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Heterologous Assembly and Rescue of Stranded Phycocyanin Subunits by Expression of a Foreign *cpcBA* Operon in *Synechocystis* sp. Strain 6803

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Light harvesting in cyanobacteria is performed by the biliproteins, which are organized into membrane-associated complexes called phycobilisomes. Most phycobilisomes have a core substructure that is composed of the allophycocyanin biliproteins and is energetically linked to chlorophyll in the photosynthetic membrane. Rod substructures are attached to the phycobilisome cores and contain phycocyanin and sometimes phycoerythrin. The different biliproteins have discrete absorbance and fluorescence maxima that overlap in an energy transfer pathway that terminates with chlorophyll. A phycocyanin-minus mutant in the cyanobacterium *Synechocystis* sp. strain 6803 (strain 4R) has been shown to have a nonsense mutation in the *cpcB* gene encoding the phycocyanin β subunit. We have expressed a foreign phycocyanin operon from *Synechocystis* sp. strain 6701 in the 4R strain and complemented the phycocyanin-minus phenotype. Complementation occurs because the foreign phycocyanin α and β subunits assemble with endogenous phycobilisome components. The phycocyanin α subunit that is normally absent in the 4R strain can be rescued by heterologous assembly as well. Expression of the *Synechocystis* sp. strain 6701 *cpcBA* operon in the wild-type *Synechocystis* sp. strain 6803 was also examined and showed that the foreign phycocyanin can compete with the endogenous protein for assembly into phycobilisomes.

Phycobilisomes from the unicellular cyanobacterium *Synechocystis* sp. strain 6803 consist of the AP and PC biliproteins. We have recently characterized a PC-minus mutant, *Synechocystis* sp. strain 6803 (strain 4R) (15). This strain synthesizes phycobilisome cores without rods because of a single-base insertion in the *cpcB* gene, producing a truncated PC β subunit that leads to a complete absence of both PC subunits. The PC-minus phenotype of 4R suggested that this transformable cyanobacterium (20) would be a suitable genetic host for the introduction of heterologous biliprotein genes in studies focused on early events of biliprotein biosynthesis. We are particularly interested in analyzing structural differences between PC and PE subunits that direct recognition-dependent processes such as subunit assembly and chromophore attachment. These questions can be addressed by site-directed mutagenesis and protein domain exchange in a heterologous transformation system that employs the PC-minus strain of *Synechocystis* sp. strain 6803 as a host for expression of the *cpc* and *cpe* genes from *Synechocystis* sp. strain 6701 (1, 2, 10, 12). This report is an analysis of *Synechocystis* sp. strain 6803 transformants that express the *cpcBA* operon from *Synechocystis* sp. strain 6701. The results validate the heterologous transformation system as a research tool and demonstrate the rescue of the endogenous PC α subunit in 4R by heterologous assembly.

MATERIALS AND METHODS

Bacterial strains and culture media. *Synechocystis* sp. strain 6803 (wild-type [WT] and 4R strains) were grown as previously described (15). Liquid and solid media were supplemented with glucose (20 mM) and kanamycin sulfate (10 μg/ml) when necessary. The bacterial strains and plasmids used in this study are listed in Table 1.

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Plasmids, DNA manipulations, and transformation of 4R. Unless specified otherwise, all plasmid and DNA manipulations were performed according to published standard procedures (16). The plasmid p1BP1 is an 8.9-kbp derivative of pBR328 and was a gift from Dzelkalns and Bogorad (Department of Biology, Harvard University). It contains a 5.7-kbp fragment of *Synechocystis* sp. strain 6803 chromosomal DNA (inserted at the *Bam*HI and *Pst*I sites of pBR328) that is separated into 0.5- and 5.2-kbp flanking regions by a chloramphenicol acetyltransferase gene driven by an *Amaranthus psbA* promoter (8). The plasmid p1BP1 was cut with *Acc*I, treated with the Klenow fragment of *Escherichia coli* DNA polymerase to fill in restriction overhangs, and ligated upon itself, removing 1.6 kbp of pBR328 DNA and creating the 7.3-kbp plasmid pSTV01 (not shown). Plasmid pSTV01 was partially digested with *Eco*RI and *Bgl*II restriction enzymes and was ligated to a *Bam*HI-*Eco*RI fragment that contained the kanamycin resistance gene from Tn*903* under the control of its own promoter. This step removed 1.3 kbp of *Synechocystis* sp. strain 6803 DNA and created the 7.2-kbp cyanobacterial transformation vector pSTV02K (Fig. 1). Construction of pSTV20 is discussed in the results section of this paper.

Synechocystis sp. strain 6803 is naturally competent for transformation by homologous recombination into the chromosome (20), and transformants are generally obtained by exposure of cells to plasmid DNA and then by expression of the selective genetic markers in nonselective media and subsequent transfer to selective media. The *cpcBA* operon from *Synechocystis* sp. strain 6701 was incorporated into *Synechocystis* sp. strain 6803 by transformation with pSTV20 (Fig. 1). Mid-log-phase cultures of 4R and WT (grown in BG-11/glucose) were harvested by centrifugation and were resuspended in 1/20 volume of fresh BG-11 (no glucose). A 5-μg aliquot of pSTV20 was added to 0.5 ml of 4R cells in a 1.5-ml microfuge tube and placed under fluorescent lighting for 6 to 8 h. The DNA-cell mixture was then diluted into 10 ml of BG-11 (no glucose), and the culture was bubbled with 1 to 2% CO_2-air under standard light conditions for 3 days at room temperature. Cells were then harvested by centrifugation and resuspended in 1 ml of BG-11 medium. Aliquots of 0.2 and 0.1 ml were spread on BG-11 plates supplemented with glucose and kanamycin sulfate. Kanamycin-resistant colonies were obtained from plates after 2 weeks in standard culture conditions and were purified and segregated to phenotypic homogeneity by serial dilution streaks on BG-11 plates with kanamycin and glucose.

