Origins of Fluorescence in Evolved Bacteriophytochromes*

Received for publication, June 16, 2014, and in revised form, September 10, 2014. Published, JBC Papers in Press, September 24, 2014, DOI 10.1074/jbc.M114.589739

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Use of fluorescent proteins to study in vivo processes in mammals requires near-infrared (NIR) biomarkers that exploit the ability of light in this range to penetrate tissue. Bacteriophytochromes (BphPs) are photoreceptors that couple absorbance of NIR light to photosimerization, protein conformational changes, and signal transduction. BphPs have been engineered to form NIR fluorophores, including IFP1.4, Wi-Phy, and the iRF series, initially by replacement of Asp-207 by His. This position was suggestive because its main chain carbonyl is within hydrogen-bonding distance to pyrrole ring nitrogens of the biliverdin chromophore, thus potentially functioning as a crucial transient proton sink during photoconversion. To explain the origin of fluorescence in these phytofluors, we solved the crystal structures of IFP1.4 and a comparison non-fluorescent monomeric phytochrome DrCBD monom. Met-186 and Val-288 in IFP1.4 are responsible for the formation of a tightly packed hydrophobic hub around the biliverdin D ring. Met-186 is also largely responsible for the blue-shifted IFP1.4 excitation maximum relative to the parent BphP. The structure of IFP1.4 revealed decreased structural heterogeneity and a contraction of two surface regions as direct consequences of side chain substitutions. Unexpectedly, IFP1.4 with Asp-207 reinstalled (IFP rev) has a higher fluorescence quantum yield (~9%) than most NIR phytofluors published to date. In agreement, fluorescence lifetime measurements confirm the exceptionally long excited state lifetimes, up to 815 ps, in IFP1.4 and IFP rev. Our research helps delineate the origin of fluorescence in engineered BphPs and will facilitate the wide-spread adoption of phytofluors as biomarkers.

Visualization of molecular processes has revolutionized the way we understand life at the cellular level. Biochemical pathways were difficult to monitor in live model systems before the advent of fluorescent biomarkers (1–4). Studying in vivo characteristics involved the use of toxic and radioactive materials and often interfered with the system being studied. Based on Dr. Frans Jöbsis’ research on tissue oxygenation (5–7), near-infrared (NIR) spectroscopy has been developed to be a highly feasible monitoring device for cellular mechanisms. Compared with visible light, NIR light is less scattered by skin, bone, or other organs and is hardly absorbed by biomolecules or water. Development of fluorescent biomarkers with NIR capabilities is, therefore, crucial to the development of in vivo imaging techniques (6, 7). Bacteriophytochromes (BPhPs) are ideally suited for this purpose for several reasons including NIR absorbance wavelength maxima, availability of the biliverdin IXa (BV) chromophore in mammalian cells, and the existence of two relatively stable photoreversible ground states (8–12). The absorption spectrum of the phytochrome class of sensory proteins is tuned by the interactions between the protein and linear tetrapyrrrole chromophore, BV, which is an intermediate in normal mammalian heme catabolism. Phytochromes are covalently linked to BV through a thioether bond to a conserved cysteine side chain; phytochromes are autocatalytic BV lyases. Available fluorescent BphPs to date, including the very recently described IFP2.0, are most successful for imaging when BV is supplemented or generated by an accessory heme oxygenase (9, 10, 13). Nonetheless, the bioavailability of BV and the lack of accessory enzymes needed to load the chromophore make BphPs an attractive design template for a mammalian NIR fluorescent biomarker (14). Moreover, the photoreversible nature of phytochromes adds another dimension of utility to these proteins as photoactivatable fluorophores (15, 16).

Phytochromes have a modular structure with the PAS, GAF, and PHY domains making up an input photosensing module (12). In many BphPs, this input module is followed C-terminally by a histidine kinase output module. Despite the thioether cys-

* This work was funded by the Draper Fund (PRJ55) and the Finnish Academy (SA Grant 138063). The atomic coordinates and structure factors (codes 408G and 41UG) have been deposited in the Protein Data Bank (http://wwpdb.org/).

† Deinococcus radiodurans.

