Microsecond Deprotonation of Aspartic Acid and Response of the \( \alpha/\beta \) Subdomain Precede C-Terminal Signaling in the Blue Light Sensor Plant Cryptochrome

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Supporting Information

**ABSTRACT:** Plant cryptochromes are photosensory receptors that regulate various central aspects of plant growth and development. These receptors consist of a photolyase homology region (PHR) carrying the oxidized flavin adenine dinucleotide (FAD) cofactor, and a cryptochrome C-terminal extension (CCT), which is essential for signaling. Absorption of blue/UVA light leads to formation of the FAD neutral radical as the likely signaling state, and ultimately activates the CCT. Little is known about the signal transfer from the flavin to the CCT. Here, we investigated the photoreaction of the PHR by time-resolved step-scan FT-IR spectroscopy complemented by UV–vis spectroscopy. The first spectrum at 500 ns shows major contributions from the FAD anion radical, which is demonstrated to then be protonated by aspartic acid 396 to the neutral radical within 3.5 \( \mu \)s. The analysis revealed the existence of three intermediates characterized by changes in secondary structure. A marked loss of \( \beta \)-sheet structure is observed in the second intermediate evolving with a time constant of 500 \( \mu \)s. This change is accompanied by a conversion of a tyrosine residue, which is identified as the formation of a tyrosine radical in the UV–vis. The only \( \beta \)-sheet in the PHR is located within the \( \alpha/\beta \) subdomain, \( \sim 25 \) Å away from the flavin. This subdomain has been previously attributed a role as a putative antenna binding site, but is now suggested to have evolved to a component in the signaling of plant cryptochromes by mediating the interaction with the CCT.

**INTRODUCTION**

Cryptochromes have been recognized as ubiquitous flavoproteins with a wide variety of functions and mechanisms in all kingdoms of life. They exhibit homology to the photolyases, flavoproteins that repair DNA upon UVA light exposure. Structurally, they all share a similar N-terminal photoactive subdomain, which may bind an antenna chromophore, as well as an \( \alpha \)-helical subdomain, which noncovalently binds a flavin adenine dinucleotide (FAD) as a cofactor. Most cryptochromes possess an additional C-terminal extension (CCT), which is poorly conserved and highly variable in sequence and length. The CCT has been shown to be essential and sufficient for signal transduction in the plant *Arabidopsis*, more precisely an 80-amino acid stretch comprising also some residues of the PHR C-terminus.

The most prominent role of cryptochromes from a historical perspective is their function as blue light receptors. As such they regulate central aspects of growth and development in plants, and are involved in the light-dependent entrainment of the circadian clock in plants and insects. Moreover, cryptochromes alter gene expression upon light induction in diatoms, green algae, and bacteria. In contrast, some cryptochromes found in insects and mammals, including humans, act as light-irresponsive components of the circadian clock. Cryptochromes have been shown to mediate light-dependent magnetosensitivity in fruit flies, a property that has been demonstrated in vitro on plant cryptochromes. These findings have fueled initial proposals that cryptochromes serve as magnetoreceptors in the magnetic compass of migratory birds.

The photoreactions of cryptochromes have turned out to be rather complex and diverse, exhibiting different reaction pathways of the flavin chromophore and involving several amino acids in electron transfer and proton transfer reactions. In plant cryptochromes, absorption of blue light induces the conversion of the oxidized FAD (FAD\( ^{\text{ox}} \)) into the neutral radical (FAD\( ^{\text{H}} \)), which likely acts as the signaling state in vivo (Figure 1). Time-resolved UV–vis spectroscopy on cryptochrome 1 from *Arabidopsis* (AtCry1) and on CPH1 (*Chlamydomonas* photolyase homologue 1) from the green alga *Chlamydomonas reinhardtii* has demonstrated that the flavin is rapidly reduced to the anion radical (FAD\( ^{-} \)) by a nearby tryptophan residue, which is part of a highly conserved tryptophan triad. The ultrafast Trp → FAD and Trp → Trp electron transfer reactions have been determined to proceed...
with time constants of 0.4 and 31 ps, respectively.\(^{22}\) The terminal tryptophan cation radical is stabilized by a biphasic proton transfer to the nearest environment within 100 ps and 500 ns\(^{2,23}\) and then decays into a tyrosine radical within a few microseconds.\(^{21}\) The fate of FAD\(^{**}\) seems to be detached from the processes within the tryptophan triad, because its protonation was reported to occur on a time scale of a few microseconds\(^{15,20}\) and is thus delayed by 6–7 orders of magnitude with respect to the initial electron transfer. This distinct separation of both processes appears paradox, given that the proton donor D396 (D393 in CPH1), as identified by infrared spectroscopy,\(^{24–26}\) is located in close proximity to the flavin in the crystal structure of AtCry1-PHR (Figure 1).\(^{27}\) However, a structural rearrangement of D396 may be prerequisite for the proton transfer, because it forms a hydrogen bond to an oxygen atom of the protein backbone.

It should be noted that this reaction model is not unanimously accepted. First, in vivo experiments on mutants in the tryptophan triad of AtCry2 have cast doubt on the biological significance of the triad for the flavin photoreduction and consequently on the role of the oxidized state of flavin as the dark form.\(^{28}\) This discrepancy might be reconciled by the recent finding that the binding of nucleotides to AtCry2 in vivo opens up alternative electron transfer pathways.\(^{29}\) Second, the sequence and time scales of the processes have been questioned by theoretical studies that predicted an ultrafast proton transfer from D396 to flavin adenine dinucleotide (FAD) in plant cryptochromes. The structure on the left represents a section from the flavin binding pocket in Arabidopsis cryptochrome 1 (AtCry1) (PDB code: 1U3C). After light absorption by oxidized FAD, the anion radical FAD\(^{**}\) is formed via an ultrafast electron transfer. A microsecond proton transfer to the flavin then yields the neutral radical FADH\(^{-}\), which likely acts as the signaling state in vivo. The tryptophan W400 and the aspartic acid D396 are located close to the flavin, and therefore are considered as likely candidates for the electron and the proton donor, respectively. The FAD is reoxidized by oxygen within minutes.

