Characterization of Two Thermostable Cyanobacterial Phytochromes Reveals Global Movements in the Chromophore-binding Domain during Photoconversion

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Photointerconversion between the red light-absorbing (Pr) form and the far-red light-absorbing (Pfr) form is the central feature that allows members of the phytochrome (Phy) superfamily to act as reversible switches in light perception. Whereas the chromophore structure and surrounding binding pocket of Pr have been described, those for Pfr have remained enigmatic for various technical reasons. Here we describe a novel pair of Phys from two thermophilic cyanobacteria, Synechococcus sp. OS-A and OS-B, that overcome several of these limitations. Like other cyanobacterial Phys, SyA-Cph1 and SyB-Cph1 covalently bind the bilin phycocyanobilin via their cGMP phosphodiesterase/adenyl cyclase/FhlA (GAF) domains and then assume the photointerconvertible Pr and Pfr states with absorption maxima at 630 and 704 nm, respectively. However, they are naturally missing the N-terminal Per/Arndt/Sim domain common to others in the Phy superfamily. Importantly, truncations containing only the GAF domain are monomeric, photoreactive, and remarkably thermostable. Resonance Raman and NMR spectroscopy show that all four pyrrole ring nitrogens of phycocyanobilin are protonated both as Pr and following red light irradiation, indicating that the GAF domain by itself can complete the Pr to Pfr photocycle. 1H-15N two-dimensional NMR spectra of isotopically labeled preparations of the SyB-Cph1 GAF domain revealed that a number of amino acids change their environment during photoconversion of Pr to Pfr, which can be reversed by subsequent photoconversion back to Pr. Through three-dimensional NMR spectroscopy before and after light photolysis, it should now be possible to define the movements of the chromophore and binding pocket during photoconversion. We also generated a series of strongly red fluorescent derivatives of SyB-Cph1, which based on their small size and thermostability may be useful as cell biological reporters.

Phytochromes (Phys) include a large and diverse superfamily of sensory photoreceptors that use a bilin (or linear tetrapyrrole) chromophore for light detection (1–3). These chromoproteins were first discovered in higher plants based on their control of many agriculturally relevant processes and have since been found by sequence searches in lower plants, algae, and numerous proteobacteria, cyanobacteria, and fungi. For photoautotrophic organisms, Phys are particularly important for optimizing photosynthetic potential where they measure the fluence rate, duration, and the direction of light and help detect shading by competitors (1, 4).

The signature feature of canonical Phys is their ability to photoconvert between two stable states, a red light-absorbing Pr form that is typically the parent state of the photoreceptor, and a far-red light-absorbing Pfr form that is often biologically active (1–3) (for examples of exceptions see Refs. 5–7). This activity is directed by a series of structural domains that play specific roles in photoperception. A large N-terminal region encompasses the chromophore-binding domain (CBD); it includes a proximal Per/Arndt/Sim (PAS) domain immediately followed by a cGMP phosphodiesterase/adenyl cyclase/FhlA (GAF) domain. The CBD autocatalytically attaches the bilin via a thioether linkage and together with the chromophore generates the unique photochromic absorption properties of Phys.

For proteobacterial (BphP) and fungal Phys, biliverdin (BV) is used as the chromophore (8). BV is synthesized by oxidative cleavage of heme by a heme oxygenase (HO) and then attached to the apoprotein through its A-pyrrole ring vinyl side chain to a positionally conserved cysteine upstream of the PAS domain (9, 10). For cyanobacterial (Cph) and plant Phys, phycocyanobilin (PCB) and phytocromobilin (PφB) are used as the chromophore, respectively (2, 11, 12). PCB/PφB are synthesized by enzymatic reduction of BV using a BV reductase (BVR) and then attached through their A-ring ethylenide side chains to a chromophore-binding domain (CBD) and then associated via their GAF domains to specific signaling domains. The signature feature of canonical Phys is their ability to photoconvert between two stable states, a red light-absorbing Pr form that is typically the parent state of the photoreceptor, and a far-red light-absorbing Pfr form that is often biologically active (1–3) (for examples of exceptions see Refs. 5–7). This activity is directed by a series of structural domains that play specific roles in photoperception. A large N-terminal region encompasses the chromophore-binding domain (CBD); it includes a proximal Per/Arndt/Sim (PAS) domain immediately followed by a cGMP phosphodiesterase/adenyl cyclase/FhlA (GAF) domain. The CBD autocatalytically attaches the bilin via a thioether linkage and together with the chromophore generates the unique photochromic absorption properties of Phys.

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‡ The abbreviations used are: Phy, phytochrome; BphP, bacteriophytochrome; BV, biliverdin IXα; CBD, chromophore-binding domain; Cph, cyanobacterial phytochrome; GAF, cGMP phosphodiesterase/adenyl cyclase/FhlA; HK, histidine kinase; HO, heme oxygenase; HSQC, heteronuclear single quantum coherence; PAS, Per/Arndt/Sim; PCB, phycocyanobilin; PφB, phytocromobilin; PHY, phytochrome domain; Pfr, red-light-absorbing form of phytochrome; RR, resonance Raman; SyA/8, Synechococcus sp. OS-A/OS-B; Syn, Synecocystis; BVR, BV reductase; FPLC, fast protein liquid chromatography; ALA, α-aminolevulinic acid; SEC, size exclusion chromatography.
Thermostable Phytochromes from Synechococcus

positionally conserved cysteine in the GAF domain. Despite these differences in binding geometry, it appears that the positioning of the bilin in the CBD is similar in both the BV- and PCB/PFB-containing Phys (13). Following the GAF domain is a phytochrome (PHY) domain that helps stabilize the Prf form (14, 15).

The C-terminal half of Phys contains one or more domains that promote signal output. The most common in microorganisms is a histidine kinase (HK) domain that allows Phys to participate in specific two-component signal transduction cascades (3). Higher plant Phys have C-terminal sequences related to HK domains (2, 3), but it has not yet been confirmed that they function as protein kinases.

Despite intensive effort, it still remains unclear how Phys photointerconvert between Pr and Pfr and how this change initiates signal transmission (2). Important insights were provided recently by determinations of the three-dimensional structure of the CBD as Pr. These high resolution x-ray crystallographic models, which were generated with proteobacterial Phys from Deinococcus radiodurans (DrBphP) (10, 13) and Rhodopseudomonas palustris BphP3 (53) assembled with BV, revealed that the bilin is deeply buried within the GAF domain in a ZZZ-syn,syn,anti configuration. The PAS domain has no direct contact with the chromophore; instead, it is connected indirectly to the bilin and the GAF domain through a figure-of-eight knot in the polypeptide. These models also identified a number of amino acid contacts potentially important for binding and photochemistry that we and others have confirmed to be critical by mutagenic analysis of recombinant chromoproteins (13, 15–19). For example, the aspartate have confirmed to be critical by mutagenic analysis of recombinant Phys involved paired amplifications that were subsequently combined, melted, and re-annealed to generate blunt-ended doubled-stranded fragments as described (37). The DNA templates for SyA-Cph1 and SyB-Cph1 full-length coding sequences were PCR-amplified directly from Synechococcus sp. OS-A and OS-B’ genomic DNA, respectively. The pBAD-C expression plasmid encoding the PAS-GAF-PHY region from Synechocystis sp. PCC6803 (Syn) Cph1 (residues 1–514) was as described (38). The codons for the C-terminal c-Myc tag attached to Syn-Cph1(PAS-GAF-PHY) were replaced with those encoding a His6 tag (underlined) followed by a stop codon by ligation the annealed primers 5’-AGCTTTGACTCATCAT-CTCATCATTTGAGC and 5’-AGCTCTTTCCTAATGATGATGATGATGATGATGATG for HindIII-digested pBAD-C plasmid containing the Syn-Cph1(PAS-GAF-PHY) construction. From this manipulation, the plasmid pBAD-6H was generated.

Construction of Recombinant Phy Expression Strains—PCR-based modifications of Phys involved paired amplifications that were subsequently combined, melted, and re-annealed to generate blunt-ended doubled-stranded fragments as described (37). The DNA templates for SyA-Cph1 and SyB-Cph1 full-length coding sequences were PCR-amplified directly from Synechococcus sp. OS-A and OS-B’ genomic DNA, respectively. The pBAD-C expression plasmid encoding the PAS-GAF-PHY region from Synechocystis sp. PCC6803 (Syn) Cph1 (residues 1–514) was as described (38). The codons for the C-terminal c-Myc tag attached to Syn-Cph1(PAS-GAF-PHY) were replaced with those encoding a His6 tag (underlined) followed by a stop codon by ligation the annealed primers 5’-AGCTTTGACTCATCAT-CTCATCATTTGAGC and 5’-AGCTCTTTCCTAATGATGATGATGATGATGATGATG for HindIII-digested pBAD-C plasmid containing the Syn-Cph1(PAS-GAF-PHY) construction. From this manipulation, the plasmid pBAD-6H was generated.

Assembly of the various Cph truncations and mutants were accomplished by PCR using appropriate primer pairs. Products for the two PCRs were combined, melted, and re-annealed, phosphorylated, and purified as described (37). Phosphorylated flush inserts were then ligated into the NcoI- and HindIII-digested pBAD-6H plasmid. Site-directed mutations were introduced into the His6-tagged SyB-Cph1(GAF) construction by the QuickChange method (Stratagene, La Jolla, CA) using Pfpx polymerase (Invitrogen) in combination with the appropriate mutagenic primers. All coding regions were sequenced in their entirety to confirm the presence of the desired mutation and the absence of secondary mutations.
The PAS-GAF-PHY truncation (residues 1–501) of DrbphP was generated by PCR amplification of the full-length coding region with the primers 5'-CGTAAGGATCCCTGACCCGCAG- 
GACCGTTGGCCC and 5'-CTGACTGAGCGCCCCGCCG-
GTCAAATGTCATCAG that were designed to add BamHI and Xho sites to the 5’ and 3’ ends, respectively. The PCR product was digested with BamHI and XhoI and ligated into the pET21a plasmid (Novagen, Madison, WI) that was similarly digested.

