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# Thiol-Based Photocycle of the Blue and Teal Light-Sensing Cyanobacteriochrome Tlr1999

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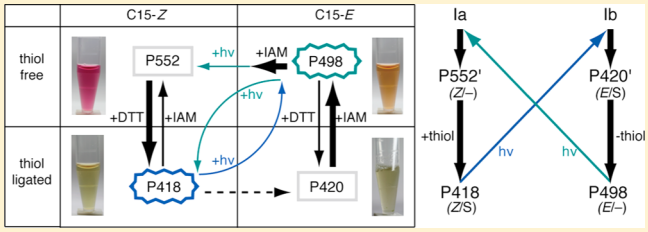
Supporting Information

**ABSTRACT:** Cyanobacteriochromes are a spectrally diverse photoreceptor family that binds a bilin chromophore. For some cyanobacteriochromes, in addition to the widely conserved cysteine to anchor the chromophore, its ligation with a second cysteine is responsible for a remarkable blue shift. Herein, we report a newly discovered cyanobacteriochrome Tlr1999 exhibiting reversible photoconversion between a blue-absorbing form at 418 nm (P418) and a teal-absorbing form at 498 nm (P498). Acidic denaturation suggests that P418 harbors C15-*Z* phycoviolobilin, whereas P498 harbors C15-*E* phycoviolobilin. When treated with iodoacetamide, which irreversibly modifies thiol groups, P418 is slowly converted to a green-absorbing photoinactive form denoted P552. The absorption spectrum of P498 appears to be unaffected by iodoacetamide, but when iodoacetamide modified, it is photoconverted to P552. These results suggest that a covalent bond exists between the second Cys and the phycoviolobilin in P418 but not in P498. Subsequent treatment with dithiothreitol converts P552 into P418, whereas dithiothreitol reduces P498 to yield P420, a photoinactive form. Site-directed mutagenesis shows that the second Cys is essential for assembly of the photoactive holoprotein and that the photoactivity of this inert mutant is partially rescued by $\beta$-mercaptoethanol. These results suggest that the covalent attachment and detachment of a thiol, although not necessarily that of the second Cys, is critical for the reversible spectral blue shift and the complete photocycle. We propose a thiol-based photocycle, in which the thiol-modified P552 and P420 are intermediate-like forms.




Phytochromes are red and far-red light receptors that regulate a wide range of physiological responses not only in plants but also in bacteria and fungi.[1−3] Their N-terminal photosensory modules consist of PAS (Per/ARNT/Sim),[4] GAF (cGMP phosphodiesterase/adenylyl cyclase/FhlA),[5] and PHY (phytochrome-specific) domains.[3] In plant and cyanobacterial phytochromes, a linear tetrapyrrole (bilin) chromophore, phytochromobilin or phycocyanobilin (PCB), respectively, is covalently bound to a conserved cysteine residue within the GAF domain.[6,7] Bacterial and fungal phytochromes bind biliverdin IX$\alpha$ at a conserved cysteine residue N-terminal to the PAS domain.[8−10] It is generally accepted that light excitation triggers a *Z*/*E* isomerization of the C15═C16 double bond between pyrrole rings C and D, leading to the reversible photoconversion between a red-absorbing form and a far-red-absorbing form.[11−14] Transient deprotonation of the chromophore has also been proposed for dark reaction that can follow the photoconversion to the far-red-absorbing form, leading to the transient spectral shift.[15,16]

Cyanobacteriochromes (CBCRs) are a recently identified group of photoreceptors that are distantly related to phytochromes and have been found only in cyanobacteria.[17] The CBCR GAF domain is multifunctional by itself *in vivo*: it acts as a PCB autolyase, a photoisomerase and in some instances as an autoisomerase.[1,17,18] The CBCR family includes regulators of the chromatic acclimation of the phycobiliproteins, RcaE[19] and CcaS,[20,21] and regulators of the phototaxis, PixJ,[22,23] PixA/UirS,[24,25] and Cph2.[26,27] CBCRs may be also involved in a light-induced reset of the circadian rhythm[28] and cell aggregation.

It is quite unexpected that CBCRs are spectrally diverse covering throughout the near-UV/vis region (Supporting Information Figure S1).[21,25,29−34] The first CBCR characterized *in vitro* was SyPixJ1, which showed reversible photoconversion between a blue-absorbing form (Pb) and a green-absorbing form (Pg).[29] Since then, Pb/Pg-type CBCRs have been investigated intensively, with studies concentrating on SyPixJ1,[35] TePixJ,[18,36−38] and Tlr0924.[39] On the other hand, AnPixJ, SyCcaS, and NpCcaS show reversible photoconversion between Pg and a red-absorbing form (Pr), denoted Pg/Pr-type

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CBCRs,[20,21,31] though these spectral properties do not necessarily reflect the chromophore configuration. More recently, comprehensive studies have revealed that CBCRs exhibit even wider variations of spectral properties.[33]

Despite such variations, these CBCRs possess the conserved Cys residue which corresponds to that in the plant and cyanobacterial phytochromes. This widely conserved "canonical" Cys binds to C3[1] of ring A to anchor the chromophore stably. Further, the Pb/Pg-type CBCRs possess another conserved cysteine residue within the conserved [D/E]XCF motif (Figure S1). This second Cys as well as the motif is also conserved in a violet/green-type PixA/UirS.[24,25] The [D/E]XCF motif corresponds to the conserved DIP motif in phytochromes, for which the aspartic acid is positioned near the bilin chromophore and is essential for photoconversion.[7,40,41] In the Pb/Pg-type CBCRs, the Cys in the [D/E]XCF motif is essential for the photoconversion.[18,24,38,39] Furthermore, the Pb/Pg-type CBCRs bind phycoviolobilin (PVB) as a chromophore.[36] Other types of CBCRs have been found, including the violet/yellow-type (SyCikA) that harnesses the Cys in a modified motif[32] and the violet/orange-type and UV/blue-type CBCRs that have a conserved cysteine in an insert sequence (named "insert Cys").[33] These CBCRs utilize PCB as a chromophore.[33]