![Diagram of photoreaction of the chromophore flavin adenine dinucleotide (FAD) in plant cryptochromes.](image)

**Figure 1.** Photoreaction of the chromophore flavin adenine dinucleotide (FAD) in plant cryptochromes. The structure on the left represents a section from the flavin binding pocket in Arabidopsis cryptochrome 1 (AtCry1) (PDB code: 1U3C). After light absorption by oxidized FAD, the anion radical FAD\(^{**}\) is formed via an ultrafast electron transfer. A microsecond proton transfer to the flavin then yields the neutral radical FADH\(^{-}\), which likely acts as the signaling state in vivo. The tryptophan W400 and the aspartic acid D396 are located close to the flavin, and therefore are considered as likely candidates for the electron and the proton donor, respectively. The FAD is reoxidized by oxygen within minutes.

Furthermore, transient grating spectroscopy revealed a large decrease in the diffusion coefficient of the protein with a time constant of 0.4 s after excitation, which was interpreted as a partial unfolding of the CCT.\(^{32}\) This region of AtCry1 has been shown to be particularly sensitive to light-dependent trypsin digestion.\(^{33}\) It contains a linear motif that may bind for inhibition to the E3 ubiquitin ligase COP1 (constitutive morphogenesis 1) after the release of the CCT from the PHR.\(^{33}\)

Therefore, we set out to probe conformational changes preceding the C-terminal unfolding by time-resolved FT-IR difference spectroscopy on the PHR of the plant cryptochrome CPH1 in the time range from submicroseconds to seconds. The FT-IR technique has proven to be a valuable tool for identifying changes in the chromophore, single amino acids, and secondary structure of proteins.\(^{34}\) The time-resolved step-scan and rapid-scan FT-IR techniques have been used to access the submicrosecond to millisecond and millisecond to minute time ranges, respectively.\(^{35}\) In the difference mode, only those contributions are resolved that change in the reaction under investigation. Vibrational modes of secondary structure elements are analyzed in the amide I (1620–1695 cm\(^{-1}\)) and amide II (1520–1590 cm\(^{-1}\)) regions,\(^{34,36}\) which contain coupled modes originating from the amide bonds. The combinations of signals of both types clearly correlate with certain secondary structure elements in statistical analyses.\(^{36}\)

To date, the only steady-state infrared difference experiments on plant cryptochromes have been performed on AtCry1\(^{20}\) and CPH1-PHR.\(^{24,25}\) In the present time-resolved FT-IR study, we identified the presence of FAD\(^{**}\) at 500 ns after the laser pulse and monitored its protonation by aspartic acid 396 with a time constant of 0.4–2 μs (AtCry1 numbering is used here and below for clarity). Furthermore, we detected a change in β-sheet structure in the late microsecond time range and assigned it to the α/β subdomain of CPH1-PHR. As we will deduce in the following, this change may play an important role in the initiation of the signal transduction in plant cryptochromes.

### EXPERIMENTAL SECTION

**Sample Preparation.** The PHR domain of CPH1 was expressed in E. coli and purified via Strep-Tactin affinity chromatography as described previously.\(^{37}\) For cell lysis, purification, and dialysis, a 50 mM sodium phosphate buffer, pH 7.8, containing 20% (v/v) glycerol and 100 mM NaCl was used. For time-resolved UV–vis spectroscopy, the concentration of the sample was adjusted to reach OD\(_{450}\) = 0.3. For FT-IR spectroscopy, the protein was washed 3 times with 20 mM sodium phosphate buffer, pH 7.8, 100 mM NaCl, 1% (v/v) glycerol and concentrated to an OD\(_{340}\) > 10 by ultrafiltration using Vivapure 50 filter devices (Sartorius Stedim Biotech) with 50 kDa cutoff. Samples were shock frozen and stored at −80 °C. Potassium ferricyanide was added to the sample solution to reach a final concentration of 10 mM. A droplet of 2 μL was placed on a CaF\(_2\) window (20 mm diameter). It was kept at 20 °C and atmospheric pressure for up to 2 min to gently reduce the water content and then sealed with a second window. Thus, we obtained a well-hydrated film with an absorbance ratio of amide I/ water (1650 cm\(^{-1}\)) to amide II (1550 cm\(^{-1}\)) of 2.3–2.5.

**FT-IR Spectroscopy.** All FT-IR experiments were conducted using an IFS 66v spectrometer (Brucker) equipped with a photconductive mercury cadmium telluride (MCT) detector at a spectral resolution of 4.5 cm\(^{-1}\). An infrared band-pass filter (Laser Components) was used to restrict the spectral range to 1794–988 cm\(^{-1}\) and to block stray light. For excitation, a 450 nm light pulse with 10 ns duration and 16–
19 mJ energy was generated by a tunable optical parametric oscillator system (Opta), which was pumped by the 355 nm third harmonic of a Nd:YAG laser (Quanta-Ray GCR-12, Spectra Physics). Single laser pulses were selected from the 10-Hz repetition rate by an optical shutter (LSTXY, nm Laser Products). The temperature of the sample was kept constant at 10 °C by a circulating water bath.

