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Biosynthesis of Cyanobacterial Phycobiliproteins in *Escherichia coli*: Chromophorylation Efficiency and Specificity of All Bilin Lyases from *Synechococcus* sp. Strain PCC 7002▽†

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Phycobiliproteins are water-soluble, light-harvesting proteins that are highly fluorescent due to linear tetrapyrrole chromophores, which makes them valuable as probes. Enzymes called bilin lyases usually attach these bilin chromophores to specific cysteine residues within the alpha and beta subunits via thioether linkages. A multiplasmid coexpression system was used to recreate the biosynthetic pathway for phycobiliproteins from the cyanobacterium *Synechococcus* sp. strain PCC 7002 in *Escherichia coli*. This system efficiently produced chromophorylated allophycocyanin (ApcA/ApcB) and α-phycocyanin with holoprotein yields ranging from 3 to 12 mg liter^{-1} of culture. This heterologous expression system was used to demonstrate that the CpcS-I and CpcU proteins are both required to attach phycocyanobilin (PCB) to allophycocyanin subunits ApcD ($\alpha^{AP\text{-}B}$) and ApcF (β^{18}). The N-terminal, allophycocyanin-like domain of ApcE (L_{CM}^{99}) was produced in soluble form and was shown to have intrinsic bilin lyase activity. Lastly, this *in vivo* system was used to evaluate the efficiency of the bilin lyases for production of β-phycocyanin.

Cyanobacteria and red algae obtain their characteristic colors from a variety of pigments, including chlorophylls and carotenoids associated with the transmembrane photosynthetic reaction centers, as well as the abundant phycobiliproteins (PBPs), which are the main components of their light-harvesting complexes, which are called phycobilisomes (PBS) (12, 27, 64). PBPs are brilliantly colored due to the presence of one to three linear tetrapyrrole prosthetic groups, called bilins, attached to their α and β subunits (28). Cyanobacteria naturally synthesize four different bilins, the most common of which are phycocyanobilin (PCB) and phycoerythrobilin; these bilins are attached to PBPs at Cys residues via thioether bonds (27, 54). Enzymes called bilin lyases are responsible for catalyzing the attachment of a bilin to the appropriate Cys residue for most PBPs (20, 46, 49, 52, 53, 72, 73, 78). The α-phycocyanin (CpcA) PCB lyase is a CpcE/CpcF heterodimer (8, 19, 20, 58, 78). Orthologs of genes encoding these proteins occur in the genomes of all cyanobacteria that synthesize phycocyanin (PC). Paralogs (e.g., *pecE*/*pecF* and *cpeY*/*cpeZ*) occur in the genomes of many cyanobacteria (note that not all *Prochlorococcus* sp. strains contain PBPs), and colocalization of these genes in operons encoding different PBPs (55) and some functional analyses have suggested that these proteins are also bilin lyases that act on different substrates (9, 34, 56, 73). This family of bilin lyases has been called the E/F family. A second family of bilin lyases was recently discovered and was called the T family (52). CpcT attaches PCB to Cys-153 on β-PC (CpcB), forming the 3^1-*S*-Cys-PCB isomer (10, 52, 76). The third group of bilin lyases is called the S/U family. This type of bilin lyase is composed of a heterodimer of two similar proteins designated CpcS-I and CpcU (46, 53) in some cyanobacteria, while in other cyanobacteria the active protein comprises a single type of subunit (designated CpcS-III, CpcS, or CpeS) (75). Some members of the S/U bilin lyase family attach PCB to Cys-82 on β-PC and to the equivalent Cys residues on allophycocyanin (AP) α and β subunits; other members attach phycoerythrobilin to Cys-82 on phycoerythrin (PE) subunits (48, 49, 63, 72, 75, 76). For the last known family of bilin lyases, the lyase reaction is catalyzed by the biliprotein itself. For example, plants, cyanobacteria, and other bacteria have phytochromes, phytochrome-like proteins, or cyanochromes, which act as bilin lyases and autoligate their bilin chromophores (37, 42, 44, 66, 67, 74). The AP-like domain of the large core membrane linker protein designated L_{CM} or ApcE contains a PCB, has a red-shifted absorbance maximum at ~665 nm, and plays a role in accepting the energy from the chromophores in the core of the PBS and transferring it to the reaction centers (1, 13, 26, 54). Autocatalytic addition of PCB was reported to occur for a truncation product of ApcE in 4 M urea (74). As it has been shown that addition of detergents can eliminate the requirement for bilin lyases for some phycobiliproteins (74), it is possible that the urea present in the reaction mixture with ApcE may have had the same effect as a detergent.

While chromophore addition is the one type of posttranslational modification that all PBPs undergo, a second type of modification is a methylation reaction that occurs specifically on the β subunits of most phycobiliproteins and is catalyzed by an *S*-adenosylmethionine-dependent methyltransferase designated CpcM (41, 51, 57). The methyltransferase reaction pro-

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TABLE 1. Plasmids used in this study

Plasmid	Recombinant protein(s) produced[a]	Parent vector	Antibiotic resistance[b]	Reference
pApcAB	*Synechococcus* sp. PCC 7002 HT-ApcA and ApcB	pET100	Ap	52
pApcDB	*Synechococcus* sp. PCC 7002 HT-ApcD and ApcB	pET100	Ap	41
pApcD	*Synechococcus* sp. PCC 7002 HT-ApcD	pET100	Ap	41
pApcF	*Synechococcus* sp. PCC 7002 HT-ApcF	pET100	Ap	41
pPcyA	*Synechocystis* sp. PCC 6803 Ho1 and *Synechococcus* sp. PCC 7002 HT-PcyA	pACYC Duet	Cm	This study
pCpcUS	*Synechococcus* sp. PCC 7002 CpcU and CpcS coexpressed on one mRNA	pCOLA Duet	Km	This study
pCpcU	*Synechococcus* sp. PCC 7002 CpcU	pCOLA Duet	Km	This study
pCpcS	*Synechococcus* sp. PCC 7002 CpcS	pCOLA Duet	Km	This study
pCpcT	*Synechococcus* sp. PCC 7002 CpcT	pCOLA Duet	Km	This study
pGST-ApcE	*Synechococcus* sp. PCC 7002 GST-ApcE (amino acids 1 to 228)	pGEX-2T	Ap	This study
pCpcBA	*Synechocystis* sp. PCC 6803 HT-CpcB and CpcA	pCDF Duet	Sp	This study
pCpcB	*Synechocystis* sp. PCC 6803 HT-CpcB	pBS150v	Sp	This study
pBS414v	*Synechocystis* sp. PCC 6803 HT-CpcA, CpcE, and CpcF	pBS350v	Sp	61
pBS415v	*Synechocystis* sp. PCC 6803 CpcE and CpcF	pBS350v	Sp	This study
pBS405v	*Synechocystis* sp. PCC 6803 HT-CpcA	pBS350v	Sp	61
pAT101	*Synechocystis* sp. PCC 6803 HO1 and PcyA	pBS350v	Km	61

[a] Proteins that were produced as fusions are indicated by using the prefixes HT- and GST-.
[b] Antibiotic resistance was used to select for the presence of the plasmid. Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sp, spectinomycin.

duces a highly conserved γ-*N*-methylasparagine residue at the β-72 position of almost all β-subunits isolated from cyanobacteria, red algae, and cryptomonads (17, 35, 36, 45, 65). This modification is thought to change the environment of the chromophore at position β-82 to minimize the rates of nonradiative energy loss within PBS (59, 60). However, characterization of *cpcM* mutants also showed that these strains contain very high levels of reactive oxygen species (51).