The precise role of the second Cys remains to be clarified. There are two contradictory models concerning the role of the Cys during Pb/Pg photoconversion. The reversible attachment model[18,39] (Figure S2) suggests that Cys is covalently ligated to C10 of PVB in Pb, whereas it is not bound to the chromophore in Pg. The stable double linkage model[38] suggests that the second Cys is stably ligated to PVB-like chromophore in both Pb and Pg during the photoconversion. It is also suggested that the chromophore is distorted between rings A and B owing to the thioether linkage between the second Cys and C4 or C5.[38] The Cys-ligated site has not yet been experimentally determined. Nonetheless, the second Cys may be involved in the Pb/Pg photocycle.

To study the photochemical and signaling mechanisms of CBCRs, we have focused on those found in the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 because its CBCRs are relatively small in number and thermostable. Of the five putative CBCRs identified according to their genomic sequences,[42] four (TePixJ, Tlr0924, Tlr0911, and Tlr1999) contain the [D/E]XCF-motif, whereas the fifth (Tll0899) is homologous to CikA. A phytochrome homologue has not been found in the *T. elongatus* genome. For the study reported herein, we expressed, purified, and characterized the spectral properties of the Tlr1999 GAF domain (Tlr1999-GAF) and studied the role of its second Cys mainly by the chemical modification of thiols.

## EXPERIMENTAL PROCEDURES

**Computational Studies.** The Tlr1999 domain composition was determined using SMART (http://smart.embl-heidelberg.de/).[43] Sequence alignment of CBCR chromophore-binding GAF domains was by CLUSTAL_X.[44]

**Plasmid Construction.** The gene encoding the GAF domain of *T. elongatus* Tlr1999 (Tlr1999-GAF, amino acid residues 191−342) was PCR amplified with Ex *Taq* DNA polymerase (TaKaRa, Ohtsu, Japan) and the primers 5′-AACATATGGAGCTTTCGACGATTCTC-3′ and 5′-GAGGATCCTATTGAGCTTTTTGATAGAGTT-3′ and was then cloned into a pT7blue T-Vector (Novagen, Madison, WI). After sequence confirmation, the DNA was excised with *Nde*I and *Bam*H1 and cloned into pTCH2031V[37] at the trc promoter site for expression in *Synechocystis* sp. PCC 6803. The gene was also cloned into pET28a (Novagen) for expression in *Escherichia coli*. The expressed protein was $(His)_6$ tagged at its N-terminus.

**Site-Directed Mutagenesis.** The genes encoding the second Cys mutant (C246A), the canonical Cys mutant (C274A), and the Cys double mutant (C274A/C246A) were created using QuikChange site-directed mutagenesis kit reagents (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Primers for the C246A construct were 5′-CGTGGGAGATTCCGCCTTTACGGAACAG-3′ and 5′-CTGTTCCGTAAAGGCGGAATCTCCCACG-3′. Primers for the C274A construct were 5′-CTCAGTTGCCCCCGCCTATCGCGATTTGC-3′ and 5′-GCAAATCGCGATAGGCGGGGGCAACTGAG-3′. The sequences were confirmed by nucleotide sequencing.

**Protein Expression in and Purification from Cyanobacterial Cells.** Cells of the cyanobacterium *Synechocystis* sp. PCC 6803 were transformed with the pTCH2031V derivatives according to ref 45. The recombinant cells were grown in 8 L of BG11 medium with 20 μg/mL chloramphenicol at 31 °C bubbled with air containing 1% (v/v) $CO_2$ in the light (intensity ca. 30 μmol photons $m^{-2}$ $s^{-1}$). Cells were harvested by centrifugation, suspended in disruption buffer (20 mM HEPES-NaOH (pH 7.5), 100 mM NaCl, 10% (w/v) glycerol), and then frozen at −80 °C. After thawing, cells were disrupted with a French press (no. 5501-M, Ohtake, Japan) three times at 1500 kg $cm^{-2}$. The homogenate was centrifuged twice at 12000*g* for 10 min and twice at 194100*g* for 30 min. The final supernatant was loaded onto a nickel-affinity His-Trap chelating column (GE Healthcare, Piscataway, NJ). Proteins were eluted with a linear gradient of 30−430 mM imidazole in disruption buffer. EDTA (1 mM) was added to the peak fractions, which were then held at 4 °C for 1 h. Pooled protein fractions were dialyzed against 20 mM HEPES-NaOH (pH 7.5), 500 mM NaCl, 10% (w/v) glycerol.

**Protein Purification from *E. coli*.** The *E. coli* strain C41 (DE3)[46] harboring the Tlr1999-GAF expression plasmid and the pKT271 plasmid for PCB[47] was precultured overnight at 37 °C in the dark in Luria−Bertani medium containing 20 μg/mL kanamycin and 20 μg/mL chloramphenicol. Fresh medium (1 L) was then inoculated with 10 mL of the culture. After incubation at 37 °C for 2 h, 1 mM isopropyl β-D-1-thiogalactopyranoside was added. The culture was incubated at 37 °C for an additional 3 h. Cells were collected by centrifugation, suspended in disruption buffer, and stored at −80 °C. Cells were thawed on ice and lysed with a French press three times at 1500 kg $cm^{-2}$. The homogenate was centrifuged at 194100*g* for 30 min at 4 °C. The protein was purified by His-Trap chromatography as described above.

