A Second Photochromic Bacteriophytochrome from *Synechocystis* sp. PCC 6803: Spectral Analysis and Down-Regulation by Light†

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ABSTRACT: It now appears that photosynthetic prokaryotes and lower eukaryotes possess higher plant phytochrome-like proteins. In this work, a second phytochrome-like gene was isolated, in addition to the recently identified Cph1 phytochrome, from the *Synechocystis* sp. PCC 6803, and its gene product was characterized photochemically. The open reading frame *sll0821* (designated *cph2* in this work) has structural characteristics similar to those of the plant phytochromes and the *Synechocystis* Cph1 with high amino acid sequence homology in the N-terminal chromophore binding domain. The predicted Cph2 protein consists of 1276 amino acids with a calculated molecular mass of 145 kDa. Interestingly, the Cph2 protein has two putative chromophore binding domains, one around Cys-129 and the other around Cys-1022. The Cph2 was overexpressed in *E. coli* as an Intein/CBD (chitin binding domain) fusion and in vitro reconstituted with phycocyanobilin (PCB) or phytochromobilin (PΦB). Both the Cph2−PCB and Cph2−PΦB adducts showed the typical photochromic reversibility with the difference spectral maxima at 643/690 and 655/701 nm, respectively. The Cys-129 was confirmed to be the chromophore binding residue by in vitro mutagenesis and Zn^{2+} fluorescence. The microenvironment of the chromophore in Cph2 seems to be similar to that in plant phytochromes. The *cph2* gene expression was dark-induced and down-regulated to a basal level by light, like the *cph1* gene. These observations suggest that *Synechocystis* species have multiple photosensory proteins, probably with distinct roles, as in higher plants.

Phytochromes are molecular photosensory switches that regulate a variety of light responses in plant photomorphogenesis (*1, 2*). Plants use them to perceive light signals in the forms of red/far-red wavelength ratio, intensity, direction, and duration and to mediate the regulation of gene expression involved in plant growth and development (*1, 3, 4*). One unique characteristic of the phytochromes is a photochromic transformation between two photochemically distinct forms, the red light absorbing Pr[1] and the far-red light absorbing Pfr forms (*5*). The photosensory Pr form is converted to the Pfr form by red light irradiation (670 nm). The physiologically active Pfr form is reverted to the Pr form by far-red light irradiation (730 nm).

The photochromic transformation accompanies intramolecular conformational changes (*6*−*8*), which subsequently trigger direct and indirect interactions of the C-terminal domains of the phytochromes with downstream signaling components, including G-proteins, cGMP, Ca^{2+}, and protein kinases/phosphatases, etc. (*9*−*12*). We recently reported that *Arabidopsis* nucleoside diphosphate kinase 2 (NDPK2) is activated by the Pfr form of phytochrome A in vitro, via the interaction with the C-terminal domain of the latter (*13*). In addition, a basic helix−loop−helix protein (PIF3) has been shown to interact with the C-terminal domain of phytochromes A and B in a light-dependent manner (*14, 15*). This suggests that a physical interaction between phytochromes and a transcriptional regulator plays a role in phytochrome-mediated light signaling.

Phytochromes are also present in lower photosynthetic eukaryotes (*16*−*18*) and prokaryotes (*19*) and nonphotosynthetic eubacteria (*20*). The bacterial phytochrome-like proteins are termed "bacteriophytochromes" (*20*). The first prokaryotic phytochrome-like sequence (*cph1*) was identified from the cyanobacterium *Synechocystis* sp. PCC 6803 (*19, 21*). The recombinant Cph1 phytochrome had histidine kinase and phosphotransferase activities, suggesting a role as a photosensory two-component signaling system with a cognate partner, Rcp1 regulator (locus *slr0474*) (*22*). The Cph1 protein has a transmitter domain of about 250 amino acids in the C-terminal region and a signal-input domain of about 500 amino acids in the N-terminal region (*23*). The N-

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[1] Abbreviations: CBD, chitin binding domain; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropylthio-β-galactoside; kDa, kilodalton(s); LB, Luria−Bertani medium; NDPK2, nucleoside diphosphate kinase 2; PCR, polymerase chain reaction; PCB, phycocyanobilin; PEB, phycoerythrobilin; PΦB, phytochromobilin; Pr, red light absorbing form of phytochrome; Pfr, far-red light absorbing form of phytochrome; PVDF, poly(vinylidene difluoride); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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terminal region also has a high amino acid sequence similarity to the chromophore binding sequences of plant phytochromes (*21*). Based on these structural characteristics, the Cph1 has been considered to be a chimeric sensory kinase with both phytochrome-like sensory and transmitter domains (*21, 23*). Further searching for phytochrome-like sequences in the genomic DNA sequence of *Synechocystis* sp. PCC 6803 identified another putative phytochrome-like sequence, the locus *sll0821* (*24, 25*). In this study, the locus *sll0821* (designated *cph2* in this work) was analyzed to see if it encodes a bona fide phytochrome-like protein.

The *cph2* gene encodes a protein of 1276 amino acids with a calculated molecular mass of 145 kDa. The recombinant Cph2 protein could ligate chromophores autocatalytically. The Cph2−chromophore adducts had the red and far-red light-dependent photoreversibility typical of plant phytochromes with the difference spectral maxima at 643/690 nm for the phycocyanobilin (PCB) adduct and at 655/701 nm for the phytochromobilin (PΦB) adduct, respectively. Interestingly, the predicted Cph2 protein has two putative chromophore binding sites, Cys-129 and Cys-1022. By in vitro mutagenesis and Zn^{2+} fluorescence, we showed that the Cys-129, not the Cys-1022, was the chromophore binding residue.