Using a combination of reverse genetics and *in vitro* biochemical analyses of recombinant proteins, the enzymes required for biogenesis of the most abundant PBPs, PC (CpcB/CpcA) and AP (ApcA/ApcB), in *Synechococcus* sp. strain PCC 7002 have been identified and characterized (41, 46, 52, 53, 78). However, the enzymes responsible for PCB addition to the two AP subunits, ApcD and ApcF, and the AP-like domain of the large core membrane linker protein, ApcE, from this organism have not been determined yet. Although low copy numbers are present in PBS, these minor biliproteins are critically important for energy transfer from the PBS to reaction centers (5, 24, 26, 38, 40, 71). In this study, a heterologous, *in vivo* biosynthetic system using *Escherichia coli* was established to study the synthesis of various holo-PBP subunits (ApcA/ApcB, ApcD/ApcB, ApcF, and ApcE) and to create partially chromophorylated subunits of PC (CpcB). It is shown here that all AP subunits except ApcE (ApcA, ApcB, ApcD, and ApcF) require the heterodimeric CpcS-I/CpcU bilin lyase for correct PCB attachment and that this system is highly efficient for making holo-AP. The AP domain of ApcE (L_{CM}^{99}), when fused to glutathione *S*-transferase (GST), was soluble in *E. coli* and was the only PBP from *Synechococcus* sp. strain PCC 7002 with intrinsic bilin lyase activity for correct attachment of PCB. Additionally, the specificity of the E/F-type and T-type lyases in this *in vivo* system was tested, and it is demonstrated here that these lyases do not attach PCB to any *Synechococcus* sp. strain PCC 7002 PBPs except their previously established substrates. Therefore, all of the enzymes required for the post-translational modification of all PBPs from the cyanobacterium *Synechococcus* sp. strain PCC 7002 have now been identified and characterized using this *in vivo* heterologous system. In addition, this *in vivo* system can be used to create large amounts of these fluorescent phycobiliproteins for bioimaging purposes.

MATERIALS AND METHODS

Construction of expression vectors. Plasmids used in this study are listed in Table 1. Some of the expression vectors used in this study were previously described (41, 52, 61). All expression constructs newly produced for this study were sequenced at the W. M. Keck Conservation and Molecular Genetics Laboratory (University of New Orleans) to confirm that no mutations had been introduced during PCR amplification and cloning.

(i) *cpcS-I* and *cpcU* expression construct. The *cpcS-I* and *cpcU* genes were cloned in the pCOLA Duet vector (Novagen, Madison, WI) to generate plasmid pCpcUS, from which these genes were cotranscribed to produce hexahistidine-tagged CpcU (HT-CpcU) and CpcS (Table 1). The *cpcS-I* gene (SYNPCC7002_A1822) was amplified by PCR from *Synechococcus* sp. strain PCC 7002 chromosomal DNA using forward primer cpcSF (5′-TCCCTGCAG AAGGAGATTTCGATATGCAAAGCTTTGC-3′) and reverse primer cpcSR (5′-ACGGTCGACCTACCAACCGCTAATAGCGTAAAG-3′) and cloned into pCOLA Duet after digestion with PstI and SalI (the restriction sites in the primers are underlined, and the forward primer contained a ribosome binding site) to create plasmid pCpcS. The *cpcU* gene (SYNPCC7002_A2053) was amplified by PCR from *Synechococcus* sp. strain PCC 7002 chromosomal DNA using primers cpcUF (5′-AGGGGATCCTATGGATATCAATGCCTTTATC-3′) and cpcUR (5′-GCCGAATTCTTAGTTACTGGCTTCAGCGGTTAC-3′) and cloned into the pCOLA Duet plasmid using restriction enzymes BamHI and EcoRI (restriction sites in the primers are underlined) to create the pCpcU plasmid. The *cpcU* gene was subcloned into the pCpcS plasmid using BamHI and EcoRI to create plasmid pCpcUS.

(ii) *cpcT* expression construct. The *cpcT* gene (SYNPCC7002_A1822) was amplified from *Synechococcus* sp. strain PCC 7002 chromosomal DNA using primers cpcTF (5′-CTCGCTTACATATGTCCCACTCTACCGATGCCCATAC-3′) and cpcTR (5′-TTCTCGAGTTAATGGGGTTGAACTTCCCCAGAGAAA TT-3′) and cloned into pCOLA Duet after digestion with NdeI and EcoRV (restriction sites in the primers are underlined) to create plasmid pCpcT.

(iii) *pcyA*/*ho1* expression construct. The heme oxygenase 1 gene, *ho1* (sll1184), was amplified by PCR from the chromosomal DNA of *Synechocystis* sp. strain PCC 6803 using primers hox1F (5′-GGTATTTTTCATATGAGTGTCAACTT AGCTTCC-3′) and hox1R (5′-AGATATCCTAGCCTTCGGAGGTGGCGA G-3′) and cloned into pACYC Duet (Novagen, Madison, WI) using NdeI and EcoRV (restriction sites in the primers are underlined). The 3*Z*-phycocyanobi-

TABLE 2. Properties of recombinant holo-PBPs

Recombinant holo-PBP (plasmids present)	Visible λ_{max}/UV λ_{max} (nm)	Visible/UV ratio	% Chromophorylation[a]
HT-CpcA (pBS414v, pPcyA)	625/370	4.64	48.1
HT-CpcA (pBS414v, pAT101)	625/370	4.5	22.4
HT-ApcA/ApcB (pApcAB, pCpcUS, pPcyA)	615/357	2.53	71.9
HT-ApcD (pApcDB, pCpcUS, pPcyA)	672/370	2.21	ND[b]
HT-ApcD (pApcD, pCpcUS, pPcyA)	642/370	0.785	ND
ApcB (pApcDB, pCpcUS, pPcyA)	616/370	2.57	ND
HT-ApcF (pApcF, pCpcUS, pPcyA)	616/370	3.37	68.1
GST-ApcE (pGST-ApcE, pPcyA)	662/370	1.64	ND
HT-CpcB (pCpcBA, pCpcUS, pPcyA)	620/367.5	5.19	37.1
HT-CpcB (pCpcBA, pCpcT, pPcyA)	592/354	2.53	17.4

[a] The percentage of chromophorylation was estimated as described in Materials and Methods.
[b] ND, not determined due to difficulties with proteolysis or the expression level.

lin:ferredoxin oxidoreductase gene, *pcyA* (SYNPCC7002_A2228), was amplified by PCR from chromosomal DNA of *Synechococcus* sp. strain PCC 7002 using primers pcyAF (5′-CAGAATTCCATGACTGCCCCTGCAACCAAGC-3′) and pcyAR (5′-AAGTCGACGATCTAGGCTGGAATATCAAACAGCACC-3′). The gene was cloned into the pACYC Duet vector containing *ho1* using EcoRI and SalI (restriction sites in the primers are underlined), and the resultant plasmid was designated pPcyA. In *E. coli* expression of these two gene products results in the production of PCB from heme (15, 21, 22).

(iv) *cpcBA* and *cpcB* expression constructs. The *Synechocystis* sp. strain PCC 6803 *cpcBA* genes (sll1577 and sll1578) (encoding the β and α subunits of PC, respectively) were amplified from an existing plasmid designated *cpcBA*/pBS150v described previously (41). CpcB could be produced with an N-terminal hexahistidine tag from this construct. The pBS150vNcoF primer (5′-AACCATGGAG ATCAGTAACAATAACTCTAGGG-3′), which anneals to the pBS150v vector sequence, and the cpcAR primer (5′-ACTAAGCTTAATTAGCTGAAGGGC G-3′) primer, which is complementary to the 3′ end of the *cpcA* gene, were used to amplify the product with a PCR. The product was cloned into the pCDF Duet vector (Novagen, Madison, WI) using the NcoI and HindIII sites (underlined in the primers). The resulting expression clone resulted in production of histidine-tagged CpcB and nontagged CpcA. Another clone expressing just HT-CpcB was created as follows. The *cpcB* gene from *Synechocystis* sp. strain PCC 6803 was amplified by PCR using the 6803cpcB.1 forward primer (5′-CAAGTAGGAGA TTAATCATATGTTCGACGTA3′) and the 6803cpcB.4 reverse primer (5′-AGAATTCCTAGGCTACGGCAGCAGCGGCG3′). The *cpcB* gene was cloned into the NdeI and EcoRI sites (underlined in the primers) of pBS150v to create pCpcB (Table 1).

