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Mechanistic Insight into the Photosensory Versatility of DXCF Cyanobacteriochromes

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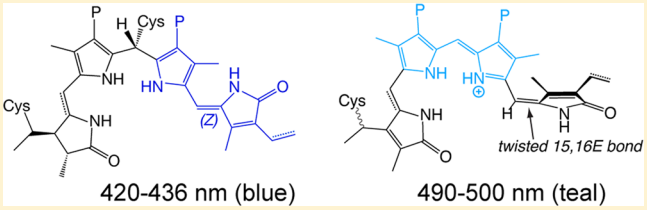
Supporting Information

ABSTRACT: Cyanobacteriochromes (CBCRs) are photosensory proteins related to the red/far-red phytochromes. Like phytochromes, CBCRs use linear tetrapyrrole (bilin) chromophores covalently attached via a thioether linkage to a conserved Cys residue also found in plant and cyanobacterial phytochromes. Unlike almost all phytochromes, CBCRs require only an isolated GAF domain to undergo efficient, reversible photocycles that are responsible for their broad light sensing range, spanning the visible to the near ultraviolet (UV). Sensing of blue, violet, and near-UV light by CBCRs requires another Cys residue proposed to form a second linkage to the bilin precursor. Light triggers 15,16-double bond isomerization as in phytochromes. After photoisomerization, elimination of the second linkage frequently occurs, thus yielding a large red shift of the stable photoproducts. Here we examine this process for representative DXCF CBCRs, a large subfamily named for the conserved Asp-Xaa-Cys-Phe motif that contains their second Cys residue. DXCF CBCRs with such dual-Cys photocycles yield a wide diversity of photoproducts absorbing teal, green, or orange light. Using a combination of CD spectroscopy, chemical modification, and bilin substitution experiments with recombinant CBCRs from *Thermosynechococcus elongatus* and *Nostoc punctiforme* expressed in *Escherichia coli*, we establish that second-linkage elimination is required for all of these photocycles. We also identify deconjugation of the D-ring as the mechanism for specific detection of teal light, at approximately 500 nm. Our studies thus provide new mechanistic insight into the photosensory versatility of this important family of photosensory proteins.



The phytochrome superfamily is one of the major groups of photosensory proteins responsible for optimizing metabolism and light harvesting in photosynthetic organisms.[2] Phytochromes of higher plants detect red and far-red light via photoisomerization of a linear tetrapyrrole (bilin) chromophore between red-absorbing P_r and far-red-absorbing P_{fr} forms. The bilin is covalently bound to a conserved Cys residue via a thioether linkage,[3] and it lies within a conserved pocket in a GAF domain that is part of a larger PAS-GAF-PHY photosensory core module containing a unique knotted architecture.[4−6] In addition to knotted phytochromes, cyanobacteria contain both knotless phytochromes with GAF-PHY architectures and the related cyanobacteriochromes (CBCRs), whose GAF domains are sufficient for assembly with bilin and for efficient, reversible photoconversion.[7,8] Because CBCRs exhibit very diverse photocycles, the mechanisms responsible for such photosensory flexibility are attracting more interest. Unlike knotted phytochromes, CBCRs are often found as modular components in varied domain architectures (Figure S1 of the Supporting Information).

Several subfamilies of CBCRs can be distinguished by primary sequence and by photocycle.[8] The DXCF subfamily of CBCRs is defined by primary sequence elements including a conserved Asp-Xaa-Cys-Phe motif. DXCF CBCRs were first reported to exhibit blue/green photocycles,[a] but other representatives of this family exhibit varied photocycles (see ref 9; examples relevant to this work are shown in Figure S2 of the Supporting Information). Most of these have blue-absorbing[b] dark states,[9−13] the formation of which requires the DXCF Cys residue.[12−15] We propose that this second Cys residue forms an additional covalent linkage to the chromophore in the blue-absorbing dark state, thereby shortening the conjugated system (Figure 1). After photoconversion, subsequent elimination of the second linkage would account for the large red shift observed for the photoproduct state. We previously showed[9] that photoproduct tuning can be achieved by GAF-dependent thermal isomerization at C5 to make phycoviolobilin [PVB (Figure S3 of the Supporting Information)] from phycocyanobilin (PCB), its precursor in cyanobacteria.[16] Unlike PVB formation in light-harvesting phycobiliproteins.[17] formation of PVB by DXCF CBCRs is a spontaneous thermal process that does not require assistance from isomerases.[9,14] In at least one case, it is also a reversible equilibrium process.[9] We propose that formation of PVB by CBCRs is an evolutionary strategy used by cyanobacteria to achieve photosensory diversity, resulting in green, yellow, and orange photoproducts. However, PVB formation does not explain the reported teal-absorbing photoproducts.[9] Such products are spectrally similar to phycourobilin (PUB) and the α-phycoerythrocyanin (α-PEC) photoproduct. Like the α-PEC photoproduct but unlike PUB, such photoproducts

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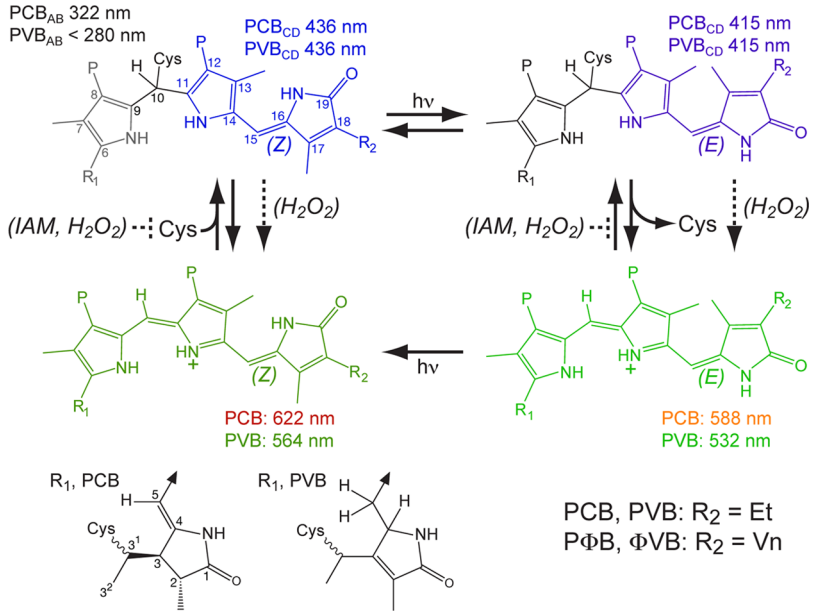