Characterization of DNA and RNA. Nucleic acids were isolated and analyzed by Southern and Northern (RNA) hybridizations according to methods described in previous publications (1, 2). A 900-bp *Nsi*I-*Sca*I fragment of the *Synechocystis* sp. strain 6701 *cpcBA* operon (1) and a 1.6-kbp *Avr*II-*Spe*I fragment of the *Synechocystis* sp. strain 6803 *cpcBAHID* operon (15) were used as gene- and transcript-specific probes for hybridization studies.

Isolation and spectrophotometric analysis of phycobilisomes and biliproteins. Cyanobacteria were harvested at cell densities of 2×10^7 to 3×10^7 cells per ml. Phycobilisomes were isolated on linear sucrose gradients according to estab-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant property(ies)	Reference or source
E. coli		
DH5α	Host strain for plasmid propagation	Gibco BRL
Synechocystis sp. strain 6803		
WT	Wild-type strain; *cpcB6803*$^+$ *cpcA6803*$^+$	V. Dzelkalns
4R	*cpcB6803* (Am)	V. Dzelkalns, 15
R20	4R:*cpcB6701*$^+$ *cpcA6701*$^+$; Kan^r by pSTV20	This study
W20	WT:*cpcB6701*$^+$ *cpcA6701*$^+$; Kan^r by pSTV20	This study
Plasmids		
pBluescriptSK$^-$	Amp^r	Stratagene
pPC02	*cpcB6701*$^+$ *cpcA6701*$^+$ in pBluescriptSK$^-$	This study
p1BP1	5.8 kbp of 6803V DNA in pBR328; Cm^r	V. Dzelkalns (8)
pSTV02K	Kan^r Cm^r; derived from p1BP1	This study
pSTV20	*cpcB6701*$^+$ *cpcA6701*$^+$ in pSTV02K; Kan^r	This study

lished procedures (3), with the exception that cyanobacterial cells were broken by agitation with 0.1-mm-diameter zirconium beads, with 1-min pulses with 1-min rest intervals performed three times in a Bead-Beater (Biospec Products). *Synechocystis* sp. strain 6803 PC was purified from a 65% ammonium sulfate precipitate of cleared supernatant by two rounds of ion-exchange chromatography on DEAE-Sephacryl (Pharmacia) according to the method described by Glazer and Fang (11) and then by chromatofocusing in phosphate on PBE 94 (Pharmacia) with a pH gradient from 7.0 to 4.0 (3). Absorbance spectra were obtained on a Shimadzu UV2101PC spectrophotometer from cell cultures that were normalized to near equivalent A_{680} values. The A_{400} to A_{800} spectra of intact cyanobacteria were measured through the frosted side of quartz cuvettes to compensate for light scattering by the cells. Room temperature fluorescence emission spectra of intact cells diluted in 0.5 M Na_2HPO_4 (pH 7.8) were obtained with a SPEX Fluoromax spectrofluorimeter equipped with a Hamamatsu R928P photomultiplier tube. Samples were excited at 580 nm (1-mm slit), and emission was measured from 595 to 700 nm (1-mm slit) and was corrected for photomultiplier tube response in the longer wavelengths.

Protein methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the buffer system of Laemmli (13) with buffer modifications and acrylamide gradient as published previously (3). Reverse-phase high-pressure liquid chromatography (HPLC) was performed on a Beckman Gold system with a Vydac C_4 column (214TP54) and the mobile phase and gradient described by Swanson and Glazer (19). Samples were prepared for HPLC by exhaustive dialysis against 50 mM sodium phosphate buffer (pH 7.2) and were denatured in acidic urea prior to injection (19). The amount of protein applied to the column was 50 to 150 μg, depending on the preparation. Protein elution was monitored at 360 nm for the absorbance of the bilin chromophores, and the Beckman Gold system software was used to integrate areas under peaks in elution profiles. Protein samples for N-terminal sequence analysis were taken directly as eluted from the column and were sequenced at the Molecular Biology Resource Facility (Ken Jackson, Director) at the University of Oklahoma Health Sciences Center.

