Light-induced Proton Release of Phytochrome Is Coupled to the Transient Deprotonation of the Tetrapyrrole Chromophore*1

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The P₃ → P₄ phototransformation of the bacteriophytochrome Agp1 from Agrobacterium tumefaciens and the structures of the biliverdin chromophore in the parent states and the cryogenically trapped intermediate Meta-Rc were investigated with resonance Raman spectroscopy and flash photolysis. Strong similarities with the resonance Raman spectra of plant phytochrome A indicate that in Agp1 the methine bridge isomerization state of the chromophore is ZZAsa in P₃ and ZZEssa in P₄, with all pyrrole nitrogens being protonated. Photoexcitation of P₃ is followed by (at least) three thermal relaxation components in the formation of P₄ with time constants of 230 μs and 3.1 and 260 ms. H₂O/D₂O exchange reveals kinetic isotope effects of 1.9, 2.6, and 1.3 for the respective transitions that are accompanied by changes of the amplitudes. The second and the third relaxation correspond to the formation and decay of Meta-Rc, respectively. Resonance Raman measurements of Meta-Rc indicate that the chromophore adopts a deprotonated ZZEs configuration. Measurements with a pH indicator dye show that formation and decay of Meta-Rc are associated with proton release and uptake, respectively. The stoichiometry of the proton release corresponds to one proton per photoconverted molecule. The coupling of transient chromophore deprotonation and proton release, which is likely to be an essential element in the P₃ → P₄ phototransformation mechanism of phytochromes in general, may play a crucial role for the structural changes in the final step of the P₄ formation that switch between the active and the inactive state of the photoreceptor.

Phytochromes are photoreceptors that utilize light as a source of information for controlling numerous biological processes (1, 2). The chromophore, a methine-bridged tetrapyrrole (Fig. 1), acts as a photoswitch between two stable, spectrally distinct forms, denoted as Pr and Pfr according to the red and far-red absorption maxima, respectively. Another group of bacterial phytochromes incorporates biliverdin (BV) as chromophore (14), which is covalently bound to a cysteine close to the N terminus of the protein (13, 16, 17). The biophysical properties of BV-binding phytochromes have not yet been analyzed in great detail. As far as P₃/P₄, photoisomerization and UV-visible spectral properties of P₃ and P₄ are concerned, these phytochromes share common features with their cyanobacterial and plant orthologs. In view of the above similarities, mechanisms found for bacterial phytochromes may well be of universal significance and also be valid for plant phytochromes.

In the present work, we have studied the recently discovered phytochrome Agp1 from Agrobacterium tumefaciens (16), which belongs to the group of BV-binding phytochromes. Site-directed mutagenesis and mass spectrometry studies showed that BV is covalently bound to Cys₂₀ of Agp1 via its ring A vinyl side chain (13, 16, 17). The BV-Agp1 adduct displays a reversible P₃ → P₄ phototransformation characteristic for all representatives of the phytochrome family. As in other bacterial phytochromes, the histidine kinase activity of Agp1 is deactivated upon conversion from P₃ to P₄ (16).

We have employed transient absorption and RR spectroscopy to ana-
lyze the $P_r \rightarrow P_{pr}$ photoconversion of BV-Agp1. Specifically, we have investigated (i) the dynamics of the chromophore-protein interaction and the protonation state of the protein on the µs and ms time scale of the $P_r/P_{pr}$ reaction cycle and (ii) the chromophore structures in the parent and intermediates states.