Figure 1. Reactivity patterns of the two-Cys photocycle. The blue-absorbing *15Z* ground state (top left) has a second covalent linkage between the C10 atom of the bilin chromophore and the "second" Cys residue of the DXCF motif. This splits the conjugated system and results in chromophores associated with the C- and D-rings (PCB_{CD}) and A- and B-rings (PCB_{AB}). Illumination results in photoequilibrium with a *15E* photoproduct[12] retaining this second linkage (top right). Subsequent light-independent elimination of the Cys restores conjugation across C10 and red shifts the photoproduct absorbance (middle right). Peroxide cleaves the second linkage in both *15Z* and *15E* photostates (ref 18 and this work). Photoconversion of this *15E* state results in a *15Z* species (middle left), which then regenerates the second linkage and completes the cycle. Iodoacetamide (IAM) and peroxide prevent re-formation of the second linkage (ref 18 and this work), presumably by reacting with the free Cys residue. This cycle can utilize either PVB or PCB chromophores,[9,14] differing at C5 and in the A-ring (bottom left). Peak wavelengths are provided for Tlr0924.[9,12] It is also possible to introduce substitutions at the C18 side chain biosynthetically (bottom right). Abbreviations: Et, ethyl; P, propionate; Vn, vinyl.

undergo a spectral shift and are indistinguishable from *15E* PVB upon acid denaturation.

We previously have shown that formation of PVB by DXCF CBCRs is quite variable. In some proteins, little or no PVB can be detected after recombinant expression in *Escherichia coli*, while other proteins are able to conduct complete isomerization on the same time scale.[9] A third group of proteins contains a mix of both PCB and PVB after expression in *E. coli*.[9,14] Such a mixture can be detected by absorbance spectroscopy, either through examination of the photochemistry of acid-denatured samples or through sequential photoconversion of each photoproduct in the native protein (Figure S4 of the Supporting Information). For the latter approach, which cannot be universally applied, difference spectra can be obtained for the parallel photocycles without denaturation [native difference spectra (Figure S4 of the Supporting Information)].

The mechanism proposed for the blue/green, blue/teal, and blue/orange photocycles of the DXCF CBCR subfamily (Figure 1) is consistent with several lines of evidence. First, the DXCF Cys residue is essential for formation of the blue-absorbing state and for efficient photochemistry.[12−15] Second, FTIR spectroscopy has demonstrated the appearance of a band in the S−H stretch region in the photoproduct state of TePixJ,[14] consistent with elimination of this linkage after photoconversion. Third, the behavior of the PCB and PVB populations observed by sequential conversion is also consistent with this mechanism (Figure S4 of the Supporting Information). Notably, the almost identical blue-absorbing dark states formed by both PCB and PVB indicate that the C5 methine bridge is not part of the conjugated π system responsible for the blue-absorbing dark state. This contrasts with the pronounced change in the photoproduct spectrum caused by PVB formation, so there must be a change in the conjugated π system of the bilin chromophore upon photoconversion.[9]

Despite our understanding of spectral tuning by formation of PVB by DXCF CBCRs, questions about the mechanism of spectral tuning by these and other dual-Cys CBCRs remain unanswered. In the insert-Cys CBCR subfamily, for example, elimination or retention of the second linkage after photoisomerization is correlated with retention or inversion of the photoproduct CD signals.[18] This implicates different facial dispositions of the D-ring in these two cases.[12,19] Only one DXCF CBCR has been characterized by CD spectroscopy to date,[12] so any possible role for stereochemical differences in determining PVB formation has not been studied. The DXCF Cys is essential for the blue-absorbing dark state,[12−15] but it is not known whether it plays any subsequent role in the photocycle. Similarly, the appearance of S−H stretch signals after photoconversion[14] is equally consistent with the loss of a disulfide bond and does not demonstrate mechanistic importance. The present studies address these points using well-characterized DXCF CBCRs from *Nostoc punctiforme* and *Thermosynechococcus elongatus* expressed in *E. coli*.[9,12,14] We demonstrate that CD is conserved during the photocycle for DXCF CBCRs using PVB, PCB, or both. Moreover, we show that a Cys residue is both protected from iodoacetamide in the dark state and essential for regeneration of that state. Finally, we use bilin substitutions to map changes in chromophore conjugation during the photocycle, revealing D-ring deconjugation to be the mechanism for formation of the teal-absorbing

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PVB photoproduct. Taken together, our results support the dual-Cys model of Figure 1 and provide new insight into spectral tuning by the versatile CBCR family of photosensors.

MATERIALS AND METHODS

Production of CBCRs. NpR2903, NpR1597g1, NpF6001, NpF1883g3, NpF1883g4, NpR5113g1, NpR5313g2, and Tlr0924 were expressed in *E. coli* strain LMG194 engineered to produce endogenous PCB, PΦB, or PEB.[9,20,21] NpF1883g2, NpF4973, and NpR5113g3 were expressed in *E. coli* strain C41[DE3][22] engineered to produce endogenous PCB.[23] Expression and purification were performed exactly as described previously.[9] Spectroscopic parameters for purified proteins are listed in Table S1 of the Supporting Information.

Characterization of CBCRs. Absorbance spectra were recorded at 25 °C on a Cary 50 spectrophotometer modified for illumination from above with a 75 W xenon source and bandpass filters as described previously.[19] The following bandpass filters were used for triggering photochemistry: 670 nm center/40 nm width (fwhm), 650 nm/40 nm, 600 nm/40 nm, 560 nm/10 nm, 550 nm/70 nm, 500 nm/25 nm, 450 nm/35 nm, 436.8 nm/10 nm, and 400 nm/70 nm. Denaturation assays used a 1:6 dilution with 6 M guanidinium chloride and 100 mM citric acid (pH 2.2).[24] Treatment with H_2O_2 was performed by addition of an equal volume of commercial 30% H_2O_2 for 2 min; spectra are corrected for dilution and for background absorbance of H_2O_2. Controls for integrity of PCB in this assay have been previously published,[18] while controls for PVB are described in this work (see below). Iodoacetamide [IAM (Sigma)] trapping experiments were performed at 1.5 mM IAM (Tlr0924) or 2 mM IAM (NpF1883g3) for 20 min at room temperature in the dark using freshly prepared stock solutions as described previously.[18] The IAM concentration was experimentally confirmed using a published extinction coefficient.[25] Triscarboxyethylphosphine [TCEP (Pierce)] was added to a final concentration of 1 mM from a freshly prepared 50 mM stock solution in water. CD spectra were recorded on an Applied Photophysics Chirascan using a 2 nm bandwidth and are reported as baseline-corrected, unsmoothed single scans. Simultaneous acquisition of fluorescence excitation, absorbance, and CD spectra used the manufacturer's fluorescence excitation accessory built with a red-enhanced photomultiplier tube and equipped with a 780 nm long-pass filter. Fluorescence spectra for Tlr0924 were recorded on a PTI QM-6/2005SE fluorometer equipped with red-enhanced photomultiplier tubes. Assembly of Tlr0924 with phycoerythrobilin in vitro was performed with apoprotein in a 1.2-fold molar excess versus bilin exactly as described previously.[9] All photochemical difference spectra are reported as *15Z* − *15E*. Native and denatured peak wavelengths are listed in Table 1.