‡ The abbreviations used are: NIR, near-infrared; Dr, Deinococcus radiodurans; BphP, bacteriophytochrome; BV, biliverdin IXα; CBD, chromophore binding domain; IRF, instrument response function.
teine location N-terminal to the PAS domain, the GAF domain houses the BV chromophore (17). These two domains thus comprise the chromophore binding domain (CBD). Absorption of a red light photon ($\lambda = 700$ nm) by the chromophore in the Pr ground state promotes an isomerization of the BV C15=C16 double bond (18). During photoconversion, the chromophore becomes transiently deprotonated. The BV D-ring movements lead to changes in the orientation and protonation state of several amino acid side chains in vicinity of the BV, which lead to local protein secondary structural changes in the PHY domain (19–23). Ultimately, the large scale movements of the output domain form the far-red light-absorbing Pfr state (23–25). Through extensive structure-guided amino acid substitutions in BphPs, it was discovered that truncation of the polypeptide chain as well as variations in the amino acid residues surrounding the chromophore significantly reduced the photoconvertability of these proteins (11, 26, 27). Absorption of red light excites the molecule, but due either to an inability to isomerize the chromophore or a disruption in the normal proton shuttle, photoconversion is blocked. In some cases, fluorescence quantum yield increases (9–11, 13, 14, 26). The utility of phytochrome-based fluorophores was explored as early as 2004 by Fischer and Lagarias (28), who introduced the apt term phytofluor. The archetypal Bph phytofluor carried a structure-based substitution of histidine for the invariant aspartic acid whose main chain carbonyl interacts with the BV A ring (26). Four rounds of directed or randomized in vitro evolution of this DrCBD-D207H led to IFP1.4 (9), which carries 12 additional substitutions (M54V, G119A, V186M, L195M, H196Q, I208T, A288V, L311K, L314G, V318R, and T135I).

Wild-type Deinococcus radiodurans BphP (DrBphP) is a dimer stabilized by a six-helix bundle interface contributed by GAF domain helices (29). To increase BphP utility as a fluorophore, residues in this GAF dimer interface have been rationally mutated in efforts to create a monomer (9, 11). Interestingly, in the case of IFP1.4, the latter rounds of random mutagenesis with selection for brighter variants led to additional changes in the dimer interface. Size exclusion chromatography demonstrated that the final IFP1.4 forms a mixed population of monomer as well as higher order oligomers (10). Our dynamic light scattering of IFP1.4 also demonstrated protein aggregation (data not shown), and recent additional directed evolution of IFP1.4 focused on stabilization of the monomeric form (13). DrCBD itself was also independently monomerized to form DrCBD$_{mon}$ by designed interruption of three favorable hydrophobic interactions in the dimer interface. Analytical ultracentrifugation confirmed the monomeric nature of DrCBD$_{mon}$ (11). The addition of D207H and a Y263F substitution both increased fluorescence of DrCBD$_{mon}$, leading to the Wi-Phy phytofluor (11).

IFP1.4 has an absorbance maximum at 684 nm and fluorescence excitation and emission maxima at 684 and 708 nm, respectively, which are blue-shifted from their respective wavelengths in the wild-type DrCBD. It was demonstrated to function as a NIR biomarker in live mice (9). However, presently available near-infrared biomarkers based on the D207H variation still have a number of properties that need to be improved, including but not limited to a propensity to photobleach and relatively low quantum yields (15).

In this paper we report the three-dimensional structures of IFP1.4 and DrCBD$_{mon}$. These structures further the collective understanding of the origin of fluorescence in BphPs. Additionally, we engineered an IFP1.4-D207 revertant, IFP$_{rev}$, and present a detailed analysis of its spectroscopic properties. In vitro, IFP$_{rev}$ is the brightest BphP-derived phytofluor to date. By measuring the fluorescence lifetime profiles of these variants we are able to distinguish site-selectively the contributions of individual amino acids to the excited state properties and, thus, to the fluorescence quenching mechanism of BV within the DrCBD ligand pocket.

