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The Cyanobacteriochrome, TePixJ, Isomerizes Its Own Chromophore by Converting Phycocyanobilin to Phycoviolobilin†

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ABSTRACT: The cyanobacterial phototaxis regulator protein, TePixJ, is a member of the subfamily of cyanobacteriochromes that binds phycoviolobilin (PVB) as a chromophore and exhibits reversible photoconversion between blue light-absorbing (Pb) and green light-absorbing (Pg) forms. We reconstituted the PVB-binding photoactive holocomplex in vivo and in vitro. Coexpression of the apoprotein and phycocyanobilin (PCB) in *Escherichia coli* (in vivo reconstitution) produced a mixture of the PCB-bound and PVB-bound holoproteins. Reconstitution in vitro of the apoprotein and synthetic PCB quickly generated a photoactive complex, which covalently bound PCB and exhibited partially reversible photoconversion between two species by UV−vis spectroscopy (with a λ_{max} values of 430 and 545 nm). Further incubation produced slow isomerization of PCB to PVB with concomitant improvement of photoreactivity. Site-directed mutagenesis confirmed that Cys522, and a second conserved Cys (Cys494), are both essential for the assembly of the photoactive complex. Fourier transform infrared (FTIR) spectroscopy revealed green light-induced cross-linking, and blue light-induced release, of a thiol group, possibly that of Cys494. These results suggest that the Pb/Pg-type cyanobacteriochrome TePixJ is assembled in at least three steps: (i) rapid and stable chromophorylation of PCB, (ii) additional photoreversible chromophorylation, and (iii) subsequent slow isomerization of PCB to PVB. In addition to its known autolyase activity with Cys522 and photoreversible isomerase activity (of the *Z* and *E* isomers at C15 and C16 of PCB), the GAF domain of TePixJ therefore appears to have other roles: as an isomerase (converting PCB to PVB) and as a photoreversible autolyase with a second conserved Cys residue.

Cyanobacteria are the only prokaryotes that perform oxygenic photosynthesis. A variety of photoreceptors have been found in cyanobacterial genomes that include most types of chromophores, such as linear tetrapyrrole, retinal, and flavin (*1*). Of these, the linear tetrapyrrole chromophore plays an important role in cyanobacteria, both in the photosynthetic antennae (phycobilisomes) and in the photoreceptors (phytochromes and cyanobacteriochromes) (*2*, *3*).

The phycobilisome is a supramolecular complex consisting of a number of phycobiliproteins (e.g., phycocyanin, phycoerythrin, phycoerythrocyanin, and allophycocyanin) and their associated linker proteins. Phycobilisomes serve as light-harvesting antennae that efficiently transfer light energy to chlorophyll in cyanobacteria and red algae photosystems (*4*). The phycobiliproteins covalently bind tetrapyrrole chromophores such as phycocyanobilin (PCB)[1], phycoviolobilin (PVB), phycoerythrobilin, and phycourobilin (*2*, *5*).

Phytochrome is a classic photoreceptor that covalently binds a tetrapyrrole chromophore, phytochromobilin, and regulates various reversible red/far-red photoresponses in plants, such as flowering, seed germination, and shade avoidance (*6*, *7*). Bacterial and fungal phytochromes that are closely related to the plant phytochrome have recently been found in many bacterial and fungal genomes (*8*, *9*), and native forms of these phytochromes covalently bind PCB or biliverdin in the conserved PAS-GAF-PHY domain (PAS, Per-ARNT-Sim; GAF, cGMP phosphodiesterase-adenylate cyclase-FhlA; PHY, phytochrome-specific). This expanded group of phytochromes, including the bacteriophytochromes, is widely distributed in a diverse array of organisms but exhibits a conserved photocycle between the red light-absorbing and far-red light-absorbing forms.

More recently, cyanobacteriochromes that bind a linear tetrapyrrole (PCB or PVB) in a single GAF domain have been discovered as a novel family of photoreceptors (*10*). These regulate, for example, phototactic cell motility (*11*), chromatic acclimation of photosynthetic phycobiliproteins (*12*), and cell aggregation. Although many putative genes have been identified, they remain to be characterized experimentally. Most cyanobacteriochromes are classified as PCB-binding or PVB-binding, exhibiting reversible green−red or blue−green photoconversion, respectively. The chromophore-binding GAF domain of the

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[1]Abbreviations: PCB, phycocyanobilin; Pb and Pg, blue and green light-absorbing forms of native TePixJ, respectively; PVB, phycoviolobilin; FTIR, Fourier transform infrared; LED, light-emitting diode; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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cyanobacteriochromes is distantly, but significantly, related to the GAF domain of phytochromes, which suggests that these two photoreceptors have diverged from a common ancestor. A key question is how the cyanobacteriochromes have developed such a wide variety of spectral properties, in marked contrast to the highly conservative phytochromes.

The blue/green light-absorbing PixJ is the first example of a cyanobacteriochrome that has been isolated from cyanobacterial cells (*13*). *SypixJ1* was originally identified as a phytochrome-like photoreceptor gene essential for positive phototaxis of a mesophilic unicellular cyanobacterium, *Synechocystis* sp. PCC 6803 (*14*). The native full-length protein product, SyPixJ1, has been partially purified from *Synechocystis* cells and exhibits reversible photoconversion between the blue light-absorbing (Pb) and green light-absorbing (Pg) forms (*13*). We then reported an improved method of preparation of a PixJ homologue by overexpression, in *Synechocystis*, of the chromophore-binding GAF domain of TePixJ from a thermophilic cyanobacterium, *Thermosynechococcus elongatus* (TePixJ_GAF). The two forms of native TePixJ_GAF exhibit major peaks in the blue or green region (Pb form, λ_{max} = 433 nm; Pg form, λ_{max} = 531 nm) with no absorption in the red region of the spectrum (*15*). These spectral properties are in contrast with the known red/far-red reversible phytochromes. Furthermore, acid and urea denaturation analysis revealed that the chromophore of TePixJ is PVB, not PCB (*16*). This intriguing result led us to ask two fundamental questions. How is the PVB produced, and is the presence of PVB responsible for the large blue shift of TePixJ, compared with the green/red light-absorbing cyanobacteriochromes?

PCB of the photosynthetic phycobiliproteins is the most abundant linear tetrapyrrole chromophore in cyanobacteria. PVB, an isomer of PCB, has one double bond in a different position: C2=C3 bond of ring A in PVB versus C4=C5 bond between rings A and B in PCB. As a result, the double bond conjugation of PVB is shorter than that of PCB; this leads to the absorption of shorter wavelengths of light (by approximately 90 nm) by PVB. PVB has been detected as a chromophore in the phycobiliprotein, phycoerythrocyanin α-subunit, in the peripheral antennae of phycobilisomes in some filamentous heterocyst-forming cyanobacteria, such as *Anabaena* and *Mastigocladus* (*17*). Although the reaction mechanism has not yet been fully elucidated in these organisms, PCB is covalently attached to α-phycoerythrocyanin, with concomitant conversion to PVB by a specific lyase−isomerase complex comprised of PecE and PecF (*18*−*20*). PecE and PecF are similar in sequence to CpcE and CpcF, respectively, these are PCB:phycocyanin-α apoprotein lyase, not isomerase, and therefore do not convert PCB to PVB. There are no genes homologous to *pecE* or *pecF* in the *Synechocystis* or *T. elongatus* genome, although there are genes homologous to *cpcE* and *cpcF* (*21*).