(v) *apcE* expression construct. The 5′ end of the *apcE* gene, which encodes the AP-like domain encompassing amino acids 1 to 228 of L_{CM}^{99} (SYNPCC7002_A2009), was amplified by PCR from *Synechococcus* sp. strain PCC 7002 DNA using primers apcEF (5′-AAACCCGGGAATGACGATTAA GGCCAGCGGTGG-3′) and apcER (5′-AGAATTCACTGCATTTCGTGATT AACACATC-3′). This gene was cloned into the SmaI and EcoRI sites (underlined in the primers) of pGEX-2T, producing plasmid pGST-ApcE, which encodes a fusion protein consisting of GST at the N terminus fused to the first 228 amino acids of ApcE.

(vi) *cpcEF* expression construct. Plasmid pBS414v (61) containing *Synechocystis* sp. strain PCC 6803 HT-*cpcA* along with *cpcE* and *cpcF* was digested with NcoI and EcoRI, the 5′ overhangs were blunt ended using DNA polymerase I and deoxynucleoside triphosphates (dNTPs), and the resulting construct was self-ligated to create plasmid pBS415v containing the *cpcE* and *cpcF* lyase genes. This expression construct was tested, and it expressed active CpcEF (not shown).

***In vivo* heterologous expression and purification of recombinant proteins.** Expression plasmids were cotransformed into *E. coli* BL21(DE3) cells, and colonies were selected on Luria-Bertani (LB) medium plates in the presence of appropriate combinations of antibiotics (Table 1) at the following concentrations: ampicillin (Ap), 100 μg ml^{-1}; chloramphenicol (Cm), 34 μg ml^{-1}; kanamycin (Km), 50 μg ml^{-1}; and spectinomycin (Sp), 100 μg ml^{-1}. To produce PCB using the pPcyA expression plasmid, a 50-ml overnight starter culture was added to 1 liter of LB medium with the appropriate combination of antibiotics. The culture was shaken at 37°C for 4 h until the optical density at 600 nm (OD_{600}) was 0.6. Production of T7 RNA polymerase was induced by addition of 0.5 mM isopropyl-β-D-thiogalactoside (IPTG). Cells were incubated with shaking at 225 rpm at 30°C for another 4 h before they were harvested by centrifugation at 10,000 × *g* for 10 min. Cells were incubated at 30°C after induction with IPTG to limit the production of inclusion bodies (data not shown). Cell pellets were stored at −20°C until they were used.

E. coli cells containing recombinant proteins were thawed and resuspended in buffer O (50 mM Tris-HCl, 150 mM NaCl; pH 8.0) using a concentration of 1 g (wet weight)/2.5 ml and then were lysed by three passages through a chilled French pressure cell at 138 MPa. The lysed cell suspension was centrifuged for 20 min at 13,000 × *g* to remove inclusion bodies, cell debris, and unbroken cells. To purify hexahistidine-tagged recombinant proteins, the supernatant was passed over a nickel-nitrilotriacetic acid Superflow affinity column (Qiagen, Inc., Chatsworth, CA) containing 5 ml of resin, and proteins were purified as previously described (52). The recombinant proteins were dialyzed with buffer O containing 10 mM 2-mercaptoethanol overnight at 4°C to remove the imidazole. For purification of GST-ApcE, cells were broken as described previously (41), except that protease inhibitor cocktail tablets (Complete Mini; Roche, Mannheim, Germany) were added. The clarified extract was passed over a 5-ml glutathione agarose column (Sigma) as described previously (41).

Fluorescence emission and absorbance spectra. Fluorescence emission and excitation spectra were recorded with an LS55 fluorescence spectrophotometer (Perkin Elmer, Waltham, MA) with slit widths for excitation and emission set at 10 nm. For recombinant phycobiliproteins, the excitation wavelength was set at 590 nm, and chromophorylated samples were diluted to obtain an optical density of ~0.05 (at λ_{max}) before their fluorescence spectra were obtained, whereas negative-control samples (e.g., samples with no lyase added) with no obvious chromophore attached were not diluted (their optical densities were generally less than 0.05). Absorbance spectra were acquired using a lambda 35 dual-beam UV-visible spectrophotometer (Perkin Elmer, Waltham, MA).

Protein and bilin analysis. Polypeptides were resolved by 15% (wt/vol) polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS), and polypeptides were visualized by staining with Coomassie blue as described previously (46). To visualize PCB linked to proteins, gels were soaked in 100 mM $ZnSO_4$ for approximately 5 min (7, 43). Zinc-enhanced fluorescence of bilins was visualized using an FX imaging system (Bio-Rad, Hercules, CA) with excitation at 550 nm. In order to calculate the percentage of chromophorylation of each protein, Bio-Rad's Quantity One software was used to determine the relative abundance of each polypeptide. (PcyA and CpcU were His tagged, but the levels of HT-CpcU expression were very low and the amount of this protein relative to the other proteins was very small). This abundance percentage was multiplied by the extinction coefficient of the protein at 280 nm (based on the Trp and Tyr content [see Table S1 in the supplemental material]) to obtain the contribution of each protein to the absorbance at 280 nm. A modified extinction coefficient at 280 nm (ε_{280}) for the total protein concentration was used, as determined as follows for purified HT-ApcA/ApcB: $\varepsilon_{280}^{total}$ = % HT-ApcA · $\varepsilon_{280}^{HT\text{-}ApcA}$ + % ApcB · ε_{280}^{ApcB} + % HT-PcyA · $\varepsilon_{280}^{HT\text{-}PcyA}$ and $\varepsilon_{280}^{HT\text{-}ApcA/ApcB}$ = (% HT-ApcA + % ApcB) · $\varepsilon_{280}^{total}$.

The PCB concentration was calculated by denaturing the recombinant protein in 8 M urea (pH 2) and using an ε_{663} of 35.4 mM^{-1} cm^{-1} (28). The concentration of the bilin was divided by the concentration of the PBP to obtain the percentage of chromophorylation (Table 2). The holo-HT-CpcA concentration was determined by using an ε_{625} of 127.6 mM^{-1} cm^{-1} (61). The yield of chromophorylated PBP (expressed in mg of PBP $liter^{-1}$ *E. coli* culture) was estimated by determining the concentration of PCB bound to protein (obtained in 8 M urea, pH 2) and multiplying this concentration by the molecular weight of the PBP subunit(s)

and the total volume of protein solution purified from the cells grown in 1 liter of culture medium.

To compare the PCB yields obtained in expression cells containing pPcyA and pAT101, 50-ml overnight cultures were transferred to 1 liter of culture medium at 37°C and grown until the OD_{600} was 0.6. IPTG (0.5 mM) was then added, and the cultures were incubated for an additional 4 h at 30°C. Cells were harvested and homogenized with 35 ml of acetone (33). The supernatant was vacuum dried to remove the acetone, and the dried pellet was dissolved in methanol (1.0 ml). The total PCB from each construct was diluted 100-fold in methanol-5% HCl, and the concentration was calculated using an ε_{680}^{PCB} of 37.9 mM^{-1} cm^{-1} (14). The PCB concentration was multiplied by the total volume in methanol and by the molecular mass of PCB (587 g/mol) (23) to determine the amount of PCB produced (in grams per liter of culture).