**EXPERIMENTAL PROCEDURES**

*Sample Preparation*—Expression and purification of Agp1 as well as the formation of the BV-Agp1 adduct have been described in detail elsewhere (16). The protein was purified by affinity chromatography and size exclusion chromatography. After chromatography, BV-Agp1 was essentially pure as checked by SDS-PAGE (see supplemental Fig. 1). The specific absorbance ratio deduced from UV-visible spectra (supplemental Fig. 2) was routinely between 0.95 and 1.0. In cases where high protein concentrations were required, the volume of the sample was reduced by ultrafiltration. Buffer exchange for measurements in D$_2$O and for de/reprotonation measurements was performed with the use of desalting columns. For low temperature UV-visible absorption spectroscopy and Resonance Raman measurements, protein solutions contained 5 mM EDTA, 300 mM NaCl, and 50 mM Tris at a pH and pD of 7.8 in H$_2$O and D$_2$O solutions, respectively. Flash photolysis experiments were performed either in 20 mM Tris at pH 7.8 and pD 7.8, respectively, or in unbuffered solutions at pH 7.8 containing 50 mM NaCl and a 0–120 µM concentration of the pH indicator dye cresol red. BV-Agp1 concentrations, determined from the absorption at 700 nm using an extinction coefficient of 90 mM$^{-1}$ cm$^{-1}$ for $P_r$, (16), were 16–22 µM and 3 mM in UV-visible absorption and RR spectroscopic measurements, respectively. Photoconversion to $P_{pr}$ and $P_{f}$ was achieved by irradiating the solution with 660 and 784 nm light, respectively, using either a light-emitting diode, a white lamp with appropriate filters, or a laser diode.

*Low Temperature UV-visible Absorption Spectroscopy*—Low temperature UV-visible absorption spectra were measured with a Cary 50 Bio, equipped with an Oxford cryostat. All experiments were carried out with a sample in aqueous buffer solution with the protein being largely (>90%) in one of the parent states. The background scattering resulting from the opaque frozen samples was removed by subtracting a fitted $\lambda^2$-curve from the measured spectrum.

*Transient UV-visible Absorption Spectroscopy*—Flash photolysis of BV-Agp1 was performed by excitation with 10-ns pulses using an excimer-pumped dye laser with an emission maximum at 695 nm (Pyridine 1) and a maximum output energy of 5 mJ/cm$^2$ (15, 18). Prior to each flash excitation the sample was re-converted to $P_r$ to more than 95% with 784 nm illumination (60 milliwatts, 20 s). We note that the remaining fraction of $P_{pr}$ was negligible for the transient absorption spectroscopic measurements in view of the lower extinction coefficient ($\sim$30 mM$^{-1}$ cm$^{-1}$ at 695 nm) and the low quantum yield ($\sim$0.004) of $P_{pr}$ (16). Absorbance changes were monitored at different wavelengths in the Soret (360–450 nm) and Q-band region (600–800 nm), from 100 ns to 5 s after flash excitation. The signals were corrected for energy fluctuations of the pulses and irreversible bleaching, which was found to be 10% after 450 laser flashes. Photoinduced proton release (and uptake) of BV-Agp1 was determined by monitoring the absorbance changes of the pH indicator dye cresol red ($pK_a = 8.2$) at 570 nm as described (15, 19). Transient absorption data at multiple wavelengths $\Delta A(\lambda,t)$ were analyzed by singular value decomposition (SVD) and simultaneous fitting (20, 21). The amplitude spectra $B_\lambda(\lambda)$ were constructed from the results of this analysis (21).

*Resonance Raman Spectroscopy*—RR spectra were obtained with 1064 nm excitation (Nd-YAG cw laser, line width < 1 cm$^{-1}$) with a Digilab Bio-Rad Fourier transform Raman spectrometer (4 cm$^{-1}$ spectral resolution) (7). All spectra were measured at $\sim$140 °C using liquid nitrogen-cooled cryostats from CRYOvac (7) or Resultec (Linkam). The laser power was between 400 and 700 milliwatts at the sample, which does not cause any laser-induced damage of the protein samples as checked by comparing the spectra obtained before and after a series of measurements. The total accumulation time was $\sim$2 h for each spectrum. In all RR spectra shown in this work, a linear background was subtracted. RR spectroscopic measurements of phyA have been described previously (7). Preparation and RR spectroscopic measurements of BV dimethyl ester (BVM) photoisomers have been reported before (22).