RESULTS

DXCF Photocycles Conserve the Facial Disposition of the Bilin D-Ring. Bilin chromophores are often CD active when bound to protein, with CD reporting the asymmetric conformation of the bilin π system in three dimensions.[26] PCB and PVB chromophores without the second linkage are particularly sensitive to the facial disposition of the D-ring relative to the B- and C-rings.[12,19] There is some evidence that the photochemical and thermal properties of different subfamilies within the phytochrome superfamily correlate with CD behavior. For example, the CD sign of the long-wavelength transition in phytochromes is negative in P_r, but the CD of P_{fr} varies depending on the class of phytochrome.[19] Members of the insert-Cys subfamily of CBCRs, which also use two-Cys photocycles, exhibit a correlation between the CD sign of the photoproduct state and the stability of the second linkage in that state.[18]

Table 1. Chromophore Substitution in DXCF CBCRs[a]

protein	bilin	native *15Z* (nm)	native *15E* (nm)	denatured *15Z*; *15E* (nm)[b]
NpF6001	PCB	316, 426	352, 578	674; 576
NpF6001	PΦB	?, 438	?, 586	682; 582
NpR1597g1	PVB	–, 420	338, 498	610; 512
NpR1597g1	ΦVB	–, 442	354, 500	624; 538
NpR5113g1	PVB	330, 564	?, 494	606; 510
NpR5113g1	ΦVB	344, 574	372, 498	614; 528
NpR5313g2	PCB	352, 550	?, 428	672; 574
NpR5313g2	PΦB	368, 560	?, 448	690; 580
NpF1883g3	PCB	320, 426	354, 546	678; 574
NpF1883g3	PVB	–, 424	336, 502	608; 514
NpF1883g3	PΦB	322, 448	366, 562	684; 578
NpF1883g3	ΦVB	–, 442	350, 502	616; 522
Tlr0924	PCB	322, 436	360, 588	674; 578
Tlr0924	PVB	–, 436	340, 532	609; 512
Tlr0924	PΦB	322, 464	380, 602	682; 572
Tlr0924	ΦVB	–, 460	354, 544	616; 514
Tlr0924	PEB	566	N/D[c]	558; N/D[c]
Tlr0924	PUB	496	N/D[c]	500; N/D[c]

[a]Native wavelengths calculated from photochemical difference spectra or sequential difference spectra for all transitions with a peak wavelength of >310 nm. Question marks denote transitions that could not be assigned with confidence. PCB and PVB values are from ref 9. [b]Denatured peak wavelengths calculated from sequential difference spectra or photochemical difference spectra for photoactive proteins. Values are reported for the long-wavelength transition as *15Z*; *15E* pairs. [c]Not determined.

We therefore used a number of recently described DXCF CBCRs[9] to test whether there might be differences in the CD spectra of examples using either PVB and/or PCB chromophores. For the sake of simplicity, we first examined DXCF CBCRs containing a single bilin. The blue/teal photocycles of NpR1597g1 and NpR5113g3 arise from PVB[9] and are associated with negative CD for both photostates (Figure 2). The blue/yellow and blue/orange photocycles of NpF6001 and NpF4973 use PCB and are also associated with negative CD for both photostates (Figure 2). The same pattern was observed for NpR5113g1 (Figure S5A of the Supporting Information), which uses PVB in an atypical green/teal photocycle.[9] NpR5113g1 is thus distinct from the photochromic phycobiliprotein α-phycoerythrocyanin (α-PEC), which exhibits inversion of CD for a very similar green/teal photocycle.[27,28]

NpR5313g2, which uses PCB in a green/blue photocycle,[9] also has negative CD in the green-absorbing dark state (Figure S5B of the Supporting Information). However, signals from the blue-absorbing photoproduct were weak (Figure S5C of the Supporting Information). We therefore acquired CD, absorbance, and fluorescence excitation spectra simultaneously to assist in assignment for this protein. Emitted fluorescence was detected with a 780 nm long-pass filter. In the *15Z* ground state (Figure S5B of the Supporting Information), the peak wavelengths for the CD band were in good agreement with those measured by absorbance and fluorescence excitation

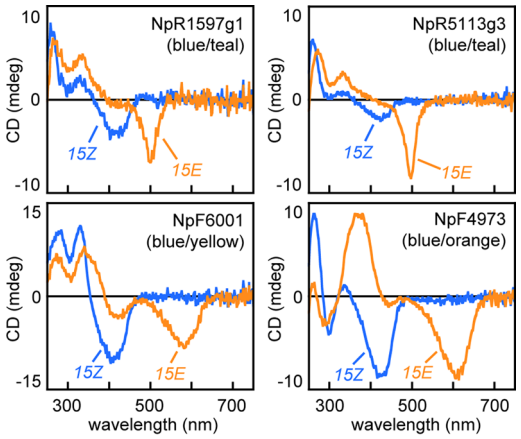

Figure 2. Characterization of DXCF CBCRs by CD spectroscopy. Indicated DXCF CBCRs with either PVB (top) or PCB (bottom) adducts were characterized in the dark state (*15Z*, blue) and photoproduct state (*15E*, orange).