RESULTS

Examination of *Synechococcus* sp. strain PCC 7002 PcyA activity in *E. coli*. The Duet vector system was used to clone genes required for the synthesis of PBP and to test the efficiency of producing large amounts of holo-PBP in *E. coli*. First, the *ho1* gene from *Synechocystis* sp. strain PCC 6803 and the *pcyA* gene from *Synechococcus* sp. strain PCC 7002 were cloned into the pACYC Duet vector as described in Materials and Methods to produce the pPcyA construct (Table 1). The *Synechocystis* sp. strain PCC 6803 *ho1* gene was used because it was possible to obtain high levels of expression and activity in *E. coli* with several different expression vectors (data not shown). However, in order to improve the level of PCB production that was obtained using the *Synechocystis* sp. strain PCC 6803 *pcyA* gene employed by Tooley et al. (61) (pAT101) (Table 1), the pPcyA plasmid was tested. Although cells containing only the pAT101 plasmid were light blue (see Fig. S1 in the supplemental material), the cells containing pPcyA for expression produced large amounts of PCB and were dull blue (see Fig. S1 in the supplemental material). The amount of PCB produced per liter of *E. coli* cells for each plasmid combination was calculated, and the yield of PCB with pPcyA was 70.8 mg $liter^{-1}$ of cells, whereas with pAT101 the yield of PCB was 20.2 mg $liter^{-1}$ of cells. Next, this new PCB expression system was compared with the system previously developed by Tooley et al. to show that holo-CpcA could be formed in *E. coli* by cotransformation with pAT101 (containing *Synechocystis* sp. strain PCC 6803 *ho1* and *pcyA*) and pBS414v (containing *Synechocystis* sp. PCC 6803 *cpcA*, *cpcE*, and *cpcF*) (61). Under the same growth conditions, 48.1% of HT-CpcA was chromophorylated with PCB when plasmids pPcyA and pBS414v were used, whereas only 22.4% of HT-CpcA was chromophorylated when the pAT101 plasmid of Tooley et al. that contained *Synechocystis* sp. strain PCC 6803 *pcyA* was used (61) (Table 2). These observations indicated that the levels of expression and/or levels of activity of *Synechococcus* sp. strain PCC 7002 PcyA are higher when pPcyA is used than when pAT101 carrying *Synechocystis* sp. strain PCC 6803 *pcyA* is used. The yield of holo-HT-CpcA (calculated using an ε_{625} of 127.6 mM^{-1} cm^{-1} [61] as described in Materials and Methods) when plasmid pPcyA was used was 3.2 mg of holo-HT-CpcA $liter^{-1}$ *E. coli cells* (see Fig. S2 in the supplemental material for the results of analyses of the HT-CpcA produced).

Development and use of a multiplasmid system for expression of holo-AP. *E. coli* cells containing plasmids pPcyA and pApcAB produced almost no colored and fluorescent product (Fig. 1A shows a sample that was not diluted before the fluorescence emission data were obtained), but *E. coli* cells containing plasmids pPcyA, pApcAB, and pCpcUS were brilliant blue (see Fig. S1 in the supplemental material). HT-ApcAB purified from these cells had an absorbance maximum at 615 nm with a small shoulder at 653 nm (Fig. 1A). This product had a fluorescence emission maximum at 634 nm, consistent with that of monomeric ($\alpha\beta$) holo-AP (Fig. 1A shows the results for a sample that was diluted to an OD_{615} of 0.05 before the fluorescence emission was measured). The ratio of the visible absorbance (615 nm) to the UV absorbance (357 nm) for HT-ApcA/ApcB was 2.53 (Table 2). Next, CpcS or CpcU alone was tested using this heterologous *E. coli* system to confirm the mutagenesis results and previous *in vitro* enzyme assay results (46, 53). Cells that were transformed with either pCpcU or pCpcS and thus expressed only one of the subunits of the heterodimeric lyase were not able to produce a highly fluorescent product, similar to the results obtained for cells containing only pApcAB and pPcyA (Fig. 1B shows the results for a sample that was not diluted before the fluorescence emission was obtained). The HT-ApcA/ApcB samples purified from the different *E. coli* cells were analyzed by SDS-PAGE (Fig. 1C). The bilin content of each protein was examined by using zinc-enhanced fluorescence of the gels, as shown in Fig. 1D; the protein content was examined after the same gel was stained with Coomassie blue (Fig. 1C). When no bilin lyase subunit was present, a small amount of covalent PCB was added to ApcB (Fig. 1C, lane 2); this observation was similar to previous observations for *in vitro* reactions (46). However, the yield of this covalent product was very low in this *in vivo E. coli* system, and the absorption and fluorescence properties suggested that the bilin that attached autocatalytically to ApcB in the absence of a bilin lyase was not the biologically correct product (3, 4, 77). When either CpcS or CpcU was present (Fig. 1D, lanes 3 and 4, respectively), the amount of PCB attached to ApcB was similar to the amount attached when no bilin lyase was present. However, when both CpcS and CpcU were present in cells (pCpcUS, pApcAB, and pPcyA), addition of bilin to both HT-ApcA and ApcB occurred. This was easily demonstrated by the zinc-enhanced fluorescence of the PCB attached to the proteins after SDS-PAGE (Fig. 2B, lane 1 [note that 3-fold-more protein was loaded in lanes 2 to 4 than in lane 1 in order to visualize the small amount of fluorescence from PCB attached to ApcB in the absence of both CpcS and CpcU]). A small amount of a proteolytic degradation product of HT-ApcA was observed (Fig. 1C, lane 1). The amount of chromophorylated ApcB in the cells containing no CpcS or CpcU or in cells containing only one of the subunits was estimated to be only ~8% of the amount of ApcB chromophorylated in the presence of CpcSU. As described in Materials and Methods, the total concentration of HT-ApcA/ApcB was calculated, and the value was compared to the PCB (linked to protein) concentration. On the basis of this calculation, approximately 72% of the HT-ApcA/ApcB polypeptides were chromophorylated. However, when the relative bilin fluorescence intensity of each polypeptide in Fig. 2B, lane 2, was evaluated, it was found that 100% of ApcB was chromophorylated, while only ~40% of HT-ApcA was chromophorylated. At sufficiently high protein concentrations and under appropriate ionic strength and pH conditions, AP forms $(\alpha\beta)_3$ trimers that result in a characteristic red shift in the absorbance (λ_{653}) and fluorescence (λ_{663})