Here, we attempted to reconstitute the PVB-bound TePixJ_GAF holoprotein, in vivo and in vitro. Expression of TePixJ_GAF in the *Escherichia coli* mutant, which produces PCB, resulted in assembly of a photoactive holoprotein with spectral properties similar to those of the native protein. Acid and urea denaturation analysis revealed that the photoactive chromophore bound to the holoprotein was a mixture of PVB and PCB, suggesting that the PCB produced by *E. coli* had been converted to PVB. To further study the isomerization, the apoprotein was incubated with synthetic PCB in vitro. The photoactive PCB-bound intermediate complex quickly assembled, and the PCB slowly isomerized to PVB, with concomitant improvement in photoreactivity. This indicates that the chromophore-binding GAF domain has inherent isomerase activity.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions. Cells of *Synechocystis* sp. PCC 6803 expressing the GAF domain of TePixJ, with an N-terminal polyhistidine tag, i.e., TePixJ_GAF, were grown and isolated to homogeneity as described previously (*15*). Harvested cells were washed and disrupted using a French pressure cell (Ohtake, Tokyo, Japan). Ni-affinity chromatography was performed using a HisTrap HP column (GE Healthcare, Piscataway, NJ), according to the manufacturer's protocol.

Expression and Purification of TePixJ_GAF Apoprotein and Holoprotein. The DNA fragment, *TePixJ_GAF*, which is identical to that expressed in *Synechocystis* (*15*), was cloned into the *Nde*I and *Bam*HI sites of pET28a (Novagen, Madison, WI) and expressed in *E. coli* BL21(DE3) cells. The apoprotein was expressed using a T7 promoter in *E. coli* BL21(DE3) cells. The holoprotein was expressed in *E. coli* BL21(DE3) cells, carrying plasmid pKT271, which encodes heme oxygenase and ferredoxin:PCB reductase for the production of PCB (*22*). *E. coli* cells were precultured overnight at 37 °C in Luria-Bertani medium (LB) in the dark, and 10 mL of preculture solution was used to inoculate 1 L of LB. When the cell density reached an OD_{600} of 0.4−0.6, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 10 μM. Cells were grown further for 3 h and 30 min at 37 °C in the dark. After being centrifuged and washed with a buffer containing 20 mM HEPES-NaOH (pH 7.5), 100 mM NaCl, and 10% (w/v) glycerol, cells were resuspended in the same buffer and then broken with the French pressure cell with three passages at 1500 kg/cm^2. The homogenate was centrifuged at 194100*g* for 30 min at 4 °C. The supernatant was applied to the HisTrap HP column (GE Healthcare) and equilibrated with 20 mM HEPES-NaOH (pH 7.5), 100 mM NaCl, and 10% (w/v) glycerol. Using a linear gradient from 30 to 500 mM imidazole over 40 min, TePixJ_GAF was eluted between 80 and 120 mM imidazole in the same buffer. The purified apoprotein was concentrated and loaded onto a Superdex 75 gel filtration column (GE Healthcare) to remove imidazole and glycerol. Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA) according to the supplier's protocol and using bovine serum albumin as the standard (*23*).

Synthesis of PCB. PCB was synthesized according to published methods (*24*, *25*). Stock solutions of PCB (approximately 1 mM) were prepared in dimethyl sulfoxide and stored at −80 °C. The concentration of each stock solution was measured using a UV-2400PC UV−visible spectrometer (Shimadzu, Kyoto, Japan) using a molar extinction coefficient, ε, for PCB in MeOH at λ_{max} of 16000 M^{-1} cm^{-1} (*24*).

In Vitro Reconstitution Analysis. PCB was added to the TePixJ_GAF apoprotein to give final concentrations of 20 μM PCB and 60 μM TePixJ_GAF apoprotein in reconstitution buffer [20 mM HEPES-NaOH (pH 7.5) and 100 mM NaCl]. The mixture was incubated in the dark at 30 or 50 °C.

UV−Vis Spectroscopy and Photoconversion. UV−vis absorption spectra were recorded using the UV-2400PC spectrophotometer. Blue light (100 μmol of photons m^{-2} s^{-1}) was generated by a light-emitting diode (LED) with a peak at 400 nm and a 15 nm half-bandwidth (SDL-5N3CUV-A, Sander, Taiwan, China). Green light (1000 μmol m^{-2} s^{-1}) was generated by an LED with a peak at 530 nm and a 25 nm half-bandwidth (DG5306XDG, Stanley Electric, Meguro, Japan).