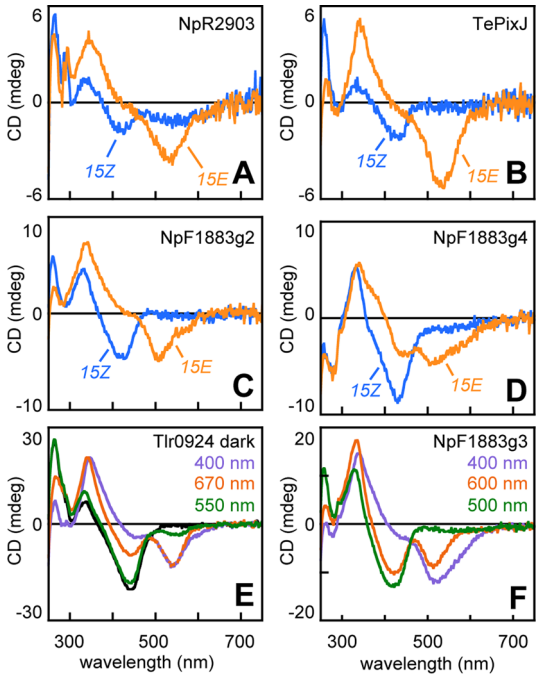

Figure 3. Characterization of mixed bilin populations by CD spectroscopy. DXCF CBCRs with mixed bilin populations were characterized by CD spectroscopy. NpR2903 (A) and TePixJ (B) do not undergo sequential photoconversion[9] and are shown in the same color scheme as in Figure 2. NpF1883g2 (C) and NpF1883g4 (D) exhibit sequential photoconversion and are shown in the same way for comparison. Full sequential photoconversion is shown for Tlr0924 (E), while the dark state is omitted for the sake of clarity in NpF1883g3 (F).

(Figure S5B of the Supporting Information). Such agreement was not observed for *15E* NpR5313g2, alone among published CBCRs (Figure S5D of the Supporting Information). Instead, the CD and excitation spectra gave maximal signals corresponding to an apparent side population at long wavelengths (Figure S5C of the Supporting Information), while the absorbance channel recorded simultaneously with the CD spectrum was in good agreement with that seen in other absorbance measurements (data not shown). We therefore conclude that the CD associated with the blue-absorbing species itself is even weaker and thus cannot be assigned.

We next characterized DXCF CBCRs that contain mixtures of PVB and PCB. Such proteins can exhibit sequential reverse photoconversion (Figure S4 of the Supporting Information), as in Tlr0924 and the three CBCR domains from the *NpF1883* locus of *N. punctiforme*.[9] Other examples, such as TePixJ and NpR2903, do not exhibit this phenomenon. This difference does not reflect differences in CD behavior, however: NpR2903, TePixJ, NpF1883g2, and NpF1883g4 all exhibit negative CD for both photostates (Figure 3). We also examined CD during sequential conversion of the individual PCB and PVB populations of Tlr0924 and NpF1883g3; both bilin populations exhibited conservation of CD (Figure 3E,F). This result extends our earlier characterization of Tlr0924,[12] which preceded the discovery of a mixed bilin composition. Finally, we observed that NpF1883g2 allowed sequential conversion of either *15E* population under kinetic control (Figure S5E of the Supporting Information), allowing us to examine both the (*15Z*-PCB; *15E*-PVB) and (*15E*-PCB; *15Z*-PVB) cases. Negative CD was obtained for all states (Figure S5F of the Supporting Information). These results demonstrate that inversion of CD is not seen in DXCF CBCR photocycles with blue-absorbing ground states, regardless of the presence or absence of PVB formation. Therefore, the facial disposition of the D-ring during the photocycle is conserved within this subfamily.

The DXCF Photocycle Requires a Chemically Labile Cys Residue. The DXCF Cys residue is essential for formation of the blue-absorbing ground state in DXCF CBCRs.[12−15] However, this could arise from a conserved structural effect rather than from the loss of formation of the second linkage. Similarly, it could be argued that the appearance of the S−H stretch in the green-absorbing state of TePixJ arises from a change in disulfide bonding rather than elimination of the C10 thioether. We therefore examined the behavior of the DXCF photocycle in response to two reagents, iodoacetamide (IAM) and hydrogen peroxide, which have been previously used to demonstrate an essential role for the second Cys in the photocycle of a different subfamily of two-Cys CBCRs.[18] IAM has also been used in combination with dithiothreitol (DTT) to demonstrate an essential Cys in the photocycle of the DXCF CBCR Tlr1999.[29]

In Tlr0924, IAM did not alter the spectroscopic properties of the blue-absorbing dark state, nor did it prevent efficient photoconversion to the photoproduct state (Figure 4A). This was not due to the steric inaccessibility of the DXCF Cys, because peroxide efficiently converted the dark state to a new species similar to the *15Z* green-absorbing dark state of NpR5113g1, but exhibiting a long-wavelength shoulder (compare Figure 4A and Figure S2 of the Supporting Information). The main peak in peroxide-treated Tlr0924 is assigned as *15Z* PVB in which the second linkage has been cleaved, while the shoulder is assigned as similarly cleaved *15Z* PCB. Control experiments confirmed that the green-absorbing dark state of NpR5113g1 is itself stable to peroxide on this time scale (Figure S6A of the Supporting Information), consistent with these assignments. The *15E* teal-absorbing photoproduct state of NpR5113g1 exhibited bleaching on this time scale (Figure S6B of the Supporting Information) but showed no evidence of a red shift as is seen in Tlr0924 and other cases with second linkages.[18]

The *15E* photoproduct state of Tlr0924 was also unaffected by IAM (Figure 4B). However, illumination of this state did not

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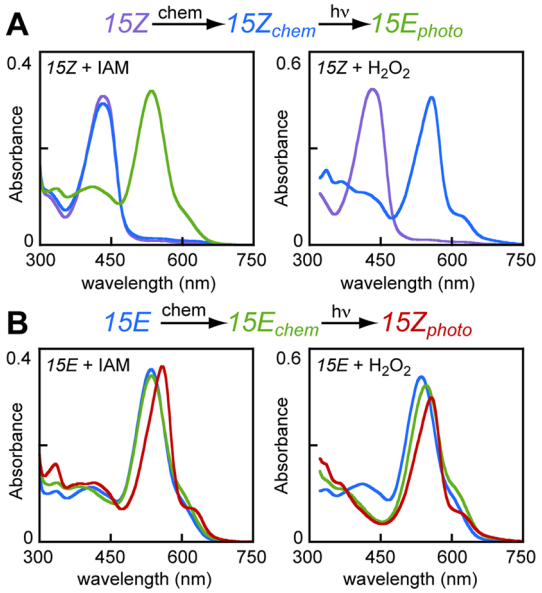

Figure 4. Chemical modification of Tlr0924. (A) Tlr0924 in the dark state (purple) was treated with IAM or hydrogen peroxide to yield the chemical reaction product (blue). For IAM, the sample was photoconverted to the *15E* photoproduct (green). (B) Tlr0924 in the *15E* photoproduct state (blue) was treated with IAM or hydrogen peroxide to yield the chemical reaction product (green), followed by photoconversion to yield a trapped state (red) that cannot regenerate the dark state.

result in regeneration of the blue-absorbing dark state but instead trapped species similar to those observed upon peroxide treatment of the dark state. These results demonstrate that a Cys residue is essential for regeneration of the blue-absorbing dark state during the photocycle and that Cys residue is protected from IAM labeling in the dark state but accessible in the photoproduct, consistent with a dynamic linkage (Figure 1).