MATERIALS AND METHODS

Reagents and Enzymes. All chemical reagents used were purchased from Sigma (St. Louis, MO) unless specified otherwise. Restriction and modifying enzymes were obtained from New England BioLabs, Inc. (Beverly, MA). PCR reagents and PfuTurbo polymerase were from Stratagene (La Jolla, CA).

Synechocystis sp. Culture and Isolation of the cph2 Gene. Unicellular *Synechocystis* sp. PCC 6803 cells were grown at 30 °C in medium C (*26*) supplemented with glucose (1% w/v). Constant illumination was provided at a total photon fluence rate of 20 μmol m^{-2} s^{-1}. White light was supplied by a white fluorescent tube (Osram FL20EX-D, 40 W), and red light by Toshiba FL20S-R-F (20 W). The far-red light was obtained from an infrared fluorescent lamp (Toshiba FL20S-FR-74, 20 W) filtered through a cutoff infrared filter (NEC IR-1). Cells grown to mid-log phase (OD_{730} = 0.8−1.2) were used for the present work.

Genomic DNA was isolated by using the method of Porter (*27*) and further purified by using the DNeasy Plant Mini Kit (Qiagen, Santa Clara, CA). Polymerase chain reaction (PCR) was employed to amplify the *cph2* gene sequence from the *Synechocystis* genomic DNA by using specific primers and a PfuTurbo polymerase with the proofreading activity (Stratagene). About 0.5 μg of genomic DNA was used for each PCR run. The N-terminal primer was 5′-CG**CATATG**AACCCTAATCGATCCTTAG and had an *Nde*I site (boldface) for cloning purpose. The C-terminal primer was 5′-CTC**CCCGGG**AACTTCCCCATCAACAT-GGGG and had an *Sma*I site (boldface) to make an in-frame fusion with the Intein/CBD sequence in the pTYB2 *E. coli* expression vector. The PCR profiles were 25 cycles each at 94 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 7 min, and one additional cycle at 72 °C for 10 min. The C-terminal end of the cloned *cph2* sequence did not have its own stop codon. This resulted in a C-terminal translational fusion of the *cph2* sequence to the Intein/CBD sequence, 5′-Cph2-Intein-CBD-3′, in the pTYB2 vector (see below). The PCR product was first blunt-end-ligated into *Sma*I-digested pGEM3Z cloning vector for DNA sequencing.

Construction of the Fusion Plasmid. PCR products (*cph2* gene sequences) were double-digested with *Nde*I and *Sma*I and ligated into the pTYB2 vector (NEB) for expression in *E. coli*, resulting in pTYB2-Cph2 or pTYB2-Cph2MT (see below). All cloning and molecular biological procedures were performed according to the standard procedures (*28*). All junctions of the expression constructs were confirmed by DNA sequencing.

Reverse Transcriptase-Mediated PCR. To examine light effects on the expression of the *cph2* sequence, *Synechocystis* cells were grown under various light conditions as described above, and total RNA was isolated. The total RNA samples were further treated with RNase-free DNase to eliminate any contaminating genomic DNA. Reverse transcriptase (RT)-mediated PCR was carried out using total RNA and *cph2*-specific primers. About 2 μg of total RNA was used for each reverse transcription in a 20 μL reaction volume, and 1 μL of the reaction mixture was subjected to PCR amplification. The PCR profile was identical to that described above but with one exception. The amplification was performed 15 cycles to more accurately quantify the *cph2* expression. The *Synechocystis cph1* and the constitutively expressed RNase P RNA genes were included as controls. The PCR products were routinely analyzed on a 1.2% agarose gel.

Expression and Concentration of the Cph2. Coexpression with a bacterial thioredoxin markedly increases the solubility and native folding of foreign proteins expressed in *E. coli* (*29*). The Cph2 expression constructs (pTYB2-Cph2 and pTYB2-Cph2MT) and the pT-Trx vector, which had the *E. coli* thioredoxin gene under the T7 promoter, were cotransformed into an *E. coli* strain, ER2566. Double transformants were selected with 100 μg/mL ampicillin for the pTYB2 constructs and 30 μg/mL chloramphenicol for the pT-Trx vector, respectively. RB medium, rather than LB, was used for expression (*21*). Two hundred fifty milliliters of RB (0.5% yeast extract, 1% tryptone, 0.5% NaCl, 0.2% glucose, pH 7.5, with NaOH) supplemented with ampicillin (100 μg/mL) and chloramphenicol (30 μg/mL) was inoculated with 3 mL of freshly grown cell culture and incubated at 30 °C with shaking at 250 rpm to an OD_{600} of 0.6. Expression was induced by adding IPTG (1 mM) and further shaken at 30 °C for 3 h. After centrifugation at 5000*g* for 5 min, the cell pellets were washed once in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.8, 500 mM NaCl, 0.1% Triton X-100, 1 mM EDTA), then resuspended in 5 mL of lysis buffer, and lysed by repeated sonications on ice (with an output of 4−5, 30 s × 4 times). The homogenate was clarified by ultracentrifugation at 100000*g* for 30 min. The crude extract was concentrated 5 times using Amicon (Centriprep YM 30, Millipore) by centrifugation at 3000*g* for 2 h for in vitro reconstitution. The Cph2 fusion proteins were self-cleaved by 30 mM DTT at 4 °C and purified using a chitin affinity column as described by the manufacturer (NEB).