**SDS-PAGE and Zinc-Enhanced Fluorescence Assay.** Purified protein was solubilized in 2% (w/v) lithium dodecyl sulfate, 60 mM dithiothreitol (DTT), and 60 mM Tris-HCl (pH 8.0) and then subjected to SDS-PAGE through a 12% (w/v) polyacrylamide gel. For the zinc-enhanced fluorescence assay,[48] the gel was soaked in 20 μM zinc acetate at room temperature for 30 min in the dark, and fluorescence was detected through a 605 nm filter with excitation at 532 nm (FMBIO II, TaKaRa). The gel was then stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Richmond, CA).

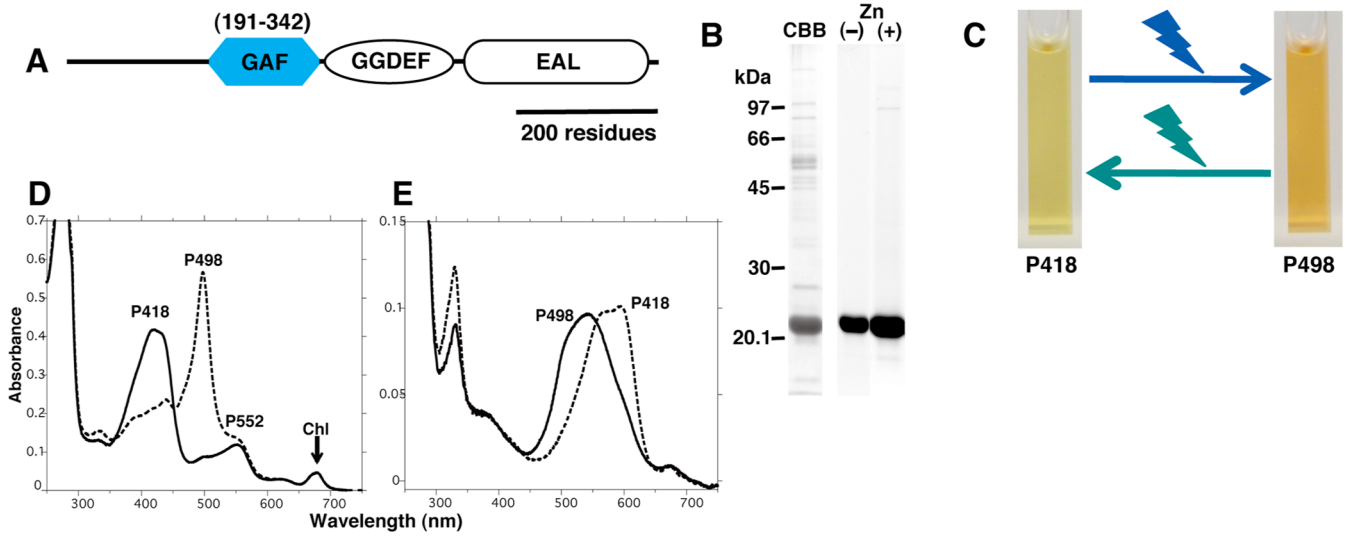


**Figure 1.** Tlr1999-GAF. (A) Domain architecture of Tlr1999 according to the motif analysis made by SMART. (B) His-tagged Tlr1999-GAF (residues 191−342) purified from cyanobacterial cells. SDS-PAGE gels of Tlr1999-GAF. Left lane: CBB, after Coomassie Brilliant Blue staining. Fluorescence emission by Tlr1999-GAF before (−, middle lane) and after (+, right lane) soaking the gel in $Zn^{2+}$. (C) Photographs of P418 and P498 in solution. (D) Absorption spectra of P418 (solid line) and P498 (broken line). Chl: absorbance peak associated with a chlorophyll contaminant. (E) Absorption spectra of denatured P498 (solid line) and P418 (broken line).

