Chromophore−Apoprotein Interactions in *Synechocystis* sp. PCC6803 Phytochrome Cph1[†]

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ABSTRACT: The secondary, tertiary, and quaternary structures of the *Synechocystis* Cph1 phytochrome were investigated by absorption and circular dichroism spectroscopy, size exclusion chromatography, and limited proteolysis. The Cph1 protein was coexpressed with a bacterial thioredoxin in *Escherichia coli*, reconstituted in vitro with tetrapyrrole chromophores, and purified by chitin affinity chromatography. The resultant Cph1 holoproteins were essentially pure and had the specific absorbance ratio (SAR) of 0.8−0.9. Circular dichroism spectroscopy and limited proteolysis showed that the chromophore binding induced marked conformational changes in the Cph1 protein. The α-helical content increased to 42−44% in the holoproteins from 37% in the apoprotein. However, no significant difference in the secondary structure was detected between the Pr and Pfr forms. The tertiary structure of the Cph1 apoprotein appeared to be relatively flexible but became more compact and resistant to tryptic digestion upon chromophore binding. Interestingly, a small chromopeptide of about 30 kDa was still predominant even after longer tryptic digestion. The N-terminal location of this chromopeptide was confirmed by expression in *E. coli* and in vitro reconstitution with chromophores of the 32.5 kDa N-terminal fragment of the Cph1 protein. This chromopeptide was fully photoreversible with the spectral characteristic similar to that of the full-size Cph1 protein. The Cph1 protein forms dimers through the C-terminal region. These results suggest that the prokaryotic Cph1 phytochrome shares the structural and conformational characteristics of plant phytochromes, such as the two-domain structure consisting of the relatively compact N-terminal and the relatively flexible C-terminal regions, in addition to the chromophore-induced conformational changes.

Genomic DNA sequence analysis of the cyanobacteria *Synechocystis* sp. PCC6803 revealed several open reading frames (ORFs) encoding proteins with amino acid sequences homologous to plant phytochromes (*1*). The locus *slr0473* (*cph1)* is the best characterized among them (*2*). The *cph1* gene encodes a protein of 748 amino acids with a molecular mass (MM) of 85 kDa.[1] The predicted Cph1 protein has structural similarities to plant phytochromes, such as the chromophore binding motif in the N-terminal region and the histidine kinase motif in the C-terminal region (*3, 4*).

The recombinant Cph1 apoprotein could be reconstituted autocatalytically with different tetrapyrrole chromophores, including phycoerythrobilin (PEB), phycocyanobilin (PCB), and phytochromobilin (PΦB) (*2, 3, 5*). The absorption spectra of the Cph1−chromophore holoproteins were similar to those of plant phytochromes in that two spectral forms, the Pr and Pfr phytochromes, are photoreversible by red and far-red light irradiation. In addition, it has been found that the Cph1 has those of Raman spectral profiles (*6*) and fluorescence and photochemical properties similar to plant phytochromes (*7*).

The Cph1−PCB holoprotein has been shown to have histidine kinase and phosphotransferase activities (*5*). The enzymatic activities are regulated in a red and far-red light-dependent manner in a similar way as has been recently demonstrated for the serine/threonine kinase activity of plant phytochromes (*8, 9*). In addition, the Cph1 protein has been implicated as the photosensor for the photomovement of *Synechocystis* (*10*). While physiological roles of the Cph1 is yet unknown, it is likely that the Cph1 is a photosensor protein acting in conjunction with the Rcp1 regulator, as a two-component signaling system similar to the bacterial binary sensory systems (*5*).

One additional phytochrome-like sequence (locus *sll0821*), which encodes a protein of 1276 amino acids (Cph2), has been expressed in *Escherichia coli* and reconstituted in vitro with chromophores (Park et al., unpublished experiment). The spectroscopic characteristics of the Cph2−PCB holoprotein are similar to those of the *Synechocystis* Cph1 and plant phytochromes. This indicates that *Synechocystis* also

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[1] Abbreviations: CD, circular dichroism; Cph1, cyanobacterial phytochrome apoprotein; EDTA, ethylenediaminetetraacetic acid; FPLC, fast-performance liquid chromatography; HPLC, high-performance liquid chromatography; kbp(s), kilobase pair(s); kDa, kilodalton(s); PCB, phycocyanobilin; PEB, phycoerythrobilin; PΦB, phytochromobilin; PMSF, pheynylmethylsulfonyl fluoride; Pr and Pfr, red and far-red light absorbing spectral forms of holoproteins, respectively; SAR, ratio of the absorbance maxima in the visible region to the absorbance at 280 nm for the Pr form; SDS, sodium dodecyl sulfate; SEC, size exclusion chromatography; [θ], mean residue molar ellipticity.

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has multiple phytochrome photoreceptors, like those in higher plants (*11, 12*).

It is of particular interest that, despite its prokaryotic origin, the Cph1 protein shares the structural, photochemical, and enzymatic characteristics with plant phytochromes. The presence of multiple phytochromes in *Synechocystis* cells mimics those in higher plants. Thus, structure−function studies of the Cph1 phytochrome are expected to provide useful information for those of plant phytochromes. The Cph1 protein (85 kDa) is smaller than that of the plant phytochrome (~125 kDa). The structural characteristics, such as the three-dimensional structure and conformational changes in photoperception and inter-domain signaling, can be more readily dissected with the Cph1 protein than with plant phytochromes. In addition, efficient expression and purification systems have been recently developed for the Cph1 protein (*2, 3*). These observations suggest that the Cph1 protein could be a useful model system for the study of crystal structure and phytochrome-mediated light signal transduction in plants (*3, 5*).

