Supporting Information

Rockwell et al. 10.1073/pnas.1107844108

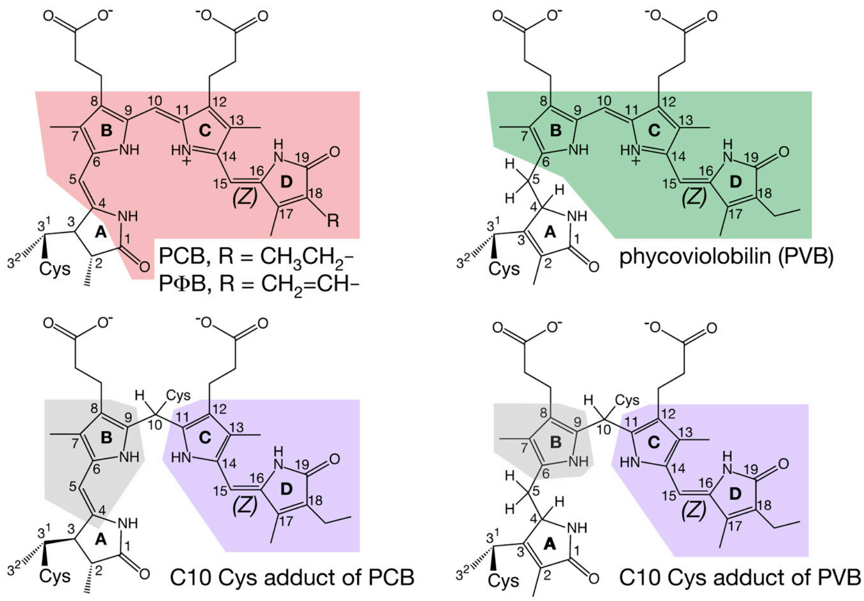

Fig. S1. Bilin chromophores of phytochromes and cyanobacteriochromes (CBCRs). Phycocyanobilin (PCB) or phytochromobilin (PΦB) precursors produce the covalent adduct shown in the *Upper Left* panel in the C5-*Z,syn* C10-*Z,syn* C15-*Z,anti* configuration of Cph1 in the P_r state (1). Phycoviolobilin (PVB) instead produces the adduct shown in the *Upper Right* panel. The second covalent linkage in dual-Cys photocycles takes place between the second Cys and the bilin C10 atom (*Lower*), and can occur with either PCB or PVB (2). Relevant conjugated systems are colored according to the color of light they absorb. Thus, unmodified PCB is colored red to match its red absorbance, and so forth.

1 Essen LO, Mailliet J, Hughes J (2008) The structure of a complete phytochrome sensory module in the Pr ground state. *Proc Natl Acad Sci USA* 105:14709–14714.
2 Ishizuka T, et al. (2011) The cyanobacteriochrome, TePixJ, isomerizes its own chromophore by converting phycocyanobilin to phycoviolobilin. *Biochemistry* 50:953–961.