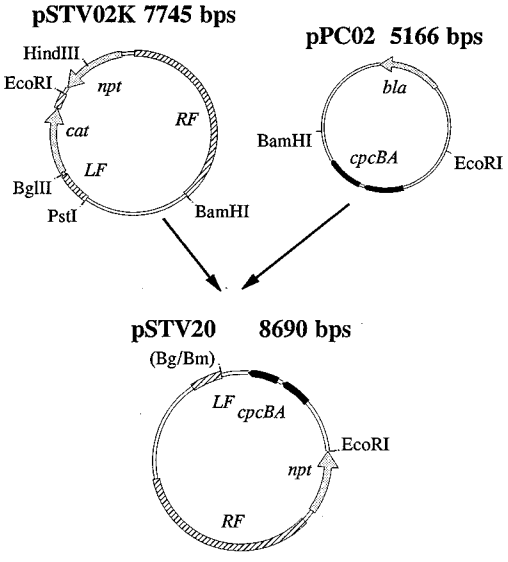

FIG. 1. Construction of transformation vector pSTV20. Plasmid pSTV02K was cut with *Bgl*II and *Eco*RI restriction endonucleases; the insert DNA containing *cpcBA6701* was obtained by cutting pPC02 with *Bam*HI and *Eco*RI and was ligated with the pSTV02K fragment, yielding pSTV20. *npt*, neomycin phosphotransferase; *cat*, chloramphenicol acetyltransferase; *bla*, β-lactamase; LF and RF, left- and right-flanking DNA regions containing chromosomal DNA from *Synechocystis* sp. strain 6803 WT, respectively.

RESULTS

Construction of cyanobacterial transformation vectors. *Synechocystis* sp. strain 6803 is naturally competent for genetic transformation, and foreign genes can be inserted into the chromosome by homologous recombination when the gene inserts are flanked by regions of *Synechocystis* sp. strain 6803 chromosomal DNA (20). We constructed the cyanobacterial transformation vector pSTV02K (Fig. 1) to provide a neutral recombination site for the expression of foreign genes in *Synechocystis* sp. strain 6803. The cyanobacterial DNA in pSTV02K is derived from a region of *Synechocystis* sp. strain 6803 DNA that was tagged by insertion of a chloramphenicol resistance gene through UV irradiation-induced nonhomologous recombination (8). Transformation of WT and 4R strains with pSTV02K produced kanamycin-resistant clones with no observable alteration of the original phenotypes. Plasmid pPC02 is a pBluescriptSK$^-$ (Stratagene) derivative that contains a 2.3-kbp fragment of *Synechocystis* sp. strain 6701 DNA that encodes the *cpcBA* operon (1). Digestion with *Eco*RI and either *Bam*HI or *Bgl*II produced vector (pSTV02K) and insert (from pPC02) fragments that were ligated to yield pSTV20 (Fig. 1), a transformation vector containing the promoters and coding region of the *cpcBA* operon from *Synechocystis* sp. strain 6701. Homologous recombination between the left- and right-flanking (LF and RF, respectively) regions of the vector (Fig. 2a) and *Synechocystis* sp. strain 6803 DNA will incorporate the *cpcBA* and *npt* genes into the host genome and will provide for selection of transformants on the antibiotic kanamycin.

Expression of *Synechocystis* sp. strain 6701 *cpcBA* in R20. The 4R strain appears olive green because of the apparent absence of PC (15). Transformation of 4R with pSTV20 produced kanamycin-resistant colonies that displayed a blue-green pigmentation similar to the WT strain of *Synechocystis* sp. strain 6803. Transformants were isolated and segregated to phenotypic homogeneity by serial transfers on solid medium, and a representative clone, R20, was selected for further anal-

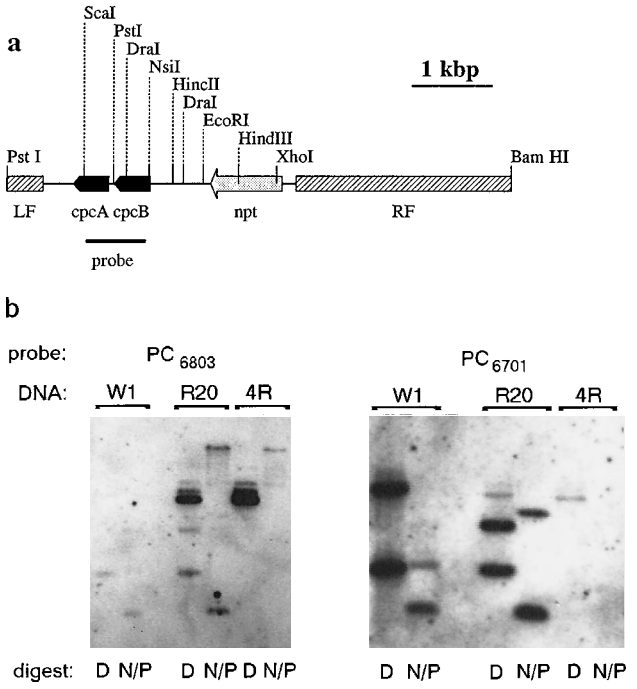

FIG. 2. Presence of *cpcBA6701* in R20. (a) Restriction endonuclease site map of insert DNA in pSTV20. The probe for PC6701 is identified by the bar under *cpcBA6701*. Diagnostic fragments for *cpcBA6701* are a 0.8-kbp *Dra*I fragment and a 0.5-kbp *Nsi*I-*Pst*I fragment. (b) Southern hybridization analyses of DNA from *Synechocystis* sp. strain 6701 (lanes W1), and *Synechocystis* sp. strain 6803 R20 and 4R with PC6803 and PC6701 probes. R20 and 4R show the homologous hybridization with PC6803, while W1 and R20 show weaker, identical signals indicating heterologous hybridization with *cpcBA6701*. The PC6701 probe hybridizes to the diagnostic *Dra*I and *Nsi*I-*Pst*I fragments in W1 and R20 that are absent in 4R. Gel images were obtained with a UVP Imagestore 7500 and were composed as figures with Aldus Photostyler software on PC-compatible hardware. Abbreviations are as defined in the legend to Fig. 1.

