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CYANOBACTERIOCHROMES: A RAINBOW OF PHOTORECEPTORS

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Abstract

Widespread phytochrome photoreceptors use photoisomerization of linear tetrapyrrole (bilin) chromophores to measure the ratio of red and far-red light. Cyanobacteria also contain distantly related cyanobacteriochrome (CBCR) proteins that share the bilin-binding GAF (cGMP-specific phosphodiesterases, cyanobacterial adenylate cyclases, and formate hydrogen lyase transcription activator FhlA) domain of phytochromes but sense other colors of light. CBCR photocycles are extremely diverse, ranging from the near-ultraviolet to the near-infrared. Photoisomerization of the bilin triggers photoconversion of the CBCR 'input,' thereby modulating the biochemical signaling state of 'output' domains such as histidine kinase bidomains that can interface with cellular signal transduction pathways. CBCRs thus can regulate several aspects of cyanobacterial photobiology, including phototaxis, metabolism of cyclic nucleotide second messengers, and optimization of the cyanobacterial light-harvesting apparatus. This review examines spectral tuning, photoconversion, and photobiology of CBCRs and recent developments in understanding their evolution and in applying them in synthetic biology.

Keywords

cyanobacteria; tetrapyrrole; light harvesting; phototaxis; second messenger; optogenetics

INTRODUCTION: WHAT IS A CYANOBACTERIOCHROME?

Cyanobacteriochromes (CBCRs) are cyanobacterial photoreceptors. CBCRs belong to one of several known superfamilies of bilin-binding proteins. The most abundant bilin-binding proteins in cyanobacteria are light-harvesting phycobiliproteins, part of a superfamily of bilin-biosynthesis-associated globins (9, 37, 69, 111). Cyanobacterial GUN4 proteins also bind bilins and stimulate the activity of magnesium chelatase for chlorophyll synthesis (50, 147). CBCRs belong to a third superfamily, phytochrome and CBCR photoreceptors defined by a bilin-binding GAF domain. Phytochromes were first discovered in land plants; such proteins have a knotted PAS-GAF-PHY photosensory tridomain. Cyanobacteria uniquely have two other lineages within this superfamily (Fig. 1A-B): knotless GAF-PHY phytochromes, and CBCRs that require only the GAF domain for covalent attachment of the linear tetrapyrrole (bilin) chromophore and perception of light (Fig. 1C). All three

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families have conserved Cys residues for covalent attachment of bilins via thioether linkages (100, 117). CBCRs are thought to have evolved from ancestral knotted phytochromes via progressive reduction from knotless phytochromes (100).

CBCRs and phytochromes share a common central dogma of photosensory function: light absorption triggers a 15,16–photoisomerization of the bilin, toggling the protein between photostates with distinct spectral and biochemical properties (5, 32, 99). Chromophore photoisomerization is also seen in photoreceptors such as rhodopsins or photoactive yellow protein, but CBCR photoproducts typically persist long enough to interact with light for reverse photoconversion. Such CBCRs sense the ratio between two colors, and illumination of such proteins generates a photoequilibrium. One photostate is stable in darkness (the dark-adapted state), whereas the other state (the photoproduct) requires light to be formed. The dark-adapted state can be regenerated with light or via thermal decay of the photoproduct (dark reversion). This review focuses on CBCR biochemistry, photobiology, and evolution, while extending earlier reports on these and other related aspects of CBCRs.

CATCHING THE RIGHT LIGHT: SPECTRAL TUNING BY THE CBCR DOMAIN.

Compared to phytochromes, CBCRs present striking diversity (Fig. 1D-G). CBCRs can respond to light ranging from the near-ultraviolet to the near-infrared using a single phycocyanobilin (PCB) chromophore precursor (102, 109). Covalent, protonated PCB adducts intrinsically absorb red or orange light (54, 145), so spectral tuning by the CBCR has a major effect on the photocycle and photobiology. Much research has focused on elucidating CBCR tuning mechanisms. We use several conventions to minimize confusion. Known CBCR chromophores adopt the 15*Z* (*cis*) configuration in the dark-adapted state; photoisomerization occurs at the 15,16–double bond to generate a 15*E* (*trans*) photoproduct. We therefore designate photocycles by the color of light detected by the 15*Z* state followed by that of the 15*E* state, as in the red/green photocycles exhibited by CBCRs such as AnPixJg2 and NpR6012g4 (86, 107). This convention allows simple identification of the dark and lit states. We also use the term ‘CBCR’ to designate the individual bilin-bound GAF domain rather than the full-length protein in which it is found. This avoids potential confusion in discussing photoreceptors with multiple CBCR domains.

Two-Cys photocycles.

The first CBCR-based photoreceptor to be spectrally characterized was SyPixJ, a phototaxis receptor from *Synechocystis* containing a single CBCR domain. The SyPixJ CBCR domain exhibits a blue/green photocycle both within the full-length protein purified from *Synechocystis* and as a recombinant GAF-only construct with PCB (144, 145). Similar blue/green photocycles were reported subsequently for its putative ortholog, TePixJ from *Thermosynechocccus elongatus*, and for the light-regulated cyclic-di-GMP synthase Tlr0924 (or SesA; Fig. 1G) from the same organism (54, 116). Work on TePixJ and Tlr0924 elucidated the basis for detection of blue light, which requires a conserved Asp-Xaa-Cys-Phe (DXCF) motif. The DXCF Cys forms a second thioether linkage to the C10 atom of the bilin in the blue-absorbing state, yielding a shorter conjugated system containing the C- and D-rings and the photoactive 15,16–bond (10, 19, 53, 54, 87, 104, 106).

Such two-Cys photocycles have evolved repeatedly (6, 49, 85, 101, 102, 110). 'Insert-Cys' CBCRs have large, Cys-containing insertion loops and exhibit 15*Z* states that detect blue, violet, or near-ultraviolet light. Photoproduct absorption ranges from blue to orange light (16, 17, 102). Second linkage formation has been directly demonstrated for one insert-Cys CBCR (74). More recently, a broad range of additional CBCR groups with known or suspected two-Cys photocycles have been described, along with specialized DXCF CBCR lineages (6, 49, 85, 101, 108, 110). This tuning mechanism has also been introduced into other CBCRs via site-directed mutagenesis, resulting in introduction of green/blue or far-red/blue photocycles into red/green or far-red/orange parent molecules (28, 33, 110, 127).