**EXPERIMENTAL PROCEDURES**

**Cloning**—Standard PCR procedures were used to perform all DNA engineering. The QuikChange™ (Stratagene, La Jolla, CA) method was used to make point mutations. IFP1.4 in pBAD was shared by Prof. Roger Tsien and Dr. Xiakun Shu (University of California San Diego). The following primers were used to transfer the IFP1.4 gene into the pET21a vector: GAAAT AATT TGTGTT ACTTT AAGAA GAAGA TATAC TGGC. The Asp-207 variant of IFP1.4 was created using the following primers: GTTTT CCGGC TAGCG ATACCC CCGGC and CCGC TCGGC CGTAT CGCTA CCGGC. The DrCBD$_{mon}$/ DrCBD$_{mon}$-Y263F M54V, V186M, and A288V variants were created using the following primers, respectively: GCGAGGT-GCTCAGGTTAGCCTAACCAGGC and GCGTAGGCGTCT-ACCTGGGACACCCTCGGC; CCAGGCCCGCGAAATGATT-GGCCAGGCCC and GGCCTCGGCAATTCAGCTTCCGGT-GGCG; GGGCCCTGATCGTGTCGACACCACAGAC and GTCTGTTGGTGGCACCACAGATCGC. Clones were verified using DNA sequencing at the University of Wisconsin-Madison DNA Biotechnology Center.

**Protein Purification**—The constructs bearing DrCBD and IFP variants were transformed into BL21 (DE3) expression cells and grown at 37 °C in LB media in the presence of 0.1 mg/ml ampicillin. At an optical density of 0.6, the cells were induced with isopropyl $\beta$-D-1-thiogalactopyranoside. The cells were harvested after 4 h by centrifugation at 5000 × g and subsequently resuspended in lysis buffer (25 mM Tris buffer, pH 8.0, 50 mM NaCl). In addition the lysis buffer contained 5 mM imidazole for the DrCBD variants. Cells were sonicated and centrifuged at 40,000 × g. Cell lysate was incubated with 200 μl of 20 mM BV in the dark for 1 h. For IFP variants approximately double the amount of BV was added to the lysate followed by an overnight incubation in the dark. Proteins were affinity-purified under green light using nickel-nitrilotriacetic acid resin (Qiagen, Valencia, CA). Further purification was performed using a hydrophobic interacting phenyl-Sepharose column (GE Healthcare) to separate apo- and holophytochrome. The purified protein was subjected to absorption scans to ascertain chromophore binding (25) as well as shifts in maximum absorption of the holoprotein.
**IFP1.4 Structure**

*Spectroscopy*—UV-visible absorption scans were collected on a Beckman Coulter DU640B spectrophotometer using 1-nm steps from 250 to 800 nm.

Fluorescence excitation and emission scans were collected on a Tecan™ Infinite M1000 monochromator-based plate reader set to a 5-nm bandwidth, with samples in Greiner FLUOTRAC 200 96-well flat-bottom black plates. Emission scans were monitored between 550 and 800 nm with excitation at 676 nm for IFP1.4 and IFP<sub>rev</sub> and 696 nm for all other DrCBD<sub>mon</sub> variants. Excitation scans were collected between 350 and 800 nm with emission monitored at 705 nm for IFP1.4 and IFP<sub>rev</sub>, and 728 nm for all other variants. There was a 15- or 18-nm gap in data collection for all scans at wavelengths where the excitation and emission wavelengths coincided. The step size used for the scans was 5 nm with an additional 2-nm step size scan across the peak for newly reported variants (IFP<sub>rev</sub>, V186M, A288V, Y263F/A288V, M54V, Y263F/V186M). All samples had an absorbance of 0.25 optical density at their absorbance maxima. Emission spectra were collected for this 0.25 optical density stock as well as three dilutions in three replicates. Integrated values under each emission curve were used to calculate the fluorescence quantum yields of the variants by comparison to Cy-5 standard dye, which has a quantum yield of 0.27 in PBS (11, 30).

Absolute quantum yields were determined using the integrating sphere method in a Hamamatsu QuantaTus™ instrument (31). Three 2.5-ml replicates of each sample were prepared in 30 mM Tris buffer, pH 8.0, at an absorbance of 0.1 at 700 nm for DrCBD<sub>mon</sub> and Wi-Phy (684 nm for IFP1.4 and IFP<sub>rev</sub>). Fluorescence was excited at a wavelength of 640 nm, 0.1M phosphate citrate, pH 4.2. Crystals were cryoprotected in 12% v/v glycerol in reservoir solution. Data were compared with the 1.7 Å resolution structure of DrCBD<sub>mon</sub>, also obtained in this study (Table 1), and to other variants of DrCBD<sub>mon</sub> (32), to obtain fluorescence lifetimes. In both N-terminal residues, C-terminal histidine and the PAS-GAF linker are disordered, and in IFP1.4 knot, is identical to previously published DrCBD variant structures (29) (PDB code 4O8G) and DrCBD<sub>mon</sub> can be accessed from the Protein Data Bank (37) using the codes 4O8G and 4IJG. The Sride server (38) was used to calculate the fluorescence quantum yields of the variants by comparison to Cy-5 standard dye, which has a quantum yield of 0.27 in PBS (11, 30).