SDS−PAGE, Western Analysis, and Zn^{2+} Fluorescence. All protein preparations and purification steps were monitored by 10% SDS−PAGE using the Hoefer Mighty Small II system (Amersham-Pharmacia, Buckinghamshire, England). The gel was stained with 0.25% Coomassie Brilliant

Blue R250 for visualization. For Western blot analysis, the crude extracts containing the fusion proteins were analyzed on a 10% SDS−PAGE and transferred onto a PVDF membrane (Hybond-P$^+$, Amersham-Pharmacia). An anti-CBD primary antibody (NEB) was used to detect the Cph2 expression in the form of fusion proteins, according to the procedure described by the manufacturer.

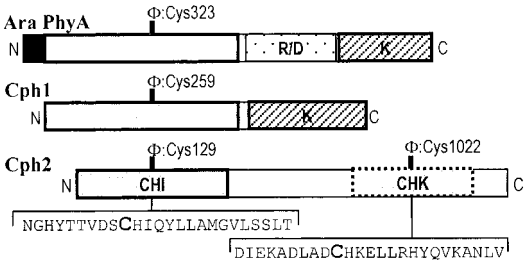
For Zn^{2+} fluorescence assays to assess the chromophore ligation, the protein samples were analyzed on a 10% SDS−PAGE and transferred onto a PVDF membrane as in Western transfers. The membrane was then soaked in 20 mM zinc acetate/150 mM Tris-HCl, pH 7.0, for 30 min at room temperature with gentle shaking and visualized under UV light (312 nm) (*30*).

In Vitro Reconstitution and Spectrophotometric Assays. Phytochromobilin (PΦB) was isolated from *Porphyridium sp*. UTEX 637 as previously described (*31*). Phycocyanobilin (PCB) was isolated from *Spirulina sp*. (Sigma) as described (*32*) but with some modifications. Crude PCB in methanol was diluted 10-fold with distilled water and mixed with chloroform. The PCB transferred into the chloroform layer was precipitated with hexane. Bilin stock solutions were prepared with DMSO and stored at −20 °C under darkness until use. All reconstitution experiments were performed under a green safety light as previously described (*33*). Apoprotein-containing crude extract was used after self-cleavage with 1 mM DTT at 4 °C overnight (*34*). The apoprotein/chromophore mixture was incubated at room temperature for 40 min in darkness, clarified by ultracentrifugation at 100000*g* for 10 min, and spectrally analyzed. Spectrophotometric measurements of the in vitro reconstituted PCB and PΦB adducts were carried out using a Cary 3 Bio UV−Vis spectrophotometer (Varian, Sugar Land, TX).

In Vitro Mutagenesis. The QuickChange kit (Stratagene) was used for in vitro mutagenesis as described by the manufacturer. Two *cph2*-specific complementary primers with base substitutions at the chromophore binding Cys-129 residue were used. The two complementary primers used were 5′-ACACAACAGTGGATAGT**TCT**CATATTCAATATCTC and 5′-GAGATATTGAATATG**AGA**ACTATCCACTGTTGTGT. The original TGT codon was mutated to the TCT (boldface) to replace the Cys-129 with serine. The His-130 was also substituted either with Phe or with Gln in the same way. The complementary primer pair for the His-130 to Phe substitution was 5′-CAACAGTGGATAGTTGT**TTT**ATTCAATATCTCCTTGCC (+ strand) and 5′-GGCAAGGAGATATTGAAT**AAA**ACAACTATCCACTGTTG (− strand). That for the His-130 to Gln substitution was 5′-CAACAGTGGATAGTTGT**CAA**ATTCAATATCTCCTTGCC (+ strand) and 5′-GGCAAGGAGATATTGAAT**TTG**ACAACTATCCACTGTTG (− strand). The original CAT codon was replaced with TTT (Phe) and CAA (Gln) (boldface). Mutated sequences were verified by DNA sequencing on both strands using an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA).

Synechocystis Cph1 Expression. The *Synechocystis cph1* gene (locus *slr0473*) was amplified from the *Synechocystis* genomic DNA by PCR, subcloned into the pTYB2 vector, and expressed in *E. coli* strain ER2566 in the same way as with the *cph2* gene. More than 90% of the expressed protein could be recovered in a soluble form. The yield was about 25 mg/L of culture in pure form (95% purity).

A. Cph2 Protein Structure



B. Alignment of Chromophore Binding Domains

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Cph2      EHS--NGHYTTVDSCHIQYLLAMGVLSSLTVPVMQDQ(129)
PHY1B     PISLAGSTLRGVHGCHAQYMANMGSVASLVMAVIIND(324)
Cph1      DLT--ESILRSAYHCHLTYLKNMGVGASLTISLIKDG(259)
AraA      DLTLCGSTLRAPHSCHLQYMANMDSIASLVMAVVVNE(323)
OatA3     DISLCGSTLRAPHSCHLQYMENMNSIASLVMAVVVNE(322)
PeaA      DLTLCGSTLRAPHSCHLQYMANMDSIASLVMAVVVND(323)
Cph2*     DIE--KADL---ADCHKELLRHYQVKANLVVPVVFNE(1022)
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FIGURE 1: (A) Cph2 protein structure. The Cph2 protein has two putative chromophore binding domains as indicated by CHI and CHK. Only the CHI domain can ligate tetrapyrrole chromophores. The typical structures of *Arabidopsis* phytochrome A (Ara PhyA) and the *Synechocystis* Cph1 are also shown. The black box indicates the N-terminal α-helix-forming motif. The gray box is the chromophore binding domain. The dashed box (K) is the histidine kinase domain. The dotted box (R/D) is the regulatory/dimerization domain, which is missing from the *Synechocystis* phytochromes. Chromophore binding cysteine residues are indicated. (B) Alignment of chromophore binding domains. Chromophore binding sequences of plant and *Synechocystis* phytochromes were aligned using ClustalW1.7. The fully conserved chromophore binding cysteine residues are in boldface. The second putative chromophore binding sequence of the Cph2 (*Cph2**) is shown in italics at the bottom. Numbers in parentheses are positions of the chromophore binding cysteine residue in each phytochrome. Dashes indicate gaps that were introduced to maximize the alignment. Amino acid residues conserved in at least three of the six sequences are shaded. Cph1 and Cph2, *Synechocystis slr0473* and *sll0821* ORFs, respectively. Other sequences were retrieved from the GenBank database. Phy1B, *M. caldariorum* phytochrome (S62714); AraA, *Arabidopsis* phytochrome A (P14712); OatA3, oat phytochrome A3 (P10931); PeaA, pea phytochrome A (P15001).

