Biosynthesis of a fluorescent cyanobacterial C-phycocyanin holo-α subunit in a heterologous host

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The entire pathway for the synthesis of a fluorescent holophyco-biliprotein subunit from a photosynthetic cyanobacterium (*Synechocystis* sp. PCC6803) was reconstituted in *Escherichia coli*. Cyanobacterial genes encoding enzymes required for the conversion of heme to the natural chromophore 3*Z*-phycocyanobilin, namely, heme oxygenase 1 and 3*Z*-phycocyanobilin:ferredoxin oxidoreductase, were expressed from a plasmid under control of the hybrid *trp-lac* (*trc*) promoter. Genes for the apoprotein (C-phycocyanin α subunit; *cpcA*) and the heterodimeric lyase (*cpcE* and *cpcF*) that catalyzes chromophore attachment were expressed from the *trc* promoter on a second plasmid. Upon induction, recombinant *E. coli* used the cellular pool of heme to produce holo-CpcA with spectroscopic properties qualitatively and quantitatively similar to those of the same protein produced endogenously in cyanobacteria. About a third of the apo-CpcA was converted to holo-CpcA. No significant bilin addition took place in a similarly engineered *E. coli* strain that lacks *cpcE* and *cpcF*. This approach should permit incisive analysis of many remaining questions in phycobiliprotein biosynthesis. These studies also demonstrate the feasibility of generating constructs of these proteins *in situ* for use as fluorescent protein probes in living cells.

The phycobiliproteins are a family of light-harvesting proteins found in cyanobacteria, red algae, and the cryptomonads. These proteins absorb strongly in the visible region of the spectrum because they carry various covalently attached linear tetrapyrrole prosthetic groups (bilins). Phycobiliproteins are tightly associated αβ heterodimers in which each subunit carries bilin(s) thioether-linked to particular cysteinyl residues (1–3).

Steps involved in bilin biosynthesis and bilin addition to apophycobiliprotein subunits have been inferred from diverse studies (e.g., refs. 4–6). However, the entire pathway from heme to a particular holophycobiliprotein subunit has not hitherto been reconstituted either *in vitro* or *in vivo*. This achievement is reported here for a pathway from endogenous heme to phycocyanin α subunit-linked phycocyanobilin (PCB) in an engineered *Escherichia coli* strain.

The studies described here are important as prototypes for experiments directed to exploring many unanswered questions in the enzymology and chemistry of phycobiliprotein synthesis. They are also of great interest in opening the way to the use of phycobiliproteins as *in vivo* fluorescent protein probes. Purified native phycobiliproteins and their subunits fluoresce strongly and, since 1982, have been widely used as external labels for cell sorting and analysis and a wide range of other fluorescence-based assays (7, 8). Fluorescent protein probes expressed *in vivo*, such as *Aequorea victoria* green fluorescent protein and its variants, have proved to be of great value in all fields of cell biology (9). Probes generated by the spontaneous addition of exogenously supplied phycoerythrobilin to recombinant apophytochrome fragments in living cells are also very promising (10). Phycobiliprotein constructs represent a broad array of spectroscopically distinctive proteins with photophysical properties superior to those of probes currently available (11). Hence, the ability to express them conveniently in functional fluorescent

form in diverse cell types would greatly expand the repertoire of tools available for examining a broad array of questions in cell biology.

Materials and Methods

Materials. Enzymes for DNA manipulation were obtained from New England Biolabs and Life Technologies (Rockville, MD), and antibiotics were obtained from Sigma. Agar and organic nutrients for LB were obtained from Difco, and other chemicals were from Sigma or Fisher Scientific. "Superflow" Ni^{2+}-nitrilotriacetic acid agarose for isolation of His-tagged proteins was purchased from Qiagen (Chatsworth, CA).

Cultures and Strains. *E. coli* strain DH5α (Life Technologies) grown in LB was used in all experiments. Plasmids conferring resistance to spectinomycin (Sp) were selected with 100 μg·ml^{-1} Sp dihydrochloride, and those conferring resistance to kanamycin (Km) were selected with 50 μg·ml^{-1} Km sulfate. For expression of P_{trc}-controlled genes, isopropyl β-D-thiogalactoside was added to a final concentration of 0.5 mM to exponentially growing cells. Induced cells were grown for 3.5 h at 37°C with shaking. Cultures were harvested and pellets were stored at −20°C until they were used.