Peroxide treatment of the photoproduct state did not result in a marked red shift of the main peak (Figure 4B). A slight red shift was observed, along with depletion of the minor blue-absorbing species and increased absorbance of the long-wavelength shoulder corresponding to *15E* PCB. These data indicate that the minor blue-absorbing species was apparently cleaved by peroxide, consistent with its assignment as a *15E* species retaining the second linkage.[12] The red shift of the main band was explained by the increased absorbance at long wavelengths, which may indicate that the *15E* blue-absorbing population is enriched in PCB relative to the total. Illumination of the peroxide-treated photoproduct again resulted in formation of trapped species similar to those seen in the other cases. This indicates that the Cys residue essential for the photocycle is sensitive to peroxide oxidation in both photostates, consistent with a labile thioether in the dark state and a reactive "free" Cys in the photoproduct state. Oxidation of a free Cys could explain the slow accumulation of similar P_g' species upon repeated cycling of DXCF CBCRs in vitro, observed for Tlr0924 and TePixJ with only tentative assignments.[11,12] Consistent with this hypothesis, repeated cycling could lead to accumulation of photochemically inert P_g' in Tlr0924 (Figure S6C of the Supporting Information), and addition of the reductant triscarboxyethylphosphine (TCEP) was able to retard P_g' accumulation (Figure S6D of the Supporting Information).

We also characterized iodoacetamide and peroxide reactivity in NpF1883g3, which also exhibits PCB and PVB populations in the native protein.[9] IAM again had no effect on the blue-absorbing dark state or its photoconversion (Figure 5A). Unlike

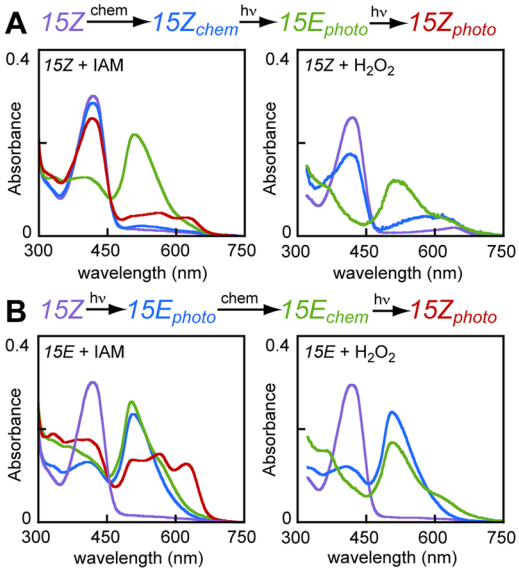

Figure 5. Chemical modification of NpF1883g3. (A) NpF1883g3 in the dark state (purple) was treated with IAM or hydrogen peroxide to yield the chemical product (blue). It was photoconverted to the *15E* state (green) and then back to the *15Z* state (IAM only, red). (B) NpF1883g3 in the dark state (purple) was photoconverted to the *15E* state (blue) and treated with IAM or hydrogen peroxide to yield the chemical product (green). It was then photoconverted back to the *15Z* state (IAM only, red).

Tlr0924, in which the stability of the second linkage in the *15E* photoproduct state results in slower photoconversion, it was also possible to examine reverse photoconversion of NpF1883g3 after iodoacetamide treatment. Regeneration of the blue-absorbing dark state was efficient, with minor trapping of unlinked *15Z* species. IAM also did not affect the photoproduct spectrum of NpF1883g3 (Figure 5B), but regeneration of the dark state was compromised and trapping of mixed red-shifted *15Z* species much more pronounced. Peroxide did not completely cleave the second linkage in the NpF1883g3 dark state, and the residual blue-absorbing population could still be photoconverted (Figure 5A). As with Tlr0924, the blue-absorbing population in the *15E* photoproduct state of NpF1883g3 was cleaved by peroxide, but the main peak was not (Figure 5B). For both proteins, these data are consistent with a stabilized second linkage between the bilin and a Cys residue, which is destabilized upon photoconversion. This Cys residue is apparently less accessible in NpF1883g3, such that inactivation of the *15E* state by IAM is not complete. In both cases, oxidation of the Cys residue or its modification by IAM specifically blocks regeneration of the blue-absorbing state. Taken together with the essential role of the DXCF Cys in the initial formation of the blue-absorbing dark state and with the appearance of a free Cys thiol in the photoproduct state of TePixJ,[12−15] the simplest interpretation is that the DXCF Cys is the essential Cys residue for regeneration of the dark state and that it forms a second linkage to the bilin in the dark state but not the photoproduct state (Figure 1).

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Teal-Absorbing Photoproducts Are Generated by Deconjugation of the D-Ring. Comparison of the PVB and PCB populations in native Tlr0924 and NpF1883g3 demonstrates that the C5 methine bridge is not involved in the π system, giving rise to the blue-absorbing dark state.[9] This leaves the C10 and C15 methine bridges as alternative sites for the second linkage. Incorporation of phytochromobilin (PΦB) as an alternative to PCB offers a means of distinguishing between these possibilities. The 18-vinyl moiety of PΦB (Figure S3 of the Supporting Information) is in conjugation with the D-ring, so transitions associated with the D-ring would show a red shift with PΦB or with its C5-saturated analogue phytoviolobilin (here abbreviated ΦVB, Figure S3 of the Supporting Information; also known as PtVB[1]) relative to PCB or PVB. A C10 adduct would leave the C- and D-rings in conjugation, giving a blue-absorbing state; a C15 adduct would instead leave the D-ring out of conjugation, and there would be no red shift upon incorporation of PΦB.