RESULTS

(a) Isolation and Expression of the Cph2 Protein. The locus *sll0821* gene sequence (designated *cph2* in this work) was isolated from the genomic DNA of *Synechocystis* sp. PCC 6803 and heterologously expressed in an *E. coli* expression system. The predicted Cph2 protein consists of 1276 amino acids with a calculated molecular mass of 145 kDa, which is somewhat larger than those of the plant phytochromes (about 1130 amino acids) and the *Synechocystis* Cph1 (748 amino acids). The deduced amino acid sequence has a sequence homology, with the fully conserved cysteine residue at position 129, to the chromophore binding domains of the plant phytochromes and *Synechocystis* Cph1 with 28% identity and 41% similarity over the range of amino acids 23−165 of the Cph2 (Figure 1B). However, the primary structure of the predicted Cph2 protein is different from those of other phytochromes. For example, the putative chromophore binding residue, Cys-129, is unusually close to the N-terminus compared to other phytochromes. In addition, one additional putative chromophore binding domain is located in the distal C-terminal region with a cysteine residue at position 1022 (23% identity and 36% similarity over the range of amino acids 925−1105 of the

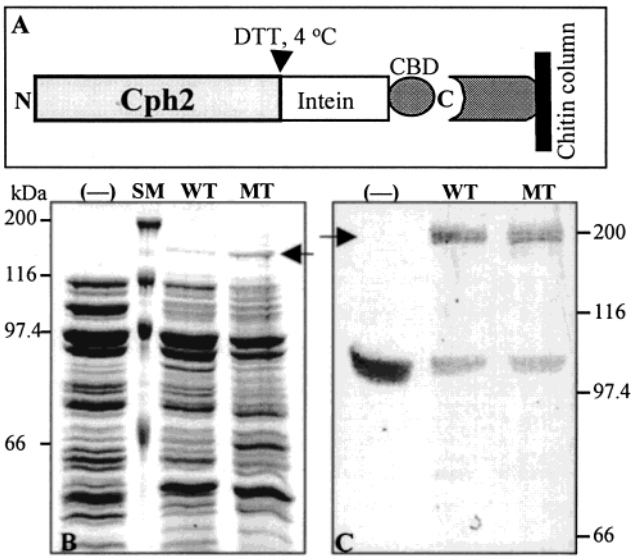

FIGURE 2: Expression of the Cph2 protein in *E. coli*. The Cph2 protein was expressed as an Intein/CBD fusion protein. (A) Structure of the Cph2−Intein/CBD tripartite fusion. The C-terminus of the Cph2 is in-frame-fused to the Intein/CBD peptide. (B) Cph2 expression in *E. coli*. Crude extracts, containing Cph2 fusion proteins, were analyzed on a 10% SDS−PAGE after DTT-induced self-cleavage (*49*). The Cph2 protein (145 kDa) is indicated by the arrow. (C) Western blot analysis. Crude extracts, containing the Cph2−Intein/CBD fusion proteins, were analyzed on a 10% SDS−PAGE and transferred onto a PVDF membrane. The Cph2 expression was examined using an anti-CBD primary antibody (NEB). The Cph2−Intein/CBD fusion (200 kDa) is indicated by the arrow. The lower molecular weight band is an intrinsic protein in *E. coli* that interacts with the anti-CBD antibody. CBD, chitin binding domain; (−), *E. coli* cells transformed with vector alone; SM, size markers; WT, wild-type Cph2; MT, mutant Cph2 (Cys129Ser); kDa, kilodaltons.

Cph2 to plant phytochromes) (Figure 1). Unlike the Cph1 phytochrome, the histidine kinase domain is not clearly identifiable in the C-terminal region of the Cph2 protein.

It was first expected, based on the efficient expression of the Cph1, that the Cph2 would be well expressed in a soluble form in *E. coli*. However, it was found that the Cph2 had a tendency to aggregate in the inclusion body when expressed in *E. coli.* Among several *E. coli* expression systems tested, the pTYB2 vector-based coexpression method gave the best result. The Cph2−Intein/CBD fusion protein was coexpressed with a bacterial thioredoxin via the pTYB2 *E. coli* expression vector (*29*). In this expression scheme, the 3′ end of the *cph2* gene was in-frame-fused to the Intein/CBD (chitin binding domain) sequence to generate a tripartite fusion, N-Cph2−Intein/CBD-C (Figure 2A). After self-cleavage, the resultant Cph2 protein had only two additional amino acids (proline and glycine) at the C-terminus as a result of the cloning procedure. Although most of the expressed Cph2 protein was precipitated in the inclusion body, about 250 μg in the crude form could be recovered from 1 L of *E. coli* culture in a soluble form after DTT-induced self-cleavage (Figure 2B). A Cph2 mutant (Cys129Ser) was also expressed in parallel to the wild-type Cph2. The mutant was generated by substituting the putative chromophore binding Cys-129 to serine to examine its chromophore ligating activity. Both the wild type and mutant Cph2 proteins were expressed to similar levels. To verify the Cph2 expression, an anti-CBD primary antibody was used in Western analysis by using the same protein preparation but without DTT-induced self-cleavage, as shown in Figure 2C. The fusion proteins of about 200 kDa were detected as expected.