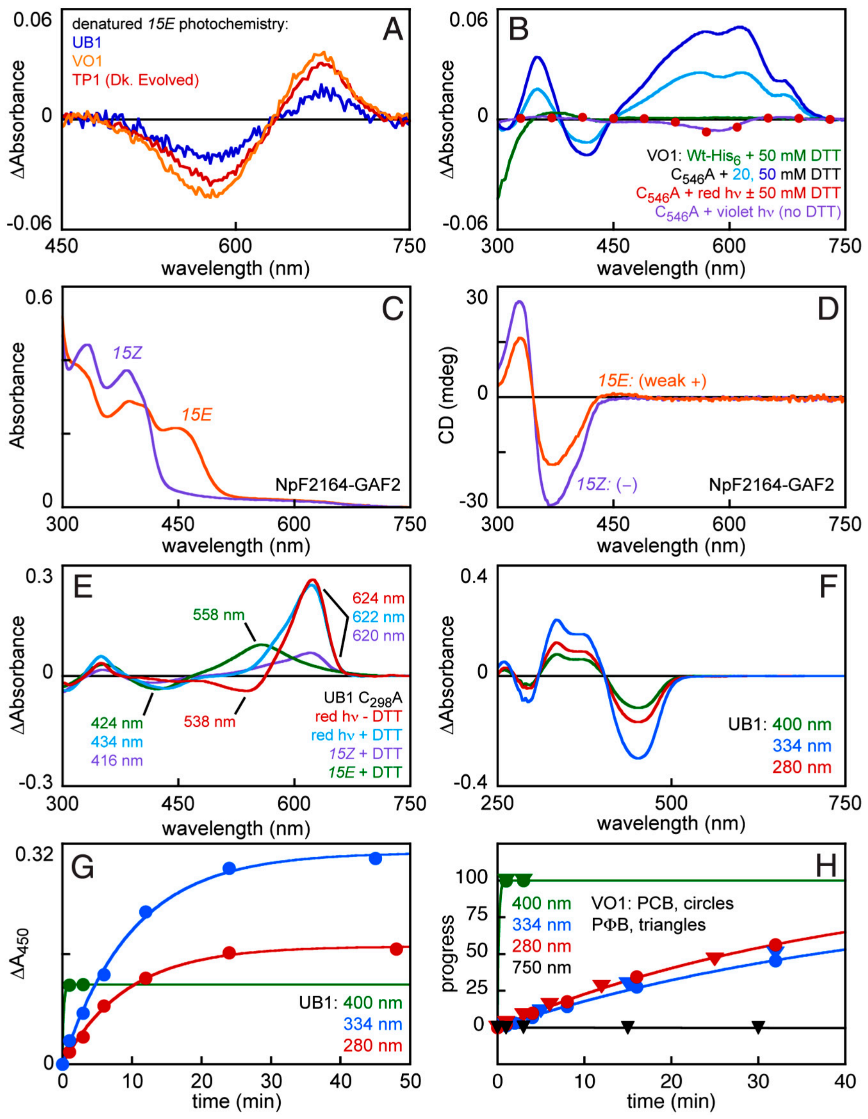

Fig. S2. Spectroscopic characterization of dual-cysteine photosensors. (*A*) Photochemical difference spectra for denatured *15E* UB1 (blue), VO1 (orange), and dark-evolved TP1 (red), shown as (*15Z-15E*). (*B*) Difference spectra for native wild-type and $C_{546}A$ VO1 under the indicated conditions. (*C*) Absorbance spectra for NpF2164-GAF2 in the *15Z* state (violet) and *15E* state (orange). (*D*) CD spectra for NpF2164-GAF2. (*E*) Difference spectra for native $C_{298}A$ UB1 under the indicated conditions. (*F*) Difference spectra for native UB1 at photoequilibrium with the indicated light sources. (*G*) Photochemical progress curves for UB1 using the conditions from (*F*). (*H*) Photochemical progress curves for VO1 with PCB (circles) or PΦB (triangles).