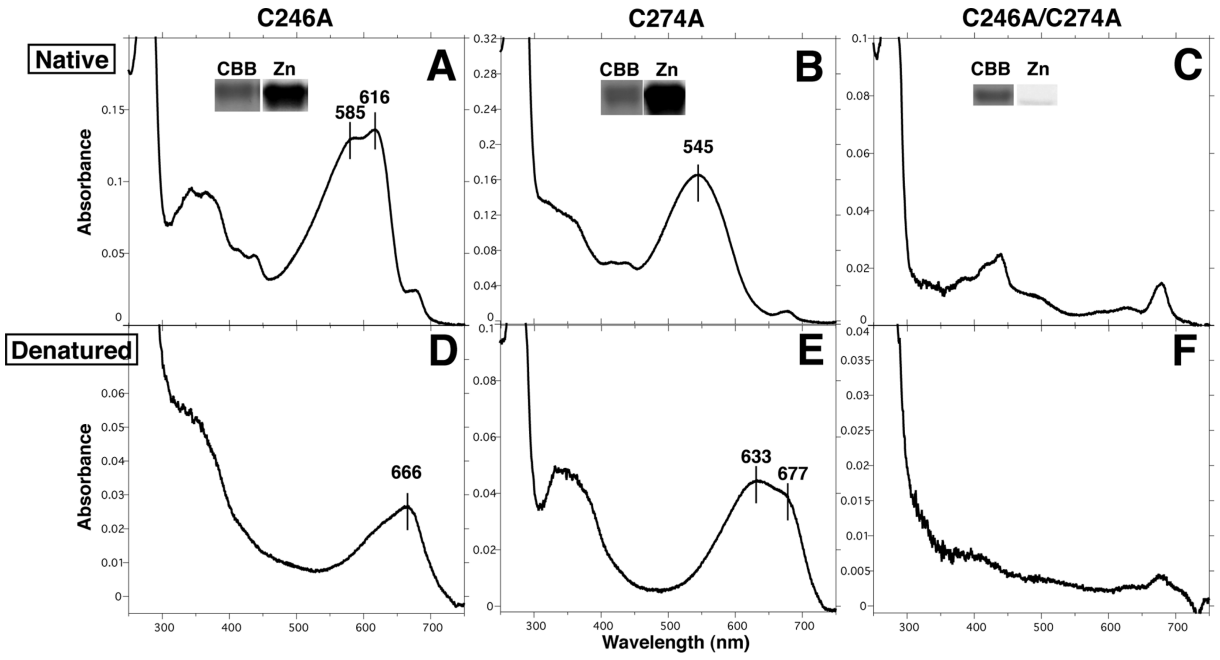


**Figure 2.** Absorption spectra of Tlr1999-GAF Cys → Ala mutants expressed in and purified from cyanobacterial cells. Absorption spectra of native (A) C246A, (B) C274A, and (C) C246A/C274A. Absorption spectra of denatured (D) C246A, (E) C274A, and (F) C246A/C274A. Insets: protein in SDS-PAGE gels after Coomassie Brilliant Blue (CBB) staining (left panels) and fluorescence after soaking the gel in a $Zn^{2+}$ solution (right panels).

**Chemical Modification and Spectral Analysis.** To modify cysteine thiols irreversibly, iodoacetamide (IAM) was added into protein solutions at a final concentration of 50 mM,[49] and the mixtures were then incubated for 60 min at room temperature in the dark. Excess IAM was removed by dialysis before treatment with DTT.

Absorption spectra were recorded at room temperature using a UV-2400PC spectrophotometer (Shimadzu, Kyoto, Japan). For blue-light irradiation, light-emitting diodes ($\lambda_{max}$ = 392 nm, half-bandwidth = 12 nm) were used. For teal-light irradiation, light from a halogen lamp was passed through an interference filter with $\lambda_{max}$ = 514 nm and a half-bandwidth = 8.5 nm. For green irradiation, light from a halogen lamp was passed through an interference filter with $\lambda_{max}$ = 567.5 nm and a half-bandwidth = 13 nm. Proteins were denatured in 8 M urea (pH 2.0) at room temperature in the dark.

## RESULTS

**Domain Architecture of Tlr1999.** According to SMART, Tlr1999 contains a CBCR-GAF domain, a GGDEF domain, and an EAL domain (Figure 1A). By comparing the sequence of Tlr1999 with those of Pb/Pg-type CBCRs, the canonical Cys and the second Cys were respectively assigned to Cys274 and


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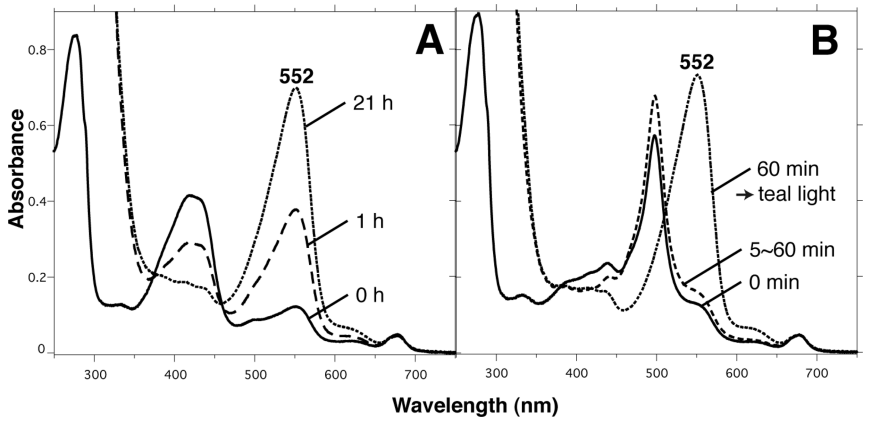


**Figure 3.** Absorption spectra of IAM-modified Tlr1999-GAF. (A) 0 h (solid line), 1 h (broken line), and 21 h (dotted line) after reaction of P418 with 50 mM IAM. (B) 0 h (solid line) and 5 min (broken line) after reaction of P498 with 50 mM IAM. No further change in the absorption spectrum was observed after 5 min. After incubation for 1 h, P498 was irradiated with teal light (dotted line).

Cys246, the latter of which is part of a [D/E]XCF motif (Figure S1).

**Photoconversion between the Blue- and Teal-Absorbing Forms.** Tlr1999-GAF was purified from cyanobacterial cells as a nearly homogeneous, soluble protein (Figure 1B). The SDS-PAGE band corresponding to Tlr1999-GAF intensely fluoresced when in the presence of $Zn^{2+}$ (Figure 1B), suggesting that Tlr1999-GAF contains a covalently bound bilin chromophore. After irradiation with blue light, the absorption spectrum of Tlr1999-GAF contained a sharp peak ($\lambda_{max}$ = 498 nm). This 498 nm light-absorbing form was denoted P498. When P498 was irradiated with teal light, the peak at 498 nm disappeared and a broader, less intense peak centered at 418 nm increased. This form was denoted P418 (Figure 1C,D). Photoconversion between P418 and P498 was reversible (data not shown).

In the absorption spectra, there was a small absorption peak at ~552 nm that was not affected by green light (Figure 1D). A similar minor, photoinactive peak has been detected in the spectra of the Pb/Pg-type CBCRs TePixJ[37] and Tlr0924.[39] When 1 mM DTT was included throughout the preparation procedure, the 552 nm peak was hardly detectable (data not shown), suggesting that the peak is associated with a form of Tlr1999-GAF that contains an oxidized cysteine(s). Another small peak at ~660 nm was assigned to a chlorophyll contaminant.