The quaternary structure of the Cph1 apoprotein has been proposed as an ellipsoid monomer with an apparent MM of 110−115 kDa in solution, as judged by size exclusion chromatography (SEC) FPLC (*3*). However, the SEC elution profile of the Cph1−PCB adduct had a predominant peak at a position of a protein with a MM of 170 kDa. When analyzed on native electrophoresis, the predominant peak has been found to be a monomer (*3*). These observations indicate that some conformational changes occur upon chromophore binding, as has been observed with plant phytochromes (*13*). In plant phytochromes, the phototransformation between the Pr and the Pfr forms accompanies subtle conformational changes (*13*). These include changes in the secondary (*14, 15*) and tertiary structures (*16*−*18*). Such conformational changes are structural prerequisites for the biological function of plant phytochromes.

Very little is known about the secondary and tertiary structures of the Cph1 phytochrome. No reports describing conformational changes upon chromophore binding and in the phototransformation between the Pr and Pfr forms appeared in the literature. In this work, we employed spectral assays, SEC, and limited proteolysis to elucidate the structural characteristics of the Cph1 protein. We found that the chromophore−apoprotein interactions, both covalent and noncovalent, induced significant conformational changes in the secondary and tertiary structures of the Cph1. The holoproteins were more resistant to proteolytic digestion than the apoprotein, indicating that the holoproteins form more compact globular conformation than the apoprotein and that the chromophore binding stabilizes the Cph1 structure.

MATERIALS AND METHODS

Construction of Expression Plasmids. The *cph1* gene (locus *slr*0473) was isolated from the *Synechocystis* sp. PCC 6803 genomic DNA by polymerase chain reaction (PCR) using specific primers and PfuTurbo polymerase (Stratagene, La Jolla, CA). The N-terminal primer was 5′-GCC**CATATG**-GCCACCACCGTACAAC and had an NdeI site (bold) for efficient cloning purpose. The C-terminal primer was 5-CTC**CCCGGG**GTTGCCAATGGGGATGGAG and had a SmaI site (bold) to make an in frame fusion to the Intein/CBD (chitin binding domain) sequence in the pTYB2 *E. coli* expression vector (NEB, Beverly, MA). The PCR was run 25 cycles each at 94 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 5 min and with 1 additional cycle at 72 °C for 10 min. The PCR product (2.3 kbp) was first blunt-end ligated into the SmaI-digested pGEM-3Z vector (Promega, Madison, WI) for DNA sequencing. The *cph1* sequence was then cut out by double-digestion with NdeI and SmaI from the pGEM-3Z and ligated into the pTYB2 vector. In this pTYB2−Cph1 expression construct, the *cph1* sequence is translationally fused to the Intein/CBD sequence, resulting in a 5′-Cph1−Intein−CBD-3′ tripartite fusion. All vector constructs were confirmed by direct DNA sequencing. All genetic manipulations were carried out according to the standard protocols (*19*).

Expression, Reconstitution, and Purification of Cph1−*Chromophore Holoproteins*. The pTYB2−Cph1 construct and the pT-Trx vector (*20*), which had the *E. coli* thioredoxin gene under the control of the T7 promoter, were cotransformed into a *E. coli* strain ER2566 (NEB). The *E. coli* cotransformants were selected with 100 μg/mL ampicillin for the pTYB2−Cph1 construct and 30 μg/mL chloramphenicol for the pT-Trx vector, respectively. The *E. coli* cells were cultured in LB medium at 37 °C overnight, and 3 mL of the culture was transferred into 250 mL of RB medium (0.5% yeast extract, 1% tryptone, 0.5% NaCl, 0.2% glucose, pH 7.5) supplemented with antibiotics and incubated at 30 °C until OD_{600} reached 0.6. The culture was then adapted to 20 °C for 1 h. Expression of the recombinant protein was induced by adding IPTG to a final concentration of 1 mM, and the culture was further incubated for 14−16 h at 20 °C. The cells were pelleted by centrifugation at 5000*g* for 5 min, washed with ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% Triton X-100, 1 mM EDTA). After centrifugation, the cell pellet was resuspended in ice-cold lysis buffer in a ratio of 2 mL/g of *E. coli* cells (wet pellet) and lysed by repeated sonications. The homogenate was clarified by centrifugation at 100 000*g* for 30 min (SW-41Ti rotor, Beckman XL-90 ultracentrifuge), and the supernatant was passed through a 0.2 mm microfilter (Nalgene) to remove any insoluble particles.

The resultant crude extract containing the Cph1 apoprotein was directly used for in vitro reconstitution without further purification, since the reconstitution efficiency of the crude form was much higher than that of the purified Cph1 apoprotein. To a 1.5 mL of the crude extract, 20 μL of the chromophore solution in DMSO with a concentration of 2−3 mM was added, and the mixture was incubated on ice for 1 h. The mixture was then loaded onto a chitin affinity column (NEB). A 2 mL volume of the column resin was routinely used for the crude extract from 250 mL of *E. coli* culture. After being washed with column buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100), the Intein-mediated on-column self-cleavage was induced by flushing the column with the same column buffer complemented with 1 mM DTT and incubation at 4 °C overnight. The eluted holoprotein was concentrated using a Centriprep 30 cartridge (Millipore, Bedford, MA). All in vitro reconstitution and purification procedures were performed under green safety light to maintain the holoproteins in Pr forms.

