High Resolution Structure of Deinococcus Bacteriophytochrome Yields New Insights into Phytochrome Architecture and Evolution*§

Phytochromes are red/far red light photochromic photoreceptors that direct many photosensory behaviors in the bacterial, fungal, and plant kingdoms. They consist of an N-terminal domain that covalently binds a bilin chromophore and a C-terminal region that transmits the light signal, often through a histidine kinase relay. Using x-ray crystallography, we recently solved the first three-dimensional structure of a phytochrome, using the chromophore-binding domain of Deinococcus radiodurans bacterial phytochrome assembled with its chromophore, biliverdin IXα. Now, by engineering the crystallization interface, we have achieved a significantly higher resolution model. This 1.45 Å resolution structure helps identify an extensive buried surface between crystal symmetry mates that may promote dimerization in vivo. It also reveals that upon ligation of the C3² carbon of biliverdin to Cys²⁶⁰, the chromophore A-ring assumes a chiral center at C2, thus becoming 2R,3(E)-phytochromobilin, a chemistry more similar to that proposed for the attached chromophores of cyanobacterial and plant phytochromes than previously appreciated. The evolution of bacterial phytochromes to those found in cyanobacteria and higher plants must have involved greater fitness using more reduced bilins, such as phycocyanobilin, combined with a switch of the attachment site from a cysteine near the N terminus to one conserved within the cGMP phosphodiesterase/adenyl cyclase/FhlA domain. From analysis of site-directed mutants in the D. radiodurans phytochrome, we show that this bilin preference was partially driven by the change in binding site, which ultimately may have helped photosynthetic organisms optimize shade detection. Collectively, these three-dimensional structural results better clarify bilin/protein interactions and help explain how higher plant phytochromes evolved from prokaryotic progenitors.

Phytochromes (Phys)³ comprise a ubiquitous superfamily of photoreceptors present in the plant, fungal, and bacterial kingdoms. They play critical roles in various light-regulated processes, ranging from phototaxis and pigmentation in bacteria to seed germination, chloroplast development, shade avoidance, and flowering in higher plants (1, 2). Phys are homodimeric complexes with each polypeptide containing an N-terminal chromophore-binding domain (CBD) that autocatalytically attaches via a thioether linkage a single linear tetrapterrole (or bilin) chromophore, a PHY domain that is important for spectral integrity, and a C-terminal domain that promotes dimerization and often signal transmission (3). Through unique interactions among the bilin and the CBD and PHY domains, Phys can exist as two metastable conformers, a red light (R)-absorbing Pr form and a far red light (FR)-absorbing Pfr form. By photoconverting between Pr and Pfr, Phys act as unique light-regulated switches in various signal transduction cascades. In bacteria and fungi, a canonical histidine kinase (HK) domain is typically present downstream of the PHY domain, thus allowing these Phys to participate in various two-component phosphorylases (1, 4, 5). Although higher plant Phys contain a C-terminal HK-related sequence that promotes dimerization (2, 3), it remains unclear if this domain has phosphotransferase activity (6) or is even involved in signal transmission (7, 8).

Despite intensive physico-chemical analysis of various Phys, we do not yet understand how contacts between the polypeptide and the bilin enable photointerconversion between Pr and Pfr, how this transformation reversibly alters the activity of the photoreceptor, or how the holoprotein dimerizes (3). Important insights were made recently with our determination of the first three-dimensional structure of the CBD domain derived from the sole bacteriophytochrome (BphP) present in the proteobacterium Deinococcus radiodurans (9). This ground state Pr structure confirmed sequence predictions that the CBD is composed of Per/Arndt/Sim (PAS) and cGMP phosphodiesterase/adenyl cyclase/FhlA (GAF) domains and revealed that the GAF domain contains a deep pocket that cradles the bilin in a...
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EXPERIMENTAL PROCEDURES

Protein Production and Crystallization—DrCBD encompassed the first 321 amino acids of DrBphP and containing an N-terminal T7 tag and a C-terminal His6 tag was as described (9). DrCBD-Y307S was generated by correcting the inadvertent point mutation that substituted Pro–240 for a Thr and converting the Tyr–307 codon to that for a serine with the QuikChange method (Stratagene, La Jolla, CA). The DrCBD-Y307S apoprotein was expressed in Escherichia coli strain Rosetta (DE3) (Novagen, San Diego, CA) with or without simultaneous expression of the heme oxygenase from Synechocystis PCC6803 (12). The crude lysates were incubated for 30 min with a >10-fold molar excess of BV (Porphyrin Products, Logan, UT), and the resulting holoproteins were purified by sequential nickel-chelate affinity and anion exchange chromatography as previously described (9). Bilin binding was assayed by zinc-induced fluorescence of the chromoprotein following SDS-PAGE (12).

Optimal crystallization conditions were identified by the hanging drop vapor diffusion method using the sparse matrix Cryoscreen (Nextal Biotechnologies, Montreal, Canada). Each trial included 2 μl of well solution and 2 μl of 20 mg/ml DrCBD-Y307S in 30 mM Tris-HCl (pH 8.0). Large crystals (0.2 × 0.18 × 0.08 mm) were generated with 0.095 M sodium citrate (pH 5.6), 19% (v/v) isopropyl alcohol, 19% (v/v) polyethylene glycol 4000, and 5% (v/v) glycerol. The crystals were briefly washed in mother liquor and then flash-frozen in liquid nitrogen.

