Unusual Spectral Properties of Bacteriophytochrome Agp2 Result from a Deprotonation of the Chromophore in the Red-absorbing Form Pr*

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Phytochromes are widely distributed photoreceptors with a bilin chromophore that undergo a typical reversible photoconversion between the two spectrally different forms, Pr and Pfr. The phytochrome Agp2 from Agrobacterium tumefaciens belongs to the group of bathy phytochromes that have a Pfr ground state as a result of the Pr to Pfr dark conversion. Agp2 has untypical spectral properties in the Pr form reminiscent of a deprotonated chromophore as confirmed by resonance Raman spectroscopy. UV/visible absorption spectroscopy showed that the pK_a is >11 in the Pfr form and ~7.6 in the Pr form. Unlike other phytochromes, photoconversion thus results in a pK_a shift of more than 3 units. The Pr/Pfr ratio after saturating irradiation with monochromatic light is strongly pH-dependent. This is partially due to a back-reaction of the deprotonated Pr chromophore at pH 9 after photoexcitation as found by flash photolysis. The chromophore protonation and dark conversion were affected by domain swapping and site-directed mutagenesis. A replacement of the PAS or GAF domain by the respective domain of the prototypical phytochrome Agp1 resulted in a protonated Pr chromophore; the GAF domain replacement afforded an inversion of the dark conversion. A reversion was also obtained with the triple mutant N12S/Q190L/H248Q, whereas each single point mutant was characterized by decelerated Pr to Pfr dark conversion.

Results:

Typical phytochromes include a protonated chromophore in the parent states (Pr and Pfr) that transiently deprotonates during photoconversion.

Background:

The phytochrome Agp2 from Agrobacterium tumefaciens belongs to the group of bathy phytochromes that have a Pfr ground state as a result of the Pr to Pfr dark conversion. Agp2 has untypical spectral properties in the Pr form reminiscent of a deprotonated chromophore as confirmed by resonance Raman spectroscopy. UV/visible absorption spectroscopy showed that the pK_a of the chromophore is lowered from >11 to 7.6 during the conversion from Pfr to Pr.

Consequence:

Chromophore protonation affects light-induced and thermal Pr to Pfr conversion.

Significance:

Agp2 can act as integrated light and pH sensor.

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3 The abbreviations used are: BV, biliverdin; RR, resonance Raman; Ptot, total phytochrome; ip, in-plane.
Protonation of Bacteriophytochrome Chromophore

BV-binding phytochromes. Quite interestingly, the chromophore-binding Cys residue of BV-binding phytochromes is located in the N terminus of the PAS domain, whereas the Cys binding site in phycocyanobilin- and phytochromobilin-binding phytochromes is in the center of the GAF domain (11).

Vibrational spectroscopy, NMR studies, and pH titrations combined with UV/visible spectroscopy have shown that the chromophore is typically protonated in the Pr and Pfr states and that Pr to Pfr photoconversion is accompanied by a transient proton release (12–14). Free phytochrome chromophores are unprotonated in neutral solution (15), and protonation under acidic conditions occurs at the nitrogen atom of ring B or ring C; i.e. a positive charge is distributed over both central rings. In the biliprotein phycocyanin, an Asp side chain serves as the counter ion for the protonated bilin chromophore (16). A comparable scenario with an Asp facing toward the chromophore has recently been observed in structures of two cyanobacteriophytochromes (17). Phytochrome crystal structures show that a highly conserved Asp residue within the conserved Pro-Ala-Ser-Asp-Ile-Pro motif is central in the chromophore pocket (8). In the structure of the bathy phytochrome PaBphP, the carboxylic acid side chain of this Asp residue is hydrogen-bonded with the ring D nitrogen of the Pfr chromophore (9). The same interaction has been confirmed by NMR measurements on cyanobacterial Cph1 (18). The Asp side chain could compensate for the positive charge of the chromophore in the Pfr state, although an interaction with the central pyrrole rings would have been expected. In the Pr form, this hydrogen bond is lost due to the Z to E isomerization around the methine bridge connecting rings C and D as shown by the crystallographic analyses in the Pr form (8, 10, 19) and temperature scan crystallography for the Pfr to Pr photoconversion of PaBphP (20). It is postulated that charge compensation is distributed over several groups including the propionic side chain and a conserved His residue. In phytochrome variants in which this His residue is mutated, the chromophore is preferentially unprotonated at pH 7.8 (21).

The soil bacterium Agrobacterium tumefaciens contains two phytochromes termed Agp1 and Agp2 (22) or AtBphP1 and AtBphP2 (2). Both have been used as model phytochromes for biochemical and biophysical studies. Agp1 may be regarded as a typical phytochrome with a C-terminal His kinase. It has a Pr ground state, and the Pfr form converts slowly to Pr in darkness. Agp2, which is characterized in the present study, has a His kinase of the HWE type. HWE stands for those amino acids that are conserved in this particular group of kinases. Unlike typical bacterial phytochromes, Agp2 has a C-terminal response regulator (see Fig. 1). The domain arrangement is thus comparable with fungal phytochromes (23). The domain arrangement suggests an intramolecular phosphorelay from the His kinase to the C-terminal response regulator. In addition, Agp2 is a bathy phytochrome with a Pfr ground state and a Pr to Pfr dark conversion.