**RESULTS AND DISCUSSION**

*Kinetics of the Photoinduced $P_r \rightarrow P_{pr}$ Transformation*—Transient absorption changes of BV-Agp1 in H$_2$O and D$_2$O, monitored after 695 nm excitation, display multiphasic behavior (Fig. 2), indicating a sequence of spectral transitions in the $P_r \rightarrow P_{pr}$ photoconversion. Since the primary photoproduct of phytochromes including BV-Agp1 is formed on the picosecond time scale (24), beyond the time resolution of the present set up, the kinetics studied in this work refer to the thermal relaxation steps on the route to $P_{pr}$ that proceed on the micro- to millisecond time scale. The formation of $P_{pr}$ is completed within seconds as concluded from the signal at 750 nm (Fig. 2C), which remains constant after about 3 s. The absorbance changes at 700 and 725 nm (Fig. 2, A and B) in the time range 1–5 s are due to $P_{pr} \rightarrow P_s$ conversion induced by the probe light. In the time range below 1 s, the effect of the measuring light is negligible and the signals exclusively reflect thermal relaxation processes following flash excitation of the $P_r$ state. As shown in Fig. 2, H$_2$O/D$_2$O exchange affects both time constants and amplitudes of the $P_r \rightarrow P_{pr}$ spectral transitions.

A quantitative analysis of the time traces obtained at 35 wavelengths was performed for the time range from 2 µs to 1 s. In this way, the noisy data below 2 µs and the effects of the measuring light for irradiation times $> 1$ s are omitted. SVD analysis revealed four relevant compo-
Additional components, with singular values of less than 1% with respect to the singular value of the first component, show very noisy time traces and were thus neglected. An excellent global fit to the traces was achieved with four exponentials (see Fig. 3). In H$_2$O time constants of 6.9 and 230 $\mu$s and 3.1 and 260 ms were obtained which are indicated by dashed vertical lines in Fig. 3 and summarized in TABLE ONE. The first component, which shows a small negative amplitude only at the emission maximum of the laser and which has essentially the same time constant in H$_2$O and D$_2$O, is most likely due to a flash artifact although a contribution from absorption changes of BV-Agp1 cannot be ruled out. The other three components, which represent true relaxation processes, display considerable deuterium kinetic isotope effects of 1.9, 2.6, and 1.3 (Fig. 3 and TABLE ONE). From the amplitudes of the fits to the weighted V-traces and the associated basis spectra, the amplitude spectra were calculated as described before (21). The amplitude spectra $B_0(\lambda)$ (Fig. 4), determined from the constants of the fits for the measurements in H$_2$O and D$_2$O, reflect the extrapolated final spectra after flash excitation of the P$_{fr}$ state. These spectra agree very well with the scaled down steady-state $P_{fr}^*$ difference spectrum (dashed lines). This agreement confirms that the relaxation cascade of the $P_{fr} \rightarrow P_{fr}^*$ photoconversion is completed within the time window of the transient measurements and that the time constant $\tau_4$ reflects the formation of the $P_{fr}$ state. From the scaling factor of the steady-state $P_{fr}^* - P_{fr}$ difference spectrum the excitation efficiency was estimated to be 20 ± 2%.

The shortest relaxation component attributable to (true) absorption changes of the chromophore ($\tau_2$) is characterized by a positive amplitude at ~725 nm and a small negative one below 670 nm (Fig. 4). When scaled to the same number of photoconverted molecules, these amplitudes are larger by ~20% in H$_2$O than in D$_2$O. The amplitudes of the subsequent relaxation component (Fig. 4) show large positive and negative values at ~680 and 760 nm, respectively. Whereas the 680 nm amplitude is solvent-independent, the 760 nm amplitude is increased in D$_2$O. The fourth component (Fig. 4) includes a negative amplitude in the 600–800 nm range (maximum at ~750 nm), which is larger by ~30% in H$_2$O than in D$_2$O, and positive (410–450 nm) contributions in the Soret band region.

