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Attachment of Noncognate Chromophores to CpcA of *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 by Heterologous Expression in *Escherichia coli*

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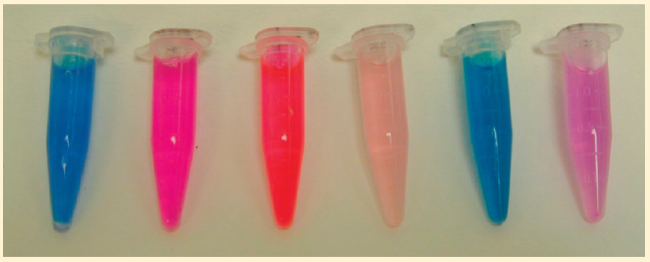
Supporting Information

ABSTRACT: Many cyanobacteria use brilliantly pigmented, multisubunit macromolecular structures known as phycobilisomes as antenna to enhance light harvesting for photosynthesis. Recent studies have defined the enzymes that synthesize phycobilin chromophores as well as many of the phycobilin lyase enzymes that attach these chromophores to their cognate apoproteins. The ability of the phycocyanin α-subunit (CpcA) to bind alternative linear tetrapyrrole chromophores was examined through the use of a heterologous expression system in *Escherichia coli*. *E. coli* strains produced phycocyanobilin, phytochromobilin, or phycoerythrobilin when they expressed 3*Z*-phycocyanobilin:ferredoxin oxidoreductase (PcyA), 3*Z*-phytochromobilin:ferredoxin oxidoreductase (HY2) from *Arabidopsis thaliana*, or phycoerythrobilin synthase (PebS) from the myovirus P-SSM4, respectively. CpcA from *Synechocystis* sp. PCC 6803 or *Synechococcus* sp. PCC 7002 was coexpressed in these strains with the phycocyanin α-subunit phycocyanobilin lyase, CpcE/CpcF, or the phycoerythrocyanin α-subunit phycocyanobilin isomerizing lyase, PecE/PecF, from *Noctoc* sp. PCC 7120. Both lyases were capable of attaching three different linear tetrapyrrole chromophores to CpcA; thus, up to six different CpcA variants, each with a unique chromophore, could be produced with this system. One of these chromophores, denoted phytoviolobilin, has not yet been observed naturally. The recombinant proteins had unexpected and potentially useful properties, which included very high fluorescence quantum yields and photochemical activity. Chimeric lyases PecE/CpcF and CpcE/PecF were used to show that the isomerizing activity that converts phycocyanobilin to phycoviolobilin resides with PecF and not PecE. Finally, spectroscopic properties of recombinant phycocyanin R-PCIII, in which the CpcA subunits carry a phycoerythrobilin chromophore, are described.



Cyanobacteria have evolved a family of water-soluble, pigmented proteins, the phycobiliproteins (PBPs), as accessory pigments for photosynthetic light harvesting. Together with nonchromophorylated "linker" proteins, PBPs form supramolecular antennae complexes known as phycobilisomes (PBS).[1,2] PBPs carry covalently bound, linear tetrapyrroles, phycobilins, which are responsible for the light-harvesting properties of these proteins.[1] Four phycobilins are known to be incorporated into cyanobacterial PBPs: phycocyanobilin (PCB) and phycoerythrobilin (PEB) and their respective Δ5-to-Δ2 double-bond isomers, phycoviolobilin (PVB) and phycourobilin (PUB).[1] Plants do not synthesize any of these four phycobilins, but unlike cyanobacteria they synthesize another linear tetrapyrrole, phytochromobilin (PΦB), which is used for light sensing. PΦB differs from PCB by the presence of an 18-vinyl moiety on the D-ring, which is an ethyl group in PCB. The resulting change in conjugation shifts the absorption maximum of PΦB to the red by ∼10 nm. Phytochrome-like photoreceptors utilizing PCB, PΦB, or PVB respond to the wavelengths of light in their environments through a photoisomerization reaction around the 15−16 double bond of the chromophore.[3,4] Although the biological significance of this reaction remains unclear, a similar photoisomerization also occurs with the PVB chromophore of PecA (i.e., phycoerythrocyanin α subunit).[5,6]

The biosynthetic pathways for the production of the four cyanobacterial phycobilins and PΦB in *Arabidopsis thaliana* have been elucidated.[7−11] These five chromophores share a common precursor, biliverdin IXα, which is the product of heme macrocycle cleavage by heme oxygenase. Biliverdin IXα is subsequently reduced by one of several possible ferredoxin-dependent bilin reductases (FDBRs). Plants utilize 3*Z*-phytochromobilin:ferredoxin oxidoreductase (HY2) to reduce the diene system of the A ring of biliverdin IXα to form PΦB.[8] Cyanobacteria utilize phycocyanobilin:ferredoxin oxidoreductase (PcyA) to perform a two-step reaction; this

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Table 1. Plasmids Used for This Study

plasmid name	recombinant proteins produced	parent vector	antibiotic[a]	references
pPcyA	*Synechocystis* 6803 Hox1 and *Synechococcus* 7002 HT-PcyA	pACYC Duet	Cm	12
pPebS	Myovirus Hox1 and HT-PebS	pACYC Duet	Cm	10
pHY2	*Synechocystis* 6803 Hox1 and *A. thaliana* HT-HY2	pACYC Duet	Cm	this paper
pBS414v	*Synechocystis* 6803 HT-CpcA, CpcE and CpcF	pBS350v	Sp	33
pBS405v	*Synechocystis* 6803 HT-CpcA	pBS350v	Sp	33
pBS405vpecEF	*Synechocystis* 6803 HT-CpcA, *Nostoc* sp. PCC 7120 PecE and PecF	pBS405v	Sp	this paper
pBS405vpecF	*Synechocystis* 6803 HT-CpcA, *Nostoc* sp. PCC 7120 PecF	pBS405v	Sp	this paper
pBS405vpecE	*Synechocystis* 6803 HT-CpcA, *Nostoc* sp. PCC 7120 PecE	pBS405v	Sp	this paper
pBS405vcpcEpecF	*Synechocystis* 6803 HT-CpcA, CpcE and *Nostoc* sp. PCC 7120 PecF	pBS405v	Sp	this paper
pBS405vpecEcpcF	*Synechocystis* 6803 HT-CpcA, CpcF and *Nostoc* sp. PCC 7120 PecE	pBS405v	Sp	this paper
pBS405vcpcE	*Synechocystis* 6803 HT-CpcA and CpcE	pBS405v	Sp	this paper
pBS405vcpcF	*Synechocystis* 6803 HT-CpcA and CpcF	pBS405v	Sp	this paper
pBS405vpecEcpcF	*Synechocystis* 6803 HT-CpcA, CpcF and *Nostoc* sp. PCC 7120 PecE	pBS405v	Sp	this paper
pPL-PΦB	*Synechocystis* 6803 Hox1 and *A. thaliana* HY2	pProLarA122	Km	39
pBSpecAEF	*Nostoc* sp. PCC 7120 PecA, PecE, and PecF	pBS405v	Sp	34, this paper
pCOLAduet-1cpcEcpcF	*Synechococcus* 7002 CpcE and CpcF	pCOLAduet-1	Km	this paper
pETduet-1cpcA	*Synechococcus* 7002 CpcA	pETduet-1	Carb	this paper

[a] Antibiotic resistance used to select for the presence of the plasmid: Carb: carbenicillin; Cm: chloramphenicol; Km: kanamycin; Sp: spectinomycin.

enzyme first reduces the 18-vinyl side chain of the D-ring and subsequently reduces the vinyl side chain of the pyrrole A ring to yield PCB.[9] PEB can be produced by either of two pathways.[9,10] In the PebA−PebB pathway found in many cyanobacteria, 15,16-DHBV:ferredoxin oxidoreductase (PebA) reduces biliverdin IXα to 15,16-dihydrobiliverdin (DHBV), and PEB:ferredoxin oxidoreductase (PebB) reduces 15,16-DHBV to PEB.[9] Alternatively, PEB synthase (PebS) from the myovirus P-SSM4 performs both reactions in a manner similar to the two-step reduction of biliverdin IXα to PCB by PcyA.[10] PVB and PUB are formed by lyases that isomerize PCB to PVB and PEB to PUB, respectively; these isomerizations occur during the attachment of these PCB or PEB chromophores to cysteines on some PBP subunits.[7,11]