Protein Expression and Purification—PCB-containing holocphs suitable for absorption, resonance Raman (RR), and/or plasmid (Novagen, Madison, WI) that was similarly digested.

To test for bilin specificity, the various Phy apoproteins were expressed in the pBAD-6H plasmid as above without co-expression of the HO and BVR genes from pL-PCB. Apo-DrbphP-(PAS-GAF-PHY) C-terminally tagged with a His6 sequence was expressed as described by Karniol et al. (15). The E. coli cells were resuspended in 30 mm Tris-HCl (pH 8.0), 100 mm NaCl, and 30 mm imidazole and lysed by sonication. The resulting extracts were purified by centrifugation as above and then mixed with 100 µM of either PCB or BV for 2 h at 4 °C. The polypeptides were purified by nickel-chelate affinity chromatography using 300 mm imidazole for elution. Presence of the covalently bound bilin was assayed by zinc-induced fluorescence of the chromoproteins following SDS-PAGE (8). PCB was purified from lyophilized Spirulina platensis as described (42) but without the final high pressure liquid chromatography step. Purified BV was obtained from Frontier Scientific (Logan, UT).

Size Exclusion Chromatography—Size exclusion chromatography (SEC) was performed by FPLC using a 24-ml Superose 6 (56) column (GE Healthcare). The chromoproteins (100 µl of a 5 mg/ml sample) were first purified through the phenyl-HP step, dissolved in 30 mm Tris-HCl (pH 8.0) and 200 mm NaCl, and either loaded onto the column as Pr or immediately following saturating irradiation of the samples with red light (620 nm). Absorption spectra were recorded before and after SEC to verify that the chromoproteins remained enriched in the desired states (Pr or Pfr).

Absorption and Fluorescence Spectroscopy—Absorption spectra were measured with a Lambda 650 UV-visible spectrophotometer (PerkinElmer Life Sciences) with the samples dissolled in 30 mm Tris-HCl (pH 8.0). Photocorsons between Pr and Pfr by red and far-red light were achieved with white light filtered through appropriate interference filters (10 nm half-bandwidth) (Andover Corp., Salem, NH), 620-nm and 690-nm filters, respectively, for SyA-Cph1 and SyB-Cph1, and 660-nm and 774-nm filters, respectively, for Syn-Cph1. Thermosability was measured in the dark for Pr samples (absorbance of the Pr absorption maximum was adjusted to 1.5) dissolved in 30 mm Tris-HCl (pH 8.0). After heating for 20 min at the appropriate temperature, the samples were clarified by centrifugation at 16,000 × g, and the amounts of soluble chromoproteins remaining were measured spectrophotometrically. Rates of Pr → Pfr photoconversion and Pfr → Pr dark reversion were measured using the absorbance of the samples at 704 nm to determine the amount of Pfr generated or lost, respectively.

Thermostable Phytochromes from Synechococcus

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Thermostable Phytochromes from Synechococcus

Fluorescence excitation and emission spectra were recorded with a QuantaMaster model C-60/2000 spectrofluorimeter (Photon Technologies International, Birmingham, NJ). Chromoprotein concentrations were adjusted to have an absorbance of 0.6 for the Pr absorbance maximum. Emission spectra were recorded during an excitation at 360 nm. Excitation spectra were recorded by measuring fluorescence at the peak emission wavelength (646–664 nm depending on the sample).

**RR Spectroscopy**—RR spectra were recorded with 1064-nm excitation (Nd-YAG cw laser, line width <1 cm⁻¹) using DigiLab (Bio-Rad) or an RFS 100/S (Bruker Optics, Ettlinger, Germany) Fourier-transform Raman spectrometer (4 cm⁻¹ spectral resolution). The near-infrared excitation line was sufficiently close to the first electronic transition to generate a strong pre-resonance enhancement of the chromophoric vibrational bands such that Raman bands of the protein matrix remained very weak in the spectra of the parent states (18, 43).

All spectra were measured at -140 °C using a liquid nitrogen-cooled cryostat (Linkam, Waterfield, Surrey, UK). The laser power at the sample was set at ~700 milliwatts, which did not damage the chromoproteins as checked by comparing the absorption spectra of the samples obtained before and after RR data acquisition. The total accumulation time was less than 2 h for each spectrum. For all RR spectra shown in this work, the background was subtracted.

To detect photoproducts that accumulate in red light, the samples were irradiated at room temperature and then rapidly frozen to liquid nitrogen temperatures. The raw RR spectra included substantial contributions from residual Pr, which was subtracted using the characteristic RR bands of Pr as a reference. Further RR experimental details have been described previously (18, 19, 43).

**NMR Spectroscopy**—Isotopically labeled forms of SyB-Cph1(GAF) assembled with PCB (~2 mM) were dissolved in 93% H₂O, 7% D₂O, 10 mM deuterated Tris-HCl (pH 8.5), and 0.15 mM NaN₃ and placed in a 280-µl Shigemi microcell. Prior to NMR analysis, the tube was heated to 65 °C for 10 min to inactivate contaminating thermosensitive *E. coli* proteases that slowly compromised the sample (data not shown). NMR spectra were collected at 25 °C using an 800-MHz 1H frequency Varian INOVA spectrometer (Varian Inc., Palo Alto, CA) equipped with a cryogenic probe. Samples with a high proportion of Pfr were obtained by irradiating the micropipet solution with saturating red light (620 nm); this photoequilibrium was maintained throughout NMR data acquisition by continuous irradiation of the micropipet with a low fluence rate of red light provided by a 1-watt, 620-nm light-emitting diode (LED) (LiteOn LED, Mouser Electronics, Mansfield, TX) channeled into the glass plunger by a fiber optic cable. This irradiation scheme maintained ~50% of the chromoprotein in the Pfr form, based on comparisons of NMR peak intensities throughout data acquisition. Photoconversion of Pfr back to Pr was completed by irradiating the micropipet with saturating far-red light (690 nm).

1H-15N HSQC spectra with 15N,13C-labeled SyB-Cph1(GAF) chromoprotein or unlabeled SyB-Cph1(GAF) protein assembled with [15N]PCB were collected as 128*(t₁, 15N)* × 1022*(t₂, 1H) data matrices. Acquisition times were 48 and 85 ms in the t₁ and t₂ dimensions, respectively. 1H-13C HSQC spectra that centered on the methyl region of the Pr absorbance maximum of SyB-Cph1(GAF) protein assembled with [13C]PCB were collected as 32*(t₁, 13C)* × 769*(t₂, 1H) data matrices. Acquisition times were 10 and 80 ms in the t₁ and t₂ dimensions, respectively. Data were processed and plotted using the NMRPipe software package (44). All NMR data were collected at the National Institutes of Health-sponsored NMR Facility at the University of Wisconsin, Madison.

**RESULTS**

*SyA-Cph1 and SyB-Cph1 Identify a New Subfamily of Phys*—During our continued effort to determine the breadth of microorganisms containing Phys (15) and to identify Phys with novel structures and/or photochemical properties (5–7), we discovered a potentially useful collection from a metagenomics project aimed at cataloging microbial communities within Yellowstone National Park hot springs (45, 46). The DNA sequences originated from two newly described cyanobacterial species designated *Synechococcus* sp. Octopus Spring (OS)-A and OS-B’, which based on the optimal growth temperature of the organisms would likely encode thermostable Phy-type photoreceptors. Included were possible orthologs of *Synecocystis* (abbreviated as Syn) Cph2 (14, 47) and TaxD1/PixJ (48, 49), suggesting that these cyanobacteria employ an array of bilin-containing photoreceptors for their light behaviors (50).

For our goals, the most relevant were two Phy genes, designated here as *SyA-Cph1* (TIGR locus number CYA_2779) and *SyB-Cph1* (TIGR locus number CYB_2465), that encode proteins with obvious GAF and PHY domains near their N termini (Fig. 1). Each of the GAF domains includes the following: (i) The positionally conserved cysteine (Cys-138 in SyB-Cph1) used by Cphs to covalently bind PCB (12, 51). (ii) The conserved aspartic acid (Asp-86 in SyB-Cph1) within the invariant DIP motif that helps coordinate the pyrrole water associated with the bilin (13). (iii) A set of conserved/similar residues within the GAF domain that have been shown to be important for bilin ligation (e.g. Arg-133, His-139, and His-169 in SyB-Cph1) and Pr → Pfr phototransformation (e.g. Tyr-54, Asp-86, Phe-82, Phe-95, Tyr-142, and His-169 in SyB-Cph1) in other Phys (13, 16–19, 52). Taken together, it was highly likely that these Cphs assemble with linear bilins and become red/far-red photochromic.

A novel structural feature that is most likely common to the CBD of canonical Phys is a figure-of-eight knot that helps tether the PAS and GAF domains (10, 13, 53). The ~33-amino acid loop sequence that forms the knot lasso is present within the GAF domains of both *SyA-Cph1* and *SyB-Cph1* (Fig. 1B). Likewise, amino acid sequence alignments revealed strong conservation within the PHY domains of both photoreceptors (Fig. 1B). Obvious HK domains are also present at their C termini (supplemental Fig. 1). Included within the HK sequence are recognizable H, N, D/F, and G boxes present in typical HKs, with the H box containing the positionally conserved histidine (His-608) expected to participate in phosphotransfer (8, 12, 54, 76).