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                     (beta1)      (beta2)                 NpF2164g3 C546          Asp-motif
Cph1                 DRVMLYRFDENNHGDVIAEDKRDDMEPYLGLH------------------------------YPESDIPQPARRLF
TePixJ               DRVIVYAFDDNYVGTVVAESVAEGWPQARD--------------------------------QVIEDPCFREHWVE
AnPixJ-GAF2          DRVAVYRFNPDWSGEFVAESVGSGWVKLVGPDIK----------------------------TVWEDTHLQETQGG
Aazo_4225            DRVVIYRFHPDWSGEFVAESVNSGWIKLWHEPNNPAQNHPQFQANINFPQECTVENFDR … NYLQDSHFQNPKGS
AmaxDRAFT_4613       DRALIYRFNSDWSGVIIKESVSPGWRKILTAN----KGEEVTTQNTVDEENCIVKKLGS … ELIEDTYLQETQGG
NIES39_J03990-GAF2   DRALIYRFNADWSGVIIKESVSPGWQKILSEN----QGEEATTQNTVDEDHCIVKKLGS … ELIEDTYLKETQGG
MC7420_3869          DRVLIYEFNPDWSGTIIAESVAPGWQALLRDG----KKSSTFAQTTTVNPQCLANQLTD … GLVQDTYLQERQGG
Sy7002A0689          DRVVIYKFNPDWSGHFVAEAMSPQWRSLMNDAS--ARQRELLKYSAVRDDRCIVKVFES … GWVEDTYLQETQGG
cce_1413             HRVAVYRFNADYSGKFIAESVAEGWRSLVQTQ-------PKDVDNAKDIKDCTIHFLNN … LGLSDTYLQETQAA
cce_4289             ERVIVYQFNPDWSGQVVAESVSEGWPTLNLIT------TPEELLTTTEEEKCTI----- … NTIEDTHLQESQGE
CY0110_23126         ERVIVYRFNPDWSGKVVAESVSEGWPSLNLIT-----TDEQLLNTTEEEEKCTI----- … NTIEDTHLQESQGE
Ct8802_1740-GAF2     DRVAIYRFNHDWSGKIISDAVGQGWTSLVEKQ-------CQYPMVDPNMGQCQVRSLCN … PQLTDTYLKAIEGK
NpF2164-GAF2         DRVAIYRFQPDWSGEFVAESVGSEWVSLLEEQ-------RNNPAIVNNVNYCSVRNLAD … SHSADSYIQHTQGK
S7335_348            DRVAIYQFNPDWSGQFVFESAGPEWISLIEAQ-------QSNDLIAKNVSDCSVRSLDI … NPTADTHLQLTYGG
Ct7822_2884-GAF2     DRVAIYRFNPDWSGQFIIESVTHPWNCLIEQQ-------LSIPEIKQNISRCSVRELKN … PQITDTYLEETEGK
Ct7822_5290-GAF2     DRVAIYRFNPDWSGEFIVDTVAHGWTSLMDLQ-------FSHLELCGNISECSVKNLVN … PQLTDTYLKNTSGG
Ct7424_1855-GAF2     DRVAIYRFNSDWSGEFIVDSVTSQWTSLMKTQ-------LIHTELCENISECSVKNLVN … PQLTDTYLQKTQGG
MC7420_107           DRVAIYRFNPDWSGEFVVESVAEGWKPLVQGQ-------YQDPQLCQNISNCSIKSLAN … PQIIDTYLQQTEGG
Aazo_0203            DRVSIYRFNPDWSGDFVFESITSGWVTLMQEQ-------LQRPELRDNISNCNLKELV- … EPPTDTYLQDTGGG
NpR1597-GAF2 (UB1)   DRVAIYRFNPDWSGEFVFESVAEGWISLIDEQ-------LQRPELSENVSECSAKDLAK … PPVADTYLQDTEGG
Ava_1210             DRVAIYRFNPDWSGEFVFESFADGWISLIDEQ-------EKKPELRKNVSECSAKDLAS … TPTVDTYLQETRGG
all4261-GAF2         DRVAIYRFNPDWSGEFVFESLADGWISLIDEQ-------EKQPELRKNVSECSAKDLAS … TPVVDTYLQETRGG
L8106_25145          SRVLVYRFNPDWSGEVLAESVMSGWKTLISKT----EYQSSLKQIAVNQADCVVKLLDS … ALIEDTYLRENQGG
L8106_05116-GAF4     DRVVIYQFLPDWSGKFVAESVSAEWRPLLDTK-----TSDSNVENAVSENECTVQEL-- … VSIQDTYLKDTQGG
MC7420_7724-GAF2     DRVAIYRFNPDWSGEFVAESVADGWVTLTLEQ----QNNPDLKADALEEERCTLKSLDG … NSLQDTYLKETRGG
NIES39_C00690        DRVLVYRFREDWSGEFMAESVAEGWVQLTAGP----LRDSKATRIAIEQEKCAFQEINY … HVISDTYLQQNQGG
L8106_24225-GAF2     DRVLIYRFNTDWSGNIVSESVAPGWKILFSDQ----ANTPELTQVTVNKDDCAVAKFQS … GLIQDTYLQNNQGG
Osci3400013          DRVGVYRFNPDWSGNFVAESVASGWKVLLDSP----INQPQRTETTIEKENCATKTLSI … EPIEDTYLQANQGG
Ct8802_4055-GAF3     DRAVIYEFNSDWTGQAVAEAVGSGWISLLVEQ----TNEEVLQSDRTNNERCILRKWYH … LVEIDTFFQQTQGG
