A Polarity Probe for Monitoring Light-induced Structural Changes at the Entrance of the Chromophore Pocket in a Bacterial Phytochrome

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Light-induced structural changes at the entrance of the chromophore pocket of Agp1 phytochrome were investigated by using a thiol-reactive fluorescein derivative that is covalently attached to the genuine chromophore binding site (Cys-20) and serves as a polarity probe. In the apoprotein, the absorption spectrum of bound fluorescein is red-shifted with respect to that of the free label suggesting that the probe enters the hydrophobic chromophore pocket. Assembly of this construct with the chromophores phytoerythrobilin or biliverdin is associated with a blue-shift of the fluorescein absorption band indicating the displacement of the probe out of the pocket. The probe does not affect the photochromic and kinetic properties of the noncovalent bilin adducts. Upon photoconversion to Pfr, the probe spectrum undergoes again a bathochromic shift and a strong rise in CD indicating a more hydrophobic and asymmetric environment. We propose that the environmental changes of the probe reflect conformational changes at the entrance of the chromophore pocket and are indicative for rearrangements of the chromophore ring A. Flash photolysis measurements showed that the absorption changes of the probe are kinetically coupled to the formation of Meta-Rc and Pfr. In the biliverdin adduct, an additional component occurs that probably reflects a transition between two Meta-Rc substates. Analogous results to that of the noncovalent phycocyanobilin adduct were obtained with the mutant V249C in which probe and chromophore are covalently attached. The conformational changes of the chromophore are correlated to proton transfer to the protein surface.

Phytochromes are red-light photoreceptors occurring in plants, bacteria, and fungi where they control important developmental processes (1–6). The discovery of microbial phytochromes from genome sequencing (7–9) provided new prospects for biochemical, spectroscopic and structural analyses of this light sensor family. Agp1 (AtBphP1) from the soil bacterium Agrobacterium tumefaciens is a typical member of the widespread family of proteobacterial phytochromes (10, 11) and is the subject of the present study.

The domain arrangement of canonical phytochromes consists of an N-terminal photosensory domain, including PAS, GAF, and PHY domains and a C-terminal regulatory kinase domain (see, e.g. Ref. 3). Bacterial phytochromes lack the N-terminal extension, and the PAS module insertion of plant phytochromes (3). In most of the bacterial phytochromes, the C-terminal regulatory domain is a histidine kinase (4). These kinases form homodimers as functional units (12) where the subunits transphosphorylate each other (13). The cofactors are linear tetrapyrroles that are covalently attached via a thioether linkage (14) to the side chains of specific conserved cysteine residues. The native chromophore of plant phytochromes is phycobilin (PÞB) (14), some cyanobacterial phytochromes incorporate phycocyanobilin (PCB) (15, 16), and all other bacterial phytochromes bind biliverdin (BV) (10, 11). Whereas the chromophore binding site of the more reduced bilins PÞB and PCB is located in the GAF domain, the binding site of BV is close to the N-terminus upstream of the PAS domain (4, 11). The two distinct binding sites apparently require a specific substituent at the C3 carbon of pyrrole ring A, either an ethyldiene (PÞB and PCB) or a vinyl (BV) group, for covalent attachment of the bilin chromophore (4). The holophytochrome assembly that includes covalent attachment of the chromophore is an autocatalytic process implying an intrinsic bilin-C-S lyase activity of the apophytochrome (17). Kinetic studies of the autoassembly in vitro showed that ligation of the chromophore is the ultimate step following incorporation in the binding pocket and internal protonation (18).

Phytochromes display photochromicity involving two either thermally stable or long-lived states, Pthr and Ptho (red and far-red absorbing forms), that can be reversibly converted by light of appropriate wavelengths. The Pthr to Ptho photoconversion is initiated by a rapid Z/E isomerization of the C-D methine bridge of the bilin chromophore (19–22) leading within picoseconds from D. radiodurans; RpBphP3, bacteriophytochrome 3 from R. palustris; PaBphP, bacteriophytochrome from Pseudomonas aeruginosa; BphP, bacteriophytochrome; BV, biliverdin IXα; PCB, phycocyanobilin; PAS domain, acronym formed from the names of the first three proteins recognized as sharing this sensory domain; GAF domain, abbreviation derived from cGMP-specific phosphodiesterases, cyanobacterial adenylate cyclases, and transcription activator FhlA; PHY domain, domain specific for phytochromes; IAF, 5-iodoacetamidofluorescein; DNTB, 5,5'-dithiobis(2-nitrobenzoic acid); ANS, 8-anilinonaphthalene 1-sulfonate; PÞB, phycobilin.
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to the formation of the Lumi-R intermediate (23, 24). The following thermal relaxations via Meta-R_A and Meta-R_C intermediates to Pfr proceed on the time scale of microseconds and milliseconds (25–28).

Assembly of Agp1 with locked BV derivatives showed that the geometry of the C-D methine bridge is 15Z_{anti} in P_{r} and 15Z_{anti} in P_{fr} (29) suggesting that this methine bridge remains in the anti conformation during photoconversion. The crystal structures of the chromophore binding domains of the bacteriophytochromes from Deinococcus radiodurans and Rhodopseudomonas palustris revealed that the BV chromophore adopts a 5Z_{syn},10Z_{syn},15Z_{anti} configuration/conformation in the P_{r} state (30–32). The 5Z_{syn} geometry of the A-B methine bridge in the P_{r} state was confirmed by assembly of Agp1 with the corresponding locked BV chromophore (33). Recently, heteronuclear NMR investigations and crystallographic studies on the complete photosensory domain of the cyanobacterial phytochrome Cph1 from Synechocystis revealed that the PCB chromophore is also in the 5Z_{syn},10Z_{syn},15Z_{anti} geometry in P_{fr} (34, 35).