The most striking feature of the *SyA-Cph1* and *SyB-Cph1* sequences is the clear absence of the N-terminal PAS domain (Fig. 1), an unusual architecture first found with *SyB*-Cph2 (14, 76).
FIGURE 1. Organization of the SyA-Cph1 and SyB-Cph1 operons and proteins from *Synechococcus* sp. OS-A and OS-B. A, diagrams of the operons and domain architectures of the encoded proteins. The *SyCp1* operon from *Synechocystis* sp. PCC6803 is included for comparison. Cph1 coding regions are shown in *black boxes*. Other coding regions within the operons and nearby separate genes are shown in *gray* and *white boxes*, respectively. Positions of the conserved RIT and DIP motifs, the GAF cysteine (C) that binds PCB, the conserved cysteine in GAF2, and the conserved histidine (H) that is the likely phosphoacceptor site in the HK domain are indicated. ORF, open reading frame. B, alignment of GAF-PHY modules in SyA-Cph1 and SyB-Cph1 with representatives from the Phy superfamily. Identical and similar amino acids are shown in black and gray boxes, respectively. Dots denote gaps. The RIT and DIP motifs are indicated by *open* and *black rectangles*, respectively, and the PCB-binding cysteine by the *arrowhead*. Asterisks identify conserved amino acids shown to be photochemically important in other Phys (16–19). The GAF lasso loop sequence that forms part of the figure-of-eight knot in DrBphP (10) is defined by the *bracket*. The GAF and PHY domains are shown by the *solid* and *dashed* lines, respectively. Sequences in the alignment include those from *SyA-Cph1* (YP_476144), *SyB-Cph1* (YP_476962), related Cphs from *N. punctiforme* PCC73102 (ZP_0011485), *Lyngbya* sp. PCC6803 (ZP_01618934), *A. variabilis* (YP_324761), *D. radiodurans DrBphP* (NP_285374), *Synechocystis* sp. PCC6803 Cph1 (NP_442237), and Cph2 (BAA10536), and *Arabidopsis thaliana* Phyk (AAC53219). The amino acid numbering is based on the SyB-Cph1 sequence.

Although the contribution(s) of the PAS domain to Phy function are not yet clear, crystal structures reveal that it is in loose contact with the GAF domain through electrostatic interactions between the knot interface and the propionate side
Thermostable Phytochromes from Synechococcus

chain of the B pyrrole ring (10, 53). Additional BLAST searches of the GenBank™ data base revealed a small collection of related Cphs also beginning with the GAF domain, including sequences from the mesophilic *Nostoc punctiforme*, *Lyngbya*, *Anabaena variabilis*, and *Acaryochloris marina* species (Fig. 1B, supplemental Fig. 2, and data not shown). Like Syn-Cph2, this new subfamily of "PAS-less" Phys contain one or two additional GAF domains downstream of the PHY domain (Fig. 1A and supplemental Fig. 2). The GAF2 sequences lack the DIP motif, the lasso loop sequence, and a number of other conserved residues present in the canonical GAF domain of Phys (see above), but they do contain a cysteine (Cys-516 in SyB-Cph1) within a Cys-His-Leu motif that could interact with bilins covalently (supplemental Figs. 1 and 2).

Although related to Syn-Cph2, this PAS-less Phy family was easily distinguished by the presence of a C-terminal HK domain and a signature 12-amino acid sequence RITX(Q/R)SLEL or RIT motif (where X is any amino acid) at the N-terminal end of the first GAF domain (residues 20–31 in SyB-Cph1 (Fig. 1B)). The function of the RIT motif is unclear. The corresponding stretch in the DrBphP CBD structure forms the α4-helix and α4-α5-helix linker domain, which includes part of the three-helix bundle that may help sister GAF domains dimerize (13).

Analysis of the surrounding genomic region revealed that the SyA-Cph1 and SyB-Cph1 coding regions are the first part of a two-locus operon in *Synechococcus* sp. OS-A and OS-B’ (Fig. 1A). The immediate 3′ open reading frames (TIGR locus numbers CYA_2781 and CYA_2484), which are 4-bp downstream or overlap with the Cph1 coding region, respectively, encode small (130 residues in OS-A and 123 residues in OS-B’), highly conserved proteins predicted to contain a domain of unknown function 309 (DUF309). This domain can be found in a number of archaeal, bacterial, fungal, algal, and higher plant species where it may bind metals via the consensus HXXEEXW(Y) sequence. Additional synteny was revealed by the presence of similar TatD homologs located 128 and 215 bp upstream from the SyA-Cph1 and SyB-Cph1 coding regions, respectively, which are predicted to be separate transcriptional units (Fig. 1A). At this time, functional connectivities between the DUF309 and TatD-related proteins and the Cphs are not obvious. *E. coli* TatD has been classified as a magnesium-dependent cytoplasmic deoxyribonuclease (55).

SyA-Cph1 and SyB-Cph1 Assemble with PCB to Generate Photochromic Phys—Given their potential thermostability and the possibility that the 200-amino acid GAF alone can complete Pr → Pfr photoconversion, we predicted that SyA-Cph1 and SyB-Cph1 might be advantageous for various physicochemical analyses of Phy-type receptors. Full-length polypeptides of both bearing a C-terminal His$_6$ tag expressed well in *E. coli* but were completely insoluble. Fortunately, truncated variants of each encompassing just the GAF domain or the GAF domain in combination with the PHY domain expressed well, were highly soluble, and could be easily purified by nickel-chelate affinity chromatography followed by hydrophobic FPLC.

To test for their ability to assemble with bilins, we incubated purified SyA-Cph1(GAF) and SyB-Cph1(GAF) apoproteins with PCB or BV *in vitro* and then assayed for covalent attachment by zinc-induced fluorescence of the products following SDS-PAGE (8). As can be seen in Fig. 2, these GAF-only constructions, like a PAS-GAF-PHY fragment from Syn-Cph1 (11, 12), readily bound PCB but not BV. In contrast, a PAS-GAF-PHY fragment from the DrBphP more effectively bound BV under identical conditions, consistent with its preference for BV (8, 56). Sequence alignments with other members of the Phy superfamily identified Cys-138 as the likely bilin attachment site for SyB-Cph1 (Fig. 1B). In support, a Cys-138 to alanine substitution in SyB-Cph1(GAF) effectively abrogated PCB ligation (Fig. 2).

To scale-up SyA-Cph1 and SyB-Cph1 chromoprotein production, we exploited the dual-plasmid *E. coli* system of Gambetta and Lagarias (38), which co-expresses the apoprotein with the HO and BVR enzymes needed to synthesize PCB from heme. Absorption spectra of the resulting *in vivo* assembled photoreceptors, either spanning the GAF-PHY region or just the GAF domain, resembled typical Phys and were photochromic following red and far-red light irradiations (Fig. 3). The Pr absorption spectra of the GAF-PHY fragments have maxima at 630 nm for the Q bands, which are blue-shifted relative to the Pr form generated by the PAS-GAF-PHY or PAS-GAF fragments.
from Syn-Cph1 with absorption maxima at 659 nm (Fig. 3) (12, 14). Saturating red light irradiations of the GAF-PHY fragment from Syn-Cph1 and SyB-Cph1 converted most Pr to Pfr with absorption maxima at 704 nm, much like Syn-Cph1 (PAS-GAF-PHY) (Fig. 3).

In contrast to truncations of other Phys that are missing the PHY domain, including Syn-Cph1 and DrBphP that are poorly photochromic (Fig. 3) (15, 19), the SyA-Cph1(GAF) and SyB-Cph1(GAF) constructions retain most of their red/far-red light photoreversibility. Saturating red light transformed a substantial portion of the PCB-bound GAF polypeptides to a species resembling Pfr. This photoconversion was more efficient for SyB-Cph1(GAF), which produced a defined Pfr absorption peak at 689 nm in saturating red light (Fig. 3).

SEC has shown that most, if not all, intact Phys are dimeric with their binding interface(s) involving one or more regions, including the GAF domain in DrBphP (13), the PAS-GAF-PHY domain in Syn-Cph1 (30, 57), and the C-terminal HK and HK-related regions in microbial and higher plants Phys (58–60). Similar SEC analysis of the GAF and GAF-PHY constructions of SyB-Cph1 assembled with PCB indicated that the PHY domain helps this chromoprotein dimerize. Whereas the GAF domain alone as Pr behaved as a monomer of 22 kDa, consistent with its calculated molecular mass of 23.3 kDa, the GAF-PHY fragment eluted as a dimeric species at 105 kDa, nearly twice the size of its calculated mass of 48.4 kDa (supplemental Fig. 3). The SEC elution profiles of both GAF and GAF-PHY constructions of SyB-Cph1 were indistinguishable following exposure to saturating red light, suggesting that their overall shapes and dimerization status are not dramatically altered upon photoconversion (supplemental Fig. 3).

**SyA-Cph1 and SyB-Cph1 Are Thermostable—Synechococcus**

SyA-Cph1 and SyB-Cph1 encompass only the GAF domain or the GAF-PHY region as Pr (solid lines) or following saturating red light (R) irradiation (mostly Pfr, dashed lines). Pr-minus-Pfr difference spectra with their maxima and minima are shown above. The absorption and difference spectra of PAS-GAF and PAS-GAF-PHY regions of Syn-Cph1 assembled with PCB are included for comparison.

**Thermostable Phytochromes from Synechococcus**

SyA-Cph1 and SyB-Cph1 are thermostable—Synechococcus sp. OS-A and OS-B’ grow well between 54 and 63 °C with an optimum growth temperature of 60 °C (45). By exposing recombinant SyA-Cph1 and SyB-Cph1 chromoproteins to a wide range of temperatures, we confirmed that the photoreceptors are likewise thermostolerant. The GAF constructions of SyA-Cph1 and SyB-Cph1 had denaturation temperatures (temperature where 50% of the protein becomes insoluble) of 76 and 83 °C, respectively, which decreased slightly to 72 and 76 °C, respectively, upon inclusion of the PHY domain (Fig. 4A). By contrast, the PAS-GAF and PAS-GAF-PHY chromoproteins from Syn-Cph1, which were derived from the mesophilic *Synechocystis* sp. PCC6803 species, denatured at 54 and 53 °C, respectively (Fig. 4A).