**Roles of the Conserved Cys Residues.** The chromophore of Tlr1999-GAF was studied by acidic urea denaturation (Figure 1E). The difference spectrum of denatured P498 minus denatured P418 ($\lambda_{max}$ = 509 nm, $\lambda_{min}$ = 602 nm; Figure S3) strongly suggested that Tlr1999-GAF covalently binds PVB and that its configuration is C15-*Z* in P418 and C15-*E* in P498, as was found for the Pb/Pg-type CBCR, TePixJ.[36]

We replaced the canonical Cys (Cys274) and the second Cys (Cys246) individually and together with alanines by site-directed mutagenesis to produce the mutants C274A, C246A, and C246A/C274. These mutants were expressed in and isolated from cyanobacterial cells (Figure 2).

The C246A mutant covalently binds a bilin as shown by its $Zn^{2+}$-enhanced fluorescence (Figure 2A). C246A showed two absorption peaks at 585 and 616 nm (Figure 2A) but was photoinactive in both cases (data not shown). After denaturation, the spectrum of C246A had a peak at 666 nm (Figure 2D), suggesting that the $C3^1$ of C15-*Z* PCB is covalently anchored to Cys274, as was found for Cph1.[36]

The C274A mutant also covalently bound a bilin, but its absorption spectrum contained a broad peak centered at 545 nm (Figure 2B). This mutant was also photoinactive (data not shown). The spectrum of denatured C274A was quite different from PCB or PVB (Figure 2E). This may correspond to isophycocyanobilin, which was suggested as a ligation intermediate in the assembly of PCB to phycocyanin apoprotein[50] or may be an aberrant ligation of PCB involving a site other than the authentic $C3^1$.

The C246A/C274A double mutant did not bind the chromophore, and its absorption spectrum was devoid of bands except for that associated with chlorophyll (Figure 2C,F).

Given their amino acid sequences, spectral properties, chromophore configurations, and our site-directed mutagenesis study, we concluded that Tlr1999 and the Pb/Pg-type CBCRs undergo the same type of photocycle and that the conserved Cys residues are involved in the photoconversion. However, the absorbance band for P498 was blue-shifted by ~35 nm compared with those of the Pg-type CBCRs ($\lambda_{max}$ = 530–540 nm). Notably, this peak is the sharpest found so far for a CBCR. The absorption spectrum of P498 is similar to that of phycourobilin, which has saturated C-4/5 and C-15/16 bonds,[51] suggesting that the PVB D ring of P498 was heavily twisted with respect to the plane formed by the B and C rings, resulting in a shorter $\pi$-conjugated system similar to that of phycourobilin. The aqueous solution of P418 is yellow as are those of the Pb forms of TePixJ and Tlr0924, whereas P498 is orange (Figure 1C), unlike the pink solutions found for the Pg forms of TePixJ[37] and Tlr0924.

The absorption spectrum of Tlr1999-GAF purified from *E. coli* had an additional peak at ~616 nm (denoted P616) (Figure S3). The absorption spectrum of denatured P616 indicated that its chromophore was ~70% PVB (603 nm) and ~30% PCB (671 nm) (Figure S3).[36] These results suggested that P616 derived from PCB. These features have also been observed in TePixJ purified from *E. coli*[18] and seem to be a common problem of PVB-harboring CBCRs. The ambience light during the protein expression may influence the PVB population as suggested recently.[52] Therefore, experiments were performed using protein prepared from cyanobacterial cells, unless stated otherwise.


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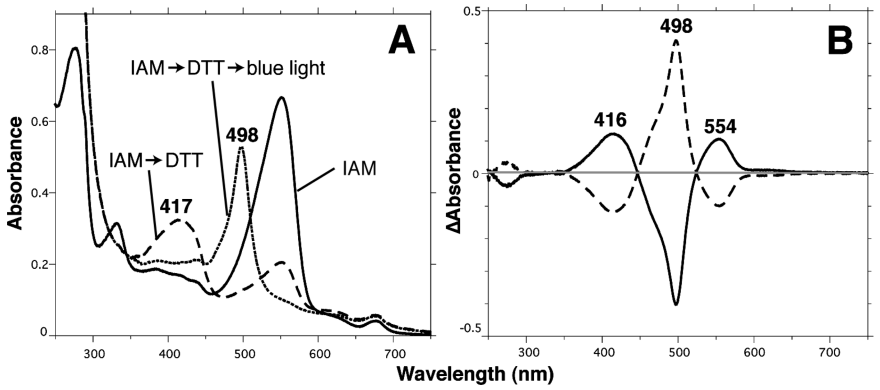


**Figure 4.** Effects of DTT on IAM-modified Tlr1999-GAF. (A) Absorption spectra. Dialyzed, IAM-modified Tlr1999-GAF (solid line) was treated with 20 mM DTT (broken line), irradiated with blue light (dotted line), and then irradiated with teal light (spectrum not shown, but it was almost the same as that shown by the broken line). (B) Difference spectra. The photoconversion by blue light shown in (A) (solid line) and the subsequent photoconversion with teal light (broken line). The spectra were shown as before−after light irradiation.