Two partial-size Cph1 constructs were also expressed and processed in *E. coli* in the same way as for the full-size Cph1

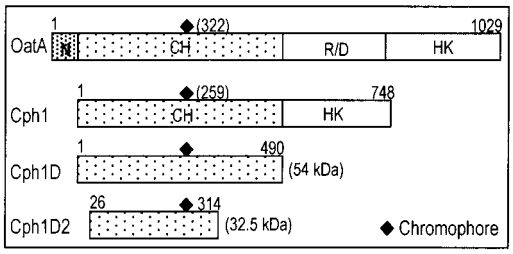

FIGURE 1: Cph1 expression constructs. The Cph1 expression constructs used in this work were compared to oat phytochrome A. The Cph1 protein lacks the N-terminal α-helix-forming motif (N) and the regulatory/dimerization domain (R/D). The Cph1D consists of the chromophore binding domain (CH, amino acids 1−490) without the histidine kinase domain (HK). The Cph1D2 includes amino acids 26−314. The Cph1 proteins were expressed in *E. coli* as Intein/CBD fusions, reconstituted in vitro with chromophores, and purified by chitin affinity chromatography. The numbers in parentheses indicate the positions of the chromophore binding cysteine residues.

(Figure 1). A 32.5 kDa N-terminal fragment of the Cph1 (amino acids 26−314, designated Cph1D2) was used for spectral analysis, and a 54 kDa N-terminal fragment (amino acids 1−490, designated Cph1D) was used for analysis of the quaternary structure.

Chromophore Isolation. Phytochromobilin (PΦB) and phycoerythrobilin (PEB) were extracted from a red alga *Porphyridium cruentum* by methanolysis and subsequently purified by chromatography as previously described (*21*). Phycocyanobilin (PCB) was isolated from *Spirulina platensis* according to the method of Terry et al. (*22*). The Waters 600 HPLC system equipped with a Waters Nova-Pac C18 column (3.9 × 300 mm) was used at the final step of purification. The pigment stocks were prepared in DMSO and stored at −70 °C until use. The chromophores were quantified by absorption spectroscopy as described (*22*).

Quaternary Structure. The quaternary structures of the Cph1 apo- and holoproteins were studied by size exclusion chromatography (SEC) and native PAGE. The SEC was performed either by the ÄKTA FPLC system (Amersham-Pharmacia, Buckinghamshire, England) on a Superose 12 column or by the Waters 600 HPLC system (Waters, Milford, MA) on a Shodex K802 column. Buffers with low ionic strength (20 mM Na phosphate, 1 mM EDTA, pH 7.8) and with high pH (190 mM glycine, 375 mM Tris-HCl, pH 9.5) and standard FPLC buffer (50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 150 mM NaCl) were used.

Native gel electrophoresis was done on a 7.5−15% gradient polyacrylamide gel using a sample loading buffer of 100 mM Tris-HCl, pH 7.5, 10% glycerol. The resolving gel buffer was 200 mM Tris-HCl, pH 9.5, and the stacking gel buffer was 100 mM Tris-HCl, pH 6.8. The running buffer was 180 mM glycine, 25 mM Tris-HCl, pH 8.3. The reference proteins used were BSA (66 kDa), yeast alcohol dehydrogenase (150 kDa), potato β-amylase (200 kDa), and horse spleen apoferritin (443 kDa).

Absorption and Circular Dichroism Spectroscopy. The absorption spectra were recorded on a Varian Cary-3 Bio double beam spectrophotometer (Sugar Land, TX). The spectra of the Pr and Pfr forms of the chromoproteins were registered after red or far-red light irradiation. A fiber optic illuminator system (Cole-Parmer) equipped with 666 and 750 nm interference filters (Oriel) was used as a light source. The light intensity was 8 W/m^2 for red light and 6 W/m^2 for far-red light. The SAR value was calculated as the ratio of absorbance at maximum in visible region versus absorbance at 280 nm for the Pr form (*3*). All experiments with the Cph1 holoproteins were performed under green safety light, which was obtained from a white fluorescent lamp equipped with a plastic filter having a maximal transmittance at 500 nm.

The circular dichroism spectra were recorded on a Jasco-700 spectropolarimeter. The Cph1 samples were passed through a Fast Desalting HR10/10 column (Pharmacia) equilibrated with 20 mM Na phosphate buffer, pH 7.8, containing 1 mM EDTA. The sample concentration was 0.1 mg/mL. Quartz cells with an optical length of 1.0 mm were used. The spectral data were scanned in the range of 260−190 nm four times, averaged, and smoothed by the instrument software. The scanning was performed at a scan speed of 20 nm/min, sensitivity 20 mdeg, resolution 1 nm, and time constant 2s. The spectropolarimeter was calibrated with *d*-10-camphorsulfonic acid ammonium salt according to the instrument manual. The secondary structures were calculated using the CDsstr program (*23*).

Limited Proteolysis. The protein digestion was performed with trypsin (Sigma, Type XI, DPCC treated, 6.000 BAEE units/mg). The Cph1 concentration used was 0.5 mg/mL, and the temperature was 20 °C. Proteolysis was stopped by adding SDS-sample buffer, and the mixture was boiled for 2 min. No degradation of the Cph1 was observed during sample preparation.

Protein Assay. The concentrations of protein samples were determined by the BCA method (*24*) using bovine serum albumin as a standard. The SDS−PAGE was performed according to the method of Laemmli (*25*), and the gel was stained with Coomassie brilliant blue dye for visualization. Chromophore-containing peptides were identified by Zn^{2+} fluorescence after chelating with Zn^{2+} (*26*). The SDS−PAGE gel was soaked for 1 h in 1 M Zn-acetate solution and photographed under UV light illumination.

RESULTS

Expression and In Vitro Reconstitution of the Cph1 Protein. In this work, we combined two expression strategies for the efficient expression and purification of the Cph1 protein, the pTYB2 vector-based *E. coli* expression system and the coexpression with a bacterial thioredoxin. Using this combined system, a recombinant protein can be purified in an extremely pure form by a single chromatographic step. The resultant recombinant protein has only 2 additional amino acids (Pro-Gly) at the C-terminus. Coexpression with the bacterial thioredoxin markedly improved the solubility and the native conformation.