Cloning of Relevant *Synechocystis* sp. PCC6803 Genes. Standard procedures were used for most DNA manipulations. Gene sequences were obtained from the Kazusa DNA Research Institute CyanoBase (refs. 12 and 13; http://www.kazusa.or.jp/cyano/index.html), and all accession numbers given below refer to that database. Using primers described below, all genes were amplified from PCC6803 genomic DNA by the PCR. The fidelity of all PCR-generated fragments was verified by direct nucleotide sequencing. A more typical *E. coli* ribosomal binding site was engineered upstream of the *cpcE*, *cpcF*, and *hox1* ORFs. DNA sequence analysis was performed with the program EDITBASE (Purdue Research Foundation and U.S. Department of Agriculture/Agricultural Research Service), and predicted amino acid sequences were deduced with LASERGENE (DNAstar, Madison, WI).

Cloning of the Gene Encoding *Synechocystis* sp. PCC6803 Phycocyanin α Subunit. Primers used to amplify the *cpcA* gene (sll1578) were 5′-CGG GAG ATA CCA GAC ATA TGA AAA CCC CTT
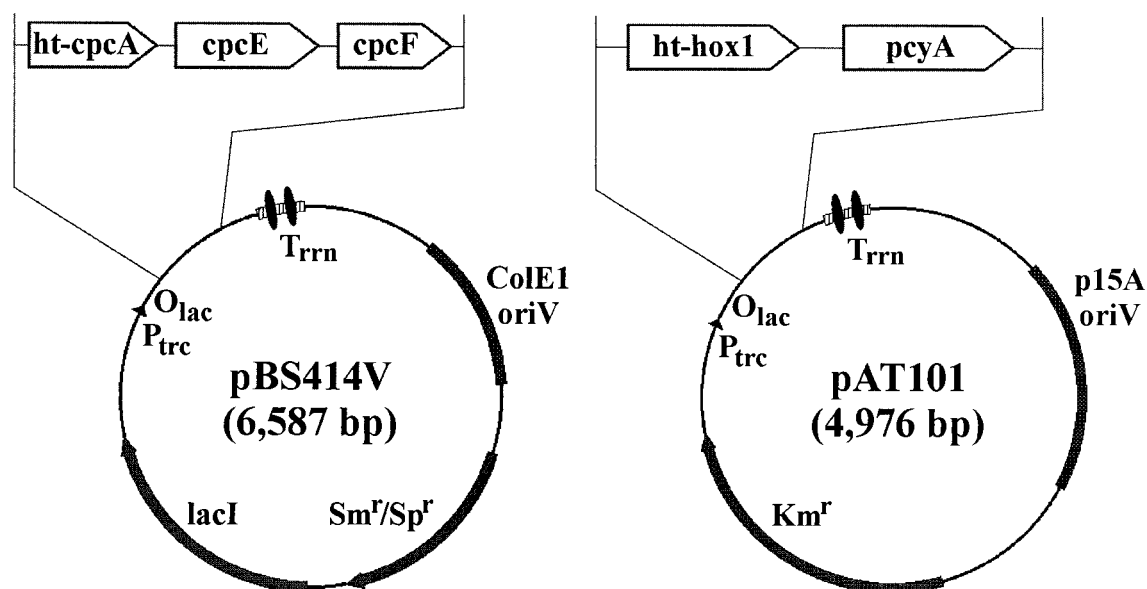
Fig. 1. Physical maps of the pBS414V and pAT101 expression vectors used for *in vivo* bilin addition to *Synechocystis* sp. PCC6803 His$_6$-tagged phycocyanin apo-α subunit (HT-CpcA) in *E. coli*. Vector pBS414V carries the phycocyanin α subunit phycocyanobilin lyase genes *cpcE* and *cpcF* and *ht-cpcA*, the gene encoding HT-CpcA. Plasmid pBS414V contains the ColE1 *oriV* for replication in *E. coli* and the *aadA* gene conferring resistance to Sp and streptomycin (Sm). Expression is controlled by the *trc* promoter, the *lac* operator, and the *lacI^q* gene encoding the Lac repressor. Two strong, bidirectional, ρ-independent transcriptional terminator structures from the *E. coli rrnB* gene are located downstream of the expression cassette. Vector pAT101 expresses the genes involved in phycocyanobilin biosynthesis from heme: HO1 (*hox1*) and 3*Z*-phycocyanobilin:ferredoxin oxidoreductase (*pcyA*). Plasmid pAT101 contains the p15A *oriV* for replication in *E. coli* and the Km-resistance gene. The use of compatible origins of replication and different selectable markers allows for both plasmids to be maintained within the same *E. coli* cell. Expression of the genes carried by pAT101 is under the same control elements as in pBS414V. Plasmid pBS405V (see text) was equivalent to plasmid pBS414V, except that the insert contained only *ht-cpcA*.

