Fluorescence of Phytochrome Adducts with Synthetic Locked Chromophores*

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We performed steady state fluorescence measurements with phytochromes Agp1 and Agp2 of Agrobacterium tumefaciens and three mutants in which photoconversion is inhibited.

These proteins were assembled with the natural chromophore biliverdin (BV), with phycoerythrobilin (PEB), which lacks a double bond in the ring C-D-connecting methine bridge, and with synthetic bilin derivatives in which the ring C-D-connecting methine bridge is locked. All PEB and locked chromophore adducts are photoinactive. According to fluorescence quantum yields, the adducts may be divided into four different groups: wild type BV adducts exhibiting a weak fluorescence, mutant BV adducts with about 10-fold enhanced fluorescence, adducts with locked chromophores in which the fluorescence quantum yields are around 0.02, and PEB adducts with a high quantum yield of around 0.5. Thus, the strong fluorescence of the PEB adducts is not reached by the locked chromophore adducts, although the photoconversion energy dissipation pathway is blocked. We therefore suggest that ring D of the bilin chromophore, which contributes to the extended π-electron system of the locked chromophores, provides an energy dissipation pathway that is independent on photoconversion.

Phytochromes are photoreceptors of plants, bacteria, and fungi, which are most sensitive to red and far-red/near-infrared light. A typical phytochrome consists of an N-terminal chromophore module that bears the PAS (Per-Arnt-Sim (1)), GAF (cGMP phosphodiesterase, adenylyl cyclase and transcriptional factor EhA (2)) and PHY (phytochrome-specific) domains and a C-terminal output module with a histidine kinase or a histidine kinase-like domain (Fig. 1). Each phytochrome protein subunit carries one bilin chromophore that is covalently bound to a conserved cysteine residue. The PAS/GAF PHY chromophore module is sufficient for chromophore incorporation and full spectral activity (3). Light absorption triggers the photoconversion between the so-called Pr and Pfr forms, which have respective absorption maxima in the red and far-red spectral regions. The first step in the photoconversion is a Z to E isomerization around the double bond between pyrrole rings C and D of the bilin chromophore. In darkness, both forms are kinetically stable or have rather long interconversion times (4). The type of bilin chromophore differs between species. In cyanobacteria, two chromophore binding modes are realized: the natural chromophore of the Cph1/CphA-type phytochromes is PCB, whereas CphB-type phytochromes incorporate BV. This bilin also constitutes the chromophore of most bacterial and probably all fungal phytochromes. A third bilin, PdB, serves as a chromophore in plant phytochromes. In BV-binding phytochromes the chromophore binding cysteine residue lies in the N-terminal part of the PAS domain, whereas PCB- and PdB-binding phytochromes have their chromophore-binding cysteine in the GAF domain (5). Apart from this major difference, the chromophore pockets of BV- and PCB-binding phytochromes are very similar as judged from the x-ray structures of recently crystallized representatives (6–11). Sequence alignments also show that amino acid residues of the chromophore pocket are highly conserved.

BV, which is synthesized from heme by a one-enzyme reaction, is regarded as the most ancient chromophore. PdB and PCB are synthesized via additional enzymes that reduce one and two double bonds of BV, respectively. These reductions result in a less conjugated system and hence in a blue shift of the respective adducts. The absorption maxima of typical BV-, PdB-, and PCB-binding phytochromes are at 700, 665, and 655 nm, respectively. This kind of spectral tuning has probably evolved as an adaptation to a chlorophyll a-rich environment. Many variations of the prototypical phytochrome have been described. Several cyanobacterial phytochrome-like proteins utilize, for example, the GAF domains for chromophore binding, which they have probably inherited from a typical phytochrome (12). Domain rearrangements have also been described for fungal and bacterial BV-binding phytochromes (13, 14). Most phytochromes have a Pr ground state, but bacterial phytochromes with a Pfr ground state, so called

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3 The abbreviations used are: Pr, red-absorbing form of phytochrome; Pfr, far-red-absorbing form of phytochrome; BV, biliverdin; PdB, phytochromobilin; PCB, phycocyanobilin; PEB, phycoerythrobilin; Agp, Agrobacterium tumefaciens phytochrome; Cph, cyanobacterial phytochrome.
bathy phytochromes, have been found in *Bradyrhizobium* sp., *Rhodopseudomonas palustris* (15), *Agrobacterium tumefaciens* (16), and *Pseudomonas aeruginosa* (17) as well as other rhizobial species (18).