Structure Determination—Two x-ray diffraction data sets were collected on DrCBD-Y307S crystals. One data set was collected at the University of Wisconsin (Madison, WI) with a Proteum CCD detector with x-rays generated by a Microstar rotating anode (Bruker AXS, Madison, WI). One degree images were captured for 360 degrees with 30-s exposure times and processed with HKL2000 and Scalepack (19) (Table 1). The second data set was collected at the Advanced Photonic Source (APS, Argonne, IL) on beamline 51DB using a MAR225 detector. One degree images were captured for 180 degrees with 1.2-s exposure times. These images were integrated and scaled to the edge of the detector to a maximum resolution of 1.45 Å (Table 1).

Phases for both x-ray data sets were determined by molecular replacement with the program AmoRe (20), using the previously described DrCBD structure (Protein Data Bank accession code 1ZTU (9)) without the chromophore or His6 tag as the search model. Unambiguous solutions for both DrCBD-Y307S data sets were nearly identical with correlation coefficients of 68.2 and 67.0 and R-factors of 42.6 and 38.5% for the low resolution and high resolution data sets, respectively. After an initial round of positional refinement with Refmac5 (21), electron density maps were constructed; nearly all of the residues from the search model correctly fit into a 2Fo−Fc electron density map. The protein–chromophore linkage was modeled by creating a modified amino acid consisting of a cysteine bonded via a thioether linkage to the C3’ carbon of the A-ring vinyl group of BV. To account for the rotation of the D-ring, the energy penalty for rotation about the C15=C16 double bond in this adduct was removed. This adduct was introduced into the structure using the 2Fo−Fc difference electron density maps, and the model was

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tion in the Pr form. The most unexpected feature of the DrCBD structure is the presence of a deep knot, where a conserved insertion within the GAF domain lassos the N-terminal 34 residues upstream of the PAS domain (9, 10). This knot bridges the PAS and GAF domains and contacts the chromophore, suggesting that it stabilizes the CBD and possibly participates in signal transmission.

The high degree of conservation for residues that form the PAS and GAF domains, contact the chromophore, and create the knot strongly suggests that the unique topology of the DrCBD is present in most, if not all, members of the Phy superfamily and probably helps generate the unique R/FR photochromic spectral properties of these photoreceptors (4, 9). Despite this similarity, there are differences in bilin preference and in the bilin attachment site between the bacterial and fungal Phys and their cyanobacterial and higher plant counterparts. In bacterial and fungal Phys, the chromophore is biliverdin IXα (BV) (11, 12), which is synthesized by oxidative cleavage of heme by a heme oxygenase. BV is attached to the CBD via a thioether linkage between a conserved cysteine upstream of the PAS domain and the A-ring C3 vinyl side chain of BV (4, 13). In contrast, the cyanobacterial and higher plant Phys use phycocyanobilin (PCB) or phytochromobilin (PΦB), respectively (14–16). Production of PCB and PΦB includes enzymatic reduction of the A-ring in BV to generate an ethylenediamine side chain at the C3 position. The conserved cysteine attachment site for these Phys is within the GAF domain (3, 15, 17, 18). Although these differences in bilin chemistry and ligation site do not appreciably alter the ability of cyanobacterial and plant Phys to photointerconvert between Pr and Pfr, they may have important adaptive consequences for maximizing light perception. Most noticeably, they blue shift the absorption spectrum of Pr to better coincide with those of chlorophylls and thus to be more efficient in detecting shade from other photosynthetic organisms (19, 20).

In an attempt to better define the structure of Phys, we have engineered the surface of DrCBD to optimize crystal packing and have obtained substantially higher resolution diffraction. The new 1.45 Å resolution structure supports conclusions drawn from the previous 2.5 Å model (9) and unequivocally confirms that BV is linked to Cys24 via the C32 carbon of the A-ring side chain. Comparisons of the crystal contacts in the 2.5 and 1.45 Å resolution structures identified an extensive buried surface between symmetry mates that we speculate represents a biologically relevant dimerization site in the photoreceptor. One degree images were captured for 180 degrees with 1.2-s exposure times. These images were integrated and scaled to the edge of the detector to a maximum resolution of 1.45 Å (Table 1).

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again refined with Refmac5 (24). Based on interpretation of both the $2F_o - F_c$ and $F_o - F_c$ electron density maps generated from data to 1.45 Å, the double bond in the A-ring was removed from between the C2 and C3 carbons and introduced between the C3 and C4 carbons, and the restraints forcing the A-ring to be planar and parallel with the B- and C-rings were released.

Both models were refined by iterative rounds of model building with Xfit (22) or COOT (23) and TLS and positional refinement with Refmac5 (24). The final models have excellent geometry and appropriate $R$-factors (Table 1). In both models, the 16 N-terminal residues (13 of which comprise the T7 tag) and the 5 C-terminal histidines of the His$_6$ tag were not modeled because of disorder. Although the model constructed from the 1.45 Å data set exhibited no gaps in the polypeptide chain, several residues forming the linker between the PAS and GAF domains were omitted in the 2.15 Å model. Data collection and refinement statistics are presented in Table 1. Structure figures were generated with PyMOL (Delano Scientific, Palo Alto, CA). Atomic coordinates and structure factors for the 2.15 and 1.45 Å resolution models have been deposited in the Protein Data Bank (25) under accession codes 2O9B and 2O9C, respectively.