Control experiments in H$_2$O solutions that contain 0.2 M sucrose, corresponding to the same solvent viscosity as D$_2$O, reveal the same kinetic data as in the absence of sucrose (data not shown). We conclude, therefore, that the changes of the time constants and amplitudes in D$_2$O represent true kinetic isotope effects.

Increasing temperatures in the range from 10 to 35 °C accelerate the decays of the three relevant components, while their amplitudes remain almost unchanged. The activation energies that are derived from the Arrhenius plots (data not shown) do not differ in H$_2$O and D$_2$O within experimental error (TABLE ONE).

Photoinduced Proton Release—To detect protonation changes in the external medium, we measured the transient absorption changes of the
pH indicator dye cresol red that was added to the unbuffered solution at different concentrations. To correct for contributions of the protein, absorption changes were measured also without pH indicator dye. The dye signal ($\Delta A$), the difference between the signals with and without pH indicator dye, displays a proton release phase followed by a proton uptake phase and can be described by two exponentials with time constants that agree very well with $\tau_3$ and $\tau_4$ determined for the photoinduced Pr$_3$Pfr transformation under the same conditions (Fig. 5A).

Thus, proton release occurs synchronously with the formation of the precursor of Pfr, whereas the subsequent proton uptake can be related to the final transition to Pfr. The kinetics and the pattern of de/reprotonation are similar to that observed with cyanobacterial phytochrome Cph1 (15). The dependence of the (maximal) absorbance changes on the dye concentration (Fig. 5B) allows determining the number of protons transiently released per photo-converted protein (19), yielding a value of $1.1 \pm 0.2$. However, proton release is not fully reversed ($<50\%$; Fig. 5A), indicating a net acidification of the protein in the Pfr state, which was confirmed by stationary dye and pH electrode measurements in Pr and in the Pr/Pfr photoequilibrium (data not shown).

**Low Temperature UV-visible Absorption Spectroscopy**—After conversion to Pr, BV-Agp1 was frozen to $-140^\circ$C and subsequently irradiated with 700 nm light. Even after prolonged irradiation for 30 min, no changes in the absorption spectrum were observed whereas at ambient temperature these irradiation conditions are more than sufficient to convert BV-Agp1 to Pfr. This finding implies that either Pr forms a photo-reversible equilibrium with the primary photoproduct that exhibits only a very small photostationary concentration or that photconversion is completely blocked at low temperature. Taking into account the band narrowing at low temperature and uncertainties due to the $\Delta A$ subtraction, the spectra of Pr and Pfr that are obtained by this procedure are very similar to those measured from the protein solution at ambient temperature (16). The experiments were repeated upon stepwise increasing the temperature at which the sample was irradiated. Only at $\sim 25^\circ$C, the absorption spectra display substantial changes indicating the accumulation of an intermediate state. Subtracting the residual contribution of unphotolyzed Pr, an absorption spectrum is

| TABLE ONE Time constants ($\tau$), amplitudes ($B_i$), and activation energies ($E_A$) of the photoinduced Pr$_3$Pfr conversion of BV-Agp1 determined in flash-photolysis experiments |
|-----------------------------------------------|
| **Solvent** | **Parameter** | **Relaxation components** |
| H$_2$O | $\tau$ | 6.9 $\mu$s | 230 $\mu$s | 3.1 ms | 260 ms |
| D$_2$O | $\tau$ | 7.3 $\mu$s | 440 $\mu$s | 8.2 ms | 340 ms |
| H$_2$O | $E_A$ (kJ/mol) | 67 $\pm$ 2 | 86 $\pm$ 4 | 109 $\pm$ 2 |
| D$_2$O | $E_A$ (kJ/mol) | 69 $\pm$ 3 | 85 $\pm$ 6 | 112 $\pm$ 5 |

**FIGURE 4.** Amplitude spectra $B_i(A)$ calculated from the amplitudes of the exponential fits and the corresponding SVD basis spectra, from measurements in H$_2$O (A) and D$_2$O (B). The related time constants are given in TABLE ONE. The dashed lines are the steady-state Pr$_3$Pfr difference spectra scaled to the $B_0$ spectra.