(b) Spectrophotometric Analysis. Amino acid sequence analysis indicated that the predicted Cph2 protein has a putative chromophore binding domain in the vicinity of the Cys-129 residue in the N-terminal domain with the highly conserved amino acid residues as observed in plant and *Synechocystis* Cph1 phytochromes (Figure 1B). The recombinant Cph2 protein was first examined to see if it could be autocatalytically reconstituted in vitro with PCB and PΦB. The Cph2 protein in a crude form was treated with 1 mM DTT at 4 °C overnight and incubated with PCB or PΦB, and the mixture was spectrophotometrically analyzed (Figure 3A). PCB and PΦB were isolated from *Spirulina sp.* and *Porphyridium sp.*, respectively, and used for in vitro reconstitution assays (*33*, *35*). Both the Cph2−PCB and Cph2−PΦB adducts showed the typical photochromic reversibility characteristic of the plant phytochromes (Figure 3A). The difference spectral maxima of the Cph2−PCB adduct were at 643 and 690 nm after red (670 nm) and far-red (730 nm) light irradiation. The Pr and Pfr forms of the Cph2−PΦB adduct absorbed maximally at 655 and 701 nm, respectively. The difference spectral maxima of the Cph2−PCB adduct were 11−12 nm blue-shifted compared to those of the Cph2−PΦB adduct, as observed with other recombinant phytochromes (*21*). These observations suggest that the Cph2 protein of *Synechocystis* sp. PCC 6803 is a bona fide phytochrome.

(c) Spectral Comparison between the Cph1 and Cph2 Phytochromes. The recombinant Cph2 protein could be in vitro reconstituted with chromophores, and the Cph2−chromophore adducts showed the red and far-red light-dependent photoreversibility (Figure 3A). However, the Cph2 protein is structurally different from the Cph1 protein in several aspects, in addition to the size difference (145 kDa vs 85 kDa, respectively). No significant amino acid sequence homology was observed beyond the highly conserved chromophore binding domain. No apparent histidine kinase domain was identified. The chromophore binding cysteine residue of the Cph2, Cys-129, is unusually closer to the N-terminus than the Cys-259 of the Cph1.

To examine whether such structural differences give rise to any spectral differences, spectral profiles of the Cph1− and Cph2−chromophore adducts were compared. The Cph1 was expressed in *E. coli* and in vitro reconstituted with chromophores in the same way as for the Cph2, and the difference spectral maxima of the Cph1−chromophore adducts were obtained (Figure 3C). The difference spectral maxima of the Cph2−PCB adduct were 643 and 690 nm, whereas the Cph1−PCB adduct showed the spectral maxima at 655 and 707 nm, consistent with the previous report (*21*) (Figure 3A,C). Similar spectral differences were also observed between the Cph1− and Cph2−PΦB adducts. These show that the difference spectral maxima of the Cph2−PCB and −PΦB adducts are about 12−17 nm blue-shifted compared to those of the Cph1−PCB and −PΦB adducts, respectively.

It was suspected that the unusually close location of the Cys-129 of the Cph2 to the N-terminus conferred the spectral shift on the Cph2 adduct. However, all the recombinant Cph2 proteins expressed via different expression constructs, including the glutathione-*S*-transferase (GST) fusion at the