In a recent study, 15 phytochromes with an Agp2-like domain arrangement have been identified in the order Rhizobiales (3). Spectral properties of six members of this group (Agp2, Avp1, Avp2, and phytochromes of Rhizobium leguminosarum, Rhizobium etli, and Azorhizobium caulinodans) have been characterized by in vivo photometry and studies on recombinant proteins (3). All of these phytochromes with the exception of Avp1, which displays unusual spectral properties, are bathy phytochromes. This correlation points to a co-evolution of phytochromes with HWE kinases and the bathy phenotype.

The bathy phytochromes characterized so far have slightly different properties. BphP1 of the photosynthetic rhizobial bacterium Bradyrhizobium ORS278 and a homologous protein of Rhodopseudomonas palustris lack the C-terminal His kinase but carry a PAS/S box at the C terminus (24). Pseudomonas aeruginosa phytochrome PabphP has the prototypical domain arrangement with a C-terminal His kinase (25).

In addition to the domain arrangement and the bathy phenotype, Agp2 also differs from prototypical phytochromes by its Pr absorption characteristics. Under standard buffer conditions (pH 7.8), the photoconversion of Pfr to Pr is characterized by only a weak absorbance change in the 700-nm region where the absorption maximum of Pr is located. By spectral studies on extracts of Agrobacterium phytochrome mutants, it has been found that compounds of the cell extract modulate the difference spectrum of Agp2; in the presence of cell extract, the difference spectrum has more balanced positive and negative parts. Titration series showed that the unknown interacting factor binds to Agp2 in a specific manner and has a concentration around 20 μM in the cell extract (4, 26).

Here we show that the unusual spectral properties of Agp2 Pr are caused by the chromophore protonation state. Whereas the Pfr chromophore is protonated up to a pH of 11, the Pr chromophore has a pK_a value of 7.6 and is thus only partially protonated at neutral pH. This conclusion was confirmed by resonance Raman (RR) spectroscopy. Flash photolysis on the Pr to Pfr half-cycle suggests that after photoisomerization the deprotonated chromophore can undergo a back-conversion from an intermediate state to Pr, resulting in a low efficiency of the net Pr to Pfr photoconversion. We also analyzed the role of protein domains and selected amino acids in the dark conversion and protonation of the Pr chromophore.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—All expression vectors are based on the plasmid pET21b (Novagen). The vector pAG2-M1 encodes for full-length Agp2 with a C-terminal His_6 tag, and pAG2-M2 encodes for the truncated protein Agp2-M2 (amino acids 1–501), which lacks the His kinase and the response regulator. Agp2-M2 also has a C-terminal His_6 tag. Cloning of pAG2-M1 and pAG2-M2 was described previously (4, 27). Expression vectors for domain swap mutants were generated by PCR of the respective fragments and blunt end ligation; the detailed positions are given in Fig. 1. For the Agp1 domains, the vector pAG1 (22) was used as template. Point mutants were generated according to the QuikChange site-directed-mutagenesis (Agilent) protocol. All expressed proteins have a C-terminal His_6 tag for affinity purification. Expression and purification were performed as described (4). Briefly, Escherichia coli BL21(DE3) cells (New England Biolabs) with the desired plasmid were grown at 37 °C in LB medium with ampicillin up to an A_600 of 0.6. Addition of 20 μg/ml isopropyl
Protonation of Bacteriophytochrome Chromophore

1-thio-β-D-galactopyranoside induced specific protein expression carried out at 16 °C until an A_{600 nm} of 3 was reached. Cells were harvested by centrifugation, washed with basic buffer (50 mM Tris-HCl, 300 mM NaCl, 5 mM EDTA, pH 7.8), centrifuged again, and extracted with a French pressure cell in the same buffer. Soluble proteins were precipitated with ammonium sulfate, suspended in low concentrated imidazole buffer (10 mm imidazole, 50 mM Tris-HCl, 300 mM NaCl, pH 7.8) and purified by Ni^{2+} affinity chromatography. After elution with highly concentrated imidazole buffer (250 mM imidazole, 50 mM Tris-HCl, 300 mM NaCl, pH 7.8), phytochrome-containing fractions were precipitated with ammonium sulfate. Proteins were usually dissolved in basic buffer containing 50 mM Tris-HCl, 5 mM EDTA, 300 mM NaCl, pH 7.8.

Chromophore Assembly and pH Titrations—All UV/visible spectra were recorded with a Jasco V550 photometer at 20 °C. Photoconversion was performed with light-emitting diodes of 655 nm/300 μmol m^{-2} s^{-1} for red light and 780 nm/1000 μmol m^{-2} s^{-1} for far-red light with typical irradiation times of 60 s. The biliverdin chromophore was purchased from Frontier Scientific and stored as a 4 mM DMSO stock solution. Purified apoproteins were incubated either with a 0.8:1 or a 2:1 molar ratio of chromophore:protein. The assembly was followed by absorption measurements and proceeded at least until no further absorption changes were detectable. Excess chromophore was removed by using desalting columns (NAP-10 columns, GE Healthcare). For varying the pH, the protein (3–5 mg/ml) was precipitated with ammonium sulfate and suspended in buffer (50 mM Tris, 300 mM NaCl, 5 mM EDTA) with the desired pH (from 4.1 to 11.9). The pH was adjusted with HCl or NaOH.