**Table 1**

| Data collection | IFP1.4 (PDB code 4O8G) | DrCBD<sub>mon</sub> (PDB code 4IJG) |
|----------------|------------------------|-----------------------------------|
| Cell dimensions | a, b, c (Å)             | 96.2, 53.3, 66.8                  |
|                | α, β, γ (°)             | 90.0, 90.6, 90.0                  |
| Resolution (Å)* | 30.0-1.6 (1.65-1.62)    | 25.0-1.7 (1.75-1.70)              |
| Mosaicity (%)   | 0.34                   | nr                                |
| I/σ             | 0.068 (0.22)            | 0.069 (0.21)                      |
| Redundancy (%)  | 93.7 (99.9)             | 95.2 (77.0)                       |
| Wilson B (Å<sup>2</sup>) | 16.5                 | 20.5                              |

*Values in parentheses represent the highest resolution shell.

**RESULTS**

**Crystal Structure**

Overall—To understand the characteristic fluorescent behavior of IFP1.4, a high resolution crystal structure of the protein was solved and refined against 1.6 Å resolution data. The structure was compared with the 1.7 Å resolution structure of DrCBD<sub>mon</sub> also obtained in this study (Table 1), and to other variants of DrCBD (11, 17, 29).

The overall architecture of IFP1.4 and DrCBD<sub>mon</sub> with canonical PAS and GAF domains and a distinctive figure-eight knot, is identical to previously published DrCBD variant structures (Fig. 1A). In both N-terminal residues, C-terminal histidines and the PAS-GAF linker are disordered, and in IFP1.4 there is an additional break in the main chain density at a connecting loop (107–108). Electron density is unambiguous for 11 of 12 expected amino acid substitutions (T135I falls within the disordered linker). Alignment of the three-dimensional structures showed that there is an overall root mean square deviation of 0.38 Å between IFP1.4 and DrCBD<sub>mon</sub> for all Ca atoms. The
integrity of the chromophore binding pocket is maintained in IFP1.4, and the D-ring tilt relative to B and C rings is within the range seen in other structures (data not shown). Polar interactions with the B and C rings are conserved; the B-ring propionate forms a salt bridge with Arg-254 and hydrogen-bonds with Tyr-216 and Ser-257, whereas the C-ring propionate hydrogen-bonds with His-260, Ser-272, and Ser-274 (Fig. 1, A and B). The plane of the His-260 imidazole also forms a perfect Van der Waals packing platform for the planar B and C rings and the methine bridge that connects them.

**D-ring Packing and Wavelength Shift**—The most striking change in the structure is the emergent tightly packed hydrophobic hub in the chromophore binding pocket of IFP1.4, formed by the novel Val-288 and Met-186 side chains, the original Met174 residue, the D-ring, and the methyl group of the C-ring. This hub locks the D-ring in place and likely prevents relaxation through photoconversion (Fig. 2A). Other amino acids undergo subtle shifts (tenths of an Å) between the two structures and contribute to the packing around the D-ring. These include Tyr-263 and Met-267. Additional amino acids, whose positions are not significantly altered between the structures, further stabilize the D-ring position. These include Tyr-176, Phe-203, and Phe-198 (Fig. 2A).

To determine the relative effects of amino acid changes within the D-ring hub on IFP1.4 fluorescence, we installed the A288V substitution in both DrCBD\textsubscript{mon} and DrCBD-Y263F and found increased fluorescence in both cases. V186M, on the other hand, had the striking effect of blueshifting the 700-nm absorption and excitation maxima of DrCBD\textsubscript{mon} to 692 nm.

**FIGURE 1.** Structure of IFP1.4. A, architecture of IFP1.4 is almost identical to previously published DrCBD structures. A protein knot keeps the PAS domain and N-terminal residues (lime) packed against the GAF domain (mint). BV (cyan), covalently attached at Cys-24, is nestled in the GAF domain. B, polar contacts to BV (dotted lines) are unchanged, whereas new and formerly observed hydrophobic contacts with the chromophore (starbursts; green for IFP1.4, blue for DrCBD\textsubscript{mon}) stabilize the D ring. In particular, Met-186 and Val-288 are absent in the wild-type counterpart DrCBD\textsubscript{mon}.