TAA CTG-3′ and 5′-TAG AAT TCG AAG ACT AGC TCA GAG CAT TGA TGG CG-3′. The resulting 0.5-kb product was digested with restriction enzymes *Nde*I and *Eco*RI and cloned into *Nde*I- and *Eco*RI-digested cloning vector, pBS350V (described in ref. 11), giving plasmid pBS405V. The α subunit of C-phycocyanin (CpcA) construct expressed from pBS405V consists of CpcA fused at the N terminus to a 24-amino acid sequence that includes a His$_6$-tag (11).

Cloning of the Genes Encoding the *Synechocystis* sp. PCC6803 Phycocyanin α Subunit Phycocyanobilin Lyase. Primers used to amplify the *cpcE* gene (slr1878) were 5′-ATT GAC GTC GAC AAG GAG CTT GCA TAT GAG TGA ACC TAA CCT CAA CCC CG-3′ and 5′-GTG CGG CCG CGA CGC TTT TAG AGT AAA CTA TCC ATT AAT TCC AAT ATT TGT CG-3′. The resulting 0.85-kb PCR fragment was digested with restriction enzymes *Sal*I and *Not*I and cloned into *Sal*I- and *Not*I-digested pBS350V, giving plasmid pBS406V. Primers used to amplify the *cpcF* gene (sll1051) were 5′-ATT AGC GGC CGC TAG GAG GGC TAA CAT ATG GAG GGT AAT AGC GTC GTA ACT CC-3′ and 5′-GAG GGA TCC TAG AAG ACT AGA TTG GGC CGA TGT TTT CCA GG-3′. The resulting 0.7-kb product was digested with restriction enzymes *Eag*I and *Bam*HI and cloned into *Eag*I- and *Bam*HI-digested pBS350V, giving plasmid pBS407V.

Cloning of the Gene Encoding *Synechocystis* sp. PCC6803 Heme Oxygenase 1 (HO1). Primers used to amplify the *hox1* gene (sll1184; ref. 14) were 5′-CAT TGG ATC CAA GGT ACC AGG GGA TTT TTC ATA TGA GTG TCA ACT TAG CTT CCC AGT TGC-3′ and 5′-GTT GGC GCG CCA GGG AAG ACT AGC CTT CGG AGG TGG CGA G-3′. The resulting 0.75-kb product was digested with restriction enzymes *Bam*HI and *Asc*I and

cloned into *Bam*HI- and *Asc*I-digested pBS350V, yielding plasmid pBS421V.

Cloning of the Gene Encoding *Synechocystis* sp. PCC6803 3*Z*-Phycocyanobilin:Ferredoxin Oxidoreductase. Primers used to amplify the *pcyA* gene (slr0116) were 5′-CTT GGC GCG CCA TAG GAG TAT TTC ATA TGG CCG TCA CTG ATT TAA GTT TGA CC-3′ and 5′-TTC GCT GCA TGC TGG AGA GTC TTT TAT TGG ATA ACA TCA AAT AAG ACT TGG CTC ATA TAC-3′. The resulting 0.8-kb product was digested with restriction enzymes *Asc*I and *Sph*I and cloned into the *Asc*I- and *Sph*I-digested pBS350V, giving the plasmid pBS422V.

Design of Expression Vectors. Two expression vectors were designed, each containing a cassette of two or more *Synechocystis* sp. PCC6803 genes. The aim was to introduce them together into *E. coli* cells and thereby produce all of the catalytic functions and components required for the formation of phycocyanin holo-α subunit *in vivo*. The cassette in one vector, pBS414V, contained *cpcA* along with *cpcE* and *cpcF*, and provides all of the components (apo-CpcA, CpcE, and CpcF) known to be both necessary and sufficient for the correct addition of PCB to apo-CpcA (15). The cassette in the other vector, pAT101, contained *hox1* and *pcyA* (6), to provide enzymes required for the conversion of heme to PCB, the proximal precursor to the polypeptide-bound bilin (15). Construction of these cassettes is described below (see also Fig. 1).