A few biliproteins, including plant and bacterial phytochromes and ApcE found in the cores of PBS, have autocatalytic chromophore ligation activity.[12−15] This capability was exploited to produce "phytofluors", fluorescent variants of phytochrome that could be produced *in vitro* and *in vivo*.[16] Apo-phytochrome can autoligate a range of bilins, including PΦB, PCB, PEB, and biliverdin.[16−18] The maturation of other PBPs requires one or more PBP lyases, which attach bilin chromophores to specific cysteine residues of appropriate, i.e., cognate apoproteins.[19,20] CpcE/CpcF, the first identified PBP lyase, is a heterodimer exclusively responsible for attachment of PCB to CpcA.[21] A paralogous lyase, PecE/PecF, isomerizes PCB to PVB and attaches it to PecA.[11] Other well-characterized lyases include the CpcS and CpcS/CpcU lyases, which attach PCB to conserved Cys residues at the 82/84 positions of CpcB, ApcA, ApcB, ApcD, and ApcF; and the CpcT lyase, which attaches PCB to the Cys-153 residue of CpcB.[12,22−26] The discovery of the CpcS/CpcU and CpcT lyases in the cyanobacterium *Synechococcus* sp. strain PCC 7002 (hereafter *Synechococcus* 7002) allowed the lyase requirements for all PBPs synthesized in this organism to be determined.[12,27] Recently RpcG, a fusion of PecE- and PecF-like domains, from the marine cyanobacterium *Synechococcus* sp. WH8102 has been reported to attach both PVB and PUB to its cognate apoprotein, RpcA.[7] Putative lyases that are probably responsible for the attachment of PEB to phycoerythrin subunits have been identified in the genomes of numerous species, but the substrate specificities of these proteins are still largely uncharacterized.[26,28−30]

The specificity of bilin attachment to apo-PBPs has recently been studied by overproducing alternative FDBRs, PebA/PebB and HY2, in *Synechococcus* 7002, an organism that naturally possesses PcyA as its sole FDBR.[31] Overexpression of PebA and PebB caused cells to become brown, resembling strains that naturally synthesize phycoerythrin, but this was not well tolerated and cells readily reverted to the wild-type pigmentation. When the chromophorylation status of the PBPs produced by this strain was examined in more detail, it was discovered that PEB was almost exclusively ligated to CpcA. Minor amounts of PEB were attached to ApcA as well, but ligation of PEB to other PBPs was not detected. Overexpression of HY2 in *Synechococcus* 7002 was much better tolerated. Similar to the results obtained when *pcyA* was used to complement a HY2 mutant in *A. thaliana*,[32] HY2 expression was sufficient in *Synechococcus* 7002 to allow insertional inactivation of *pcyA*, a mutation that was otherwise lethal. The absorption maxima of all holo-PBPs produced by this strain were red-shifted ∼10 nm due to the additional vinyl group of PΦB compared to PCB.

Recent advances in knowledge of PBP biogenesis have allowed researchers to produce holo-PBPs in heterologous hosts.[7,12,26,33−38] These systems generally utilize genes encoding a cyanobacterial heme oxygenase (*hox1*), a FDBR (either *pcyA* or *pebAB*) to produce bilin chromophores from heme, genes for apo-PBPs, and the cognate lyase(s), if required. In the studies reported here, apo-CpcA from *Synechococcus* 7002 and *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803), cyanobacteria that only synthesize PCB, was produced heterologously in *Escherichia coli*. The goals of these studies were to understand lyase function better by determining the extent to which alternative chromophores could be ligated to apo-CpcA and to create unique PBPs, which have novel properties and do not occur naturally.

MATERIALS AND METHODS

Plasmid Construction. Several plasmids used in the studies reported here have been used in previous studies, and their

construction is described elsewhere (see Table 1 for references). For the expression of $[His]_6$-tagged- (HT-) CpcA from *Synechococcus* 7002 in *E. coli* the lyase genes (either *cpcE* and *cpcF* or *pecE* and *pecF*) were cloned into the pCOLAduet-1 vector (Novagen-EMD, La Jolla, CA). The *cpcA* gene was cloned into the compatible plasmid, pETduet-1 (Novagen-EMD, La Jolla, CA). The *Synechococcus* 7002 *cpcA* gene was amplified by PCR using primers 7002cpcAF and 7002cpcAR (see Supporting Information Table 1 for the sequences of the primers used in this study), digested with *Bam*HI and *Eco*RI and ligated into similarly digested pETduet-1. *cpcE* was amplified by PCR using primers 7002cpcEFB and 7002cpcER, digested with *Nco*I and *Pst*I, and ligated into similarly digested pCOLAduet-1. *cpcF* was amplified by PCR using primers 7002cpcFF and 7002cpcFR, digested with *Nde*I and *Xho*I, and ligated into similarly digested pCOLAduet-1 with *cpcF* to make pCOLAduet-1cpcEF.

The HY2 gene was amplified from the plasmid pPL-PΦB[39] using the primers ECHY2F (an A to G silent mutation (bold italicized "G" in the primer sequence) was introduced to eliminate an *Eco*RI site, and a separate *Eco*RI site was added near the 5′ end of the primer) and ECHY2R. The corresponding HY2 amplicon was cloned into pPcyA by removing *pcyA* (by digestion with *Eco*RI and *Sal*I) and inserting the appropriately digested HY2 amplicon to yield plasmid pHY2.

The plasmid containing *cpcA* from *Synechocystis* 6803 and *pecE* and *pecF* genes from the genome of *Nostoc* sp. strain PCC 7120 was made by PCR-amplification of *pecE* (using primers pecEF and pecER), which contain *Sal*I and *Not*I sites, respectively, and *pecF* (using primers pecFF and pecFR), which contain *Eag*I and *Bam*HI sites, respectively[40] (GenBank accession no. AF178757). Along with *cpcE*, *cpcF* (both from pBS414v), these DNA fragments were cloned into pBS405v to produce plasmids pBS405vpecEF, pBS405vpecE, pBS405vpecF, pBS405vcpcE-pecF, pBS405vpecEcpcF, pBS405vcpcE, and pBS405vcpcF (see Table 1).

In order to create a plasmid with the *Nostoc* sp. PCC 7120 *pecA*, *pecE*, and *pecF* genes, *pecA* was amplified by PCR using primers pecAF and pecAR, digested with *Nde*I and *Eco*RI and cloned into similarly digested pBS405v, which removed the existing *Synechocystis* 6803 *cpcA* gene. The *pecE* and *pecF* genes were then subcloned into this new vector from pBS405vpecEF using *Sal*I and *Bam*HI, to create pBSpecAEF. *pecE* and *pecF* were also cloned into pCOLA duet-1 by amplifying *pecE* by PCR using pecE2F and pecE2R, digesting the product with *Nco*I and *Pst*I, and cloning it into similarly digested pCOLAduet-1. *pecF* was obtained by digestion from pBS405vpecEF using *Nde*I and *Bam*HI and subcloned into similarly digested pCOLAduet-1 containing *pecE*, to create pCOLAduet-1pecEF.

Heterologous Expression and Purification of Recombinant Proteins. Plasmids for the expression of various combinations of bilins, lyases, and apo-PBPs were transformed into *E. coli* BL21 (DE3) cells. To ensure the maintenance of the plasmids for the production of bilins, apoproteins, and lyases, transformants were grown on media containing chloramphenicol (34 μg mL^{-1}) and spectinomycin (50 μg mL^{-1}) for the two plasmid expression system or chloramphenicol (34 μg mL^{-1}), kanamycin (50 μg mL^{-1}), and carbenicillin (50 μg mL^{-1}) for the three-plasmid system. For expression studies, a 5 mL overnight starter culture was added to 1.0 L of Luria−Bertani medium containing appropriate antibiotics, and the cultures were shaken at 150 rpm at 37 °C for 3−4 h until the $OD_{600\ nm}$ reached 0.6, at which time gene expression was induced by the addition of isopropyl β-D-thiogalactoside (final concentration 1.0 mM). Cultures were incubated with shaking at 18 °C overnight, and cells were harvested by centrifugation at 10000*g* for 10 min and washed once in Buffer O (50 mM Tris-HCl, 150 mM NaCl, pH 8.0). Cell pellets were stored at −20 °C until required.