Isomerization of PCB to phycoviolobilin.

Characterization of the first DXCF CBCRs also identified an additional tuning mechanism. Biliproteins can be denatured using acidic urea or acidic guanidinium chloride to ablate spectral tuning. Application of this assay to SyPixJ and TePixJ demonstrated the presence of a majority phycoviolobilin (PVB) adduct in both photostates, despite the presence of PCB as chromophore precursor (53, 54, 104, 145). Whereas PVB can be made from PCB during biosynthesis of some phycobiliproteins (69, 133), *in vitro* assembly of TePixJ or Tlr0924 apoprotein with PCB resulted in rapid formation of a photoactive blue-absorbing state followed by slower formation of PVB (53, 104). These CBCRs are thus able to carry out PVB formation by themselves (Fig. 2A-B). The two bilins differ structurally at C5 and in the bilin A-ring (Fig. 2B), so effects of this isomerization on the blue-absorbing state are only minor (106). Photoconversion and elimination of the labile linkage at C10 result in spectrally distinct 15*E* photoproducts: 15*E* PCB absorbs orange light, whereas 15*E* PVB absorbs green light (53, 104, 106). The combination of second linkage formation and PVB formation thus generates the blue/green photocycles of TePixJ and Tlr0924.

PVB formation can be complete upon expression in cyanobacteria but incomplete when the same protein is expressed in *E. coli* (52). Recombinant expression of CBCRs or phytochromes in *E. coli* is performed for under 24 hours in darkness, whereas cyanobacterial expression systems require longer growth and use continuous, bright light to maximize biomass. PVB formation is sometimes more efficient in the photoproduct state (104), so additional time and light could result in more complete PVB formation. The extent to which PVB is formed by different DXCF CBCRs also varies considerably (44, 76, 104); PVB formation is absent in some cases but is complete in others, and at least one DXCF protein from *N. punctiforme* equilibrates between PCB and PVB depending on the photostate (104). Formation of PVB seems much rarer in other CBCR lineages but has been reported (79, 110). Like DXCF CBCRs, such cases contain second Cys residues. Both site-directed mutagenesis and model compound studies indicate that formation of the linkage at C10 may facilitate PVB formation (44, 53, 76, 104, 130). It has also been possible to engineer PVB formation into a red/green CBCR alongside introduction of a second linkage and to modulate PVB formation and second linkage formation in a DXCF CBCR (28, 29, 110).

Trapped-twist photocycles.

DXCF CBCRs can exhibit other photocycles. Some members of this group have a photoproduct with a characteristically narrow, sharp lineshape (Fig. 2C) in the teal region of

the spectrum (6, 104, 136). This photoproduct is associated with PVB and with green- or blue-absorbing dark states. Site-directed mutagenesis has identified a pair of Phe residues that are necessary for formation of the teal-absorbing photoproduct, because variant proteins lacking one or both of these residues instead form green-absorbing photoproducts (105). Equivalent Phe residues are also conserved in red/green CBCRs, which use PCB chromophores (Fig. 2D). Similar substitutions again result in red-shifted photoproduct states with little to no effect on the properties of the dark-adapted state.

Characterization of one red/green CBCR using solution NMR spectroscopy demonstrated that the 15*E* photoproduct adopts a twisted configuration about the C15 methine bridge (113). These studies were then extended to yield atomic resolution solution structures for both photostates of this protein (Fig. 1C; (75)), revealing an additional twist about the A-ring in the photoproduct. Both structural features were also present in the green-absorbing photoproduct crystal structure for the red/green CBCR slr1393g3 from *Synechocystis* (138). This conserved, twisted photoproduct geometry results in a shorter effective conjugation for the chromophore, blue-shifting the photoproduct state (135). Loss of the conserved Phe residues is believed to result in a less twisted photoproduct. The similar role of these Phe residues in green/teal or blue/teal CBCRs indicates that a similar ‘trapped-twist’ mechanism should apply to the photoproduct of these proteins as well (105). The trapped-twist mechanism can also be modulated by site-directed mutagenesis in combination with controlled formation of a second linkage and of PVB, generating a broad range of photocycles from a single protein scaffold (28, 33).

Protochromic photocycles.

In red/green CBCRs such as NpR6012g4, all four bilin nitrogens are protonated in both photostates (114). The chromophore thus has a cationic π system. However, other CBCRs use bilin protonation/deprotonation as a tuning mechanism. The recently described ‘ee23’ (‘earliest extant as of 2023’) CBCRs use PCB chromophores and can exhibit green- or red-absorbing 15*Z* states (101). These spectral differences arise due to differences in protonation of the PCB chromophore: the green-absorbing species is deprotonated, whereas the red-absorbing state is protonated. Modulating the bilin pK_a thus provides an additional tuning mechanism, with deprotonated neutral bilins exhibiting a substantial blue shift because the ground-state electronic structure of their π systems is less conjugated than those of protonated cationic bilins (94).

Most ee23 CBCRs possess two-cysteine photocycles with blue-absorbing photoproduct states, so bilin protonation tunes only the dark state. Green/red CBCRs such as RcaE or CcaS (Fig. 1F) take this tuning mechanism further. In such proteins, the green-absorbing 15*Z* dark state is deprotonated but the red-absorbing 15*E* photoproduct is protonated (46–48, 94). Hence, the spectral separation between the two photostates is driven by changes in protonation state. This also means that such proteins are not effective photoreceptors outside the physiological pH range. For example, at high pH both photostates of RcaE are deprotonated and there is little spectral difference between them (101). Detailed vibrational and theoretical studies of RcaE demonstrate that the B-ring nitrogen is deprotonated in the 15*Z* state (94). The crystal structure of RcaE has recently been determined in the 15*E*

photostate, revealing a C15-*E,syn* configuration rather than the C15-*E,anti* configuration seen in DXCF and red/green CBCR photoproduct chromophores (10, 19, 75, 79, 81, 87, 138). RcaE can be reconstituted with a synthetic bilin that is locked in the 15-*Z,anti* configuration (47). If this configuration is also present in the authentic 15*Z* state of RcaE, then photoconversion would take place between 15-*Z,anti* and 15-*E,syn* configurations, exhibiting a formal hula-twist reaction not previously seen in biliproteins.