Thermostable enzymes typically work more efficiently at higher temperatures, with optimal performance often matching the preferred growth temperature of the host organism (61). This expectation may also hold true for the Pr/Pfr interconversion of SyA-Cph1 and SyB-Cph1. Both the GAF and GAF-PHY constructions of SyB-Cph1 were more efficient at Pr → Pfr photoconversion at 65 °C versus 23 °C using the same fluence rate of red light (initial rates increased by ~3- and 2-fold, respectively) (Fig. 4B and supplemental Fig. 4A). Because the primary photochemical event should be relatively temperature-insensitive, much of this increased rate likely reflects accelerated thermal relaxation steps from the Lumi-R intermediate to Pfr. Subsequent Pfr → Pr thermal reversion of the GAF chromoprotein was also faster at 65 versus 23 °C, with the initial rate increased by 9-fold at the higher temperature (Fig. 4C). Surprisingly, little thermal reversion occurred at 23 °C for the GAF-PHY construction of SyB-Cph1 even after 12 h of dark incubation.
Thermostable Phytochromes from Synechococcus

**A**

- SyA-Cph1(GAF)
- SyB-Cph1(GAF)
- Syn-Cph1(PAS-GAF)

**B**

Pr → Pfr Photoconversion

| Temperature (°C) | Absorbance at 704 nm (%) |
|-----------------|--------------------------|
| 23°C            | 100                      |
| 65°C            | 100                      |

**C**

Pfr → Pr Dark Reversion

| Temperature (°C) | Absorbance at 704 nm (%) |
|-----------------|--------------------------|
| 23°C            | 100                      |
| 65°C            | 100                      |

**FIGURE 4. Thermostability of the SyA-Cph1 and SyB-Cph1 chromoprotein.** Recombinant GAF and GAF-PHY fragments of SyA-Cph1 and SyB-Cph1 were assembled with PCB in vivo and purified by nickel-chelate affinity chromatography. A, solubility of the chromoproteins upon exposure to increasing temperatures. PAS-GAF and PAS-GAF-PHY fragments of Syn-Cph1 from the mesophilic Synechocystis sp. PCC6803 species are included for comparison. B and C, effect of temperature (23 versus 65 °C) on Pr → Pfr photoconversion by red light (B) and Pfr → Pr dark reversion (C) of SyB-Cph1(GAF) assembled with PCB.

A very strong red fluorescent chromoprotein was obtained when the SER-86 (D86H) variant was replaced in SyB-Cph1(GAF-PHY D86H) variant showing especially strong fluorescence output. Excitation maxima were evident at 357, 379, 591, and 620 nm with a single emission peak at 650 nm. By comparing fluorescence emission using equivalent amounts of recombinant (Fig. 5B), suggesting that even lower temperatures might effectively stabilize the Pfr form of this fragment.

**SyB-Cph1 Mutant Affecting Asp-86 Emits Intense Red Fluorescence**—Random and structurally guided mutagenesis of Phys has identified a number of conserved residues important for photochemistry. As examples, replacement of Tyr-176 in Syn-Cph1 with a histidine generates a highly red fluorescent chromoprotein that cannot photoconvert to Pfr (17, 52), whereas various substitutions of Asp-207 (e.g. D207H variant) block Pr → Pfr photoconversion in DrBphP (19). To further compare the binding pocket of these PAS-less Phys relative to more typical Phys, analogous mutants (Y54H and D86H) were introduced into the GAF and GAF-PHY constructions of SyB-Cph1.

Both sets of Y54H and D86H variants in SyB-Cph1 were soluble, retained their ability to bind PCB (Fig. 5A), and generated near normal absorption spectra for the Pr state with Q band maxima at or near 630 nm (Fig. 5B), in agreement with prior studies with other Phys showing that these residues do not significantly affect Pr assembly (17–19). However, absorption spectra recorded after red light irradiation revealed substantial photochemical defects in the variants (Fig. 5B). For the SyB-Cph1(GAF) chromoprotein, both substitutions generated partially bleached photoproducts as judged by the loss of absorbance for the Pr form at 630 nm that was not accompanied by a commensurate absorbance increase for Pfr at 689 nm. Upon inclusion of the PHY domain, a similar mild effect was evident for the Y54H substitution. However, the SyB-Cph1(GAF-PHY D86H) substitution was strongly blocked in its ability to photoconvert. The absorption spectrum of this variant was only slightly affected by saturating red light. The small loss of absorbance at 630 nm that was observed was not associated with a concomitant increase in absorbance in the far-red wavelength region for Pfr, indicating that most Pr → Pfr photoconversion was inhibited.

Similar photochromic defects were evident in comparable substitutions of Syn-Cph1 (both PAS-GAF and PAS-GAF-PHY truncations), but the effects were stronger with the tyrrosine variants (Fig. 5). In agreement with Fischer *et al.* (17, 52), the Y176H variant introduced into both Syn-Cph1 constructions strongly compromised Pr → Pfr photoconversion with saturating red light generating little or no Pfr. By contrast, the absorption spectrum of the Syn-Cph1(PAS-GAF D207H) variant after treatment with saturating red light produced a “bleached” photoproduct similar to its wild-type counterpart. The Syn-Cph1(PAS-GAF-PHY D207H) mutant also retained its ability to photoconvert in red light, but instead of generating the far-red light-absorbing Pfr-like state, it transformed to an unexpected blue-shifted species with a well defined absorption maximum at 592 nm. The mechanism responsible for this unorthodox hypsochromic shift is not known, but it could resemble the Pnr state of the novel PnB (RpbPbP3) recently described from *R. palustris* (7, 53). *RpbPbP3 does not photoconvert from Pr to Pfr in red light but instead generates this blue-shifted PnB species.

When the fluorescent properties of the set of SyB-Cph1 and Syn-Cph1 variants were analyzed, similar differential defects were observed that roughly paralleled the effects of the variants on Pr → Pfr photoconversion (Fig. 6). In accord with previous studies (17, 52), introduction of the Y176H mutation into either the PAS-GAF or PAS-GAF-PHY constructions of Syn-Cph1 generated red fluorescent chromoproteins with strong emission maxima at 662 nm. The PAS-GAF-PHY Y176H variant was approximately three times more fluorescent than the equivalent PAS-GAF construction. Neither the wild-type chromoproteins nor constructions bearing the D207H mutation were fluorescent.

The opposite trend occurred for SyB-Cph1 in addition to a much greater overall fluorescence yield (Fig. 6). Like the wild-type GAF-PHY constructions of SyB-Cph1, the corresponding Y54H substitutions were moderately fluorescent, with emission maxima at 646–654 nm. In contrast, the D86H substitutions were substantially more fluorescent with the SyB-Cph1(GAF-PHY D86H) variant showing especially strong fluorescence output. Excitation maxima were evident at 357, 379, 591, and 620 nm with a single emission peak at 650 nm. By comparing fluorescence emission using equivalent amounts of recombinant (based on equal absorbance of the Pr Q band), the SyB-Cph1(GAF-PHY D86H) chromoprotein was ~3.5 times more fluorescent than the comparable chromoprotein without the PHY domain and was five times more fluorescent when compared with the Syn-Cph1(PAS-GAF-PHY Y176H) chromoprotein.

**Resonance Raman (RR) Spectroscopy of SyB-Cph1**—The sequence homology within the GAF domains of SyA-Cph1 and
SyB-Cph1 relative to more typical Phys strongly suggested that these PAS-less Phys adopt a similar bilin geometry and employ a similar reaction mechanism to generate Pfr from Pr, including the use of a deprotonation/reprotonation cycle during the relaxation steps from Lumi-R to Pfr (2, 18, 19). To help confirm this expectation, we employed RR spectroscopy to assess bilin conformation and protonation state. In particular, RR bands in the region between 1500 and 1700 cm\(^{-1}\) can (i) help predict the methine bridge configurations and conformations (i.e. Z/E and syn/anti geometries), (ii) reveal the protonation state of the pyrrole nitrogens in the Pr and Pfr states, and (iii) detect the accumulation of transformation intermediates if complete photoconversion stalls (e.g. deprotonated Meta-R intermediate, 18, 24, 43, 62, 63). Moreover, by comparing the RR spectra of the GAF and GAF-PHY constructions, we could define the importance of the PHY domain during Pr \(\rightarrow\) Pfr photoconversion for these thermostable PAS-less Phys.

The RR spectra of the Pr state of SyB-Cph1(GAF) and SyB-Cph1(GAF-PHY) displayed very similar vibrational band patterns (supplemental Fig. 5). These data agreed with previous studies demonstrating that the PHY domain has little impact on Pr absorption (15, 19, 26) and bilin geometry (10, 19). In particular, RR bands attributed to the methine bridge geometry of PCB (1600–1650 cm\(^{-1}\)) (63) and subtle details of the bilin structure (600–900 cm\(^{-1}\)) were identical or very similar for SyB-Cph1(GAF) and SyB-Cph1(GAF-PHY) (Fig. 7 and supplemental Fig. 5). The N-H in-plane bending mode of pyrrole rings B and C in Pr, assigned based on its disappearance in D\(_2\)O (43), was at similar positions for both chromoproteins (1573 versus 1575 cm\(^{-1}\) (Fig. 7)). These data imply that PCB retained its protonation (cationic) state and that its hydrogen bond interactions with the protein environment were for the most part unaffected by removal of the PHY domain. Only subtle differences were evident in the region between 1400 and 1500 cm\(^{-1}\), which could reflect slightly greater contributions from protein Raman bands in the SyB-Cph1(GAF-PHY) (supplemental Fig. 5).