For the step-scan experiments, 449 mirror positions were recorded with two coadditions at an excitation rate of 0.25 Hz. 1000 equidistant spectra were sampled up to 5 ms at each mirror position. In the time range −129 to −4 μs before the emission of the laser pulse, 25 dark spectra were recorded, averaged, and used for calculating the light-induced difference spectra. Each protein sample was used for up to five step-scan experiments, corresponding to ~4500 excitations per sample. The stability of the protein for this number of excitations was checked by rapid-scan experiments with the same excitation rate. For the first spectrum at 0.5 μs (−4 to 1 μs), 258 coadditions with a strong 1545 cm⁻¹ flavin band were selected and averaged. The resulting spectrum was scaled in intensity by a factor of 5, which was calculated by extrapolating the global fit of the 1354 cm⁻¹ band to 0.5 μs. A scaling factor of 5 would be expected theoretically because the illuminated state was recorded only for 1 μs in the first 5 μs time window. The deviation is attributed to the short integration time, which may lead to some nonlinearities in the absolute band intensities. For the spectra beginning at 3.5 μs, 246 coadditions were selected, in which the amide I region could be resolved. Except where explicitly stated otherwise, spectra of intermediates were scaled with respect to the band of oxidized flavin near 1354 cm⁻¹, which does not overlap with amide I/II bands and has been found not to be compensated by contributions from the product states.

The rapid-scan experiments were performed with a mirror velocity of 150 kHz in a splitted single sided, forward–backward mode. To overcome a general inaccuracy of ±30 μs in the timing because of the independence in frequency of interferometer mirror (10.52 Hz) and laser (10 Hz), synchronization was achieved by integrating a 10 μs TTL pulse from the spectrometer signaling the readiness for after emission in a home-built electronic device. An output TTL pulse was only generated if both incoming pulses overlapped for ≥10 μs. TTL pulses were generated before each experiment to renew the synchronization between spectrometer and laser, which was confirmed by a simulation (see Supporting Information). The first spectrum was recorded from (4.3 ± 1.2) ms to (40.2 ± 1.2) ms with a midpoint of (22.3 ± 1.7) ms after the laser pulse, and further 81 spectra were recorded until 3.883 s. The excitation rate was 0.17 Hz. For each rapid-scan experiment, 512 cycles were performed and averaged afterward. 38 data sets were selected and averaged, resulting in 19 456 scans per spectrum.

Data Analysis. The time-resolved spectra were analyzed with MATLAB (The Mathworks). Step-scan data recorded after 126 μs and rapid-scan data taken after 330 ms were summarized on a logarithmic time scale to increase the signal-to-noise ratio. Contributions of the broad water band at around 1650 cm⁻¹ were subtracted from the individual spectra (see Supporting Information). For the kinetic analysis, the step-scan data (3.5 μs-4.62 ms) and rapid-scan data (22.3 ms-3.63 s) were concatenated (matrix A). A weighted global fit was applied with weighting factors calculated from the variance along the wavenumber dimension and using a kinetic model of sequential first-order reactions with four intermediates. A fit with fewer intermediates did not fully account for all significant spectral differences within the data set, whereas a fit with more intermediates yielded pairs of similar lifetimes. To account for an observed decrease in the yield in the sequence of intermediates, the kinetic model was extended by back reactions from intermediates to the dark state (see Supporting Information). The nonlinear fits were performed with the program SolvOpt for MATLAB. The concentration profiles C resulting from the global fits were used to calculate the SADS D via matrix division according to $D = AC^\dagger$ (with $C^\dagger$ being the pseudoinverse of C). Further kinetic analysis was performed with OriginPro 9.1 (OriginLab).

UV–Vis Spectroscopy. The experimental setup for time-resolved UV–vis difference spectroscopy has been described previously. For excitation, 450 nm light pulses were generated by the laser system described above with an energy density of 2 mJ/cm² at the sample at a rate of (1.6 s)⁻¹. The setup was further improved for the study of slowly recovering systems. Multiple excitations were minimized by using a magnetic stirrer driving a 5 mm stirring bar inside the 10 × 10 mm cuvette for 750 ms after each detection. A horizontal geometry of the experiments with 4 × 10 mm (height × width) ensured a fast sample exchange within a total volume of 2 mL. A mirror was placed behind the exit window to obtain a homogeneous excitation. The opening time of the probe light shutter was set to 200 μs or 1 ms depending on the measuring time window. By alternating the recording of reference and signal spectra, contributions from previous excitations were avoided. All experiments were conducted at 20 °C.

The integration time was set to 10, 100, and 500 μs for difference spectra recorded at 17 μs, 0.3–1.5 ms, and 5–5000 ms, respectively. Each spectrum of CPH1-PHR was obtained by averaging 6–39 separate experiments, in which each sample was excited 3 times. Additionally, difference spectra of FADH⁺ in CPH1-PHR and TyrO⁻ in water were recorded as a reference (see Supporting Information). The kinetics of Trp⁺ and TyrO⁻ in CPH1-PHR were analyzed by a simultaneous fit on the basis of the reaction scheme $\text{Trp}^+ \rightarrow \text{TyrO}^- \rightarrow \text{OH}$. Accordingly, $[\text{Trp}^+] = \text{rate law } y = y_0 + A_1 \exp(-k_1 t)$ and $[\text{TyrO}^-]$ by the rate law $y = y_0 + A_2 (k_2/k_1) (\exp(-k_1 t) - \exp(-k_2 t))$, with $k_1, k_2$ as rate constants, $A_0$ as the initial $\text{Trp}^+$ concentration, and $y_0$ as the residual concentration. The fit was performed with shared parameters $k_1$ and $A_0$.