**IAM Modification: P552 and P420.** To determine the roles of the Cys residues, especially that of the second Cys, we treated Tlr1999-GAF with IAM, which irreversibly modifies thiols.[49] When P418 was incubated with 50 mM IAM in the dark, the P418 peak was gradually disappeared (>21 h) as the peak at 552 nm increased (Figure 3A). Green-light irradiation did not affect the 552 nm peak, indicating that it is a photoinactive form (denoted P552). The spectrum of denatured P552 confirmed that C15-*Z* configuration of its PVB had been maintained during and after the modification with IAM (data not shown). The slow IAM-induced conversion of P418 into P552 suggested that the covalent linkage between Cys and the chromophore is not so stable; i.e., Cys exists in a dynamic equilibrium involving its ligated and free forms. Perhaps, IAM modified Cys to yield P552. After incubating P498 with 50 mM IAM in the dark for 5 min, the small peak near 420 nm (which we associated with a form of Tlr1999-GAF denoted P420) had decreased and the peak at 498 nm had increased (Figure 3B). We assumed that P420 is a photoinactive form of Tlr1999-GAF because the 420 nm peak in the spectrum of P498 was unaltered by saturating blue-light irradiation. The IAM-induced conversion of P420 into P498 suggested that in P420 C15-*E* PVB is ligated to Cys and in P498 C15-*E* PVB is not ligated to Cys. Further incubation with IAM did not affect the spectrum of P498. However, teal-light irradiation converted P498 into P552 (Figure 3B). P498-derived P552 seems to be identical to P418-derived P552 because the PVB chromophore is in the C15-*Z* configuration in both forms, neither of which responds to a green-light irradiation. Because teal-light irradiation could induce C15-*E* → *Z* isomerization in IAM-modified P498, it seemed that the second Cys is not necessary for the isomerization. In summary, the results suggested that in P418 C15-*Z* PVB is ligated to the second Cys; in P552, C15-*Z* PVB and Cys are not ligated; in P420, C15-*E* PVB is ligated to Cys; and in P498, C15-*E* PVB and Cys are not ligated. The 552 nm peak in the native protein may also be P552, which contains the oxidized second Cys.

**Effects of DTT on P552 and P498.** After excess IAM had been removed by dialysis, treatment with 20 mM DTT in the dark reverted P552 into P418 within 1 min (Figure 4A). P418 could then be converted into P498 by the blue-light irradiation, together with reduction of P552. P552 itself, however, could not be converted into P498 by green-light irradiation. Perhaps, P552 was indirectly converted into P498 via P418 during the blue-light irradiation. Irradiation of IAM-modified P498 with teal light generated both P552 and P418 (Figure 4). L-Cysteine and DTT had similar effects (Figure S4). These results suggested that instead of the IAM-modified Cys, the thiol of DTT (and L-cysteine) reversibly ligated to the chromophore because an IAM-modified thiol is unaffected by DTT.[49]

When Tlr1999-GAF purified from *E. coli* was treated with IAM and then DTT, the amount of not only P552 but also P616 was increased by IAM and decreased by DTT (Figure S5), suggesting that P616 contains a C15-*Z* PCB that was not ligated to a thiol just like C246A (Figure 2A). The presence of DTT decreased the amount of P616 to a lesser extent than it did P552, suggesting that thiols were ligated more easily to PVB than to PCB.

When an excess of DTT (1 M) was added in the dark to a solution of P498, the protein was completely converted to a photoinactive blue-absorbing form peaking around 420 nm in the dark (Figure 5). Denaturation analysis confirmed that the configuration of its PVB (C15-*E*) was maintained (data not shown). These results indicate that P420 carrying C15-*E* PVB ligated with the thiol of DTT was produced by 1 M DTT. Notably, although 20 mM DTT largely converted P552 to P418, little P420 was produced (Figure 4).

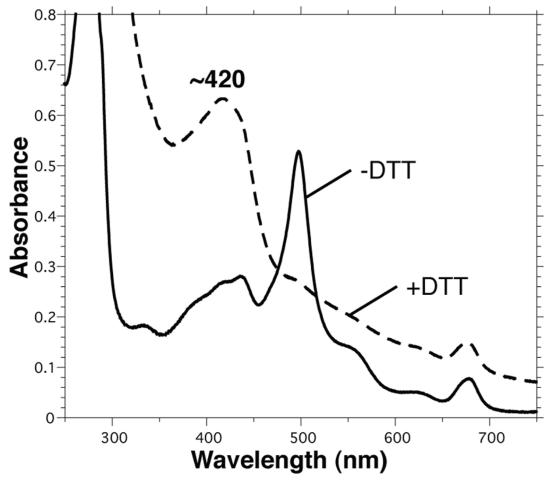


**Figure 5.** Treatment of unmodified P498 with 1 M DTT. Absorption spectra before (solid line) and after (broken line) DTT treatment.


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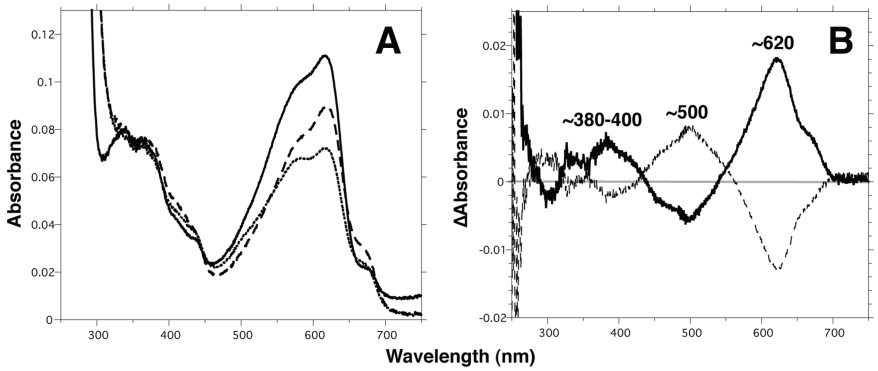


**Figure 6.** Treatment of C246A with βME. (A) Absorption spectra of C246A (solid line) after treatment with 1% βME (broken line) followed by blue-light irradiation (dotted line) and then teal-light irradiation (spectrum not shown, but it was almost the same as that shown by the broken line). (B) Difference spectra. The photoconversion by blue light shown in (A) (solid line) and the subsequent photoconversion with teal light (broken line). The spectra were shown as before−after light irradiation.