The *Synechocystis* Cph1 proteins expressed in this scheme were recovered nearly 100% in a soluble form and electrophoretically pure. The Cph1−chromophore adducts had the specific absorbance ratio (SAR) of 0.8−0.9 (Table 1), which is substantially higher than the previously reported value of 0.55 (*2*, *3*, *5*), indicative of the higher spectral purity of our preparations. In addition, the recombinant Cph1 protein was more stable than those expressed by other systems examined in this work even after long storage.

Spectral Analysis. In 20 mM Na phosphate buffer with 1 mM EDTA, the absorption spectra of the Cph1 holoproteins

Table 1: Comparative Photochemical Properties of the Recombinant Cph1 Holoproteins

	Cph1−PCB (nm)	Cph1−PΦB (nm)	Cph1−PEB (nm)
	Absorption Spectra[a]		
Pr λ_{max}	656	671	579
Pfr λ_{max}	703	712	
Pfr-$A_{\lambda_{max}}$/Pr-$A_{\lambda_{max}}$	0.523	0.452	
	Difference Spectra[b]		
$\lambda(\Delta A_{max})$	655	671	
$\lambda(\Delta A_{min})$	703	713	
$\Delta A_{max}/\Delta A_{min}$	1.028	1.778	
SAR[c]	0.868	0.828	0.875

[a] The parameter Pfr-$A_{\lambda_{max}}$/Pr-$A_{\lambda_{max}}$ indicates the ratio of the peak maxima on the absorbance spectra of the Pr and Pfr forms. [b] The parameter $\Delta A_{max}/\Delta A_{min}$ represents the ratio of the peak absorbance values of the maxima and minima peaks for the difference spectrum. [c] The SAR value is a parameter of spectral purity and refers to the ratio of the Pr absorbance maxima to the 280 nm absorbance peak.

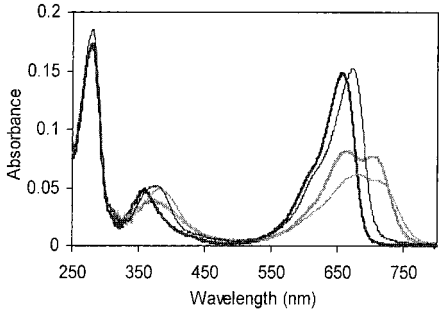

FIGURE 2: Absorption spectra of the Pr and Pfr forms of the Cph1 holoproteins. The Cph1−PCB (thick line) and −PΦB (thin line) holoproteins were prepared in 20 mM Na phosphate and 1 mM EDTA, pH 7.8. The Pr (solid line) and Pfr (dotted line) forms are indicated. Spectral measurements were obtained using the Cary 3 UV−vis spectrophotometer.

had the characteristic absorption maxima in the visible region (Figure 2). The absorption maxima for the Pr forms of the PCB, PΦB, and PEB adducts were at 656, 671, and 579 nm, respectively. The Pfr forms of the PCB and PΦB adducts had the absorption maxima at 703 and 712 nm, respectively. Although the spectral profiles of the Cph1 holoproteins were similar to the previously reported, and the absorbance maxima were somewhat different (*3*). For example, the absorbance maxima of our Cph1−PΦB adduct were 671 and 712 nm (Table 1), whereas those of the previous report were 670 and 719 nm, respectively. One plausible reason for this difference might be due to slightly different conformations of the protein preparations used, as indicated by different SAR values. The spectral change ratios, Pfr-$A_{\lambda_{max}}$/Pr-$A_{\lambda_{max}}$ and $\Delta A_{max}/\Delta A_{min}$, of the Cph1−PCB and −PΦB adducts were different (Table 1), unlike the recombinant plant phytochrome A (*27*). This result suggests that the environment of the prosthetic groups in the Cph1 holoproteins and their chromophore−apoprotein interactions are different from those of plant phytochromes.

These observations, together with the higher SAR value than the previously reported one, indicate that the Cph1 protein prepared here has a conformation closer to the native folding and is suitable for further structural and photochemical analysis.

Quaternary Structure. Because of the significant differences in the spectral characteristics of our Cph1 preparations

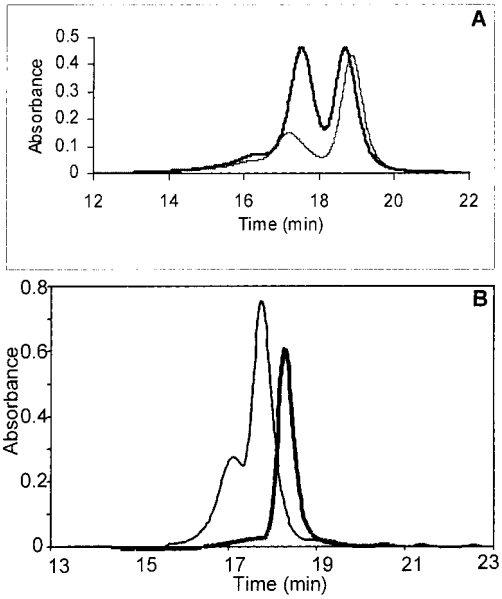

FIGURE 3: Quaternary structures of the Cph1 proteins. (A) The SEC HPLC elution profiles of the Cph1 apoprotein (thin line) and the Cph1−PCB holoprotein (thick line) on a Shodex-802P column. The running buffer used was 20 mM Na phosphate and 1 mM EDTA, pH 7.8. (B) The SEC FPLC of the full-size and a partial-size Cph1 proteins. The full-size Cph1 (thin) and a partial-size construct (thick line; Cph1D, aa 1−490) apoproteins were analyzed on the ÄKTA FPLC system (Superose 12 column). The running buffer and conditions were the same as in Figure 3A.