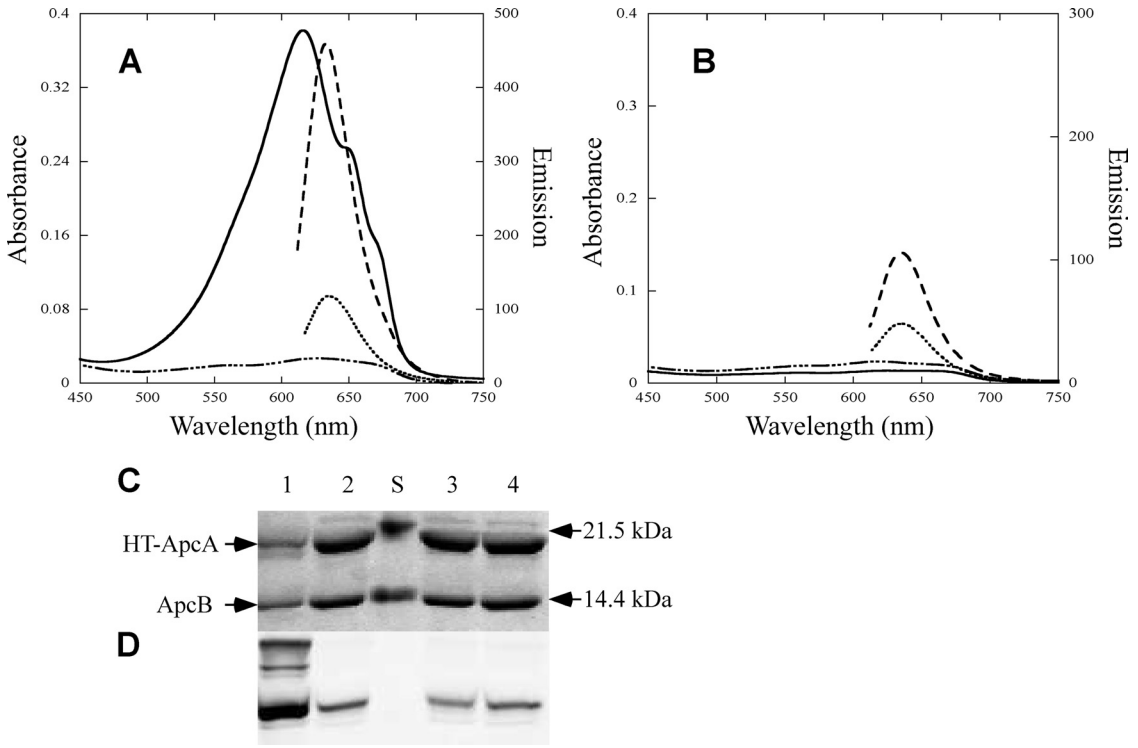


FIG. 1. Analyses of major AP subunits ApcA and ApcB synthesized in *E. coli*. (A) Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-ApcA/ApcB purified from cells with pPcyA but containing pApcAB and pPcyA with pCpcUS and absorbance (dashed and dotted line) and fluorescence (dotted line) spectra of HT-ApcA/ApcB purified from cells with pPcyA but without pCpcUS. (B) Absorbance (solid line) and fluorescence emission (dashed line) spectra of HT-ApcA/ApcB purified from cells containing pApcAB, pPcyA, and pCpcS and absorbance (dashed and dotted line) and fluorescence (dotted line) spectra of HT-ApcA/ApcB purified from cells containing pApcAB, pPcyA, and pCpcU. In order to acquire the fluorescence emission spectra of the AP subunits produced in the presence of pCpcUS (panel A, dashed line), the sample was diluted to obtain an OD_{615} of 0.05, whereas to acquire the fluorescence emission spectra of HT-ApcA/ApcB produced without CpcS-I/CpcU (panel A, dotted line) or produced with pPcyA and pCpcS (panel B, dashed line) or with pPcyA and pCpcU (panel B, dotted line), the samples were not diluted. (C) Coomassie blue-stained SDS-polyacrylamide gel containing HT-ApcA/ApcB purified from cells containing pApcAB and pPcyA with (lane 1) or without (lane 2) pCpcUS and HT-ApcA/ApcB purified from cells containing pApcAB, pPcyA, and either pCpcS (lane 3) or pCpcU (lane 4). Lane S contained molecular mass standards, and selected molecular masses are indicated on the right. (D) Zinc-enhanced fluorescence image of the gel shown in panel C.

properties of the protein (6, 30, 31, 39). When the linker protein ApcC was present, it also sharpens the absorbance spectrum of trimers (47). Although there is a small shoulder at ~650 nm in the absorbance spectrum (Fig. 1A), the fluorescence emission spectrum showed no emission at 660 nm (Fig. 1A). After analysis of the sample by size exclusion high-performance liquid chromatography (HPLC), two peaks were observed for this holo-HT-ApcA/ApcB. One peak was at a molecular mass consistent with single α or β subunits, while the other was at a molecular mass consistent with that of AP (αβ) protomers; no peak consistent with the elution properties of AP $(\alpha\beta)_3$ trimers was observed (data not shown). Because of the presence of a significant proportion of apo subunits in the purified mixture, this was expected. Assuming that the association of chromophorylated and nonchromophorylated subunits is random and that the chromophorylation rates are 40% for the α subunit and 100% for the β subunits, the probability of obtaining αβ monomers with both subunits chromophorylated is ~40% (1.0×0.40) and the probability of obtaining trimers in which all subunits are chromophorylated is ~6.4% ($1.0^3 \times 0.4^3$).

The specificity of the other two known bilin lyases was also tested to see if either the CpcE/CpcF lyase or the CpcT lyase could attach PCB to HT-ApcA/ApcB in this *in vivo* system. As judged by absorbance and fluorescence emission spectra (see Fig. S4A and B, respectively, in the supplemental material), no holo-AP was produced when either pCpcEF or pCpcT was introduced together with pApcAB and pPcyA. In addition, only a small amount of PCB added to ApcB was observed; the level was similar to that observed when no lyase was present (compare to Fig. 1D, lane 2). Therefore, the specificity of the lyases for their PBP substrates was maintained in this *E. coli* system and was completely consistent with previous *in vitro* biochemical and mutational analyses (19, 20, 46, 52, 53, 78).

Chromophorylation requirements for HT-ApcD. ApcD is a variant α-AP subunit ($\alpha^{AP\text{-}B}$) that pairs with β-AP (ApcB), forming AP-B, which has an extremely red-shifted absorbance at 670 nm and is an important terminal emitter of the PBS involved in energy transfer to photosystem I; two copies of ApcD are present per PBS molecule (5, 16, 24, 29, 38, 40, 71). An alignment of ApcD with other allophycocyanin subunits is shown in Fig. S3 in the supplemental material. The *apcD* gene was cloned together with *apcB* in order to produce HT-ApcD and ApcB, as this construct produced a more soluble recom-

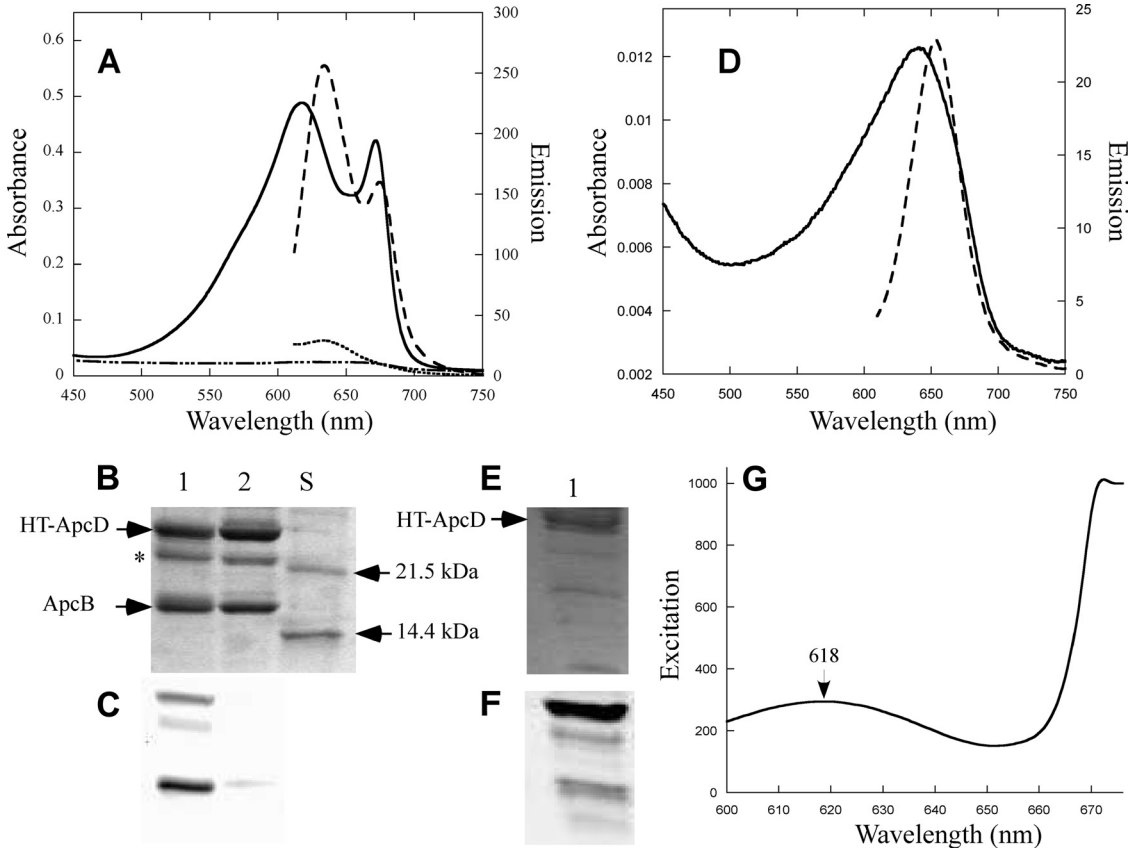


FIG. 2. Analyses of AP-B α-subunits (ApcD) synthesized in *E. coli*. (A) Absorbance (solid lines) and fluorescence emission (dashed lines) spectra of HT-ApcD/ApcB purified from cells containing pApcDB and pPcyA with pCpcUS and absorbance (dashed and dotted line) and fluorescence (dotted line) spectra of HT-ApcD/ApcB purified from cells without pCpcUS. (B) Coomassie blue-stained SDS-polyacrylamide gel containing HT-ApcD/ApcB purified from cells containing pApcDB and pPcyA with (lane 1) or without (lane 2) pCpcUS. Lane S contained molecular mass standards, and selected molecular masses are indicated on the right. The asterisk on the left of panel B denotes the position of a proteolytic degradation product of HT-ApcD. (C) Zinc-enhanced fluorescence from the bilins for the gel shown in panel B. (D) Absorbance (solid lines) and fluorescence emission (dashed lines) spectra of HT-ApcD purified from cells containing pApcD and pPcyA with pCpcUS. (E) Coomassie blue-stained SDS-polyacrylamide gel containing HT-ApcD purified from cells containing pApcD and pPcyA with pCpcUS. (F) Zinc-enhanced fluorescence of the bilins for the gel shown in panel E. (G) Excitation spectrum of HT-ApcD/ApcB purified from cells containing pApcBD and pPcyA with pCpcUS. The emission wavelength was set at 676 nm. The excitation peak is indicated by an arrow.