We first examined Tlr0924 and NpF1883g3 with PΦB coexpression (Figure 6). Both proteins exhibited red-shifted

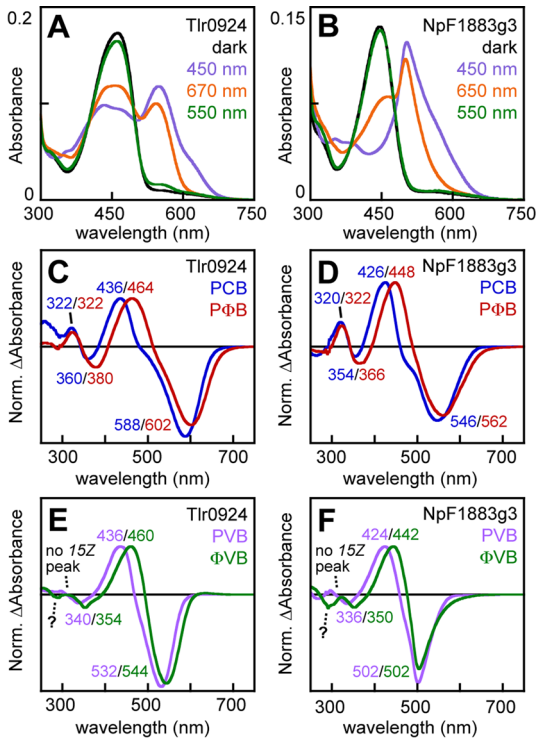

Figure 6. Chromophore conjugation in DXCF CBCRs with parallel photocycles. The PΦB adducts of Tlr0924 (A) and NpF1883g3 (B) exhibited sequential reverse photoconversion. Normalized difference spectra are shown for the C5-unsaturated populations with 18-ethyl or 18-vinyl moieties [PCB (C) and PΦB (D)] and C5-saturated populations with 18-ethyl or 18-vinyl moieties [PVB (E) and ΦVB (F)] for Tlr0924 (C and E) and NpF1883g3 (D and F).

dark states (Table 1). Forward photoconversion was less efficient in Tlr0924 (Figure 6A), but sequential reverse photoconversion of the PΦB and ΦVB populations was still possible in both proteins. Denaturation analysis confirmed the presence of the 18-vinyl moiety in samples coexpressed with PΦB (Table 1). The PCB and PΦB populations of both proteins show a strong red shift of the blue-absorbing *15Z* band and of both photoproduct bands possessing the 18-vinyl moiety (i.e., PΦB-derived population), but such a shift is not seen in the second *15Z* transition at approximately 320 nm (Figure 6C,D). This transition is absent in the PVB and ΦVB populations of both proteins as expected, while the main blue-absorbing band is present and shows a red shift (Figure 6E,F). Red shifts were observed for both bands of the PVB and ΦVB photoproduct states of Tlr0924, but the teal-absorbing photoproduct state of C5-saturated NpF1883g3 did not show such a shift. On balance, the pattern of red shifts observed in the *15Z* states is in good agreement with a C10 adduct.

We next examined two DXCF CBCRs that do not exhibit PVB formation. NpF6001 exhibits a blue/yellow photocycle, while NpR5313g2 exhibits a green/blue photocycle.[9] Incorporation of PΦB into NpF6001 resulted in a dark state with a substantial red shift (Figure S7A,B of the Supporting Information). Formation of the red-shifted photoproduct state was less efficient in the presence of the 18-vinyl moiety (compare Figures S2 and S7A of the Supporting Information). This did not arise from a defect in primary photochemistry, because denaturation analysis showed efficient formation of the *15E* state (Figure S7C of the Supporting Information). The PΦB adduct of NpF6001 must therefore exhibit a more stable second linkage in the *15E* photostate than does the PCB adduct. This overlapped population complicated analysis of the difference spectrum, so we could not assign the higher-energy transitions for the PΦB adduct. NpR5313g2 with PΦB incorporated exhibited a green/blue photocycle much more comparable to that of the PCB adduct (Figure S7D of the Supporting Information), and red shifts were observed for both peaks in the green-absorbing state as well as for the long-wavelength transition of the blue-absorbing photoproduct (Figure S7E of the Supporting Information). Primary photoconversion was again efficient (Figure S7F of the Supporting Information), but the second transition of the *15E* state was at approximately 292 nm. Such a band could arise from the protein or from the chromophore, and hence, it could not be assigned to the bilin with confidence.

The absence of a red shift in the teal-absorbing PVB photoproduct state of NpF1883g3 prompted us to examine NpR1597g1, which exhibits a blue/teal photocycle, and NpR5113g1, which exhibits a green/teal photocycle. In both cases, the 18-vinyl moiety introduced a significant red shift into the dark state, but no significant change in the photoproduct (Figure 7A–D). Denaturation analysis confirmed the presence of ΦVB (Figure 7E). Such small red shifts were not observed in other CBCR photostates (Figure 7F), indicating that the absence of a red shift upon introduction of the 18-vinyl moiety is specific to the teal-absorbing state. Such a situation could arise through a twisted PVB photoproduct, in which the D-ring is out of conjugation because of torsion about the C15 methine bridge[30] or because of formation of phycourobilin [PUB (Figure S3 of the Supporting Information)] in the native protein. The presence of only PVB in the denaturation assay is not consistent with the latter interpretation, and PUB has not been described in any CBCR to date. However, the teal-absorbing photoproducts of CBCRs have a strong resemblance to both the α-PEC photoproduct and PUB,[9,27,28,31,32] so either case seemed plausible.

We therefore examined the adduct(s) formed by coexpression of Tlr0924 with phycoerythrobilin [PEB (Figure S3 of the Supporting Information)]. While incorporation of PEB into Tlr0924 is quite poor, we were able to obtain both native and denatured spectra.[9] Comparison of those spectra reveals a minor peak at approximately 490–500 nm that is stable to

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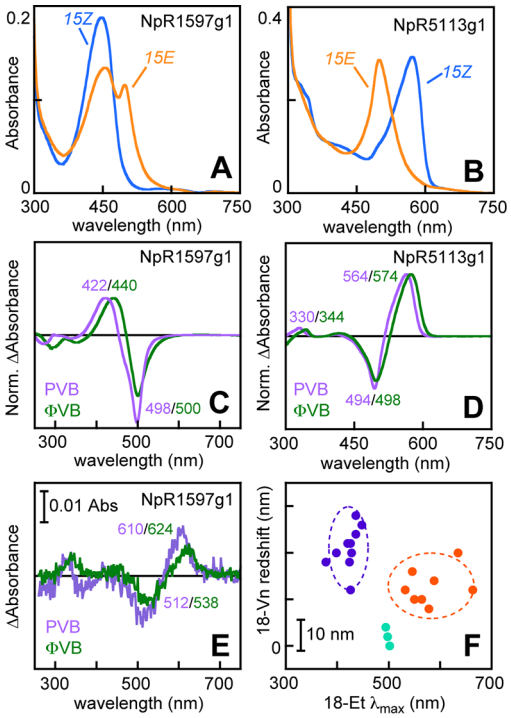