Native Size Determinations—The coding sequence for DrCBD-PHY (amino acids 1–501) was PCR-amplified from the full-length DrBphP construction by using primers designed to introduce BamHI and XhoI sites to the 5’ and 3’ ends, respectively. The BamHI-XhoI-digested PCR product was cloned into pET21b (+) (Novagen), which was similarly digested, to direct the expression of DrCBD-PHY bearing an N-terminal T7 tag and a C-terminal His$_6$ tag. Nickel-chelate affinity-purified full-length DrBphP, DrCBD-PHY, DrCBD, and DrCBD-Y307S were concentrated by ammonium sulfate precipitation and resuspended in 150 mM NaCl and 50 mM Tris-HCl (pH 7.5) to a final concentration of ~5 mg/ml. Dynamic light scattering was performed using a DynaPro (Protein Solutions/Wyatt Technology Corp., Santa Barbara, CA) multiangle light scattering instrument with a 5 mg/ml concentration of each DrBphP dissolved in 30 mM Tris-HCl (pH 8.0). Approximate molecular radii assuming spherical proteins were calculated by the Dynamics software package (Wyatt Technology Corp.). Size exclusion fast protein liquid chromatography was performed using a Superdex 200 (Amersham Biosciences) column equilibrated with 150 mM NaCl and 50 mM Tris-HCl (pH 7.5) and a flow rate of 200 µl/min. The column was calibrated using the gel filtration standards from Bio-Rad. Nondenaturing PAGE was performed as described (26) using bovine serum albumin (U. S. Biochemical Corp.) and ovalbumin (Sigma) as standards (16).

Analysis of Site-directed Mutants—The PAS and GAF cysteine codon mutations were generated by the QuikChange method using the full-length DrBphP construction bearing a C-terminal His$_6$ tag (12). To assess chromophore preference, the apoproteins were expressed as above without co-expression of Synechocystis heme oxygenase (12) and assembled with the chromophore in vitro, using a >10-fold molar excess of either BV, PCB purified from Spirulina platensis (27), or various ratios of BV and PCB. The assembled proteins were purified from the crude lysates by nickel-chelate affinity chromatography. Absorption and difference spectra were obtained with a PerkinElmer Life Sciences Lambda 650 spectrophotometer following saturating irradiations with R (690 or 660 nm) and FR (770 nm) enriched by interference filters. To quantitate binding efficiency of BV relative to PCB, the absorbance maxima of BV- and PCB-bound proteins were identified in the Soret and Q bands for the Pr spectrum generated by each BV/PCB mixture. By incorporating these absorbance values in the ratio (Q$_{BV}$/Q$_{PCB}$)/(Soret$_{BV}$/Soret$_{PCB}$), the relative contributions of BV-bound and PCB-bound holoproteins in the mixtures were calculated. These ratios were normalized by subtracting the value calculated for the 100% PCB sample (100% PCB-bound sample now represented 0% BV incorporation) from all other values and scaling all of the adjusted values so that the values from samples generated solely with BV equaled 100%.

RESULTS

Crystal Lattice Engineering Leads to a High Resolution Structure of DrCBD—To better define the structure of BV and its interactions with the CBD and to generate crystals suitable for time-resolved x-ray analysis of the Pr to Pfr phototransformation, we sought to improve the diffraction resolution of DrCBD crystals. We noticed a chemically impossible overlap of symmetry-related Tyr-307 side chains in the 1ZTU structure (9) that could create local disorder detrimental to crystal formation (Fig. 1A). To reduce this conflict, we replaced Tyr-307 with Ser (Fig. 1B), reasoning that a substitution of residue 307 should not appreciably affect DrCBD folding or photochemical activity, given its surface location and considerable distance from the chromophore (Fig. 1C). Indeed, the DrCBD-Y307S apoprotein was stable and soluble and readily assembled with BV to generate a chromoprotein spectrophotometrically indistinguishable from DrCBD following R or FR irradiation. DrCBD-Y307S holoprotein did not crystallize under the previously described condition (9), but in subsequent unbiased screens, we identified new conditions that readily generated bright blue crystals in the space group C2. Importantly, the DrCBD-Y307S crystals yielded data sets to maximum resolutions of 2.15 and 1.45 Å using x-rays generated from a rotating anode and synchrotron radiation, respectively (Table 1). From this pair of data sets, two models for DrCBD-Y307S were created by molecular replacement and subsequent refinement (Table 1).

Whereas the prior 2.5 Å model lacked several residues in two disordered loops (9), the 1.45 Å model for DrCBD-Y307S includes all 318 residues of the CBD with no breaks in the polypeptide chain from amino acid 4 to 322 (Fig. 1D). Alternate conformations were modeled for 25 mostly solvent-exposed residues; however, even in this high resolution structure, seven disordered side chains were truncated to alanine. Although DrCBD-Y307S crystallized in a different space group than DrCBD, the 1ZTU model and the new structure were remarkably coherent, with a least squares alignment of all protein backbone atoms yielding a root mean square deviation of 0.49 Å$^2$. Even the structure of α8 surrounding residue 307 was not substantially altered, indicating that the Tyr to Ser substitution in DrCBD-Y307S had minimal impact on CBD folding. The adjacent α4 was slightly distorted, presumably to accommodate

4 J. R. Wagner, J. Zhang, R. D. Vierstra, and K. T. Forest, unpublished data.
the altered crystal packing (Fig. 1, A–C). In contrast to the clash at Tyr-307 with its symmetry mate in the DrCBD structure, the Ser at this position in the DrCBD-Y307S structure pointed away from its symmetry mate, which we presume improved crystal packing (Fig. 1 B).

The higher resolution model confirmed the presence of the knot between the GAF and PAS domains (Fig. 1D), along with the positioning of Ile35 and Gln36 at its center (9). As recently noted by Virnau et al. (10) in a large scale survey of knotted protein structures, the knot in DrCBD has four crossover points and is thus defined as a figure-of-eight knot (Fig. 1, D and E).