binant apoprotein than a construct expressing *apcD* alone (L. Harrison, Jr., and W. M. Schluchter, unpublished data). *E. coli* cells containing plasmids pApcDB and pPcyA were faint dull blue (data not shown), whereas cells containing plasmids pApcDB, pPcyA, and pCpcUS were brilliant blue (see Fig. S1 in the supplemental material). The absorbance and fluorescence emission spectra for HT-ApcD/ApcB after purification of the proteins by metal affinity chromatography from cells containing these two plasmid combinations are shown in Fig. 2A. HT-ApcD/ApcB produced in the absence of the pCpcUS plasmid had very little absorbance or fluorescence, but HT-ApcD/ApcB produced in the presence of pCpcUS had absorbance peaks at 616 and 672 nm. When excited at 590 nm, the recombinant-produced HT-ApcD/ApcB had fluorescence emission maxima at 634 nm and 675 nm. When these proteins were separated by SDS-PAGE and evaluated by using zinc-enhanced fluorescence, the results showed that both subunits carried PCB chromophores (Fig. 2C, lane 1), but based on quantitation of the fluorescence intensities (compare lanes 1 in Fig. 2B and 2C), the chromophorylation level for HT-ApcD was only ~40% (when the level for the ApcB subunit was 100%). However, in the absence of pCpcUS, no bilin addition to HT-ApcD occurred, and very little bilin added to ApcB was detected (Fig. 2B and 2C, lane 2). A degradation product of HT-ApcD was apparent in some preparations (Fig. 2B).

In order to determine the absorbance spectrum of holo-HT-ApcD alone, HT-ApcD was produced in the presence of pPcyA and pCpcUS. As shown by the absorbance and fluorescence spectra in Fig. 2D and the SDS-PAGE and zinc-enhanced bilin fluorescence in Fig. 2E and F, a small amount of chromophorylated product was obtained. This holo-HT-ApcD had an absorbance maximum at 642 nm and a fluorescence emission maximum at 653 nm. This likely means that when holo-HT-ApcD associates with holo-ApcB, there are interactions between chromophores that red shift the absorbance maximum to 672 nm. The 616-nm absorption maximum shown in Fig. 2A is probably due to holo-ApcB (paired with apo-HT-ApcD), because there is an excess of chromophorylated ApcB relative to the amount of holo-HT-ApcD (Fig. 2C). In support of this idea, the fluorescence excitation spectrum of holo-HT-

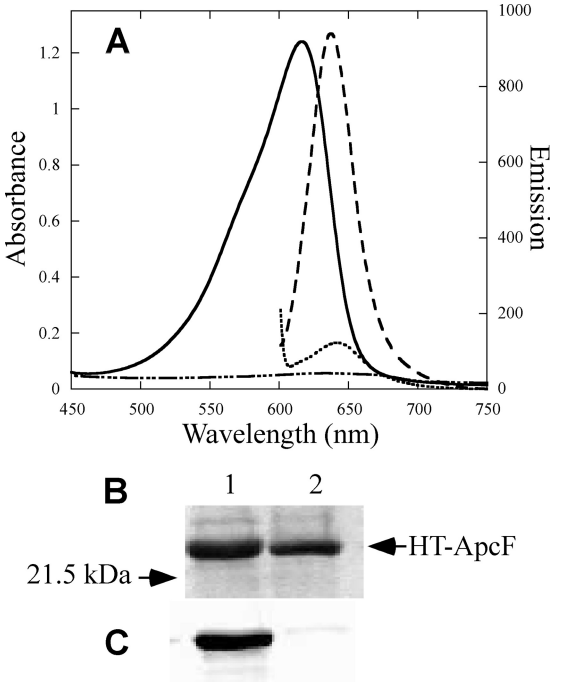


FIG. 3. Analyses of AP β^{18}-subunit (ApcF) synthesized in *E. coli*. (A) Absorbance (solid lines) and fluorescence emission (dashed lines) spectra of HT-ApcF purified from cells containing pApcF and pPcyA with pCpcUS and absorbance (dashed and dotted line) and fluorescence (dotted line) spectra of HT-ApcF purified from cells without pCpcUS. (B) Coomassie blue-stained SDS-polyacrylamide gel containing HT-ApcF purified from cells containing pApcF and pPcyA with (lane 1) or without (lane 2) pCpcUS. The molecular mass of a standard is indicated on the left. (C) Zinc-enhanced fluorescence of the bilins in the gel shown in panel B.

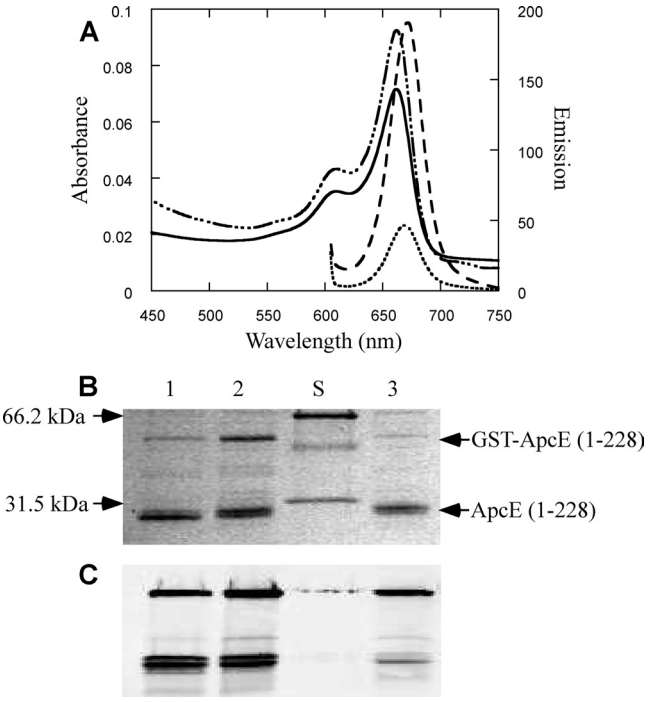


FIG. 4. Analyses of GST-ApcE purified from *E. coli* cells. (A) Absorbance (solid line) and fluorescence emission (dashed line) spectra of GST-ApcE purified from *E. coli* cells containing pGST-ApcE, pPcyA, and pCpcUS and absorbance (dashed and dotted line) and fluorescence (dotted line) spectra of GST-ApcE produced without pCpcUS. (B) Coomassie blue-stained polyacrylamide gel containing purified GST-ApcE from *E. coli* cells containing pGST-ApcE and pPcyA with (lane 1) or without pCpcUS (lane 2). Lane S contained molecular mass standards, and selected molecular masses are indicated on the left. The identities of polypeptides are indicated on the right. (C) Zinc-enhanced bilin fluorescence of the gel shown in panel B.