Ct8802_4055-GAF4     DRTLIYQFNTDWTGSVVAESVGSGWVSLLVEQ----TNDRILSGDRVATERCILRKWSQ … LLEPDTYFESTGGG
Ct8802_4055-GAF2     DRAVIYQFNSDWTGQPVAESVGSGWNSLFIEQ----TNDPILQSNRTNSDRCILRKWAQ … IVGTDTYFQSTAGG
NpF2164-GAF3 (VO1)   DRVVVYQFHPDWSGEVIAESVGAGWSSLVEMQ----EQDGILKTGLISSERCNIKDYGS … KADADTYLKETQGG
CwDRAFT_2358         DRAVVYHFNADWTGEVLAESAGAEWVSVMEIQ---ETDPTLFSKEMNAHEQCTLKNMQA … ALDQDTYFINTQGG
Ct8802_4055-GAF5     DRTVVYRFNADWSGEVIAESVNPQWVSVMELQ---QMDQQLYRTITTNDERCTLKKLSS … TFDIDTYLQETKGG
                         Cph1 C259     (beta5)        (beta6)
Cph1                 … ESILRSAYHCHLTYLKNMGVGASLTISLIKDGHLWGLIACH
TePixJ               … DIFKAGLTECHLNQLRPLKVRANLVVPMVIDDQLFGLLIAH
AnPixJ-GAF2          … DIYEAGHFSCHLEILEQFEIKAYIIVPVFAAEKLWGLLAAY
Aazo_4225            … IYYTAGFNDCYQQLLEKIQARAYTIVPIFNSGKLWGLLAVY
AmaxDRAFT_4613       … I-YKADFSDCYLRFLEQFQIRSYIISPIFQGYQLWGLLCVY
NIES39_J03990-GAF2   … I-YNSGFSDCYLKFLEQFQIRAYIISPIFQGYQLWGLLCVY
MC7420_3869          … V-EQANFSECYLNLLKGMQAQAYIIAPIFCGQQLWGLLGVY
Sy7002A0689          … I-YKAGFPPCYIELLENFQAKAYITLPIFLGDRLWGLLGIY
cce_1413             … I-YQAEFSHCYLQLLERYQAKAYIIVAIYKNKSLWGLFAAY
cce_4289             … I-YEAGFTPCYINLLEQLQAKAYIVVPIFCSNQLWGLILTY
CY0110_23126         … I-YEADFTPCYINLLEQLQAKAYIVVPIFCNNQLWGLILAY
Ct8802_1740-GAF2     … I-YEAGFSPCYIEILEEYEAKAYIIVAIYHQDTLWGLLAAY
NpF2164-GAF2         … I-YSAGFSDCYIKILEAYQAKAYIIVAIFQGEKLWGLLAAY
S7335_348            … I-ENAGFSDCYVDVLKSYQARSYTIIAVYLDNHLWGLLAAY
Ct7822_2884-GAF2     … I-YEQKFSQCYINILEHYQVRAYIIVAIYEGEKLWGLLAAY
Ct7822_5290-GAF2     … I-YNAGFSECYIKILEICQAKAYVIVAIYEGEKLWGLLATY
Ct7424_1855-GAF2     … I-YHAGFSDCYINILELCQARAYVIVAIYEGEKLWGLLATY
MC7420_107           … I-YTAGFSDCYIQSLERYQARAYTIIAIYKGKNLWGLLAAF
Aazo_0203            … I-YSAGFNECYIKILESYQARAYVIVAILYNHKIWGLLAVY
NpR1597-GAF2 (UB1)   … I-YNSGFSDCYIKILEIYQARAYVIIAIYHGQKLWGLLAVY
Ava_1210             … I-YNAGFSDCYIKILETYQARAYVIIAIYHGQKLWGLLAIY
all4261-GAF2         … I-YNAGFSDCYIKVLETYQARAYVIIAIYHGQKLWGLLAIY
L8106_25145          … I-YTAGFDECYLNFLEQLQARSYMIAPIFCGTNLWGLLCAY
L8106_05116-GAF4     … I-YEANFSDCYVELLEQFQARSYITVPIFCGTQLWGLLATY
MC7420_7724g-GAF2    … I-YNAGFTDCYIELLEQFQARSYIIAPIFCGKKLWGLLATY
NIES39_C00690        … I-YEMGFDACYLELLKQFQARAYIITPIFSGYQLWGLLAVY
L8106_24225-GAF2     … I-YQAGFDSCYLEFLEQLQARAYTITPIFCGKKLWGLLGVY
Osci3400013          … I-YQAGFDTCYIDLLEQFQARAYIITPIFCSSKLWGLLATY
Ct8802_4055-GAF3     … I-YATDFPSCYIESLEKYQAKAYIIVPIFKDEQLWGLLGCY
Ct8802_4055-GAF4     … V-YAMDFPECYIESLEKYQAKAYIIVPIFQGRKLWGLMGCY
Ct8802_4055-GAF2     … V-YAANFPTCYIESLEKYQATAYIIVPIFQNQKLWGLLGCY
NpF2164-GAF3 (VO1)   … I-SAAGFSSCYLESLEKFQAKAYIIVPVFRAGNLWGLLAAY
CwDRAFT_2358         … I-YNAGFSSCYLRSLEKYQAKAYMIVPIFQNNQLWGLFAVY
Ct8802_4055-GAF5     … I-YKAGFSPCYIEFLEKYQAKAYIIIPIFRNEDFWGLLAVY
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Fig. S3. Sequence alignment of insert-Cys CBCRs. Proteins in this study are highlighted. Both Cys residues and the Asp-motif are indicated, and β strand colors match Fig. 1*A*.