Figure 7. Bilin C18 side chain not in conjugation in the teal-absorbing photoproduct state. PΦB adducts of NpR1597g1 (A) and NpR5113g1 (B) are shown in the *15Z* dark state (blue) and at photoequilibrium or in the *15E* photoproduct state (orange). Normalized difference spectra are shown for NpR1597g1 (C) and NpR5113g1 (D) with 18-ethyl or 18-vinyl moieties (green for PVB and purple for ΦVB). (E) Photochemical difference spectra for the acid-denatured PVB and ΦVB adducts of NpR1597g1, using the color scheme of panels C and D. (F) Red shift associated with an 18-vinyl side chain relative to an 18-ethyl side chain plotted vs the 18-ethyl peak wavelength for proteins in this study (Table 1) and other published cases.[9,18,21] Error arcs are drawn at two standard deviations for two clusters: one for which λ_{max} < 480 nm (n = 10; red shift of 21 ± 5 nm) and another for which λ_{max} > 510 nm (n = 9; red shift of 13 ± 4 nm).

denaturation (Figure S8A of the Supporting Information). This is consistent with autocatalytic PUB formation from PEB by Tlr0924, presumably proceeding via the same isomerization pathway that generates PVB from PCB. The two species were photoinactive and fluorescent, as has been reported for PEB adducts of phytochrome.[33] The mixed population of PEB and PUB species complicated fluorescence measurements, but the PUB population could be detected in excitation scans of the native protein (Figure S8B of the Supporting Information). Both PEB and PUB peaks failed to exhibit red shifts after peroxide treatment (Figure S8C of the Supporting Information), and the minor peak was notably more stable to peroxide than the teal-absorbing photoproduct of NpR5113g1 (Figure S6B of the Supporting Information). Finally, the minor population in Tlr0924 was not observed in Tlr0924 assembled with PEB in vitro (Figure S8D of the Supporting Information). Taken together, these experiments demonstrate that Tlr0924 is able to form a small amount of PUB over time and show that PUB adducts of CBCRs, if present, would be stable to denaturation. This contrasts with the teal-absorbing photoproduct states formed by several CBCRs, which are not stable to denaturation. We therefore conclude that teal-absorbing photoproducts arise from deconjugation of the bilin D-ring in the teal-absorbing state because of torsion about C15 (Figure 8), although we cannot dismiss the formation of an acid labile, peroxide stable adduct at present.

DISCUSSION

We have used CD spectroscopy, chemical modification, and bilin substitution experiments to map changes in the conjugated π system during the photocycles of DXCF CBCRs. No evidence of a correlation between PVB formation and D-ring dynamics as assayed by CD spectroscopy was found. It can be argued that CBCRs produced as isolated GAF domains in *E. coli* do not faithfully reproduce the properties of the actual cyanobacterial photosensory proteins, but those CBCRs expressed in both *Synechocystis* and *E. coli* have not exhibited significant differences in their photochemical properties.[15,34,35] TePixJ expressed in *Synechocystis* contains only PVB, while that expressed in *E. coli* contains a variable mix of PVB and PCB. However, the photocycle of the PVB population is apparently identical in the two expression systems, and the difference is in the amount of PCB that is present after purification.[14] On the basis of PVB isomerization in Tlr0924 and NpF1883g3, we have proposed that this difference arises because of the longer growth period and high light exposure needed for growth of cyanobacterial cultures.[9] Similarly, there is good evidence from several systems that the photochemical behavior of isolated CBCR GAF domains is comparable to that of larger fragments and even to that of full-length proteins.[9,10,12,34,36] We are thus confident that the insights gained from our mechanistic studies should prove to be applicable to other DXCF CBCRs and to the larger, complex cyanobacterial signaling proteins in which they are often found (Figure S1 of the Supporting Information).

Our results validate several aspects of the proposed two-Cys photocycle (Figure 1). Upon introduction of the 18-vinyl moiety, only the longest blue-absorbing *15Z* dark state showed a red shift, while the shorter-wavelength transition of the PCB population (approximately 320−322 nm) was unaffected (Figure S9 of the Supporting Information). The PVB and PCB populations show nearly identical blue bands, but the second transition is absent in the PVB population. These results clearly show a split-conjugated system, with the blue band associated with the D-ring and the second transition associated with the C5 methine bridge. IAM does not shift the absorbance spectrum or prevent normal photochemistry, while peroxide shifts the blue band to long-wavelength species. Taken together with the known reactivity of the C10 atom toward thiol nucleophiles and the spectral properties of rubins and C10 adducts,[37−43] the simplest interpretation of these data is that the blue-absorbing state arises because of the presence of a second covalent linkage between the DXCF Cys residue and the bilin C10 atom.

Green, yellow, or orange photoproducts are seen for DXCF CBCRs depending on whether the CBCR in question contains PVB or PCB.[9] Comparison of PCB and PVB populations within the same protein shows a clear blue shift of the photoproduct band (Figure S10 of the Supporting Information), and denaturation analysis confirms that green-absorbing states arise from PVB and yellow- or orange-absorbing states arise from PCB.[9] Our studies show that introduction of an 18-vinyl moiety shifts both bands in the photoproduct state. Therefore, there is a single conjugated system after photoconversion. Neither iodoacetamide nor peroxide affects the photoproduct spectrum, but both prevent regeneration of the normal dark state. Therefore, a Cys residue is essential for