Verdin binding for sensing far-red light.

Most CBCRs incorporate PCB, with some then generating PVB. However, some red/green CBCRs can incorporate the PCB precursors biliverdin (BV) or 18^1,18^2-dihydrobiliverdin (DHBV; Fig. 2A). BV is synthesized from heme by heme oxygenase (HO). Subsequent reduction of BV by the ferredoxin-dependent bilin reductase PcyA generates DHBV as an intermediate in the synthesis of PCB (72). BV and DHBV lack the reduced A-ring found in PCB, resulting in a spectral red shift. Two CBCR lineages have been shown to bind BV efficiently. One group from *A. marina* is able to bind both BV and PCB (31, 88). Incorporation of PCB chromophore results in a normal red/green photocycle, whereas incorporation of BV results in a far-red/orange photocycle. *A. marina* is well adapted to growth under far-red light but also exhibits physiological responses under orange light (59), potentially matching this far-red/orange photocycle. *A. marina* also has two *pcyA* genes, one of which encodes a protein that accumulates DHBV at unusually high levels (77, 78); hence, BV, DHBV, and PCB are all potentially relevant chromophores in this unusual cyanobacterium. The affinity for BV is high enough to permit applications in mammalian cells synthesizing BV and can be conferred on other red/green CBCRs with only a few substitutions, expanding the range of possible applications (31, 70).

The other CBCR lineage known to have bind verdins is more widespread and is also red-shifted (Fig. 2E). This ‘DPYL-oar’ lineage is distinct from the BV-binding CBCRs of *A. marina* in phylogenetic analysis; these proteins can bind BV or DHBV but cannot bind PCB (79). Crystal structures demonstrate that an engineered CBCR mimicking the *A. marina* examples forms a covalent ‘exo’ linkage to $C3^2$ of BV, in contrast with the $C3^1$ ‘endo’ linkages seen for PCB in the parent molecule and for BV or DHBV in a DPYL-oar CBCR (31, 79, 87). Identification of amino acid determinants sufficient for bilin specificity in DPYL-oar CBCRs has not been reported to date, although a critical Pro residue is known to be necessary for exclusion of PCB in one such protein (79).

Tuning mechanisms in need of elucidation.

The tuning mechanisms for some CBCR photocycles have not been elucidated. In one example, some CBCRs lack the canonical Cys residue and instead ligate the chromophore to the bilin A-ring using a ‘second Cys’ aligned with the DXCF second Cys residue (34). Characterized representatives exhibit green/green photocycles with little spectral distinction between the two states, and some cases exhibit unstable photoproducts. Denaturation analysis reveals a spectrum that does not match the known features of PCB, PVB, or mixtures thereof. Hence, this CBCR group generates an unknown bilin adduct to detect green light.

Another poorly understood tuning mechanism is seen in far-red CBCRs such as Anacy_2551g3 (Fig. 2F) and Anacy_4718g3, which possess red-shifted PCB adducts that detect light at extremely long wavelengths (725–745 nm) in their dark-adapted states (109). Incorporation of phycoerythrobilin yields a red-shifted species compared to other CBCRs incorporating this bilin. Phycoerythrobilin has a saturated C15 methine bridge and cannot undergo photoconversion (80, 106), so this result indicates that the tuning mechanism does not require the D-ring to be in conjugation. Remarkably, the crystal structure of Anacy_2551g3 in the far-red-absorbing state revealed a C15-*Z,syn* configuration distinct from the C15-*Z,anti* structures of other CBCRs and phytochromes (3, 10, 19, 24, 31, 75, 79, 87, 131, 138). This twisted conformation would be expected to be blue-shifted relative to free PCB (3). Hence, even with a crystal structure, the tuning mechanisms underlying this remarkable red shift remain unknown, although electrostatic effects are likely to be important.

FROM LIGHT INTO DARKNESS: PHOTOISOMERIZATION, THERMAL REACTION PATHWAYS, AND SIGNAL TRANSMISSION BY CBCRs.

Photoisomerization is the first step in a series of events ultimately leading to a photobiological response such as phototaxis or changes in gene expression. This section examines what is currently known about the processes that occur within CBCR domains and full-length photoreceptors after light excitation.

Photon absorption and evolution of the excited state.

Photon absorption by a bilin chromophore results in formation of an electronic excited state. The initial ground states frequently have significant heterogeneity (1, 75, 104), so the excited-state ensemble can also be heterogeneous. After excitation, the excited-state population undergoes vibrational Franck-Condon cooling on a sub-picosecond timescale (38, 66). Several competing processes can then occur. Formation of the photoproduct state typically occurs via passage through a conical intersection to give an isomerized primary photoproduct (13, 39, 65, 125). Other de-excitation processes typically reduce the quantum yield for photoconversion and the overall efficiency of the photoreceptor. Evolution of the excited state is typically not monotonic, and this behavior has been interpreted in terms of chromophore solvation dynamics or parallel evolution of a heterogeneous ensemble arising from different ground-state sub-populations (26, 39, 41, 63, 64, 132). Given the diversity exhibited by CBCRs, it is not clear whether future studies will provide a single, general answer to this question. Interestingly, the quantum yield of CBCRs is not always determined by de-excitation of the excited state (65). Application of ultrafast pump-dump-probe spectroscopy has demonstrated that the red/green CBCR NpR6012g4 can undergo photoproduct formation after de-excitation in forward photoconversion (15*Z* to 15*E*). Such second-chance initiation dynamics proceed via a vibrationally excited ground-state intermediate matching the starting 15*Z* configuration; this intermediate then partitions between 15*Z* and 15*E* populations during vibrational cooling, resulting in photochemical quantum yields as high as 50% (13, 65).

De-excitation due to fluorescence is known in both the 15*Z* and 15*E* configurations of CBCRs. Red/green CBCRs such as NpF2164g5 from *N. punctiforme* that fail to undergo photoconversion and exhibit fluorescence quantum yields of 15–25% are comparable to engineered phytochromes or CBCRs developed for far-red imaging applications (25, 92, 93, 115). In such naturally fluorescent CBCRs, photoisomerization is prevented by three conserved residues (115). These residues have been introduced into NpR6012g4 to create a red-fluorescent variant resembling NpF2164g5, and the reverse substitutions restore photoconversion in NpF2164g5. Such red-absorbing, photoinactive CBCRs are typically found at identical positions within tandem CBCR arrays (see Fig. 1B for placement of NpF2164g5 within the full-length NpPtxD protein). This conserved placement suggests that these domains may play a functional, as yet unknown, role in the responses of the full-length photoreceptor. NpF2164g5 has also been engineered for BV binding, allowing its use as an imaging reagent in mammalian cells (31).