and those of the previously reported (*3*), it was suspected that the quaternary structures were also different between the two preparations. To examine this possibility, the Cph1 apo- and holoproteins were analyzed on two different SEC systems under the same experimental conditions as used earlier, a Superose 12 FPLC column and a Shodex K802 HPLC column. Elution profiles of the Cph1 proteins on the two SEC systems were basically identical. One such result on the Superose 12 HPLC is shown in Figure 3A. The Cph1 apoprotein was eluted in two peaks. The calculated MM of the first peak was 290−300 kDa (a presumptive dimer form), and that of the second peak was 150−175 kDa (a presumptive monomer form). These elution profiles were slightly different from those of the previous one (*3*). The two elution peaks were better separated, and the ratio of the first/second peaks was higher. These results suggest that our Cph1 apoprotein more readily form dimers, probably because the conformation of the Cph1 protein prepared in this work is more stabilized in a native conformation and/or it contains less competitively binding impurities, as indicated by the higher SAR values.

Both the Cph1 apo- and the Cph1−PCB holoproteins were eluted in two major peaks on both the Superose 12 FPLC and the Shodex K802 HPLC systems. However, the ratios of the two peaks were drastically different. The apoprotein was eluted mainly in the second peak, whereas the holoprotein was eluted almost equal amounts in two peaks (Figure 3A). It appears that chromophore binding affected the distribution of these two size exclusion peaks, presumably resulting from some chromophore-induced conformational changes as suggested in plant phytochrome A (*28, 29*).

To examine whether the presumed dimer formation is mediated by the C-terminal region as in plant phytochromes (*30*−*33*), a partial-size N-terminal fragment (Cph1D), which

lacks the C-terminal region, was expressed (Figure 1). The Cph1D apoprotein was eluted exclusively as a single peak at the position of the presumed monomer (Figure 3B). This suggests that the dimerization is mediated by some structural motifs in the C-terminal region, as in plant phytochromes.

When the Cph1 apo- and Cph1−PCB holoproteins were analyzed on native gel electrophoresis under the published condition (*3*), two bands were detected, one prominent band with an apparent MM of 85 kDa (monomer) and the other faint band with an apparent MM of 160 kDa (dimer) for both the apo- and holoproteins (data not shown). However, the intensity ratios of the two bands were similar for both the apo- and holoproteins, which is inconsistent with the result from SEC. A possible reason for this inconsistency is a high pH value of the resolving gel of the native electrophoresis used (pH 9.5), at which dimers may dissociate into monomers.

Taken together, these results indicate that the Cph1 apoprotein exists primarily as monomers in an equilibrium with dimers and that the chromophore binding causes the equilibrium to shift toward the dimers.

The MM estimations of the presumed monomer and dimer peaks of the Cph1 on SEC are much larger than the predicted MM of 85 kDa for the monomer and 170 kDa for the dimer. The similar discrepancy has been observed in the previous reports on oat phytochrome A (*32*). This might be due to the high axis ratio of the ellipsoid Cph1 monomer (see Discussion).

Determination of Secondary Structures. Spectral and SEC studies indicate that the Cph1 proteins prepared in this work are basically similar to but slightly different from those of the previously reported in terms of photochemical activities and quaternary structures. To further investigate conformational changes induced by chromophore binding, the secondary structures of the Cph1 apo- and holoproteins were probed by CD spectroscopy. Both the Pr and Pfr forms of the Cph1−PCB and −PΦB holoproteins were examined, while only the Pr form of the nonphotorevertible PEB holoprotein was examined. The results are presented in Figure 4 and Table 2.

The CD spectral profiles of both the apo- and holoproteins were similar to each other with a negative extremum of molar ellipticity at 220 nm and a positive maximum at 190 nm (Figure 4). The ratio of $[\theta]_{190}/[\theta]_{220}$ values indicated that the predominant secondary structural component in all Cph1 proteins analyzed was the α-helical conformation (Table 2), as in plant phytochromes (*33, 34*). The α-helical content of the apoprotein was calculated to be 37%. That of all holoproteins was determined to be 42−44%. The sum of all helices (3.6_{13} and 3_{10} helices) was 46% for the apoproteins and 52−54% for the holoproteins, respectively. However, the content of β-sheet fold did not show any significant changes upon chromophore binding. The content of β-sheet fold was 5−8% for all Cph1 apo- and holoproteins. This is different from plant phytochromes. The β-sheet content of plant phytochrome A was reported to be 27% for the Zn^{2+}-chelated phytochrome and 0% for the native phytochrome (*34, 35*).

There was no significant difference in the secondary structures between the Pr and Pfr forms of the Cph1 holoproteins unlike plant phytochrome A, although 1−2% difference of some structural components was always