Early studies on plant phytochromes have shown that the fluorescence quantum yield of Pr is low at ambient temperature but is increased significantly under cryoconditions where photoconversion is blocked (19). Adducts that fluoresce strongly at ambient temperature have been obtained by chromophore replacement. Phycoerythrobilin (PEB), a bilin chromophore of the phycoerythrin antenna pigment of cyanobacteria and red algae, lacks the C15 = C16 double bond between pyrrole rings C and D (Fig. 2). This bilin is incorporated into apophytochromes, but the adducts are photoinactive and highly fluorescent (20). A quantum yield of 0.82 has been reported for the PEB adduct of cyanobacterial phytochrome Cph1 (20). Feeding PEB to plant mutants with an impaired chromophore biosynthesis enabled the detection of phytochrome by fluorescence microscopy within the cell (20, 21) and studies on dimer subunit interactions of Cph1 by means of fluorescence resonance energy transfer (22). An adduct of *P. aeruginosa* phytochrome PaBphP with 15,16-dihydrobiliverdin, which also lacks the ring C-D-connecting double bond, is also fluorescent (17). The inhibition of photoconversion by low temperature or chromophore replacement correlates with an increase of Pr fluorescence, indicating that photoisomerization provides a major route of energy dissipation. Fluorescence is also increased in phytochrome mutants with impaired photoconversion; the Y166H mutant of cyanobacterial phytochrome Cph1, in which photoconversion is inhibited, has a fluorescence quantum yield of >0.1 (23). The homologous mutation resulted in the same fluorescence increase in plant phytochromes but not in BV-binding bacterial phytochromes (24). Increased fluorescence has been reported for mutants of *Deinococcus radiodurans* and *P. aeruginosa* phytochromes DrBphP and PaBphP, respectively, in which a highly conserved Asp residue (Asp-207 in DrBphP) was replaced by Ala (9, 25). The D197A mutant of the DrBphP PAS/GAF fragment has been used for directed evolution employing DNA shuffling. A 13-residue mutant resulting from that approach had an increased fluorescence quantum yield of 0.07. This variant has been successfully employed as a marker protein with advantageous properties in mammalian organs (26).

Adducts have been prepared with locked chromophores in which rings C and D are held in a fixed geometry by an additional carbon bridge. They cannot undergo photoconversion...
and are thus comparable with the above mentioned PEB and 15,16-dihydrobiliverdin adducts. However, such adducts with locked chromophores have longer wavelength absorption maxima because of their extended π-system, a property that is in line with the demand for novel fluorescent dyes. In this article, we have characterized the fluorescence properties of several different adducts with bacterial phytocromes and locked chromophores. These studies show that the lock increases fluorescence significantly but not to the same extent as the loss of a C15=C16 double bond. This difference suggests that the D ring provides a radiationless energy dissipation pathway.

**MATERIALS AND METHODS**

**Phytochrome Expression Vectors, Protein Expression, and Purification**—All proteins (Cph1, Agp1, Agp1-V249C, Agp1-M15-D197A, Agp1-H280A, Agp2, and Agp2_Phyl) were expressed in *Escherichia coli* X11blue or BL21(DE3) cells. The expression vectors encode for proteins with a C-terminal poly-His tag. Expression vectors pF10.his, pAg1, and pAg2, which encode for Cph1, Agp1, and Agp2, respectively, are described elsewhere (27–29). The expression vectors for the Agp1-V249C mutant, which binds phycoerythrobilin in a covalent manner, and for the Agp1-M15-D197A mutant are described elsewhere (30, 31). An expression vector for the Agp1-H280A mutant was made with the QuikChange site-directed mutagenesis kit (Stratagene). The expression vector pAg2-Phy1 encodes for a domain-swap mutant termed Agp2_Phyl. In this protein, the PHY domain of Agp2 (amino acids 315–495) is replaced by the PHY domain of Agp1 (amino acid 315 to 495). The vector pAg2-Phy1 was cloned as follows. The sequence coding for the PHY domain of Agp2 was amplified by PCR using *Phusion* polymerase (Finnzymes, Espoo, Finland) and the following phosphorylated primers: BZ22.1 5'-CTCGC-ACCATCAACAGCCCATG-3' and BZ22.2 5'-CTCGC-TGTGGTGGAACGCCAC-3'. Then, another PCR reaction was performed with the pET21b (+) (Novagen, Madison, WI)-based Agp2 expression plasmid, pAg2 (3), using the primers BZ22.1 5'-TTGATGGCAAGGGAAGAGACG-3’ and BZ22.2 5'-GTCGAGGGCGCCTTCTGCTTC-3'. In this reaction, the entire plasmid DNA except for the sequence coding the PHY domain of Agp2 was amplified. Both blunt end PCR products were ligated with the T4 ligase (New England Biolabs, Beverly, MA) overnight at 15 °C (32) and transformed directly into the *E. coli* expression strain BL21(DE3). The correctness of the new expression vector was confirmed by DNA sequencing.