ApcD/ApcB with emission at 675 nm has an excitation peak that is centered at 618 nm, indicating that some of the ApcB-PCB chromophore transfers energy to ApcD (Fig. 2G). An alternative hypothesis to explain the difference in the spectra of HT-ApcD produced with and without ApcB is that the folding of ApcD was adversely affected in the absence of ApcB, leading to the differences in the absorbance and fluorescence emission spectra observed.

Chromophorylation requirements for HT-ApcF. The requirements for synthesis of holo-HT-ApcF were examined next. ApcF is a variant β-AP subunit (also designated β^{18}) that partners with ApcE, a terminal emitter, and that in *Synechococcus* sp. strain PCC 7002 influences energy transfer from PBS to photosystem II (5, 26, 70, 71). This His-tagged subunit was expressed in the presence of pPcyA or both pPcyA and pCpcUS. The absorbance and fluorescence spectra of purified HT-ApcF from cells with and without pCpcUS are shown in Fig. 3A. Holo-HT-ApcF was produced only in the presence of the CpcS-I/CpcU bilin lyase and had an absorbance maximum at 616 nm with fluorescence emission at 637 nm. The protein purified from cells not expressing *cpcS-I* and *cpcU* exhibited very little absorbance or fluorescence (Fig. 3A). This was also apparent when we examined the zinc-enhanced bilin fluorescence after SDS-PAGE of HT-ApcF purified from the two cell types (Fig. 3B and 3C, compare lanes 1 and 2). With this *in vivo* system, 68% of HT-ApcF was estimated to be chromophorylated (Table 2).

Chromophorylation requirements of ApcE. The AP-like domain of ApcE from *Synechococcus* sp. strain PCC 7002 contains a PCB at Cys-186 (see Fig. S3 in the supplemental material). The expression construct pGST-ApcE fused GST to amino acids 1 to 228 of the AP-like domain of ApcE (Table 1). The pGST-ApcE construct was transformed into *E. coli* cells with pPcyA with or without pCpcUS to determine whether the CpcS-I/CpcU bilin lyase was required for correct and efficient PCB addition at Cys-186. Figure 4A shows the absorbance and fluorescence spectra of the GST-ApcE purified from the two cell types. The ApcE domain alone was sufficient to attach PCB in the correct manner to produce a strongly red-shifted PCB product with an absorbance maximum at 662 nm and a fluorescence emission maximum at 675 nm. GST alone did not react with PCB (data not shown). GST-ApcE synthesized in cells containing pCpcUS also had absorbance and fluorescence properties similar to those of the native protein (13, 26, 74), but given that the correct product can be formed in the absence of any additional enzyme, the CpcS-I/CpcU bilin lyase apparently is not required for bilin ligation to ApcE. The GST-ApcE proteins were then separated by SDS-PAGE, and the zinc-enhanced bilin fluorescence was analyzed before the

protein was stained with Coomassie blue. It was necessary to lyse the cells in the presence of a mixture of protease inhibitors in order to avoid formation of degradation products. The results of this purification are shown in Fig. 4B and 4C (and correspond to the spectra shown in Fig. 4A). Three polypeptides carried a bilin chromophore. One polypeptide likely corresponded to the full-length fusion (GST-ApcE; predicted molecular mass, 51,445 Da), whereas the other two polypeptides had estimated molecular masses consistent with those of degradation products containing ApcE (amino acids 1 to 228) with an expected molecular mass of 24.5 kDa. As judged by zinc-enhanced fluorescence (Fig. 4C, lanes 1 and 2) and supporting the conclusion that ApcE has intrinsic bilin lyase activity, approximately equivalent levels of added PCB were observed when pCpcUS was present and when pCpcUS was not present.

Creation of partially chromophorylated PBPs in *E. coli*. The efficiency of this *in vivo* system for creating partially chromophorylated CpcB was examined in order to determine the order for posttranslational modifications in CpcB (41). The CpcS-I/CpcU lyase and the CpcT lyase were tested separately to confirm that each could attach PCB to CpcB in this system, and the efficiency of PCB addition to Cys-82 and Cys-153 in *E. coli* was estimated (see Table S2 in the supplemental material). Cells containing pCpcBA, pPcyA, and either pCpcUS or pCpcT produced strongly colored PBPs (see Fig. S1 in the supplemental material). The spectra of the two partially chromophorylated PCs are shown in Fig. S5A and S6A in the supplemental material. The PC produced by chromophorylation at Cys-82 of CpcB by CpcS-I/CpcU was deep blue (see Fig. S1 in the supplemental material) and had an absorbance maximum at 620 nm and a fluorescence emission maximum at 642 nm for the CpcSU product at Cys-82 (see Fig. S5A in the supplemental material, solid lines). The purple PC (see Fig. S1 in the supplemental material) produced by chromophorylation of Cys-153 by CpcT had an absorbance maximum at 592 nm and a fluorescence emission maxima at 618.5 nm (see Fig. S6A and Table S2 in the supplemental material). SDS-PAGE analyses of these two PCs were also performed, and Coomassie blue-stained gels containing the purified proteins are shown in Fig. S5C and S6B in the supplemental material, respectively. The zinc-enhanced bilin fluorescence analyses (see Fig. S5D, lane 3, and Fig. S6C, lane 1, in the supplemental material) showed that CpcB was the subunit to which PCB was added. As observed previously (3, 46, 52, 53, 75), no significant bilin addition was observed in the absence of the lyase. An estimated 37% of the CpcB was chromophorylated by CpcS-I/CpcU, whereas CpcT chromophorylated only ~17% of the CpcB (Table 2). Thus, although both bilin lyases displayed activity with CpcB in this *in vivo E. coli* system, the chromophorylation levels were much lower than those observed with CpcS-I/CpcU for AP subunit substrates. To determine whether the presence of the CpcA subunit might interfere with the chromophorylation by CpcSU, *cpcB* was expressed from the plasmid pCpcB construct (Table 1) together with pCpcUS and pPcyA. Although the yields of holo-HT-CpcB produced in the absence of CpcA were much less than the yields when CpcB was coexpressed with CpcA (about 10-fold less), CpcSU was able to chromophorylate the HT-CpcB protein at a similar level. When equal amounts of HT-CpcB were loaded on an SDS-PAGE gel (see Fig. S5C, lanes 3 and 4, in the supplemental material) and the bilin fluorescence was examined (see Fig. S5D in the supplemental material), the levels of bilin fluorescence were roughly the same (within 10%, as estimated by the Quantity One software). These observations indicated that CpcA did not interfere with chromophorylation of CpcB by CpcSU.

DISCUSSION

In the studies reported here, an *in vivo*, heterologous expression system was developed to determine the biosynthetic requirements for synthesis of holo-ApcD, ApcF, and ApcE, to test the efficiency and specificity of various bilin lyases in chromophorylating substrates, and to produce large quantities of holo-PBPs that might be used as fluorescent probes. Other workers have used a similar approach to produce single subunits in *E. coli* (25, 61, 62, 72), but the system described here produced the best yield of holo-PBPs in *E. coli* reported to date: 3 to 12 mg holo-PBP liter^{-1} of *E. coli* culture (compared to 0.86 to 1.0 mg liter^{-1} for AP subunits [25, 68]). When the system of Tooley et al. for production of PCB (pAT101 containing *Synechocystis* sp. strain PCC 6803 *ho1* and *pcyA*) (61) was directly compared with the system described here for production of PCB (pPcyA containing *Synechocystis* sp. strain PCC 6803 *ho1* and *Synechococcus* sp. strain PCC 7002 *pcyA*), 3.5-fold more PCB was obtained in the same culture volume when pPcyA was used than when pAT101 was used. This could have been due to higher solubility, higher activity, and/or a higher level of expression of PcyA from *Synechococcus* sp. strain PCC 7002. It could also have been due to a difference in plasmid copy number or the T7 promoter activity levels for the Duet plasmid pPcyA construct (pAT101 uses the *trc* [*trp-lac*] promoter). The *Synechocystis* sp. strain PCC 6803 heme oxygenase was used in both constructs, and the heme oxygenase enzyme produced by the Duet vector containing just *ho1* (parent of the pPcyA vector) was assayed *in vitro* and was very active (Y. M. Vasquez and W. M. Schluchter, unpublished results). It is possible that because *Synechococcus* sp. strain PCC 7002 grows optimally at nearly the same temperature as *E. coli* (38 to 40°C), its enzymes may be more stable and exhibit higher activities when they are expressed in *E. coli* than those of *Synechocystis* sp. strain PCC 6803, which has a lower optimum growth temperature. In fact, it may be possible to improve the PCB production levels if the *ho1* gene of *Synechococcus* sp. strain PCC 7002 is used instead of the *Synechocystis* sp. strain PCC 6803 *ho1* gene.