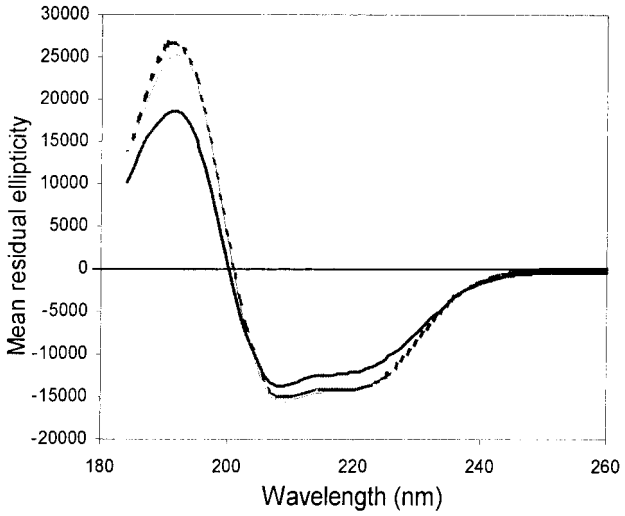

FIGURE 4: CD spectra of the Cph1 proteins in solution. The far-UV CD spectrum was acquired from 260 to 190 nm. The path length of the quartz cuvette used was 1 mm. The spectra were registered on a Jasco-700 spectropolarimeter, and four scans were averaged. The concentration of the protein samples was 0.1 mg/mL. The Cph1 apoprotein (solid line) and the Pr (thin line) and Pfr (dotted line) forms of the Cph1−PCB holoprotein were prepared in 20 mM K phosphate buffer, pH 7.8, containing 1 mM EDTA.

Table 2: Secondary Structures of the Cph1 Apo- and Holoproteins[a]

	contents of secondary structures (%)[c]					
proteins[b]	H	G	E	T	P	O
apo	37 ± 5	9 ± 1	8 ± 2	12 ± 2	5 ± 1	30 ± 3
PEB	43 ± 5	9 ± 1	6 ± 3	11 ± 1	5 ± 1	27 ± 2
PCBr	42 ± 5	9 ± 1	7 ± 3	11 ± 1	4 ± 1	28 ± 2
PCBfr	42 ± 4	10 ± 2	7 ± 3	10 ± 2	5 ± 1	26 ± 4
PΦBr	44 ± 4	9 ± 1	5 ± 2	11 ± 1	5 ± 1	26 ± 3
PΦBfr	44 ± 5	10 ± 1	5 ± 3	13 ± 3	3 ± 2	25 ± 4

[a] Calculated from the CD spectra used in Figure 4 using the CDsstr program (*23*). Ellipticities from 190 to 260 nm at 1-nm intervals were used in the calculations. [b] Apo, Cph1 apoprotein; PCBr and PCBfr, PCB holoproteins in Pr and Pfr forms; PΦBr and PΦBfr, PΦB holoproteins in Pr and Pfr forms; PEB; PEB holoprotein. [c] H; α-Helix; G, 3_{10} helix, E, extended β-strand; T, β-turn; P, polyproline-like 3/1-helix, O, others.

registered (Table 2). It was reported that the α-helical content of plant phytochrome A increased from 45.5% to 50.5% upon the Pr → Pfr phototransformation (*34*).

Limited Proteolysis. Limited trypsin treatment was employed to see whether the chromophore-induced changes in the secondary structure are accompanied by any changes in the tertiary structure and whether such conformational changes also occur in the Pr ↔ Pfr phototransformation. The results are shown in Figure 5.

We first compared the proteolytic maps of the apo- and holoproteins (Figure 5A). The Pr forms of all holoproteins (PCB, PΦB, and PEB adducts) were subjected to proteolysis, since the PEB adduct exists only in a Pr-like spectral form. Proteolysis of the apo- and holoproteins gave drastically different peptide patterns. In the case of the apoprotein, a 5-min treatment with trypsin resulted in two major peptides with MM of 50 and 54 kDa and a few minor peptides (Figure 5A). The holoproteins treated under the same conditions generated one additional dominant peptide with a MM of 61 kDa. After 2-h digestion, the apoprotein was almost completely digested, whereas one peptide with a MM of 45

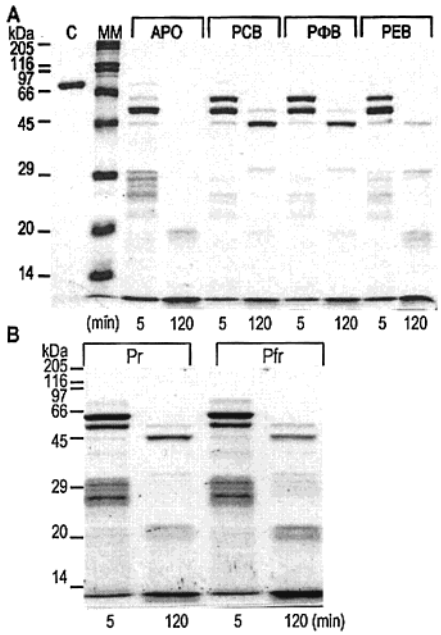

FIGURE 5: Limited proteolysis of the Cph1 proteins with trypsin. (A) Proteolysis of the Cph1 proteins. The Cph1 apo- and holoproteins were treated with trypsin at 20 °C either for 5 or 120 min as indicated. The holoproteins were Cph1−PCB, −PΦB, and PEB adducts in the Pr forms. The treated samples were analyzed on a 10% SDS−PAGE and stained with Coomassie brilliant blue dye. Lane C: undigested Cph1 apoprotein, MM; molecular mass markers (from the top: 205, 116, 97, 66, 45, 29, 20, and 14 kDa). The 5 and 120 indicate the digestion time in minutes. (B) Proteolysis of the Pr and Pfr forms of the Cph−PCB holoprotein. The protein samples were treated with trypsin as in (A) and analyzed on a 10% SDS−PAGE. The Cph1−PCB holoprotein in the Pr form used is essentially identical to that used in (A). Protein mass markers are indicated in kilodaltons on the left.

kDa still dominated even after the 2-h digestion of the holoproteins. These results indicate that chromophore binding induced some conformational changes, resulting in more resistant structures against proteolytic digestion. However, no significant difference was observed among the PCB, PΦB, and PEB adducts (Figure 5A) and between the Pr and Pfr spectral forms (Figure 5B).