Because the locked 5Z_{syn} adduct of Agp1 did not show a P_{fr}-like photo-product, conformational changes of the A-B methine bridge in the thermal relaxation cascade have been predicted (33). Flash photolysis experiments with this adduct suggested that these changes occur in the Meta-R_A to Meta-R_C transition (36). The stereochemistry of the A-B methine bridge in the P_{r} state and in the preceding intermediates could not be determined unambiguously yet. Recent studies with doubly locked chromophores suggest that the C5–C6 single bond undergoes a thermal rotation from syn to anti in the photoconversion of Agp1, whereas an additional Z/E isomerization around the C4=C5 double bond (hula-twist mechanism) was postulated for Agp2 (37). However, the crystal structure of the photosensory domain of the bacteriophytochrome PaBphP in its P_{fr}-enriched dark-adapted state favors the 5Z_{syn} configuration of the BV chromophore (38). Structural changes of the A-B methine bridge were excluded for the PCB chromophore of Cph1 on the basis of heteronuclear NMR (34), whereas low temperature Fourier transform IR studies on plant phytochrome suggested an environmental change of the ring A carbonyl group and/or a twist of the A-B methine bridge (39).

The mechanism by which the signal is transmitted from the bilin chromophore to the protein is still obscure. The recent three-dimensional structures of the complete photosensory domains of Cph1 (35) and PaBphP (38) reveal key interactions between GAF and PHY domains in the corresponding dark states reflecting P_{r} and P_{fr}, respectively. In view of the intrinsic differences between the two phytochromes, it is not trivial to differentiate which of the numerous structural differences arise from light-induced conformational changes and are thus potentially important for signal transmission. We note that many approaches to provide a clue on the mechanism of signal transmission from the bilin chromophore to its proximate environment imply that this process is exclusively coupled to the photo-isomerization localized at ring D and its environment and that the chromophore then remains a passive element in the thermal relaxation cascade. This point of view is supported by recent results from femtosecond stimulated Raman spectroscopy suggesting that the chromophore structures in Lumi-R and P_{fr} are very similar (24). On the other hand, size exclusion chromatography experiments demonstrated that the global conformational changes observed for the P_{fr} state of Agp1 WT are absent in constructs (locked 5Zs adduct and mutants D197A and H250A), where the formation of P_{fr} is inhibited but the primary photoreaction proceeds (33, 40). These results are difficult to explain in terms of an ultra-fast signal transmission from the chromophore to the surrounding residues in its pocket.

Light-induced conformational changes at the surface of plant phytochrome were observed by using covalently attached labels that are sensitive to the polarity of the microenvironment (41, 42). Due to the accessibility of several binding sites (i.e. the sulphydryl groups of cysteines) in these experiments, the labeling was unspecific preventing further assignment of the observed changes to particular regions of the protein. Time-resolved absorption measurements with a covalently attached fluorescein derivative showed that the changes occur in the Meta-R_C to P_{fr} transition (41). In the present work with Agp1 phytochrome, we take advantage of the highly reactive sulphydryl group of Cys-20, the genuine binding site of the BV chromophore, to specifically attach a fluorescein derivative. We observed that this construct assembles with PCB and BV forming noncovalent photochromic adducts, spectrally and kinetically undisturbed by the fluorescein label. Upon photo-conversion, the absorption band of the label displays a bathochromic shift and increase in ellipticity suggesting that the label moves into more hydrophobic and asymmetric environment in the P_{fr} state. The label thus serves as a polarity probe at the entrance of the binding pocket. We postulate that these polarity changes reflect conformational changes of the A-B methine of the bilin chromophore and/or the microenvironment of ring A at the entrance of the binding pocket. Time-resolved measurements reveal that the changes occur in the Meta-R_C to Meta-R_C and Meta-R_C to P_{fr} transitions. Analogous results were obtained with the V249C mutant of Agp1 in which both the fluorescein probe and the PCB chromophore are covalently attached.

EXPERIMENTAL PROCEDURES

Preparation of Agp1 Samples with Covalently Attached Polarity Probe—Protein expression and purification of the apoprotein of Agp1 phytochrome from Agrobacterium tumefaciens were performed as described (43–45). 5-Iodoactadidamido-fluorescein (IAF) was used as a polarity probe binding stoichiometrically to the cysteine that is the binding site of the chromophore in the native holoprotein (Cys-20 in Agp1). Most of the experiments with noncovalently attached chromophore were performed with the Agp1-M15 variant that includes the PAS, GAF, and PHY domains but lacks the C-terminal histidine kinase. The Agp1 apoprotein was incubated with IAF from a 5 mM stock solution at moderate molar excess for <30 min and was subsequently passed through a NAP-5 or NAP-10 desalting column (Amersham Biosciences) to remove the unbound IAF. The labeled Agp1 apoprotein was then titrated in the dark with microliter shots of PCB or BV dissolved in MeOH until the absorption spectrum indicated saturation of the respective noncovalent bilin adduct. Excessive PCB was removed by a
desalting column. The unlabeled reference samples for the noncovalent adducts were prepared analogously. To prevent covalent attachment of BV in this case, the chromophore binding site was chemically blocked with 5,5′-dithiobis-(2-nitrobenzoid acid) (DTNB) (11).

The spectral data of the labeled samples show that the polarity probe is specifically bound to Cys-20 of the protein and that IAF binding to one of the other Cys residues, which are located at positions 279 and 295, is negligible (see “Results” and “Discussion” for details). According to the crystal structures of other bacterial phytochromes, both Cys residues are not exposed to the surface. However, when IAF at 10-fold molar excess was added to the Agp1-BV adduct and incubated over night, the label was also incorporated in sub-stoichiometric amounts. Such a sample was used for control measurements (see “Results” and “Discussion”).

The Agp1-V249C mutant was used to attach PCB covalently in addition to the polarity probe. Because PCB binds selectively to this introduced Cys residue in the GAF domain (45), the apoprotein was first assembled with PCB. Nearly complete regeneration (formation of the covalent PCB adduct) was achieved by addition of 1–2 mM dithiothreitol to the protein-chromophore mixture. Excessive PCB was again removed by a desalting column. Afterward, the covalent PCB adduct of Agp1 was incubated with IAF as described above, and the unbound IAF was removed by a desalting column.