The RR spectra of the Pr state of SyB-Cph1(GAF) and SyB-Cph1(GAF-PHY) differed from that obtained with Syn-Cph1(PAS-GAF-PHY), suggesting that subtle differences in bilin geometry exist between the two Cph classes. The marker band region for protonated bilins (1600–1650 cm\(^{-1}\)) is usually

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**FIGURE 5.** Effects of positionally conserved tyrosine and aspartic acid residues on assembly of SyB-Cph1 and Syn-Cph1 with PCB and the absorption spectra of the resulting chromoproteins. GAF and GAF-PHY polypeptides from SyB-Cph1 and PAS-GAF and PAS-GAF-PHY polypeptides from Syn-Cph1 bearing histidine substitutions for the tyrosine (residue 54 and 176 in SyB-Cph1 and Syn-Cph1, respectively) or aspartic acid (residue 86 and 207 in SyB-Cph1 and Syn-Cph1, respectively) were co-expressed with PCB and purified by nickel-chelate affinity chromatography. Samples were subjected to SDS-PAGE and either assayed for the bound bilin by zinc-induced fluorescence (WT) or stained for protein with Coomassie Blue (Prot), B, UV-visible absorption spectra of the mutants shown in A as Pr (solid lines) or following saturating red light (R) irradiation (mostly Pfr, dashed lines). Pr-minus-Pfr difference spectra with their difference maxima and minima are shown above. WT, wild type.

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**TABLE 1.**

| Protein        | Absorption Maxima (nm) |
|----------------|------------------------|
| WT SyB-Cph1(GAF) | 630, 659               |
| Y54H SyB-Cph1(GAF) | 630, 659               |
| D86H SyB-Cph1(GAF) | 630, 659               |
| Y176H SyB-Cph1(GAF) | 630, 659               |
| D207H SyB-Cph1(GAF) | 630, 659               |
| WT Syn-Cph1(PAS-GAF) | 630, 659               |
| Y54H Syn-Cph1(PAS-GAF) | 630, 659               |
| D86H Syn-Cph1(PAS-GAF) | 630, 659               |
| Y176H Syn-Cph1(PAS-GAF) | 630, 659               |
| D207H Syn-Cph1(PAS-GAF) | 630, 659               |
| WT Syn-Cph1(PAS-GAF-PHY) | 630, 659               |
| Y54H Syn-Cph1(PAS-GAF-PHY) | 630, 659               |
| D86H Syn-Cph1(PAS-GAF-PHY) | 630, 659               |
| Y176H Syn-Cph1(PAS-GAF-PHY) | 630, 659               |
| D207H Syn-Cph1(PAS-GAF-PHY) | 630, 659               |

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**FIGURE 6.** Absorbance spectra of WT SyB-Cph1(GAF) and its Y54H, D86H, Y176H, and D207H mutants. A, UV-visible absorption spectra of the Pr state of WT SyB-Cph1(GAF) and its Y54H, D86H, Y176H, and D207H mutants. B, UV-visible absorption spectra of the Pr state of WT Syn-Cph1(PAS-GAF-PHY) and its Y54H, D86H, Y176H, and D207H mutants.
dominated by two species originating from the C=C stretching modes of the ring-A-B and -C-D methine bridges (63); these can be seen as a prominent band (1630 cm\(^{-1}\)) and a shoulder (1649 cm\(^{-1}\)) in the RR spectrum of Syn-Cph1(PAS-GAF-PHY) (Fig. 7). However, for SyB-Cph1(GAF) and SyB-Cph1(GAF-PHY), the 1630 cm\(^{-1}\) band seemed to be upshifted to nearly coincide with the higher frequency mode, thus creating a single symmetric band at 1645 cm\(^{-1}\) (Fig. 7). This shift most likely reflects a conformational difference at the C-D bridge as compared with Syn-Cph1(PAS-GAF-PHY). This structural difference may also affect hydrogen bonding of the ring-C N-H group as indicated by the 7 cm\(^{-1}\) higher in-plane bending frequency of the N-H group for SyB-Cph1(GAF-PHY) (1575 cm\(^{-1}\)) compared with Syn-Cph1(PAS-GAF-PHY) (1568 cm\(^{-1}\)). In contrast, RR spectra provide no indication for structural differences at the A-B and B-C methine bridges because the corresponding stretching bands at 1649 cm\(^{-1}\) (A-B) and 1609 cm\(^{-1}\) (B-C) were largely unchanged in SyB-Cph1(GAF-PHY) versus Syn-Cph1(PAS-GAF-PHY) (Fig. 7).

a 0.6 absorbance at the Pr absorption maximum. Top panels, purified chromoprotein solutions upon irradiation with UV light (UV) or white light (WL). Bottom spectra, fluorescence excitation (dashed lines) and emission spectra (solid lines) of the chromoprotein samples shown at top. Excitation and emission maxima are indicated.
Differences in absorption spectra of SyB-Cph1(GAF) and SyB-Cph1(GAF-PHY) following red light suggested that the GAF domain by itself is not fully competent in forming Pfr (Fig. 3). However, in contrast to other prokaryotic Phys that lack the PHY domain (19, 43, 62), RR spectra revealed the SyB-Cph1(GAF) fragment does not arrest in a Meta R-like intermediate upon red light irradiation. Instead, several signature RR bands of the SyB-Cph1(GAF) photoproduct were consistent with the accumulation of Pfr. Included were a drastic downshift of the C-D methine bridge stretching from 1645 cm\(^{-1}\) in Pr to 1614 cm\(^{-1}\) and ring-B and -C N-H in-plane bending mode at 1554 cm\(^{-1}\), which is characteristic of the protonated Pfr state (43, 62) (Fig. 7). Only the shoulder at \(\sim 1595\) cm\(^{-1}\) in the RR spectrum of the Pfr-like state of SyB-Cph1(GAF) is reminiscent of a Meta-R-like species (24) (Fig. 7). However, this band was not observed in D\(_2\)O leaving its assignment ambiguous.

The most significant difference in the RR spectra of the photoconversion products between the GAF and GAF-PHY constructions of SyB-Cph1 was a large intensity reduction in the band at \(\sim 813\) cm\(^{-1}\) (supplemental Fig. 5). This band originates from the C-H out-of-plane mode of the C-D methine bridge (43, 64); its high RR intensity, which is a typical feature of the Pfr chromophore, may reflect torsion of the C-D methine bridge. Furthermore, the A-B stretching could not be identified as a distinct peak or shoulder in SyB-Cph1(GAF) as it may overlap with the dominant 1613-cm\(^{-1}\) band. Instead, the RR spectra of the Pfr states for SyB-Cph1(GAF-PHY) and Syn-Cph1(PAS-GAF-PHY) display two bands at \(\sim 1620\) and 1640 cm\(^{-1}\), which both are possible candidates for the A-B stretching, thus pointing to a conformational heterogeneity at the A-B methine bridge. These differences suggest that the PHY domain subtly affects the chromophore-protein architecture of SyB-Cph1 in the Pfr state with respect to the structural details of the A-B and C-D methine bridges.

**One-dimensional and Two-dimensional NMR Analysis of PCB Bound to SyB-Cph1(GAF)**—The fact that the 200-amino acid GAF domain of SyB-Cph1 retains its bilin lyase activity and most of its red/far-red photochromicity and behaves as a monomer indicated that this fragment could help visualize by NMR spectroscopy global movements of a Phy chromophore and its binding pocket during phototransformation. Toward this goal, we synthesized and assembled SyB-Cph1(GAF) holoproteins in which either PCB or the polypeptide were labeled individually with \(^{15}\)N and/or \(^{13}\)C. Incorporation of isotopically labeled PCB into unlabeled protein was accomplished by replacing the bilin precursor ALA, added in excess to the growth medium, with precursor ALA, added in excess to the growth medium, with \(^{13}\)C into the polypeptide was achieved by replacing NH\(_4\)Cl and either \(^{15}\)N- or \(^{13}\)C-labeled derivatives. Incorporation of \(^{15}\)N\(^{-}\) and \(^{13}\)C into the polypeptide was achieved by replacing NH\(_4\)Cl and glycerol in the medium with \(^{15}\)N\(^{-}\) and \(^{13}\)C-labeled counterparts, respectively, together with an excess of unlabeled ALA. For a visual aid, Fig. 8A shows the predicted position of the \(^{13}\)C-labeled carbons incorporated into PCB upon addition of 1,2-\(^{13}\)CALA to the medium. Prior one-dimensional \(^{15}\)N NMR analysis of Syn-Cph1(PAS-GAF-PHY) assembled with \(^{13}\)C\(^{-}\) \(^{15}\)N PCB \textit{in vitro} detected all four pyrrole ring nitrogens in the Pr conformer, with three new distinct peaks appearing after red light irradiation (32, 33). These spectra implied that at least three of the chromophore

**FIGURE 8.** One-dimensional \(^{15}\)N spectra and two-dimensional \(^1\)H-\(^{15}\)N and \(^1\)H-\(^{13}\)C HSQC NMR spectra of SyB-Cph1(GAF) assembled with isotopically labeled \(^{15}\)N\(^{-}\)PCB or \(^{13}\)C PCB. A, location of the PCB carbons (circled in red) labeled with \(^{15}\)C using the heme precursor 1,2-\(^{13}\)CALA. PCB is diagrammed in the predicted ZZZ-syn,syn,anti conformation for Pr (34). The blue arrow shows the predicted \(Z\) to \(E\) rotation around the C-15–C-16 double bond during phototransformation from Pr to Pfr. B, one-dimensional \(^{15}\)N spectra of \(^{15}\)N\(^{-}\)PCB SyB-Cph1(GAF) as Pr (blue) or following saturating red light (mostly Pr, red), and a two-dimensional \(^1\)H-\(^{15}\)N HSQC spectrum of the same sample in Pr, C, \(^1\)H-\(^{13}\)C and \(^1\)H-\(^{15}\)N HSQC spectra of SyB-Cph1(GAF) containing isotopically labeled PCB either as Pr (blue) or following saturating red light (mostly Pfr, red). PCB was either uniformly labeled with \(^{13}\)N (inset) or assembled with \(^{13}\)C PCB isotopically labeled at the positions shown in A. Arrows identify new peaks that appeared during red light irradiation.
nitrogens significantly change their geometry/chemical environment during Pr → Pfr photoconversion. A similar one-dimensional NMR spectrum was obtained here for the Pr conformer of SyB-Cph1(GAF) assembled with uniformly labeled [15N]PCB (Fig. 8B). Four distinct peaks were evident in the 15N spectrum representing each of the four pyrrole nitrogens. The positions of three of the four one-dimensional 15N NMR peaks (131, 155, and 160 ppm) from SyB-Cph1(GAF) were unaffected by red light irradiation, implying that the environments of these pyrrole nitrogens do not detectably change upon photoconversion to Pfr (Fig. 8B). A decrease in signal was observed for the fourth 15N peak at 144 ppm coincident with the appearance of a new 15N peak at 142 ppm, suggesting that this pyrrole nitrogen experiences a new environment upon Pr → Pfr photoconversion. Surprisingly, two-dimensional 1H-15N HSQC of [15N]PCB-labeled SyB-Cph1(GAF) as Pr revealed only a single H-N correlation peak at 131 ppm (Fig. 8B), which did not change position or intensity upon red light exposure (Fig. 8C, inset). This failure to detect additional H-N correlation peaks implied that the protons associated with three of the four pyrrole nitrogens readily exchange with the solvent.