## RESULTS

Decay of FAD Anion Radical in Plant Cryptochrome. The first measurement window by time-resolved infrared spectroscopy on CPH1-PHR monitored processes up to 1 μs after the laser pulse. The lifetime of FAD⁺ had previously been determined to 1.7 μs by UV–vis spectroscopy. Therefore, the resulting spectrum at 500 ns was directly compared with the steady-state light-minus-dark difference spectrum of FAD⁺ (Figure 2A), which had been generated in the D396C mutant of CPH1 by blocking the proton transfer to the FAD. Negative bands in the difference spectra originate from the dark state. Both spectra exhibit the characteristic signature of oxidized flavin with bands at 1577, 1547, and 1354 cm⁻¹, which are typically found in difference spectra of flavoproteins. The carbonyl bands of the C(4)═O group at 1708 and 1692 cm⁻¹ are not resolved in the 500 ns spectrum of the wild type, but show up as a broad contribution at ~1698 cm⁻¹. Additionally, a band at 1672 cm⁻¹ is found in the time-resolved spectrum, which is assigned to the C(2)═O group.

Positive bands in the difference spectra represent the state formed directly after illumination. In the spectrum of the D396C mutant, the bands at 1655, 1626, 1518, and 1490 cm⁻¹ are considered as marker bands of FAD⁺. In comparison, the 500 ns spectrum features essentially the same band pattern at these positions, the difference being that the carbonyl bands at 1655 and 1626 cm⁻¹ are slightly downshifted by 4–5 cm⁻¹. This difference can be explained by the presence of a cysteine instead of the aspartic acid at position 396 of the mutant and thus an altered environment in the flavin binding pocket. It is concluded that the 500 ns spectrum of the wild type contains the major contribution from FAD⁺.

In addition to the marker bands of FAD⁺, the 500 ns spectrum shows some difference bands at positions similar to those in the later spectra ($t > 8.5$ μs), in which FAD⁺ completely decayed into FADH⁺. These bands can therefore be assigned to the formation of FADH⁺. The pronounced positive band at 1641 cm⁻¹ is a marker band of FADH⁺ in CPH1-PHR. The negative bands at 1405, 1354, and 1251
cm⁻¹ originate from oxidized flavin and have been observed in difference spectra of other flavoproteins as well, while the assignment of the 1414 cm⁻¹ band to FADox remains tentative. Similarly, a small but significant negative band at 1730 cm⁻¹ is observed in the 500 ns spectrum of the wild type but not in the spectrum of the mutant, indicating a partial deprotonation of the aspartic acid D396.

To obtain the lifetime of the FAD⁺⁺ protonation, the difference absorbance of the marker band of FAD⁺⁺ at 1518 cm⁻¹ as well as those of FADH⁺ at 1530 cm⁻¹ and of the aspartic acid at 1730–1733 cm⁻¹ were analyzed for the first 10 spectra after the laser pulse (0.5–43.5 μs) (Figure 2B). Additionally, the ratio between ΔA₁₆₃₁ and ΔA₁₆₄₁ was determined as an indicator for the relative concentration of FAD⁺⁺ and FADH⁺, because both exhibit a band at 1622–1625 cm⁻¹. A simultaneous monoeponential fit of all four band kinetics yielded a lifetime of (1.1 ± 0.7) μs. The significance of this value is lowered by the lack of time points in the early microsecond time region. Nonetheless, lower and upper limiting values for the lifetime of τ = 0.4 μs and τ = 2 μs, respectively, can be given. A faster decay of FAD⁺⁺ would render its detection as the major species at 500 ns practically impossible (Figure S1, Supporting Information), whereas a slower decay of FAD⁺⁺ would lead to a detectable contribution of FAD⁺⁺ to the second spectrum at 3.5 μs (>22% of the signal at 500 ns). These considerations lead to a more substantiated range for the lifetime of 0.4 μs < τ < 2 μs, which is in sufficient agreement with the reported lifetime for FAD⁺⁺ of 1.7 μs. As illustrated by the kinetics of the band at 1730–1733 cm⁻¹, D396 is completely deprotonated within 3.5 μs after illumination.

In addition to the flavin-specific bands, the spectrum of the D396C mutant also contains a band at 1673 cm⁻¹, which has been attributed to a light-induced change in turn elements in the wild type. The 500 ns spectrum of the wild type contains two bands at 1683 and 1664 cm⁻¹ in this region, separated by the negative carboxyl band of FADox at 1672 cm⁻¹. The origin of these bands remains unclear. It may be speculated that they function as precursors of the amide I band at 1667–1671 cm⁻¹ in the later spectra.

In summary, the spectral features resolved in the spectra of the wild type imply that the anion radical is present to a major extent at 500 ns. The kinetics of selected marker bands allowed for an approximate determination of the FAD⁺⁺ lifetime to 0.4–2 μs. Within the same time window, deprotonation of the proton donor D396 was detected.

Changes in Conformation upon and after Formation of the FAD Neutral Radical. We obtained a data set covering a continuous range of more than 6 orders of magnitude in time by including step-scan experiments from 0 μs to 4.6 ms and rapid-scan experiments from 4.3 ms to 3.9 s. The difference spectra from 1 μs onward were concatenated and analyzed by a global monoeponential fit using a kinetic model of sequential first-order reactions with four intermediates. The fit yielded lifetimes of the intermediates I₁, I₂, I₃, and I₄ of τ₀ = 36 μs, τ₁ = 290 μs, τ₂ = 29 ms, and τ₃ = 3.3 s, respectively. The resulting concentration profiles were used to calculate the species-associated difference spectra (SADS). SADS and concentration profiles (Figures S2 and S3, Supporting Information) were multiplied to obtain a fitted data set for illustration (Figure 3A). The SADS obtained from the model of sequential first-order reactions exhibit a decrease in the yield from I₁ to I₄, indicating their partial relaxation to the dark state. Therefore, back reactions from I₄ to I₃ and I₂ to the dark state need to be included in the kinetic model to provide for additional decay pathways (Scheme 1) (Figure S4, Supporting Information). Thus, I₁ and I₂ each exhibit two decay channels with the apparent rate constants k₃₁ = k₄₁ = (290 μs)⁻¹ and k₃₂ = k₄₂ = (29 ms)⁻¹, respectively. A nonlinear fit using this model yielded rate constants of k₃₁ = (500 μs)⁻¹ and k₃₂ = (690 μs)⁻¹ for I₁ and k₃₂ = (33 ms)⁻¹ and k₃₃ = (270 ms)⁻¹ for I₂. The resulting SADS of I₃, I₄, I₃, and I₄ are shown in Figure 3B.