Preparation of the samples and spectroscopic measurements were done in 50 mM Tris, 200 mM NaCl, and pH 7.8–7.9 unless otherwise specified. In this pH range, fluorescein is predominantly in its deprotonated form.

CD Spectroscopy—Stationary CD spectra were collected with a Jasco 500A instrument modified and updated with a homemade data acquisition system as described (18, 46, 47). Data were collected at room temperature, with cells of 10-mm path length and averaged over 5–15 scans. For baseline corrections reference spectra recorded with buffer were subtracted.

Calculation of Pure Pr and Pfr Spectra—CD and absorption spectra of the pure Pr and Pfr forms were extrapolated from the respective spectra after red light and far-red light illumination by variation of the estimated relative contributions of Pr and Pfr in the mixed states (see also Ref. 47). For the noncovalent BV adduct, the dark state (after assembly in the dark or thermal dark reversion) was identified with the pure Pr form. The contributions of the P state after red light illumination are typically 30% for the PCB adducts and 20% for the noncovalent BV adduct.

Transient Absorption Spectroscopy—Flash photolysis was performed as described (27, 28, 47, 48). Absorbance changes with respect to the Pr state were recorded at 20 °C in a cuvette of 5-mm path length, from 100 ns to 5 s after laser-flash excitation at 695 or 640 nm. The intrinsic time resolution of the detection system is <1 μs. Before each flash-excitation the sample was reconverted by far-red light from appropriate light sources to the P state (see Ref. 47). Time traces at multiple wavelengths were analyzed by singular value decomposition using Mathematica 3.0 and subsequent simultaneous fitting of the relevant components using Origin 5.0 (49). Amplitude spectra and extrapolated initial difference spectra \( \Delta A_{int} \) were calculated as described (18, 47). Protonation changes at the entrance of the chromophore pocket were monitored by the covalently attached fluorescein probe in the covalent PCB adduct (Agp1-V249C-PCB-AF) at ~pH 6.7, i.e. close to the \( pK_a \) of fluorescein. The protonation signal is the difference of the traces in the absence and presence of buffer (10 mM NaPP). The use of fluorescein derivatives as covalently attached pH-indicator dyes is documented for bacteriorhodopsin (50–52).

RESULTS

Covalent Attachment of Fluorescein to Cys-20 and Formation of Noncovalent Bilin Adducts—Addition of IAF to the Agp1-M15 apoprotein and subsequent elution on a desalting column demonstrates the formation of a protein-dye complex where the fluorescein derivative is covalently attached. The spectra of free IAF in aqueous solution and the protein-dye complex (Agp1-M15-AF) differ by their absorption maxima (493

\[ \text{versus} \]

\[ \text{versus} \]
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511 nm, see Fig. 1A) indicating a clearly more hydrophobic environment of the covalently attached fluorescein. The ratio of the absorbances at 511 and ~280 nm of ~0.75 in Agp1-M15-AF (Fig. 1A) furthermore suggests that the fluorescein derivative (extinction coefficient $\varepsilon \approx 78,000 \text{ M}^{-1} \text{ cm}^{-1}$) binds almost stoichiometrically to a single cysteine of the Agp1-M15 apoprotein. Upon titration of Agp1-M15-AF in the dark with the bilin chromophores PCB and BV, the absorption maximum of the fluorescein label displays a progressive hypsochromic shift to the final values of 497 and 498 nm, respectively (Fig. 1, B and C), reflecting a less hydrophobic environment of the label. The absorbances of the Q bands of the respective bilin chromophores simultaneously increase with absorption maxima at ~685 nm for PCB and ~700 nm for BV, respectively, indicative of the formation of the corresponding noncovalent bilin adducts of Agp1-M15 (see Ref. 47). The noncovalent nature of the BV adduct, which can directly be assigned from the light-induced difference spectra (see next paragraph), shows that the fluorescein label is covalently attached to Cys-20 of the Agp1-M15 apoprotein, thereby preventing covalent attachment of BV to its canonical binding site. Analogue experiments with the apoprotein of full-length Agp1 led to almost identical results (see supplemental Fig. S1) indicating that the kinase domain is irrelevant for attachment of the label and the bilin chromophore. We note that the presence of the label does not affect the affinity of the bilin chromophore to its actual binding pocket. Because the absorption maximum of fluorescein is sensitive to the polarity of its environment, the fluorescein label acts as a polarity probe and will be designated accordingly in the following.

Environmental Changes of the Fluorescein Probe upon Photoconversion of the Noncovalent Bilin Adducts—The noncovalent PCB and BV adducts of Agp1-M15 with the covalently attached fluorescein probe exhibit photochromicity involving $P_r$ and $P_{fr}$ states spectrally almost indistinguishable in the Q-band range from that of the respective unlabeled constructs (Figs. 2A and 3A). The absorption maximum of the polarity probe displays a bathochromic shift of ~5 nm upon $P_r$ to $P_{fr}$ photoconversion indicating an environmental change of the probe. This shift is more obvious in the $P_{fr}$ minus $P_r$ difference spectra (Figs. 2B and 3B) showing a maximal decrease at 490 and 491 nm and a maximal increase at 510 and 514 nm, for the noncovalent PCB and BV adducts, respectively. The relative absorption changes of the polarity probe are larger in the BV adduct than in the PCB adduct. Direct comparison with the respective difference spectra of the unlabeled constructs in the Q-band range confirms that the spectral properties of the bilin chromophores in the $P_r$ and $P_{fr}$ states are unaffected by the polarity probe (Figs. 2B and 3B). The excellent agreement for the BV adducts moreover demonstrates that the fluorescein label completely blocks covalent attachment of BV to Cys-20: the light-induced difference spectra of the covalent and noncovalent BV adducts are significantly distinct mainly by the lower and blue-shifted absorption of the $P_r$ band in the noncovalent adduct (11, 47). No light-induced absorption changes of the fluorescein label were observed in control measurements with the covalent BV adduct of Agp1 (wild type) where the label was attached to another residue, most likely to one or both of the other two cysteines, Cys-279 and Cys-295 (see supplemental Fig. S2). This control provides further evidence that the light-induced absorption changes of the polarity probe exclusively arise from the fluorescein label attached to Cys-20.