Frozen cells containing recombinant proteins were thawed, resuspended in Buffer O (20 mL of buffer was used for the cells from 1.0 L of culture), and lysed by three passages through a chilled French pressure cell at 138 MPa. The resulting whole-cell lysate was centrifuged for 35 min at 35000*g* to remove unbroken cells and large cellular debris. $[His_6]$-tagged recombinant proteins were purified by affinity chromatography on columns (1.0 mL bed volume) containing Ni-NTA His-bind resin (Novagen-EMD, La Jolla, CA); proteins were eluted with Buffer O containing 250 mM imidazole.[23] Recombinant proteins were dialyzed against buffer O overnight at 4 °C to remove the imidazole. Purified proteins were stored at −20 °C until analyzed.

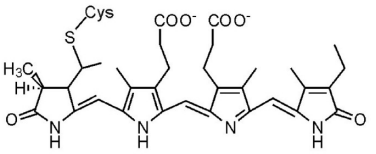
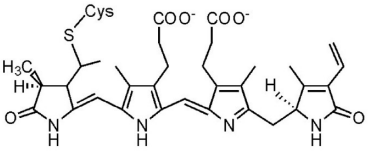
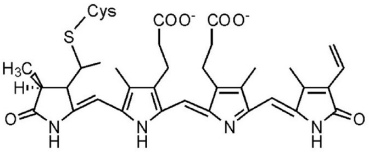
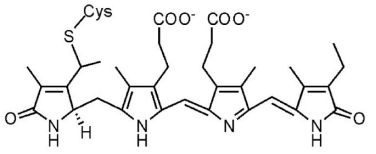
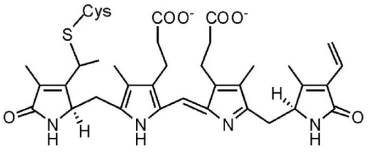
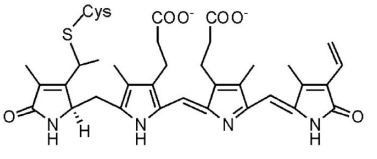
Absorbance and Fluorescence Measurements. Absorbance spectra were acquired using a Genesys 10 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Fluorescence emission spectra were recorded using an SLM 8000C spectrofluorometer modernized for computerized data acquisition by Olis, Inc. (Bogart, GA). The excitation monochromator was set to 570 nm for CpcA with PCB or PΦB chromophores and to 530 nm for CpcA with PEB, PVB, or PtVB chromophores. Fluorescence quantum yield measurements were made using the comparative methods described by Nanoco Technologies (www.nanocotechnologies.com/download.aspx?ID=77) and Jobin Yvon Ltd. (www.jobinyvon.com/usadivisions/Fluorescence/applications/quantumyieldstrad.pdf). Quantum yield measurements determined this way reliable to about ±10%. The standard used for these measurements was cresyl violet perchlorate (Sigma Chemical Co., St. Louis, MO), which has an absorption maximum at 603 nm, an emission maximum at 622 nm, and a quantum yield of 0.59 in ethanol.[41]

To determine if protein samples were photochemically active, absorbance spectra were recorded immediately before subjecting as-isolated samples to continuous illumination at their absorbance maxima for up to 2 h. Illumination was provided from 400 W tungsten−halogen lamp (model 66057, Oriel Corp., Stratford, CT) using a 1/4 m monochromator (Model 82-410, Jarrell-Ash Co., Waltham, MA), which has a 0.2 nm bandwidth resolution. The absorption spectra of the samples were immediately measured after illumination to determine whether any light-induced changes had occurred. Samples exhibiting changes in absorption were subsequently treated with monochromatic light at the newly formed absorption maximum to determine if the change was photoreversible.

Polyacrylamide Gel Electrophoresis and Fluorescence Imaging. Proteins were analyzed by polyacrylamide gel electrophoresis in the presence of SDS as previously described.[42] Gels containing chromophorylated proteins were treated with a solution of 25 mM $ZnCl_2$ to enhance the bilin fluorescence,[43] and gels were scanned with a Typhoon 8600 variable mode imager (GE Healthcare Lifesciences, Pittsburgh, PA) using an excitation wavelength of 532 nm. After fluorescence imaging, gels were stained with Coomassie Brilliant Blue R-250.

Separation of Phycocyanin Subunits from *Synechococcus* 7002. Phycocyanin (PC) was purified as described previously.[44] Briefly, *Synechococcus* 7002 cells were suspended in K-phosphate buffer at pH 7.0. The cells were passed though a chilled French pressure cell, the extract was centrifuged to remove the cellular

Table 2. Chromophores Attached to *Synechocystis* 6803 and *Synechococcus* 7002 CpcA in This Study and Features of the Resulting Holoproteins[a]

Chromophore	Structure	Abs[2] Max	Em[2] Max	Abs[2] Max Urea	QY[2]
PCB		625 / 623	646 / 645	665 / 665	0.39 / 0.31
PEB		556 / 556	568 / 568	561 / 558	0.98 / 0.94
PΦB		637 / 637	656 / 655	676 / 674	0.18 / 0.14
PVB		561 / 561	577 / 578	569 / 565	0.14 / 0.18
PUB		497	ND[3]	502	ND[3]
PtVB		575	590	579	0.23

[a] Data provided as values for *Synechocystis* 6803/values for *Synechococcus* 7002 or just *Synechocystis* 6803 when the corresponding *Synechococcus* 7002 variant was not produced. [2]Abs max, absorption maximum; Em max, emission maximum; QY, quantum yield. [3]ND, not determined.

debris, and PC and allophycocyanin were separated by chromatography on a DEAE-cellulose (DE-52) column.[44] CpcA and CpcB (i.e., PC α and β subunits) were separated by chromatography on a Biorex-70 column as described by Glazer and Fang.[45] CpcB was collected and dialyzed against water to remove the urea.[45]

Refolding and Reconstitution of Phycocyanin. HT-CpcA carrying a PEB chromophore (HT-CpcA-PEB) and CpcB carrying two PCB chromophores (i.e., holo-β-PC subunit) were refolded using the protocol described by Glazer and Fang[45] with minor changes. Briefly, the HT-CpcA-PEB and holo-β-PC subunits were lyophilized. Protein concentrations were calculated by denaturing the proteins in 8.0 M urea at pH 3.0 and by using the molar extinction coefficients for PEB or PCB and molecular weights of HT-CpcA-PEB and holo-β-PC.[44] Aliquots of the two subunits (1.3 mg each) (holo HT-CpcA and holo-β-PC have similar molecular weights of 19.69 and 19.57 kDa, respectively) were dissolved in 12.5 mL of 8.0 M urea, 5 mM 2-mercaptoethanol at pH 8.0. The resulting solution was dialyzed against 400 mL of 3.0 M urea, 6.0 mM Na-phosphate, 5.0 mM 2-mercaptoethanol at pH 6.7 for 4 h. The solution was then dialyzed against 500 mL of 10 mM Na-phosphate, 5.0 mM 2-mercaptoethanol at pH 6.5 for 20 h at 4 °C, with one change of dialysis buffer. A progressive gain in color was observed during the dialysis. The solution was finally dialyzed against 5.0 mM Na-phosphate, 1.0 mM 2-mercaptoethanol at pH 7.0 and then

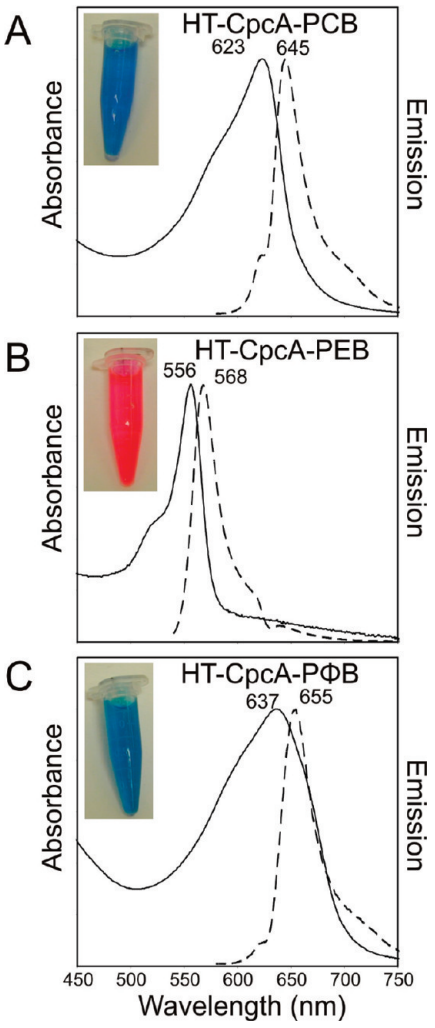


Figure 1. *Synechococcus* 7002 CpcA with PCB, PEB, and PΦB chromophores. Absorption and fluorescence emission spectra of *Synechococcus* 7002 HT-CpcA with the three different 3-ethylidine chromophores along with a color photograph of the purified protein. (A) HT-CpcA-PCB, (B) HT-CpcA-PEB, and (C) HT-CpcA-PΦB.