The 45 kDa peptide was a chromophore-containing peptide, as judged by Zn^{2+} fluorescence (Figure 6). Interestingly, a 30 kDa chromopeptide still dominated even after longer proteolysis. This chromopeptide was partially purified by ion-exchange chromatography. It was photoreversible, and the spectral maxima were similar to those of the full-size Cph1 holoprotein but with a small blue shift (see below). To ensure that this chromopeptide was derived from the N-terminal chromophore-containing domain, the N-terminal 32.5 kDa region of the Cph1 (amino acids 26−314, Cph1D2) was expressed in *E. coli* and in vitro reconstituted with PCB (Figure 1). This recombinant peptide could be reconstituted with chromophores and was photochemically fully active. Its absorption spectra were essentially identical to those of the 30 kDa peptide, again with the characteristic blue shift of λ_{max} in both the Pr and Pfr forms relative to the λ_{max} values of the full-size Cph1 protein (Figure 7).

DISCUSSION

The *Synechocystis* Cph1 phytochrome can be an invaluable model system for the study of the phytochrome-mediated

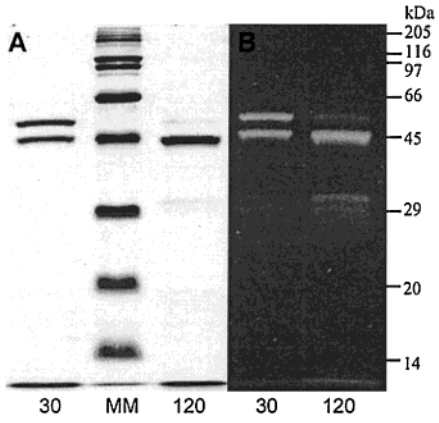

FIGURE 6: Identification of the chromopeptides by Zn^{2+} fluorescence. The Pr form of the Cph−PCB holoprotein was treated with trypsin either for 30 or 120 min. The treated samples were analyzed on two 10% SDS−PAGE gels. One was stained with Coomassie brilliant blue (A), and the other was transferred to a PVDF membrane (Hybond-P^+, Amersham) (B). The membrane was gently shaken in 1 M zinc acetate for 1 h at room temperature and visualized under UV light (312 nm).

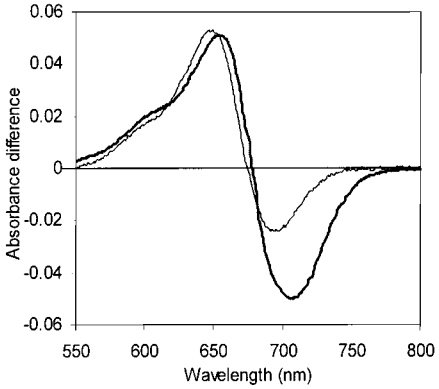

FIGURE 7: Difference spectra of the full-size Cph1−PCB (thick line) and the 32.5 kDa peptide−PCB (thin line, Cph1D2, aa 26−314) adducts. Crude extracts containing the apoproteins were in vitro reconstituted with PCB and purified by a chitin affinity column. The spectral measurements were obtained as in Figure 2.

light signaling in plants. It is the first phytochrome molecule that has been proven to have kinase and phosphotransferase activities, which subsequently triggered the reexamination of the enzymatic activity of plant phytochromes. The Cph1 protein can be efficiently expressed to a high level in the soluble form in *E. coli* and reconstituted in vitro with chromophores (*2*, *6*, *7*), resulting in photochemically active holoproteins. This is a prerequisite for the structural and functional studies of the phytochromes. In addition, analysis of the light signaling functions could provide a mechanistic similarity and/or contrast to plant phytochromes due to the unicellular nature of *Synechocystis*.

Despite the earlier expectation that the three-dimensional structure of the Cph1 phytochrome could be easily determined, no Cph1 crystal obtained has been reported so far. This is probably due to the intrinsic properties of phytochrome protein molecules, such as the tendency to precipitate in aggregates, structural heterogeneity (a mixture of differentially phosphorylated forms), presence of two spectrally different forms (Pr and Pfr), and light-sensitive degradation problem. Also, structural characterization of the Cph1 in solution is desirable for the elucidation of the structure−

function relationship. However, little is known about the relationship between the Pr ↔ Pfr phototransformation and the associated conformational changes in the Cph1 protein. To expand our knowledge on the structure of the Cph1 phytochrome, we investigated the secondary and tertiary structures of the Cph1 in both apo- and holoproteins.

The quality of protein samples is critical to obtain accurate data for structural analysis. Among the recombinant expression systems tested, the pTYB2-based Intein fusion system (NEB) gave the best result for the Cph1 expression. In addition, we also employed the coexpression strategy with the bacterial thioredoxin to further improve the solubility and native folding of the expressed protein in *E. coli*.

The recombinant Cph1 apoprotein prepared in this work was reconstituted with chromophores at a higher index of spectral purity than those of the previously reported ones. The ratio of the Pr absorbance to the protein absorbance peak at 280 nm (SAR) is a frequently used spectroscopic parameter to assess the spectral purity of the phytochromes, in addition to the classical electrophoretic purity. Our recombinant Cph1 protein was essentially pure on SDS−PAGE. The SAR values for the Cph1 preparations were routinely 0.85−0.90. These values are significantly higher than the previously reported one and even comparable to that of the pure oat phytochrome A. The SAR of the pure oat phytochrome has been measured to be 1.05−1.10 (*36*), whereas that of the recombinant Cph1−PCB holoprotein has been reported to be 0.55 (*3*). Although their Cph1 preparations were electrophoretically pure, the protein samples seemed to contain some nonproteineous impurities. The low SAR might be also due to the partial unfolding of the apo- or holoproteins during sample preparations, which has been known to bleach the chromophores and decrease the SAR as it has been observed in plant phytochromes (*36*). The coexpression strategy used in this work appears to stabilize the native conformation of the recombinant Cph1 in *E. coli* as it has been proven with other recombinant proteins (*20*). The Cph1 sample prepared in this work will be an excellent material for X-ray crystallographic studies.