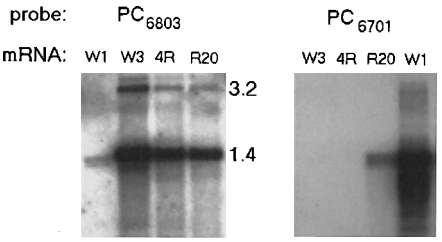

FIG. 3. Northern hybridization analysis of transcripts from *cpcBA6701* and *cpcBA6803*. DNA probes are identical to those used in Southern hybridizations. mRNA was loaded at approximately 5 μg/lane. W1, mRNA from *Synechocystis* sp. strain 6701 WT; W3, mRNA from *Synechocystis* sp. strain 6803 WT; 4R, mRNA from 4R; R20, mRNA from the R20 transformant. Approximate sizes of transcripts in W3 are indicated at 3.2 and 1.4 kb. Gel images were obtained with a UVP Imagestore 7500 and were composed as figures with Aldus Photostyler software on PC-compatible hardware.

ysis. R20 has maintained its phenotype on solid medium for more than 2 years through many serial transfers.

Southern hybridizations were conducted with R20 DNA to establish that the *Synechocystis* sp. strain 6701 *cpcBA* operon was successfully incorporated into the genome. Figure 2a shows the restriction endonuclease map for the insert DNA (*cpcBA* and *npt*) flanked by the LF and RF genomic recombination targets. Restriction digests of 4R and R20 chromosomal DNA were hybridized at high stringency with two different probes: an *Nsi*I-*Sca*I fragment of *Synechocystis* sp. strain 6701 DNA that included all of the *cpcB* gene and the first 70% of *cpcA* (1), and a 1.6-kbp *Avr*II-*Spe*I fragment that encodes most of *cpcBA* from *Synechocystis* sp. strain 6803 (15). The hybridization patterns with the PC6803 probe identified major signals specific to the endogenous *cpcBA6803* in 4R and R20 and showed heterologous hybridization to the *cpcBA6701* operon in *Synechocystis* sp. strains 6701 and R20 (Fig. 2b). The PC6701 probe lights up diagnostic fragments for *cpcBA6701* in the R20 transformant and not in 4R. The same DNA probes were used to examine mRNA levels in the WT, 4R, and R20 strains of *Synechocystis* sp. strain 6803 and in *Synechocystis* sp. strain 6701. The PC6803 probe hybridized to the expected 1.4- and 3.2-kb transcripts in WT, 4R, and R20 (15) and also displayed a heterologous hybridization signal with the *cpcBA6701* transcripts (Fig. 3). When probed with PC6701, WT and 4R showed no signal, whereas R20 and *Synechocystis* sp. strain 6701 both showed the dual transcripts that are characteristic of the *cpcBA6701* operon (1). The rRNA bands in each RNA sample were used for normalization of gel loads, and the heterologous signal (Fig. 3, lane W1; 6803 probe) and very strong homologous signal (lane W1; 6701 probe) indicate that *cpcBA* transcripts in *Synechocystis* sp. strain 6701 are present at levels higher than those of any *cpcBA* transcripts in the 6803 strains. Thus, the genetic evidence allows that the blue-green phenotype of R20 might derive from the presence and expression of the *cpcBA6701* operon in the 4R background.

Characterization of R20. The blue-green phenotype of the R20 transformant suggested that the *cpcBA6701* operon was complementing the PC-minus mutation in 4R. A comparison of whole-cell absorbance spectra for WT, 4R, and R20 cultures (Fig. 4a) showed that the PC content in R20 (as measured by the signal near 625 nm) was clearly increased relative to that in 4R but was less than the PC level in *Synechocystis* sp. strain

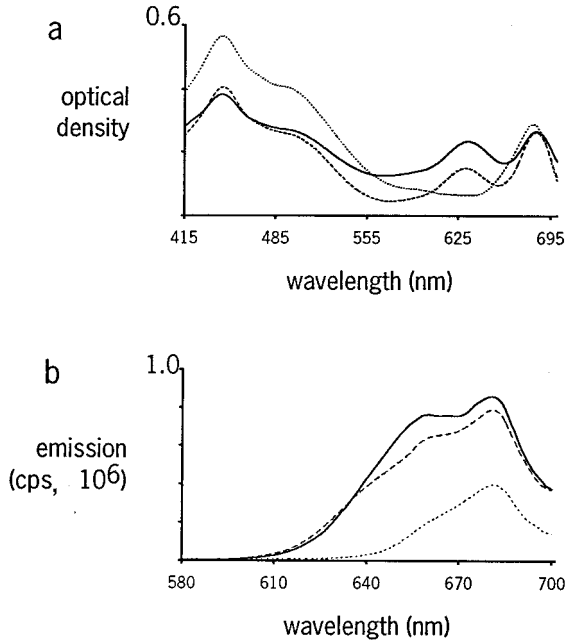

FIG. 4. Absorbance and fluorescence emission spectra for intact cells. (a) Absorbance spectra for WT (——), 4R (·····), and R20 (– – – –) cells. Cell densities for spectra were 2.1×10^7, 3.4×10^7, and 2.1×10^7 cells per ml for WT, 4R, and R20 cells, respectively. (b) Fluorescence emission spectra of intact cells and curve identifiers are the same as in panel a. Cell densities for spectra were 2.1×10^5, 4.3×10^5, and 2.6×10^5 cells per ml for WT, 4R, and R20 cells, respectively. cps, counts per second.