centrifuged at 13000*g* to remove insoluble protein. Trimeric and hexameric PC fractions were obtained from the reconstituted protein solution by size-exclusion chromatography as described previously.[22] Mass standards (BioRad) and purified hexameric PC from *Synechococcus* 7002 were used to calibrate the column as described.[22]

RESULTS

Heterologous Production of HT-CpcA-PCB in *E. coli*. Two- and three-plasmid systems were used to reconstitute the pathway for $[His]_6$-tagged CpcA (HT-CpcA) synthesis from *Synechocystis* 6803 and *Synechococcus* 7002, respectively, in *E. coli*. To produce *Synechocystis* 6803 HT-CpcA-PCB, *E. coli* was cotransformed with pPcyA, which contained *hox1* and *pcyA*, and pBS414v, which contained *cpcA*, *cpcE*, and *cpcF*. To produce the *Synechococcus* 7002 HT-CpcA-PCB, *E. coli* was cotransformed with pETduet7002cpcA, which contained *cpcA*, pCOLAduet7002cpcEF, which contained *cpcE* and *cpcF*, and pPcyA. Cultures of these strains became blue-green after induction with IPTG and incubation at 18 °C for 5−18 h. The resulting *E. coli* cell pellets were intensely blue-pigmented after removal of the medium by centrifugation (Figure S1). *Synechocystis* 6803 HT-CpcA-PCB (i.e., HT-CpcA carrying a PCB chromophore) had an absorption maximum at 625 nm, a fluorescence emission maximum at 646 nm, and a fluorescence quantum yield of 0.39 (Table 2). Recombinant *Synechococcus* 7002 HT-CpcA-PCB was very similar to an absorption maximum at 623 nm, a fluorescence emission maximum at 645 nm, and a fluorescence quantum yield of 0.31 (Table 2). The absorption and fluorescence emission properties of these proteins were similar to those reported for other PC α subunits.[33,46] These results showed that, with both the two-plasmid and three-plasmid systems, Hox1 and PcyA produced PCB from endogenously synthesized heme and that the CpcE/CpcF lyase attached the synthesized PCB to apo-HT-CpcA, forming HT-CpcA-PCB (i.e., holo-HT-CpcA).

Production of HT-CpcA-PEB Using the Alternative FDBR, Phycoerythrobilin Synthase (PebS). To determine if the CpcE/CpcF lyase could attach PEB to Cys82 of CpcA, *E. coli* cells were cotransformed as above but with pPebS, encoding *hox1* and *pebS* (Table 1), in place of pPcyA. After IPTG induction and overnight incubation at 18 °C, these cultures turned pink and the *E. coli* cells became intensely red (Figure S1). After affinity purification of the recombinant HT-CpcA-PEB variants, absorption and fluorescence emission spectra were recorded to determine the effects of the coexpression of the alternative bilin synthesis enzyme (Figure 1B). The results showed that the CpcE/CpcF lyase attached PEB to apo-HT-CpcA. HT-CpcA-PEB had spectroscopic properties similar to those of naturally occurring phycoerythrins; the *Synechocystis* 6803 and *Synechococcus* 7002 HT-CpcA-PEB variants had an absorption maximum at 556 nm and a fluorescence emission maximum at 568 nm. These proteins were intensely fluorescent (see Figure 1B) and had quantum yields of 0.94 and 0.98 for the *Synechococcus* 7002 and *Synechocystis* 6803 CpcA variants, respectively (Table 2).

Production of HT-CpcA-PΦB Using the Alternative FDBR, Phytochromobilin Synthase (HY2). To determine if CpcE/CpcF was also capable of ligating PΦB to Cys82 of apo-HT-CpcA, *E. coli* cells were cotransformed with the same set of lyase and apoprotein plasmids as described above, but with pHY2, which encodes both *hox1* and HY2 and directs the synthesis of PΦB, in place of pPcyA or pPebS.[8] After induction and purification of the resulting HT-CpcA from the highly pigmented, greenish-blue cells (Figure S1), the absorption and fluorescence spectra of the purified proteins were recorded (Figure 1C and Table 2). The two HT-CpcA-PΦB variants had absorption maxima at 637 nm, fluorescence emission maxima at 655−656 nm, and quantum yields of 0.14−0.18 (Table 2). The absorption and fluorescence emission maxima of the two HT-CpcA-PΦB variants were red-shifted (relative to HT-CpcA-PCB) by ∼10 nm because of the additional conjugated double bond contributed by the vinyl substituent on the D-ring of PΦB (Figure 1A,C and Table 2). The fluorescence quantum yields of the HT-CpcA-PΦB variants were much lower than for HT-CpcA-PCB.

Coexpression of CpcA with the Isomerizing PecE/PecF Lyase Resulted in Attachment of Alternative Chromophores to CpcA. The results described above showed that the CpcE/CpcF lyase could attach PCB, PEB, and PΦB chromophores to HT-CpcA. These observations suggested that apo-HT-CpcA has a bilin-binding site that could accommodate a variety of linear

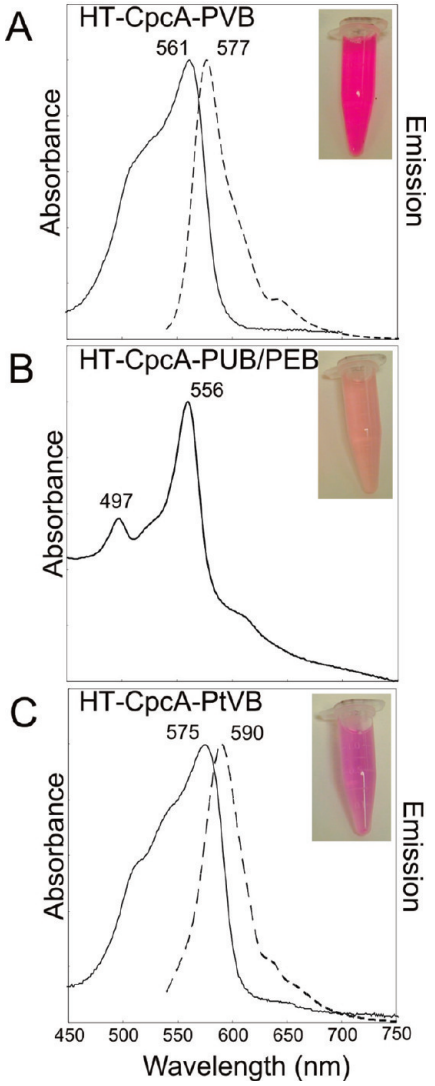


Figure 2. *Synechocystis* 6803 HT-CpcA with PVB, PUB, and PtVB chromophores. Absorption spectra and fluorescence emission spectra of *Synechocystis* 6803 HT-CpcA with the three different 3-vinyl chromophores along with a color photograph of the purified protein. (A) HT-CpcA-PVB, (B) HT-CpcA-PUB, and (C) HT-CpcA-PtVB.

tetrapyrroles with different numbers of conjugated double bonds. In order to determine whether CpcA could bind other bilin chromophores, HT-CpcA was coproduced with an isomerizing lyase, PecE/PecF from *Nostoc* sp. PCC 7120. Variant forms of HT-CpcA from *Synechocystis* 6803 were synthesized from *E. coli* cells cotransformed with pBS405vpecEF and pPcyA while variant forms of HT-CpcA from *Synechococcus* 7002 were synthesized from *E. coli* cells cotransformed with pETduet-CpcA, pCOLAduet-pecEF, and pPcyA. The *E. coli* cells producing these combinations of proteins were an intense, reddish-violet color after IPTG induction and overnight incubation at 18 °C (Figure S1). The PecE/PecF isomerizing lyase produced PVB from PCB and attached this isomerized chromophore to HT-CpcA (Figure 2A). The resulting HT-CpcA-PVB variants had an absorbance maximum at 561 nm and a fluorescence emission maximum at 577−578 nm (Figure 2A and Table 2). The fluorescence quantum yields were only 0.14 and 0.18 for the proteins from *Synechocystis* 6803 and *Synechococcus* 7002, respectively (Table 2). These low fluorescence quantum yield values were probably due to light-induced isomerization of the chromophore (see below). These properties of HT-CpcA-PVB were very similar to those of native holo-PecA (phycoerythrocyanin α subunits) synthesized in cyanobacteria.[47]