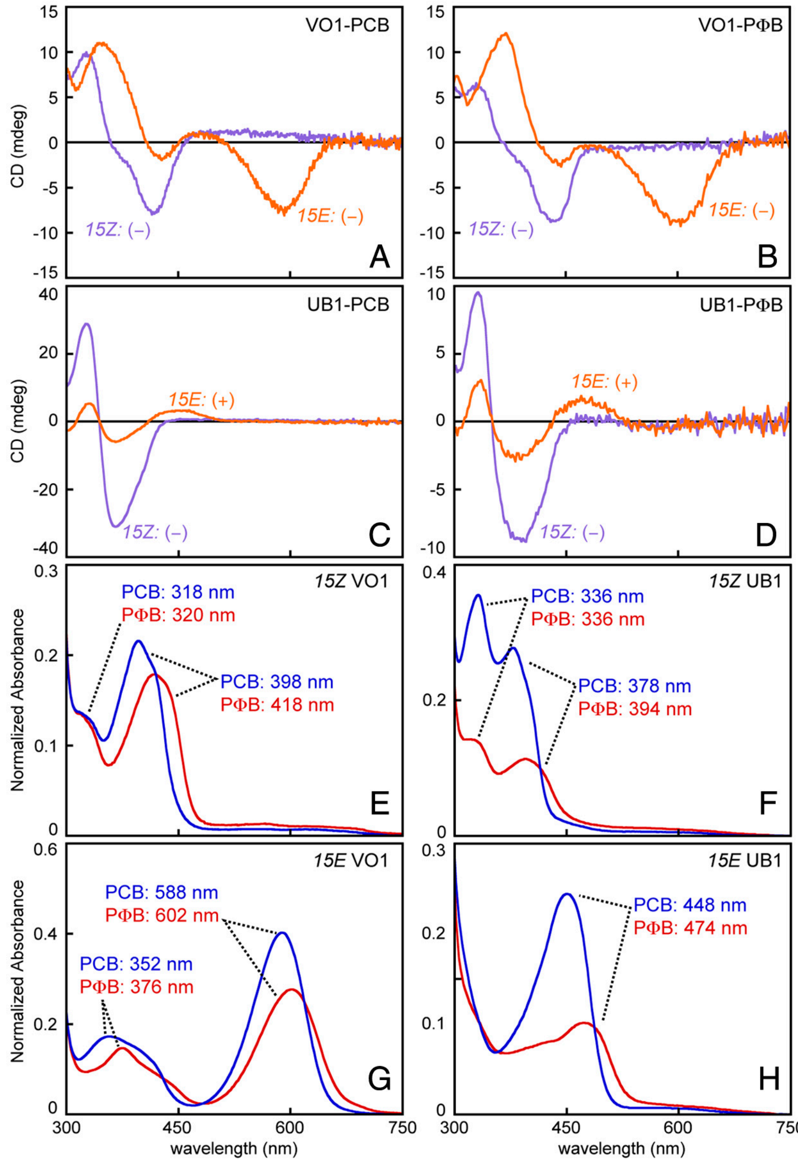

Fig. S4. Comparison of PCB and PΦB adducts of VO1 and UB1. (*A*)–(*D*) Characterization of VO1 and UB1 by CD spectroscopy. PCB and PΦB adducts are shown in the *15Z* (purple) and *15E* (orange) states. VO1 does not invert CD upon photoconversion, like the CBCR Tlr0924 (1) or BphPs. UB1 does invert CD, like Cph1 and plant phytochrome (2). (*E*)–(*H*) Absorption spectra are shown for PCB (blue) and PΦB (red) adducts, with peak wavelengths for the two observed bilin transitions indicated. The second transitions of both proteins show no redshift with PΦB in the *15Z* state, unlike the first transitions and unlike both transitions of *15E* VO1.

1 Rockwell NC, et al. (2008) A second conserved GAF domain cysteine is required for the blue/green photoreversibility of cyanobacteriochrome Tlr0924 from *Thermosynechococcus elongatus*. *Biochemistry* 47:7304–7316.

2 Rockwell NC, Shang L, Martin SS, Lagarias JC (2009) Distinct classes of red/far-red photochemistry within the phytochrome superfamily. *Proc Natl Acad Sci USA* 106:6123–6127.