Plasmid pBS414V Containing the *cpcA-cpcE-cpcF* Cassette. The *cpcF* gene, as a 0.75-kb *Bam*HI–*Eag*I fragment from pBS407V, was cloned into the *Bam*HI- and *Eag*I-digested pBS405V containing *ht-cpcA*, giving plasmid pBS412V. Subsequently, the *cpcE* gene, as a 0.85-kb *Sal*I–*Not*I fragment from pBS406V, was cloned into
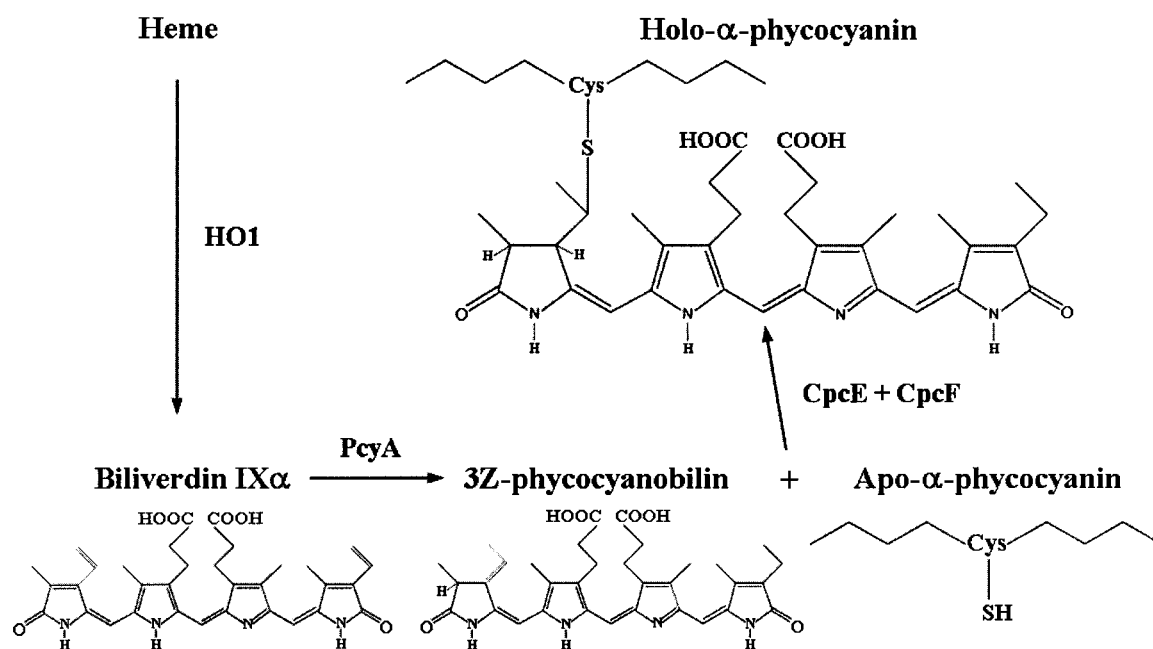
Fig. 2. Minimal biosynthetic pathway for the production of phycocyanobilin from heme and its addition to the C-phycocyanin apo-α subunit.

the *Sal*I- and *Not*I-digested pBS412V containing *ht-cpcA* and *cpcF*, giving the final cassette plasmid, pBS414V.

Construction of Plasmid pAT101 Containing the PCB Biosynthesis Cassette. The *hox1* gene, as a 0.7-kb *Nde*I–*Asc*I fragment from pBS421V, was cloned into the *Nde*I- and *Asc*I-digested pBS350V, giving plasmid pBS425V. Unlike pBS421V, pBS425V allows for expression of an N-terminal His₆-tagged HO1 (HT-HO1) fusion protein. Subsequently, the *pcyA* gene, as a 0.8-kb *Asc*I–*Sph*I fragment from pBS422V, was cloned into the *Asc*I- and *Sph*I-digested pBS425V containing *hox1*, giving plasmid pBS426V. As noted above, a more typical *E. coli* ribosomal binding site is present upstream of each of the ORFs in this cassette and for the ORFs in plasmid pBS414V.

Because plasmids pBS414V and pBS426V, containing the two distinct gene cassettes, were derived from within the same parental pBS350V with the ColE1 origin of replication, they are incompatible within the same *E. coli* cell. To maintain and express these two cassettes within the same *E. coli* cell, the use of different but compatible origins of replication was required. To achieve this goal, the 2.2-kb fragment generated from pBS426V by *Ssp*I (partial) and *Bsp*HI digestion, containing the *trc* promoter, the *lac* operator, and the genes encoding HO1 and 3Z-phycocyanobilin:ferredoxin oxidoreductase (PcyA), was cloned into the 2.77-kb *Bsp*HI- (partial) and *Psi*I-digested plasmid pACYC177 (GenBank accession no. X06402), which contains the p15A origin of replication (16) and confers resistance to Km. The β-lactamase gene in pACYC177 was completely removed in the cloning. The resulting pACYC177 derivative, pAT101, carries the genes required for PCB synthesis (*ht-hox1* and *pcyA*) and is compatible with pBS414V, which carries the genes encoding the heterodimeric phycocyanin α subunit PCB lyase (*cpcE* and *cpcF*) and HT-CpcA (*ht-cpcA*) (Fig. 1).