Evolution of the primary photoproduct.

CBCRs typically undergo photoisomerization with varying efficiency to produce primary photoproducts. Photoisomerization typically occurs on a timescale of picoseconds (26, 39–41, 125). After formation of the primary photoproduct, most CBCRs evolve through one or more ground-state intermediates to generate the new photostate, with some intermediates able to decay back to starting photostate (75). Detailed work on a series of red/green CBCRs has shown that the timescales and intermediates for these processes can vary widely even for closely related CBCRs (39, 57, 58, 67), with appearance of the final photostate on a timescale of milliseconds. Two-Cys photocycles often require the slower formation or elimination of the thioether linkage at C10. These processes have recently been examined in TePixJ using time-resolved circular dichroism and vibrational techniques (18, 118), but without clear agreement on intermediates and timescales. The diversity observed for red/green CBCRs is also likely to be present in other groups, so we do not yet know whether there are conserved steps in CBCR reaction pathways or how any such changes might be related to conserved changes in signaling state.

Signal transmission and domain-domain interactions within photoreceptors.

It is unclear whether photoconversion triggers conserved structural changes that modulate adjacent protein domains (either in the same molecule or as part of a complex). An early attempt at identifying such changes used solution NMR studies of the insert-Cys CBCR NpF2164g3 (74). Forward photoconversion yielded a longer α-helix at the C-terminus of the GAF fold. Such a change could propagate to a C-terminally adjacent domain with a helical element at its N-terminus, allowing a signal to be passed to an adjacent domain and potentially facilitating oligomerization of the full-length photoreceptor. By contrast, studies of an N-terminally truncated Tlr0924/SesA construct having only the CBCR domain and the C-terminal GGDEF domain found no evidence for a similar structural change or for such changes in oligomerization state, even though this construct exhibited robust light-dependent synthesis of the bacterial second messenger cyclic-di-GMP (5).

CBCR domains can regulate the signaling state of output domains in large, multi-domain photoreceptors. For example, PPHK (or JSC1_41510) has a complex structure including an

N-terminal REC domain, a CBCR domain, a histidine kinase bi-domain, and a C-terminal REC domain (103, 124). Biochemical studies demonstrated that the N-terminal REC domain and CBCR domain both regulate histidine kinase activity, forming a logical OR gate (124). Atomic resolution crystal structures of the N-terminal REC:CBCR fragment provided evidence that different signaling states of these two domains produced structural changes leading to greater or lesser degrees of asymmetry within a dimeric structure, including a light-induced bending of the helical spine that provides the dimerization interface. Such changes would not be expected in blue/orange CBCRs, a specialized DXCF lineage in which the full-length protein consists only of the CBCR domain (Fig. 1B; (108)). Such proteins have not yet been examined structurally, but structures of the isolated DXCF CBCR domain of TePixJ have demonstrated that photoconversion leads to a change in exposed residues in the vicinity of the D-ring (10, 19, 87). Blue/orange CBCRs have an unusually hydrophobic stretch of residues in this region, so it is possible that a similar change would expose a hydrophobic surface to support new protein-protein interactions.

In multi-domain photoreceptors, neighboring domains can theoretically affect the behavior of the CBCR domain as well (cross-talk). Full-length Tlr0924/SesA exhibits substantial temperature-dependent conversion of the 15*E* state between blue-absorbing and green-absorbing forms due to second linkage formation, but equivalent changes are only minor in the absence of the N-terminus (104, 116). Multi-photoreceptor proteins provide another case of possible cross-talk. Characterized examples include those having multiple CBCRs in the same protein and those having a knotless phytochrome and a CBCR domain. Such studies show that photoconversion of both domains can be required for maximum signaling output when assayed, but the observed photocycles do not differ from those seen in the individual, isolated photosensory domains (6, 15, 112). Reported cross-talk between a knotless phytochrome and a CBCR in IflA from *Fremyella diplosiphon* (11) stands as a possible exception, albeit with spectral overlap of the photocycles.

CBCR responses beyond color.

Many CBCR domains can be viewed as integrators of light and some other physiological cue. For example, two-Cys photocycles often have a reactive Cys residue that is attached to the bilin in one photostate but not the other (44, 76, 104, 116). Second linkage formation can be incomplete, resulting in a minor population that is unlinked in the presence of a linked majority or vice versa. Such photocycles are intrinsically redox sensitive, because molecular oxygen or reactive oxygen species can modify the unlinked Cys residue and prevent its reattachment to the chromophore (16, 102, 104, 106). Two-Cys photocycles can also exhibit pronounced temperature effects in the 15*E* state (104, 116). An orange-absorbing, nonproductive side population has been reported in the red/green CBCR NpR6012g4 (75). The equilibrium between such sub-populations in red/green CBCRs can be sensitive to temperature or pH (1, 75), so the extent to which photoconversion takes place is again sensitive to physiological cues beyond light. CBCRs using bilin protonation as a tuning mechanism are sensitive to intracellular pH. Such effects are not limited to tuning one or more photostates; recent work has shown that the efficiency of photoconversion is also sensitive to pH in ee23 CBCRs (101).

These examples show that CBCRs can integrate physiological cues while sensing the ratio between two colors of light. Some CBCRs have instead evolved to detect light intensity (34, 44, 107). This function relies on the thermal relaxation of the photoproduct to the dark-adapted state (dark reversion). Dark reversion is a well-known feature of phytochromes but is variable in CBCRs, with some 15*E* states persisting for days and others reverting to the 15*Z* state rapidly (107, 117). NpF2164g7 has an orange/green photocycle, but the photoproduct decays in seconds. The extent of photoproduct formation is approximately constant under orange light with or without green light, demonstrating that dark reversion is so fast that reverse photoconversion is no longer significant (68, 107). NpF2164g7 is in the same XRG clade as red/green CBCRs like NpR6012g4 (see below), but transient absorption spectroscopy has shown that its primary and secondary dynamics are distinct from such proteins (68). Other reported power sensors are not closely related to NpF2164g7 (27, 34, 44), so we do not know whether there are common adaptations for sensing light intensity. Studies have identified key residues that can be introduced into stable red/green CBCRs to produce faster dark reversion, allowing control of adenylate cyclase activity by red light intensity (27, 55).