To examine movements of the PCB carbons in SyB-Cph1(GAF), we used 1,2-[13C]ALA as the PCB precursor to label all six methyl groups and C82 and C122 methylene carbons present in the B- and C-ring propionate side chains, respectively (67) (Fig. 8A). Two-dimensional NMR 1H-13C HSQC spectra of the sample as Pr detected peaks for the expected six methyl groups (Fig. 8C). However, we did not observe peaks for the two propionate methylene carbons, suggesting that they are highly mobile in the Pr conformation. The positions of the 1H-13C peaks for the methyl groups as Pr were similar to those described by Strauss et al. (32) for Syn-Cph1(PAS-GAF-PHY). Taken together with similar RR spectra (supplemental Fig. 5), it appears that the Pr conformer of SyB-Cph1 has a bilin geometry similar to that for Syn-Cph1 even without the PAS domain. Upon saturating red light irradiation, several new peaks appeared in the 1H-13C HSQC spectra of [13C]PCB SyB-Cph1(GAF) that can be readily seen by overlaying this spectrum with that for Pr (Fig. 8C). These new peaks likely reflect movement of several PCB methyl groups to new chemical environments during Pr → Pfr photoconversion.

Two-dimensional NMR Analysis of 15N-13C-Labeled SyB-Cph1(GAF) Reveals Global Movement in the GAF Domain during Photoconversion—To study global movements of the GAF domain polypeptide during Pr → Pfr photoconversion, we synthesized 15N and 13C double-labeled SyB-Cph1(GAF) chromoprotein. Presumably because of its stability and small size (208 amino acids), this sample generated highly resolved two-dimensional 1H-15N HSQC spectra as Pr (Fig. 9A). The number of detected 1H-15N peaks was reasonably close to the expected number generated from the predicted 208-amino acid His6-tagged SyB-Cph1(GAF) chromoprotein with the addition of some observable side-chain resonances.

When the 15N-13C-labeled SyB-Cph1(GAF) sample was photoconverted to Pfr by saturating red light and then maintained in this photoequilibrium during data collection by continuous red light irradiation, a remarkably distinct 1H-15N HSQC spectrum was captured. When overlaid with the original Pr plot, the two-dimensional NMR Pr/Pfr spectrum not only contained the same 1H-15N peaks observed with Pr (Fig. 9A, peaks colored in blue), but also contained numerous new 1H-15N peaks that reflected the movement of various amide groups specific to Pfr (Fig. 9A, peaks colored in red). Close inspection identified chemical shift changes (reflecting chemical and magnetic environmental changes) for ~50% of the residues within the GAF domain.
upon photoconversion. Based on preliminary residue assignments, many of these changes are small to moderate, but a few large (2–3 ppm \(^{13}\text{C}\) or \(^{1}\text{H}\)) backbone chemical shift changes could be observed (data not shown). Moreover, when red light-irradiated samples were converted back to Pr, either by extended darkness or by subsequent irradiation with saturating far-red light, all Pfr-associated peaks returned back to their original Pr positions (Fig. 9B and data not shown). This photo-reversibility demonstrated that the new peaks observed after red light irradiation were not generated by denaturation or photo-bleaching of the sample, but likely reflected the signature photochromicity of Phys. This measurement thus represents the first assessment of the global movements of a Phy CBD during Pr → Pfr photoconversion in solution and indicates that the conformation of the GAF domain in the Pr and Pfr conformers could differ substantially.

**DISCUSSION**

Novel Features of SyA-Cph1 and SyB-Cph1 Enable Structural Analysis of Phys by NMR—The structural resolution of both Pr and Pfr will be essential to fully understand how Phys function at the atomic level. Although the Pr structures of two CBDs have recently been determined using x-ray crystallography (10, 13, 53), those for Pfr have not yet been solved for a variety of technical reasons. Crystallization of the Pfr state has been challenged by substantial contamination of Pr even after saturating red light and the instability of Pfr once formed, which for most Phys will revert thermally back to Pr (Fig. 4C) (2). Generating Pfr by irradiating diffraction quality Pr crystals with red light has also failed thus far. The crystals have either failed to photoconvert or dissolved (53, 68, 69), implying that the crystal lattice containing Pr cannot accommodate the structural rearrangements that occur during Pr → Pfr photoconversion. Exploiting naturally occurring Phys that prefer Pfr as the most stable state is an alternative for crystallizations, but it remains unclear if these bathy-Phys structurally resemble the canonical Phys in which Pr is the most stable state (5, 6).

Solving the solution structure of Pfr using NMR is an attractive alternative, given the ability of this approach to resolve chemical shifts generated solely from Pfr by “subtracting” the NMR spectrum generated with Pr from that obtained after saturating red light irradiation. NMR approaches work best if the size of the protein complex in solution is below 35 kDa (35, 36). Unfortunately, the Phy CBDs studied to date exceed this size range, needing at least the 35-kDa PAS-GAF fragment to generate the Meta-R state from Pr and at least the ∼55-kDa PAS-GAF-PHY fragment for full photoconversion (15, 18, 19, 70). These sizes are further increased by dimerization (19, 57), thus putting most previously described Phys outside the acceptable size range required for conventional NMR spectroscopy techniques.

Here we described two thermotable Cphs, SyA-Cph1 and SyB-Cph1, that belong to a new sub-class of Phys with unique features that are particularly amenable to NMR studies. They were discovered by a metagenomic sequencing project aimed at classifying thermotolerant species (45, 46). Like canonical Phys, SyA-Cph1 and SyB-Cph1 contain an obvious GAF domain that binds the bilin, followed by a PHY domain often essential for complete Pr → Pfr photoconversion, and end with an HK domain similar to those found in many two-component signaling receptors. The GAF domains of both contain all of the strongly conserved amino acids shown to be important for bilin binding and photochemistry (13, 15–19). SyA-Cph1 and SyB-Cph1 both have a positionally conserved cysteine, which like others within the Cph family (11, 12) appears to serve as the bilin linkage site. As expected this pair binds PCB effectively to generate red/far-red light photochromic photoreceptors. Unfortunately, given the insolubility of the full-length recombinant polypeptides, we do not yet know how Pr → Pfr photoconversion affects the phosphotransferase activity conferred by the distal HK domain.

Conservation of the GAF domain is further supported by the analysis of site-directed mutants affecting Tyr-54 and Asp-86 in SyB-Cph1, which have been shown to hold key functional roles in other Phys (16–19, 52). Like similar mutations in SynCph1 and DrBphP, replacement of these residues does not affect Pr assembly but can substantially affect Pfr formation. The D86H chromoproteins in particular are substantially blocked in Pr → Pfr photoconversion and are highly red fluorescent. The effect of the Y54H and D86H mutations contrasts those for Syn-Cph1 in which the comparable Y176H is strongly fluorescent, whereas the D207H mutant is poorly fluorescent (Fig. 6) (17, 52).

One unique feature of SyA-Cph1 and SyB-Cph1 is that they belong to a previously unknown subfamily of Cphs present in at least several cyanobacteria (including mesophiles) that lack the N-terminal PAS domain. This subfamily is distinct from Syn-Cph2 and its relatives by having a strongly conserved RIT motif at the beginning of the GAF domain and ending in an HK domain. Although the function(s) of the PAS domain in Phy signaling are not yet clear, its presence in most Phys studied to date across a wide range of species, including bacteria, cyanobacteria, fungi, and plants, implied that it serves an essential function (15).

Three-dimensional structures of the CBD (10, 13, 53) revealed that the PAS domain is tethered to the adjacent GAF domain by a figure-of-eight knot, created by threading the residues N-terminal to the PAS domain through a lasso loop between B9 and a7 of the GAF domain (based on the DrBphP CBD structure (10)). The resulting anti-parallel three-helix bundle also contacts the B-ring propionate acid side chain of the bilin, thus connecting the PAS domain indirectly to the chromophore. Strikingly, even though the PAS domain is absent in the PAS-less RIT and Syn-Cph2-type subfamilies, their GAF domains have retained the extra sequence that forms the lasso loop and several conserved residues within that are central to the knot core (e.g. Ile-113, Leu-127, and Arg-133 in SyB-Cph1 (10, 19)). Why the loop sequences have been retained is unclear. Without the upstream sequences, these loops should be more flexible and extend into the solvent. One possibility is that the GAF domains of PAS-less Cphs still use this loop sequence to interact with PAS-like domains synthesized separately.

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\(^4\) J. R. Wagner, K. T. Forest, and R. D. Vierstra, unpublished data.
Thermostable Phytochromes from Synechococcus

Regardless of its function, it is clear from ours and others studies (14, 47) that the PAS domain is not essential for photochromicity. Like Syn-Cph2 (14, 47), SyA-Cph1 and SyB-Cph1 can bind PCB covalently in vitro and become red/far-red light photochromic with the only substantive difference being a blue shift of the Pr absorption maxima. Similarly, SyA-Cph1 and SyB-Cph1 also contain a second GAF domain downstream of the PHY domain. For Syn-Cph2, this GAF2 sequence can bind PCB when expressed alone, but the resulting chromoprotein is not red/far-red photochromic (14, 47). By inference, it is possible that SyA-Cph1 and SyB-Cph1 can incorporate two bilin groups simultaneously.