Isolation of His-Tagged Proteins by Immobilized Metal Affinity Chromatography. Cell pellets were thawed and resuspended in 20 ml of cold (0–4°C) buffer 0 (20 mM Tris·HCl, pH 8.0/50 mM NaCl/50 mM KCl). Phenylmethylsulfonyl fluoride and 2-

mercaptoethanol were added to final concentrations of 1 mM and 10 mM, respectively, immediately before breakage of cells by passage through a French pressure cell three times at 18,000 psi (1 psi = 6.89 kPa). Cell debris was removed by centrifugation at 4°C in a Beckman JA20 rotor at $30,000 \times g$ for 1 h. The supernatant solution was mixed with 2–3 ml of Ni²⁺-nitrilotriacetic acid agarose at 4°C for 15 min before the agarose was loaded onto a column. The agarose then was washed with 10 column volumes each of cold buffer A1 (20 mM Tris·HCl, pH 8.0/50 mM NaCl/50 mM KCl/20 mM imidazole/5% vol/vol glycerol), buffer B (20 mM Tris·HCl, pH 8.0/500 mM NaCl/500 mM KCl), and buffer A2 (20 mM Tris·HCl, pH 8.0/50 mM NaCl/50 mM KCl/30 mM imidazole). His-tagged proteins were eluted from the agarose with buffer C (20 mM Tris·HCl, pH 8.0/50 mM NaCl/50 mM KCl/200 mM imidazole) and then were dialyzed overnight with 50 mM sodium phosphate (pH 7.0).

Absorbance and Fluorescence Spectrometry. Absorbance spectra were acquired on a computer-controlled, dual-beam λ6 UV/VIS spectrophotometer (Perkin–Elmer). Corrected fluorescence spectra were obtained on a FP-750 spectrofluorometer (Jasco, Easton, MD). Excitation and emission slits were set at 5 nm for all measurements.

Results

A minimal biosynthetic pathway in cyanobacteria leading from heme to the formation of the cysteinyl adduct of PCB with CpcA (Fig. 2) is consistent with substantial *in vivo* and *in vitro* data (4, 6, 14, 15, 17–21).