**FIGURE 2.** The evolved hydrophobic hub leads to excitation wavelength shift. A, hydrophobic interactions of conserved and novel (Met-186, Val-188) side chains restrict the D ring rotation. Notably, Val-288 adopts two rotamers, one of which interacts preferentially with Met-174, whereas the other stabilizes the C-ring methyl group (13). B, a single amino acid change of V186M is sufficient to blue-shift the excitation wavelength maximum to 692 from 701. Scans shown are from 5-nm step-size excitation (monitored at 713 nm) and emission (excited at 645 nm) scans.
Quantum yield measurements and fluorescence lifetimes

| Protein variant                  | Absorbance maximum | Excitation maximum | Emission maximum | Φ* | ε at absorbance max | Brightness | Lifetime $^b$ |
|---------------------------------|---------------------|--------------------|------------------|----|---------------------|------------|--------------|
|                                | nm                  | nm                 | nm               | % | µmol/cm$^2$ s$^{-1}$ | %          | ps           |
| DrCBD$_{mon}$                  | 698                 | 698                | 718              | 2.9 ± 0.1 | 133,199             | 100        | 390 ± 30 (77%) |
| DrCBD$_{mon}$Y263F$^c$          | 701                 | 700                | 722              | 4.0 ± 0.1 | 128,250             | 132        | 620 ± 70 (33%) |
| Wi-Phy$^d$                      | 701                 | 700                | 722              | 6.3 ± 0.2 | 117,947             | 192        | 670 ± 10      |
| IFP1.4                         | 684 ± 1             | 685 ± 5            | 708 ± 5          | 7.0 ± 0.3 | 130,533             | 236        | 800 ± 10      |
| IFP1.4A$^e$                     | 684 ± 1             | 685 ± 5            | 708 ± 2          | 8.7 ± 0.1 | 131,473             | 296        | 815 ± 11      |
| DrCBD$_{mon}$A288V              | 701 ± 1             | 700 ± 5            | 722 ± 2          | 4.2 ± 0.2 | 126,566             | 138        | ND           |
| DrCBD$_{mon}$Y263F/A288V        | 701 ± 1             | 700 ± 5            | 722 ± 2          | 4.6 ± 0.1 | 130,210             | 155        | ND           |
| DrCBD$_{mon}$Y263F/V186M        | 692 ± 1             | 692 ± 2            | 708 ± 2          | 6.2 ± 0.1 | 135,619             | 91         | ND           |
| DrCBD$_{mon}$M54V               | 701 ± 1             | 700 ± 5            | 720 ± 2          | 3.0 ± 0.1 | 91,000              | 101        | ND           |

$^a$ Fluorescence quantum yield measurements were obtained by integration in two independent laboratories and by the absolute quantum yield method for samples 1–5 (rankings were all the same, and absolute quantum yield values are reported) and by integration for samples 6–9.

$^b$ The weighted mean square deviations (the $\chi$ value), providing information about the goodness of fits, stayed in all cases in the range 1.02–1.06. The error margins are the result of fitting.

$^c$ Absorbance, excitation, and emission maxima as reported in Ref. 11.