Circular dichroism spectra corroborate that the fluorescein probe does not affect the spectral properties and the geometries of the noncovalently bound bilin chromophores (Figs. 2C and 3C). Specifically, the CD spectra of the $P_r$ states are characterized by negative ellipticity in the Q band and positive ellipticity in the Soret range with band ratios of ~1:1 for the noncovalent PCB adduct and ~1:2 for the noncovalent BV adduct (47). Upon photoconversion to the $P_{fr}$ state, the ellipticity of the Q band reaches almost zero in these adducts (47).

The CD signal of the fluorescein probe in the $P_r$ state is identical to zero for the noncovalent PCB adduct and of moderate
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positive magnitude for the noncovalent BV adduct (Figs. 2C and 3C). In the Pfr state, the fluorescein probe exhibits a strong positive CD signal for both adducts. These observations indicate that the largely planar fluorescein probe moves from a more symmetric to a highly asymmetric environment upon Pr to Pfr photoconversion of the bilin chromophore. Induced CD of fluorescein is known, e.g. from IAF bound to the highly reactive thiol of myosin fragment I (53). Interestingly, the CD signal of the probe is weak and negative in the absence of the bilin chromophore (Figs. 2C and 3C), while the corresponding absorption maximum is red-shifted compared with the bilin adduct in the Pr state. This indicates that the environment of the polarity probe in the apoprotein is more hydrophobic but less asymmetric than in the Pfr state of the bilin adduct. The photochromic and spectral properties of the labeled constructs are unaffected by the kinase domain (see supplemental Figs. S3 and S4).

Kinetics of the Fluorescein Probe during Photoconversion of the Noncovalent Bilin Adducts—Flash photolysis experiments with the noncovalent bilin adducts of the fluorescein labeled Agp1-M15 show that the kinetics and the spectral transitions of the Pr- to Pfr photoconversion are only slightly affected by the covalently attached probe (Figs. 4A and 5A). Analogous measurements with full-length Agp1 showed that the kinase domain does not affect the kinetic properties (see supplemental Figs. S5 and S6). Time traces at characteristic wavelengths of the respective bilin chromophores are shown for the PCB adduct (Fig. 4A) and the BV adduct (Fig. 5A) in the presence (solid lines) and absence (dashed lines) of the polarity probe. The transient absorption changes in the wavelength range of the polarity probe (i.e. at 490 and 510 nm) show that this approach is also appropriate to time-resolve the environmental changes of the probe (Figs. 4B and 5B). For both noncovalent bilin adducts, the contributions from the bilin chromophore to the transient signals are minor (dashed lines). Due to the differences in the kinetics of these bilin adducts (see Ref. 47), deviations in the transients of the polarity probe have been anticipated. However, the transient signals from the probe reveal not only kinetic differences but also discrepancies in the qualitative progression (Figs. 4B and 5B). Whereas the signals at 490 and 510 nm for the noncovalent PCB adduct exhibit strictly monotonic decrease and increase, respectively, the signals for the noncovalent BV adduct display reversal in the time range of >200 ms. These discrepancies will be analyzed in the following.

The noncovalent PCB adduct is much more photostable than the noncovalent BV adduct allowing measurements at multiple wavelengths. Only minor corrections for photobleaching during the course of the flash photolysis measurements were necessary. The four relevant time traces from a singular value decomposition could be fitted satisfactorily with three kinetic components, two exponentials with time constants of 80 and 300 ms and a power law with a time constant of 2.7 ms (Table 1) leading to reasonable amplitude spectra (Fig. 4C). Fitting with four kinetic components (exponentials) as in the case of the unlabeled noncovalent PCB adduct (see Ref. 47) led neither to a significant improvement of the fit nor to a qualitative change of the amplitudes of the three main components and was therefore discarded. The spectral characteristic of the amplitude spectra is very similar to that of the principal components (first three exponentials) of the unlabeled noncovalent PCB adduct (see Ref. 47) and is reminiscent to that of the well characterized covalent BV adduct (28) allowing assignment to the Lumi-R to Meta-RA, the Meta-RA to Meta-RC, and the Meta-RC to Pfr transition, respectively. The amplitude spectra and the extrapolated initial difference spectrum in the spectral range of the polarity probe clearly show that the polarity changes mainly occur in the Meta-Rα to Meta-Rc transition and to a somewhat lower extent in the Meta-Rc to Pfr transition (amplitude spectra B2 and B3 in Fig. 4D). In the Lumi-R state, the polarity in the environment of the probe remains unchanged with respect to the Pfr state ($\Delta A_{init}$ in Fig. 4D), and the Lumi-R to Meta-Rα transition is associated with a minor contribution (B1 in Fig. 4D).

FIGURE 3. Spectral properties of noncovalent BV adduct of IAF labeled Agp1. Absorption (A) and CD spectra (C) of Agp1-M15-AF (solid line) and its noncovalent BV adduct in the Pr and Pfr states (dashed and dotted lines, respectively). The Pr spectrum is that of the dark state, and the Pfr spectrum was calculated from the spectrum after red light illumination and the dark spectrum. Absorption and CD spectra were measured at path lengths of 5 and 10 mm, respectively, the former were scaled to 10-mm path length. B, light-induced absorption changes of the noncovalent BV adducts of Agp1-M15-AF (labeled, solid line) and Agp1-M15-DTNB (unlabeled, dashed line) scaled to the same $\Delta A$ at 745 nm. The arrows mark the wavelengths of maximal changes.