BEYOND THE BILIN: CBCRs IN CYANOBACTERIAL PHOTOBIOLOGY.

Whether the photoproduct persists for seconds or weeks, photoconversion provides a light-sensitive ‘input’ that can modulate the activity of a biochemical ‘output’ to control various aspects of photobiology via cellular signal transduction pathways. CBCRs are associated with different outputs (Fig. 1B), with the most common being two-component histidine kinases, GGDEF and EAL domains that regulate cyclic-di-GMP levels, or MCP domains that regulate taxis. This section examines currently known CBCR functions in cyanobacterial photobiology. This subject has been recently reviewed (137), so our coverage here is more selective.

Regulation of light harvesting: chromatic acclimation.

Almost all cyanobacteria harvest light using bilin-bearing phycobiliproteins, which have long been recognized for their striking colors (129). It has also long been recognized that some cyanobacteria regulate phycobiliprotein composition in response to the ambient light color (35), a process known as chromatic acclimation (CA). For example, *F. diplosiphon* will up-regulate green-absorbing phycoerythrin and down-regulate red-absorbing phycocyanin when grown under green light, with the reverse pattern under red light (60, 61). There are several types of CA (8, 45, 61). *F. diplosiphon* exhibits Type III CA (CA3), whereas *N. punctiforme* regulates phycoerythrin but not phycocyanin (CA2) and *Leptolyngbya* sp. PCC 6406 regulates a different green-absorbing phycobiliprotein, phycoerythrocyanin (CA7). In a groundbreaking study, CA3 in *F. diplosiphon* was found to be regulated by a gene named *rcaE* with distant homology to phytochrome (60). *RcaE* encodes a photoreceptor that senses light through its green/red CBCR domain. Indeed, all three of these physiological processes are controlled by CBCRs (45, 46, 48, 60): RcaE controls CA3, whereas the closely related CBCR CcaS regulates CA2 and CA7. Both RcaE and CcaS exhibit light-regulated histidine kinase activity, but with opposite polarity: CcaS is activated by green light, whereas RcaE is activated by red light (46, 47). In both cases, a

phosphotransfer relay leads to phosphorylation of DNA-binding transcription factors (46, 61). In *F. diplosiphon*, phycoerythrin expression is regulated not only by RcaE but also by DpxA (136). The DpxA CBCR domain uses a combination of PVB formation and a trapped-twist photoproduct to generate a yellow/teal photocycle that provides a 'fine tuning' mechanism for perception of green light by RcaE. Other types of CA are not controlled by CBCRs. Far-red-light photoacclimation (FaRLiP or CA6) is controlled by the knotless phytochrome RfpA, whereas CA4 is only found in cyanobacteria that lack phytochromes and CBCRs and must use a different photoreceptor (36, 42, 119, 148).

Life in the slow lane: cyanobacterial phototaxis.

The study of phototaxis in cyanobacteria has a long history (91). Detailed studies have used the unicellular coccoid *Synechocystis* (4, 90, 123, 146), the unicellular rods *Synechococcus elongatus* PCC 7942 *Thermosynechococcus* (84, 140), and the filamentous *N. punctiforme* (12). This research has identified proteins required for phototaxis or for gliding motility (98, 142), including proteins responsible for production and regulation of Type IV pili (T4P). One locus resembling multigene bacterial taxis loci is essential for T4P formation and movement and has apparently been vertically inherited during cyanobacterial evolution (12, 101, 142, 146). A second such locus is specifically required for phototaxis in both *Synechocystis* and *N. punctiforme*, but these loci are not closely related to each other (101). However, both of them contain photoreceptor proteins with one or more CBCR domains combined with a C-terminal MCP (methyl-accepting chemotaxis protein) domain. The CBCR domains in these proteins are variable: a broad range of CBCR lineages and photocycles have been found in CBCR:MCP proteins, as have both color and power sensors (16, 34, 44, 79, 102, 107). CBCRs also need not be associated with MCP domains to play a role in phototaxis: phototaxis in *Synechocystis* is regulated by the CBCR:MCP protein SyPixJ and by the histidine kinase PixA/UirS (89, 126, 142–144, 146). This organism also provides an example of a CBCR that regulates motility indirectly via the bacterial second messenger cyclic-di-GMP.

To sink or to float: cyclic-di-GMP metabolism.

Cyclic-di-GMP can control sessile/motile transitions, biofilm formation, flocculation, and pathogenicity in diverse bacteria (23). It is synthesized from two GTP molecules by diguanylate cyclase (DGC) GGDEF domains and is degraded in specific phosphodiesterase (PDE) reactions via GGDEF/EAL bidomains or HD-GYP domains. In cyanobacteria, both GGDEF and EAL domains can be light regulated by CBCRs. In *Synechocystis*, the Cph2 protein combines an N-terminal red/far-red knotless phytochrome, a blue/green DXCF CBCR, and GGDEF and EAL domains. Blue light stimulates cyclic-di-GMP synthesis, and Cph2 inhibits positive phototaxis under blue light (121).

Cyclic-di-GMP regulation has been characterized in more detail in *Thermosynechococcus*. In this organism, cyclic-di-GMP levels are regulated by three DXCF CBCRs (20–23). Tlr0924/SesA is a large, multi-domain protein having a blue/green CBCR and a C-terminal GGDEF domain. As in Cph2, blue light stimulates DGC activity. Tlr1999/SesB has a blue/teal CBCR and a C-terminal GGDEF:EAL bidomain. It exhibits teal-stimulated PDE activity. Tlr0911/SesC again has a blue/green photocycle. This protein combines both DGC

and PDE activities; blue light stimulates DGC activity, whereas green light stimulates PDE activity. This three-protein system illustrates the extent to which multiple CBCRs can combine to regulate a single biochemical readout. However, cyclic-di-GMP itself has multiple roles in *Thermosynechococcus*, including regulation of cell-cell adhesion and determining the direction of phototaxis (23). CBCR:GGDEF proteins have also been expressed heterologously to control cyclic-di-GMP levels in *E. coli* (5, 6).