To determine if the PecE/PecF lyase could similarly isomerize PEB to PUB and attach it to CpcA, *cpcA*, *pecE*, *pecF*, *hox1*, and *pebS* were coexpressed in *E. coli* cells that were cotransformed with pPebS and pBS405vpecEF to produce the CpcA variant from *Synechocystis* 6803 or with pPebS, pETduet-cpcA, and pCOLAduetpecEF to produce the CpcA variant from *Synechococcus* 7002 (see Table 1). For the *Synechocystis* 6803 CpcA variant, the resulting *E. coli* cells were a pale yellow-orange color, but the cells expressing the *Synechococcus* 7002 *cpcA* gene were similar in color to *E. coli* control cells. Suggesting that a mixture of products was produced, the purified HT-CpcA product from cells producing the *Synechocystis* 6803 CpcA variant had absorption maxima at 497 nm and at 560 nm. The former value is characteristic of PUB while the latter is similar to that observed for PEB (see above and Table 2). Thus, the absorption spectrum suggested that both HT-CpcA-PUB and HT-CpcA-PEB were produced from the cells producing the *Synechocystis* 6803 CpcA variant. No chromophorylated product was observed in cells expected to produce the equivalent *Synechococcus* 7002 CpcA variant.

Lastly, HY2 was coexpressed with *hox1*, *cpcA*, *pecE*, and *pecF* to determine if the Δ5-to-Δ2 double-bond isomer of PΦB could be produced and attached to HT-CpcA by the PecE/PecF lyase. To produce the *Synechocystis* 6803 CpcA variant, *E. coli* cells were cotransformed with pHY2 and pBS405vpecEF; to produce the *Synechococcus* 7002 CpcA variant, *E. coli* cells were cotransformed with pHY2, pETduet-cpcA, and pCOLAduetpecEF. After IPTG induction and overnight incubation at 18 °C, the cells producing the *Synechocystis* 6803 HT-CpcA variant were a pale violet color, while cells expressing *Synechococcus* 7002 *cpcA* resembled *E. coli* control cells (i.e., were not pigmented). The holo-HT-CpcA isolated from the cells expressing the *Synechocystis* 6803 *cpcA* gene had an absorption maximum at 575 nm, a fluorescence emission maximum at 590 nm, and a fluorescence quantum yield of 0.23. These characteristics as well as those for the chromophore after denaturation of the protein in acidic urea (see below) were consistent with this chromophore being the Δ5-to-Δ2 double-bond isomer of PΦB. This is a novel linear tetrapyrrole chromophore, which has been named phytoviolobilin (PtVB); this bilin has not yet been described from natural sources. PtVB is probably the same chromophore that was attached to PecA by PecE/PecF when PΦB was provided as a substrate *in vitro;* the resulting holoprotein had a similar absorbance maximum at 577 nm.[48] Although a detailed structural characterization of the bound chromophore was not made, the properties of this holoprotein were consistent with structural assignment made in Table 2 and were clearly different from the properties of all of the other well-characterized chromophores (see Table 2).

All Alternative Chromophores Are Covalently Attached to HT-CpcA. In order to verify that the bilins produced in *E. coli* were covalently bound to HT-CpcA, each of the purified proteins was subjected to analytical SDS-PAGE. After electrophoresis, gels were first soaked in $ZnCl_2$ to enhance the bilin fluorescence and to visualize whether a bilin was covalently bound to the polypeptide. The gels were scanned using a 532 nm excitation laser. As shown in Figure 3A, all CpcA variants were fluorescent, and thus the results showed that a bilin chromophore was

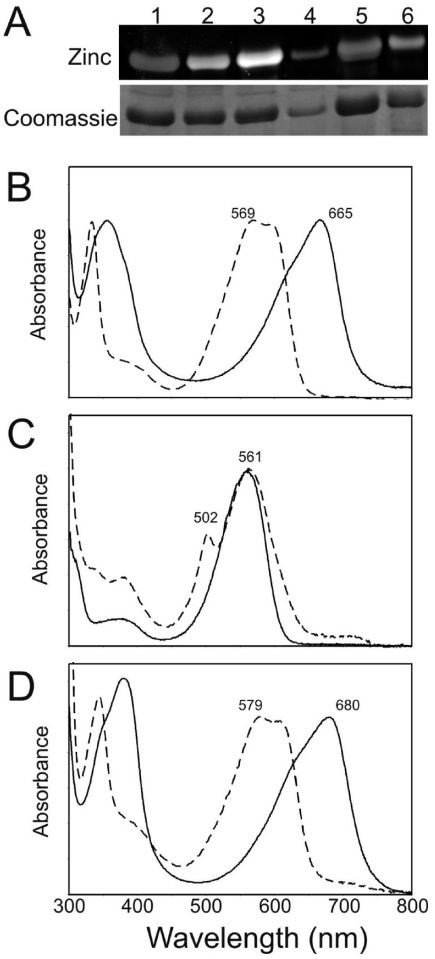


Figure 3. Chromophore analysis of each form of *Synechocystis* 6803 HT-CpcA. (A) Images of zinc-stained (top) and Coomassie blue-stained (bottom) polyacrylamide gel containing each form of HT-CpcA. Lane 1: HT-CpcA-PCB; lane 2: HT-CpcA-PVB; lane 3: HT-CpcA-PEB; lane 4: -HT-Cpc-PUB/PEB; lane 5: HT-CpcA-PΦB; lane 6: HT-CpcA-PtVB. (B) Absorption spectra of HT-CpcA-PCB (solid line) and HT-CpcA-PVB (dashed line) denatured in acidic urea. (C) Absorption spectra of HT-CpcA-PEB (solid line) and HT-CpcA-PUB/PEB (dashed line) denatured in acidic urea. (D) Absorption spectra of HT-CpcA-PΦB (solid line) and HT-CpcA-PtVB (dashed line) denatured in acidic urea.

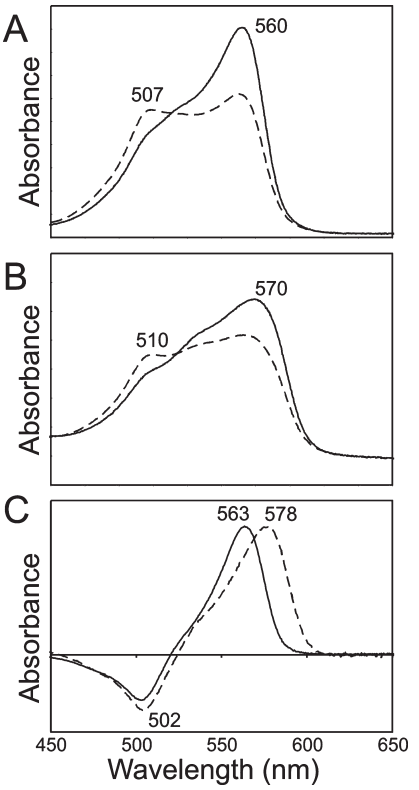


Figure 4. Demonstration of photochemistry of *Synechocystis* 6803 HT-CpcA-PVB and HT-CpcA-PtVB. Absorbance profile of HT-CpcA-PVB (A) and HT-CpcA-PtVB (B) before (solid line) and after (dashed line) treatment with saturating light at its pretreatment absorption maximum and the difference spectra (C) of the two forms of HT-CpcA-PVB (solid line) and HT-CpcA-PtVB (dashed line).

covalently attached to the HT-CpcA variant for each chromophore and lyase combination.