Although we have not explicitly addressed the mechanism of protein folding in our current work, we note parenthetically that this accurately refined CBD structure revealed a cis peptide bond at Asp235-Pro236 within the extended GAF insertion that forms the knot crossover (Fig. 1D). Cis-trans isomerization of prolines is well known to be a rate-limiting step in protein folding in general (28), and is important for the folding of at least one knotted protein (29). For this reason, we speculate that the proline-rich nature of the GAF insertion (5 prolines within 15 residues) and in particular the cis conformation of Pro236 could be critical in stabilizing intermediate folded states until the knot is threaded and the hydrophobic amino acids in the GAF domain insertion condense around the invariant Ile35 within the N-terminal extension.

**Dimerization of DrCBD—** Our previous 2.5 Å resolution structure of DrCBD contained a large contact surface between 2-fold symmetry mates. Although we altered a residue in this crystallographic interface and obtained a different space group (C2 versus P21212), the same overall dimer contacts were retained in DrCBD-Y307S crystals (Fig. 1C). No other packing interactions were the same between the two space groups. The buried surface in both crystal forms was extensive, representing 1,070 and 1,417 Å² (or 7% and 10% of the total solvent-accessible area) per monomer for the DrCBD and DrCBD-Y307S models, respectively (Fig. 2, A and B). The persistence of the interface between the two crystal forms and the large buried surface area suggest that this contact represents a DrBphP dimerization site in solution.

The extensive contact between the symmetry mates involves a noncanonical four-helix bundle, which is formed by the largely hydrophobic association of key residues. In particular, Leu140 and Met144 from GAF helix α4, and Ser307, aliphatic atoms of Arg310 (truncated to Ala in refined structure), Leu311, Leu314, and Val318 from GAF helix α6 pack in parallel fashion against their symmetry mates (Figs. 1C and 2, A and B). On
either side of this central hydrophobic interaction, polar contacts between residues within PAS domain β3 (Gln198 and Arg306), GAF α4 (His138 and Arg141), and GAF α8 (Asp300, Glu301, and presumed side chain of Arg310) complete the interface. Although the residues involved in this interface are not strongly conserved within the Phy superfamily, the hydrophobic nature of the strong α8 interactions is (4).

To confirm that the N-terminal region of DrBphP can dimerize in solution, we determined the native size of the DrCBD and DrCBD-Y307S chromoproteins by several methods. Dynamic light scattering of both CBDs and a longer fragment that included the PHY domain (DrCBD-PHY) were consistent with these N-terminal portions behaving as dimers in solution.4 Preceding native size measurements by size exclusion fast protein liquid chromatography demonstrated that full-length BV-DrBphP adduct is a dimer (12). Similar measurements of DrCBD-PHY and DrCBD fragments yielded the same conclusion (Fig. 3A). Upon assembly with BV, full-length DrBphP had a native size of 256 kDa, whereas the DrCBD-PHY fragment was 125 kDa, and the two DrCBD fragments were ~67 kDa, each of which are at least twice the predicted monomeric sizes of 84, 56.5, 37.3, and 37.3 kDa, respectively. The larger than predicted size of the full-length holoprotein is consistent with small angle x-ray scattering and electron microscopic images of bacterial and plant Phys, which revealed nonspherical shapes (30–32). Dimers and larger complexes of the chromoproteins were also detected using nondenaturing PAGE. In each case, the apparent native mass of the most abundant species was at least twice the actual mass calculated for the BV-bound monomeric polypeptide (Fig. 3B). The presumed monomer was apparent only in the DrCBD-PHY preparations, suggesting that this DrBphP fragment is less stable as a dimer than the other chromoproteins, at least under the conditions tested here.

Table 1

| TABLE 1 | Data collection and refinement statistics |
|---------|------------------------------------------|
| Data collection: | Rotating anode | Synchrotron |
| Wavelength (Å) | 1.54 | 0.972 |
| No. of unique reflections | 17,484 (1,401)* | 56,868 (4,813) |
| Resolution (Å) | 24.2-1.5 (2.2-2.15) | 20-1.45 (1.5-1.45) |
| Completeness (%) | 96.4 (78.8) | 97.5 (83.3) |
| Redundancy | 6.2 (2.6) | 3.7 (2.9) |
| Rmerge (%) | 10.4 (33.3) | 3.9 (27.6) |
| I/σI | 10.0 (2.8) | 12.6 (3.0) |
| Space group | C2 | C2 |
| Cell dimensions | a, b, c (Å) | 89.5, 51.6, 80.9 | 89.3, 51.8, 80.4 |
| α, β, γ (degrees) | 90.0, 116.4, 90.0 | 90.0, 116.3, 90.0 |

Refinement statistics:
- Resolution range (Å): 24.0-2.15 (2.2-2.15) | 20-1.45 (1.49-1.45)
- R-factor (%): 18.9 (21.8) | 16.5 (21.3)
- Free R-factor (%): 23.7 (25.1) | 19.2 (27.3)
- Protein atoms: 2,445 | 2,531
- Solvent molecules: 111 | 301
- Heteroatoms: 43 | 43
- Dual conformers: 10 | 25
- Isotropic average temperature factors (Å²): Overall 32.4 | 24.2
- Protein atoms 32.6 | 23.2
- Heteroatoms 22.9 | 21
- Water molecules 31.8 | 32.1
- Root mean square deviations:
  - Bond lengths (Å): 0.014 | 0.012
  - Bond angles (degrees): 1.8 | 1.8
  - Estimated coordinate error (Å²): 0.14 | 0.044

*The value for the highest resolution shell is shown in parentheses.
set. Given the invariant nature of Asp\textsuperscript{207}, its proximity to BV, and its importance for proper photoconversion (4, 9, 33), this contact could be photochemically relevant. The only other amino acid in the bilin-binding pocket whose position was appreciably altered in the 1.45 Å resolution structure was Cys\textsuperscript{24} (see below).