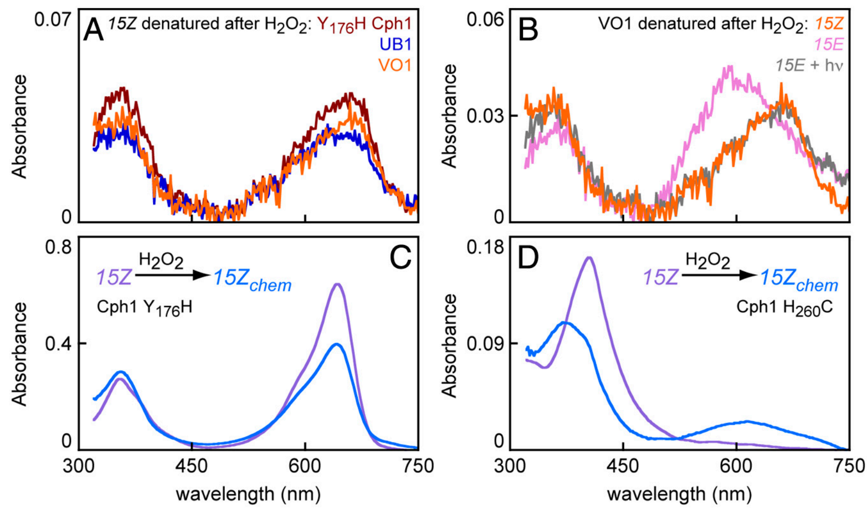

Fig. S5. Controls for reaction of CBCRs with hydrogen peroxide. (*A*) *15Z* H_2O_2 products were denatured with acidic guanidinium chloride and characterized by absorbance spectroscopy. (*B*) H_2O_2 products of VO1 in the indicated states were denatured with acidic guanidinium chloride. (*C*)–(*D*) The response of *15Z* $Y_{176}H$ and $H_{260}C$ mutant Cph1 to H_2O_2 was assessed. The *15Z* state (purple) was treated with H_2O_2 in darkness to yield chemical products (blue). Blue traces are scaled to reflect the 1:1 dilution of sample upon peroxide addition.

A

Protein	amino acids
NpF2164-GAF2	251-456
NpF2164-GAF3 (VO1)	463-664
VO1-His_6	463-664 +20 aa
NpR1597-GAF2 (UB1)	214-407
NpF1183 (NpCph1a, TP1)	2-513

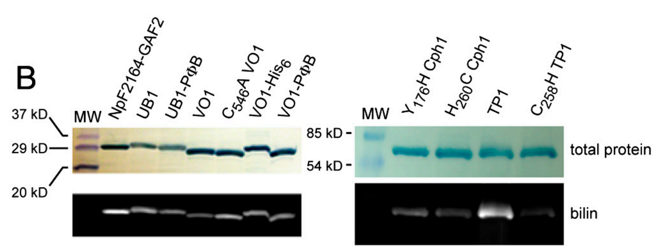

Fig. S6. Overview of novel 2-Cys photosensors. (*A*) Amino acids amplified for expression are indicated. (*B*) Characterization of purified proteins by SDS-PAGE and zinc blotting.