A

Agp1-AF···BV

B

Agp1···BV

C

Agp1-AF···BV

Wavelength (nm)

Absorbance (OD)

$\Delta A$ (OD)

CD (m$^\circ$)

300 400 500 600 700 800

0.0

0.2

-0.4

-20

0

0.8

0.2

0.1

-10

514 nm

491 nm

1.6

510 nm

1.2

0.0

300 400 500 600 700 800

At 745 nm. The arrows mark the wavelengths of maximal changes.
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FIGURE 4. Photoconversion kinetics of noncovalent PCB adduct of IAF-labeled Agp1. A and B, flash-induced absorption changes of the noncovalent PCB adducts of Agp1-M15-AF (labeled, solid lines) and Agp1-M15-DTNB (unlabeled, dashed lines) measured at 680, 705, and 730 nm (PCB chromophore) and 490 and 510 nm (fluorescein probe). The traces of Agp1-M15-PCB were scaled to match the initial (−10 μs) absorption changes of Agp1-M15-AF−BV at 700 and 750 nm. The dashed vertical lines mark the time constants of the main transitions of the fluorescein probe (790 μs, 46 ms, and 430 ms). Conditions: 50 mM Tris, 200 mM NaCl, pH 7.8, 20 °C.

C and D, amplitude spectra of Agp1-M15-AF−PCB. ∆A init is the sum of the amplitude spectra B0 to B3. The dashed line is the steady-state Pfr−P difference spectrum scaled to the B3 spectrum. Panel D highlights the spectral range of the fluorescein probe using the same symbols as in Panel C.

FIGURE 5. Photoconversion kinetics of noncovalent BV adduct of IAF-labeled Agp1. Flash-induced absorption changes of the noncovalent BV adducts of Agp1-M15-AF (labeled, solid lines) and Agp1-M15-DTNB (unlabeled, dashed lines) measured at 700, 725, and 750 nm (BV chromophore, panel A) and 490 and 510 nm (fluorescein probe, panel B). The traces of Agp1-M15-DTNB−BV were scaled to match the initial (−10 μs) absorption changes of Agp1-M15-AF−BV at 700 and 750 nm. The dashed vertical lines in panel B mark the time constants of the main transitions of the fluorescein probe (790 μs, 46 ms, and 430 ms). Conditions: 50 mM Tris, 200 mM NaCl, pH 7.8, 20 °C.

TABLE 1

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<th>Kinetics of P to Pfr photoconversion of the bilin adducts of Agp1 with covalently attached fluorescein probe</th>
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<td>The time constants for the PCB adducts were determined from simultaneous fits with two exponentials and one power law to the four relevant singular value decomposition traces obtained from singular value decomposition of time traces at multiple wavelengths in the range of 360–750 nm (Agp1-M15-AF−PCB) and 340–750 nm (Agp1-V249C-PCB-AF), respectively. The time constants for the noncovalent BV adduct (Agp1-M15-AF−BV) were determined from simultaneous fits with four exponentials to traces at 8 wavelengths (380, 420, 490, 510, 700, 725, 735, and 750 nm). Conditions: 50 mM Tris, 200 mM NaCl, pH 7.8, 20 °C.</td>
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Due to the low photostability, transient absorption changes of the noncovalent BV adduct were measured at a limited number of wavelengths (380, 420, 490, 510, 700, 725, 735, and 750 nm) passing at least through two cycles. Simultaneous fits with four exponentials to these traces afforded time constants of 220 μs, 790 μs, 46 ms, and 430 ms (see Table 1). The third component (t3 = 46 ms) is absent in the unlabeled control sample (BV adduct of DTNB-blocked Agp1-M15) and is associated with only minor amplitudes at the wavelengths of the BV chromophore. Adapting the assignment from the (noncovalent) BV adduct of the C20A mutant of Agp1 that is spectrally and kinetically almost indistinguishable from the BV adduct of DTNB-blocked Agp1, the first, second and fourth component of the noncovalent BV adduct of Agp1-M15-AF reflect the Lumi-R to Meta-R A, the Meta-R A to Meta-R C, and the Meta-R C to Pfr transitions.
transition, respectively. The third transition associated with considerable amplitude in the signal of the polarity probe is thus most likely a transition between two substates of the Meta-Rc state. The time traces at 490 and 510 nm show that the polarity probe experiences an increase in the hydrophobicity of its environment during the second (Meta-Rc to Meta-Rc, and third (Meta-Rc, to Meta-Rc, transition followed by a decrease of smaller amplitude in the fourth (Meta-Rc, to Pr, transition.

Experiments with Covalently Attached Polarity Probe and Bilin Chromophore—To extend the methodological approach, the possibility of covalent attachment of both polarity probe and bilin chromophore was explored. This was realized with the V249C mutant of Agp1 in which PCB can bind to the additionally introduced cysteine at its canonical position in the GAF domain leaving the cysteine close to the N terminus (Cys-20) for subsequent attachment of the polarity probe. The presence of covalently attached PCB does not affect the affinity of IAF to the highly reactive sulfhydryl of Cys-20 leading to almost 100% labeling stoichiometry. The absorption spectra of this construct confirm that the spectral integrity and the photochromicity of the covalent PCB adduct are sustained in the presence of the covalently attached fluorescein probe (Fig. 6, A and B). Upon photoconversion to the Pr state, the absorption maximum of the fluorescein probe shifts in the same manner as in the noncovalent PCB adduct (compare Figs. 6A and 6B to Figs. 2A and 2B). Moreover, the CD signals of the probe in the Pr and Pc states are essentially the same in the covalent and noncovalent PCB adducts (compare Figs. 6C and 2C). The CD spectra of the unlabeled PCB adduct are overlaid in Fig. 6C demonstrating that the presence of the probe induces only negligible deviations in the Q and Soret bands. In particular, the sign reversal of the Q band CD, the hallmark of the covalent PCB adduct (47), is nicely reproduced in the construct with covalently attached polarity probe. Flash photolysis measurements at characteristic wavelengths show that the Pr, to Pc photoconversion in the covalent PCB adduct is kinetically and spectrally unaffected by the probe (Fig. 7A). The transient signals of the probe are very similar to that of the noncovalent PCB adduct showing strictly monotonic decrease and increase at 490 and 510 nm, respectively (Fig. 7B). As expected, the covalent PCB adduct of the fluorescein labeled Agp1 is even more photostable than the respective noncovalent PCB adduct. Analysis of the time traces at multiple wavelength leads to very similar results as with the noncovalent PCB adduct including two exponential components and one power law with time constants of 110 ns, 1.1 ms, and 2.2 ms (see Table 1). Due to the very similar spectral shape of the amplitude spectra (compare Figs. 4C and 7C) the assignment of the kinetic components is analogous to that of the noncovalent PCB adduct. The amplitude spectra in the spectral range of the fluorescein probe indicate that the polarity changes in this construct also develop in the Meta-Rc to Meta-Rc, and Meta-Rc, to Pr, transitions (amplitude spectra B, and B, in Fig. 7D). No changes occur in the formation of the Lumi-R and Meta-Rc states (initial difference spectrum ∆Ainit and amplitude spectrum B, in Fig. 7D).