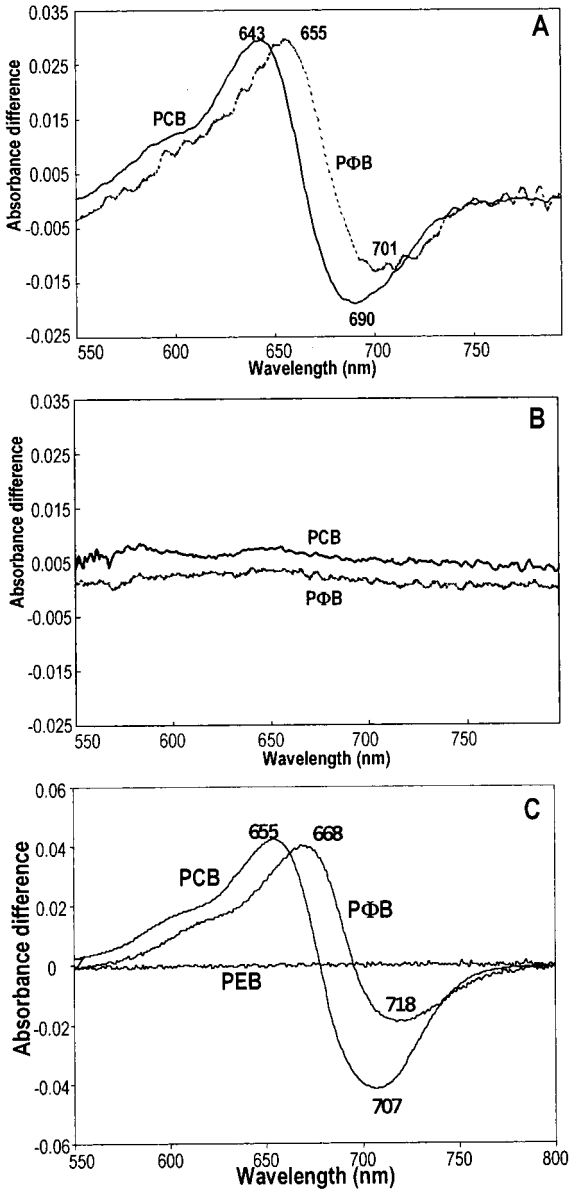

FIGURE 3: Spectrophotometric analysis of the Cph2− and Cph1−chromophore adducts. Crude extracts were incubated at 4 °C overnight in the presence of 1 mM DTT to remove the Intein/CBD peptides and in vitro reconstituted with PCB or PΦB as described (*33*, *35*). Spectrophotometric measurements were performed by using a Cary 3 UV−Vis spectrophotometer. (A) Wild-type Cph2 adducts. (B) Mutant Cph2 adducts (Cys129Ser). (C) Cph1 adducts. The Cph1 was expressed in *E. coli* and processed in an identical way to the Cph2 proteins. Numbers are wavelengths (nm) of the difference spectral maxima. PCB, phycocyanobilin; PΦB, phytochromobilin; PEB, phycoerythrobilin.

N-terminus, showed identical spectral profiles to those expressed via the pTYB2 vector (data not shown). The spectral difference between the Cph1 and Cph2 adducts may not be simply due to the unusual location of the Cys-129 of the Cph2 protein but reflect a Cph2-specific chromophore−apoprotein interaction (see Discussion) (*36*, *37*).

(d) Identification of the Chromophore Binding Residue. Unlike other phytochromes characterized so far (*38*), the Cph2 protein has an additional putative chromophore binding site in the vicinity of the Cys-1022 in the distal C-terminal domain, in addition to the Cys-129 (Figure 1B). Although the Cys-129 residue seemed to be more likely to be the chromophore binding residue, based on the highly conserved amino acid residues around the fully conserved cysteine residue (*38*), some of the highly conserved amino acids among different phytochromes were also identified in the vicinity of the Cys-1022 residue. To determine which cysteine residue is the chromophore binding residue, the Cys-129 was substituted with serine by in vitro mutagenesis. The mutant Cph2 protein was expressed, concentrated, and in vitro ligated with chromophores, in parallel to the wild-type Cph2. The mutated Cph2 holoprotein did not exhibit any photochromic activity (Figure 3B), indicating that the Cph2 apoprotein did not ligate the chromophore and that the Cys-129 is the chromophore binding residue in the Cph2 protein, as previously observed in similar experiments with plant phytochromes (*4*, *39*, *40*).

However, it was still possible that the Cys-1022 in the mutant Cph2 protein could actually bind chromophores but did not show the typical phytochrome-like photochromic activity. To examine this possibility, the wild-type and mutant Cph2 apoproteins in crude extracts were incubated with PCB under the same conditions, and the Cph2−PCB adducts were partially purified by chitin affinity chromatography. About 150 μg of the Cph2−PCB adducts could be recovered from 1 L of *E. coli* culture with a purity of about 50%, as judged by SDS−PAGE and Coomassie Brilliant Blue dye staining. The resultant Cph2 adducts were either analyzed by SDS−PAGE or visualized by Zn^{2+} fluorescence after transfer onto a PVDF membrane (Figure 4A), as previously described (*30*). The wild-type Cph2 adduct emitted strong Zn^{2+} fluorescence under the UV light. However, the Zn^{2+} fluorescence of the mutant Cph2 adduct drastically decreased, about one-fortieth of that of the wild-type Cph2. These results confirmed that the Cys-1022 did not have any significant chromophore ligating activity.

The recently identified bacteriophytochromes (BphPs) are unique in that they do not contain the conserved cysteine residue in the chromophore binding domains and that a histidine is used for the chromophore attachment through a Schiff base linkage (*20*). Although the mutated Cph2 apoprotein did not show any significant chromophore ligating activity, it was suspected that the His-130 of the Cph2 might be the site for the chromophore ligation and that the Cys-129 to Ser replacement abrupts the ligating activity of the His-130. To examine this possibility, the His-130 was replaced with Phe and Gln residues, resulting in H130F and H130Q, respectively. The two residues were chosen since substitutions of the corresponding histidine residue to Gln and Phe did not affect the chromophore ligating activity of the Cys residue in plant phytochromes (*37*). The mutant Cph2 proteins were ligated with PCB as with the wild-type Cph2, and their chromophore ligating activity was examined by Zn^{2+} fluorescence. Both mutant apoproteins efficiently assembled PCB in vitro (Figure 4B). The H130Q assembled the chromophore about 10% more efficiently than the wild-type Cph2. However, the chromophore ligating activity of the H130F mutant was 30−40% lower than that of the wild-type Cph2. This result clearly shows that the Cys-129, rather than the His-130, is the chromophore binding site. It also indicates that the His-130 to Phe and Gln replacements do not significantly affect the chromophore ligating activity of the Cys-129. In addition, these results are similar to those obtained with His-324 to Gln and Phe mutants of plant phytochromes (*37*).