For protein expression, *E. coli* cells containing the desired plasmid were grown in LB medium with ampicillin at 37 °C until the *A_600 nm* reached about 0.6. Then specific protein expression was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside (20 μg/ml). The cultures were further grown at 16 °C until the *A_600 nm* reached about 3. Thereafter, cells were collected by centrifugation, washed with basic buffer (50 mM Tris–HCl, 300 mM NaCl, 5 mM EDTA, pH 7.8), and extracted with basic buffer. Proteins of the soluble fraction were precipitated with ammonium sulfate and resuspended in 10 mM imidazole, 50 mM Tris–HCl, 300 mM NaCl, pH 7.8. The His-tagged phytochrome proteins were then purified by Ni²⁺ affinity chromatography. Following elution from the column with a buffer containing 250 mM imidazole, the phytochrome-containing fractions were again subjected to ammonium sulfate precipitation, and the protein was finally suspended in basic buffer. Details on the purification steps are given in earlier publications (28, 33–35).

**Chromophores and Chromophore Assembly**—BV was obtained from Frontier Scientific. PEB was extracted from *Porphyridium cruentum* and purified by high pressure liquid chromatography as described elsewhere (35, 36). Synthesis of the locked biliverdin chromophores 15Za and 15Ea (37, 38), of the doubly locked chromophores 5Za15Ea and 5Ea15Ea (39), and of 5Zs15Za (40) is described elsewhere. All chromophores were dissolved in dimethyl sulfoxide at concentrations of about 4 mM. The exact stock concentrations were estimated by measuring the absorption of the samples diluted in methanol/HCl. The following extinction coefficients (at λ_max) were used: *ε_BV* = 30,800 M⁻¹ cm⁻¹ (41), *ε_PEB* = 25,200 M⁻¹ cm⁻¹ (42), *ε_15Za* = 40,300 M⁻¹ cm⁻¹, *ε_15Ea* = 51,300 M⁻¹ cm⁻¹, *ε_61Za15Ea* = 61,488 M⁻¹ cm⁻¹, *ε_5Ea15Ea* = 41,461 M⁻¹ cm⁻¹, and *ε_5Zs15Za* = 83,400 M⁻¹ cm⁻¹. The values for locked chromophores were obtained from exactly weighed, pure, solid material after dissolution in methanol/HCl.

Chromophore assembly was initiated by adding 2–3 μl of chromophore from the stock solution to 1 ml of protein, which was adjusted to about 10 μM to achieve an equimolar concentration of protein and chromophore. The assembly process was followed by measuring absorption spectra at different time points until no further absorption changes were evident. Thereafter, free chromophore was separated from the protein by using desalting columns (NAP-10 columns, GE Healthcare). Most if not all chromophore remained bound to the protein after the separation. The columns were equilibrated with basic buffer, and 1 ml of holoprotein was loaded on the column. The flow-through was discarded. Thereafter, 1.5 ml of basic buffer was applied on the column, and the flow-through, containing the protein, was collected. Free chromophore was not eluted because of its low molecular weight and its tight interaction with the matrix. Spectra were recorded before and after the separation.

**UV-visible Spectroscopy, Fluorescence Spectroscopy, and Quantum Yield**—All UV-visible measurements were performed at 20 °C using a JASCO V-550 photometer. Fluorescence spectra were recorded by using a Yobin-Yvon FluoroMax2 spectrofluorimeter at 20 °C. To avoid cross-talk and scattering artifacts, the emission monochromator was shielded with an appropriate cut-off filter (RG570, RG665, or RG695, thickness of 3 mm; Schott, Mainz, Germany). Slit widths of excitation and emission were set between 2 and 7 nm according to the fluorescence intensities. Corrected excitation and emission spectra were obtained considering the spectral characteristics of the lamp, the filter, and the detector. The fluorescence quantum yield Φ of each sample was calculated by integrating its emission spectrum and referring it to fluorescein as a standard (Φ = 0.95 (43)). Agp2-BV was irradiated with far-red light (780 nm from a light-emitting
TABLE 1

Absorption and fluorescence characteristics of free chromophores in aqueous buffer at pH 7.8 and after the addition of 0.5 M HCl from a 10 M stock solution at pH 1.