IFP1.4 Structure

### Table 2

Quantum yield measurements and fluorescence lifetimes

| Protein variant                  | Absorbance maximum | Excitation maximum | Emission maximum | Φ* | ε at absorbance max | Brightness | Lifetime $^b$ |
|---------------------------------|---------------------|--------------------|------------------|----|---------------------|------------|--------------|
|                                | nm                  | nm                 | nm               | % | µmol/cm$^2$ s$^{-1}$ | %          | ps           |
| DrCBD$_{mon}$                  | 698                 | 698                | 718              | 2.9 ± 0.1 | 133,199             | 100        | 390 ± 30 (77%) |
| DrCBD$_{mon}$Y263F$^c$          | 701                 | 700                | 722              | 4.0 ± 0.1 | 128,250             | 132        | 620 ± 70 (33%) |
| Wi-Phy$^d$                      | 701                 | 700                | 722              | 6.3 ± 0.2 | 117,947             | 192        | 670 ± 10      |
| IFP1.4                         | 684 ± 1             | 685 ± 5            | 708 ± 5          | 7.0 ± 0.3 | 130,533             | 236        | 800 ± 10      |
| IFP1.4A$^e$                     | 684 ± 1             | 685 ± 5            | 708 ± 2          | 8.7 ± 0.1 | 131,473             | 296        | 815 ± 11      |
| DrCBD$_{mon}$A288V              | 701 ± 1             | 700 ± 5            | 722 ± 2          | 4.2 ± 0.2 | 126,566             | 138        | ND           |
| DrCBD$_{mon}$Y263F/A288V        | 701 ± 1             | 700 ± 5            | 722 ± 2          | 4.6 ± 0.1 | 130,210             | 155        | ND           |
| DrCBD$_{mon}$Y263F/V186M        | 692 ± 1             | 692 ± 2            | 708 ± 2          | 6.2 ± 0.1 | 135,619             | 91         | ND           |
| DrCBD$_{mon}$M54V               | 701 ± 1             | 700 ± 5            | 720 ± 2          | 3.0 ± 0.1 | 91,000              | 101        | ND           |

$^a$ Fluorescence quantum yield measurements were obtained by integration in two independent laboratories and by the absolute quantum yield method for samples 1–5 (rankings were all the same, and absolute quantum yield values are reported) and by integration for samples 6–9.

$^b$ The weighted mean square deviations (the $\chi$ value), providing information about the goodness of fits, stayed in all cases in the range 1.02–1.06. The error margins are the result of fitting.

$^c$ Absorbance, excitation, and emission maxima as reported in Ref. 11.

(Fig. 2B, Table 2) without any positive impact on fluorescence quantum yield (Table 2).

**Polar Chromophore Water Interactions**—The Asp-Ile-Pro motif is a highly conserved structural motif found in canonical phytochromes. The main chain carbonyl of Asp-207 has been implicated as a proton sink in both the proton release and uptake required for normal photoconversion as well as during non-radiative decay via excited state proton transfer due to its proximity to all three protonated ring nitrogens and the highly ordered pyrrole water (Fig. 3) (26, 27, 40, 41). The water-mediated hydrogen-bonding network also extends between the pyrrole ring nitrogens of the chromophore and the Nδ1 of His-260 (Fig. 3A). The partial negative charge of the carbonyl oxygen of Asp-207 and the Nδ1 of His-260 would aid in stabilizing the protonated chromophore (41, 42). In IFP1.4, amino acids 207 and 208 have been altered to His and Thr, respectively. The impact of His-207 has been previously discussed and could be on positioning of Tyr-263 and/or interactions with the D-ring hydroxyl in the Pfr form (11). Additionally in IFP1.4 the novel hydroxyl group of Thr-208 makes a hydrogen-bond to the main chain carbonyl of residue 207 (Fig. 3B). This polar contact potentially affects the excited state proton transfer pathway. The direct interaction between Thr-208 and the chromophore is weak, consisting only of a long (3.5 Å) hydrogen-bond between the Thr-OH and the C-ring nitrogen. In DrCBD$_{mon}$ a second water molecule was found in the binding pocket (Fig. 3B) in space made available by the repositioning of Tyr-263 to the most distal position in which it has been observed (not shown).

**Increased Rigidity and Compactness**—The M54V substitution was isolated in the context of the dimeric phytochrome in the second stage of the in vitro evolution of IFP1.4 and provided a 32% increase in fluorescence quantum yield (9). By aligning seven DrCBD structures we are able to conclude that the parental Met at position 54 can adopt one of three rotamers, placing the Cγ of the side chain in one of two positions with the consequence that the short PAS domain helix α2 can also occupy one of two positions (Fig. 4A). The Val substitution in IFP1.4 mimics only one of these two and thus promotes a more compact and less heterogeneous structure (Fig. 4A).

Limiting conformational alternatives of the α2 helix only indirectly improves fluorescence quantum yield in IFP1.4. We found upon installing the M54V substitution in DrCBD$_{mon}$ there was no increase in brightness (Table 2). Thus increased rigidity of the protein decreases the chance of thermal dissipation of energy and leads to increased fluorescence only in a
phytochrome that has a higher than background probability of non-radiative decay.