The construct with covalently attached polarity probe and PCB chromophore (Agp1-V249C-PCB-AF) was also used to detect transient protonation changes during photoconversion.

We expected that the covalently attached fluorescein derivative would be an appropriate pH-indicator dye to monitor putative proton release from the chromophore in an early time range below the diffusion limit of the solvent. For this purpose the pH of the sample was lowered to ~6.7, i.e. in the range of the pKs of fluorescein in aqueous solution. The light-induced difference spectra of unbuffered and buffered samples at pH 6.7 and their scaled difference show that the absorption from the deprotonated fluorescein species somewhat decreased indicating a contribution of a small proton release component to the dye signal in unbuffered solution (Fig. 8A). We note that the shape of the difference signal from the fluorescein label becomes asymmetric at this pH value (Fig. 8A) probably due to a pKs shift of the fluorescein derivative upon the polarity change. Consistent with the stationary difference spectra, the transient absorp-
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Figure 7. Photoconversion kinetics of covalent PCB adduct of IAF labeled Agp1. A and B, flash-induced absorption changes of Agp1-V249C-PCB-AF (labeled, solid lines) and Agp1-V249C-PCB (unlabeled, dashed lines) measured at 655, 675, and 700 nm (PCB chromophore) and 490 and 510 nm (fluorescein probe). The traces of Agp1-V249C-PCB were scaled to match the initial (∼10 μs) absorption changes of Agp1-V249C-PCB-AF at 655 nm. The dashed vertical lines in panel B mark the time constants of the main transitions of the fluorescein probe (1.1 and 2.2 ms). Conditions: 50 mM Tris, 200 mM NaCl, pH 7.8, 20 °C. C, and D, amplitude spectra of Agp1-V249C-PCB-AF. ΔA \(_{\text{init}}\) is the sum of the amplitude spectra B \(_{\text{t}}\) to B \(_{\text{f}}\). The dashed line is the steady-state \(B_0 - P\) difference spectrum scaled to the \(B_0\) spectrum. Panel D highlights the spectral range of the fluorescein probe using the same symbols as in Panel C.

Figure 8. Protonation changes monitored by covalently attached pH-indicator dye. A, light-induced absorption changes of Agp1-V249C-PCB-AF measured at pH 6.7 in the absence of buffer (unbuffered, solid line) and in the presence of 10 mM NaPP buffer (buffered, dotted line), scaled to the same \(\Delta A\) at 700 nm. The difference of both changes (protonation signal) is scaled up by a factor of 10 for comparison (dashed line). B, flash-induced absorption changes of Agp1-V249C-PCB-AF measured under the same conditions as in panel A at 490 and 510 nm. C, differences unbuffered minus buffered (transient protonation signal) of the traces of panel B, wavelengths as indicated.

Mechanism of Probe Attachment and Chromophore Incorporation—The present labeling studies show that the thiol-reactive probe IAF binds almost stoichiometrically to Cys-20 of the Agp1 apoprotein, the genuine binding site of the BV chromophore. Upon covalent attachment, the absorption maximum of the fluorescein label displays a considerable
bathochromic shift (511 versus 493 nm for free IAF in aqueous solution) indicating that the probe has moved into a highly hydrophobic environment. This hydrophobic environment is most likely the chromophore pocket, which is lined with a cluster of hydrophobic amino acids facing inward (30, 35, 38). The crystal structure of the related phytochrome PsBphP did not reveal other clusters of hydrophobic amino acids in the region 8 Å around the chromophore binding Cys (38). In view of the highly hydrophobic environment of the fluorescein probe in the apoprotein, it appears very unlikely that the chromophore pocket is not yet present in the apoprotein.

Assembly of the IAF labeled Agp1 apoprotein with either PCB or BV in the dark results in the formation of noncovalent adducts spectrally identical to the respective adducts of the unlabeled Agp1 apoprotein. Intriguingly, the assembly process is associated with a hypochromic shift of the absorption band of the fluorescein probe and a substantial change in its ellipticity. The most plausible explanation for these observations is that the bilin displaces the fluorescein probe from its hydrophobic environment because of its higher affinity to the chromophore pocket. The polarity probe is probably now in a less hydrophobic environment at the entrance of the chromophore pocket. Competition experiments with Agp1 showed that even an incorporated bilin chromophore can be replaced by another bilin that is added subsequently (45). The CD signals of the fluorescein probe for the noncovalent PCB and BV adducts in the Pr state reflect different symmetries in the probe environment. The cancellation of the CD signal in the noncovalent PCB adduct may suggest that the displacement of the probe out of the chromophore pocket is more rigorous in this construct.