| Chromophore         | Absorption $\lambda_{max}$ | Excitation $\lambda_{max}$ | Emission $\lambda_{max}$ | Stokes shift | $\phi$  |
|---------------------|-----------------------------|-----------------------------|---------------------------|--------------|--------|
| Fluorescein         | 490                         | 488                         | 574                       | 86           | 0.95   |
| PEB, pH 7.8         | 531                         | 535                         | 579                       | 44           | 0.0011 |
| PEB, pH 1           | 587                         | 580                         | 613                       | 33           | 0.0016 |
| BV, pH 7.8          | 673                         | ND                          | ND                        | ND           | ND     |
| BV, pH 1            | 629                         | ND                          | ND                        | ND           | ND     |
| 15Za, pH 7.8        | 618                         | ND                          | ND                        | ND           | ND     |
| 15Za, pH 1          | 678                         | ND                          | ND                        | ND           | ND     |
| 15Ea, pH 7.8        | 631                         | 632                         | 708                       | 76           | 0.000003 |
| 15Ea, pH 1          | 679                         | 640                         | 683                       | 43           | 0.000013 |
| 5Za15Ea, pH 7.8     | 653                         | 654                         | 661                       | 7            | 0.000026 |
| 5Za15Ea, pH 1       | 656                         | 655                         | 672                       | 17           | 0.000006 |
| 5Ea15Ea, pH 7.8     | 609                         | 647                         | 669                       | 22           | 0.000046 |
| 5Ea15Ea, pH 1       | 644                         | 640                         | 668                       | 28           | 0.000099 |
| 5Za15Za, pH 7.8     | 653                         | 660                         | 674                       | 14           | 0.000004 |
| 5Za15Za, pH 1       | 706                         | ND                          | ND                        | ND           | ND     |

The absorption, excitation, and emission maxima of the Q-band are given. Fluorescein (43) was used as a reference for calculating the quantum yields ($\phi$). ND, not detectable.

RESULTS

Fluorescence Properties of Free Chromophores—Because of the free mobility of the pyrrole rings, the fluorescence of free bilin molecules is very weak (44, 45). Incorporation into the chromophore pocket of a biliprotein such as phycocyanin or phytochrome restricts this mobility and results in an increase of the fluorescence quantum yield. We initially measured the fluorescence properties of free chromophores under the buffer conditions that were later been used for measurements of phytochrome adducts (Table 1). The fluorescence of BV and the locked chromophore 15Za was not measurable under these conditions, we assume that the fluorescence quantum yields are below $10^{-6}$. The other chromophores had quantum yields in the range of $10^{-5}$ to $10^{-4}$, and the quantum yield of free PEB was around $10^{-3}$. Because in the chromophore pocket of phytochromes the bilins are in the protonated form, the fluorescence of free chromophores was also measured after acidification by HCl. This treatment changed the absorption and fluorescence properties of all tested chromophores. As stated by Falk (45), acidification of bilins is generally accompanied by a red shift of the absorption maximum. All chromophores in the present study, with the exception of BV, follow this rule, although the red shifts of 5Za15Ea and 5Zs15Za are only 3 and 13 nm, respectively. The blue shift of BV upon acidification is in contrast to other work (46) but might be related to the aqueous solvent used in the present control measurements.

The fluorescence quantum yields of the locked chromophores are generally higher than that of BV but lower than that of PEB. The fluorescence increase over BV is most likely due to the lock and the enhanced rigidity. Note that the introduction of a lock does not reduce mobility completely; it is assumed that seven-member rings still provide some flexibility (47), and the same is probably true for eight-member rings. Acidification led to a (slight) fluorescence increase of three chromophores, whereas two showed a decrease. Hence, there was no general pattern for the effect of protonation on fluorescence yields.

Phycocerythrobilin and Biliverdin Adducts—As a reference for our new phytochrome chromophore adducts, we measured absorption and fluorescence spectra of the PEB adducts of Cph1 and Agp1 (Fig. 3). In our hands the quantum yield of PEB-Cph1 was 0.54 (Table 2) and thus slightly lower than the 0.82 value reported by others (20, 48). The bacterial phytochrome Agp1 contains BV as a natural chromophore, bound via the vinyl side chain of ring A to the protein; the protein can also incorporate PEB into its chromophore pocket. However, PEB, PCB, and PB, which carry an ethylidene side chain on ring A, are incorporated in a noncovalent manner, and the PEB adduct has an atypical, broad absorption spectrum (28). The mutant V249C of Agp1 incorporates PCB (30) as well as PEB$^4$ in a covalent manner. The absorption and fluorescence spectra of the PEB-Agp1-V249C adduct (Fig. 3$^4$) are almost indistinguishable from those of PEB-Cph1. The excitation and emission maxima for the Cph1 and Agp1-V249C adducts lie at 579/580 and 590/588 nm (Table 2), respectively. Hence, the broad spectrum of the PEB adduct with wild type Agp1 (28) results from the noncovalent binding

$^4$ B. Borucki and D. Opitz, personal communication.
TABLE 2
Absorption and fluorescence characteristics of the phytochrome adducts used in the present study