The very well ordered “pyrrole water” that contacts BV was maintained in the \textit{DrCBD-Y307S} structure (Fig. 4, A and B). This water participates in a remarkable hydrogen-bonding network with the pyrrole nitrogens of the BV-rings A, B, and C; the main chain oxygen of Asp\textsuperscript{207}; and the Ne of His\textsuperscript{260} (9). In this position, the pyrrole water could participate in the deprotonation/protonation cycle proposed for Phys during phototransformation (3, 34). The previously identified water (9) that appears to hydrogen-bond with the Ne of His\textsuperscript{290} and the propionate carbonyl of ring C can be resolved in the 1.45 Å resolution structure of \textit{DrCBD-Y307S} as two fully occupied water positions (Fig 4B), which together mediate the hydrogen bond network between the C-ring propionate carbonyl and His\textsuperscript{290}. A new water interaction is observed with the hydroxyl of Tyr\textsuperscript{176} (Fig. 4C). Perhaps surprisingly, we could not refine any convincing waters in the small cavity between Tyr\textsuperscript{176} and the BV D-ring (Fig. 4C). As a consequence of the higher resolution diffraction data, a total of 301 waters were found in the \textit{DrCBD-Y307S} structure versus the 25 reported for the \textit{DrCBD} structure (9).

Ten residues in the \textit{DrCBD-Y307S} structure adopt unusual side chain rotamers; most can be explained by crystal packing interactions. Arg\textsuperscript{222} is an exception; its buried side chain curls above both BV propionate side chains and Tyr\textsuperscript{216} but not in direct contact with the chromophore. The amine moiety of Arg\textsuperscript{222} is not neutralized by a countercharge but does interact with two Ser O\textsubscript{HAT}/H\textsubscript{274} atoms (274 and 276) as well as two main chain atoms (Leu\textsuperscript{222} O and His\textsuperscript{219} O).

Our earlier model proposing that BV is bound to Cys\textsuperscript{24} via the C32 carbon of the A-ring vinyl side chain was based on the best interpretation of our electron density maps (9). However, the geometry of Cys\textsuperscript{24} was nonideal, and we could not rule out radiation damage effects. Refinement of the covalent linkage between Cys\textsuperscript{24} and BV against our higher resolution data sets led to a much more satisfying fit to the electron density and standard Φ/Ψ angles and rotamer conformation for the cysteine. In these models, the thiether linkage to Cys\textsuperscript{24} within the A-ring vinyl is unambiguous. This bacterial Phy linkage is distinct from those in cyanobacteria and higher plant Phys, which are proposed to bind PCB and P\textsubscript{Φ}B via the C31 carbon (3, 15, 17).

Reinterpretation of the \textit{DrCBD} structure based on the higher resolution data sets also revealed a surprise with respect to the structure of the bilin A-ring within the binding pocket. Although there was a slight reorientation of the C-ring propionate carboxyl group, the overall orientations of the B-, C-, and D-rings were not significantly altered in the higher resolution models (Fig. 4A). Importantly, the high resolution data confirmed the 44° rotation of ring D with respect to the plane formed by rings B and C. However, it became evident early in the new refinement that the planar geometry used previously to describe the A-ring was incomplete. Inspections of high resolution difference electron density maps calculated using a planar A-ring model revealed areas of strong negative density surrounding the N, C21, and O atoms of ring A and areas of strong positive density above these atoms (Fig. 5A). To help eliminate
these anomalies, we released the planar and rotational constraints on the A-ring and removed the proposed double bond between the C3 and C2 carbons of the A-ring. Subsequent refinements with this modified Cys24-BV adduct led to an approximate rotation of the A-ring and its substituents relative to the B- and C-rings (Figs. 4, A and B, and 5B). In the absence of planar restrictions, the C2 carbon of the A-ring assumed a tetrahedral geometry. This arrangement allowed the C21 methyl carbon to rotate toward Met259, which agreed well with the Fo/Fc difference electron density map in this area (Fig. 5B). Given the red-shifted Pr absorption spectrum of BV-bound DrBphP relative to those assembled with PCB and PФB (4, 12, 35), we predicted that an additional double bond is present in the BV-DrCBD adduct to extend the π conjugation system. Within the constrains of the electron density, the best way to achieve this extension in the presence of a tetrahedral C2 carbon was to model a double bond between the C3 and C31 carbons of BV (Fig. 5C). Thus, BV is predicted to convert to 2(R),3(E)-PФB when conjugated to the DrBphP apoprotein. Remarkably, this BV adduct is a stereoisomer of that proposed for PCB/PФB (3, 15, 17) but with opposite chirality at the C2 position (Fig. 5C).

Evolution of Phys—The evolution of cyanobacterial and higher plant Phys from prokaryotic progenitors required not only enzymes to produce PCB/PФB but also a way to discriminate them from the BV precursor during chromophore ligation (36). A possible mechanism to generate such a bias became readily apparent in light of our three-dimensional structures of DrCBD (9). One important step was the switch of the binding site cysteine from the PAS domain to the GAF domain. This switch must position the sulfur moiety of the apoprotein cysteine to extend toward the C31 position of PCB/PФB instead of toward the C32 position of BV as observed in DrCBD. In this way, cyanobacterial and plant Phys can maintain ligation to the C3 side chain double bond despite the change in the position of this double bond from the C31–C32 (vinyl) to the C31–C32 (ethylidene) in BV versus PCB/PФB (Fig. 5C). A second step was a change in the chirality of the C2 carbon in PCB/PФB versus BV once bound. In DrBphP, the out of plane C2 methyl group points toward and forms van der Waals interactions with Met259, as other carbons of the C3 side chain. Within the BphP family, Met or other hydrophobic residues (Leu, Ile, and Val (4)) invariably occupy this position, which should promote similar van der Waals interactions with the A-ring of 2(R),3(E)-PФB. However, for PCB/PФB, the C2 methyl group would point away from position 259. A cysteine inserted at position 259 could then attack the C31 carbon of the ethylidene side chain without steric clash.