The rest of the pack: other outputs.

Other output domains are sometimes associated with CBCRs. There are some examples of CBCRs associated with protein domains that can add and subtract methyl groups to MCP domains, potentially providing another avenue for controlling phototaxis responses (43, 73, 95). Photocycles for two such proteins were found to be variable, even though the CBCRs themselves were closely related (101). There is also an unusual example of a CBCR-controlled adenylate cyclase, cPAC (7). In this case, the DXCF domain confers stimulation of cyclic AMP (cAMP) synthesis under blue light in the presence of an N-terminal REC domain. Deletion of the REC domain resulted in a loss of cAMP synthesis, a situation reminiscent of PPHK (124). Both cPAC and engineered variants incorporating different CBCRs with other photocycles are also able to carry out light-regulated cAMP synthesis in *E. coli* (7).

There are also examples of CBCRs lacking apparent output domains. The IflA protein from *F. diplosiphon* has an N-terminal knotless phytochrome and a green/blue CBCR, but the short C-terminus lacks the histidine kinase bidomain seen in related photoreceptors such as NpR5313/Npun_R5313 from *N. punctiforme* (11, 104, 108). Deletion of *iflA* resulted in delayed growth at low cell density (11). In the absence of an output domain, IflA may exert a photobiological effect via protein-protein interactions. A similar effect could explain the unknown role of blue/orange CBCRs, a DXCF subtype in which the full-length photoreceptor is a solitary GAF domain (76, 104, 120).

FROM HUMBLE BEGINNINGS: EVOLUTION AND DIVERSIFICATION OF THE CBCR DOMAIN.

CBCRs are thought to have arisen from knotted PAS-GAF-PHY phytochromes via knotless GAF-PHY phytochromes (Fig. 1A; (100)). Recent studies have provided potential insight into this process, with identification of the ee23 CBCRs as the first known CBCR lineage (96, 101). Branching of ee23 CBCRs was followed by branching of GGR (greater green/red) CBCRs and then the DXCF CBCRs. DXCF CBCRs in turn gave rise to a number of other lineages, such as the late-evolving XRG clade including the insert-Cys CBCRs, red/green CBCRs such as NpR6012g4, AnPixJg2, and slr1393g3, and other groups (27, 30, 101, 107, 110, 139). One of these studies identified late-evolving CBCR lineages that were present in the last common ancestor of the earliest known cyanobacterial branch (101), providing good evidence that CBCRs arose from phytochromes early in cyanobacterial evolution.

WIDGETS AND BUILDING BLOCKS: CBCRs AS REAGENTS FOR SYNTHETIC BIOLOGY.

Optogenetic applications of natural photoreceptors allow regulation of cellular processes by light with precise spatial and temporal specificity. Owing to their broad wavelength range for light sensing and their use of a common PCB chromophore precursor, CBCRs represent an excellent choice for multiplexing light-regulated processes within single cells and in multicellular tissues. One of the first such applications combined the green/red CBCR CcaS with the red/far-red cyanobacterial phytochrome Cph1 to regulate orthogonal gene expression circuits in *E. coli* cells (128). More recent studies by the same group have further streamlined this two-color regulatory system, exploiting fluorescent protein readouts to develop a second-generation system with improved dynamic range and decreased leakiness (122). In a further extension, this group similarly repurposed the UV-violet/green CBCR UirS from *Synechocystis* sp. PCC 6803, improving its dynamic regulatory range and yielding the most blue-shifted photoswitchable transcriptional regulatory tool to date (97).

Other groups have expanded the optogenetic applications of CcaS, beginning with use of the CcaSR system to regulate heterologous gene expression in another cyanobacterium (2). For potential bioprocessing applications, the CcsA-CcaR regulatory circuit was used to control the surface-displayed autotransporter Antigen 43 (82), selectively inducing cellular aggregation under green light. Removal of a linker region and two PAS domains located between the photosensory CBCR and histidine kinase output domains generated a miniaturized CcaS sensor with a reversed light signal output (83), underscoring the understudied roles of domains that transmit signals between CBCR domains and regulatory outputs. In this regard, a recent optogenetic application of CcaS in plants demonstrated that the deleted PAS domains are necessary for flavin binding by the full-length sensor (71). The engineered Highlighter variant of CcaS exhibited a striking blue-light regulatory response *in planta*. Most recently, CcaS was repurposed to develop a green-light activated CRISPR system with high dynamic range (14).

CBCRs have also been used to regulate production of cyclic nucleotide second messengers in heterologous systems. These include the blue-green CBCRs SesA/Tlr0924 (5) and cPAC (7), which respectively regulate production of cyclic di-GMP and c-AMP. Engineered chimeras between the red/green CBCRs AnPixJg2 and AnPixJg4 and the adenylate cyclase domain of the blue-light sensor CyaB1 from *Anabaena* sp. PCC7120 (27) have also been used to control cAMP levels with light intensity (134). Finally, application of CBCRs as optogenetic tools in mammalian cells requires CBCR re-engineering for biliverdin binding. This has been accomplished for several CBCRs (31, 51, 127), including the CBCR Amg2 (56) from *Acaryochloris marina*. The latter study is notable for its development of both green-ON/red-OFF and red-ON/green-OFF variants of bidirectional, cyanobacteriochrome-based light-inducible dimers for controlling transcription and subcellular protein targeting that can be multiplexed with existing blue-light tools in mammalian cells (see (62) for a recent compendium of methodologies).

CONCLUSIONS AND FUTURE PERSPECTIVES.

Relative to phytochromes, CBCRs were identified only recently. Their extreme diversity means that new CBCR lineages are still being discovered and also makes them attractive targets for synthetic biology applications. Despite the relative youth of CBCR research, much progress has been made. We now understand many of the tuning mechanisms that these proteins use to generate such diverse behavior, and in several cases these mechanisms can be controllably introduced into other CBCRs. We have atomic resolution structures for multiple CBCRs in one or both photostates, and more structures will appear in the future. We also have several examples of complete CBCR-based photobiological systems, such as CA2, CA3, and cyclic-di-GMP metabolism in *Thermosynechococcus*. However, much still remains to be understood. The very diversity that makes CBCRs so appealing also makes it hard to ascertain whether there are conserved structural changes or processes underlying their signal transduction mechanisms. CBCR applications in synthetic biology are beginning to emerge, but they have yet to find widespread use. Lastly, most work on CBCRs has focused on the properties of isolated CBCR domains or of small fragments. The revolution in structure determination using cryo-electron microscopy may permit study of full-length CBCR-based photoreceptors and their signaling partners.