The absorption, excitation, and emission maxima of the Q-band are given. Fluorescein (43) was used as a reference for calculating the quantum yields (Φ).

| Chromophore Adduct | Absorption maxima (nm) | Excitation maxima (nm) | Emission maxima (nm) | Stokes shift (nm) | φ |
|--------------------|------------------------|------------------------|----------------------|------------------|----|
| Fluorescein        | 490                    | 488                    | 574                  | 86               | 0.95 |
| PEB-Chp1           | 580                    | 579                    | 590                  | 11               | 0.6706 |
| PEB-Agp1-V249C     | 580                    | 580                    | 588                  | 8                | 0.0002 |
| BV-Agp1            | 703                    | 700                    | 701                  | 1                | 0.0002 |
| BV-Agp2            | 703                    | 703                    | 713                  | 10               | 0.0013 |
| BV-Agp1-M15-D197A  | 700                    | 684                    | 714                  | 30               | 0.0022 |
| BV-Agp1-H280A      | 701                    | 705                    | 713                  | 8                | 0.0012 |
| BV-Agp2_Phy1       | 693                    | 686                    | 711                  | 25               | 0.0016 |
| 15Za-Agp1          | 715                    | 714                    | 718                  | 4                | 0.0131 |
| 15Za-Agp2          | 713                    | 712                    | 718                  | 6                | 0.0140 |
| 15Za-Agp1-M15-D197A| 713                    | 715                    | 716                  | 1                | 0.0158 |
| 15Za-Agp1-H280A    | 714                    | 705                    | 713                  | 8                | 0.0123 |
| 15Za-Agp2_Phy1     | 742                    | 715                    | 715                  | 0                | 0.0024 |
| 15Ea-Agp1          | 736                    | 712                    | 721                  | 9                | 0.0027 |
| 5Za15Ea-Agp1       | 685                    | 682                    | 696                  | 14               | 0.0196 |
| 5Za15Ea-Agp2       | 704                    | 702                    | 707                  | 5                | 0.0006 |
| 5Fa15Ea-Agp1       | 689                    | 670                    | 697                  | 27               | 0.0015 |
| 5Fa15Ea-Agp2       | 701                    | 683                    | 697                  | 14               | 0.0022 |
| 5Za15Za-Agp1       | 711                    | 709                    | 712                  | 3                | 0.0099 |
| 5Za15Za-Agp2       | 707                    | 705                    | 712                  | 7                | 0.0118 |
| 15Ea-Agp1          | 749                    | ND                     | ND                   |                 |     |

mode. The fluorescence quantum yield of PEB-Agp1-V249C is 0.67 (Table 2), which is even higher than that of our PEB-Chp1 control.

As expected, the fluorescence of wild type phytochromes Agp1 and Agp2 assembled with the natural chromophore BV is very weak (Fig. 4). The fluorescence of both adducts was measured in the Pr form, because thus far no Pfr fluorescence has been detected (19). The bathy phytochrome Agp2 converts into Pfr in darkness; hence this sample was photoconverted to Pr by 780 nm light prior to the fluorescence measurements. We obtained quantum yields for BV-Agp1 and BV-Agp2 of 0.0002 and 0.0013, respectively (Table 2). The BV adduct of Agp1 showed not only the weakest fluorescence of all, but excitation and emission maxima were also indistinguishable from each other.

Several phytochrome mutants have been described that exhibit inefficient photoconversion combined with an elevated fluorescence quantum yield (23, 24, 26). Here, we tested the fluorescence properties of three different mutants, Agp1-M15-D197A (31) and two new mutants, Agp1-H280A and the domain-swap mutant Agp2_Phy1 (Fig. 4). In the Agp1-M15-D197A mutant, the highly conserved Asp residue at position 197, which is located close to rings B and C of the tetrapyrrole chromophore, is mutated to Ala. The mutation results in a slower chromophore incorporation, a weaker chromophore, is mutated to Ala. As a result of this mutation, BV assembly was slowed down (Fig. 5A). Red light induces only a very weak photoconversion (compare the difference spectrum with the absorption spectrum (Fig. 5A)). The fluorescence excitation spectrum matches well with the absorption spectrum, and the fluorescence quantum yield is 0.0012, which is 6 times higher than that of wild type BV-Agp1.