**FIGURE 5.** A, transient absorbance changes of cresol red at 570 nm at different dye concentrations corrected for contributions of the protein ($\Delta A$ measured without dye), monitored after photoexcitation of BV-Agp1 (16 M) in H$_2$O (pH 7.8). B, absolute value of the dye signal at different dye concentrations read from the traces at 20 ms (vertical dashed line) in A (symbols). The solid line is a fit to the data with Equation 1 of Ref. 12. The dotted line indicates the predicted dependence in the absence of saturation effects.
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![Diagram](Image)

**FIGURE 6.** A, low temperature UV-visible absorption spectra of P₆ (solid line) and P₆ (dashed line) from BV-Agp1 measured at −140 °C. The spectrum of Meta-Rₙ(Agp1) (dotted line) was obtained after irradiating the sample at −25 °C with 700 nm light. B, difference spectrum “P₆” minus “Meta-Rₙ(Agp1)” obtained from steady-state low temperature absorption data (solid line), compared with difference spectra constructed from transient absorption data (—), using a mixture of 85% of B₄ and 15% of B₃.

obtained that is reminiscent of the Meta-Rₙ state found in phyA (Fig. 6) (25). The difference spectrum “intermediate minus P₆,” reveals close similarities to the amplitude spectrum of the fourth relaxation component (B₄) obtained in the time-resolved measurements and an even better agreement is achieved for a mixture of 85% of B₄ and 15% of B₃. Thus, the cryogenically trapped state mainly corresponds to the precursor of P₆ and is, therefore, denoted as Meta-Rₙ(Agp1) in analogy to the P₆ precursor of phyA stabilized at the same temperature (25).

**Resonance Raman Spectroscopic Analysis of the Chromophore Structures in the Parent States**—The RR spectra of the P₆ and P₆ states in H₂O and D₂O were obtained by photoconversion of BV-Agp1 into the respective state at ambient temperature and subsequent cooling to −140 °C in the dark. For both states, the overall vibrational band patterns display far reaching similarities with the RR spectra of the respective states of phyA (Fig. 7). In particular, the prominent bands between 1500 and 1650 cm⁻¹ of both P₆ and P₆ show a one-to-one correspondence between phyA and BV-Agp1. Slightly different frequencies and relative intensities mainly originate from the different chemical constitutions of the chromophores (phytochromobilin versus BV; Fig. 1), as indicated by preliminary normal mode calculations.

For the P₆ state of phyA, it has been demonstrated that the chromophore adopts the ZZZasa configuration with all pyrrole nitrogens being protonated (7, 10). The double bond configuration of the P₆ chromophore, which is also in the protonated form, has been shown to be ZZE (10). Theoretical calculations furthermore suggest a (partial) rotation around the A-B single bond in P₆. This conclusion is based on the substantial downshift of the C=C stretching of the A-B methine bridge from 1643 (P₆) to 1618 cm⁻¹ (P₆), which is only reproduced by the calculated spectrum for the ZZZasa configuration (10). In BV-Agp1, the downshift of this mode compared with the P₆ state is about the same as in phyA. The most characteristic marker band for the (protonated) cationic form of the tetrapyrole originates from the N-H in-plane bending of the rings B and C (7), which leads to relatively intense bands at 1574 (P₆) and 1555 cm⁻¹ (P₆) in phyA and at similar frequencies in BV-Agp1. Upon H/D exchange, these bands disappear concomitant to a 5-7 cm⁻¹ downshift of the bands at 1626 and 1600 cm⁻¹ of P₆ and P₆, respectively. This band originates from the C=C stretching of the C-D methine bridge and exhibits the strongest RR intensity in cationic and neutral tetrapyroles in general (7, 10). However, this band shows an appreciable frequency downshift upon H/D exchange only in the cationic form.