The absorption spectrum of each protein was additionally recorded after the protein had been denatured in acidic 8.0 M urea. This allows the spectral properties of the attached chromophore to be determined without interference from the protein. Representative results are shown for each of the bilin chromophores in Figure 3B−D; in each panel, the solid line shows the product obtained with the CpcE/CpcF lyase and the dashed line shows that obtained with the PecE/PecF lyase. When PcyA was the FDBR and CpcE/CpcF was the lyase, the denatured protein had an absorption maximum at 665 nm, which indicated the attached bilin was PCB.[49,50] Similarly, when PcyA was the FDBR and the lyase was PecE/PecF, the denatured product had an absorption maximum at 569 nm with a strong shoulder at 597 nm. This spectrum showed that the attached bilin was PVB,[47] and from the ratio of the absorption peaks, the chromophore was predominantly in the 15*Z* configuration.[51,52] The spectra from samples produced using PebS as the FDBR are shown in Figure 3C. When CpcE/CpcF was used as the lyase, the denatured protein had an absorbance maximum at 561 nm characteristic of PEB.[50,53] However, when PecE/PecF was the lyase, the product had a major absorption maximum at 561 nm and a minor peak at 502 nm, characteristic of PEB and PUB, respectively.[50] The spectra of products generated with HY2 as FDBR are shown in Figure 3D. The spectrum of the PΦB product, produced with HY2 and CpcE/CpcF, was similar to that of PCB, but the maximum was red-shifted 15 nm to 680 nm.[31] The product produced with HY2 and PecE/PecF had a spectrum that closely resembled that of PVB, but the maxima were red-shifted to 579 and 609 nm. As discussed above, these properties are consistent with this variant carrying the Δ5-to-Δ2 double-bond isomer of PΦB, PtVB. By making the reasonable assumption that the spectroscopic properties of PtVB are similar to those of PVB,[51,52] the ratio of the absorption peaks indicated that the chromophore was predominantly in the 15*Z* configuration.

CpcA with PVB or PtVB Is Photochemically Active. Naturally produced PecA (i.e., the α subunit of phycoerythrocyanin) undergoes fully reversible photochemistry similar to phytochrome-type photoreceptors.[5] In order to determine if HT-CpcA-PVB, HT-CpcA-PΦB, and HT-CpcA-PtVB were similarly photoactive, samples of these proteins were exposed to monochromatic light at their absorbance maxima for up to 2 h. Illumination of the HT-CpcA-PVB sample with 560 nm light

induced a change in the absorption spectrum that caused a loss of absorption at 560 nm and a simultaneous increase in absorption at 507 nm (Figure 4A). Similarly, illumination of the HT-CpcA-PtVB at its absorption maximum (570 nm) caused a loss of absorption at 570 nm and an increase in absorption at 510 nm (Figure 4B). The difference spectra for these samples are shown in Figure 4C. The observed changes could be fully reversed by illumination of the sample with light at the lower absorption maximum. Under the conditions employed, no light-induced absorption changes were observed for HT-CpcA-PΦB (data not shown).

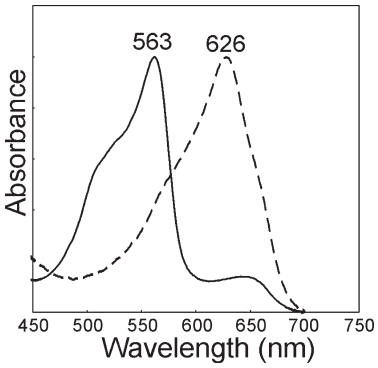


Figure 5. *Synechocystis* 6803 CpcA with PcyA and chimeric lyases. Absorbance spectra of isolated proteins from *E. coli* strains expressing *hox1* and *pcyA* together with either *cpcA*, *pecE*, and *cpcF* (dashed line) or *cpcA*, *cpcE*, and *pecF* (solid line).

Chimeric Lyases Show That the Isomerization Activity Is Associated with PecF. The CpcE/CpcF and PecE/PecF lyases are clearly related by a gene duplication event,[40] but only the latter is capable of both isomerizing and attaching chromophores to PecA or CpcA. To determine whether the isomerization activity is associated with the PecE or PecF subunit of the lyase, two chimeric lyases, PecE/CpcF and CpcE/PecF, were recombinantly produced in *E. coli* cells together with apo-HT-CpcA and PCB. Although the yields of chromophorylated HT-CpcA were much lower with these chimeric lyases than with the CpcE/CpcF and PecE/PecF lyase combinations, sufficient bilin attachment occurred to answer the isomerization question definitively. The chimeric lyase PecE/CpcF produced a holo-HT-CpcA

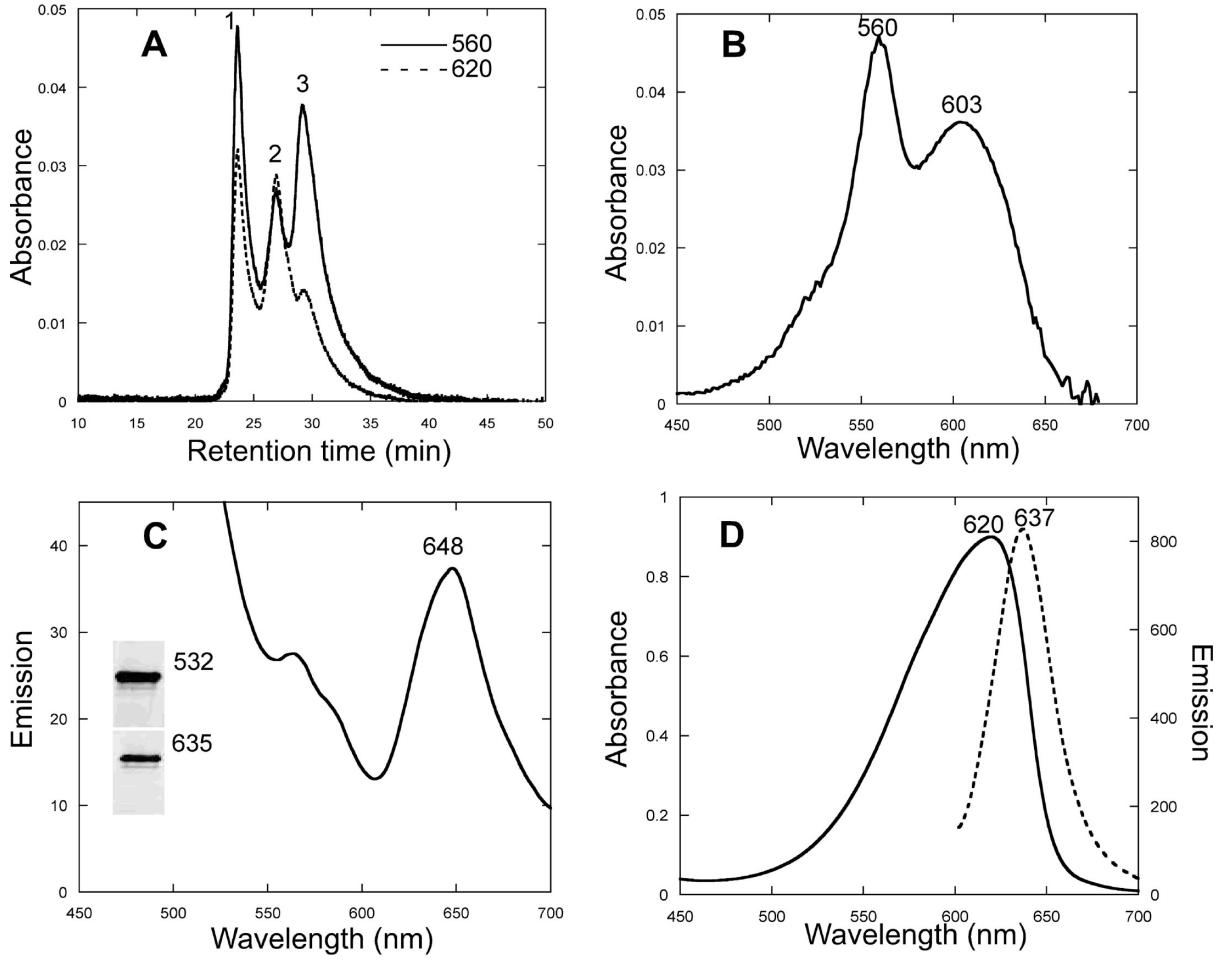


Figure 6. Characteristics of reconstituted R-PCIII with HT-CpcA-PEB and holo-CpcB from *Synechococcus* 7002. (A) Representative chromatogram for the size-exclusion HPLC of PC that had been refolded and reconstituted in vitro from HT-CpcA-PEB and holo-CpcB. The chromatograms at 560 nm (solid line) and 620 nm (dashed line) are shown, and numbered peaks were collected for other analyses. (B) Absorbance spectrum of the reconstituted hexameric PC collected from peak 1. (C) Fluorescence emission spectrum of the reconstituted hexameric PC from peak 1 after excitation at 520 nm. The inset showing Zn-enhanced fluorescence after SDS-PAGE of the reconstituted hexameric PC complexes collected from peak 1 after excitation at 535 nm and 635 nm. The HTCpcA-PEB and holo-CpcB migrated to the same position on the gel. (D) Absorbance (solid line) and fluorescence emission (dashed line) for native, hexameric PC obtained from *Synechococcus* 7002.