In the domain-swap mutant Agp2_Phy1, the PHY domain of Agp2 is replaced by the PHY domain of Agp1 (Fig. 1). The PHY domain is known to be required for the spectral integrity of the phytochrome and for the complete photoconversion into Pfr (3). The replacement of the PHY domain switched the bathy phytochrome Agp2 (with a Pfr ground state) to a “normal” phytochrome with a Pr-like ground state (Fig. 5). The absorption profile is flat and broad, suggesting that the chromophore is misplaced. The BV assembly of this mutant is characterized by the transient appearance of a long wavelength absorption peak. The maximum of this peak is at 744 nm, whereas the maximum of the final Pr adduct is at 693 nm. Because the position of the assembly intermediate absorption maximum coincides with that of Pfr, one may speculate that the protein might transiently go through a Pfr state during chromophore incorporation. However, the formation of Pfr during the dark assembly of BV-Agp2 or during dark conversion from Pr takes hours to reach completion. Hence, a rapid formation of a Pfr chromophore during BV assembly of Agp2_Phy1 is very unlikely. The long wavelength intermediate might thus represent, e.g. a noncovalent Pfr-like adduct.

Red light irradiation of BV-Agp2_Phy1 resulted in only a weak photoconversion of the BV adduct of this mutant. The shape of the difference spectrum (Pr minus Pfr) has the same characteristics as that of Agp2, i.e. a small Pr peak and a large Pfr valley (30, 51). The fluorescence excitation maximum of Agp2_Phy1 was also blue-shifted as compared with the absorption maximum, in this case by 7 nm. Again, the fluorescence might be related to a subtraction with a deprotonated chromophore. The quantum yield of the adduct at 0.0016 was within the range of BV-Agp1-M15-D197A and BV-Agp1-H280A (Table 2).

Fluorescence of Adducts with Singly Locked Chromophores—
In the next step, we tested the fluorescence properties of adducts with singly locked chromophores. In the 15Za and 15Ea...
chromophores, the C15=C16 methine bridge of the chromophore is fixed in either Z anti or E anti stereochemistry, which correspond to the Pr and Pfr form of phytochrome, respectively (Fig. 2). It has been shown that both chromophores assemble with Agp1 and Agp2 and produce adducts with Pr- and Pfr-like properties. As in the PEB adducts, photoisomerization of the 15Za and 15Ea adducts is blocked (29).

The 15Za chromophore was assembled with Agp1, Agp2, Agp1-M15-D197A, Agp1-H280A, and Agp2_Phy1. In all cases the fluorescence quantum yield was higher than that of the corresponding BV adducts. The increase was only minimal for Agp2_Phy1, whereas 15Za-Agp1-M15-D197A and 15Za-Agp1-H280A reached quantum yields of 0.016 and 0.012, respectively. The quantum yields of the 15Za adducts with wild type Agp1 and Agp2 were within the same range, namely 0.013 and 0.014, respectively (Table 2). In all of the 15Za adducts the excitation and emission spectra overlap with indistinguishable peak positions (Fig. 6). The fluorescence spectra of the Agp1, Agp2, Agp1-M15-D196A, and Agp1-H280A adducts match well with the absorption spectra, whereas in the Agp2_Phy1 adduct, absorption and excitation...
spectra are again clearly different; the excitation maxima lie at shorter wavelengths than the absorption maxima, respectively (Fig. 6). In addition, the fluorescence spectra of 15Za-Agp1-M15-D197A and 15Za-Agp1 resemble each other closely. This spectral coincidence speaks for a fluorescence of the protonated subfraction of 15Za-Agp1-M15-D197A.

We obtained a weak fluorescence for the 15Ea-Agp1 adduct, but the excitation maximum (712 nm) and the emission maximum (721 nm) were significantly blue-shifted compared with the absorption maximum (736 nm). We could not measure a fluorescence signal in 15Ea-Agp2. Other 15Ea adducts were not analyzed.

Fluorescence of Adducts with Doubly Locked Chromophores—We also tested phytochrome adducts with doubly locked chromophores in which both the C5 and C15 methine bridges are locked by additional carbon chains (52). The 5Za15Ea and 5Ea15Ea chromophores do not form a covalent link with the protein, because of the missing vinyl side chain of the ring A (Fig. 2). Both chromophores form adducts with Agp1 and Agp2, which absorb in the red region of the spectrum, whereas adducts with two other doubly locked chromophores, 5Es15Ea and 5Zs15Ea, absorb in the blue spectral range only (52). Adducts with the latter two chromophores were not considered further here.