To test the importance of the switch in binding site cysteines, a set of PAS and GAF domain cysteine substitution mutants were generated in full-length DrBphP and assayed for bilin binding and preference. The set included wild-type DrBphP with only the N-terminal cysteine, M259C with both cysteines (35), C24A/M259C with only the GAF cysteine, and DrC24A with neither cysteine present. The apoproteins were expressed recombinantly; assembled with BV, PCB, or mixtures of the two; purified; and then analyzed for chromophore ligation and
spectral properties. (The full R and FR absorption and R-FR difference spectra for each can be found in supplemental Fig. 1.)

As in previous studies (4, 12, 35), wild-type DrBphP efficiently assembled covalently with both BV and PCB (as judged by zinc-induced fluorescence of the holoprotein following SDS-PAGE) (Fig. 6A). DrBphP-M259C, previously shown to bind PCB (35), also covalently bound BV, although BV ligation was less efficient (Fig. 6A). The C24A/M259C apoprotein could covalently bind BV and PCB, indicating that bilin binding at position 259 was possible in the absence of Cys24. The cysteine-less C24A variant failed to ligate either bilin. Taken together, the data show that a cysteine at either position can participate in covalent bilin ligation of BV and PCB and that alternative sites do not become uncovered in the absence of both residues. Surprisingly, the C24A protein mixed with BV or PCB generated relatively normal Pr spectra (maxima at 705 and 686 nm, respectively; Fig. 6B and supplemental Fig. 1), indicating that bilins can still associate with the CBD in the absence of covalent binding. Such noncovalent interactions have also been noted for Agrobacterium tumefaciens BphP1 (Agp1) (37) and Calothrix CphB (38).

The BV-DrBphP holoprotein is R/FR photochromic with strong absorption for both Pr and Pfr conformers (maxima at 698 and 750 nm, respectively (9, 12) (Fig. 6B and supplemental Fig. 1)). In contrast, the Pr absorption spectrum for the PCB adduct was markedly less intense and blue-shifted to 653 nm. The blue shift was consistent with two fewer double bonds in its π conjugation system, one due to the more reduced D-ring vinyl moiety of PCB versus the ethyl moiety of BV and the other due to the predicted loss of the A-ring ethyldiene double bond when PCB is conjugated to the apoprotein (9, 12, 15, 17) (see Fig. 5C). The PCB adduct following saturating R irradiation was also substantially bleached (supplemental Fig. 1), indicating that PCB adducts would not photoconvert to Pfr but instead generated a photoreversibly bleached intermediate. Likewise, the C24A protein-BV and -PCB complexes would not photoconvert to Pfr but appeared to become photoreversibly bleached following saturating R (supplemental Fig. 1).

Each of the novel BV-holoproteins was affected in the ratio of the R to blue absorbance of Pr, although the difference was only ~2-fold compared with wild type (R/B ratios of 1.3, 1.6, and 1.5 for M259C, C24A/M259C, and C24A, respectively, versus 2.7 for BV-DrCBD (supplemental Fig. 1)), suggesting that each bound bilin retained a relatively linear conformation (39). For the PCB complexes, the B/R ratio varied from 1.9 for wild type to 2.6 for the C24A/M259C protein. Collectively, the data imply that the ability of DrBphP to generate photochemically active Pfr requires both a hydrophobic residue at position 259 and covalent attachment of BV to Cys24.

To examine the bilin binding efficiency of each variant more closely, we developed a competition assay in which various ratios of BV and PCB within a fixed total concentration were incubated with each apoprotein (ratios ranging from 64:1 to 1:64). The ratios of BV/PCB bound were then calculated spec-
trophotometrically from the resulting holoprotein mixtures based on the assumption that each bilin could attain full occupancy of the chromophore pocket. As shown in Fig. 6C, the Pr spectra of the BV- and PCB-bound forms were sufficiently distinct to allow us to estimate the percentage of BV and PCB bound for each polypeptide. In fact, for wild-type DrBphP and the M259C and C24A/M259C variants, two Pr peaks were evident when equivalent amounts of the BV- and PCB-bound forms were generated.

Based on this assay, we calculated that the wild-type apoprotein covalently bound BV 4 times more effectively than PCB; i.e. a nearly equal ratio of the two chromoproteins was created upon incubation with a 1:4 molar ratio of BV/PCB (Fig. 6C). For the M259C variant in which both binding sites are available, BV and PCB bound to the apoprotein with nearly equal affinity. The C24A/M259C apoprotein, by contrast, preferred PCB and bound it almost 4-fold better than BV with a 1:4 molar ratio of PCB/BV needed for equal occupancy (Fig. 6C). Thus, by switching the bilin binding site from the N-terminal region to the GAF domain, an 16-fold increase in PCB preference was achieved versus BV. Surprisingly, the preference for BV was largely eliminated in the C24A variant (Fig. 6C), implying that the bilin-binding pocket in DrBphP poorly discriminates between BV and PCB in the absence of covalent attachment. Collectively, the data show that by exchanging the N-terminal cysteine with one in the appropriate position of the GAF domain, we could generate a DrBphP with a greater preference for PCB but that this switch alone did not generate a properly photoreversible holoprotein.