UV/visible spectra of the pure Pr form were calculated under the following assumptions. (i) Spectra of the pure Pfr form are given by the non-irradiated samples. (ii) The irradiated sample contains only Pr and Pfr. (iii) The Pr form has an absorbance ratio A_{705 nm}/A_{755 nm} of 0.1 (28). The wavelength positions correspond to the absorption maxima of Pr and Pfr. The ratio is based on the spectrum of an Agp2 adduct with a locked 15Za-BV chromophore, which has a Pr-like spectrum. Pure Pr spectra of Agp2 can otherwise not be constructed. With these assumptions, data evaluation gave consistent results. When lower or higher A_{705 nm}/A_{755 nm} values were chosen, the calculated spectra had a more or less aberrant shape.

Thus, pure Pr spectra were calculated according to

$$A_{Pr} = \frac{A - f \cdot A_{Pfr}}{1 - f} \quad (Eq. 1)$$

where f is the fraction of Pfr/Ptot. This value was adjusted until $A_{Pr, 705 nm}/A_{Pr, 755 nm} = 0.1$.

Calculated maxima of the Pr spectra were plotted versus the pH. To determine the $pK_a$, the Henderson-Hasselbalch equation was fitted to the data (OriginPro 8.5).

$$y = \frac{10^{pH - pK_a}}{1 + 10^{pH - pK_a}} \times (A1 - A2) + A2 \quad (Eq. 2)$$

Here A1 and A2 refer to the absorbance values of the protonated and deprotonated form, respectively, and y is the measured absorbance.

Flash Photolysis—Flash photolysis experiments on the Pr half-cycle at room temperature were performed using excitation pulses of 10-ns duration and ~1.5-mJ energy at λ = 660 nm (high energy part of Pr absorption spectrum, i.e. little spectral overlap with Pfr; see Fig. 2, A and C), generated by an optical parametric oscillator (GWU Versa-Scan) pumped by a neodymium-doped yttrium aluminum garnet laser (Quanta-Ray-INDI). The spectrally dispersed continuous wave probe light from a xenon lamp (Hamamatsu, L2273) was detected via a photomultiplier (Hamamatsu, R928). For the experiments, Agp2 was suspended in standard buffer solution (50 mM Tris, 300 mM NaCl, 5 mM EDTA, 6 mM DTT, pH adjusted with HCl or NaOH). The concentration corresponded to ~0.7 OD units at 750 nm in a cuvette with 1-mm path length at maximum Pfr enrichment. Immediately before each exciting laser shot the sample was pre-illuminated at 785 nm for 10 s. The dose of pre-illumination and the choice of excitation wavelength ensured maximum Pr population and predominantly Pr excitation, respectively. Control experiments with blocked excitation pulse were performed to quantify the contributions of the Pr dark reaction to the photoinduced transient absorption signals within 4 s after photoexcitation.
Transient absorption data, $\Delta A(t, \lambda)$ (i.e. the time-dependent absorption difference between sample absorbance at time $t$ and the unphotolyzed ground state), were analyzed by a global fit using a sum of exponentials according to Equation 3.

$$\Delta A(t, \lambda) = A_0(\lambda) + \sum_{j=1}^{N} A_j(\lambda) e^{-t/\tau_j} \quad \text{(Eq. 3)}$$

yielded time constants $\tau_j$, decay-associated spectra $A_j(\lambda)$, and the difference spectrum at long delay times $A_0(\lambda)$. The minimum number of time constants was determined by evaluation of fit residuals and singular value decomposition.

Resonance Raman Spectroscopy—For RR experiments, protein solutions were prepared in Tris-HCl buffer (100 mM Tris, 300 mM NaCl, 5 mM EDTA) at the desired pH. Complementary measurements were performed in D$_2$O (Deutero GmbH) under otherwise identical buffer conditions. The protein concentration corresponded to an A of 50 at 280 nm.

RR spectra were measured at $-140^\circ$C either with 1064- or 413-nm excitation using the setups described previously (21, 29). The total accumulation times were 1 h (780 milliwatts) and 5 min (3–4 milliwatts) at 1064 and 413 nm, respectively. The spectrum of the Pfr state was obtained without additional illumination. After 10 s of far-red irradiation with 785 nm (20 milliwatts) at ambient temperature, the RR spectra of the Pr-enriched sample were recorded. Pr to Pfr back-conversion was monitored by measuring the spectrum after red light irradiation (2 min, 664 nm, 3 milliwatts) at ambient temperature prior to the analysis of the protonation equilibrium of the Pr species. The contributions of the Pfr state (and in the case of the 1064-nm measurements also the spectral contribution of the protein matrix) were subtracted on the basis of characteristic marker bands. In all spectra shown in this work, the background was removed by polynomial subtraction. For the determination of the PK$_a$, we have measured both the Pr and Pfr spectrum within pH 6.0 and 10.0 (at 0.5 pH steps). The raw Pr spectra were then subjected to a component analysis (30). Here complete spectra rather than individual bands were fitted to the RR spectra measured as a function of the pH. As only four components (corresponding to four variables) are used, the error of the fitting procedure itself is very low. The four components are the protonated and deprotonated forms of the Pr state, the Pfr state, and the apoprotein. Although the spectra of the latter two components were measured in separate experiments, initial component spectra of the protonated and unprotonated species were obtained by mutual subtraction of the spectra measured at the extreme pH values. These initial spectra were further refined to achieve the best possible global fit to all measured spectra. The relative spectral contributions of the protonated and deprotonated Pr ($I_p$ and $I_d$) as determined from the component analysis are proportional to the relative concentrations ($c_p$ and $c_d$) according to