It was also possible to produce large quantities (5 to 12.4 mg of holo-AP subunit liter^{-1} of *E. coli* culture) of holo-HT-ApcA/ApcB, holo-HT-ApcF, and holo-HTApcD/ApcB in *E. coli*. The CpcS-I/CpcU bilin lyase was required for PCB addition to all of these proteins, but it appears that slightly smaller quantities of the holo-α-subunits (HT-ApcA and HT-ApcD) than of their holo-β-subunit counterparts were produced (Fig. 2B and 2D). When produced in *E. coli*, AP subunits have previously been reported to have different levels of solubility (25, 41, 68, 69, 72). Similarly, very high levels of apo-HT-ApcF were not produced unless ApcA was coexpressed with it (A. Fletcher and W. M. Schluchter, unpublished results). However, HT-ApcF was stabilized by chromophorylation, which

permitted high levels of production to be obtained in the absence of ApcA; up to ~12 mg of this PBP was produced per liter of *E. coli* culture. However, the levels of HT-ApcD (produced alone) were low whether it was produced in the apo or holo form (Fig. 2D). Other studies have reported that holo-ApcB can be produced in *E. coli* (0.86 mg liter^{-1}) (25) and in *Streptomyces* sp. (38 mg liter^{-1}) (32) and that holo-ApcA can be produced in *E. coli* (1 g liter^{-1}) (68). Although one report claimed that holo-ApcA could be produced without any bilin lyase (33), the results presented here and in other studies show that either the heterologous CpcS-I/CpcU-type bilin lyase or the CpcS-III single subunit-type bilin lyase (also called CpeS1 by Zhao et al. [75]) is required for maximal and correct addition of PCB to ApcA (25, 46, 72, 75). The results of analyses of bilin lyase mutants also strongly support the conclusion that bilin lyases are essential for AP biogenesis (53). However, small amounts of AP subunits could be detected in a *cpcSUT* triple mutant by immunoblotting (53). This suggests that some autocatalytic addition of bilin may occur in the absence of bilin lyases in cyanobacteria. In the studies reported here, neither CpcE/CpcF nor CpcT attached PCB to ApcA or ApcB (see Fig. S4 in the supplemental material).

The synthesis of holo-HT-ApcD/ApcB resulted in a product with a sharp, red-shifted absorption peak at 672 nm and a fluorescence emission maximum at 675 nm, both of which are characteristic of native AP-B ($\alpha\beta$) (29, 38). In studies by Zhao et al. (72) that showed that the single-subunit bilin lyase CpcS-III (CpeSI) from *Nostoc* sp. strain PCC 7120 was required for addition of bilin to ApcD, the absorbance spectrum of the product had a broad peak centered at 650 nm, possibly due to poor solubility. In the system described here, holo-HT-ApcD had an absorbance maximum at 642 nm. Coproducing ApcD with its partner subunit, ApcB, apparently improved its solubility, which resulted in a chromoprotein with red-shifted absorbance and fluorescence emission maxima very similar to those of native allophycocyanin B (29, 38). ApcD may be particularly useful as a fluorescent tag due to its far-red absorbance and fluorescence emission.

For the production of chromophorylated AP and PC subunits, it appears that a major limitation may be the folding and/or conformation of the PBP subunit. For example, the rates of chromophorylation by the CpcS-I/CpcU bilin lyase for ApcA/ApcB are much higher than those for CpcB, and the only difference is the PBP substrate. Because both substrates were expressed as the α and β subunits and because of the overall structural similarity of the two proteins (11, 50), presumably there is little difference in the accessibility of the Cys-82 chromophorylation sites.

As first reported by Zhao et al. (74), the only PBP synthesized by *Synechococcus* sp. strain PCC 7002 that is capable of autocatalytic PCB attachment is ApcE. However, because of solubility problems with the truncation product of ApcE, Zhao et al. had to perform *in vitro* bilin addition reactions with the PBP domain of ApcE (residues 1 to 240) in 4 M urea, which would affect not only the conformation of the protein substrate but also potentially of that of the PCB chromophore. Addition of detergents, such as Triton X-100, has been shown to affect chromophore conformation and to facilitate autocatalytic addition of bilins to PBPs in the absence of lyases (77). Therefore, a critical role for the bilin lyases is to bind the correct chromophore (in organisms producing more than one bilin) in the correct conformation to achieve the appropriate stereochemical attachment of the bilin to the apoprotein (77). At high bilin concentrations, which are probably never encountered inside cyanobacterial cells, autocatalytic attachment does occur *in vitro*, but the products of these reactions do not produce the naturally occurring holoproteins (3, 18, 49). For all of these reasons, it was important to show that a soluble form of ApcE, in the absence of detergents or urea, had intrinsic bilin lyase activity.

The amino-terminal domain of ApcE, including amino acids 1 to 228, contains an AP-like domain that interacts with the AP-β-like subunit, ApcF, within an AP trimer-like disk of the PBS core (see Fig. S3 in the supplemental material). This domain probably has a largely alpha-helical, globin-like fold that is similar to the structures of other AP and PC subunits (11). This AP-like domain of ApcE is interrupted by an insertion consisting of 50 amino acid residues called the PB loop (2), and this insertion is readily apparent when an alignment of the amino-terminal domain with other AP subunits is examined (see Fig. S3 in the supplemental material). Because no other PBP subunit can efficiently autocatalytically ligate PCB to Cys residues, it is possible that this insertion includes residues that are important in the autocatalytic PCB ligation activity of ApcE. However, Ajlani and Vernotte showed that deletion of this PB loop within ApcE did not affect its ability to attach a PCB chromophore (2). Because the chromophore on ApcE is bound to the opposing helix of the binding pocket relative to every other biliprotein, the requirements for binding may be modified so that autocatalytic binding is possible. ApcE (residues 1 to 200) from *Synechococcus* sp. strain PCC 7002 and *Synechocystis* sp. strain PCC 6803 were aligned with the bilin lyase containing domains of Cph1 and Cph2, two cyanobacteriochromes (66). One small motif that is conserved among these four proteins is the sequence DXXLE corresponding to amino acids 32 to 36 in ApcE from *Synechococcus* sp. strain PCC 7002 and to amino acids 185 to 189 in Cph1 or Slr0473. The glutamic acid at the end of this region (underlined in the sequence above) is the glutamic acid that was shown by mutagenesis to have a function in bilin binding in Cph1 (66). The possibility that E36 in ApcE is also responsible for PCB binding will be tested in future studies.

In summary, the maturation pathway for each PBP in *Synechococcus* sp. strain PCC 7002 has now been completely defined, and which of the four families of bilin lyases is responsible for addition of PCB to the eight discrete chromophore-binding sites on the seven different PBP subunits has been established (see Table S2 in the supplemental material) (19, 20, 46, 52, 53, 78). A heterologous, *in vivo* expression system using PcyA from *Synechococcus* sp. strain PCC 7002 and Ho1 from *Synechocystis* sp. strain PCC 6803 appears to be very efficient and generates large amounts of PCB in *E. coli*. Use of a multiplasmid system allows workers to test biosynthetic requirements of uncharacterized PBPs from other organisms rapidly, to produce partially chromophorylated biliproteins for studies of the order of posttranslational modifications in *in vitro* reactions, and to generate holo-PBPs for use as fluorescent probes for bioimaging.

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