DISCUSSION

By crystal design, we have achieved a 1.45 Å resolution model of DrCBD that provides additional insights into the three-dimensional structure of the CBD of Phys. Our ability to change a single residue in the symmetry mate interface to substantially improve the crystallization properties of a protein is an unusually successful case of crystal engineering. Although there are numerous cases where site-directed mutagenesis led to better crystallization properties (40, 41), there are fewer examples involving the intentional design of crystallization interfaces (42).

The improved models of DrCBD developed with these engineered crystals highlighted an extensive contact among symmetry mates that promotes dimerization of the N-terminal region, confirmed the bilin linkage site, and provided novel information about the chemical structure and orientation of the bilin within the binding pocket. Although nearly all amino acids in both our new 1.45 Å resolution DrCBD-Y307S and previous 2.5 Å resolution DrCBD structures are in equivalent positions, a notable few are significantly different. The new positions of Tyr\textsuperscript{263} and

Figure 6. Bilin binding and photochemical properties of DrBphP variants affected in chromophore ligation. Full-length wild-type (WT) DrBphP and substituted apoproteins with altered Cys\textsuperscript{24} and/or Met\textsuperscript{259} were purified by nickel-chelate affinity chromatography, and equal amounts (as determined by \(A_{280}\)) were assembled with BV, PCB, or various ratios of BV and PCB. A, assay of covalent bilin binding. Following a 1-h incubation with either BV or PCB, the chromoproteins were subjected to SDS-PAGE and either assayed for the bound bilin by zinc-induced fluorescence (Zn Prot) or stained with Coomassie Blue (Prot). B, Pr absorption spectra of equivalent amounts of DrBphP incubated with BV or PCB alone or the indicated ratios of the two bilins. C, bilin competition assays for holo-DrBphP assembly. The various DrBphP apoproteins were incubated with the given ratios of BV and PCB and assayed spectrophotometrically for the percentage of each chromophore that bound.
Asp\textsuperscript{207} near the chromophore reveal a polar interaction between these side chains (Fig. 4, A and B) that could impinge on the bilin deprotonation/protonation cycle during photoconversion (3, 33, 34). In this context, it is interesting to note the proposed similar role for a polar interaction between an acidic residue and the hydroxyl of Tyr\textsuperscript{176} in the control of chromophore protonation for Cph1 (39). Tyr\textsuperscript{176} lies on the opposite side of the chromophore from Tyr\textsuperscript{263} (Fig. 4, B and C), and in one standard rotamer conformation of the Asp\textsuperscript{207} side chain, an \( \sim 2.8 \) \( \text{Å} \) polar interaction would occur with Tyr\textsuperscript{176}.OH.

Our analyses of the two crystal forms of DrCBD identified a previously unanticipated dimer interface, whose stability was confirmed in solution by several methods (Fig. 3, A and B). The observations that the interface is similar in two unrelated crystal forms, is stable \textit{in vitro}, buries \( \sim 2,100–2,800 \) \( \text{Å}^2 \) (7–10% of the total solvent-accessible surface area of the monomer), clusters most of the lattice interactions in a large patch (43), and is characterized by a hydrophobic core surrounded by polar interactions (Fig. 2, A and B) (44) all lead us to suggest that this surface participates in DrBphP dimerization \textit{in vivo}. The hydrophobic residues in the \( \alpha \)4 and \( \alpha \)8 helices that form the interface (e.g. Leu\textsuperscript{146}, Leu\textsuperscript{311}, Leu\textsuperscript{314}, and Val\textsuperscript{318}) are only moderately conserved within the Phy superfamily (4, 9). However, based on the helical bundle arrangement of the \( \alpha \)4/\( \alpha \)8 interactions and the fact that the \( \alpha \)8 helix in general is enriched in hydrophobic residues, it is possible that adjacent hydrophobic contacts between symmetry mates suffice in promoting CBD dimerization of other Phys. It is also possible that the CBD interface is conserved within the Phy superfamily using different mechanisms that reflect the unique requirements of downstream signaling pathways. Within the CBD dimer, the pyrrole rings of the sister bilins are between 42 \( \text{Å} \) (C-rings) and 68 \( \text{Å} \) (A-rings) away from each other, which agrees well with an interchromophore distance measured for \textit{Synechocystis} Cph1 by fluorescence resonance energy transfer (49–63 \( \text{Å} \)) (45).

Although numerous studies have demonstrated that the C-terminal region of plant Phys contains one or more contacts for dimerization (\textit{e.g.} see Refs. 17, 46–48), our data with Dr-BphP and recent analyses of a CBD-PHY fragment from \textit{Synechocystis} Cph1 (49, 50) are the first to indicate that N-terminal dimerization site(s) also exist. Although the N-terminal interface in Cph1 has not been mapped (45), it appears to be hydrophobic and thus could involve the same \( \alpha \)4/\( \alpha \)8 helices identified here in DrCBD. Although it is likely that the HK domain promotes dimerization, it is possible that prokaryotic Phys also use the CBD for a second dimerization contact. For higher plant Phys, no contacts between sister CBDs have been detected (46, 48). Whether this distinction reflects structural differences between higher plant and microbial Phys or the need for both N- and C-terminal contacts to stabilize the quaternary structure of the plant forms is not yet known. Clearly, a reexamination of the quaternary structures of various Phy types is needed to address this issue. For example, the Y-shaped quaternary structures revealed by small angle x-ray scattering (30, 31) and electron microscopy (32) of higher plant and bacterial Phys have been interpreted solely on the basis of C-terminal contacts.