The results of the SEC and native PAGE show that the Cph1 protein can also form dimers and that the dimerization is mediated by the C-terminal region, as in plant phytochromes (*30*−*32*). Although the extent of the dimerization is significantly lower than that of plant phytochromes, it is an unexpected result. Sequence analysis revealed that the Cph1 protein lacks most of the C-terminal structural motifs that have been suggested to be involved in the dimerization of plant phytochromes (*4*, *31*, *32*). It seems that other structural components have complementary roles in the dimer formation. The Cph1 may form dimers more readily after associating with other factor(s), such as the Rcp1 partner.

The discrepancy of the MM estimations of the presumed monomer and dimer peaks on SEC could be explained by the high axis ratio of the ellipsoid monomer of the Cph1 protein. It has been shown that the proteins on the Superose 12 column are eluted according to their Stokes radii (*37*). By comparison of the SEC data of the Cph1 protein and the previous data (*34*), the Stokes radius of the Cph1 protein eluted with a MM of 170 kDa is calculated to be about 47 Å. The simple geometric calculations for the Cph1 monomer indicate that the ratio of ellipsoid axis could be as high as 1:5. These considerations suggest that the high ratio of ellipsoid axis of the Cph1 protein seems to be the reason for the discrepancy of the MM estimations on SEC.

The predominant secondary structural motif in all the Cph1 apo- and holoproteins was the α-helical component (Figure 4 and Table 2) as has been observed with plant phytochromes (*34*, *35*). The contents of the other structural motifs of the Cph1 were also similar to those of plant phytochromes. In addition, the α-helical content significantly increased to 42−44% in the holoproteins from 37% in the apoprotein upon chromophore binding. However, this increase is less than that (15−20%) of plant phytochromes. The difference may be explained by the fact that the Cph1 lacks the characteristic α-helical forming motif (about 60 amino acids) in the far N-terminus, which has been well characterized in plant phytochromes (*38*) (see below).

The holoproteins contained less unfolded structure compared to the apoprotein, while the contents of β-structures were about the same (Table 2). These observations indicate that chromophore binding induces an additional folding of the unfolded structure into the α-helical conformation. This is also consistent with the earlier finding that disruption of chromophore−apoprotein interactions in plant phytochromes caused marked changes in the secondary structure, namely decrease in the helical content and increase in the random coil conformation (*34*).

The secondary structures did not significantly change during the Pr ↔ Pfr phototransformation in the Cph1 phytochrome, probably due to the absence of the amphiphilic α-helix forming motif in the N-terminus (*35*). A prominent conformational change upon the Pr ↔ Pfr phototransformation in plant phytochromes occurs in the N-terminal 60-amino acid sequence. This short amino acid sequence forms an α-helical conformation in the Pfr form but exists as a random coil conformation in the Pr form. When this peptide was removed or when its structure was fixed by specific antibodies, it could not form the α-helical conformation (*14*, *39*). Thus, we speculate that lack of similar changes in the secondary structures of the Cph1 during the Pr ↔ Pfr phototransformation may be explained by the absence of the amphiphilic α-helix forming N-terminal motif (*38*).

The changes in the secondary structure of the Cph1 upon chromophore binding coincide with significant conformational changes in the tertiary structures, as confirmed by limited proteolysis. The Cph1 apoprotein was almost completely digested by 2-h trypsin treatment. However, the holoproteins were much more resistant to the proteolysis, as a few chromophore-containing peptides still persisted even after longer treatment (Figure 5). It is known that at least 12 amino acid residues around the trypsin recognition sequence are required for full accessibility to trypsin (*40*). This implies that the conformation of the Cph1 apoprotein is relatively flexible, and most of the 61 trypsin cleavage sites are accessible to trypsin (Figure 5A). The chromophore binding would induce the conformational changes in ways to stabilize the structure of the Cph1 holoproteins, resulting in a more compact structure. These conformational changes seem to occur mainly in the N-terminal chromophore-containing domain. The most trypsin-resistant peptide is the 30 kDa chromopeptide as confirmed by limited proteolysis and Zn^{2+} fluorescence (Figure 6A ,B). It exhibited spectroscopic features similar to those of the full-size Cph1 holoprotein

(Figure 7), although its reconstitution efficiency was lower than that of the full-size Cph1.

Interestingly, no detectable changes in the tertiary structure during the Cph1 Pr ↔ Pfr phototransformation were observed by proteolysis. It has been well documented that the tertiary structures of plant phytochromes change during the Pr ↔ Pfr phototransformation (*16*, *17*, *41*). A more sensitive method is necessary to probe the subtle conformational changes that may be induced by the Cph1 phototransformation.

In conclusion, our results show that the *Synechocystis* Cph1 phytochrome undergoes conformational changes in secondary and tertiary structures upon chromophore binding in similar ways to those in plant phytochromes. However, the Cph1 is different from plant phytochromes in that no detectable conformational changes occur during the Pr ↔ Pfr phototransformation. The Cph1 lacks the N-terminal α-helix-forming motif. Also, it lacks the 300-residue region in the proximal C-terminal domain that corresponds to the regulatory domain in plant phytochromes. The 300-residue region also contains the putative dimerization motif. These observations, in combination with our results, suggest that the mechanisms of photoperception and upstream signaling are different for plant and Cph1 phytochromes.

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