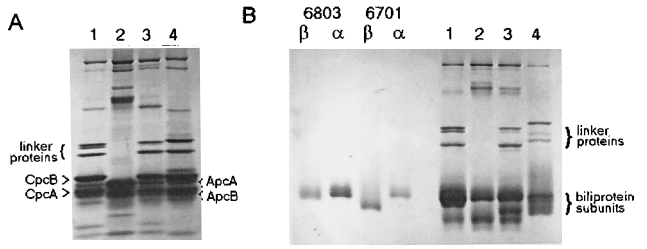

FIG. 5. SDS-PAGE analysis of phycobilisome preparations. (a) Standard SDS-PAGE of phycobilisome from *Synechocystis* sp. strain 6803 WT (lane 1), 4R (lane 2), and R20 (lane 3) and *Synechocystis* sp. strain 6701 SLGM (lane 4). Linker proteins and biliprotein subunits are identified. (b) SDS-PAGE in 8 M urea for isolated PC subunits, as indicated, and phycobilisomes. Lane designations are the same as in panel a. Gel images were obtained with a UVP Imagestore 7500 and were composed as figures with Aldus Photostyler software on PC-compatible hardware.

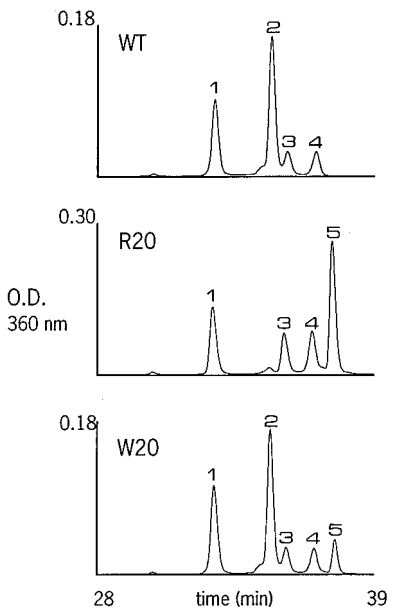

FIG. 6. Reverse-phase HPLC of phycobilisomes. Protein elution was monitored at 360 nm for phycocyanobilin absorbance. Elution profiles are presented for phycobilisomes from WT, R20, and W20 strains (see text). Peak 1, CpcA6701 and CpcA6803; peak 2, CpcB6803; peaks 3 and 4, ApcB and ApcA; peak 5, CpcB6701. OD, optical density.

6803 WT cells. Energy transfer from PC to AP in R20 cells was similar to that in WT cells, with prominent emission in the 685-nm region and minimal fluorescence near 638 nm, indicating the absence of significant amounts of uncoupled PC (Fig. 4b). Phycobilisomes isolated from R20 on linear sucrose density gradients migrated slightly behind the band of WT phycobilisomes on an identical gradient, suggesting less mass in the R20 complexes. The protein compositions of the R20 phycobilisomes were compared with those of WT, 4R, and a phycoerythrin-depleted mutant of *Synechocystis* sp. strain 6701, strain SLGM (Fig. 5a). Phycobilisomes from 4R completely lacked both PC subunits, and all of the rod-associated linker proteins in the 27- to 34-kDa mass range (the two major proteins in the 60- to 70-kDa range are not associated with phycobilisomes, but are contaminant proteins that cosediment with the 4R complexes on the sucrose gradient). R20 phycobilisomes contained both PC subunits (CpcA and CpcB) and all three rod linkers. The 31-kDa rod linker protein was decreased relative to the WT phycobilisomes and likely reflects the structural consequences of a decreased PC level in the R20 phycobilisomes.

Our analysis of the R20 strain was developed without knowledge of the mutation in *cpcB* in 4R (15), and a complete characterization required the ability to differentiate the endogenous PC subunits, encoded on the *cpcBA6803* operon, from the introduced PC encoded on *cpcBA6701*. Standard SDS-PAGE does not clearly distinguish the PC subunits of *Synechocystis* sp. strains 6803 and 6701 because of their similar masses (Fig. 5a). The addition of 8 M urea in SDS-PAGE can cause anomalous migration and can be used to separate proteins that comigrate under standard electrophoresis conditions (5). PC was purified from *Synechocystis* sp. strains 6803 and 6701, and the individual subunits were separated by reverse-phase HPLC (19). The subunits were then subjected to SDS-PAGE in 8 M urea along with phycobilisomes from strains WT, 4R, R20, and SLGM (Fig. 5b). These results demonstrate that the R20 phycobilisomes do not contain any *CpcB6803* and that the PC β subunits in these complexes must originate from the *cpcBA6701* operon. This result corresponds to subsequent work which established that intact CpcB cannot be synthesized in 4R because of a mutation in *cpcB* (15). Complementation of the PC-minus phenotype in R20 must be due, in part, to the synthesis of CpcB from the *cpcBA6701* operon. The urea gel also confirms that the linker proteins in R20 phycobilisomes derive from the endogenous *cpcBAHID* operon, since they comigrate with linkers in WT phycobilisomes (Fig. 5b). SDS-PAGE in urea does not allow a distinction between CpcA6701 and CpcA6803.