In contrast to several other Phys (15, 19, 62), SyB-Cph1 (and likely SyA-Cph1) also appears less dependent on the PHY domain for photoconversion to Pfr. Absorption, RR, and NMR spectroscopy indicate that most of the SyB-Cph1 (GAF) truncation photoconverts to the protonated Pfr-type state upon red light irradiation with only slight modifications of its spectral properties (e.g. 15-nm blue shift of Pfr absorption maximum). Combined with its monomeric size, the SyB-Cph1 (GAF) fragment is well within the acceptable size range for NMR analysis of Pfr.

Another important feature of these Phys is their remarkable heat stability, being capable of withstanding temperatures above 70 °C. In fact, NMR data collection was facilitated by our ability to protect the recombinant photoreceptors against contaminating E. coli proteases simply by heating the preparations to 65 °C before use. These “sterilized” preparations were surprisingly stable both in terms of solubility and photochemistry, and easily survived long term three-dimensional data collection. This thermostability was also reflected by its Pr → Pfr photoconversion and Pfr → Pr dark reversion kinetics, which are both faster at high temperatures. Taken together, we expect that these Phys rapidly interconvert between Pr and Pfr within the host Synechococcus OS-A and OS-B’ organisms when they are grown in their natural daylight environment.

Finally, we show that SyB-Cph1 (GAF) can be easily labeled isotopically for NMR analyses. By either introducing 15N or 13C isotopes into amino acids or feeding the 15N- or 13C-labeled heme precursor ALA to SyB-Cph1 (GAF)-expressing cells, we could independently introduce isotopes into the polypeptide and PCB moieties, respectively. The resulting preparations generated two-dimensional NMR spectra with sufficient clarity to discern peaks associated with Pfr from those associated with Pr.

NMR Analysis of the SyB-Cph1 Chromophore PCB—Initial NMR analyses confirmed the utility of SyB-Cph1 (GAF) preparations for structural studies. One-dimensional NMR spectra of [15N]PCB-labeled preparations in the Pr state detected all four of the pyrrole ring nitrogens (Fig. 8B). Surprisingly, only one of these nitrogens (144 ppm) apparently changed its chemical environment after red light irradiation. Collectively, the spectra confirmed RR data that the GAF fragment of SyB-Cph1 completes the protonation cycle of the chromophore and implies that the environment/position of three of the four pyrrole N-H groups are similar in the Pr and Pfr states. 1H-15N HSQC spectra both before and after red light irradiation detected only a single 1H-15N chemical shift at 131 ppm, indicating that one of the four bound protons is tightly held and exchanges slowly with the solvent while the remaining three protons readily mix with the surrounding water.

Where are the exchangeable and retained N-H pyrrole-associated protons located in SyB-Cph1, and which one of these protons changes its environment upon photoconversion? Prior RR, infrared, and NMR spectroscopic studies with a variety of Phys, including Synechocystis Cph1, have suggested that the B and C pyrrole rings move little during photoconversion with most of the movement involving rings A and D (23, 32–34, 62, 71–73). Contrary to the expected large movements of the D-ring following the Z to E isomerization of the C15–C16 methine linker double bond, it has been proposed further that the A-ring undergoes the more pronounced conformational changes, potentially via a Z-syn to Z-anti rotation around the C5–C6 methine bridge between rings A and B.

Our 15N and 13C NMR data with SyB-Cph1 (GAF) are consistent with these interpretations. The high resolution crystal structure of DrBphP assembled with BV showed that the pyrrole nitrogens present in the A–C-rings are held in a ZZ-syn,syn configuration as Pr and hydrogen bond with the centrally positioned pyrrole water (see Fig. 8A), whereas the D-ring N-H group is contorted 44° out-of-plane in Z-anti configuration and held in place by a second hydrogen bond network involving His-290 and water 2 (13). Assuming this configuration holds true for SyB-Cph1 assembled with PCB, we predict that the exchangeable protons (detected at 160, 156, and 144 ppm) are all bound to the A–C-rings. This assignment is consistent with the RR data, which unambiguously show that the pyrrole rings B and C are protonated with the protons rapidly exchanging with the solvent (13). The free exchange of the ring-A–C protons with the solvent is also plausible in view of the deprotonation/protonation cycle of the chromophore during Pr → Pfr photoconversion (18, 19).

Only one of the exchangeable protons in SyB-Cph1 (GAF) changed environment/location upon photoconversion to Pfr. It appeared in our one-dimensional 15N NMR spectra as a peak at 144 ppm, which diminished in height during red-light irradiation concomitant with the appearance of a new peak at 142 ppm. If we assume that the B- and C-rings are in similar chemical environments and are more rigidly held via their proprio- side chains (10, 19), then the moving pyrrole ring is best assigned to the A-ring. In support, Rohmer et al. (33) tentatively assigned a similar 15N chemical shift, which moved from 146.8 to 142.8 ppm during photoconversion, to the A-ring nitrogen of Synechocystis Cph1 (PAS-GAF-PHY) fragment.

The remaining slowly exchangeable proton nitrogen in SyB-Cph1 (GAF), which had a cross-peak at 131 ppm (15N chemical shift), would then be assigned by default to the D-ring pyrrole N-H group (in agreement with Ref. 33). The absence of additional H-N cross-peaks in our 1H-15N HSQC Pr/Pfr spectra overlay further implies that the amide of this ring does not change upon photoconversion (see Fig. 8C, inset). Why we failed to detect the expected movement of the D-ring by NMR spectroscopy is unclear. It could reflect a role for the PHY domain in stabilizing D-ring in the Pfr conformer and/or the possibility that the Z to E isomerization does not dramatically affect the environment of the D-ring N-H group in SyB-Cph1.
Examination of SyB-Cph1(GAF) bearing [13C]PCB by 1H-13C HSQC detected all six predicted methyl groups, and revealed that four of the six experience a different environment after red light irradiation (Fig. 8C). Such movements closely parallel those obtained by Strauss et al. (32) with Syn-Cph1(PAS-GAF-PHY), who showed that all but one of the six methyl groups acquire alternate peaks after red light saturation. The peak of one methyl in particular (15.5 ppm) for SyB-Cph1(GAF) moved a considerable distance during photoconversion, implying that it encounters a radically different chemical environment as Pfr. An interesting possibility is that this moving methyl group (like the moving nitrogen) is also associated with the A-ring.

SyB-Cph1(GAF) Is Amenable to 1H-15N-13C Three-dimensional NMR—Although several studies have demonstrated that various regions of the Phy polypeptide move during Pr → Pfr phototransformation (25–31), their exact motions remain to be determined. The small size and thermostability of the SyB-Cph1(GAF) truncation coupled with its ability to effectively complete the Pr → Pfr photocycle strongly suggests that this species may help resolve movements in the GAF domain by NMR approaches. Toward this objective we have found that SyB-Cph1(GAF) generates excellent three-dimensional NMR spectra using preparations incorporating 15N and 13C into the chromophore or polypeptide. Overlays of either 1H-15N or 1H-13C HMQC spectra from 15N,13C-labeled SyB-Cph1(GAF) samples obtained before and during continuous saturating red light irradiations identified numerous new reversible peaks (Fig. 9 and data not shown). These new peaks presumably reflect changing chemical environments of specific amino acid-associated amides during Pr → Pfr photoconversion. Moreover, many of these peaks remained well defined as Pfr, indicating that the corresponding amides now occupy new and stable environments in this state. Clearly, the large number of unique Pfr peaks implies that the GAF domain by itself undergoes a more robust conformational change during phototransformation than we anticipated. Based on these preliminary data, solving the solution structures of both the Pr and Pfr states of the SyB-Cph1(GAF) chromoprotein may be within reach.

Potential Use of SyB-Cph1(GAF) Chromoproteins as Fluorophores—Our discovery of a set of SyB-Cph1 mutants that emit red fluorescence may have utility in various cell biological and molecular assays as small portable fluorescent tags. In particular, the SyB-Cph1(GAF-PHY D86H) mutant emits strong fluorescence and when directly compared was significantly brighter than the previously reported Y176H mutant generated with the PAS-GAF-PHY fragments of Synchocystis Cph1 (Fig. 6) (17, 52). The D86H mutant retains its red fluorescence in a monomeric truncation that encompasses only the 200-amino acid GAF domain, thus making this fluorophore even smaller than the commonly used green fluorescent protein (GFP) (70). Potential advantages over other fluorophores such as GFP include the following: (i) its remarkable thermostability, (ii) the large separation of the electronically excited state (Soret transition at 380 nm) from the emitting state (650 nm), which would minimize light contamination, and (iii) the ability to modify fluorescence in both time and space by controlling PCB availability (74). The D86H mutant may also be excited in the first electronic transition with orange light to produce an identical emission peak (data not shown), thus circumventing potential damages/effects induced by UV or blue light excitation. Expression vectors that exploit SyB-Cph1(GAF D86H) as a fluorescent reporter either by itself or when fused to other proteins are currently under development.