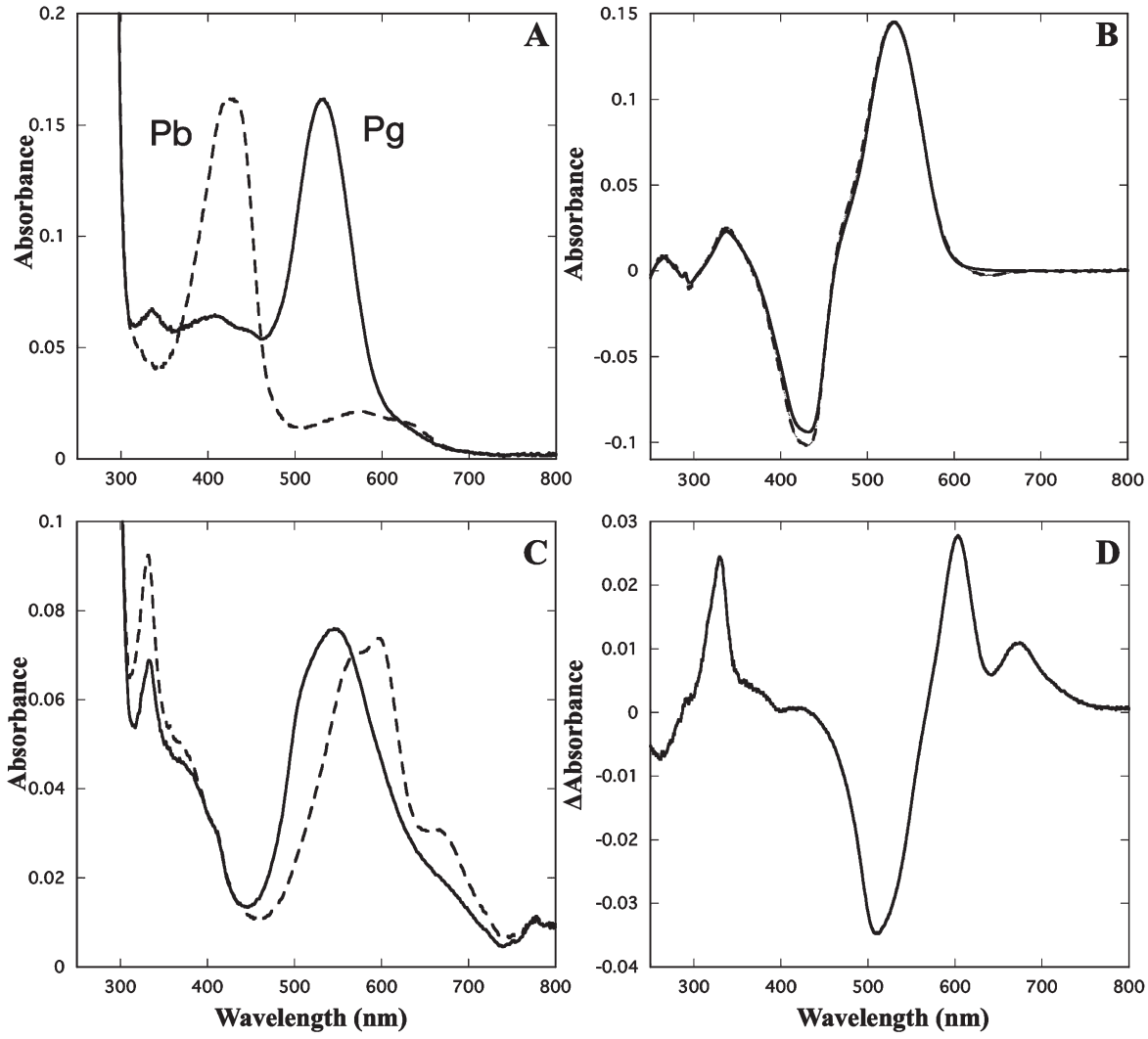

FIGURE 1: Absorption spectra of holo-TePixJ_GAF, expressed in *E. coli* that coproduced PCB (in vivo reconstitution). (A) Absorption spectra of the blue light-absorbing form (– – –) and green light-absorbing form (—) of holo-TePixJ_GAF. (B) Difference absorption spectra of the assembled holo-TePixJ_GAF (– – –) and native TePixJ_GAF [Pg minus Pb (—)]. (C) Absorption spectra of denatured green light-absorbing form, before (—) and after (– – –) irradiation with white light. (D) Difference absorption spectrum from panel C.

Fourier Transform Infrared (*FTIR*) *Spectroscopy*. Following the Ni-affinity chromatography step, a solution of *Synechocystis*-expressed TePixJ_GAF in buffer [20 mM HEPES-NaOH (pH 7.5) and 100 mM NaCl] was concentrated by ultrafiltration (Ultrafree0.5 for a molecular mass cutoff of 10000, Millipore) until a protein concentration of approximately ~20 mg/mL was reached. An aliquot (7 μL) of this sample solution was dried on a 25 mm diameter CaF_2 plate under a flow of N_2 to produce an elliptically shaped film (6 mm × 9 mm). The dry film was hydrated using 2 μL of a 40% (v/v) glycerol/H_2O solution placed near, but not touching, the sample, to control humidity in a previously described method (*26*). The sample was sealed with a second CaF_2 plate separated by a greased 0.5 mm Teflon spacer. The sample was incubated for 1 h at 10 °C so that hydration was complete before spectroscopic measurement. The temperature was maintained by circulating cold water in a copper cell holder.

Light-induced FTIR difference spectra were recorded using a Bruker IFS-66/S spectrophotometer, equipped with a mercury–cadmium–telluride (MCT) detector (Infrared D316/8) at 4 cm^{-1} resolution. After illumination of the sample with the green LED light (λ_{max} = 530 nm) for 50 s to fully populate the Pb form, single-beam spectra (50 s scans) were recorded before and after illumination with a blue LED light (λ_{max} = 452 nm, 4000 μmol m^{-2} s^{-1}; Luxeon Star, Lumileds Lighting) for 50 s, and a Pg − Pb difference spectrum was calculated. Similarly, a Pb − Pg difference spectrum was measured by illumination with green LED light. This cycle was repeated three times, and the spectra were averaged.

Denaturation Analysis. The Pb and Pg forms of TePixJ_GAF were denatured by the addition of 8 M urea-HCl (pH 2.0) at room temperature, in the dark. The *E*-configured chromophore was then converted to the *Z*-configured form by white light irradiation (*16*, *27*).

RESULTS

In Vivo Reconstitution: Assembly in E. coli. We expressed the TePixJ_GAF apoprotein in *E. coli* that coproduced PCB to investigate in vivo reconstitution of the holoprotein. From the UV–vis spectra (Figure 1), the affinity-purified protein exhibited a bound chromophore that displayed reversible photoconversion between the blue light-absorbing and green light-absorbing forms, similar to those of native TePixJ_GAF prepared from cyanobacterial cells. There were some differences, however, and

Table 1: Ratio of PVB to PCB of Photoactive TePixJ

in vivo		in vitro				
			50 °C		30 °C	
Synechocystis	*E. coli*	0 h	20 h	72 h	41 h	60 days
100:0	80:20	0:100	50:50	80:20	10:90	80:20

although the positions of the peaks were almost identical, their relative heights were somewhat different: the peak height at 530 nm was comparable to the peak height at 433 nm in the *E. coli*-expressed protein (Figure 1A), although the Pb peak is approximately 80% as large as the Pg peak for native TePixJ_GAF (*15*). Moreover, a significant light absorption was observed above 600 nm (Figure 1A), and the difference spectrum consistently exhibited a small but obvious negative peak at this location (Figure 1B, Pg minus Pb).

The chromophore species was identified by acid/urea denaturation and subsequent photoconversion. Some linear tetrapyrroles can be photoconverted between the *Z* and *E* configurations at the C15═C16 bond and show distinct absorption even after denaturation, although the equilibrium is greatly in favor of the *Z* configuration. This directional photoconversion allowed us to identify the *E*-configured chromophore species: we denatured the Pg form of the in vivo-reconstituted TePixJ_GAF and photoconverted the chromophore. The difference spectrum of the *E. coli*-expressed holoprotein after photoconversion following denaturation clearly showed two positive peaks at 603 and 673 nm, and these correspond to PVB and PCB, respectively (Figure 1C,D). From the peak ratios, we roughly estimated the PVB:PCB ratio to be 4:1 (Table 1).

An interesting question is how red light-absorbing PCB can be modified to absorb the blue light at 430 nm. The DNA sequences of the chromophore-binding GAF domains of cyanobacteriochromes show two conserved Cys residues: one a canonical Cys residue (Cys522 in TePixJ), conserved in all members, and the other Cys494 in TePixJ, conserved only in the Pb- and Pg-type cyanobacteriochromes (Figure S1 of the Supporting Information). Using site-directed mutagenesis, we substituted Cys494 with Ala and expressed the protein in PCB-producing *E. coli*. The Cys494Ala mutant was clearly not photoactive and exhibited an absorption peak at 638 nm indicative of bound PCB (Figure S2 of the Supporting Information). Acid/urea denaturation showed that the chromophore was exclusively *Z*-configured at C15. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Zn-induced fluorescence confirmed the covalent ligation of PCB to the Cys494Ala apoprotein. Further incubation at 50 °C did not change the chromophore, suggesting that Cys494 is essential for photoconversion and isomerization to PVB. We additionally confirmed, by site-directed mutagenesis, that the canonical Cys522 is also essential for the assembly of the photoactive holocomplex (Figure S3 of the Supporting Information).