The three-dimensional structures of DrBphP and RpBphP3 phytochrome fragments in which the PAS and GAF domains are present imply accessibility of the chromophore pocket and the binding site from the solvent (30–32). More recent results on the PAS/GAF/PHY-domain containing phytochrome fragments of Cph1 and PaBphP suggest that a tongue-like protrusion of the respective PHY domains restricts the accessibility (35, 38) (see also supplemental Fig. S7). However, the arrangements of this protrusion in the crystals of Cph1 and PaBphP are completely different (see supplemental Fig. S7) suggesting structural differences between the two classes of phytochromes (PCB versus BV binding phytochromes) or light-induced changes at the entrance of the chromophore pocket (Pr versus Pfr). Alternatively, these differences might reflect a certain flexibility of the tongue in solution. Our data show that the thiol-reactive compound cannot only bind to Cys-20 of the Agp1 apoprotein but also to Cys-20 of a functional Agp1 holoprotein in which the PCB chromophore is covalently attached to Cys-249. We therefore conclude that the chromophore binding site and the entrance of the chromophore pocket are not obstructed by the PHY domain in the holoprotein. We propose that thermal protein motions in solution, which are not seen in the crystal structures, provide space for attachment of the probe at the entrance of the chromophore pocket. The same thermal flexibility could allow for the entry of the chromophore into the binding pocket of the apoprotein. Although it could be that the binding pocket in the apoprotein is more exposed to the solvent than in the holoprotein, comparable accessibilities of the Cys binding site for the thiol-reactive IAF leading to almost stoichiometric binding argue against such a structural difference. Intramolecular cross-linking also suggests that overall domain arrangements do not significantly differ between Agp1 apoproteins (43).

We note in this context that (i) the covalent binding of thiol-reactive probes to the genuine chromophore attachment site has also been demonstrated for Cph1 (18); (ii) even cyclic porphyrins are able to intrude into the binding pocket of phytochromes (54, 55); and (iii) the chromophore is incorporated in the binding pocket of Cph1 and Agp1 with similar time constants, which are 150 ms (18) and 100 ms, respectively, suggesting adequate accessibility of the pocket.

**Mechanistic Implications of Polarity Changes upon Photoconversion**—Stationary absorption and CD measurements of the Agp1 constructs with a covalently attached fluorescein probe reveal that the probe experiences a change of its environment upon Pr to Pfr photoconversion. The bathochromic shift of the absorption maximum of the fluorescein probe indicates a more hydrophobic environment in the Pfr state. The strong increase in the ellipticity of the fluorescein band moreover shows that this environment is highly asymmetric. The environmental changes of the probe suggest that conformational changes occur upon photoconversion allowing the probe to intrude partially into the chromophore pocket. We exclude that the observed changes are due to an electrostatic effect originating from charge displacements during photoconversion without substantial movement of the polarity probe. The contribution from surface potential changes (see e.g. Refs. 50, 56) is negligible, because control experiments with wild-type Agp1 labeled at Cys-279 and/or Cys-295 did not show stationary and transient signals (see supplemental Fig. S2). The present conclusions are consistent with results from previous binding studies on plant phytochrome using the hydrophobic probe 8-anilinonaphthalene-1-sulfonate (ANS) (57). These experiments suggested competitive binding of ANS to the chromophore binding site showing a higher affinity of ANS for the Pfr form than for the Pr form. However, the binding of ANS to phytochromes led to a bleach of the chromophore absorption (57). It is important to note that the polarity probe induces only negligible deviations in the absorption and CD spectra of the bilin chromophores with respect to that of the unlabeled control samples. In particular, the invariance of the CD spectra ensures that the geometry of the bilin chromophore is unaffected by the presence of the probe. The probe is thus an excellent monitor for the light-induced conformational rearrangements at the “entrance” of the chromophore binding pocket.

The supposed proximity of the probe to pyrrole ring A of the bilin chromophore suggests that the probe is sensitive to possible changes of the conformation/configuration of the A-B methine bridge and/or the microenvironment of ring A at the entrance of the chromophore binding pocket. The present results do not allow discrimination between these two possibilities. One possible scenario could be that the postulated syn to anti rotation of the C5–C6 single bond (33, 36) is associated with a...
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with a relocation of pyrrole ring A allowing the probe to occupy the initial position of ring A. Alternatively, a partial rotation of ring A could produce a cavity such that the probe slips beneath. These rearrangements could occur for example in the proposed hula-twist motion at C5 that involves a concerted syn to anti rotation and Z to E isomerization (33, 58). We note, however, that the results from recent structural investigations on other phytochromes argue against substantial conformational changes of the A-B methine bridge and the surrounding protein matrix (34, 35, 38, 59).

Very similar results have been obtained with all three adducts in which the probe is covalently attached to Cys-20 indicating that the conformational changes monitored by the fluorescein probe upon photoconversion depend neither on the substituent at C3 of ring A (ethyldiene versus vinyl group in the noncovalent adducts) nor on the presence of the covalent attachment of the bilin chromophore (covalent versus noncovalent PCB adduct). This finding is consistent with the conclusion from recent work that provides an integrative interpretation of the CD spectra of the covalent and noncovalent bilin adducts of Agp1 in terms a common mechanism of photoactivation, including the same changes in the geometry of the bilin chromophore (47). On the other hand, the congruent results from the three adducts demonstrate that the labeling position at Cys-20 is particularly suitable to attach the polarity sensitive probe. In previous experiments, Cph1 was labeled at Cys-259 with the same fluorescein derivative (IAF) and subsequently assembled with PCB to form a noncovalent photochromic adduct (18). In this adduct, no spectral changes of the probe and thus no polarity changes could be observed upon photocconversion suggesting that the labeling position is inappropriate. To check an analogous geometry in Agp1, the V249C mutant was first assembled with BV and then incubated with IAF assuming covalent attachment of BV to Cys-20 and binding of the fluorescein probe to Cys-249. Upon photocconversion, this construct showed only small absorption changes of the fluorescein band and complete absence of changes in the CD (see supplemental Fig. S8). However, the spectral properties of the BV adduct of the V249C mutant in the far-red absorbing photoprodut deviate considerably from that of the Pₚ form of wildtype Agp1, similar to the homologous mutant of DrBphP (31). These deviations, already observed with the unlabeled construct, challenge the above assumptions on the covalent attachment of BV and the polarity probe at the specific positions and thus do not allow further conclusions.