Table S1. Characterization of phytochromes and CBCRs*

Protein	Native SAR	Denatured SAR	% conversion	% recovery
NpF2164-GAF2	0.18	0.24	50	81
NpF2164-GAF3 (VO1)	0.22	0.26	85	98
VO1-PΦB	0.18	0.23	N/D	89
VO1 $C_{546}A$†	0.20	0.15	55	N/D
VO1-His_6	0.23	0.29	90	101
NpR1597g2 (UB1)	0.28	0.50	75 (40)‡	97
UB1-PΦB	0.11	0.10	N/D	103
UB1 $C_{298}A$	0.65	0.4	70	82§
NpF1183 (NpCph1a, TP1)	0.20	0.22	85	83
TP1 $C_{258}H$¶	0.03	0.03	<10	N/D
Cph1 $H_{260}C$	0.18	0.11	60	24

*Specific absorbance ratio (SAR) was calculated from peak absorbances of the bilin band and the protein band at 280 nm. Denatured SAR (in acidic guanidinium chloride) permits more direct comparison of proteins with different peak wavelengths in the native state. Percent conversion to *15E* photoproduct for PCB adducts was estimated from the denatured spectra relative to standards, but the absence of suitable PΦB standards precluded a similar approach for those samples. Percent recovery of the ground state was estimated from the *15Z* maxima of the forward and reverse difference spectra. N/D, not determined.

†Values are reported for photoconversion with violet light in the presence of 50 mM DTT.

‡Value in parentheses is for 400 nm photoequilibrium.

§Recovery is for 500 nm light without DTT. Recovery with 436 nm light and 50 mM DTT, 62%. Recovery in the presence of DTT is not corrected for presence of any *15Z* DTT adduct.

¶Forward photochemistry was too inefficient to allow characterization of the reverse reaction.

Table S2. Peak wavelengths*

Protein	Native λ_{max} (nm)	Denatured λ_{max} (nm)
NpF2164-GAF2	382 (–); 446 (+)	672; 580
NpF2164-GAF3 (VO1)	398 (–); 588 (–)	677; 582
VO1-PΦB	418 (–); 602 (–)	680; 586
VO1 $C_{546}A$†	614; 592	676; 582
VO1-His_6	398; 588	676; 578
NpR1597-GAF2 (UB1)	378 (–); 448 (+)	676; 576 (674; 578)‡
UB1-PΦB	396 (–); 474 (+)	682; 578
UB1 $C_{298}A$§	622; 558	674; 578 (674; 586)
NpF1183 (NpCph1a, TP1)¶	392; 598, 670	674; 578
TP1 $C_{258}H$	612; –	662; –
Cph1 $H_{260}C$	406; 570	682; 582
Cph1‖	662; 704	674; 582
α-PEC**	563; 506	599; 508

*Peak wavelengths are reported for the bilin transition of longest wavelength (S^1) as *15Z; 15E*. All proteins were expressed as intein-CBD fusion proteins with coexpression of PCB unless otherwise stated. Where measured, the CD sign for each transition is reported in parentheses for the native protein. Values for denatured samples were derived from difference spectra.

†Values are reported for photoconversion with violet light in the presence of 50 mM DTT.

‡Values in parentheses are for 334 nm illumination.

§Values in parentheses are in the presence of DTT. The free *15E* value was derived from the DTT-addition difference spectrum to avoid overlap with the free *15Z* state.

¶Multiple values for the *15E* state of TP1 reflect thermal evolution of photoproduct, which proceeded as a first-order process with an apparent rate constant of 0.2 min^{-1}.

‖Peak wavelengths (1) are references for covalent PCB adducts.