In Vitro Reconstitution with Synthetic PCB. To follow the time course of the assembly of the holocomplex and isomerization of PCB to PVB, we performed in vitro reconstitution using chemically synthesized PCB and TePixJ_GAF apoprotein. Mixing of PCB with the apoprotein in the dark resulted in an immediate change in its absorption spectrum (Figure 2A, thin solid and dashed lines). The difference spectrum shows a decrease in absorption at 380 nm and an increase at 430 nm (Figure 2B). Approximately half of this blue light-absorbing holoprotein was photoactive and converted to a green light-absorbing form upon irradiation with blue light (Figure 2A, dotted line). The difference spectrum shows an intense negative peak at 430 nm (blue) and a broad positive peak at 545 nm (green), accompanied by a small negative peak near 642 nm (Figure 2B, dashed line). The negative peak at blue wavelengths is very similar to that for native TePixJ_GAF, whereas the peak at green wavelengths is much wider than that of the native species (Figure 2B, dotted line). Interestingly, irradiation for approximately 10 min was needed for full photoconversion, suggesting that the quantum yield is rather low compared with that of the native species. The green light-absorbing form of the reconstituted protein was mostly photoactive; approximately 86% of the peak at green wavelengths was converted back to the peak at blue wavelengths upon irradiation with green light (Figure 2A, thick solid line). Further irradiation gave almost fully reversible photoconversion between the blue light-absorbing and green light-absorbing forms, although the peak at green wavelengths was broader (Figure 2C,D).

Because the reconstituted preparation contained both photoactive and inactive chromophores, the photoactive chromophore was identified by acid/urea denaturation and subsequent photoconversion (Figure 2E). Both PCB and PVB exhibit similar *E*-to-*Z* photoconversion, but their peak positions differ (*16*). The chromophore of the blue light-absorbing form (reconstituted in the dark) was assigned to *Z*-PCB at C15 as judged by its acid-denatured absorption spectrum and its inability to photoconvert. The chromophore of the green light-absorbing form, photogenerated from the blue light-absorbing form, was assigned to *E*-PCB at C15 (Figure 2E,F). Interestingly, no PVB was detected in the initially reconstituted holoprotein, despite it exhibiting partial photoconversion between the blue light-absorbing and broad green light-absorbing forms that are similar to the Pb and Pg forms of native TePixJ_GAF. The covalent ligation of PCB immediately after reconstitution in the dark was confirmed by Zn-induced fluorescence following SDS−PAGE (Figure S3 of the Supporting Information). These results suggest that the covalently ligated PCB (i.e., *Z*-PCB at C15) bound to TePixJ_GAF is the chromophore of the partially photoactive blue light-absorbing form (λ_{max} = 430 nm), although the photoreactivity was not as strong as for PVB in the native protein.

In Vitro Isomerization of PCB to PVB. The holoprotein that had been reconstituted in vitro was incubated in the dark to monitor the isomerization of PCB to PVB. The reversibility of the photoconversion was improved, and the difference spectrum of the photoconversion became closer to that of the native protein upon incubation at 50 °C for 72 h (Figure 3A,B). Specifically, the peak at 545 nm was blue-shifted to 530 nm and had narrowed compared to that of the initially reconstituted holoprotein (Figure 3A, dashed line). Acid/urea denaturation and subsequent photoconversion revealed that the active chromophore of the incubated protein was approximately 80% PVB and 20% PCB (Figure 3C,D). After incubation at 50 °C for 1 week, the active chromophore became 100% PVB, although a considerable amount was precipitated from solution (Table 1). The conversion from PCB to PVB in TePixJ_GAF was much slower at 30 °C, however (Figure S4 of the Supporting Information): incubation at 30 °C for 60 days produced nearly 80% PVB, but incubation for 41 h produced only 10% PVB. By comparison, free PCB was not converted to PVB at 30 or 50 °C in the absence of apoprotein. These results clearly demonstrate that TePixJ_GAF has intrinsic