Both 5Ea15Ea adducts show weak fluorescence with excitation maxima, which are blue-shifted as compared with their absorption maxima (Fig. 7). The 5Za15Ea-Agp2 adduct exhibits a very weak fluorescence, which is even lower than that of the BV adduct, whereas 5Za15Ea-Agp1 has a rather high quantum yield of 0.02 (Table 2). This is surprising, as the other 5Za15Ea/5Ea15Ea adducts showed only very weak fluorescence as well. We had proposed earlier that the stereo-chemistry of the Agp1 Pfr chromophore could be ZZZEasa (i.e. the double bonds around C5, C10, and C15 are in the Z, Z, E configuration, and the single bonds are in the anti, syn, anti conformation) (29, 52). The 5Za15Ea chromophore thus might fit well into the chromophore pocket of Agp1, where it appears to be kept in a more rigid conformation. Another possibility is that the chromophore is oriented in a reversed manner within the chromophore binding pocket of Agp1, i.e. as 5Ea15Za. This would be in line with the rather high fluorescence of the other 15Za adducts, although the fluorescence of the Agp2 adduct with the doubly locked chromophore is not as strong.

The new doubly locked chromophore 5Zs15Za has a ring A vinyl side chain (Fig. 2) and could thus bind covalently to Agp1 and Agp2. Both adducts exhibit a characteristic Pr-like absorption spectrum with maxima at 711 nm for 5Zs15Za-Agp1 and 707 nm for 5Zs15Za-Agp2. Covalent binding of the chromophore to Agp1 and Agp2 was demonstrated using desalting columns and SDS (0.1%)-treated protein adducts (53) (data not shown).

The fluorescence excitation maximum of both 5Zs15Za adducts matches closely with the absorption maximum, and the excitation-emission red shift was 3 and 7 nm for the Agp1 and Agp2 adducts, respectively. The quantum yields were in the range of 1% (Table 2). The Agp2 adduct again revealed a higher quantum yield than that of the Agp1 adduct.

DISCUSSION

Phytochromes are biliproteins that exhibit only very weak fluorescence under ambient conditions. This property distinguishes them from the strongly fluorescent phycobiliproteins of cyanobacteria and red algae (34). However, phytochrome fluorescence intensity can be increased by lowering the temperature, by chromophore replacement, or by site-directed mutagenesis, which leads to an inhibited photoconversion. One future aim is to use phytochrome derivatives as fluorescence markers for cellular studies in a similar manner as the green fluorescent protein. One of the advantages of phytochromes lies in their long wavelength excitation and emission maxima. The use of biliverdin-binding bacterial phytochromes might be a superior choice over PCB- or PFB-binding cyanobacterial and plant phytochromes (26), because the
heme degradation product, biliverdin, is present in many cells including mammalian cells, whereas the other chromophores are found only in plant and cyanobacterial cells.

In this study we investigated the fluorescence properties of the two phytochromes Agp1 and Agp2 from *A. tumefaciens* and mutants thereof, which were assembled with different natural and synthetic chromophores. According to the fluorescence quantum yield the resulting phytochrome adducts may be divided into four groups: (i) adducts of the wild type proteins with the natural chromophore, which exhibit only very weak fluorescence; (ii) BV adducts of mutants with an impaired Pr to Pfr photoconversion, which have a fluorescence quantum yield in the range of 0.1 to 0.2%; (iii) adducts with locked chromophores, in which the quantum yield is in the range of 1%; and (iv) PEB adducts with a high fluorescence quantum yield of around 50% (Table 2).

We were surprised to see that the fluorescence of all locked chromophore adducts is weaker than that of the PEB adducts. It is generally agreed that the fluorescence of PEB adducts is

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**FIGURE 6. Spectra for Agp1 and Agp2 wild type and mutant adducts with singly locked chromophores.** A, 15Za-Agp1; B, 15Za-Agp2; C, 15Za-Agp1-M15-D197A; D, 15Za-Agp1-H280A; E, 15Za-Agp2_Phy1; F, 15Ea-Agp1. Dotted lines, absorption spectra; dashed and solid lines, excitation and fluorescence emission spectra, respectively.
high, because photoisomerization as the proposed major route of energy dissipation is blocked. However, the same argument also holds for all locked chromophore adducts investigated here. The forced conformation of the locked chromophores could impose structural restraints, and the qualitative difference between PEB adducts and locked chromophore adducts could result from different arrangements of the bilins within the chromophore pocket. In many of the adducts with doubly locked chromophores, the chromophore indeed appears distorted and thus more flexible, as indicated by the low extinction coefficients and broad absorption spectra. (We assumed that distortions should generally result in lowered extinction coefficients, because the high extinction coefficients established during the evolution of phytochromes reflect an optimized situation.) Notably, however, the 15Za adducts of Agp1 and Agp2, and of all PEB adducts used here, are characterized by sharp absorption peaks and high extinction coefficients. Therefore, these chromophores are most likely firmly integrated into the chromophore pocket. The only plausible explanation for the lower quantum yield of 15Za-Agp1 as compared with PEB-Agp1 is to suppose that the energy in the 15Za adducts is dissipated via ring D of the

![FIGURE 7. Spectra for Agp1 and Agp2 adducts with doubly locked chromophores.](image-url)
Phytochrome Adduct Fluorescence with Locked Chromophores

chromophore, which contributes to the extended $\pi$-system in 15Za but not in PEB. It is interesting to note that an energy dissipation pathway via ring D has been proposed for phycoerythrocyanin. The phycoviolobilin chromophore of phycoerythrocyanin undergoes light-triggered $Z$ to $E$ isomerization around the C15 methine bridge. The fluorescence yield with the $E$-configured chromophore is very low (55).