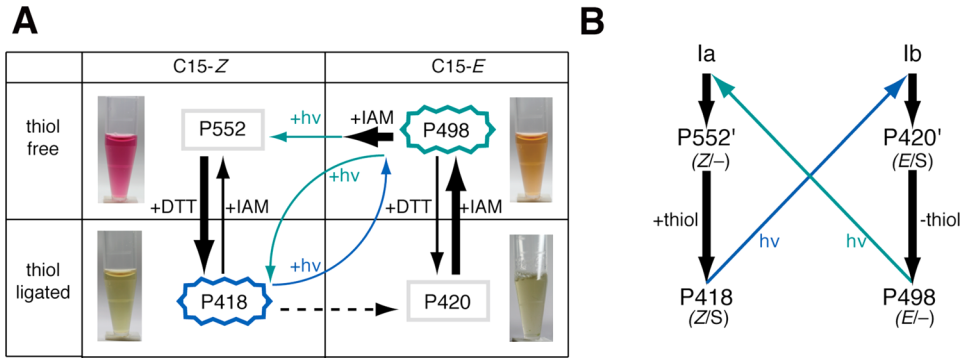


**Figure 7.** (A) Summary of IAM modification and DTT treatment of Tlr1999-GAF. Blue/teal arrows indicate photoconversions. Thick black arrows indicate dark reactions that occur rapidly, and thin black arrows indicate dark reactions that occur slowly or are unfavorable. Note that P418 and P498 are photoactive species, whereas P552 and P420 are photoinactive species. (B) A model for the Tlr1999-GAF photocycle. *Z* or *E*, C15-*Z* or C15-*E*, respectively; S or −, thiol-ligated or thiol-free PVB, respectively. See text for descriptions of Ia, Ib, P552′, and P420′.

**Effects of β-Mercaptoethanol (βME) on C246A.** The DTT-induced blue spectral shift of P552 to P418 (Figure 4) and P498 to P420 (Figure 5) prompted us to examine the effect of DTT on C246A, which possibly binds PCB at C3[1] with the canonical Cys274 (Figure 2A). DTT had little effect on the absorption spectrum of C246A (data not shown). However, when βME, which is smaller compound than is DTT, was added to C246A, the absorbance at ∼620 nm was decreased and the absorption at ∼380−400 nm was increased slightly (Figure 6). Blue-light irradiation reduced the 380−400 and 620 nm absorption peaks and increased the absorbance at ∼500 nm slightly. Subsequent teal-light irradiation reduced the 500 nm absorbance and increased the absorbance at ∼380−400 and 620 nm. Namely, blue and teal light induced a reversible photoconversion. The spectrum of the denatured teal-absorbing form indicated that the protein contained C15-*E* PCB, but isomerization of PCB to PVB had not occurred (data not shown). These results suggested that the photoinactive peak at ∼620 nm (C15-*Z* PCB, free from thiol) was converted into the photoactive blue-absorbing form (C15-*Z* PCB, thiol-ligated) by βME and then photoconverted to the photoactive teal-absorbing form (C15-*E* PCB, free from thiol). These reactions (Figure 6) are similar to the photoconversions shown in Figure 4. Notably, the absorption-peak maximum of PCB C15-*E* form (∼500 nm) was at nearly the same position as that of PVB (498 nm), suggesting that PCB is heavily twisted at C5, leading to a shorter π-conjugated system similar to that of PVB.

## DISCUSSION

**Reversible Attachment of the Second Cys to PVB during Photoconversion.** For this report, we studied Tlr1999, which is a new type of CBCR that contains a [D/E]XCF motif and undergoes a reversible photoconversion between a blue-absorbing form (P418) and a teal-absorbing form (P498). Modification of Tlr1999-GAF with IAM in the dark converts P418 into P552 and P420 into P498, without C15-*Z*/*E* isomerization (Figure 3). These results suggest that P418 and P420 contain a second Cys-PVB adduct, whereas P552 and P498 contain PVB free from Cys. This suggests that the covalent ligation of the second Cys to PVB is unstable; i.e., the ligated and free forms of Cys are in dynamic equilibrium. The possibility that the second Cys−chromophore bond is thermally labile has been discussed for Tlr0924.[39] Our study characterized four forms of Tlr1999-GAF: photoactive P418 containing C15-*Z* PVB ligated to the second Cys [P418 (*Z*/S)], photoactive P498 containing C15-*E* PVB that is not ligated to Cys [P498 (*E*/−)], photoinactive P552 containing C15-*Z* PVB that is not ligated to Cys [P552 (*Z*/−)], and photoinactive P420 containing C15-*E* PVB ligated to Cys [P420 (*E*/S)] (Figure 7A).

For the [D/E]XCF-type CBCRs, there are two conflicting hypotheses concerning the role of the second Cys. One hypothesis is the reversible attachment model for which the second Cys is ligated to PVB in Pb, but free in Pg (Figure S2).[18,39] The other is the stable double linkage model for which


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the second Cys is ligated to PCB in both Pb and Pg, forming a stable PVB-type chromophore.[38] By modification of Tlr1999-GAF wild-type protein with IAM, we demonstrated that the second Cys reversibly binds PVB during the P418 (*Z*/S)/ P498 (*E*/−) photoconversion.

**An Active Thiol, Not Necessarily That of Cys, Is Critical for the Spectral Blue Shift.** Once modified by IAM, a cysteine thiol cannot be regenerated by DTT.[49] However, treatment of the photoinactive P552 (*Z*/−) with DTT in the dark regenerated the photoactive blue-absorbing P418 (*Z*/S) (Figure 4). Similarly, the photoactive P498 (*E*/−) was converted to the photoinactive blue-absorbing P420 (*E*/S) by the treatment with 1 M DTT in the dark (Figure 5). These results suggest that DTT efficiently ligates the chromophore, instead of the second Cys thiol, causing the remarkable spectral blue shift. We also showed that the treatment of C246A with $\beta$ME in the dark, but not with DTT, generates a small amount of a photoactive blue-absorbing form from a photoinactive red-absorbing form (Figure 6). This may imply that $\beta$ME, which is smaller than DTT, can access the chromophore-binding pocket and covalently ligate the chromophore. Although the thiol-ligation site on the chromophore has not been characterized, we assume that it is C10 of PVB as was suggested previously,[39] because the blue spectral shift found for the thiol adducts fits with rubinoid species that contain a disconnected $\pi$-conjugated system between rings B and C[53,54] and the nucleophilic reactivity of the C10 of the chromophore.[50,55]