$$c_p = f_p \cdot I_p \quad \text{(Eq. 4)}$$

and

$$c_d = f_d \cdot I_d \quad \text{(Eq. 5)}$$

where $f_p$ and $f_d$ are proportionality factors that are proportional to the reciprocal Raman cross-sections.

The relative concentrations were normalized to 1 according to Equation 6.

$$1 = f_p \cdot I_p + f_d \cdot I_d = c_p + c_d \quad \text{(Eq. 6)}$$

Then the Henderson-Hasselbalch equation adopts the form

$$\text{pH} = \text{pK}_a + \log \left( \frac{c_d}{c_p} \right) = \text{pK}_{\text{a,app}} + \log \left( \frac{I_d}{I_p} \right) \quad \text{(Eq. 7)}$$

where $\text{pK}_{\text{a,app}}$ is given by Equation 8.

$$\text{pK}_{\text{a,app}} = \text{pK}_a + \log \left( \frac{I_d}{I_p} \right) \quad \text{(Eq. 8)}$$

The ratio $I_d/I_p$ is approximated by the reciprocal intensity ratio of the strongest cofactor bands relative to the Phe band at 1005 cm$^{-1}$ (as an internal standard) of a deprotonated and protonated species that has been determined to be $\sim$2.

**RESULTS**

Titration of Agp2: UV/Visible Spectral Properties—It is generally agreed that chromophore deprotonation is indicated by a reduced oscillator strength to the first electronic transition. We thus assumed that the low Pr absorbance of Agp2 as measured at pH 7.8 indicates partial or complete deprotonation of the Pr chromophore, whereas the spectrum of Pfr suggests a protonated chromophore. To determine the $\text{pK}_a$ of the Pr and Pfr chromophores, we performed titration experiments between pH 5 and 11. Within this range, Pfr spectra were pH-independent (Fig. 2, A and C), indicating that the Pfr chromophore is protonated up to a pH of 11 or more, corresponding to a $\text{pK}_a$ $>$11. For the analysis of the Pr form, we irradiated Agp2 with saturating 780 nm light (far-red) before the measurements. After far-red irradiation, the relative Pfr content of prototypical phytochromes is only around 5% Pfr (22). However, this value was much higher in Agp2 (Fig. 2B), and the relative Pfr content was found to be pH-dependent. We estimated that at low pH values around 5 the Pfr/Ptot ratio after saturating far-red irradiation is 0.7 (Fig. 2E). This unexpected high ratio could be due to two effects. (i) Despite the low absorption of Pr at 780 nm, Pr to Pfr photoconversion could contribute to Pfr formation. (ii) Dark conversion occurs already during the 40-s delay between the switch off of the actinic light and the beginning of the absorbance measurement. The Pfr/Ptot ratio decreased with increasing pH and reached 0.08 for high pH values of 9 and above (Fig. 2E). This pH-dependent Pfr/Ptot ratio was further characterized by flash photolysis experiments (see below).

From the raw spectra, we calculated pure Pr spectra (Fig. 2C), which show that the extinction coefficient of Pr is pH-dependent. This value is high for low pH and low for high pH (Fig. 2D), indicative for a protonated and deprotonated chromophore, respectively. A Henderson-Hasselbalch fit yielded a $\text{pK}_a$ of 7.59 $\pm$ 0.07. The ratio of absorbance changes that are observed after photoconversion from Pfr to Pr at 705 and 755 nm ($-\Delta A_{705 \text{ nm}}/\Delta A_{755 \text{ nm}}$) can be taken as a quantitative measure for the protonation state of the Pr chromophore. This
“absorbance change ratio” varies from 0.5 for low pH values to
0.2 for high pH values (Fig. 2, F and G).

Titration of Truncated Agp2-M2—The truncated Agp2-M2
contains only the PAS, GAF, and PHY domains and lacks the
HWE His kinase and the response regulator (Fig. 1). Usually,
spectral properties of such truncated phytochromes are similar
to those of the full-length proteins (31). In Agp2, spectra of the
truncated Agp2-M2 are considerably different from Agp2 (4).
The Pfr spectra of the truncated protein, measured between pH
4 and 12, varied only slightly (Fig. 3, A and D), apparently due
to minor scattering effects. Again, the Pfr chromophore appears
to be protonated at least up to a pH of 12. After far-red irradi-
ation, the spectral contribution of Pr is not visible (Fig. 3B)
because the absorbance of Agp2-M2 Pr is even lower than that
of the full-length adduct. Pure Pr spectra were again obtained by
calculations (Fig. 3C). These spectra were also pH-dependent (Fig. 3D).
However, the pKₐ of the Pr chromophore was
determined to be 6.66 ± 0.08. The fraction Pfr/Ptot after far-
red irradiation varied between 0.9 at pH 6 and 0.2 at pH 12. The
comparison between the full-length protein and the truncated
Agp2-M2, therefore, shows that the His kinase/response regu-
lator affects the protonation state of the Pr chromophore.