The SADS of I₄ is similar to that of I₃. The only difference is that the SADS of I₄ exhibits broad negative contributions at around 1560 and 1700 cm⁻¹ (Figure S3, Supporting Information). The bandwidth of this contribution is far too...
high for an assignment to specific molecular modes, which indicates that \(I_1\) contains some heat artifact caused by the laser irradiation. Its time dependence is very similar to that of the heat relaxation in bacteriorhodopsin films.\(^{43}\) Therefore, \(I_1\) is not considered to be a separate species and was omitted in the following analysis.

All intermediates show the characteristic band pattern of FADH\(^*\) with bands at 1641, 1625, 1611, and 1530 cm\(^{-1}\). Another distinct positive band is found at 1667–1671 cm\(^{-1}\), which has been ascribed to an amide I mode of a turn element.\(^{25}\) The intensity of this band remains essentially constant throughout the SADS, suggesting a homogeneous decay together with the FADH\(^*\) and with the recovery of protonated D396 as observed at 1733 cm\(^{-1}\). The band shift from 1667 to 1671 cm\(^{-1}\) in \(I_2\) might lead to a compensation and to the decreased intensity of the negative flavin C=O band at 1693 cm\(^{-1}\).

In \(I_2\), the prominent appearance of the 1641 cm\(^{-1}\) band is striking with the adjacent shoulder at 1649 cm\(^{-1}\), which becomes the band maximum in \(I_3\). The band intensity at 1641 cm\(^{-1}\) decreases significantly from \(I_1\) to the later intermediates. These observations imply that the 1641/1649 cm\(^{-1}\) band does not exclusively originate from a C=O vibration of flavin, but contains an additional contribution from an amide I band of helical elements.\(^{44}\)

Aside from the positive difference bands, distinct negative contributions are observed, too, which change drastically in the scaled comparison and therefore do not originate from oxidized flavin but rather from either changes in secondary structure or specific amino acids. The most noticeable features in the frequency region of amide I and amide II are found at 1693, 1632, and 1545 cm\(^{-1}\), all occurring in the SADS of \(I_2\). The 1632 cm\(^{-1}\) band has previously been reported for steady-state light-minus-dark spectra of CPH1-PHR and has been preliminarily assigned to an amide I mode of the protein moiety.\(^{25}\) Judging from the transition from \(I_1\) to \(I_2\), the band seems to become so strong that it significantly reduces the intensity of the two prominent, adjacent bands.

As for its assignment to specific secondary structure elements, the frequency ranges 1641–1623 cm\(^{-1}\) (average 1633 cm\(^{-1}\)) and 1695–1674 cm\(^{-1}\) (average 1684 cm\(^{-1}\)) have unanimously been associated with \(\beta\)-sheets in the literature,\(^{44}\) whereas the frequency of 1545 cm\(^{-1}\) is typical of a less specific amide II contribution. In a more recent FT-IR study of a 50-protein database built for secondary structure prediction,\(^{36}\) maximum associations with \(\beta\)-sheets were observed for the frequencies 1691 and 1634 cm\(^{-1}\), i.e., these frequencies predicted the occurrence of \(\beta\)-sheets in proteins best. Hence, the marked negative contributions at these very specific positions in the SADS of \(I_2\) are interpreted as evidence for a significant loss of \(\beta\)-sheet structure in this intermediate. Of note, these features are lost in the following intermediate \(I_3\).

To illustrate the rise and decay of the change in \(\beta\)-sheet structure, associated band kinetics are compared to those of other contributions. First, the negative marker band of FADox at
1350–1354 cm\(^{-1}\) shows a monotonous decay, indicating the recovery of FAD\(_{ox}\) from FADH\(^*\) (Figure 3C). In parallel with this back reaction, the positive band of turn elements at 1666–1671 cm\(^{-1}\) also decays monotonously (Figure 3C), whereas the negative band at 1630–1632 cm\(^{-1}\) of \(\beta\)-sheet structure becomes more pronounced and reaches a local extremum in the early millisecond time range. It then starts to decay again, remaining almost constant in the later millisecond to second time range. Consequently, changes in turn elements and \(\beta\)-sheet structure exhibit completely different kinetics. The increase in turn elements is already fully developed at the earliest observation of FADH\(^*\) at 3.5 \(\mu\)s and decays completely afterward in parallel with the recovery of FAD\(_{ox}\). By contrast, the loss of \(\beta\)-sheet rises together with I\(_2\) much later in the submillisecond region and decays again within tens of milliseconds, and is thus not related to the kinetics of the chromophore.

In summary, three distinct intermediates were identified in the time-resolved data set. The most noticeable feature in the SADS is a conformational change identified as a loss of \(\beta\)-sheet structure. This change occurs in the second intermediate I\(_2\), which rises and decays with time constants of 500 \(\mu\)s and 29 ms, respectively, and differs significantly from the kinetics of changes in turn elements.

**Kinetics of Tryptophan and Tyrosine Radicals.** Previous time-resolved UV–vis experiments showed the formation of tryptophan and tyrosine radicals in the photoreactions of CPH1 and AtCry1.\(^{20–23}\) Trp\(^*\) is formed by ultrafast electron transfer to the flavin and deprotonation in the picosecond and nanosecond time regions.\(^{22,23}\) Trp\(^*\) appears to decay into a tyrosine radical TyrO\(^*\) with a half-life of \(\sim 1\) ms as demonstrated for AtCry1.\(^{21}\) For CPH1-PHR, evidence for the formation of TyrO\(^*\) is missing.