To test the predictions of this scheme, two strains of *E. coli*, each carrying two expression vectors, were generated. To maintain the two plasmids simultaneously, double transformants were selected for resistance to both Sp and Km and were grown with both Sp and Km. *E. coli* strain DH5α(414V,101) carries plasmids pBS414V (*ht-cpcA-cpcE-cpcF* cassette, Sp^r) and pAT101 (*ht-hox1-pcyA* cassette, Km^r) and expresses all of the known genes believed to be involved in PCB biosynthesis and its addition to apo-CpcA (Fig. 2). The other *E. coli* strain, DH5α(405V,101),
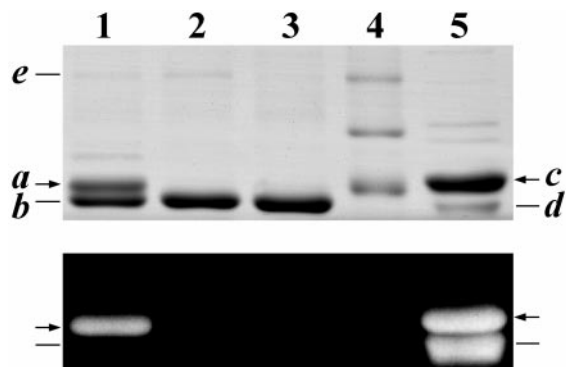
Fig. 3. SDS/PAGE analysis of *Synechocystis* sp. PCC6803 His-tagged proteins expressed in various *E. coli* strains and purified by immobilized metal affinity chromatography. Proteins were visualized by Coomassie blue stain (*Upper*) and by the UV-excited fluorescence of bilin-bearing polypeptides in the presence of Zn^{2+} (ref. 22; *Lower*). Lane 1, HT-CpcA (holo- and apo-HT-CpcA, bands a and b, respectively) expressed in *E. coli* strain DH5α(414V,101), which also expresses CpcE and CpcF, HT-HO1 (band e), and PcyA (see Fig. 1). Lane 2, HT-CpcA expressed in strain DH5α(405V,101), which also expresses HT-HO1 (band e) and PcyA, but not CpcE or CpcF. Lane 3, HT-CpcA expressed in strain DH5α(pBS405V), which does not express HO1, PcyA, CpcE, or CpcF. Lane 4, molecular mass standards (from the top): 30, 25, and 20 kDa. Lane 5, holo-HT-CpcA (21.9 kDa; band c) and the phycocyanin holo-β subunit (19.5 kDa; band d), both from *Anabaena* sp. PCC7120 (11). The difference in the relative intensities of the Coomassie blue-stained HT-α and β bands and of the fluorescence of these bands in the presence of Zn^{2+} is due to the fact that Coomassie blue stains the β subunit less strongly than the His-tagged α subunit, and that the β subunit carries two phycocyanobilins, whereas the α carries one.
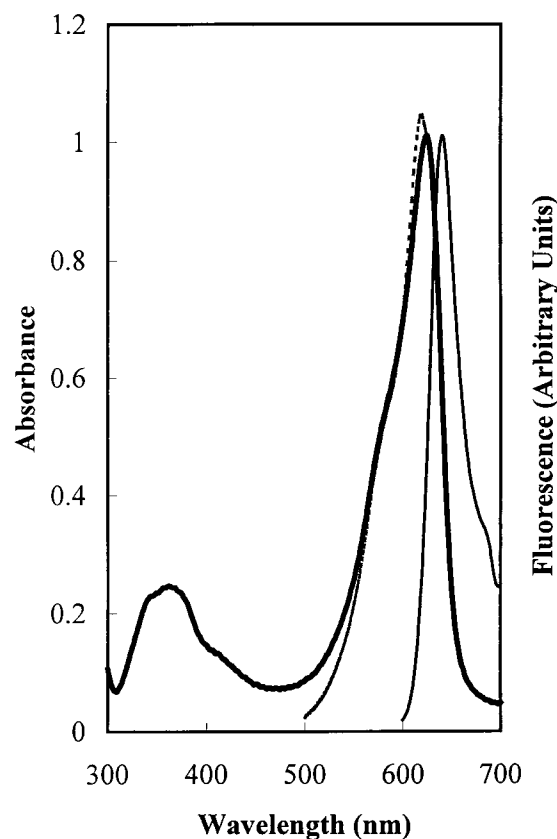

Fig. 4. Spectroscopic properties of *Synechocystis* sp. PCC6803 holo-HT-CpcA expressed in *E. coli*. Shown are the absorbance (λ_{max} 625 nm; thick solid line), fluorescence emission (λ_{max} 641 nm; λ_{exc} 580 nm; thin solid line), and fluorescence excitation (λ_{em} 645 nm; fluorescence excitation λ_{max} 619 nm; dashed line) spectra for HT-CpcA purified from *E. coli* by affinity chromatography. The fluorescence excitation spectrum was normalized to the absorbance spectrum at 625 nm.

carries plasmid pBS405V (expressing CpcA but lacking CpcE and CpcF, Sp^r) and plasmid pAT101. Comparison of the properties of the His-tagged CpcA proteins recovered from these two *E. coli* strains tests the stringency of the requirement for CpcE and CpcF for PCB addition *in vivo*. Previous studies have shown that HT-CpcA behaves just like untagged CpcA in cyanobacteria (11).

Expression of the gene cassettes in these two strains of *E. coli* led to two very different phenotypes. Upon induction with isopropyl β-D-thiogalactoside, the *E. coli* strain DH5α(414V,101) culture acquired a pronounced blue tint. This color change was not seen in the culture of DH5α(405V,101). HT-CpcA was purified from strain DH5α(414V,101) by affinity chromatography. Analysis of Coomassie blue-stained gels after SDS/PAGE of the purified protein showed two distinct bands in a ratio of $\approx$1:2, of 21.1 kDa (band a) and 20.5 kDa (band b), corresponding to the calculated molecular masses of His-tagged holo- and apo-CpcA, respectively (Fig. 3, lane 1). Upon exposure to Zn^{2+} (22) and UV illumination, only band a was fluorescent, indicating that it contained covalently attached bilin (Fig. 3, lane 1, *Lower*).

The ratio of bands a:b was unaltered in HT-CpcA preparations from DH5α(414V,101) cultures grown in medium supplemented with 5 μM δ-aminolevulinic acid (a precursor of heme biosynthesis), with 0.5 μM ferric ammonium citrate (enhances heme biosynthesis in *E. coli*), or with both (data not shown). The yield of holo-HT-CpcA, calculated from the 625-nm ($\varepsilon_M = 127,600$ $M^{-1}\cdot cm^{-1}$; see below) absorbance was $\approx$0.4 mg/g wet weight of *E. coli* cells.