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ABBREVIATIONS LIST

BV	biliverdin
CA	chromatic acclimation, occurring in different sub-types (e.g., CA2 is type 2 CA)
CBCR	cyanobacteriochrome
DHBV	18^1,18^2-dihydrobiliverdin
DGC	diguanylate cyclase
DPYL-oar	CBCR lineage characterized by Asp-Pro-Tyr-Leu (DPYL) motif and specificity for bilins with oxidized A-rings (oar)
DXCF	an Asp-Xaa-Cys-Phe motif found in (and identifying) a type of CBCR
EAL	protein domain named for a conserved Glu-Ala-Leu (EAL) motif
ee23	CBCR lineage (‘earliest extant as of 2023’) that is the earliest known branch in CBCR evolution

GAF	protein domain named for cGMP-specific phosphodiesterases, cyanobacterial adenylate cyclases, and formate hydrogen lyase transcription activator FhlA
GGDEF	protein domain named for a Gly-Gly-Asp-Glu-Phe (GGDEF) motif
GGR	CBCR lineage (the Greater Green/Red clade) including green/red CBCRs and other groups
MCP	protein domain found at the C-termini of Methyl-accepting Chemotaxis Proteins
PAS	protein domain named for period clock protein, aromatic hydrocarbon receptor nuclear translocator, and single-minded
PHY	phytochrome-specific domain
PCB	phycocyanobilin
PVB	phycoviolobilin
PPHK	A CBCR based photoreceptor named for its phosphorylation-responsive photosensitive histidine kinase activity (also designated JSC1_41510)
XRG	Extended Red/Green, designating a CBCR clade

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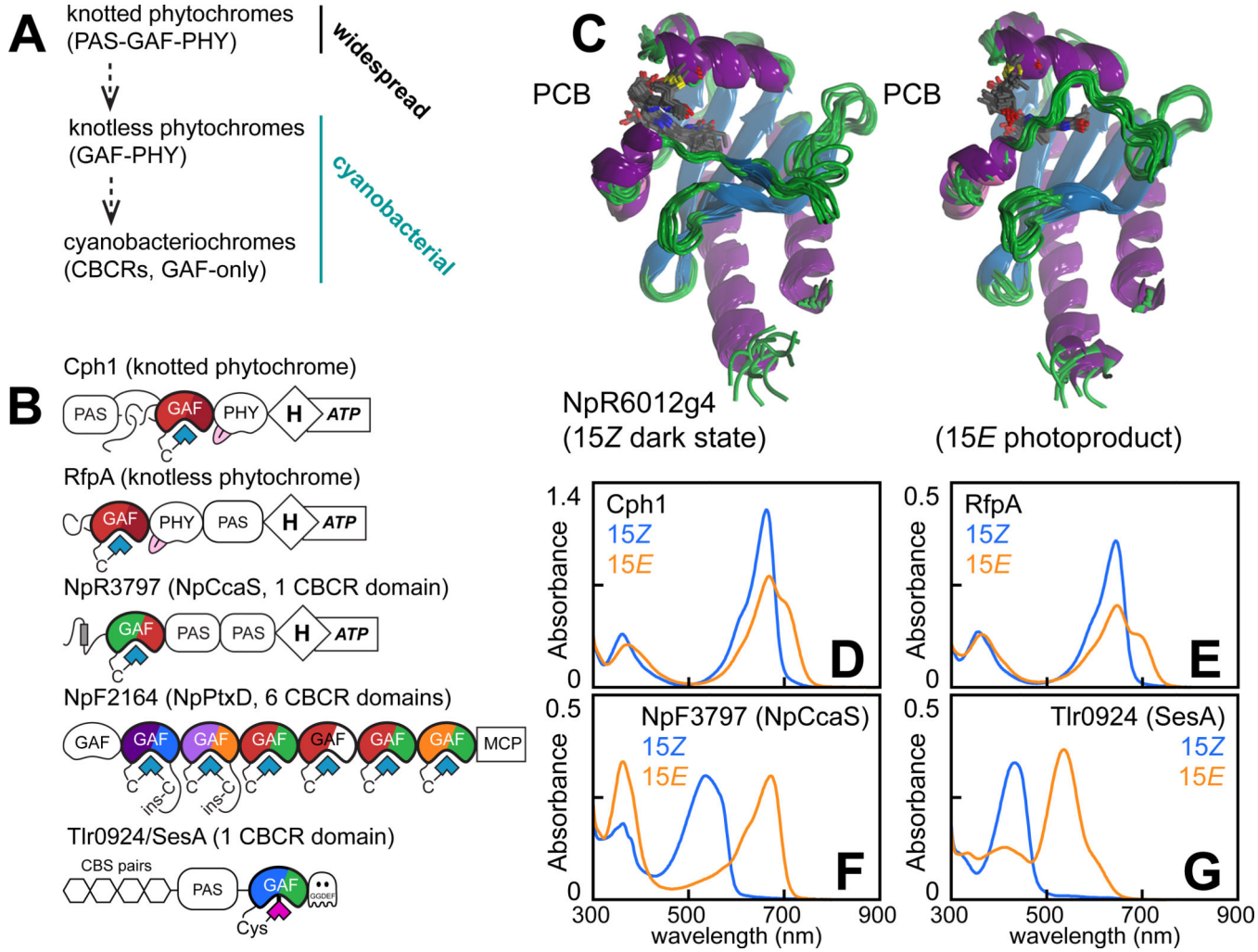

Figure 1. Cyanobacteriochromes are diverse biliprotein photoreceptors.