Time-resolved UV–vis difference spectra of CPH1-PHR were recorded in the time range of 17 \(\mu\)s to 5 s (Figure S5, Supporting Information). Both Trp\(^*\) and TyrO\(^*\) are readily accessible via UV–vis spectroscopy and exhibit distinct band patterns. The Trp\(^*\) minus TrpH difference spectrum exhibits a very broad band centered at around 510 nm,\(^{45}\) whereas the TyrO\(^*\) minus TyrOH spectrum was determined in water to contain two sharp, adjacent bands at 388 and 408 nm (Figure 4A), in agreement with previous recordings at 140 K.\(^{46}\) Additionally, protein-bound FADH\(^*\) minus FAD\(_{ox}\) shows a characteristic positive contribution in the 500–600 nm region. All these three reference spectra were necessary to fully reconstruct the time-resolved spectra of CPH1-PHR. For each time-resolved spectrum, the amount of FADH\(^*\), Trp\(^*\), and TyrO\(^*\) was varied in order to obtain the best match between the recorded spectrum and the fitted sum spectrum (Figure 4A). The fractions were transformed into molar concentrations using the absorption coefficients of the species.

The resulting kinetics of FADH\(^*\) was fitted using the time constants \((\tau_1 = 290 \mu\)s, \(\tau_2 = 29\) ms) obtained from the global fit of the FT-IR data set (Figure 4B). The agreement of the fit with the experimental data supports the proposed kinetic model with back reactions (Scheme 1) and points to a similar behavior of CPH1-PHR under the conditions of the FT-IR and UV–vis experiments. The decay kinetics of Trp\(^*\) can be fitted well by a single monoexponential function (Figure 4C). By contrast, the concentration of TyrO\(^*\) rises to a maximum at \(\sim 1\) ms before decaying completely. The simplest kinetic model for this pathway can be formulated as a decay of Trp\(^*\) into TyrO\(^*\) with a rate constant \(k_1\) and a consecutive decay of TyrO\(^*\) with a rate constant \(k_2\). A global fit with shared parameters yielded

![Figure 4](https://example.com/figure4.png)
Trp end of its reported frequency range of 1235 cm$^{-1}$ between 5 and 20 ms. The fact that this band occurs at the very end suggests a slower decay as compared to the UV donor band at 1235 cm$^{-1}$.

In contrast to the UV donor band, the absorption of TyrO$^-$• as obtained by the spectral interference bands from the reduced amino acids in the dark state may be identified as TyrO$^-$• in the infrared remains mostly elusive because of the unresolved ambiguity in the assignment of the terminal tryptophan in cryptochromes by EPR, transient grating, and UV–vis spectroscopy. It has been demonstrated that taking the distances as the only criterion for the assignment of the electron transfer pathway may be misleading.

In summary, the time-resolved UV–vis data show the formation of TyrO$^-$• from Trp$^+$ within ~1 ms and its decay on the same time scale. Complementary FT-IR data imply that the tyrosine residue in question acts as a strong hydrogen bond donor in its reduced state.

## DISCUSSION

Time-resolved infrared spectroscopy on plant cryptochrome demonstrated the presence of FAD$^{1+}$ at 500 ns after the laser pulse. FAD$^{1+}$ was subsequently formed concomitant with the deprotonation of D396. In the late microsecond region, major structural changes were observed, which were identified as a loss of $\beta$-sheet content. These findings have been integrated into a photocycle for plant cryptochromes (Figure 5) and will be discussed below with a focus on the proton transfer and on the potential role of the $\beta$-sheet unfolding for the signal propagation in these light receptors.

### Protonation of FAD$^{1+}$ and Comparison to Theory.

Time-resolved UV–vis spectroscopy on plant cryptochrome CRY$\alpha$ (I$_1$), CRY$\beta$ (I$_2$), and CRY$\gamma$ (I$_3$), on the basis of time-resolved FT-IR spectroscopy (orange) on CPH1-PHR (this work) as well as present and previous time-resolved UV–vis spectroscopy (blue) on CPH1-PHR, and transient grating spectroscopy (gray) on AtCRY$\alpha$. The structure on the left represents the PHR of AtCRY$\alpha$ (PDB code: IU3C). The location of FAD and important amino acids in the $\alpha$-helical subdomain as well as the only $\beta$-sheet of the PHR within the $\alpha/\beta$ subdomain are highlighted. The lifetimes of CRY$\beta$ and CRY$\gamma$ have to be considered preliminary because of the acceleration of the back reaction by ferricyanide, and have therefore been set in brackets.

Deviations of fitted curves from experimental data that are evident in the kinetics of TyrO$^-$• might be explained by deviations in the reported absorption coefficients of Trp$^+$* and TyrO$^+$*. As an example, the determined lifetime of Trp$^+$* of 1.2 ms is in good agreement with a reported half-life of 1.1 ± 0.4 ms.

In contrast to the UV–vis spectral range, the absorption of Trp$^+$* and TyrO$^+$* in the infrared remains mostly elusive because of the small absorption coefficients and a lack of reference spectra. Negative deviations from the experimental data that are evident in the kinetics might be explained by deviations in the reported absorption coefficients of Trp$^+$* and TyrO$^+$* as well as by inaccuracies in the concentration fractions of the species as obtained by the spectral fit. The determined lifetime of Trp$^+$* of 1.2 ms is in good agreement with a reported half-life of 1.1 ± 0.4 ms for the conversion of Trp$^+$* into TyrO$^+$* in AtCRY$\alpha$. In contrast to AtCRY$\alpha$, however, TyrO$^+$* exhibits a faster decay of 1.1 ms as compared to 1.2 ms in AtCRY$\alpha$, and there is no indication of a significant contribution from a slower decay with $t_{1/2} > 100$ ms. Instead, both radicals seem to decay with almost the same lifetime of ~1 ms in CPH1-PHR.