**Thiol Attachment/Detachment: A Thermal and Dark Reaction during the Photocycle.** Upon photoconversion, isomerization at C15 and covalent attachment/detachment of the thiol to PVB occur. IAM modification and treatment with DTT in the dark induced the thiol-attachment/detachment reactions without isomerizing C15 (Figures 3−5). Because a relatively small concentration of DTT converted P552 (*Z*/−) into P418 (*Z*/S) (Figure 4), the conversion of the P552 (*Z*/−) into P418 (*Z*/S) seems to be a naturally occurring dark reaction. Because excess DTT was needed to cause the opposite reaction (P498 (*E*/−) into P420 (*E*/S)) (Figure 5), the conversion of the P420 (*E*/S) into P498 (*E*/−) also seems to be naturally occurring dark reaction. The latter suggestion is consistent with the observation that IAM-induced conversion of P420 (*E*/S) into P498 (*E*/−), which occurs within a few minutes, is much faster than that of P418 (*Z*/S) into P552 (*Z*/−) (>21 h) (Figure 3). These reactions are summarized in Figure 7A. The photoconversion of P498 (*E*/−) to P418 (*Z*/S) can be split into two steps: the photoreaction P498 (*E*/−) → P552 (*Z*/−) and the dark reaction P552 (*Z*/−) → P418 (*Z*/S) when aided by IAM and DTT. We postulate that the reverse photoconversion of P418 (*Z*/S) to P498 (*E*/−) as in Figure 7A, although we could not demonstrate the efficient production of P420 (*E*/S) from P418 (*Z*/S) under reducing conditions.

In this context, it should be noted that the Pb/Pg-type CBCR, Tlr0924, showed temperature-induced changes in equilibrium implicated in the dark reactions.[39] However, we did not observe such temperature-induced spectral changes in the equilibrium of P498 (*E*/−) and P420 (*E*/S) at 45 or 4 °C (data not shown). At present, we do not know the biochemistry that underlies the difference in the temperature dependency in the equilibrium of the dark reaction between Tlr1999 and Tlr0924. How or if temperature induces a change in the equilibrium positions of P498 (*E*/−) and P420 (*E*/S) awaits determination.

**A Proposed Thiol-Based Photocycle.** Given our results, we can propose a complete photocycle for Tlr1999 (Figure 7B). We can assume a P552-like intermediate [P552′ (*Z*/−)] transiently exists during the photoconversion of P498 (*E*/−) to P418 (*Z*/S). Similarly, we can assume that a P420-like intermediate [P420′ (*E*/S)] transiently exists during the photoconversion of P418 (*Z*/S) to P498 (*E*/−). Furthermore, we assume that the initial photoproducts (Ia and Ib) decay to the metastable P552′ (*Z*/−) and P420′ (*E*/S). A time-resolved photoconversion study of the related Pb/Pg-type CBCR, TePixJ, detected intermediates peaking at 560−570 nm after <50 ns and 870 $\mu$s of excitation of the Pg form [Fukushima, Y., and Itoh, S., personal communication]. These slightly red-shifted intermediates of TePixJ might be analogous to P552′ (*Z*/−) of Tlr1999. Similarly, at least two intermediates (Ib and P420′) can be assumed to be present during the reverse photoconversion.

In the two-step dark reactions from Ia to P418 (*Z*/S), we can assume that only slight conformational changes occur in the apoprotein in the absence of thiol attachment, whereas large conformational changes coupled to the thiol attachment occurring during the P552′ (*Z*/−) → P418 (*Z*/S) reaction. These conformational changes in the apoprotein suppress the reverse photoreaction from P418 (*Z*/S) to P498 (*E*/−) via P552′ (*Z*/−). Instead, P418 (*Z*/S) can be photoconverted into Ib, which decays first to P420′ (*E*/S) and then to P498 (*E*/−) in the dark (Figure 7B).

In this study, we demonstrated that the thiol of the second Cys or an exogenous thiol such as those of DTT supports full photoconversion of Tlr1999, suggesting that DTT promotes conformational changes similar to those induced by the Cys. Tlr1999 also contains GGDEF and EAL domains (Figure 2A), which may serve as a diguanylate cyclase[56] or a phosphodiesterase[57] for the bacterial second messenger bis(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), respectively.[58,59] Measuring these activities would determine if the DTT-adduct P418 (Z/S) acts in a manner equivalent to that of the native P418 (Z/S) during c-di-GMP signaling. It also would be interesting to compare the signaling activity of P552 (*Z*/−) and P498 (E/−) after the IAM modification. By measuring these signaling activities, we can assess the effect of Cys and exogenonus thiols on the protein conformational changes.

## ■ ASSOCIATED CONTENT

### Supporting Information

Sequence alignment of the chromophore-binding GAF domains of CBCRs (Figure S1), the chromophore structure and the reversible Cys-attachment model for Pb/Pg photoconversion (Figure S2), absorption and difference spectra for Tlr1999-GAF purified from cyanobacterial cells and *E. coli* cells (Figure S3), treatment of IAM-modified Tlr1999-GAF with L-cysteine (Figure S4), and effects of IAM and DTT on Tlr1999-GAF from *E. coli* in comparison with those from cyanobacterial cells (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

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## ■ ABBREVIATIONS

CBCR, cyanobacteriochrome; DTT, dithiothreitol; IAM, iodoacetamide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Pb, blue-absorbing form; PCB, phycocyanobilin; Pg, green-absorbing form; PVB, phycoviolobilin; $\beta$ME, $\beta$-mercaptoethanol.

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