Further analysis employed reverse-phase HPLC to isolate and compare the PC subunits in phycobilisome preparations from R20 and WT. Purified PC preparations from *Synechocystis* sp. strains 6803 and 6701 were used to establish elution times for CpcA and CpcB from each of these organisms. CpcB6803 and CpcB6701 are separated by approximately 2.5 min, whereas CpcA6803 and CpcA6701 elute at the same time (data not shown). The WT phycobilisomes produced four major peaks on the basis of the A_{360} of the phycocyanobilin chromophores (Fig. 6). Peaks 1 and 2 represent the CpcA6803 and CpcB6803 proteins, respectively, while peaks 3 and 4 are the AP subunits. The R20 phycobilisomes do not contain CpcB6803 (peak 2) but do yield major peak 5, which is CpcB6701. Integration of peak areas allowed calculation of the relative PC content in each preparation and showed that the R20 phycobilisomes had 47% of the PC content found in the WT complexes.

Coelution of CpcA6803 and CpcA6701 in the HPLC gradient meant that peak 1 in the R20 profile could possibly contain both α subunits. Peak 1 was subjected to an N-terminal sequence analysis that would identify protein in this peak on the basis of the predicted amino acid sequences of MKTPL for CpcA6803 (14) and MSKTPL for CpcA6701 (1, 4). Initial analysis of the first five residues produced the sequence SKTPL, which corresponds with CpcA6701 on the assumption that the N-terminal methionine is cleaved after translation of the protein. In this case, the loss of methionine would follow the N-terminal cleavage rule as determined for *E. coli*, by which a second-site serine is associated with a high probability of scission for the primary residue (17). However, a secondary Met signal in the first cycle was of sufficient strength (about 10% relative to Ser) to warrant a more extensive sequence analysis. The PC α subunits from *Synechocystis* sp. strains 6701 and 6803 are more than 80% identical, with only seven differences in the first 50 amino acids. Cycle-specific heterogeneities for simultaneous sequence analysis of both subunits were predicted by

cycle #:	1	11	26	31
$CpcA_{6701}$:	SKTPLTEAVSAADSQGRFLSSTELQVAFGRF			
$CpcA_{6803}$:	M	T	I	L

FIG. 7. Predicted amino acid sequences for CpcA6701 and CpcA6803. Heterogeneity in the simultaneous N-terminal sequence analysis of CpcA6701 and CpcA6803 is expected to occur at cycles 1, 11, 26, and 31 in the first 31 cycles. All other displayed residues are identical between the two proteins.

alignment of the first 31 residues and assuming cleavage of N-Met for CpcA6701 (Fig. 7). The N-terminal residue for protein in peak 1 from R20 phycobilisomes was determined through 31 cycles. Significant secondary signals were observed in cycles no. 1, 11, 26, and 31 and were identified as Met, Thr, Ile, and Leu, respectively, by the sequencer operator without any knowledge of the CpcA6803 sequence, thus corresponding with the predicted heterogeneities in a simultaneous sequence analysis of CpcA6701 and CpcA6803. The minor signals averaged about 10% of the total signal in cycles no. 11, 26, and 31 (Table 2). These results establish that R20 phycobilisomes contain two types of CpcA: 90% derived from the imported *Synechocystis* sp. strain 6701 *cpcA* and 10% derived from the endogenous *Synechocystis* sp. strain 6803 *cpcA*.

In summary, expression of the *cpcBA6701* operon in R20 results in a complementation of the PC-minus phenotype of the parent 4R strain. CpcB6701 and CpcA6701 are capable of assembling with the endogenous rod linkers of R20 to produce a functional phycobilisome, although PC content in R20 is lower than in WT. A low level of CpcA6803 was rescued by heterologous assembly with the 6701 PC subunits, proving the functionality of this protein.

Expression of *cpcBA6701* in the WT strain. Analysis of the R20 strain showed that CpcA6701 and CpcB6701 could assemble with endogenous linker proteins in a PC-minus mutant background, but can these foreign subunits compete with intact endogenous PC for assembly sites in the phycobilisome? We transformed *Synechocystis* sp. strain 6803 WT with pSTV20 and selected for kanamycin-resistant clones. After a number of serial transfers on solid selective medium, clone W20 was selected for analysis, and phycobilisomes were prepared according to standard protocols. Standard SDS-PAGE of phycobilisome preparations (Fig. 8) shows that W20 phycobilisomes are similar in composition to the WT complexes. With regard to function, fluorescence energy transfer spectra for W20 phycobilisomes were not substantially different from those for the WT phycobilisomes (data not shown). Reverse-phase HPLC of W20 phycobilisomes (Fig. 5) indicates the presence of

TABLE 2. Amino acid sequence heterogeneity in PC α subunits isolated by HPLC

Source	Cycle no.	Amino acid	%[a]
R20	11	Alanine	90.3
		Threonine	9.7
R20	26	Valine	91.5
		Isoleucine	8.5
R20	31	Phenylalanine	89.5
		Leucine	10.5
R20	Avg[b]	Major peak	90.4
		Minor peak	9.6
W20	11	Alanine	21.0
		Threonine	79.0

[a] Calculated as percentage of area in net picomoles (both signals) after subtraction of background signals in the subsequent or previous cycle.
[b] Average of percentages over cycles 11, 26, and 31 for R20.