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In contrast, the evolution of anisotropy in ultrafast UV–vis experiments on CPH1-PHR indicated that electron transfer within the tryptophan triad occurs on the subnanosecond time scale. In the present study, a lifetime of FAD•− of 0.4–2 μs was determined by time-resolved FT-IR spectroscopy (Figure 2). The infrared signature of FAD•− was separated from that of the concomitant species FADH* and could be unambiguously assigned using the spectrum of the CPH1-D396C mutant. More importantly, the bleaching of the CO stretching vibration of the proton donor D396 was observed to be directly linked to the FAD•− → FADH* transition. The corresponding band at \( \sim 1730 \text{ cm}^{-1} \) is only weakly developed at 500 ns but reaches its full extent in the subsequent spectrum at 3.5 μs. D396 then remains deprotonated for the whole experimental time window and is finally reprotonated concomitant with flavin reduction. We cannot exclude that a minority of FADH* is formed via an ultrafast channel, as pointed out recently,25 because of the limited time resolution. However, it should be noted that our study is a clear extension to previous findings insofar as we directly monitored the protonation state of D396.

In conclusion, the photoreaction model postulated by Solovyov et al.30,31 fails to explain experimental findings in plant cryptochromes, especially concerning the role of D396. Contrary to this model, the time-resolved FT-IR data corroborate the notion of a distinct temporal separation of electron and proton transfer to flavin. Thus, the reorientation of D396 toward the flavin, which is prerequisite for a D396 → FAD•− proton transfer, is likely to occur in the submicrosecond time region rather than within 1 ns. In a recent multiscale computational approach, the inclusion of the nuclear dynamics in the environment of the active site led to a much better agreement of electron transfer rates with the experiment.25 In contrast, the free energy barrier of \( \Delta G = 7.5 \text{ kcal/mol} \) as determined for the proton transfer remains too low to account for the observed microsecond process.25 The delay of the proton transfer has been proposed to be caused by a necessary movement of the α15 helix,25 which is in a hydrogen-bonding distance to D396 via M381.27 In contrast, molecular dynamics simulations show a direct hydrogen bond between flavin and protonated D396 in the dark in 70% of the simulation time in absence of ATP, which is similar to our experimental conditions.53 Evidently, more extensive theoretical and experimental studies are required to elucidate the structural aspects and concomitant activation barriers of the proton transfer in plant cryptochrome.

Location and Possible Role of Conformational Changes. The global fit of the time-resolved FT-IR data set yielded three separate intermediates I1 to I3, which are characterized by an increase in α-helices (I1), and by a loss of β-sheet (I2), respectively. I3 essentially only represents the recovery of the protein into the dark state. As a consistent notation for the reaction intermediates in cryptochromes is not yet available, the intermediates I1, I2, and I3 will be referred to as CRYα, CRYβ, and CRYγ henceforth, in analogy to the major structural changes in I1 and I2.

The loss of β-sheet structure in CRYβ rises with a time constant of 500 μs and decays concomitantly with the formation of CRYγ with an apparent time constant of 29 ns, as can be deduced from the corresponding negative marker bands at 1693, 1632, and 1545 cm\(^{-1}\) (Figure 3B). The kinetics of the 1632 cm\(^{-1}\) band shows that the loss of β-sheet structure becomes maximal at around 1 ms (Figure 3C). The detection of a change in β-sheet structure raises the question as to where this secondary structure element is located in the protein. It can be deduced from the characteristic fold of the PHRs of the photolyase/cryptochrome family that the only candidate for the β-sheet is located in the N-terminal α/β subdomain with its Rossmann-like fold that contains a five-stranded parallel β-sheet. In the crystal structure of AcCRY1-PHR,27 the α/β subdomain comprises residues 13–139, whereas the residues 217–495 form the purely α-helical subdomain binding the FAD. The α/β subdomain has not previously been considered to be involved either in the signaling process of sensory cryptochromes or in the photorepair of DNA by photolyases. Its commonly assigned role has been limited to providing a scaffold for the antenna molecule, which is to current knowledge much less tightly bound in sensory cryptochromes than in photolyases.27 The latter finding is in line with the fact that plant cryptochromes can act as efficient light sensors without an antenna molecule,23 considering the sufficiently high absorption coefficient of the oxidized flavin. Therefore, the reason for the high conservation of this α/β subdomain in sensory cryptochromes is unclear.

The function of this β-sheet and its unfolding in the signaling process is not directly evident, considering that the signaling is effected by the CCT.3 CCTs show robust binding to the cognate PHRs and seem to undergo a folded/unfolded transition upon light excitation of the PHR.32,33 The unfolded part of the CCT may then be ready to interact with downstream signaling partners. On the basis of our results, we reason that the β-sheet of the PHR α/β subdomain may play a central role in the interaction between PHR and CCT. The α/β subdomain of AcCRY1 exhibits a positively charged surface patch in the region around the α1 helix, β5 strand, and α5 helix, which may provide an interaction site for the largely acidic CCT (Figures S6 and S7, Supporting Information). This charge distribution is conserved in PHR and CCT of CPH1 but not in PHR and CCT of mouse cryptochrome154 in agreement with the light-independent function of the latter protein (Figure S6, Supporting Information). Other charged surface regions of the mouse cryptochrome-1 PHR have been shown to be involved in the binding of the clock protein PERIOD 2.54 In plant cryptochromes, the C-terminal part of the CCT is considered to be long enough to extend to the α/β subdomain of the PHR (Figure 5). Consistent with this structural model, the C-terminal part of the CCT is particularly sensitive to light-induced trypsin digestion and is therefore likely to undergo the largest structural changes, whereas the N-terminal part seems to be tightly bound to the PHR.33 In contrast, the CCT of Drosophila cryptochrome comprises only few residues and directly binds to the α-helical subdomain close to the FAD binding pocket.54,55 The relative orientation of the CCT and the exact nature of the PHR-CCT interaction in plant cryptochromes are unclear because a crystal structure of the full-length receptor is not available. We postulate that the β-sheet provides a scaffold for proximal, charged PHR-CCT interaction sites on the protein surface. Then, the light-induced β-sheet unfolding may represent a key step in the dissociation of the PHR-CCT complex and the subsequent unfolding of the CCT.