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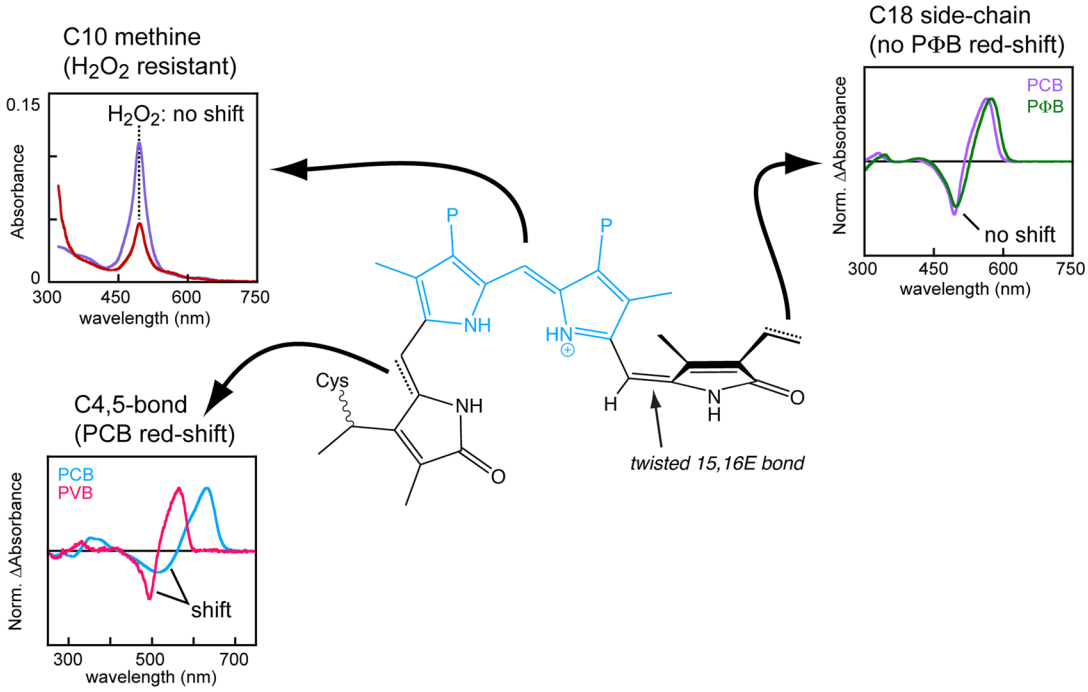

Figure 8. Mapping the conjugated system in the teal-absorbing photoproduct state. A PCB population generated by in vitro assembly is red-shifted relative to the PVB population (bottom left[9]). Desaturation of the C18 side chain does not produce a red shift of the teal-absorbing band (bottom right). H_2O_2 does not shift absorption (top). These results indicate an intact C10 methine bridge in conjugation with the C5 methine bridge but not with the C15 methine bridge. This could be achieved by twisting about C15 (center), as is seen in α-PEC,[30] or by a labile adduct (not shown).

regeneration of the dark state, is protected from IAM in that dark state, but is accessible to IAM in the photoproduct state. It is also known that the DXCF Cys is required for initial formation of the blue-absorbing state, that the Tlr0924 photoproduct exhibits temperature-dependent spectral shifts between a blue-absorbing state and the green-absorbing state, and that at least one Cys residue becomes a free thiol upon photoconversion of TePixJ to the *15E* state.[12−15] The simplest interpretation of this body of results is that the second linkage is eliminated after photoconversion, resulting in species with absorbance ranging from green to orange depending on isomerization of the C5 methine bridge.[9]

Our work provides new information about how CBCRs can sense teal light. Acid denaturation red shifts this species, allowing identification of PVB as the denatured bilin. The fact that this does not happen with the PUB population of Tlr0924 shows that the teal-absorbing states are not PUB adducts. The initial red/green photocycle of NpR5113g1 assembled with PCB shows a red shift relative to the teal-absorbing state,[9] so it is possible the A-ring would be in conjugation in the teal-absorbing photoproduct were C5 not saturated. However, the D-ring is not in conjugation, because introduction of an 18-vinyl moiety into several CBCRs with teal-absorbing photoproducts produced no significant red shift. Therefore, in the native mature protein, we envisage that teal chromophores adopt a PUB-like conjugated system via twisting the D-ring (Figure 8). Alternatively, teal states could arise because of some type of labile adduct at C15. Such an adduct is unlikely to be a thioether, both because of the different reactivity of C15 and C10 toward nucleophiles[44] and because of the stability of the teal-absorbing state to peroxide. A water molecule or a nucleophilic side chain near the C15 methine bridge could attack, resulting in the teal-absorbing state; however, C15 is not typically electrophilic,[44] and ether adducts of bilins are not acid-stable.[43] We thus favor a twisted photoproduct with the D-ring out of conjugation (Figure 8). Such a highly twisted photoproduct has been trapped and characterized crystallographically in α-PEC,[30] providing a plausible model for such bilin geometries. The lack of an 18-vinyl effect has also been seen in phycobiliproteins incorporating ΦVB or PVB,[1] making it possible that such twisted species may be intrinsically less disfavored in C5-saturated species such as PVB than in more conjugated bilins like PCB or biliverdin.

In conclusion, this work reveals the use of C10 adducts as a powerful evolutionary strategy for spectral tuning that has evolved repeatedly. The ability of CBCRs to sense blue and violet light may also explain the proliferation and radiation of the phytochrome superfamily in cyanobacteria, which already contain high levels of bilin chromophores for production of the light-harvesting phycobiliproteins. The ready availability of biliprotein photosensors spanning the visible spectrum would also explain the smaller number of flavin-based and retinal-based photosensory proteins in cyanobacteria:[45] two-Cys CBCRs are able to provide comparable spectral coverage with UV/blue, blue/teal, or blue/green photocycles, and others yet to be discovered.

■ ASSOCIATED CONTENT

Supporting Information

Ten figures and one table. 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

ΔAbsorbance, change in absorbance (in difference spectra, which are reported as *15Z* − *15E*); α-PEC, α-phycoerythrocyanin; CBCR, cyanobacteriochrome; DXCF, Asp-Xaa-Cys-Phe motif defining a subfamily of dual-Cys CBCRs; ΦVB, phytoviolobilin (also known as PtVB[1]); GAF, domain acronym derived from vertebrate cGMP-specific phosphodiesterases, cyanobacterial adenylate cyclases, and formate hydrogen lyase transcription activator FhlA; PCB, phycocyanobilin; PVB, phycoviolobilin; PΦB, phytochromobilin; TES, *N*-[tris-(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Xaa, any amino acid.

■ ADDITIONAL NOTES

[a]Throughout, we designate photocycles by the *15Z* state followed by the *15E* state. Thus, we designate DXCF CBCRs such as TePixJ or Tlr0924 as blue/green: a blue-absorbing *15Z* ground state photoconverting to and from a green-absorbing *15E* photoproduct.

[b]Color definitions used in this study are as follows: near-UV, 300−395 nm; violet, 395−410 nm; blue, 410−480 nm; teal, 480−520 nm; green, 520−570 nm; yellow, 570−580 nm; orange, 580−615 nm; red, 615−685 nm; far-red, 685−750 nm.

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