis, the PecE/PecF lyase has been shown to bind bilins weakly, and some bilin absorption can be detected after purification of His-tagged PecE/PecF lyase subunits through a nickel-nitrilotriacetic acid column (73). Triton X-100 experiments by Zhao *et al.* (32) suggest that bilin conformation is critical during approach and attachment. However, if the second hypothesis were true, then the PBP subunit should contain the catalytic residues required for bilin lyase activity. When one compares PBP and phytochromes (74), there is very little similarity around the cysteine residues to which the bilins are attached. With the exception of ApcE, all PBPs tested appear to require separate enzymes or some other cofactor for chromophore attachment (16, 20, 25, 33). Zhao *et al.* (32) proposed that enzymes might not be required when Triton X-100 is present to elicit the appropriate PCB conformation; however, even though PCB was the product formed at β153 on PC in the presence of Triton X-100 as assayed by absorption spectroscopy, Zhao *et al.* (32) did not determine the x-ray structure of the CpcB addition product to verify that the appropriate *R* or *S* isomer was formed at C3[1]. Interactions of the detergent with the surface of PC may modify the chromophore binding pocket in such a manner that PCB has easier access to Cys-β153.

The two previously characterized α-subunit PCB lyases function as heterodimers, and it is possible that CpcT functions as a homodimer. However, no additional proteins were required to obtain the observed enzymatic activity and specificity. We were also able to obtain activity and specificity without exhaustively testing cofactor requirements, but it should be noted that addition of 1 mM $MgCl_2$ increased activity as judged by the amount of product formed (data not shown); $MgCl_2$ was previously shown to enhance the activities of CpcE/CpcF and PecE/PecF (19, 20, 25, 27). These details will be more exhaustively explored in future experiments.

CpcT is not related in sequence to the CpcE/CpcF α-PC lyase nor to the lyase/isomerase PecE/PecF, which both attach bilins to α-subunits. Therefore, CpcT constitutes a new class of bilin lyase. There are *cpcT* and *cpeT*-like genes present in the genomes of all sequenced cyanobacteria except *P. marinus* MED4 (see Fig. 1), and the number of *cpcT*/*cpeT* paralogs present in each genome roughly correlates to the number of PBP β-subunits encoded in these genomes and present in the rods of the PBS (PC, PE, and/or PEC). One reason for the different classes of CpcT/CpeT proteins is that some marine strains produce PC variants, known as R-PCs, in which cysteine β153 carries a PEB chromophore rather than a PCB chromophore (75–77). Although it is possible that the CpeT paralogs might have other functions within PE-containing organisms, given their sequence similarity to CpcT, is seems likely that CpeT functions in chromophore attachment at the position equivalent to β153 near the C terminus of PE. In support of this contention, the *P. marinus* MED4 encodes and synthesizes β-PE but does not possess the *cpeT* gene (78). However, the β-PE of *P. marinus* MED4 lacks the β165 (β153 equivalent) chromophore-binding cysteine and thus would not require CpeT for its biogenesis (79). Other lyases, such as the CpcE/CpcF-related CpeY/CpeZ (80), probably attach bilins to the α-PE subunits. Finally, a bacteriophage that infects marine strains of *Synechococcus* sp. carries a *cpeT* ortholog, which may be a way for this phage to increase the "vigor" of the host by increasing its ability to synthesize PE for light harvesting (55).

When an alignment of all CpcT and CpeT sequences is examined, several highly conserved amino acid residues are apparent (see supplemental Fig. 2). Residues that may be important in bilin binding include Glu-54, Asp-165, Gln-55, and Asn-20 (using *Synechococcus* sp. PCC 7002 CpcT numbering), which could interact with the pyrrole nitrogens of the bilin, and arginine residues at positions 39, 66, 68, 143, and/or 166 could provide a salt linkage(s) to the bilin propionate group(s). A glutamic acid residue within the region of phytochrome important for lyase activity and bilin binding was shown to be important to these activities, possibly through its interaction with the pyrrole nitrogens of phytochromobilin (8). There are two highly conserved cysteines (Cys-118 and Cys-139) and a histidine (33) that could play roles in catalysis or in transient bilin binding. A cysteine has been shown to be critical for PecF activity (73), and a histidine transiently and covalently binds heme in the heme chaperone CcmE (81). However, there is no conserved histidine next to either of these cysteines as one finds in phytochrome and PecF (8, 73). No covalently bound bilin was detected by zinc-enhanced fluorescence of CpcT in the experiments reported in this study (see Fig. 8, *lane A*). Because there was a large amount of CpcB substrate present in the assays performed in these studies, it is possible that a transient, covalently bound, bilin intermediate would not have been detected on CpcT (see Fig. 9*A*). In future experiments we will determine directly whether CpcT is able to bind bilins.

The combined approaches of comparative bioinformatics, reverse genetics, and biochemistry have proven to be powerful tools in the identification and analysis of bilin lyases. We are continuing to use these approaches to identify and characterize additional bilin lyases in cyanobacteria.

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[5] N. Saunée, S. R. Williams, and W. M. Schluchter, unpublished results.

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