Thus, we conclude that (i) the respective methine bridge isomerization state of the chromophores in P₆ and P₆ is the same in BV-Agp1 and phyA, i.e. ZZZasa in P₆ and ZZZEssa in P₆, and (ii) in both parent states the chromophore is protonated. Subtle details in the chromophore structures may be different for each of the parent states, as it can be concluded from the RR spectra of BV-Agp1 and phyA in the frequency range below 1500 cm⁻¹.

**Chromophore Structure of the Meta-Rₙ(Agp1) Intermediate**—Following the protocol employed in low temperature UV-visible absorption measurements, Meta-Rₙ(Agp1) was accumulated and studied by RR spectroscopy. The RR spectra obtained under these conditions also include contributions from the unphotolyzed parent state P₆, which were subtracted using characteristic bands of P₆ (e.g. at 1626 and 1570 cm⁻¹ in H₂O) as a reference (7).

The most prominent band in the spectrum of Meta-Rₙ(Agp1) in H₂O (Fig. 8A) that is attributable to the C=C stretching of the C-D methine bridge is found at 1590 cm⁻¹, which is lower in frequency by ~10 cm⁻¹ than the corresponding band of the P₆ spectrum (Fig. 7), despite the putatively same (E) configuration of the C-D methine bridge. This band remains largely unchanged in D₂O in contrast to the corresponding modes in P₆ and P₆ (Figs. 7 and 8A). Moreover, no band attributable to the N-H in-plane bending of the rings B and C is observed in the region between 1500 and 1590 cm⁻¹, which only displays weak bands that are invariant upon H/D exchange. Only the 1619 cm⁻¹ band that originates from a mode involving the C=C stretching of the A-B methine bridge and a small contribution from the N-H in plane bending of ring A exhibits a 4 cm⁻¹ downshift and an intensity decrease in D₂O. Thus, the lack of the marker bands for cationic tetrapyroles indicates that the chromophore in Meta-Rₙ(Agp1) adopts a ZZE configuration with one of the inner rings B or C being deprotonated. This interpretation is confirmed by comparison with the RR spectra of the ZZE isomer of deprotonated BVM that can be obtained by photoconversion of the helical BVM (ZZZsss) (Fig. 8B) (22). The frequencies and the relative band intensities as well as the spectral changes brought about by H/D exchange are very similar to those in the spectra of Meta-Rₙ(Agp1).

**Mechanisms of the P₆ → P₆ Phototransformation**—On the basis of the available RR spectra, we conclude that in BV-Agp1, phyA and Cph1 (7, 8), the chromophore adopts a protonated ZZZasa and a protonated ZZZEssa configuration in P₆ and P₆, respectively. Whereas in Cph1 and phyA the corresponding chromophore structures agree even in details, minor differences exist in BV-Agp1 due to slightly different interactions with the protein environment that may be related to the specific chromophore-binding site in Agp1 and the different chemical constitution of the chromophore. Nevertheless, the gross chromophore structural changes during the P₆ → P₆ conversion are likely to be the same, including the Z/E double bond isomerization at the C-D methine bridge in the primary photoprocess (3, 10) and a thermal rotation around the single bond of the A-B methine bridge. Also the number of the thermal chromophore relaxations and their decay times after photoexcitation of the P₆ state are similar in BV-Agp1 (TABLE ONE), Cph1 (8, 15), and phyA (26, 27). Adopting the nomenclature established for phyA (25), we therefore assign τₖ, τ₄, and τ₅ to the decay times of Lumi-R(Agp1), Meta-Rₙ(Agp1), and Meta-Rₙ(Agp1), respectively. However, the amplitude changes of BV-Agp1 in D₂O cannot be reconciled with a simple sequential reaction scheme but indicate the involvement of branching and/or back reactions (equilibria).