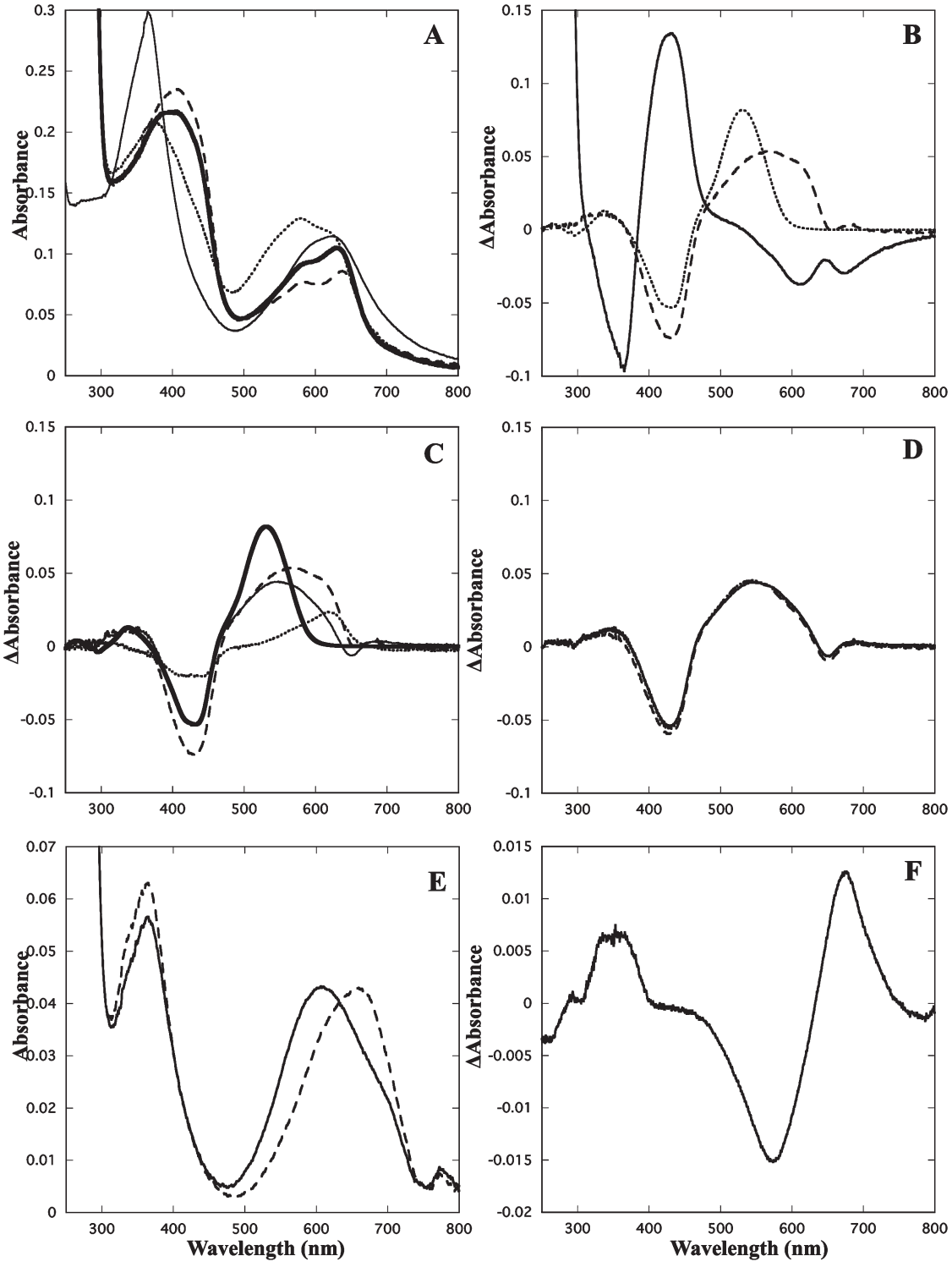

FIGURE 2: Absorption spectra of the photoconversion of holo-TePixJ_GAF reconstituted in vitro with PCB. (A) Free synthetic PCB (20 μM) in reconstitution buffer (thin solid line), holo-TePixJ_GAF immediately after reconstitution in the dark (dashed line), holo-TePixJ_GAF irradiated first with blue light (dotted line), and holo-TePixJ_GAF then irradiated with green light (thick solid line). (B) Difference spectra for reconstituted holo-TePixJ_GAF, after reconstitution minus before reconstitution (—) and after first blue light irradiation minus under dark conditions (− − −), and native TePixJ_GAF, Pg form minus Pb form (· · ·). (C) Difference spectra of sequential photoconversions. The first photoconversion was after first blue light irradiation minus under dark conditions (dashed line), the second photoconversion after first blue light irradiation minus first green light irradiation (thin solid line), the difference between the first and second photoconversions (dotted line), and photoconversion of native TePixJ_GAF (thick solid line). (D) Difference spectra of sequential photoconversions. The third photoconversion was second blue light irradiation minus first green light irradiation (—), the fourth photoconversion second blue light irradiation minus second green light irradiation (− − −), and the fifth photoconversion third blue light irradiation minus second green light irradiation (· · ·). (E) Absorption spectra of the denatured green light-absorbing form generated by first irradiation with blue light: before (—) and after (− − −) irradiation with white light. (F) Difference spectrum of the photoconversion of the denatured green light-absorbing form.

PCB to PVB isomerase activity, and that the holoprotein, especially the green light-absorbing form, shows increased reactivity and photoconversion reversibility compared to that of the PCB holoprotein.

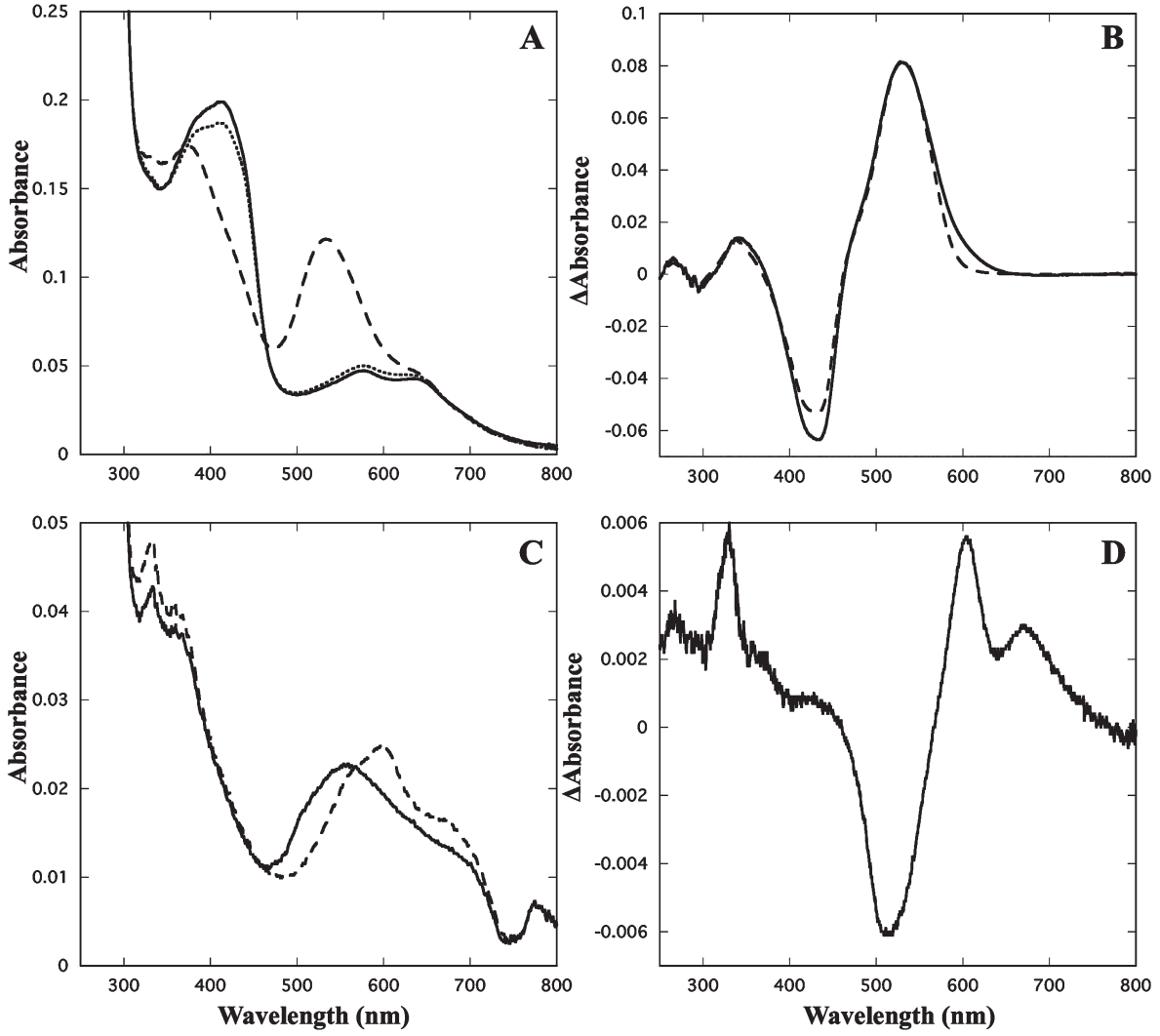

FIGURE 3: Absorption spectra of holo-TePixJ_GAF reconstituted in vitro after incubation at 50 °C for 72 h. (A) Absorption spectra of holo-TePixJ_GAF after incubation for 72 h (—), after irradiation with blue light (– – –), and after irradiation with green light (· · ·). (B) Difference spectra of the assembled holo-TePixJ_GAF incubated for 72 h: green light-absorbing form minus blue light-absorbing form (—) and native TePixJ_GAF, Pg form minus Pb form (– – –). (C) Absorption spectra of the denatured green light-absorbing form before (—) and after (– – –) irradiation with white light. (D) Difference spectrum of the photoconversion of the denatured green light-absorbing form.