**Values for α-phycoerythrocyanin (α-PEC) calculated from spectra provided by Kai-Hong Zhao (Huazhong Agricultural University) as references for covalent PVB adducts.

1 Shang L, Rockwell NC, Martin SS, Lagarias JC (2010) Biliverdin amides reveal roles for propionate side chains in bilin reductase recognition and in holophytochrome assembly and photoconversion. *Biochemistry* 49:6070–6082.

Table S3. Accession information for insert-Cys CBCRs*

Name[†]	Locus tag	Organism	Amino acids
Aazo_4225	Aazo_4225	*Nostoc azollae* 0708	650–791
AmaxDRAFT_4613	AmaxDRAFT_4613	*Arthrospira maxima* CS-328	387–510
NIES39_J03990-GAF2	NIES39_J03990	*Arthrospira platensis* NIES-39	349–472
MC7420_3869	MC7420_3869	*Microcoleus chthonoplastes* PCC 7420	378–501
Sy7002A0689	SYNPCC7002_A0689	*Synechococcus sp.* PCC 7002	211–337
cce_1413	cce_1413	*Cyanothece sp.* ATCC 51142	219–337
cce_4289	cce_4289	*Cyanothece sp.* ATCC 51142	236–350
CY0110_23126	CY0110_23126	*Cyanothece sp.* CCY0110	214–327
Ct8802_1740-GAF2	Cyan8802_1740	*Cyanothece sp.* PCC 8802	225–343
NpF2164-GAF2	Npun_F2164	*Nostoc punctiforme* ATCC 29133[‡]	287–415
S7335_348	S7335_348	*Synechococcus sp.* PCC 7335	47–165
Ct7822_2884-GAF2	Cyan7822_2884	*Cyanothece sp.* PCC 7822	250–368
Ct7822_5290-GAF2	Cyan7822_5290	*Cyanothece sp.* PCC 7822	275–392
Ct7424_1855-GAF2	PCC7424_1855	*Cyanothece sp.* PCC 7424	259–377
MC7420_107	MC7420_107	*Microcoleus chthonoplastes* PCC 7420	264–382
Aazo_0203	Aazo_0203	*Nostoc azollae* 0708	294–411
NpR1597-GAF2 (UB1)	Npun_R1597	*Nostoc punctiforme* ATCC 29133[‡]	254–372
Ava_1210	Ava_1210	*Anabaena variabilis* ATCC 29413	252–370
all4261-GAF2	all4261	*Nostoc sp.* PCC 7120	252–370
L8106_25145	L8106_25145	*Lyngbya sp.* PCC 8106	564–688
L8106_05116-GAF4	L8106_05116	*Lyngbya sp.* PCC 8106	954–1072
MC7420_7724-GAF2	MC7420_7724	*Microcoleus chthonoplastes* PCC 7420	499–650
NIES39_C00690	NIES39_C00690	*Arthrospira platensis* NIES-39	407–529
L8106_24225-GAF2	L8106_24225	*Lyngbya sp.* PCC 8106	385–508
Osci3400013	OSCI_3400013	*Oscillatoria sp.* PCC 6506	567–690
Ct8802_4055-GAF3	Cyan8802_4055	*Cyanothece sp.* PCC 8802	492–617
Ct8802_4055-GAF4	Cyan8802_4055	*Cyanothece sp.* PCC 8802	701–824
Ct8802_4055-GAF2	Cyan8802_4055	*Cyanothece sp.* PCC 8802	285–408
NpF2164-GAF3 (VO1)	Npun_F2164	*Nostoc punctiforme* ATCC 29133[‡]	499–622
CwDRAFT_2358	CwatDRAFT_2358	*Crocosphaera watsonii* WH 8501	106–230
Ct8802_4055-GAF5	Cyan8802_4055	*Cyanothece sp.* PCC 8802	901–1026

*Accession information is provided in the form of the GenBank locus tag. The numbers of the aligned amino acids are shown, corresponding to the first through sixth GAF beta strands. This alignment is intended to show the sequence diversity in this subfamily; therefore, some identical CBCR sequences from closely related *Cyanothece* and *Arthrospira* species are not shown.

[†]Name given in Fig. S3.

[‡]Also known as *Nostoc punctiforme sp.* PCC 73102.