Given the importance of the N-terminal region for photochemistry and possible enzymatic output by the C-terminal HK domain (1–3), the CBD contact could play an essential role in signal transmission. The reorientation of the dimer interface we observe between the two crystal forms (Figs. 1C and 2, A and B) may be a harbinger of the kind of motions that occur during photoconversion, where changes in contact strength promote Pfr formation. In support, Strauss \textit{et al}. (49) reported that dimerization of the CBD-PHY fragment from \textit{Synechocystis} Cph1 is light-dependent, with the Pfr dimer being significantly more stable. An intriguing possibility is that the tendency of the CBD to dimerize is diminished by the presence of the PHY and/or HK domains and that this effect is augmented by phototransformation to Pfr. Secondary structure predictions of many bacterial Phys suggest that \( \alpha \)8 continues after the CBD into the PHY domain for an additional several helical turns.\textsuperscript{5} These long \( \alpha \)8 helices could reorient during photoconversion to shift the relative contributions of the CBD and HK domains to the Phy dimerization interface.

With respect to the chromophore, the higher resolution model provides a clearer picture of its structure and configuration within the bilin-binding pocket. BV ligation at the C3\textsuperscript{2} carbon of the A-ring vinyl side chain generates a saturated A-ring with a tetrahedral C2 carbon and introduces a double bond between the C3 and C3\textsuperscript{1} carbons to maintain an extended \( \pi \) conjugation system. Remarkably, this BV adduct structure is more like those proposed for PCB/PF\textsubscript{B} bound to cyanobacterial and plant Phys than previously thought, further supporting spectroscopic studies suggesting that plant and bacterial Phys are photochemically similar (3, 34).

One step in the evolution of Phy was the ability of cyanobacterial and plant Phys to discriminate between the BV precursor and its more reduced products PCB and PF\textsubscript{B} (36). Our refinement of the CBD structure, along with analysis of site-directed mutants, suggests that several steps were involved. Obviously, one prominent change was the switch of the binding site from the N-terminal cysteine upstream of the PAS domain to a cysteine within the GAF domain. We demonstrated here that this switch is sufficient to substantially alter the affinity of DrBphP for PCB \textit{versus} BV. Strikingly, a double mutant of DrBphP (C24A/M259C) bearing a cysteine at position 259 and missing the N-terminal cysteine bound PCB \( \sim 16 \) times more effectively as compared with the wild-type CBD. For DrBphP and presumably other bacterial and fungal Phys, the sulfur moiety of the N-terminal cysteine points directly toward the C3\textsuperscript{2} carbon of the vinyl side chain that ultimately forms the thioether bond with BV. In contrast, we predict based on the model for DrCBD that the sulfur moiety of the GAF cysteine in cyanobacterial and plant Phys points toward the C3\textsuperscript{1} ligation site of the PCB/PF\textsubscript{B} ethylidene side chain.

A second discriminator in bilin preference may relate to the stereochemistry of the C2 carbon. For PCB/PF\textsubscript{B}, this chirality directs the C2\textsuperscript{1} methyl group away from the GAF cysteine, which in turn could improve access of the cysteine to the C3\textsuperscript{1} position. Packing of this methyl group with residue 259 may also be important for BphPs based on the conservation of a

\textsuperscript{5} D. M. Anstrom, unpublished data.
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hydrophobic residue in this position within the BphP family (4) and our observations that the M259C mutant binds bilins but fails to generate photochemically normal Pfr. Based on the three-dimensional structural analysis of DrBphP (this report) and binding studies with modified bilin (4, 13), we do not expect the D-ring ethyl in PCB versus the vinyl in BV to be an important discriminator in bilin preference for cyanobacteria, since both side chains are similar in size and should rotate equally well in the D-ring-binding pocket.

Clearly, given that the CBD mutants tested here can conjugate bilins using either the N-terminal or GAF cysteines but that the resulting chromoproteins are poorly photochromic (supplemental Fig. 1), additional changes were also essential to evolve PCB/Phe-bound Phys with proper photochemistry. We presume that some of these changes involve slight alterations of the chromatophore-binding pocket that would facilitate light-induced rotation of PCB or Phe ligated within the GAF domain, would be needed to maintain the more linear configuration of the bilin (39), and/or would promote the deprotonation/protonation cycle essential to form Pfr (3, 34). Indeed, the R/FR spectral and photochemical properties of Phys are highly sensitive to the amino acid side chains surrounding the bilin (33).4 Hopefully, as more structures of Phys become available, especially from cyanobacteria and higher plants, the identities of these key features will become evident.

Our binding studies also address the enzymology underpinning bilin attachment. Given that both the wild type and C24A/M259C CBDs can bind bilins (at least in vitro), we propose that a special geometry of specific residues near the binding site cysteine is not needed for catalysis. Coupled with the fact that a cysteineless apoprotein (C24A) can interact and form relatively normal Pr, we propose that covalent binding is driven primarily by proper orientation of the bilin in the binding pocket. It is interesting to note that the C24A mutant of DrBphP, which cannot ligate bilins, interacts equally well with BV and PCB. Consequently, it is possible that the ability to covalently bind the bilin and not its association with the binding pocket confers much of the bilin specificity.

Our higher resolution structure of a Phy CBD now provides entry into a number of experimental approaches to help define the photochemistry and subsequent signaling by Phys. In particular, the coordinates presented here should better resolve the structure and orientation of the bilin during the Pr to Pfr phototransformation using resonance Raman spectroscopy (34, 51). The more ordered crystals may also enable the use of time-resolved x-ray studies (e.g. see Ref. 52) to define the movements of the chromophore and protein during the initial fast photochemical events predicted to follow R absorption by Pr (3, 34, 53). Our surface engineering strategy to generate well ordered crystals by minimizing conflicts between symmetry mates also illustrates the utility of this approach to improve x-ray diffraction resolution.

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