Flash Photolysis Experiments—The impact of pH on the Pr
photoreaction and Pr dark conversion of Agp2 was further
characterized by transient absorption experiments on the pho-
toinduced Pr to Pfr conversion. The transient spectra clearly
show that the photoconversion patterns at high and low pH
differ significantly.

Fig. 4 (left) shows selected transient absorption difference
spectra after Pr photoexcitation at pH 6, 7.8, and 9 in the time
range up to 4 s after photoexcitation (see “Experimental Proce-
dures”). Negative signals below 725 nm at late times reflect the
pump pulse-induced depletion of the Pr state; positive signals
above 725 nm display the formation of Pfr on the time scale up
to several seconds. The absence of an isosbestic point indicates
the presence of more than two molecular species involved as
expected for the Pr half-cycle in phytochromes (Agp1 (14),
PhyA (32, 33), and Cph1 (12, 34)). The global fit (Equation 3)
of the data at each pH revealed
four time constants, τ₁ to τ₄, and associated amplitude spectra
A₁ to A₄ (Fig. 4, middle), the latter describing the spectral
absorbance changes associated with the corresponding time
constants. A₀ stands for the amplitude of total changes, i.e. the
difference spectrum of all product states formed 4 s after pho-

![FIGURE 2. UV/visible spectral properties of full-length Agp2 at different pH values. A, spectra of dark-adapted form (Pfr). B, spectra after far-red irradiation showing a mixture of Pr and Pfr. C, calculated Pr spectra. D, absorption of Pfr and Pr at their respective maximum at different pH values. The Henderson-Hasselbalch (HH) fit to the Pr data yielded a pKₐ of 7.59. E, estimated fraction (f = Pfr/Ptot) of far-red-irradiated samples as shown in B. F, difference spectra of far-red-irradiated minus dark-adapted Agp2. G, absorbance change ratios from these difference spectra.](image)

![FIGURE 3. UV/visible spectral properties of Agp2-M2 (PAS-GAF-PHY domains of Agp2 without C-terminal His kinase) at different pH values. A, spectra of dark-adapted form (Pfr). B, spectra after far-red irradiation showing a mixture of Pr and Pfr. C, calculated Pr spectra. D, absorption of Pfr and Pr at their respective maximum at different pH values. The Henderson-Hasselbalch (HH) fit to the Pr data yielded a pKₐ of 6.68. E, estimated fraction (f = Pfr/Ptot) of far-red-irradiated samples as shown in B.](image)
to excitation (including the contributions of the Pr dark conversion) and the spectrum before time 0. At each pH, $A_4$ is at least 5 times larger than each of the remaining three time constants. By means of control experiments with blocked excitation pulses, it was possible to assign $A_4$ to the dark conversion in terms of amplitude and spectral shape. Thus, the strong amplitude of $A_0$ in the region of Pfr absorption is mainly caused by dark conversion. This separation of time scales and the control experiments allow adding $A_4$ to $A_0$ (Fig. 4, right). Thereby, in good approximation, $A_0$ reflects the difference spectrum of exclusively the photoinduced amount of Pfr (4 s after photoexcitation) and the initial amount of Pr.

The observed time constants show some variations with increasing pH. In particular, $\tau_3$ increases, and $\tau_2$ decreases. The value of $\tau_4$, the time constant characterizing the dark reaction within the first 4 s (after switching off the pre-illumination at 785 nm), is almost doubled between pH 6 and 9.

The negative amplitude of $A_3$ at 755 nm is assigned to the photoinduced Pfr formation. The similarity of the negative features of $A_3$ at 705 and 650 nm (at pH 7.8 and 9) with the (pure) Pr spectrum is remarkable and is thus assigned to the formation of meta-Rc and, at low pH, the formation of Pfr. The change of $A_3$ with increasing pH (see above) strongly suggests that the deprotonated form undergoes a considerably altered photocycle (Pr half-cycle) with an increased recovery of the Pr state in a shunt reaction and, in consequence, less formation of Pfr. Note, however, that independently of the chromophore protonation state, Pr undergoes a photoisomerization followed by a number of intermediates, i.e. thermally unstable states. The overall amplitude of $A_2$ is very small and difficult to implement into the half-cycle. It could represent a back-reaction within the cycle or a side reaction to another intermediate (5). It is not further discussed here.