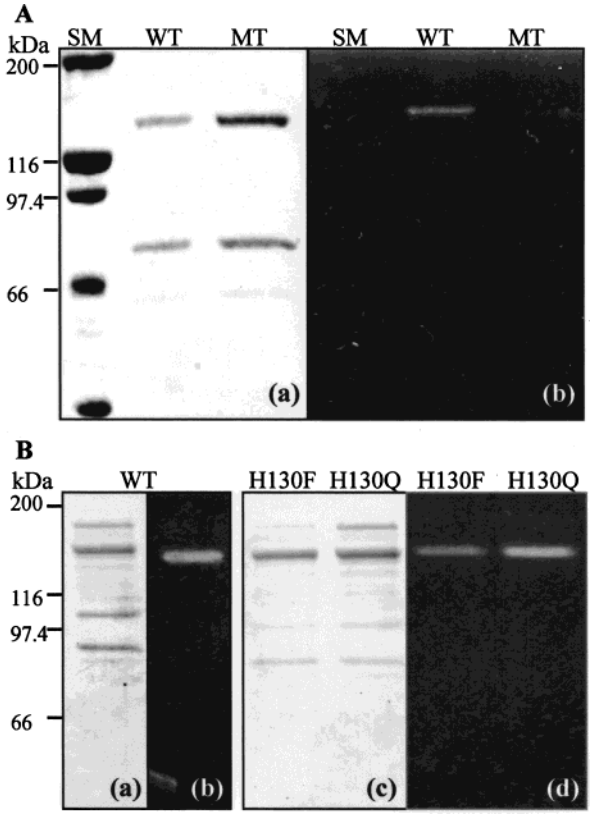

FIGURE 4: Zn^{2+} fluorescence of the mutant Cph2−PCB adducts. Crude extracts were in vitro reconstituted with PCB, and the Cph2 holoproteins were partially purified by chitin affinity chromatography and analyzed by Zn^{2+}-induced fluorescence. (A) Cys-129 to Ser mutant. (a) is the Coomassie Brilliant Blue dye-stained SDS−PAGE gel, and (b) is the Zn^{2+}-induced fluorescence. For Zn^{2+} fluorescence, the gel was transferred onto a PVDF membrane, and the membrane was soaked in 20 mM zinc acetate/150 mM Tris-HCl (pH 7.0) at room temperature for 30 min and visualized under UV light (312 nm) (*30*). SM, size marker proteins; WT, wild-type Cph2; MT, mutant Cph2 (Cys-129 to Ser). (B) His-130 to Gln and Phe mutant Cph2 proteins (H130Q and H130F, respectively). The mutant proteins were expressed and processed as in (A). (a) and (c) are SDS−PAGE gels, and (b) and (d) are Zn^{2+}-induced fluorescence. kDa, kilodaltons.

(e) Light-Dependent Expression of the cph2. To examine if the expression of the *cph2* sequence takes place in *Synechocystis* cells and if the *cph2* expression is regulated by light, total RNA was isolated from *Synechocystis* cells grown under various light conditions and subjected to RT-PCR. The total RNA was further treated with RNase-free DNase before RT-PCR runs to completely eliminate any contaminating genomic DNAs that frequently cause some false signals. The result showed that the *cph2* sequence was expressed in *Synechocystis* cells and that the *cph2* expression was down-regulated by white light and red and far-red light illuminations (Figure 5). The expression level was extremely low under the white light and highest in the dark. Red and far-red lights repressed the *cph2* expression. The light-responsive expression pattern of the *cph2* is similar to that of the *cph1* gene (*41*). The results suggest that the Cph2 is also involved in an adaptation process in light↔dark transitions of the *Synechocystis* cells, like the Cph1.

DISCUSSION

We showed here that the locus *sll0821 (cph2)* in the cyanobacterium *Synechocystis* sp. PCC 6803 encodes a phytochrome-like protein with the characteristic photochromic activity. The Cph2 protein shows highly conserved structural motifs of the plant and *Synechocystis* Cph1 phytochromes (*21, 23*). In particular, the chromophore binding domain was clearly recognized in the N-terminal region of the Cph2 (Figure 1B). The recombinant Cph2 apoprotein had the capacity to autocatalytically ligate chromophores, and the Cph2−chromophore adducts showed the photochromic transformation, a distinct characteristic typical of the phytochrome photoreceptors.

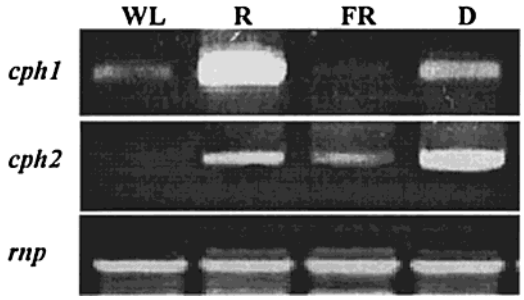

FIGURE 5: Expression of the *cph2* sequence in *Synechocystis* cells. Unicellular *Synechocystis* sp. PCC 6803 cells were grown under various light conditions as described under Materials and Methods, and total RNA was isolated. Light effects on the expression of the *cph2* sequence were examined by RT-PCR. About 2 μg of total RNA was used for reverse transcription in a 20 μL reaction volume, and 1 μL of the reaction mixture was subjected to PCR. The PCR products were analyzed on a 1.2% agarose gel and stained with ethidium bromide. The light-regulated *cph1* gene and the constitutively expressed RNase P RNA gene (*rnp*) were included as controls. WL, white light; R, red light; FR, far-red light; D, dark.