Because apo-HT-CpcA does not absorb light above 320 nm, the spectroscopic properties of holo-HT-CpcA were determined without fractionation of the apo- and holo-HT-CpcA mixture. The absorbance spectrum of holo-HT-CpcA had a λ_{max} at 625 nm and a ratio of $A_{625\ nm}$:$A_{369\ nm}$ of 4.14 (Fig. 4). The corresponding values for *Synechococcus* sp. PCC7002 holo-CpcA are 620 nm and 4.22 (see figure 3 in ref. 5). The λ_{max} for *Anabaena*

sp. PCC7120 holo-HT-CpcA is at 622 nm with $\varepsilon_M = 109,000$ $M^{-1}\cdot cm^{-1}$ (Y.A.C., J. T. Murphy, G. J. Wedemayer, and A.N.G., unpublished data). The absorbance properties of native holo-CpcA preparations from other cyanobacteria are similar (e.g., ref. 23). The holo-HT-CpcA produced in *E. coli*, denatured in 8 M urea at pH 2, had a λ_{max} of 661 nm and a ratio of A_{352}:A_{661} of 1.03, values characteristic of polypeptide-bound PCB in the denatured cyanobacterial C-phycocyanin α subunit (24). From the acid urea spectrum, an ε_M of 127,600 $M^{-1}\cdot cm^{-1}$ at 625 nm in 50 mM sodium phosphate (pH 7) was calculated for the native holo-HT-CpcA. The fluorescence emission maximum of the holo-HT-CpcA produced in *E. coli* was at 641 nm (Fig. 4), as compared with 642 nm for *Synechococcus* sp. PCC7002 holo-CpcA (12), and 637 nm for *Anabaena* sp. PCC7120 holo-HT-CpcA (Y.A.C., J. T. Murphy, G. J. Wedemayer, and A.N.G., unpublished data). The excitation spectrum of the holo-HT-CpcA produced in *E. coli* corresponded well to the absorbance spectrum (see Fig. 4), indicating that only one red fluorescence-emitting species was present. The absorbance spectrum was determined at a holo-HT-CpcA concentration of 9.2×10^{-6} M, whereas the excitation spectrum was measured at 4.6×10^{-7} M; the small red shift in the peak of the absorbance spectrum relative to that of the excitation spectrum reflects the concentration-dependent red shift that accompanies dimerization of holo-CpcA (24). The fluorescence quantum yield of holo-HT-CpcA produced in *E. coli* was 0.31, as compared with 0.23 for *Anabaena* sp. PCC7120 holo-HT-CpcA (Y.A.C., J. T. Murphy, G. J. Wedemayer, and A.N.G., unpublished data).

Upon induction, DH5α(405V,101) expressed a level of HT-CpcA similar to that of DH5α(414V,101). SDS/PAGE analysis of HT-CpcA purified from DH5α(405V,101) showed a single major band with a molecular weight corresponding to apo-HT-CpcA, and exposure of the gel to UV illumination after exposure to Zn^{2+} indicated the complete absence of any fluorescent bands (Fig. 3, lane 2). When 10 times as much protein was loaded on the gel, a very weak fluorescence was detected in the corresponding band (data not shown). Spectroscopic analysis of the affinity-purified HT-CpcA fraction from DH5α(405V,101) indicated an upper limit of <1% to any bilin-bearing CpcA in the preparation (data not shown). The analysis of HT-CpcA purified from an *E. coli* strain expressing only CpcA is shown for comparison in Fig. 3 (lane 3). Thus, overall, the results show at most a trace of bilin addition to HT-CpcA in the absence of CpcE and CpcF.

Of the proteins produced upon induction of the *E. coli* strains described here, both CpcA and HO1 were His-tagged (see Fig. 1). HO1 was tagged in this study solely to check for protein expression from pAT101. It was anticipated that HT-HO1 (calculated mass 29.9 kDa) would copurify on the Ni^{2+} affinity column along with HT-CpcA, which indeed is the case. A weak band *e* with a mass of 30 kDa is seen in Fig. 3, lanes 1 and 2, where the purified His-tagged protein preparation was derived from strains carrying the *hox1* and *cpcA* genes, but is absent in lane 3, where the protein was obtained from an *E. coli* strain expressing solely the *cpcA* gene.