Involvement of Cysteine Residues in Photoconversion. Light-induced changes were monitored at the thiol group of the Cys residues by FTIR spectroscopy. The thiol S−H vibrational stretching mode is detected in the wavenumber region of 2650−2520 cm^{-1}. During photoconversion from Pb to Pg of native TePixJ_GAF, the FTIR difference spectrum exhibited a new peak generated at 2567 cm^{-1}, although no corresponding negative peak was detected (Figure 4, solid line) and an inverted signal was detected upon conversion from Pg to Pb (Figure 4, dashed line). This suggests that the signal in Pg can be assigned to a free SH group, and its disappearance in the Pb form corresponds to adduct formation, possibly with the chromophore, suggesting that a second Cys residue, perhaps Cys494, forms an adduct with PVB in the Pb form and is released in the Pg form.

Reversion of the Pg Form to the Pb Form of TePixJ_GAF under Dark Conditions. The stability of the Pb and Pg forms of native TePixJ_GAF was investigated under dark conditions at the physiological temperature (for *T. elongatus*) of 50 °C. The Pb form was clearly stable, and the Pg form had mostly reverted to the Pb form after 1 day. Nonspecific denaturation appeared to have also taken place during the incubation

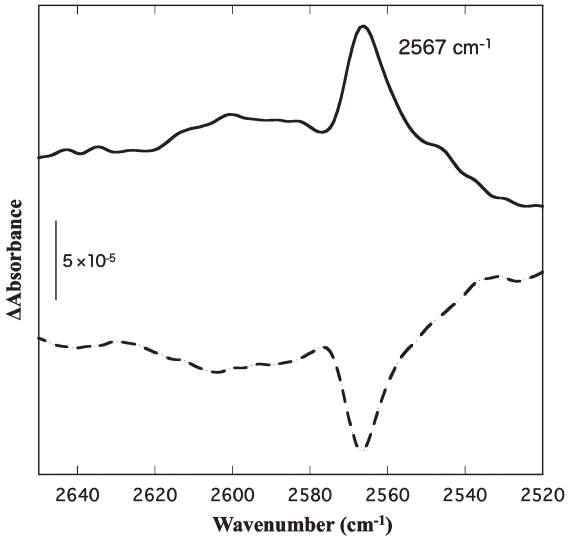

FIGURE 4: FTIR difference spectra in the S−H stretching region for native TePixJ_GAF. Pg form minus Pb form after blue (—) and green (– – –) light irradiation.

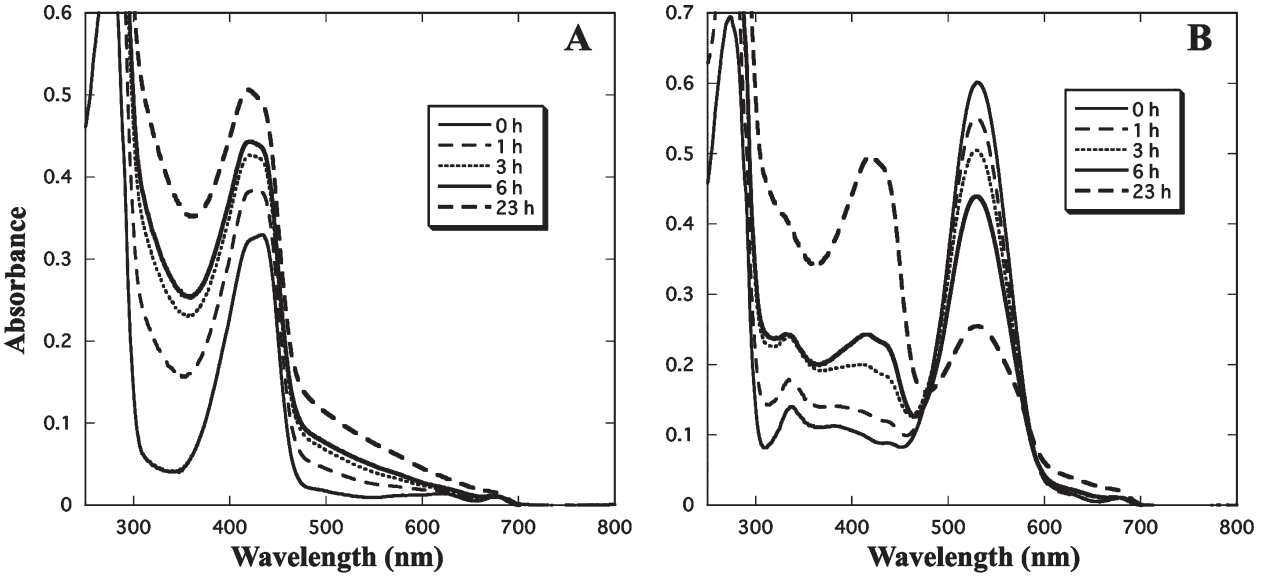

FIGURE 5: Absorption spectra of the reversion of native TePixJ_GAF under dark conditions at 50 °C for (A) the Pb form and (B) the Pg form.

period, irrespective of the spectral forms (Figure 5). The Pb form generated from the Pg form was very similar to the photochemically generated Pb form. This result strongly suggests that the Pb form is the nonsignaling state, and the Pg form is the signaling state. Spontaneous dark reversion from Pg to Pb was not observed at 0 °C (*15*).

DISCUSSION

We have demonstrated that the GAF domain of TePixJ isomerizes PCB (C4═C5) to PVB (C2═C3) in vivo and in vitro. Although it has been reported previously that Pb- and Pg-type cyanobacteriochromes have been prepared in PCB-producing *E. coli*, the bound chromophores have not been carefully studied or compared to cyanobacteriochromes isolated from cyanobacterial cells (*28*−*30*). In this report, we also expressed several other Pb- and Pg-type cyanobacteriochromes in PCB-producing *E. coli* and confirmed that the photoactive chromophore was not 100% PVB but was comprised of 10−20% PCB. The isomerization reaction in vitro was rather slow and required a relatively high temperature (50 °C), which is close to the growth optimum (55 °C) for the source organism, *T. elongatus*. Alternatively, other (co)factors may facilitate the reaction in cyanobacterial cells. The GAF domain alone was also sufficient for fast ligation between $C3^1$ of ring A of PCB and the canonical Cys522, and photoisomerization occurred between the *Z* and *E* isomers at C15. Among members of the GAF superfamily, the GAF domain of TePixJ and related PVB-binding GAF domains possesses unusual multifunctionality.