(A) Scheme for evolution of cyanobacteriochromes (CBCRs). (B) Domain architectures are shown for representative phytochromes and CBCRs. Bilin-binding GAF domains are colored by photocycle. (C) Ensemble solution structures are shown for the red/green CBCR NpR6012g4 from *Nostoc punctiforme* in the 15*Z* dark-adapted state *(left)* and 15*E* photoproduct *(right)*. Structures are colored by secondary structure with the phycocyanobilin (PCB) chromophore in ball-and-stick representation. (D) The red/far-red photocycle of the knotted phytochrome Cph1 from *Synechocystis* sp. PCC 6803 (141) is shown with the 15*Z* dark-adapted state in blue and the 15*E* photoproduct in orange. (E) The red/far-red photocycle of the knotless phytochrome RfpA from *Leptolyngbya* sp. JSC-1 (36) is shown. (F) The green/red photocycle of CBCR NpF3797 (NpCcaS; (46)) is shown. (G) The blue/green photocycle of CBCR Tlr0924 (SesA; (22, 116)) is shown.

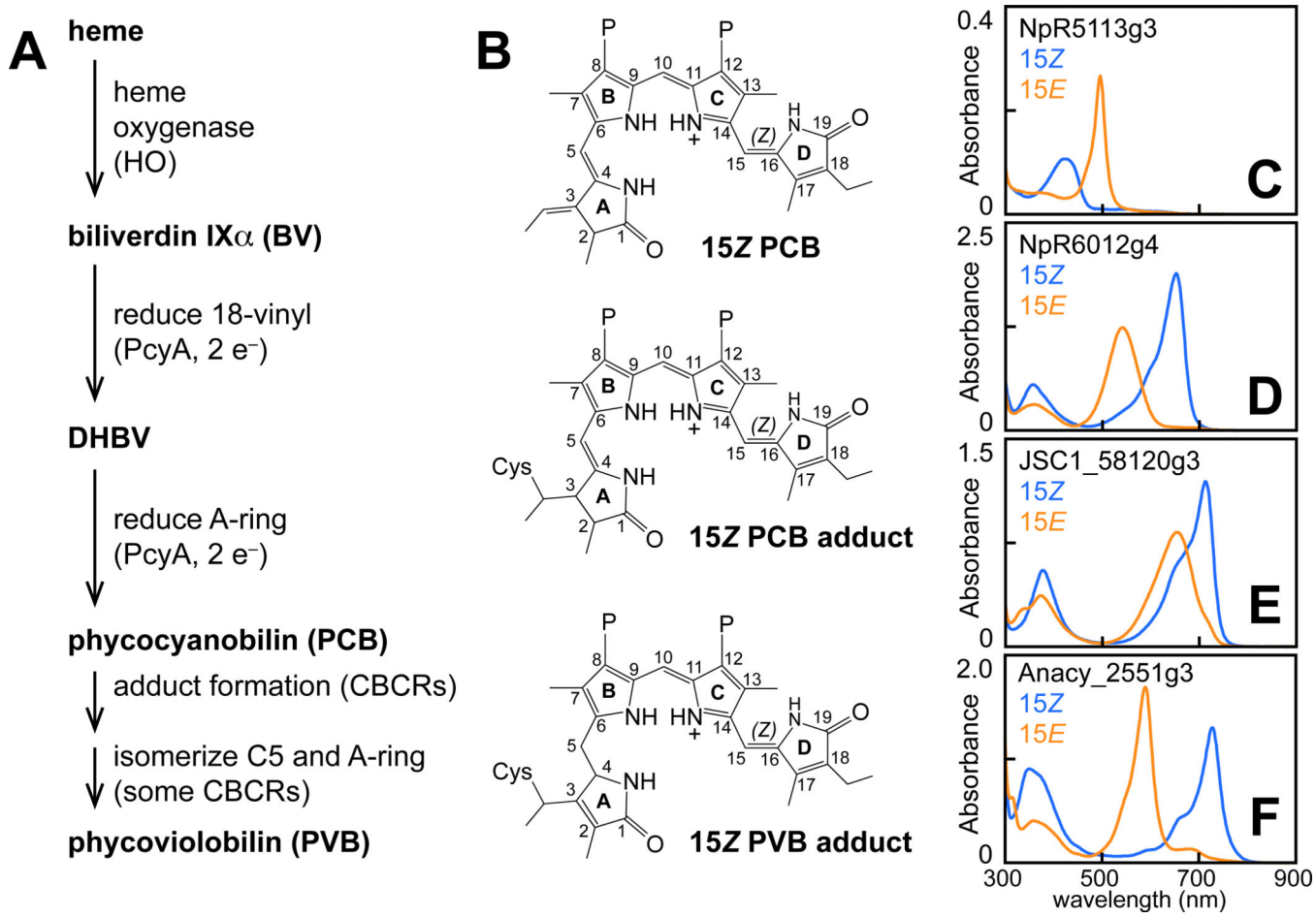

Figure 2. Bilin chromophores and photosensory diversity of cyanobacteriochromes.

(A) Cyanobacterial bilin biosynthesis pathway and cyanobacteriochrome (CBCR) chromophorylation are shown (52, 111). Heme oxygenase (HO) converts heme into biliverdin IXα (BV). BV is converted into phycocyanobilin (PCB) by the ferredoxin-dependent bilin reductase PcyA. This reaction proceeds via 2-electron reduction of the C18 side chain to yield 18^1,18^2–dihydrobiliverdin (DHBV), followed by 2-electron reduction of the A-ring to yield PCB. BV, DHBV, and PCB can all be used as CBCR chromophores, but PCB is by far the most common. Some CBCRs then isomerize PCB to phycoviolobilin (PVB). (B) Free PCB (*top)* is shown in the C5–*Z,syn*, C10–*Z,syn*, C15–*Z,anti* configuration found in the dark-adapted states of red/green CBCRs. Covalent attachment of the canonical Cys to the C3 side chain generates a covalent adduct *(middle*). PVB formation *(bottom)* proceeds via isomerization of the A-ring and the C5 methine bridge. (C) The photocycle of the DXCF CBCR NpR5113g3 (104) is shown, with a PVB chromophore. (D) The photocycle of the red/green CBCR NpR6012g4 with PCB is shown, with the 15*Z* spectrum in blue and 15*E* spectrum in orange (107). (E) The photocycle of the DPYL-oar CBCR JSC1_58120g3 is shown, with a DHBV chromophore (79). (F) The photocycle of the far-red CBCR Anacy_2551g3 is shown, with a PCB chromophore (3, 109).