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REFERENCES

1. Quail, P. H. (2002) Nat. Rev. Mol. Cell Biol. 3, 85–93
2. Rockwell, N. C., Su, Y. S., and Lagarias, J. C. (2006) Annu. Rev. Plant. Biol. 57, 837–858
3. Vierstra, R. D., and Karnieli, B. (2005) in Handbook of Photosensory Receptors (Briggs, W. R., and Spudich, J. L., eds) pp. 171–196, Wiley-VCH Press, Weinheim, Germany
4. Smith, H. (1995) Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 269–315
5. Karnieli, B., and Vierstra, R. D. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2807–2812
6. Giraud, E., Fardoux, J., Fourrier, N., Hannibal, L., Genty, B., Bouver, P., Dreyfus, F., and Vermeglio, A. (2002) Nature 417, 202–205
7. Giraud, E., Zappa, S., Vuillet, L., Adriano, J. M., Hannibal, L., Fardoux, J., Berthomieu, C., Bouver, P., Pignol, D., and Vermeglio, A. (2005) J. Biol. Chem. 280, 32389–32397
8. Bhoo, S. H., Davis, S. J., Walker, J., Karnieli, B., and Vierstra, R. D. (2001) Nature 414, 776–779
9. Lamparter, T., Michael, N., Caspani, O., Miyata, T., Shirai, K., and Inomata, K. (2003) J. Biol. Chem. 278, 33786–33792
10. Wagner, J. R., Brunzelle, J. S., Forest, K. T., and Vierstra, R. D. (2005) Nature 438, 325–331
11. Lamparter, T., Mittmann, F., Gartner, W., Birner, T., Hartmann, E., and Hughes, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11792–11797
12. Yeh, K. C., Wu, S. H., Murphy, J. T., and Lagarias, J. C. (1997) Science 277, 1505–1508
13. Wagner, J. R., Zhang, J., Brunzelle, J. S., Vierstra, R. D., and Forest, K. T. (2007) J. Biol. Chem. 282, 12298–12309
14. Wu, S. H., and Lagarias, J. C. (2000) Biochemistry 39, 13487–13495
15. Karnieli, B., Wagner, J. R., Walker, J. M., and Vierstra, R. D. (2005) Biochim. J. 392, 103–116
16. Hahn, J., Strauss, H. M., Landgraf, F. T., Gimenez, H. F., Lochnit, G., Schneider, P., and Hughes, J. (2006) FEBS J. 273, 1415–1429
17. Fischer, A. J., and Lagarias, J. C. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 17334–17339
18. von Stetten, D., Seibeck, S., Michael, N., Scheer, P., Morginski, M. A., Murgida, D. H., Krauss, N., Heyn, M. P., Hildebrandt, P., Borucki, B., and Lamparter, T. (2007) J. Biol. Chem. 282, 2116–2123
19. Wagner, J. R., Zhang, Z., von Stetten, D., Gunter, M., Murgida, D. H., Morginski, M. A., Walker, J. M., Forest, K. T., Hildebrandt, P., and Vierstra, R. D. (2008) J. Biol. Chem. 283, 12212–12226
20. Rüdiger, W., Thümmler, F., Cmel, E., and Schneider, S. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 6244–6248
Thermostable Phytochromes from Synechococcus

21. Mizutani, Y., Tokutom, S., and Kitagawa, T. (1994) Biochemistry 33, 153–158
22. Andel, F., III, Lagarias, J. C., and Mathies, R. A. (1996) Biochemistry 35, 15997–16008
23. Foersterdorff, H., Benda, C., Gärnter, W., Storf, M., Scheer, H., and Siebert, F. (2001) Biochemistry 40, 14952–14959
24. Borucki, B., von Stetten, D., Seibeck, S., Lamparter, T., Michael, N., Mroginski, M. A., Otto, H., Murgida, D. H., Heyn, M. P., and Hildebrandt, P. (2005) J. Biol. Chem. 280, 34358–34364
25. Lagarias, J. C., and Mercurio, F. M. (1985) J. Biol. Chem. 260, 2415–2423
26. Noack, S., Michael, N., Rosen, R., and Lamparter, T. (2007) Biochemistry 46, 4164–4176
27. Grimm, R., Ecker, R., Lottspeich, F., Zenger, C., and Rüdiger, W. (1998) Planta 174, 396–401
28. Quail, P. H. (1997) Plant Cell Environ. 20, 657–665
29. Lapko, V. N., Jiang, X. Y., Smith, D. L., and Song, P. S. (1998) Biochemistry 37, 12526–12535
30. Estevez, B., Carrascal, M., Abian, J., and Lamparter, T. (2005) Biochemistry 44, 450–461
31. Natori, C., Kim, J. I., Bhoo, S. H., Han, Y. J., Hanzawa, H., Furuya, M., and Song, P. S. (2007) Photochem. Photobiol. Sci. 6, 83–89
32. Strauss, H. M., Hughes, J., and Schmieder, P. (2005) Biochemistry 44, 8244–8250
33. Rohmer, T., Strauss, H., Hughes, J., de Groot, H., Gärnter, W., Schmieder, P., and Matsyk, J. (2006) J. Phys. Chem. B. 110, 20580–20585
34. Hahn, J., Kuhnhe, R., and Schmieder, P. (2007) ChemBioChem 8, 2249–2255
35. Yu, H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 332–334
36. Rick, R., Wider, G., Pervushin, K., and Wüthrich, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4918–4923
37. Ulijasz, A. T., Grenader, A., and Weisblum, B. (1996) J. Bacteriol. 178, 6305–6309
38. Gam beetta, G. A., and Lagarias, J. C. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 10566–10571
39. Wang, L., Elliott, M., and Song, P. S. (2000) Biochemistry 39, 10840–10847
40. Edgerton, M. D., and Jones, A. M. (1992) Plant Cell 4, 161–171
41. Blumenstein, A., Vienken, K., Tasler, R., Purschowitz, J., Vieth, D., Franken berg-Dinkel, N., and Fischer, R. (2005) Curr. Biol. 15, 1833–1838
42. Shlyk-Kerner, O., Samish, I., Kaftan, D., Holland, N., Sai, P. S., Kless, H., and Scherz, A. (2006) Nature 442, 827–830
43. Mrogrinski, M. A., Murgida, D. H., and Hildebrandt, P. (2007) Acc. Chem. Res. 40, 258–266
44. Murgida, D. H., von Stetten, D., Hildebrandt, P., Schwinte, P., Siebert, F., Sharda, S., Gärnter, W., and Mrogrinski, M. A. (2007) Biophys. J. 93, 2410–2417
45. Fischer, A., J. Rockwell, N. C., Jang, A. Y., Ernst, L. A., Waggner, A. S., Duan, Y., Lei, H., and Lagarias, J. C. (2005) Biochemistry 44, 15203–15215
46. Yang, X., Stojkovic, E. M., Kuk, J., and Moffat, K. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 12571–12576
47. Stock, A. M., Robinson, V. L., and Goudreau, P. N. (2000) Annu. Rev. Biochem. 69, 183–215
48. Wexler, M., Sargent, F., Jock, R. L., Stanley, N. R., Bogsch, E. G., Robinson, C., Berks, B. C., and Palmer, T. (2000) J. Biol. Chem. 275, 16171–16172
49. Davis, S. J., Vener, A. V., and Vierstra, R. D. (1999) Science 286, 2517–2520
50. Scheerer, P., Michael, N., Rosen, R., and Lamparter, T. (2007) FEBS Lett. 579, 3970–3974
51. Scheer, H., and Vierstra, R. D. (1993) Plant Cell 5, 565–575
52. Edgerton, M. D., and Jones, A. M. (1992) Plant Cell 4, 161–171
53. Blumenstein, A., Vienken, K., Tasler, R., Purschowitz, J., Vieth, D., Franken berg-Dinkel, N., and Fischer, R. (2005) Curr. Biol. 15, 1833–1838
54. Shlyk-Kerner, O., Samish, I., Kaftan, D., Holland, N., Sai, P. S., Kless, H., and Scherz, A. (2006) Nature 442, 827–830
55. Mrogrinski, M. A., Murgida, D. H., and Hildebrandt, P. (2007) Acc. Chem. Res. 40, 258–266
56. Murgida, D. H., von Stetten, D., Hildebrandt, P., Schwinte, P., Siebert, F., Sharda, S., Gärnter, W., and Mrogrinski, M. A. (2007) Biophys. J. 93, 2410–2417
57. Fodor, S. P., Lagarias, J. C., and Mathies, R. A. (1990) Biochemistry 29, 11141–11146
58. Limbach, H. H., Hennig, J., Kendrick, R., and Yannoni, C. S. (1984) J. Am. Chem. Soc. 106, 4059–4060
59. Falk, H. (1989) The Chemistry of Linear Oligopyrroles and Bile Pigments, Springer-Verlag, New York
60. Rivera, M., and Walker, F. A. (1995) Anal. Biochem. 230, 295–302
61. Scheer, P., Michael, N., Park, J. H., Noack, S., Forster, C., Hamman, M. A., Inomata, K., Choe, H. W., Lamparter, T., and Krauss, N. (2006) J. Struct. Biol. 153, 97–102
62. von Stetten, D., Gunther, M., Kaminiski, S., Scherer, P., Krauss, N., Lamparter, T., Forest, K. T., Vierstra, R. D., Gärnter, W., Murgida, D. H., Mrogrinski, M. A., and Hildebrandt, P. (2008) Annu. Chem., in press
63. Li, X., Zhang, G., Ngo, N., Zhao, X., Kain, S. R., and Huang, C. C. (1997) J. Biol. Chem. 272, 28545–28549
64. van Thor, J. J., Macke, M., Kupov, L., Dwek, R. A., and Wormald, M. R. (2006) BioPhy. J. 91, 1811–1822
65. Mrogrinski, M. A., Murgida, D. H., von Stetten, D., Kniep, C., Mark, F., and Hildebrandt, P. (2004) J. Am. Chem. Soc. 126, 16734–16735
66. Inomata, K., Hamman, M. A. S., Kimoshita, H., Murata, Y., Khawn, H., Noack, S., Michael, N., and Lamparter, T. (2005) J. Biol. Chem. 280, 24491–24497
67. Miller, A. E., Fischer, A. J., Laurence, T., Hollars, C. W., Saykally, R. J., Lagarias, J. C., and Huser, T. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 11136–11141
68. Remberg, A., Lindner, I., Lamparter, T., Hughes, J., Kniep, C., Hildebrandt, P., Braslavsky, S. E., Gärnter, W., and Schaffner, G. (1997) Biochemistry 36, 13389–13395
69. Korniol, B., and Vierstra, R. D. (2004) J. Bacteriol. 186, 445–453