Following the results of similar experiments on Agp1 (35) in terms of observed relaxation time constants and shape of the associated spectra, $\tau_1$ is assigned to the decay of meta-Ra and the formation of meta-Rc, and $\tau_3$ is assigned to the decay of meta-Rc and, at low pH, the formation of Pfr. The change of $A_3$ with increasing pH (see above) strongly suggests that the deprotonated form undergoes a considerably altered photocycle (Pr half-cycle) with an increased recovery of the Pr state in a shunt reaction and, in consequence, less formation of Pfr. Note, however, that independently of the chromophore protonation state, Pr undergoes a photoisomerization followed by a number of intermediates, i.e. thermally unstable states. The overall amplitude of $A_2$ is very small and difficult to implement into the half-cycle. It could represent a back-reaction within the cycle or a side reaction to another intermediate (5). It is not further discussed here.

**Resonance Raman Spectroscopy**—RR spectroscopy can be used to monitor the structure and protonation state of phytochrome chromophore. RR spectra of the Pr state of Agp2 were measured in the pH range from 6.0 to 10.0 using 1064- and 413-nm excitation lines, which are in (pre-) resonance with the first and second electronic transitions of the tetrapyrrole cofactor, respectively. The pH-dependent spectral changes correspond to the acid-base reaction of the cofactor as reflected in the UV/visible absorption spectra (Fig. 2C). A selection of RR spectra is shown in Fig. 5A. A careful inspection reveals that several bands, indicated by the vertical dotted lines, are only present at pH 6.0 (e.g. bands at 756, 806, 1295, and 1305 cm$^{-1}$), whereas others are characteristic features at pH 9.0 (e.g. bands
at 711, 1071, 1166, and 1251 cm\(^{-1}\)). The Pr spectra at higher pH values (9.5 and 10.0) were reminiscent of deprotonated species at pH 9.0 species (data not shown). The spectrum measured at an intermediate pH of 7.8 includes contributions of both groups of bands, indicating a two-state pH-dependent equilibrium consistent with the UV/visible spectroscopic data (see above).

More information about the structure and protonation state of the chromophore in the two species involved can be obtained from the region between 1500 and 1700 cm\(^{-1}\) that is dominated by the C=C stretching modes of the methine bridges and the individual pyrrole rings (Fig. 5B). In addition, this region includes a unique marker band for the protonation state originating from the in-phase in-plane bending of the N–H groups of rings B and C (N–H ip) (36, 37) (for the notation, see Fig. 5C). In the spectrum of the “acidic” form, this mode is readily assigned to the band at 1571 cm\(^{-1}\) that disappears in D\(_2\)O (Fig. 5B). Hence, the state prevailing at pH 6.0 includes a protonated chromophore.

For protonated tetrapyrroles in the ZZZssa (Pr) and ZZEssa (Pfr) configuration, vibrational assignments are well established based on theoretical calculations and supported by isotopic labeling (36–41). In general, the vibrational band pattern of the protonated Pr state of Agp2 is quite similar to that of Deinococcus radiodurans, indicating the same ZZZssa geometry of the tetrapyrrole. The strongest RR band originates from the C=C stretching of the C-D methine bridge, which because of the coupling with the N–H ip coordinate of ring C undergoes a downshift in D\(_2\)O. Hence, this mode is assigned to the band at 1621 cm\(^{-1}\) in H\(_2\)O (1610 cm\(^{-1}\) in D\(_2\)O) (40). The nearby hydrogen/deuterium-insensitive band at 1625 cm\(^{-1}\) originates from
Protonation of Bacteriophytochrome Chromophore

Domain Swap Mutants and Dark Conversion—To find out which region of the protein is relevant for Pr to Pfr dark conversion and for BV protonation, we generated domain swap mutants in which one domain of Agp2 was replaced by the equivalent domain of Agp1 as outlined in Fig. 1. All proteins were assembled with BV for 15 h and measured after removal of free chromophore at a pH of 7.8 (Fig. 6, A–F). Under these conditions, Agp2 is completely in the Pfr state. A second spectrum was recorded after photoconversion by red or far-red light (Fig. 6, A–F). Fig. 6, G–K, show spectra of irradiated samples that were taken in 10-min intervals during dark conversion. The spectra of Agp2_Gaf1 (Fig. 6C) in which the GAF domain of Agp2 was replaced by that of Agp1 are very similar to those of Agp1 (Fig. 6F). The protein remained in the Pr form during assembly and after photoconversion into Pfr, it converted back to Pr in darkness (Fig. 6l). The absorbance change ratio was 1.29, which is much higher than the value of 0.24 for Agp2 and almost as high as the value of 1.45 for Agp1. This finding suggests that the Pr chromophore is fully protonated. In Agp2_Pas1, the PAS domain of Agp2 is replaced by that of Agp1. This mutant undergoes Pr to Pfr dark conversion during assembly or after far-red irradiation (Fig. 6, B and H), but dark conversion was very slow and was incomplete even after 15 h such that both Pr and Pfr were present (Fig. 6E). The absorbance change ratio of 0.74 is indicative for a high fraction of the protonated Pr chromophore. Spectra of Agp2_Phy1 in which the PHY domain of Agp2 is replaced by that of Agp1 have also been shown in an earlier study (43). These spectra are reminiscent of a bleached Pr; irradiation induces only minor spectral changes (Fig. 6D). We could not observe dark conversion after irradiation with red or far-red light (Fig. 6f). An absorbance change ratio of 0.06 indicates that only a minor fraction of the Pr chromophore is protonated. When the His kinase/response regulator was replaced by the His kinase of Agp1 (mutant Agp2_Hiskin1), spectra (Fig. 6E) were similar to those of the Agp2 wild type (Fig. 6A) but different from those of the truncated version Agp2-M2 (Fig. 3). Thus, the above effect of the Agp2 His kinase on spectra and chromophore protonation can be replaced by another His kinase and is apparently not specific. Dark conversion of Agp2_Hiskin1 was more rapid than that of Agp2 (Fig. 6K).