Kinetics of Polarity Changes in the Pₛ to Pₚ Photoconversion—Flash photolysis measurements demonstrate that the polarity changes observed in stationary spectra can be time-resolved. The more photostable PCB adducts allowed measurements at multiple wavelengths. The chromophore of the noncovalent BV adduct irreversibly bleaches during flash photolysis. Thus, measurements were performed at few wavelengths characteristic for the polarity probe and the bilin chromophore. The kinetics and the spectral signature of the Pₛ to Pₚ photocconversion are not significantly affected by the covalently attached probe. In contrast, the use of ANS as a hydrophobic probe led to acceleration of the Pₛ to Pₚ photoconversion and inhibition of the light-induced and thermal reconversion (57).

For all of the three adducts, no significant polarity changes with respect to the Pₛ state occur in the early time range reflecting the photoisomerization of the C-D methine bridge and the formation of Lumi-R. This is consistent with the supposed geometry in which the probe is remote from the C-D methine bridge. Moreover, our data suggest that the process of photoisomerization is not associated with additional conformational changes of the A-B methine bridge located close to the probe. In contrast, changes in Lumi-R near C5 from ultrafast mid-infrared spectroscopy of Cph1 have been interpreted as low bond order torsional angle changes of the C5–C6 bond (60). We note that putative rapid changes were also absent in our experiment with the covalent PCB adduct in which the coupling between chromophore and probe is expected to be stronger and thus more efficient for rapid signal transmission than in the noncovalent PCB adduct.

The first transition resolved in the flash photolysis experiments reflecting the Lumi-R to Meta-Rₛ transition is also not associated with substantial polarity changes in all three adducts. These changes clearly occur in the subsequent transitions reflecting the Meta-Rₛ to Meta-Rₐ and the Meta-Rₐ to Pₚ transitions, respectively. In an adduct of Agp1 with a locked 5Zs-BV chromophore the Meta-Rₛ to Meta-Rₐ transition was inhibited (36). The present results provide further evidence for conformational changes of the A-B methine bridge during the Meta-Rₛ to Meta-Rₐ transition. Moreover, the approach using the polarity probe reveals that additional changes occur at this position in the Meta-Rₐ to Pₛ transition. Control measurements with the unlabeled adducts make sure that structural changes of the bilin chromophore and the binding pocket during photocconversion are almost unaffected by the polarity probe. In this respect, the approach with the polarity probe is clearly advantageous over the more indirect method using the locked chromophore. The temporal progression of the observed conformational changes is consistent with results from low temperature Fourier transform IR suggesting that ring A is only affected in the late steps of the Pₛ to Pₚ photoconversion (39).

The signals of the fluorescein probe differ qualitatively in the PCB and BV adducts. Whereas a biphasic strictly monotonous rise was observed in both the noncovalent and covalent PCB adducts (Figs. 4B and 7B), the signal in the (noncovalent) BV adduct displays a biphasic rise that is followed by a partial decay (Fig. 5B). The first rise component of the fluorescein signal is kinetically synchronized with the formation of the Meta-Rₐ intermediate in all three adducts with time constants in the same range (~1 ms). The second rise component in the BV adduct (τ = 46 ms) is not associated with a major transition in the relaxation cascade of the chromophore most likely reflecting a transition between two Meta-Rₐ substates. This component is not apparent in the signals of the PCB adducts and is probably hidden by the main transitions (Meta-Rₐ to Meta-Rₐ and Meta-Rₐ to Pₛ) with time constants nearby. Note that the formation of Pₛ in the PCB adducts is accelerated by two orders of magnitude with respect to the BV adduct (2.7/2.2 ms versus 430 ms). Provided that the assignment to the canonical transitions is correct, the Meta-Rₐ to Pₛ transition in the PCB adducts is associated with a rise component of the fluorescein.
signal, whereas a decay component of smaller amplitude is present in the respective transition of the BV adduct. This apparent discrepancy suggests that the conformational changes of the A-B methine bridge of the bilin chromophore and/or the microenvironment of ring A differ in the PCB and BV adducts in the final transition of the Pπ to Pn photoconversion. However, the data of both adducts may be reconciled by assuming that the second rise component and the smaller decay component present in the BV adduct merge to one rise component of reduced amplitude in the PCB adducts. Further investigations are necessary to resolve this issue.

Kinetic coupling of polarity changes and proton release during photoconversion was observed with the V249C mutant of Agp1 in which both the fluorescein probe and the PCB chromophore are covalently attached. Moreover, the kinetics of proton release monitored with the covalently attached fluorescein probe is essentially the same as observed with pyranine in solution. These observations suggest that the conformational changes, responsible for the observed polarity changes, initiate proton release to the surface of the protein and ultimately to the solvent. Apparently, these proton transfer steps proceed in a time range where diffusion is not rate-limiting. Because the conformational changes most likely involve the chromophore, direct coupling to the transient deprotonation of the bilin chromophore in the Meta-Rc intermediate (28) seems reasonable and supports the assumption that the chromophore is the source of the released proton (61).

Conclusions and Outlook—The present study demonstrates that polarity probes, covalently attached at the entrance of the chromophore pocket, are valuable tools to monitor conformational changes of the A-B methine bridge of the bilin chromophore and/or the microenvironment of ring A at the entrance of the binding pocket during Pπ to Pn photoconversion. These changes occur in the thermal relaxation cascade after photoisomerization, kinetically correlated with the Meta-Rc to Meta-Rp and the Meta-Rp to Pn transitions, and may be essential for transmission of the signal from the chromophore to the photoreceptor protein.

In future work, the constructs of Agp1 presented here will be utilized to investigate the Pπ to Pn photoconversion and dark reversion. Preliminary results with the V249C mutant of Agp1 showed that the major polarity changes of the probe environment are coupled to the slow phase in the biphasic dark reversion. One particular challenge will be the preparation of the covalent BV adduct of Agp1 with the polarity probe attached at an alternative position. Modeling studies will be performed to assign the observed polarity changes to specific conformational changes. The experimental approach using a covalently attached polarity probe is probably appropriate for other phytochromes and will be tested, e.g., with Agp2 and the L18C mutant of Cph1, the analogue of the V249C mutant of Agp1.

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A Polarity Probe for Monitoring Light-induced Structural Changes at the Entrance of the Chromophore Pocket in a Bacterial Phytochrome

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