At the initial stage of in vitro reconstitution, PCB-bound TePixJ_GAF exhibited unique photocycles with limited reversibility (Figure 2). Approximately half of the initial reconstituted blue light-absorbing form (λ_{max} = 430 nm) was photoactive and converted to the green light-absorbing peak (λ_{max} = 545 nm); the remaining blue light-absorbing form was inactive. Subsequently, most of the green light-absorbing form was photoconverted back to the blue light-absorbing form (λ_{max} = 430 nm). Also present was a small amount of red light-absorbing form (λ_{max} = 618 nm), which was inactive upon photoconversion (Figure 2C, dotted line). Additional illumination gave almost reversible photoconversion between the blue light- and green light-absorbing forms (Figure 2D). Both the active and inactive blue light-absorbing forms show absorption spectra very similar to that of the native Pb form, in terms of peak position (at approximately 430 nm) and narrow bandwidth. This result suggests that there are at least two steps for the correct assembly of the active Pb form: conversion of the red light-absorbing PCB to a bilirubin-like blue light-absorbing chromophore and proper positioning of the chromophore (possibly rings C and D) in the protein pocket.

The initially photogenerated green light-absorbing form exhibited a broad peak, which reflected a mixture of active green light-absorbing and inactive red light-absorbing forms. The denaturation analysis revealed that the chromophore was mostly C15 *E*-PCB. Because the absorption of this inactive form resembles that of free PCB, the chromophore may be ejected from the chromophore-binding pocket on photoconversion of the blue light-absorbing form that was reconstituted in vitro. It is also possible that the inactive form consisted of C15 *Z*-PCB, cleaved from Cys494 upon illumination. In any case, it is of note that the active green light-absorbing form containing C15 *E*-PCB exhibited a rather broad peak at green wavelengths, compared with the native Pg form containing C15 *E*-PVB. Interestingly, the photoactive green light-absorbing form appears to bind red light-absorbing C15 *E*-PCB and displays a broad peak. This suggests that PCB, in the green light-absorbing form, does not fit optimally into the PVB-binding pocket and is somewhat distorted in the PVB-binding pocket.

Perhaps unexpectedly, the peak of the initially reconstituted protein that binds PCB at blue wavelengths is very similar to that of the native PVB-bound Pb form, despite the different double bond conjugation. This can be explained if the double bond conjugation of PCB is interrupted at C10 by bonding via the thiol group of a (second) Cys residue (probably Cys494); this explanation was originally postulated for another Pb- and Pg-type cyanobacteriochrome, Tlr0924, by Rockwell et al. (*29*). Unstable binding of a methanol molecule was consistently observed at C10 of biliverdin during its chemical synthesis (*31*). On the basis of various spectroscopic measurements and molecular modeling, alternative bonding via the thiol group of a second Cys residue at the C5 methine bridge of PCB has also been proposed (*30*). Our FTIR results show a free thiol signal was present in the Pg form, but not in the Pb form, of native TePixJ_GAF protein; this is

consistent with the hypothesis that a second Cys residue is involved in binding. This putative reversible covalent binding of the second Cys residue to PCB seems to be labile compared to the stable covalent bonding of the canonical Cys522 to $C3^1$ of PCB, because no such blue absorption was detected after acid/urea denaturation or SDS denaturation at neutral pH (*15*). From the site-directed mutagenesis experiments reported here, we propose that it is Cys494, conserved only in Pb- and Pg-type cyanobacteriochromes, that reversibly bonds to the chromophore, possibly at C10.

Clearly, the GAF domain alone of TePixJ is able to isomerize PCB to PVB, although the efficiency of the reaction was lower when TePixJ_GAF reconstitution occurred in vitro compared to in vivo. Cys494 is essential for isomerization from PCB to PVB, and we suggest that the putative adduct between Cys494 and C10 in the blue light-absorbing form may facilitate the conversion from a C4=C5 bond to a C2=C3 bond. Such a bilirubin-like adduct was also suggested as a transient intermediate from PCB to PVB in the chromophore attachment of phycoerythrocyanin (*32*). We attempted to compare the isomerization of PVB for the blue light- and green light-absorbing forms of the PCB-bound holo-TePixJ_GAF, but spontaneous reversion from the green light- to blue light-absorbing form (at 50 °C in the dark) prevented this. This is consistent with the observation that native TePixJ_GAF exhibited unidirectional dark reversion from Pg to Pb (Figure 5). Interestingly, a Cys residue is required for isomerization of PCB to PVB by PecE/F (*18*), and although there is no sequence homology between TePixJ and PecE/F, a related chemical reaction may underlie the isomerization.

Finally, we note that the blue form was assembled in vitro in the dark, and the unidirectional reversion of native Pg to Pb also takes place in the dark, at physiological temperatures. We have also observed that the Pb form of TePixJ_GAF assembles in *E. coli* when grown in the dark (data not shown). These results are consistent with our observations for SyPixJ1 (*13*) and strongly suggest that TePixJ, like SyPixJ1, is a blue light sensor.

In conclusion, in addition to its known autolyase activity with Cys522 and photoreversible isomerase activity (of the *Z* and *E* isomers at C15 and C16 of PCB), the GAF domain of TePixJ appears to have other roles: as an isomerase (converting PCB to PVB) and as a photoreversible autolyase with a second conserved Cys residue.

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SUPPORTING INFORMATION AVAILABLE