The Cph2−PCB adduct showed difference spectral maxima at 643 and 690 nm, and the Cph2−PΦB adduct at 655 and 701 nm, after red and far-red light irradiation, respectively. These spectral profiles are similar to those of the previously characterized *Synechocystis* Cph1 phytochrome (*19, 21*). However, the difference spectral maxima were about 12−17 nm blue-shifted compared to those of the Cph1−chromophore holoproteins. The Cph1−PCB holoprotein absorbed maximally at 655 and 707 nm, whereas the Cph2−PCB adduct absorbed maximally at 643 and 690 nm after red and far-red light irradiation, respectively. A similar spectral difference was also observed between the Cph1−PΦB and Cph2−PΦB adducts (Figure 3A,C). The spectrophotometric results of the Cph1 adducts were essentially identical to those of the previous report (*21*), indicating that the blue shifts were not derived from any experimental variations, such as different expression and purification systems used, but a genuine property of the Cph2 protein. In addition, both recombinant Cph2 proteins expressed by the pTYB2 and pRSETA vectors, which had different numbers of additional amino acids at either N- or C-termini, showed identical spectral profiles. These observations suggest that the blue shifts are derived from the Cph2-specific chromophore−apoprotein interactions as observed with the substitution mutants of pea phytochrome A (*36, 37*). Substitutions of the highly conserved amino acids, such as Tyr-327 to Trp, in the vicinity of the chromophore binding cysteine residue caused blue shifts in pea phytochrome A, probably as a result of altered chromophore−apoprotein interactions.

Some of the highly conserved amino acid residues around the chromophore binding cysteine residues of plant phyto-

chromes and *Synechocystis* Cph1 are not present in the Cph2 protein (see Figure 1B). For example, Arg-318 and His-321 in pea phytochrome A correspond to Thr-124 and D-127 in the Cph2 protein, respectively. In addition, Leu-325 and Asn-340 correspond to Ile-131 and Ala-136 in the Cph2, respectively (Figure 1B). These amino acid residues have been suggested to be involved in the chromophore−apoprotein interactions in plant phytochromes (*37*). The mechanism of the blue shifts could be resolved by substitutions of these amino acids.

The mutated Cph2 apoprotein (Cys-129 to Ser) could not efficiently bind chromophores, as judged by Zn^{2+} fluorescence (Figure 4A) and spectrophotometric analysis (Figure 3B). This observation is similar to those observed with the chromophore binding Cys to Ser mutants of plant phytochromes (*4, 40*). In addition, the Zn^{2+} fluorescence analysis confirmed that the Cys-1022 did not have significant chromophore ligating activity, at least in the full-size Cph2 apoprotein. It is still possible that there may be another noncysteine amino acid residue, such as histidine, as recently identified in the nonphotosynthetic eubacteria (*20*), which might link the chromophore and that its chromophore ligating activity is disrupted by the substitution at Cys-129. However, it is unlikely since the H130Q and H130F Cph2 mutant proteins can efficiently assemble the chromophore (*8, 37*). Furthermore, the H130Q more efficiently assembled the chromophore than the wild-type Cph2. These results are analogous to those obtained with H324Q and H324F mutants of the pea phytochrome A, suggesting that the microenvironments of the chromophores in the *Synechocystis* Cph2 and plant phytochromes are similar.

It is interesting that the fully conserved chromophore binding cysteine and the adjacent histidine residues as well as other highly conserved residues (but not all) in the vicinity of the Cys-1022 residue are insufficient for chromophore ligation. It would be interesting to introduce or mutate critical amino acid residue(s) that can induce chromophore binding at the Cys-1022 residue. The present results suggest that the locus *sll0821* indeed encodes a phytochrome-like protein, which is the second phytochrome identified from the *Synechocystis* sp. PCC6803 genome.

The presence of at least two phytochrome-like proteins in *Synechocystis*, Cph1 and Cph2, suggests that *Synechocystis* has multiple photosensory proteins as in higher plants (*42, 43*). They may have either overlapping or distinct roles. The *Synechocystis* Rcp1 (locus *slr0474*) is the cognate partner of the Cph1 phytochrome (*22*) and is closely related to the CheY bacterial response regulator that is involved in bacterial chemotaxis (*44*). It is therefore possible that the Cph1 is the photoreceptor for the cyanobacterial phototaxis as has been suggested (*45*). The Cph2 may have a complementary role in the phototaxis, depending on light conditions, analogous to the differential and complementary functions of plant phytochromes A and B (*1, 46, 47*). However, it is more likely for the Cph2 protein to have a distinct role because of its unique domain structure. Unlike the Cph1 and plant phytochromes, no discernible histidine kinase motif was identified from the C-terminal region of the Cph2 (*38*). Also, the Cph2 protein lacks the characteristic N-terminal α-helix-forming motif (*7, 48*), and the fully conserved Cys-129 is unusually close to the N-terminus. In addition, the sequence homology is restricted to the chromophore binding N-terminal region. These structural characteristics support the uniqueness of the Cph2 phytochrome and the hypothesis for distinct roles.

Both the *cph1* and *cph2* genes are expressed in *Synechocystis* cells in a light-dependent manner (*41*, this work). They are highly expressed in the dark, but their expression levels are very low in the light. However, the expression pattern of the *cph1* gene in our work is different from the previous one (*41*) in that it is most highly expressed in the red light. This difference may reflect different culture conditions. Although their physiological functions are currently unknown, the expression patterns of the *cph1* and *cph2* suggest some roles in light↔dark adaptation process or in the protection of *Synechocystis* cells against light. Generation and functional analysis of the *cph1* and *cph2* knock-out mutants will address this question. Work is currently under way to segregate the *cph1*, *cph2*, and *cph1/cph2* double knock-out mutants for a genetic analysis of the light responses.

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