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Table 3. Retention Times and Calculated Molecular Masses of Reconstituted PC Complexes Produced from HT-CpcA-PEB and holo-CpcB Subjected to Size-Exclusion Chromatography by HPLC

peaks	retention time (min)	calcd molecular mass (kDa)
1	23.6[a]	283.2
2	26.8	103.2
3	29.2	39.2

[a] Hexameric PC purified from *Synechococcus* 7002 had a retention time of 23.7 min with a calculated molecular mass of 277.3 kDa.

product with an absorption spectrum nearly identical to that produced with CpcE/CpcF (Figure 5); this result showed that the HT-CpcA carried a PCB chromophore that had not been isomerized. The HT-CpcA produced in cells with the chimeric lyase CpcE/PecF had an absorption spectrum very similar to that produced by PecE/PecF (Figure 5). This indicated that HT-CpcA carried a PVB chromophore, which was produced by isomerization of PCB. Thus, PecF was responsible for the isomerization activity associated with the PecE/PecF lyase. When only a single subunit of these heterodimeric lyases was coproduced with HT-CpcA, no lyase activity was observed (data not shown).

Reconstitution and Analysis of Hexameric PC from HT-CpcA-PEB and Holo-CpcB from *Synechococcus* 7002. HT-CpcA-PEB purified from *E. coli* cells expressing the *Synechococcus* 7002 *cpcA* and holo-CpcB (i.e., the β subunit of PC), purified directly from *Synechococcus* 7002, were isolated, refolded from purified denatured subunits, and reconstituted using equal molar quantities of each protein subunit. The refolded and reconstituted product sample was subjected to size-exclusion HPLC, and the resulting chromatogram is shown in Figure 6A. The reconstituted PC eluted as three peaks, which indicated that the sample contained a mixture of different complexes (labeled 1−3 in Figure 6A). Standard proteins were used to estimate the molecular masses of the three complexes as described previously.[45] Holo-PC isolated from *Synechococcus* 7002 was also analyzed as a control; this complex eluted at 23.7 min, a retention time consistent with the hexameric aggregation state.[41,54] The calculated masses for the complexes in Figure 6A are consistent with the following assignments: peak 1, hexameric PC; peak 2, trimeric PC; and peak 3, monomers, dimers, or protomers of HT-CpcA-PEB or CpcB (see Table 3). The estimated mass of the reconstituted hexameric PC, $(CpcA\text{-}PEB/CpcB)_6$ (peak 1, retention time 23.6 min, 283 kDa), was virtually identical to that for PC isolated from native cells (with a retention time of 23.7 min and a calculated mass of 277.3 kDa).

The recombinant hexameric PC $(CpcA\text{-}PEB/CpcB)_6$ collected from peak 1 was further characterized by SDS-polyacrylamide gel electrophoresis. The gel was incubated with $ZnCl_2$ prior to examining the fluorescence of bilins after excitation at 532 nm (Figure 6C) and 635 nm (Figure 6D). The HT-CpcA-PEB subunit migrated to the same position on the gel as the holo-CpcB subunit, but a 635 nm laser preferentially excites PCB, whereas a 532 nm laser excites both PEB and PCB. The samples collected from peaks 1 and 2 contained both subunits. The absorbance of the recombinant PC $(HT\text{-}CpcA\text{-}PEB/CpcB)_6$ from peak one is shown in Figure 6B, and the fluorescence emission spectrum is shown in Figure 6C. The recombinant $(CpcA\text{-}PEB/CpcB)_6$ had absorbance maxima at 560 and 603 nm (Figure 6B), and the fluorescence emission spectrum showed a major emission peak at 648 nm after excitation at 520 nm (Figure 6C). These data indicate that energy transfer occurred from the PEB chromophore on HT-CpcA to the PCB chromophores present on the CpcB subunits. The fluorescence emission maximum of $(CpcA\text{-}PEB/CpcB)_6$ was red-shifted about 11 nm relative to the emission maximum of native, hexameric PC (Figure 6D).

DISCUSSION

Besides their cognate PCB chromophore, the CpcA apoproteins studied could accept five alternative bilin chromophores from the CpcE/CpcF and PecE/PecF lyases. *In vitro* assays previously demonstrated that CpcE/CpcF from *Synechococcus* 7002 has a preference for PCB over PEB in both binding affinity and catalytic rate,[55] but in these heterologous *in vivo* systems, CpcE/CpcF was nevertheless very effective in adding PEB to HT-CpcA. As previously observed with the phytofluors, HT-CpcA carrying the PEB chromophore was also significantly more fluorescent than proteins carrying the cognate chromophore.[16] Our measurements show that these proteins had much higher quantum yields than phytofluors, and thus the proteins described here may be more useful as biological labels. Phytofluors, however, have the advantage of autocatalytic chromophore attachment, and thus they do not require the coexpression of a lyase.[24]

The ready ability to bind different chromophores seems to be primarily a characteristic of the α subunits of PBPs. For example, it has been reported that both PecA and RpcA also have the ability to bind alternative chromophores,[7] and the ApcA subunit of *Synechococcus* 7002 was found to bind a small amount of PEB *in vivo* when PebA and PebB were overproduced in this cyanobacterium.[31] The CpcS/CpcU bilin lyase, which is responsible for attaching PCB to ApcA, ApcB, ApcF, ApcD, and Cys84 of CpcB,[12] showed much lower activity when PEB was provided as the bilin substrate in a heterologous expression system (data not shown). In cyanobacterial cells that naturally synthesize both PCB and PEB, if the CpcS/CpcU or CpeS/CpeU bilin lyase inappropriately attached PEB to CpcB or the various allophycocyanin subunits of the cores of PBS, there would likely be severe consequences on efficient energy transfer and light harvesting. Because the attachment of noncognate bilins to apo-PBPs has not generally been observed in wild-type cyanobacteria, it has generally been assumed that PBP lyases must exhibit a high degree of substrate specificity *in vivo*. However, misincorporation of bilins into PBPs has been achieved artificially *in vitro*[55] and *in vivo*.[7,31,40]

With the exception of the PUB and PtVB samples, coproduction of various phycobilin chromophores and lyases led to excellent yields of chromophorylated HT-CpcA in *E. coli*. It is possible that the minor differences between the two CpcA apoproteins was due more to the different expression systems than to actual differences in the proteins themselves, which are 81% identical over 162 amino acids. The mixed chromophore content and lower yields of chromophorylated CpcA from the strains coexpressing *cpcA*, *pecE*, *pecF*, and *pebS* was surprising. PEB was very efficiently attached to HT-CpcA in the strains coexpressing *cpcA*, *cpcE*, *cpcF*, and *pebS*. However, when the structure of PUB is compared to those of the other phycobilins that could be attached, PUB differs most from the native PCB chromophore because of changes in conjugation at both the A- and D-rings of the

chromophore and by the presence of the 18-vinyl group (Table 2). These changes apparently combine to produce a chromophore that was less efficiently ligated to apo-CpcA. This is likely due to a bias against PUB by CpcA rather than an inability of PecE/PecF lyase to bind PEB because HT-PecA-PUB is efficiently produced in a strain coexpressing *pecA*, *pecE*, and *pecF* along with *hox1* and *pebS* (Figure S2). This combination also resulted in the production of a small amount of HT-PecA-PEB, however (Figure S2).

A previous report suggested that PEB was not a suitable bilin substrate for the PecE/PecF lyase and its cognate substrate PecA *in vitro*.[48] However, the same authors subsequently reported different results when the same components were expressed heterologously in *E. coli*.[7] The results presented here show that the PecE/PecF lyase does not even require the cognate acceptor, PecA (Figure 2). The reasons for these discrepancies are presently unclear. Expression levels, gene/enzyme sources, and perhaps even *E. coli* strain differences may conspire to overcome the apparent substrate specificities previously observed *in vitro* and in cyanobacteria.