Sequence alignment of the chromophore-binding GAF domains of cyanobacteriochromes and phytochromes (Figure 1), absorption spectra and SDS−PAGE (Coomassie staining and zinc-induced fluorescence) of holo-TePixJ_GAF (Cys494Ala) assembled in *E. coli* that coproduced PCB (Figure 2), absorption spectra and SDS−PAGE (Coomassie staining and zinc-induced fluorescence) of holo-TePixJ_GAF (Cys522Ala) assembled in *E. coli* that coproduced PCB (Figure 3), SDS−PAGE of holo-TePixJ_GAF reconstituted with PCB in vitro (Figure 4), absorption spectra of holo-TePixJ_GAF reconstituted in vitro after incubation at 30 °C for 41 h (Figure 5). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. van der Horst, M. A., and Hellingwerf, K. J. (2004) Photoreceptor proteins, "star actors of modern times": A review of the functional dynamics in the structure of representative members of six different photoreceptor families. *Acc. Chem. Res. 37*, 13–20.
2. Sidler, W. A. (1994) Phycobilisome and phycobiliprotein structures. In The molecular biology of cyanobacteria (Bryant, D. A., Ed.) pp 139−216, Kluwer Academic, Dordrecht, The Netherlands.
3. Montgomery, B. L., and Lagarias, J. C. (2002) Phytochrome ancestry: Sensors of bilins and light. *Trends Plant Sci. 7*, 357–366.
4. Liu, L. N., Chen, X. L., Zhang, Y. Z., and Zhou, B. C. (2005) Characterization, structure and function of linker polypeptides in phycobilisomes of cyanobacteria and red algae: An overview. *Biochim. Biophys. Acta 1708*, 133–142.
5. MacColl, R. (1998) Cyanobacterial phycobilisomes. *J. Struct. Biol. 124*, 311–334.
6. Kuno, N., and Furuya, M. (2000) Phytochrome regulation of nuclear gene expression in plants. *Semin. Cell Dev. Biol. 11*, 485–493.
7. Rockwell, N. C., Su, Y. S., and Lagarias, J. C. (2006) Phytochrome structure and signaling mechanisms. *Annu. Rev. Plant Biol. 57*, 837–858.
8. Sharrock, R. A. (2008) The phytochrome red/far-red photoreceptor superfamily. *Genome Biol. 9*, 230.
9. Rodriguez-Romero, J., Hedtke, M., Kastner, C., Muller, S., and Fischer, R. (2010) Fungi, hidden in soil or up in the air: Light makes a difference. *Annu. Rev. Microbiol. 64*, 585–610.
10. Ikeuchi, M., and Ishizuka, T. (2008) Cyanobacteriochromes: A new superfamily of tetrapyrrole-binding photoreceptors in cyanobacteria. *Photochem. Photobiol. Sci. 7*, 1159–1167.
11. Yoshihara, S., and Ikeuchi, M. (2004) Phototactic motility in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. *Photochem. Photobiol. Sci. 3*, 512–518.
12. Hirose, Y., Shimada, T., Narikawa, R., Katayama, M., and Ikeuchi, M. (2008) Cyanobacteriochrome CcaS is the green light receptor that induces the expression of phycobilisome linker protein. *Proc. Natl. Acad. Sci. U.S.A. 105*, 9528–9533.
13. Yoshihara, S., Katayama, M., Geng, X., and Ikeuchi, M. (2004) Cyanobacterial phytochrome-like PixJ1 holoprotein shows novel reversible photoconversion between blue- and green-absorbing forms. *Plant Cell Physiol. 45*, 1729–1737.
14. Yoshihara, S., Suzuki, F., Fujita, H., Geng, X. X., and Ikeuchi, M. (2000) Novel putative photoreceptor and regulatory genes required for the positive phototactic movement of the unicellular motile cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Cell Physiol. 41*, 1299–1304.
15. Ishizuka, T., Shimada, T., Okajima, K., Yoshihara, S., Ochiai, Y., Katayama, M., and Ikeuchi, M. (2006) Characterization of cyanobacteriochrome TePixJ from a thermophilic cyanobacterium *Thermosynechococcus elongatus* strain BP-1. *Plant Cell Physiol. 47*, 1251–1261.
16. Ishizuka, T., Narikawa, R., Kohchi, T., Katayama, M., and Ikeuchi, M. (2007) Cyanobacteriochrome TePixJ of *Thermosynechococcus elongatus* harbors phycoviolobilin as a chromophore. *Plant Cell Physiol. 48*, 1385–1390.
17. Bishop, J. E., Rapoport, H., Klotz, A. V., Chan, C. F., Glazer, A. N., Füglistaller, P., and Zuber, H. (1987) Chromopeptides from phycoerythrocyanin. Structure and linkage of the three bilin groups. *J. Am. Chem. Soc. 109*, 875–881.
18. Zhao, K. H., Wu, D., Zhou, M., Zhang, L., Bohm, S., Bubenzer, C., and Scheer, H. (2005) Amino acid residues associated with enzymatic activities of the isomerizing phycoviolobilin-lyase PecE/F. *Biochemistry 44*, 8126–8137.
19. Scheer, H., and Zhao, K. H. (2008) Biliprotein maturation: The chromophore attachment. *Mol. Microbiol. 68*, 263–276.
20. Bohm, S., Endres, S., Scheer, H., and Zhao, K. H. (2007) Biliprotein chromophore attachment: Chaperone-like function of the PecE subunit of α-phycoerythrocyanin lyase. *J. Biol. Chem. 282*, 25357–25366.
21. Nakamura, Y., Kaneko, T., Sato, S., Ikeuchi, M., Katoh, H., Sasamoto, S., Watanabe, A., Iriguchi, M., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa, C., Kohara, M., Matsumoto, M., Matsuno, A., Nakazaki, N., Shimpo, S., Sugimoto, M., Takeuchi, C., Yamada, M., and Tabata, S. (2002) Complete genome structure of the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1. *DNA Res. 9*, 123–130.
22. Mukougawa, K., Kanamoto, H., Kobayashi, T., Yokota, A., and Kohchi, T. (2006) Metabolic engineering to produce phytochromes with phytochromobilin, phycocyanobilin, or phycoerythrobilin chromophore in *Escherichia coli*. *FEBS Lett. 580*, 1333–1338.

23. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*, 248–254.
24. Kakiuchi, T., Kato, H., Jayasundera, K. P., Higashi, T., Watabe, K., Sakamoto, D., Kinoshita, H., and Inomata, K. (1998) Total synthesis of (±)-phycocyanobilin and its derivatives bearing a photoreactive group at D-ring. *Chem. Lett. 27*, 1001–1002.
25. Kakiuchi, T., Kinoshita, H., and Inomata, K. (1999) Total Synthesis of (±)-Phytochromobilin Starting from Two Pyrrole Derivatives. *Synlett,* 901–904.
26. Noguchi, T., and Sugiura, M. (2002) Flash-induced FTIR difference spectra of the water oxidizing complex in moderately hydrated photosystem II core films: Effect of hydration extent on S-state transitions. *Biochemistry 41*, 2322–2330.
27. Zhao, K. H., and Scheer, H. (1995) Type I and type II reversible photochemistry of phycoerythrocyanin α-subunit from *Mastigocladus laminosus* both involve Z, E isomerization of phycoviolobilin chromophore and are controlled by sulfhydryls in apoprotein. *Biochim. Biophys. Acta 1228*, 244–253.
28. Yoshihara, S., Shimada, T., Matsuoka, D., Zikihara, K., Kohchi, T., and Tokutomi, S. (2006) Reconstitution of blue-green reversible photoconversion of a cyanobacterial photoreceptor, PixJ1, in phycocyanobilin-producing *Escherichia coli*. *Biochemistry 45*, 3775–3784.
29. Rockwell, N. C., Njuguna, S. L., Roberts, L., Castillo, E., Parson, V. L., Dwojak, S., Lagarias, J. C., and Spiller, S. C. (2008) A second conserved GAF domain cysteine is required for the blue/green photoreversibility of cyanobacteriochrome Tlr0924 from *Thermosynechococcus elongatus*. *Biochemistry 47*, 7304–7316.
30. Ulijasz, A. T., Cornilescu, G., von Stetten, D., Cornilescu, C., Velazquez Escobar, F., Zhang, J., Stankey, R. J., Rivera, M., Hildebrandt, P., and Vierstra, R. D. (2009) Cyanochromes are blue/green light photoreversible photoreceptors defined by a stable double cysteine linkage to a phycoviolobilin-type chromophore. *J. Biol. Chem. 284*, 29757–29772.
31. Chen, L., Kinoshita, H., and Inomata, K. (2009) Synthesis of doubly locked 5*Z*s15*Z*a-biliverdin derivatives and their unique spectral behavior. *Chem. Lett. 38*, 602.
32. Tu, J. M., Zhou, M., Haessner, R., Ploscher, M., Eichacker, L., Scheer, H., and Zhao, K. H. (2009) Toward a mechanism for biliprotein lyases: Revisiting nucleophilic addition to phycocyanobilin. *J. Am. Chem. Soc. 131*, 5399–5401.