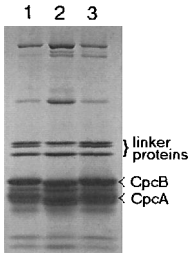

FIG. 8. SDS-PAGE analysis of phycobilisomes from WT, R20, and W20. Lanes: 1, WT phycobilisomes; 2, R20 phycobilisomes; 3, W20 phycobilisomes. The linker proteins and PC subunits are identified. Gel images were obtained with a UVP Imagestore 7500 and were composed as figures with Aldus Photostyler software on PC-compatible hardware.

CpcB6701 and CpcB6803 (peaks 2 and 5, respectively). Peak areas for PC subunits relative to AP show that W20 phycobilisomes have 12% greater PC content than the WT phycobilisomes and that 17% of the total CpcB in these structures is derived from the *cpcBA6701* operon. The CpcA fraction from HPLC (peak 1) was subjected to N-terminal sequence analysis through 11 cycles to determine if CpcA heterogeneity corresponded with the CpcB results. The major sequence indicated the presence of CpcA6803 (MKTPLTEAVST); however, cycles 1 and 11 had minor signals that corresponded to the CpcA6701 sequence (SKTPLTEAVSA). The heterogeneity in cycle 11 was calculated at 79% (Thr) and 21% (Ala) (Table 2). Since threonine recovery is not quantitative in N-terminal sequence analysis, it is possible that the relative amount of CpcA6701 in W20 phycobilisomes is overestimated at 21% and may be closer to the 17% composition determined for CpcB6701.

The isolation of phycobilisomes in linear sucrose gradients can produce a band of biliprotein material that is less massive than the intact phycobilisomes and contains AP and PC aggregates that may be assembly intermediates or dissociation products caused by the isolation procedure. We reasoned that this gradient fraction might contain a disproportionate amount of CpcB6701 if it represented assembly intermediates and if CpcB6701 is less competitive for assembly with endogenous phycobilisome components. Reverse-phase HPLC of this fraction yielded a relative CpcB6701 content of 15% of the total CpcB (data not shown), which is comparable to the amount of CpcB6701 found in the intact phycobilisomes.

Thus, expression of *cpcBA6701* in *Synechocystis* sp. strain 6803 WT can produce phycobilisomes with increased PC content and heterogeneous compositions in which approximately one-sixth of the PC is derived from the foreign operon.

DISCUSSION

Heterologous and competitive assembly in vivo. In the absence of assembly competition from the endogenous PC in R20, expression of CpcB6701 and CpcA6701 compensates for the mutation in *cpcB* of 4R (15) and complements the PC-minus phenotype of the parental strain. Heterologous assembly of the PC6701 subunits with the endogenous 28-, 31-, and 33-kDa linker proteins produces functional phycobilisomes with half of the PC content seen in the WT strain.

The relative levels of CpcB6701 and CpcA6701 in transformant phycobilisomes are significantly reduced in strain W20, in which the presence of an intact *cpcBA6803* operon results in two species of PC competing for assembly sites in the phycobilisome. The quantitative nature of the HPLC profiles allows determination of a structural stoichiometry based on the ratio

between peaks 1 (CpcA) and 4 (ApcA). The average phycobilisome rod contains 3.2 PC hexamers in WT and 3.6 PC hexamers in W20. The amount of PC6701 in W20 phycobilisomes is equivalent to 0.6 hexamers per rod (0.17 × 3.6 hexamers per rod), or about one trimer, and represents only 40% of the assembly potential that the PC6701 demonstrates in the R20 transformant (1.5 PC hexamers per rod). The lower level of PC6701 in W20 phycobilisomes relative to the R20 complexes could result from a number of factors. The transcription or translation of the *cpcBA6701* operon in W20 may be affected by expression of the intact *cpcBAHID6803* operon, minor structural differences between the two PC species may favor homogenous over heterologous assembly, or cotranscription and proximal translation of subunits and linkers from the *cpcBAHID6803* operon may promote the rapid formation of PC6803-linker complexes. Competitive assembly between two species of allophycocyanin has also been observed in transformants of *Synechococcus* sp. strain 7002, whereby expression from plasmids containing the *apcAB* operon from the plastid of *Cyanophora paradoxa* yielded phycobilisomes in which 9 to 13% of the allophycocyanin was derived from the foreign genes (7).

Rescue of CpcA6803 in R20. The 4R strain of *Synechocystis* sp. strain 6803 has been characterized as a PC-minus strain due to a mutation that causes premature termination of CpcB6803 (15). It is remarkable that CpcA6803 cannot be detected in 4R, given the high level of transcripts from the *cpcBA6803* operon. Analysis of 4R and other mutants indicates that a biliprotein subunit is not stably expressed when it cannot associate with its assembly partner. The mechanistic reasons for complete loss of stranded biliprotein subunits in mutants such as 4R may involve translational controls or the rapid proteolysis of unassembled material. The expression of CpcA6803 and its rescue by heterologous assembly with CpcB6701 in R20 eliminates a requirement of CpcB6803 expression for CpcA6803 synthesis. While the low level of CpcA6803 rescue in R20 phycobilisomes (about 5% of the CpcA content of WT phycobilisomes) does not exclude autogenous regulation, the rapid proteolysis of stranded biliprotein subunits remains the simplest explanation for their complete absence in mutants such as 4R (6, 15, 18).