The activation energies for the P₆ → P₆ photoconversion of BV-Agp1 increase in the sequence of the relaxation processes and, at least, for the
first decay (Lumi-R(Agp1)) the activation energy is higher (~67 kJ/mol) than in the case of phyA (50–60 kJ/mol; (28)). Thus, it is surprising that unlike in phyA, it was not possible to trap cryogenically either Lumi-R(Agp1) or Meta-R_A(Agp1), and the only relaxation product that is stabilized at low temperatures is Meta-R_C(Agp1), the precursor of Pfr. This discrepancy suggests that in BV-Agp1 either the primary photo-process is blocked up to a temperature of ~25 °C, or the intermediates Lumi-R(Agp1) and Meta-R_A(Agp1) can be efficiently photo-converted back to Pr.

The thermal relaxation processes of Lumi-R(Agp1) and Meta-R_A(Agp1) are associated with relatively large kinetic isotope effects of 1.9 and 2.6, respectively (TABLE ONE), which are nearly as large as those observed for the Pr → Pfr photoconversion of Cph1 (8). Kinetic isotope effects of this magnitude are consistent with a proton transfer as the rate-limiting step. Whereas the molecular event associated with the isotope effect of the Lumi-R(Agp1) decay cannot be identified, the subsequent transition to Meta-R_C(Agp1) correlates with the proton release of Agp1. Since in Meta-Rc(Agp1) the tetrapyrrole is deprotonated, it is concluded that proton release is coupled to the deprotonation of the chromophore which is assigned to be the rate-limiting step in the formation of Meta-Rc(Agp1). A transient deprotonation of the tetrapyrrole may result from the reorganization of the hydrogen bond network associated with a rotation around the A-B methine bridge single bond, which has to occur during the thermal relaxation steps to Pfr. Thus, it is tempting to assume that this rotation is linked to the chromophore deprotonation in Meta-Rc(Agp1), which in turn is coupled to the transient release of one proton to the solvent. The subsequent reprotonation of the chromophore in Pfr only leads to a substoichiometric proton re-uptake of less than 0.5 H^+ implying that the protein structural changes associated with the Pfr formation cause a ΔpK_a alteration of one (or more) protonable residue(s).

The far reaching similarities with respect to the chromophore structures in the parent states and the kinetics of the photoinduced processes in Agp1, Cph1, and phyA suggest that the Pr → Pfr conversion proceeds...
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via the same mechanism in all phytochromes. As shown in this work, the transient deprotonation of the chromophore represents a crucial reaction step in this mechanism. Proton release and (partial) uptake associated with the rise and decay of Meta-RC could be observed with Agp1 (this study) as well as with Cph1 (15). However, so far, an intermediate with a deprotonated chromophore has only been unambiguously identified in Agp1. For phyA, RR spectroscopic studies have led to conflicting conclusions about the protonation state of Meta-RC (5, 7), although these discrepancies could be due to the involvement of the chromophore in an acid-base equilibrium. Since the positions of such equilibria sensitively depend on the local protein environment of the various phytochromes, accumulation of the deprotonated Meta-RC form that is sufficient for a safe spectroscopic identification may not be possible in each case. In fact, H/D effects on the amplitudes of the thermal relaxation steps of the P_r → P_a phototransformation observed for Agp1 in this work are consistent with acid-base equilibria. Thus, we conclude that the coupling of chromophore structural changes and proton translocation revealed for BV-Agp1 is an essential element for the photoinduced reaction mechanism of phytochromes in general, and only details of the kinetics and positions of the acid-base equilibria may be different among the various representatives of this photoreceptor family.

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FIGURE 8. RR spectra of Meta-RC from BV-Agp1 (A) in H_2O (solid) and D_2O (dotted), compared with the spectra of the ZZE isomer of BVM (B) in the neutral non-deuterated (solid line) and deuterated form (dotted line) (22), all measured with 1064 nm excitation at −140 °C. The spectra in A were obtained after subtracting the contributions from P_r as described in the text. The bands at ∼1650 cm⁻¹ originate from the amide I modes of the apoprotein, which come out more strongly in A than in the RR spectra of the parent states due to the lower resonance enhancement of Meta-RC (Agp1).