PΦB synthesis has not yet been shown to occur naturally in cyanobacteria. In *A. thaliana*, PCB can substitute for PΦB to produce functional phytochromes, and *pcyA* can thus largely complement a HY2 deficient mutant that is unable to synthesize PΦB.[32] Conversely, it has recently been demonstrated that overexpression of HY2 in the cyanobacterium *Synechococcus* 7002 creates a background that permits the inactivation of the otherwise essential *pcyA*.[31] Because PΦB only differs from PCB by the presence of a vinyl group instead of an ethyl group on the D-ring (Table 2), it is perhaps not surprising that these structurally similar chromophores can be used interchangeably. It has been demonstrated here that HT-CpcA-PΦB can readily be synthesized; the product has absorbance and fluorescence emission maxima that are red-shifted by about 10 nm relative to HT-CpcA-PCB (Table 2). Surprisingly, this protein had a very low fluorescence quantum yield relative to other HT-CpcA/bilin combinations, but initial attempts to demonstrate photochemical activity failed. One possible explanation is that HT-CpcA-PΦB is photochemically active, but the back-conversion to the red-absorbing form occurs very rapidly. The methods used here would not have been able to detect such behavior, and thus additional, more detailed investigations into the reasons for the very low fluorescence quantum yield observed for HT-CpcA-PΦB will be required. It is worth noting that, when the PCB chromophores of all PBPs were replaced by PΦB in *Synechococcus* 7002, the resulting PBPs carrying PΦB chromophores transferred energy inefficiently as well.[31]

The PecE/PecF isomerizing lyase had not previously been shown to attach chromophores to the noncognate substrate, HT-CpcA, and neither *Synechococcus* 7002 nor *Synechocystis* 6803 naturally synthesizes PBPs with isomerized chromophores. However, the PecE/PecF lyase was recently shown to use RpcA as an acceptor substrate for the attachment of both PVB and PUB.[7] A previous report on the structure of PecA had suggested that the specificity of chromophore−apoprotein recognition was due to only a few subtle amino acid differences between PecA and CpcA.[56] However, the results presented here demonstrate that these differences alone are insufficient to ensure absolute specificity in chromophore attachment. The PecE/PecF lyase was also capable of attaching the isomerized form of PΦB, PtVB, to HT-CpcA. Because HY2 is only known to occur in plants and PecE/PecF-type isomerases are only known to occur in cyanobacteria, PtVB is a chromophore that has not yet been reported from a natural source. Because the conjugation states of the chromophores are altered in the same manner, it was hypothesized that PtVB would have absorption and fluorescence emission maxima that would be blue-shifted relative to PΦB to a similar degree as the values for PVB compared to PCB (i.e., ca. 66 nm; Table 2), and this was indeed the case.

The finding that HT-CpcA carrying either PVB or PtVB exhibits photochemistry like PecA-PVB is particularly interesting. This photochemistry is due to a light-induced photoisomerization of the chromophore around the 15,16 double bond.[52] It has been somewhat perplexing to explain why this sort of photochemistry does not occur when CpcA (or other PBP subunits) is chromophorylated with either PCB or PΦB because both chromophores have 15,16 double bonds and are clearly capable of light-induced isomerization when bound by phytochrome-like proteins.[57,58] Schmidt et al.[59] compared the structures of PecA from *Mastigocladus laminosus* and CpcA from *Thermosynechococcus vulcanus*. They suggested that small differences between the proteins, including a loop connecting helix 4 and helix 5 in the CpcA, may be responsible for creating a less flexible environment that prohibits light-induced isomerization of the bound PCB.[59] The results presented here suggest that the role of the chromophore is probably more important than the role of the protein because PVB is clearly still capable of photoisomerization even when attached to HT-CpcA. RpcA-PVB synthesized in *E. coli* also showed weak photoisomerization activity;[7] however, CpcB-PVB synthesized *in vitro* reportedly lacked photochemistry.[60] A comparison of the structures of CpcA-PVB and CpcA-PCB using the same apoprotein may provide insights into factors that modulate photoisomerization in PBPs.

It is a relatively recent development that chromophore attachment to PBPs can be studied in heterologous *in vivo* systems. This approach appears to have many advantages over studies that had previously been performed *in vitro*. For example, it is not necessary to cleave the bilins from PBPs by methanolysis and to purify these substrates by preparative HPLC. Furthermore, *in vitro* assays often produced mixed protein populations that carried oxidized bilin products.[61−63] On the basis of the absorption and fluorescence profiles of the proteins produced in this system, it appears that most of these proteins contained uniformly chromophorylated products that carried a single type of covalently bound bilin. The obvious exception to this was HT-CpcA isolated from a strain coproducing *Synechocystis* 6803 CpcA, PEB, and the PecE/PecF lyase, which clearly produced a mixture of HT-CpcA-PUB and HT-CpcA-PEB.

Most studies performed to date on the PecE/PecF lyase/isomerases have been conducted *in vitro*.[11,48,64,65] The results presented here extend our understanding of how this lyase works by showing that it can also utilize CpcA as an acceptor/substrate and that it can isomerize three different bilin substrates: PCB, PEB, and PΦB. Previous studies, which reported a small (6−7%) residual isomerase activity for PecF alone *in vitro* with PecA and PCB as substrates, had suggested that the isomerase activity was associated with the PecF subunit of this isomerizing lyase.[65,66] By forming chimeric lyases, it was unambiguously shown here that CpcE/PecF attached PVB to HT-CpcA, and other chromophorylation products were not produced. Individually, CpcE, CpcF, PecE, or PecF had no detectable lyase activity *in vivo* (data not shown). However, when CpcF was combined with PecE, the resulting chimeric lyase only attached PCB to HT-CpcA, and no PVB was observed. The combination of these results conclusively

established that the isomerization activity of the PecE/PecF lyase is associated with the PecF subunit.

The *in vitro* reconstitution of PC trimers and hexamers from HT-CpcA-PEB and native holo-CpcB effectively created the unique form of PC termed R-PCIII. R-PCIII is a type of PC with a PCB:PEB ratio of 2:1 and in which PCB occupies the two chromophorylation sites on CpcB and PEB the single site on CpcA. This form of PC was reported to occur in *Synechococcus* sp. strain WH7805 (and may also occur in *Synechococcus* sp. RCC307[67]). R-PCIII differs from R-PCI and R-PCII, which also carry PEB chromophores. R-PCI also has a PCB:PEB ratio of 2:1, with PEB only occurring on the β-155 Cys residue.[50] In R-PCII, the PCB:PEB ratio is 1:2, and PEB occurs on both Cys-82 of the α subunit and Cys-155 of the β subunit.[68,69] Although the absorption spectrum for R-PCIII has not been reported, it was described as having an absorption maximum at 555 nm and a shoulder at 590 nm.[68] This is quite different from native R-PCI, which has a major absorption peak at 618 nm and a lesser one at 555 nm.[50,68] The reconstituted R-PCIII hexamers described here had absorption maxima at 560 and 603 nm (Figure 6B). These values are more red-shifted than those reported for the PC of *Synechococcus* sp. strain WH7805. This could obviously be due to differences in the protein environments surrounding the chromophores.

In conclusion, the studies reported here provide new insights into the binding specificity, and the lack thereof, exhibited by apo-PBPs and PBP lyases for bilin chromophores. By combining bilin biosynthetic enzymes, isomerizing and nonisomerizing lyases, and an appropriate apo-PBP subunit in various combinations, it has been possible to create a suite of fluorescent proteins—all based on a single polypeptide, CpcA. The resulting proteins are stable, are easily purified, and efficiently absorb light over a wavelength span of ~140 nm, and some of them have very high fluorescence quantum yields. Preliminary studies have shown that CpcA can still be chromophorylated when this domain is added to other proteins produced recombinantly (data not shown). Thus, the protein/chromophore combinations described here could have biotechnological applications, which could include facile and highly sensitive detection of recombinant proteins and other analytes.

■ ASSOCIATED CONTENT

Supporting Information. Photographs of cell pellets of *E. coli* that have produced each of the six chromophorylated forms of HT-CpcA mentioned in this study (Figure 1); *Nostoc* sp. PCC 7120 HT-PecA-PUB produced with PecE/PecF, Hox1, and PebS (Figure 2); primers used in this study (Table 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

HPLC, high-performance liquid chromatography; HT, histidine-tagged; PAGE, polyacrylamide gel electrophoresis; PBP(s), phycobiliprotein(s); PBS, phycobilisome(s); PC, phycocyanin; PCB, phycocyanobilin; PCR, polymerase chain reaction; PEB, phycoerythrobilin; PS, photosystem; PtVB, phytoviolobilin; PUB, phycourobilin; PVB, phycoviolobilin; PΦB, phytochromobilin; SDS, sodium dodecyl sulfate.

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