It is clear that the synthesis of both β and α subunits is required for stable biliprotein expression. The rapid formation of a monomer, possibly promoted by the cotranscription-proximal translation of both subunits, would prevent degradation, and the low level of CpcA6803 in R20 phycobilisomes must result, in part, from the exposure of this protein as a stranded subunit immediately after synthesis. Since assembly of heterologous PC subunits into functional PC complexes has been demonstrated in vitro (11), it is reasonable to expect that CpcB6701 can form a monomer with CpcA6803 in vivo. Rescue of CpcA6803 probably occurs through subunit exchange with the pool of PC6701 complexes produced from *cpcBA6701* in R20. Subunit exchange could result from the dissociation equilibrium between monomer and individual subunits in the cell, or it could be promoted during assembly events, such as chromophore attachment, that may perturb subunit structure (9) and increase the probability of dissociation in a homogeneous monomer.

Conclusion. The rescue of CpcA6803 and the endogenous linkers by heterologous assembly in R20 phycobilisomes proves that absence of these proteins in 4R is not due to loss of protein function or inability to translate the *cpc* transcripts. This provides further evidence that the *cpcB* mutation is the basis for the PC-minus phenotype in 4R and that subunit interactions are necessary for biliprotein stability. The complementation of the 4R phenotype with the *cpcBA6701* operon provides a heterologous genetic system that can be used to examine molecular recognition in processes that are specific to PC or PE. Substitution of PE6701 residues or domains into the PC6701 subunits by site-specific mutagenesis and subsequent expression in 4R or its derivatives will identify changes that affect chromophore attachment or subunit binding.

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REFERENCES

1. **Anderson, L. K., and A. R. Grossman.** 1990. Genes for phycocyanin subunits in *Synechocystis* sp. strain 6701 and assembly mutant UV16. J. Bacteriol. **172:**1289–1296.
2. **Anderson, L. K., and A. R. Grossman.** 1990. Structure and light regulated expression of phycoerythrin genes in wild type and phycobilisome assembly mutants of *Synechocystis* sp. strain PCC 6701. J. Bacteriol. **172:**1297–1305.
3. **Anderson, L. K., M. C. Rayner, and F. A. Eiserling.** 1987. Mutations that affect structure and assembly of light-harvesting proteins in the cyanobacterium *Synechocystis* sp. strain 6701. J. Bacteriol. **169:**102–109.
4. **Apt, K. E., J. L. Collier, and A. R. Grossman.** 1995. Evolution of the phycobiliproteins. J. Mol. Biol. **248:**79–96.
5. **Bryant, D. A.** 1981. The photoregulated expression of multiple phycocyanin species. Eur. J. Biochem. **119:**425–429.
6. **Bryant, D. A.** 1991. Cyanobacterial phycobilisomes: progress towards a complete structural and functional analysis via molecular genetics, p. 257–300. *In* L. Bogorad, and I. K. Vasil (ed.), The photosynthetic apparatus: molecular biology and operation. Academic Press, Inc., San Diego, Calif.
7. **de Lorimier, R., G. Guglielmi, D. A. Bryant, and S. E. Stevens, Jr.** 1987. Functional expression of plastid allophycocyanin genes in a cyanobacterium. J. Bacteriol. **169:**1830–1835.
8. **Dzelkalns, V. A., and L. Bogorad.** 1986. Stable transformation of the cyanobacterium *Synechocystis* sp. strain PCC 6803 induced by UV irradiation. J. Bacteriol. **165:**964–971.
9. **Fairchild, C. D., and A. Glazer.** 1994. Oligomeric structure, enzyme kinetics, and substrate specificity of the phycocyanin a subunit phycocyanobilin lyase. J. Biol. Chem. **269:**8686–8694.
10. **Gingrich, J. C., L. K. Blaha, and A. N. Glazer.** 1982. Rod substructure in cyanobacterial phycobilisomes: analysis of *Synechocystis* sp. 6701 mutants low in phycoerythrin. J. Cell Biol. **92:**261–268.
11. **Glazer, A. N., and S. Fang.** 1973. Formation of hybrid proteins from the a and b subunits of phycocyanins of unicellular and filamentous blue-green algae. J. Biol. Chem. **248:**663–671.
12. **Glazer, A. N., D. J. Lundell, G. Yamanaka, and R. C. Williams.** 1983. The structure of a "simple" phycobilisome. Ann. Inst. Pasteur Microbiol. **134B:** 159–180.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
14. **Plank, T., and L. K. Anderson.** Unpublished data.
15. **Plank, T., C. Toole, and L. K. Anderson.** 1995. Subunit interactions and protein stability in the cyanobacterial light-harvesting proteins. **177:**6798–6803.
16. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
17. **Sherman, F., J. W. Stewart, and S. Tsunasawa.** 1985. Methionine or not methionine at the beginning of a protein. BioEssays **3:**27–31.
18. **Su, X., P. G. Fraenkel, and L. Bogorad.** 1992. Excitation energy transfer from phycocyanin to chlorophyll in an *apcA*-defective mutant of *Synechocystis* sp. 6803. J. Biol. Chem. **267:**22944–22950.
19. **Swanson, R. V., and A. N. Glazer.** 1990. Separation of phycobiliprotein subunits by reverse-phase high-pressure liquid chromatography. Anal. Biochem. **188:**295–299.
20. **Williams, J. G. K.** 1988. Transformation in the unicellular cyanobacterium *Synechocystis* sp. 6803. Methods Enzymol. **167:**766–778.