Point Mutants—To study the role of single amino acids on the protonation status and dark conversion of Agp2, we produced a series of point mutants that were measured by UV/visible spectroscopy. By studying phytochrome sequence homologies, we had noticed that Asn-12 of Agp2 is conserved in ~50% bathy phytochromes, whereas other phytochromes have different amino acids at that position. Asn-12 is located next to the chromophore-binding Cys-13, and dark conversion depends on the formation of a covalent bond between this Cys residue and the chromophore (44). Therefore, we reasoned that the amino acid could have an impact on the dark conversion of Agp2. We replaced Asn-12 by Ser, the homologous amino acid of Agp1, to yield the mutant N12S. The second mutation, H248Q, was made according to Karniol et al. (45) who proposed that the H248Q mutant of truncated Agp2 has a Pr ground state. His-248 interacts with rings B and C of the chromophore. It is involved in a hydrogen bonding network and in
the protonation of the chromophore. The third mutant, Q190L, is equivalent to the mutant Q188L of *P. aeruginosa* phytochrome PaBphP from Yang *et al.* (46). According to crystal structure data, the Gln side chain forms ionic interactions with ring D of the Pfr chromophore and stabilizes the C15 E configuration. In that study, the Q188L mutant was reported to have a slower Pr to Pfr dark conversion. Spectra of all mutants measured at pH 7.8 after dark assembly and after photoconversion are given in Fig. 7. Dark conversion after saturating far-red irradiation was followed at 750 nm (close to the Pfr maximum) at pH 6.8, 7.8, and 8.8 for 4000 s. The dark conversion of red pre-irradiated samples was monitored at pH 7.8. The higher initial Pfr level adjusted by red light shows whether the sample undergoes Pr to Pfr dark conversion. All curves were simulated by exponential or linear functions. Time constants and amplitudes of these fit functions are given in Table 1. From the spectra (Fig. 7), it becomes clear that wild type Agp2 is the only variant that is completely in the Pfr form after assembly and dark incubation. All mutants include a residual fraction of Pr, which could be due to either incomplete Pr to Pfr dark conversion or incomplete Pfr to Pr dark conversion (Fig. 7). The dark conversions of Agp2 could well be fitted with a triexponential function (Table 1 and data not shown). As also seen in the flash photolysis measurements (Fig. 4), the time constants increase with increasing pH. This pH dependence was also found for almost all mutants in this study. In Agp2, the pH effect was strongest for the shortest time constant, which increased from 7.8 s at pH 6.8 to 52 s at pH 8.8. Dark conversion of N12S could again be described by triexponential functions. Time constants were slower than those of the wild type, confirming that the amino acid Asn-12, which is close to ring A of the chromophore, has an impact on dark conversion. The rather high absorbance change ratio of 0.8 shows that the chromophore is in the protonated state. The Q190L mutant revealed a mixture of Pr and Pfr after overnight assembly of about equal amounts (Fig. 7). Absorbance measurements after red or far-red irradiation indicate that dark conversion proceeds from Pr to Pfr. However, the time curves could only be fitted with monoexponential functions. All time constants are larger than the corresponding largest time constant of Agp2 (Table 1). The absorbance measurements after red or far-red irradiation indicate that dark conversion proceeds from Pr to Pfr.
ance change ratio of 0.7 again indicates protonation of the Pr chromophore. In the H248Q mutant, the Pfr form dominated after assembly (Fig. 7). Pr to Pfr dark conversion curves could be simulated by biexponential (for pH 6.8) or monoexponential (for pH 7.8 and 8.8) functions; dark conversion was slower than that of N12S, Q190L, and the wild type (Table 1). For the H248Q mutant of the truncated version of Agp2, the Pfr ground state is in contrast to the Pr ground state reported by Karniol et al. (45). After irradiating H248Q with either red or far-red light, the spectral pattern developed in an unusual way as the overall absorbance in the red or far-red wavelength range decreased; the initial ground state recovered slowly in the dark (Fig. 7). We propose that after irradiation the chromophore is deprotonated in the Pr and Pfr forms and that dark conversion switches back to a protonated Pfr chromophore with a higher absorbance.