We discovered two alternative main chain configurations for the loop region between residues 192 and 201 containing the Met-195 and the Gln-196 variants. In the more clearly defined main chain alternative, this region is 1.5 Å closer to the chromophore in comparison to DrCBDmon (Fig. 4B). Met-195 and Gln-196 are thus a second example of a change that increases phytofluor fluorescence by stabilizing a single rigid protein conformation.

Time-resolved Fluorescence

Excited state lifetime measurements provide information about fluorescence properties of phytofluors independent of the concentration of the sample. The fluorescence decay properties of BV molecules in the binding pocket were studied by a photon-counting method with an excitation wavelength of 660 nm and monitoring wavelength of 714 nm (Fig. 5). The fluorescence decays were fitted with either mono- or biexponential functions (32) to obtain the excited state lifetimes of particular DrCBD constructs. We found two categories; in the first, the decay profile of the non-fluorescent monomer required two exponential components, whereas in the case of fluorescent variants Wi-Phy, DrCBD-Y263F, IFP1.4, and IFPrev, monoexponential fits were sufficient to describe the fluorescence decay (Table 2). Thus, more decay components are present in the first category than in the second. As predicted, the excited state lifetime measurements yield longer lifetimes, up to 815 ps, for the variants with the highest independently measured fluorescence quantum yields (Table 2). However, we did not find a linear dependence between the excited state lifetimes and fluorescence quantum yields across the entire family of phytochrome variant proteins measured in this study. The lack of such a correlation indicates a variation in the radiative lifetimes that apparently depends on the chromophore environment created by individual amino acid substitutions.

DISCUSSION

Taking advantage of time-resolved and steady state fluorescence spectroscopy as well as the time-averaged technique of protein crystallography, we present evidence for three pathways by which fluorescence quantum yield is increased in BphP-based phytofluors. In the first, the chromophore is rigidified by van der Waals packing interactions with neighboring side chains, particularly in the D-ring. The excited state chromophore was then prevented from C15=C16 double bond rotation, and fluorescence quantum yield was increased (Fig. 2). This interpretation agrees perfectly with the recent computational and spectroscopic results on fluorescence in the RpB-phP2-derived phytofluors (43). In the second mechanism changes to the local network of polar interactions between solvent, protein, and BV lessen the likelihood for excited state proton transfer, thus increasing fluorescence quantum yield (Fig. 3). The third mechanism is less direct but also minimizes non-radiative decay. It involves the structural repositioning of amino acids not in direct contact with the chromophore. Such repositioning can have the effect of lessening structural heterogeneity and/or increasing rigidity of the protein, thus decreasing thermal relaxation (Fig. 4A). It also has the consequence of reducing solvent and ion access to the chromophore binding pocket. For example, residues 195–196 are pulled inward 1.5 Å...
in IFP1.4 compared with DrCBD<sub>mon</sub>, excluding a solvent glycerol (Fig. 4B).

One consequence of the selection scheme used to optimize IFP1.4 was that the procedure optimized for fluorescence based on FACS sorting using a shorter excitation wavelength than the absorption maximum of DrCBD (9). It is thus not surprising that the resulting phytofluor has an excitation maximum of 684 nm. We have shown that a single change (V186M) in the hydrophobic environment around the BV D-ring was sufficient to cause blue-shifting (Fig. 1) and moreover that there is no gain of fluorescence intensity when this side chain is added to DrCBD<sub>mon</sub>-Y263F (Table 2). This is a welcome result given the impetus to find NIR phytofluors with high quantum yield and long wavelength optima.

On replacement of Asp-207 by His, DrCBD exhibits near-IR fluorescent capabilities (9, 11, 26). Recent work has demonstrated that reversal of the His residue to Asp in the Asp-Ile-Pro motif had no impact on the fluorescent quantum yield of the DrCBD<sub>mon</sub>-Y263F variant (11). *Rhodopseudomonas palustris* BphP3 is naturally fluorescent with an Asp at this position (27, 44, 45). We thus studied the solution behaviors of DrCBD variants with either Asp or His at position 207 and discovered that IFP1.4-Asp-207 (IFP1.4<sub>Asp</sub>) is ~30% more fluorescent than its progenitor with a quantum yield of nearly 9% in comparison to the 7% of IFP1.4 (Table 2). The underlying reason for the increased quantum yield of IFP<sub>rev</sub> is not obvious. pK<sub>a</sub> differences between the His and Asp side chains could have differential effects in the subtly different chromophore environments of the now multiply described NIR phytofluors and warrant further investigation. Alternatively, the steric blocking of the second water in the center of the BV by the interaction of Tyr-263 with His-207 may be the relevant variable. This interaction has been noted previously (11). We found IFP1.4 and Wi-Phy showed higher variability in quantum yield measurements between experimental techniques and investigators than IFP1.4<sub>rev</sub> or DrCBD<sub>mon</sub>, which were consistent. We concluded that the presence of His-207 position leads to sensitivity to even slight changes in pH, ion concentration, illumination flux, or other possible experimental variations. In any case, our steady state and time-resolved fluorescence measurements clearly indicate the His-207 residue is not necessary for enhanced fluorescence of BphPs.