For Agp1, we propose two possibilities for energy dissipation via ring D. (i) Thermal motions of ring D: As noted above, the seven- and eight-member rings of the locked chromophores still provide some flexibility (25, 47). It is unclear whether inside the protein this flexibility is high enough to provide an energy dissipation pathway for $>60\%$ of absorbed photons. (ii) Excited state proton transfer: In a recent publication on bacteriophytochrome BphP3 from R. palustris, it has been put forward that the excited state of phytochrome might be deactivated by a rapid (350 ps) proton transfer that finally ends up in a back-conversion to Pr (56). Such a reaction would compete with fluorescence that has a lifetime in the ns range (22). This excited state proton transfer could be a major route of energy dissipation in phycobilins that has not been considered previously, although such a possibility has been discussed for bilins in general (45). The difference between locked chromophore and PEB adducts could result from differences in their excited state proton transfer; in PEB adducts this effect could be lost but still be present in the locked chromophore adducts. In this view, the excited state proton transfer would originate from ring D of the chromophore, which is decoupled from the major conjugated $\pi$-system in PEB.

In the H280A mutant, the hydrogen-bonding network around ring D should be disturbed, but a water molecule close to the ring D (7) might serve as proton acceptor for the excited state proton transfer in this mutant. Newly designed chromophores can help to decide between these possibilities of energy dissipation.

The effect of chromophore distortion can be studied on the Agp2_Phy1 mutant. This protein has been assembled with BV and 15Za, and in both adducts the absorption profiles are broad and flat as compared with the equivalent Agp1 or Agp2 adducts. In the BV adduct of Agp2_Phy1, these distortions are accompanied by an inhibition of photoisomerization. This inhibition is most likely the reason for the elevated fluorescence quantum yield of BV-Agp2_Phy1 over BV-Agp1 or BV-Agp2. The quantum yield of 15Za-Agp2_Phy1 is slightly higher than that of BV-Agp2_Phy1 but lower than that of 15Za-Agp1 or 15Za-Agp2. Thus, distortions of the 15Za chromophore result in a lower fluorescence quantum yield in this case.

In the Agp1-M15-D197A mutant, the situation is different; the 15Za adduct fluoresces stronger than 15Za-Agp1 or 15Za-Agp2, although the comparison of absorption and fluorescence spectra shows that only a subfraction of the adduct is fluorescent. The chromophore of the Agp1-M15-D197A mutant is only partially protonated at the slightly basic pH used here, whereas in wild type Agp1 the chromophore is completely protonated under these conditions (57). Because the excitation and emission spectra of 15Za-Agp1-M15-D197A are remarkably similar to those of 15Za-Agp1, we propose that fluorescence is related to the protonated chromophore.

In BV-Agp1-M15-D197A, the excitation spectrum is, however, blue-shifted relative to the absorption spectrum. This speaks for a fluorescence of the deprotonated chromophore. Thus, the Agp1-M15-D197A mutation increases the fluorescence of BV and 15Za in two different ways. The BV fluorescence increases because photoisomerization of the deprotonated chromophore is inhibited. The larger 15Za fluorescence in Agp1-M15-D197A versus Agp1 cannot result from an inhibition of photoisomerization. We assume that the fluorescence increase is related to changes in the hydrogen-bonding network. In wild type Agp1, Asp-197 is part of a hydrogen-bonding network that involves rings B and C of the chromophore (7, 11, 31). Disturbing this network by an Asp-197 replacement could also impact the hydrogen-bonding network around ring D and, in this way, inhibit the energy dissipation pathway proposed above (56).

Altogether, our data may be interpreted in the following manner: in adducts with the natural chromophore, BV, fluorescence can be increased by inhibiting photoisomerization. Such inhibition can, for example, result from a disturbance of the chromophore hydrogen-bonding network, deprotonation of the chromophore, or chromophore displacement. These same effects, which finally result in increased quantum yields, can, however, lower fluorescence by other means. Such effects become evident from the locked chromophore adducts, in which photoisomerization is always inhibited. We thus propose that the combination of mutagenesis and synthesis of new locked chromophores can result in high fluorescence quantum yields of bacterial phytochrome derivatives.

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