By analyzing double and triple mutants, we found that the mutant effects on dark conversion are largely additive. In all

FIGURE 7. Absorption spectra of point mutants. Agp2 wild type and mutants were purified as apoproteins and assembled with BV. After a 16-h dark incubation, free chromophore was removed from the protein, and spectra were recorded. Samples were then irradiated with red light (655 nm, 300 μm m⁻² s⁻¹) for 60 s, measured, irradiated with far-red light (780 nm, 1000 μm m⁻² s⁻¹) for 60 s, and measured again.
double mutants, the Pr form dominated (Fig. 7), and the triple mutant was completely in the Pr form after overnight assembly (Fig. 7). Dark conversions were always slower than in each single mutant or the wild type protein. Monoexponential functions were fitted to the curves of N12S/Q190L and N12S/H248Q, and the time constants were always above 1000 s (Table 1). Dark conversions of Q190L/H248Q and the N12S/Q190L/H248Q triple mutant seemed to be associated with a linear rather than an exponential time dependence. This indicates that dark conversion in these mutants is so slow that during the measuring time of 4000 s the sample is still in the initial phase of the exponential progression. The pH dependence was no longer given for those two mutants: rates at pH 8.8 were slightly higher than at pH 7.8. The triple mutant converted from Pfr to Pr after red irradiation. The rate constant of 3.7 ± 10^{-6} s^{-1} was still low but 50 times higher than that of the Pr to Pfr conversion after far-red irradiation. The equilibrium was clearly shifted to the Pr side. A reversion of dark conversion was thus achieved by mutation of one amino acid interacting with ring A of the chromophore, a second amino acid interacting with rings B and C, and a third amino acid interacting with ring D.

**DISCUSSION**

*Agrobacterium tumefaciens* has two phytochromes, Agp1 and Agp2, which have both been investigated biochemically. Agp1 is a prototypical bacterial phytochrome, and Agp2, containing an HWE His kinase and a C-terminal response regulator, is a member of the bathy phytochromes with a Pfr ground state. Titration experiments combined with UV/visible and RR spectroscopy have shown that Agp2 differs from other phytochromes by its untypical chromophore protonation. In Agp2, the pK_a of the Pfr chromophore lies above 11, and this value shifts down to 7.6 upon photoconversion into Pr. These observations raise two questions. (a) What is the molecular origin for the deprotonated Pr chromophore and the dramatic pK_a change during photoconversion? (b) Is this pK_a change associated with a specific biological function?

A homology model for Agp2 (Fig. 8) suggests that Asp-196 interacts with ring D of the Pfr chromophore as given by the LIGPLOT program. Bottom, three-dimensional arrangement of the amino acids interacting with the chromophore. Superposition of the Agp2 model with the crystal structure of PaBphP (Protein Data Bank code 3C2W) is shown. The chromophore is drawn with green carbon atoms. Amino acids of PaBphP are drawn with blue carbon atoms. Amino acids of Agp2 are drawn in purple if they are identical with PaBphP or orange if they are different from PaBphP. Asp-196 of Agp2 is drawn with pink carbon atoms. Dashed lines indicate hydrogen bonds.
residues that constitute the chromophore pocket might give a clue to this question. However, 14 of the 18 amino acids of the Agp2 chromophore pocket are identical with PaBphP (Fig. 8), which has a normal Pr spectrum with a protonated chromophore. The four chromophore-interacting amino acids by which Agp2 differs from PaBphP are found in many other phytochromes with normal Pr spectra. Thus, the different protonation of the Pr chromophore cannot be attributed to a single amino acid interacting with the chromophore. A replacement of the GAF domain of Agp2 by the GAF domain of Agp1 resulted in a protonated Pr chromophore. The replacement of the PAS domain yielded also a protonated Pr chromophore (Fig. 6). Substitution of Asp-12 and Glu-190 caused chromophore protonation, whereas the His-248 substitution led to a deprotonated BV. It is also relevant in this context that a compound of the bacterial cellular extract increases the absorbance of the Pr form of Agp2 (4, 26) most likely due to a differential protonation of the Pr chromophore. Finally, the C-terminal part of Agp2 has an impact on the chromophore protonation status (Figs. 2 and 3). It appears that Pr protonation is sensitive to subtle structural changes. Besides Asp-196 and His-248, helix 5 (residues 245–255) within the GAF domain seems to be most relevant for protonation. This helix is located next to the knot between the GAF and PAS domains and might be reoriented by either the GAF or PAS domain swap. This helix contains the highly conserved Tyr-255, which interacts with the chromophore, and a Glu residue next to it facing away from the chromophore.

What is the effect of the deprotonated Pr chromophore on (i) the photocycle and (ii) the capability of photoinduced or thermal chromophore double bond isomerization? Measurements at pH 6 (protonated chromophore) point to a normal conversion to Pfr. At pH 9 where the chromophore in its electronic ground state is deprotonated, a flash induces photoconversion to the intermediate states lumi-R, meta-Ra, and meta-Rc, but from the meta-Rc state, the major fraction seems to convert back to Pr or a Pr-like state. Agp1 mutants with deprotonated chromophore were also unable to complete the transition from meta-Rc to Pfr (29), although in this case, no back-conversion was observed. In Agp2, this futile cycle explains in part why the bacteria “see” the plant root. Plant stems and roots function as light vessels, and long wavelength light around 750 nm transmits more deeply than shorter wavelengths (51), and next to a plant root. Plant infection process (53). ChvG is a His kinase sensor protein of A. tumefaciens that is required for acid induction of virulence genes (54).

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