Typically, DrCBD wild-type samples show biexponential fluorescence decay profiles (32), suggesting either a heterogeneous population of molecules in the ground state or a second fluorescent species that appears in the excited state of the BV. The fluorescence lifetime of a molecule is extremely sensitive to local environmental changes. Such heterogeneity can be manifest on very small distance and time scales, for example slight repositioning of the pyrrolye water and/or a small rotation of the D-ring. Comparing DrCBD<sub>mon</sub> constructs to more rigid IFPs reveals some interesting behavior. Quantum yields and fluorescence lifetimes are increased (Table 2). Any disturbance of the π-system, e.g. blocking the torsional vibrations of the C15=C16 double bond of the methine bridge between rings C and D, causes these values to increase. This can be explained by the increase in the σπ-interaction of and the number and strength of the hydrogen bonds between the protein and D-ring. Spectroscopic studies confirmed the spatial observations of x-ray crystallography. The excited state lifetimes for fluorescent DrCBD derivatives are consistently longer and monoexponential compared with the value found for CBDs from *Deinococcus radiodurans* (32) and *R. palustris* (45). However, Cph1 lifetimes have been reported as long as 1.8 ns at room temperature (46, 47). The longer decay lifetime and monoexponential decay behavior in the case of the DrCBD<sub>mon</sub>-Y263F variant indicates greater rigidity compared with the wild type and/or that the wild type lacks any type of fluorescent photoproduc. In the case of IFP1.4 and IFP<sub>rev</sub>, the time constants are longer than observed in any other Dr phytochrome variants. The considerably longer lifetimes together with the blue-shifted absorption and emission wavelengths of the IFP1.4 proteins compared with the other DrCBD systems arise from the hydrophobic hub and thus confirm the hub forms in solution as well as in a crystalline environment.

Both IFP1.4 and Wi-Phy contain the D207H mutation. Interestingly, pairwise comparison of the corresponding constructs with and without D207H replacement (IFP1.4 versus IFP1.4<sub>rev</sub> and Wi-Phy versus DrCBD<sub>mon</sub>-Y263F) reveals similar excited state lifetimes yet different fluorescent quantum yields. Such an effect suggests changes in the quenching mechanism between members of each matched pair. In agreement with our crystal structures, small changes in the charge distribution in the BV binding pocket may lead to variation, for example in the access of solvent ions to the binding pocket, and therefore change the nature of the quenching pathway.

In all of the phytofluors studied here, the quantum yield for *bona fide* photoconversion is essentially zero (Ref. 11 and data not shown), yet the fluorescence quantum yield does not exceed 10%. This discrepancy indicates a large contribution for non-radiative decay processes of the excited state of the BV molecule. Our structural and spectroscopic data indicate there are several avenues for further improvement in the desirable properties of NIR biomarkers based on bacterial phytochromes. High temporal and spatial resolution will be needed to understand and minimize the pathways for excited state proton transfer and static and dynamic quenching mechanisms.

**Acknowledgments—**We thank Professor Felix Castellano and Dr. Rony S. Khnayzer for generous assistance with absolute quantum yield measurements, Professor Robert Landick for equipment access, and Dr. Kenneth Satyshur for technical assistance. Use of the Advanced Photon Source, an Office of Science User Facility operated for the United States Dept. of Energy (DOE) Office of Science by Argonne National Laboratory, was supported by the United States DOE under Contract DE-AC02-06CH11357. Use of the LS-CAT Sector 21 was supported by the Michigan Economic Development Corp. and the Michigan Technology Tri-Corridor (Grant 08SP1000817).

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