Discussion

The entire genome of *Synechocystis* sp. PCC6803 has been sequenced (12). This organism is particularly convenient for exploratory studies on phycobiliprotein biosynthesis, because PCB is the sole bilin attached to the phycobiliproteins in this cyanobacterium. The *Synechocystis* sp. PCC6803 genes encoding the phycocyanin α subunit, *cpcA*; both of the genes required for the conversion of heme to PCB, *hox1* and *pcyA*; and the two genes required for bilin attachment, *cpcE* and *cpcF*, encoding the heterodimeric phycocyanin α subunit PCB lyase, have all been identified (6, 12–14). These advances allowed the design described here of an *E. coli* strain wherein endogenous heme could be converted to PCB that would then be attached to apo-CpcA (Fig. 2). The ability to generate PCB within heterologous cells is critical. Earlier studies with yeast expressing CpcA, CpcE, and CpcF showed that even though soluble and active proteins were produced, no holo-CpcA formed upon PCB addition to the cells under a wide variety of conditions (ref. 25; B. Schroeder, J. T. Thorner, and A.N.G., unpublished observations). Evidently, intracellular PCB concentrations adequate for bilin addition to CpcA were not achieved.

We show here that upon induction, *E. coli* strain DH5α(414V,101) produces HT-HO1, PcyA, CpcE, and CpcF in active form and converts about one-third of the soluble apo-HT-CpcA produced into holo-HT-CpcA with endogenous heme. By SDS/PAGE and spectroscopic analyses, the holo-HT-CpcA is in all respects similar to the holo-CpcA produced in wild-type cyanobacteria. Because the addition of δ-aminolevulinic acid and/or iron did not affect the extent of PCB addition, the yield

of holo-CpcA appears not to be limited by the availability of heme. Rather, the lack of complete conversion may be due in part to codon usage (26) unfavorable for *E. coli* to generate large amounts of HO1, PcyA, CpcE, and CpcF. Codon usage does not seem to be a problem for the expression of *cpcA* in either *E. coli* or yeast (25). Another likely contributing factor is that in strain DH5α(414V,101) the copy number of the plasmid pAT101 is significantly lower than that of plasmid pBS414V (data not shown). Yet another unexplored issue is the fraction of each HO1, PcyA, CpcE, and CpcF that aggregates into inclusion bodies. Earlier studies of the expression of CpcA, CpcE, and CpcF in *E. coli* showed that a substantial amount of these proteins is found in the cell pellet (15). In a more complete list, there may yet be other, unidentified concerns.

The evidence that CpcE and CpcF are needed for the correct addition of PCB to apo-CpcA, both *in vivo* in various cyanobacteria (17–19) as well as *in vitro* (5, 15), is very strong. *In vitro*, nonenzymic addition of PCB to CpcA leads to the formation of an unnatural, oxidized adduct, mesobiliverdin-CpcA, with distinctive spectroscopic properties (20, 21); however, in the presence of CpcE and CpcF, normal holo-CpcA is produced (5, 15). The *E. coli* strain DH5α(405V,101) produces HO1, PcyA, and CpcA, but not CpcE and CpcF. In this strain no significant bilin addition of any kind takes place. Here, CpcE and CpcF are absolutely required for PCB addition to CpcA. Little, if any, of the spontaneous mesobiliverdin-CpcA adduct (20, 21) is formed, presumably because of the highly reducing conditions within the *E. coli* cell (e.g., ref. 27).

Two other bilin lyases with specificities different from those of CpcE and CpcF have been described to date. PecE plus PecF catalyzes the addition of PCB to phycoerythrocyanin apo-α subunit and the isomerization of the bound bilin to phycoviolobilin (28, 29). CpeY plus CpeZ has been reported to catalyze the addition of phycoerythrobilin to one of the bilin attachment sites on the α subunit of C-phycoerythrin (30). Additional phycobiliprotein-bilin lyases remain to be discovered. In addition to PcyA, which converts biliverdin to PCB, Frankenberg *et al.* (6) have described PebA, which converts biliverdin to 15,16-dihydrobiliverdin, and PebB, which converts 15,16-dihydrobiliverdin to 3Z-phycoerythrobilin.

Phycobiliprotein subunits are highly versatile as fusion partners (11). Consequently, the expression in prokaryotic or eukaryotic cells of an appropriate selection of genes encoding enzymes and apophycobiliprotein subunit-containing fusion proteins will lead to the intracellular production of constructs carrying specific bilins at unique locations, with broad utility in addressing a variety of questions in cell biology.

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