Rise and Decay of Conformational Changes. When considering the time scales of the aforementioned processes, the β-sheet unfolding in CPH1-PHR was found to proceed within a millisecond time scale with \( \tau = 500 \mu s \), whereas the unfolding of the CCT in AcCRY1 was reported to occur in the late millisecond time region with \( \tau = 400 \text{ ms} \).27 The time
constants of these processes are consistent with a photoreaction model in which (a) the molecular changes induced by the photoexcitation of FAD have to migrate within hundreds of microseconds from the flavin chromophore to the secondary structure element, the β-sheet, located at a distance of ~25 Å, and in which (b) resulting changes in the tertiary structure proceed much later within hundreds of milliseconds because of the time-consuming dissociation and unfolding of large parts of the CCT.

In CPH1-PHR, the conformational changes revert together with the decay of the tyrosine radical by formation of the final intermediate CRYγ. Of note, the lifetimes of CRYγ and CRYγ with \( r = 29 \text{ ms} \) and \( r = 3.3 \text{ s} \), respectively, are very likely strongly dependent on the concentration of the oxidant ferricyanide in the protein solution, which was necessary to achieve a suitable repetition rate for the experiment. Without this oxidant, FADH⁺ is reoxidized by oxygen with a time constant of ~200 s. The ferricyanide ions are expected to diffuse into the flavin binding pocket and oxidize the neutral radical on a millisecond time scale. Hence, in the absence of ferricyanide, the decay of CRYβ and the formation of CRYγ might be delayed into the seconds to minutes time range. In contrast, CRYβ formation in the submillisecond time range does not seem to be affected by the addition of oxidants, which is demonstrated by the good agreement between the kinetics of tyrosine conversion in the infrared and UV–vis spectral range, as determined with and without ferricyanide, respectively (Figures 4C and D).

From these considerations it follows that steady-state FT-IR difference spectra recorded without ferricyanide over a period of a few minutes might represent a mixture of CRYβ and CRYγ. Indeed, the comparison between the steady-state difference spectrum and the SADS of CRYβ and CRYγ shows that the loss of β-sheet as the characteristic feature of CRYβ is still pronounced in the steady-state spectrum (Figure S8, Supporting Information). Therefore, the conformational changes in the β-sheet seem to be persistent enough to maintain the rupture of the PHR-CCT interface on a time scale of a few minutes, which represents the relevant time scale for the lifetime of the signaling state FADH⁺ in vivo.¹⁹

CONCLUSIONS

Time-resolved infrared spectroscopy on a plant cryptochrome PHR enabled us (a) to detect the deprotonation of the proton donor D396 concomitantly with the microsecond protonation of FAD⁺⁺, and (b) to reveal the submillisecond unfolding of a β-sheet element in the PHR and specifically trace it to the α/β subdomain. This subdomain has previously not been considered to be involved in the signaling of sensory cryptochromes despite its evolutionary conservation. Interestingly, in the nonsensory mouse cryptochrome 1, the point mutation R109Q within the α/β subdomain leads to disruption of its binding to other core components of the clock, BMAL1 and CLOCK.⁶⁶ The role of the α/β subdomain might therefore have changed in both plant and mammalian cryptochromes, away from antenna binding.

We infer from our findings that the unfolding event in the α/β subdomain may constitute a key step in the initiation of the signaling cascade in plant cryptochromes by enabling the subsequent dissociation of the PHR-CCT complex. This emerging view of the signal progression resembles an activation via a protein quake as it has been coined for the photocative yellow protein (PYP).⁵⁷ Such a quake would be opposed to a model in which a succession of redox reactions finally activates a signaling partner.⁵⁸ As in PYP, the protein quake may originate from the generation of a charge, either of FAD⁺⁺ or of the deprotonated D396. A prominent role of charges in the signaling process has been suggested for plant cryptochromes in a recent hypothesis. Moreover, the formation of FAD⁺⁺ has already been demonstrated to be sufficient for the stimulation of a response in the protein conformation of PHR.²⁴ The specific interaction sites between PHR and CCT of plant cryptochromes remain yet to be identified. This study will motivate an extension of the focus of this search to the α/β subdomain remote from the flavin chromophore.

ASSOCIATED CONTENT

Supporting Information

Experimental section, simulation of spectra for partial radical conversion, concentration profiles and SADS of the intermediates from the global fit using a model of sequential reactions, concentration profiles using a model of sequential reactions with additional back reactions to the dark state, time-resolved UV–vis difference spectra, electrostatic surface potentials of PHRs, sequences of CCTs, and comparison of the steady-state FT-IR difference spectrum with the SADS of CRYβ (I₂) and CRYγ (I₃). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b01404.

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Notes

The authors declare no competing financial interest.

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Supporting Information

Microsecond Deprotonation of Aspartic Acid and Response of the α/β Subdomain Precede C-terminal Signaling in the Blue Light Sensor Plant Cryptochrome

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Experimental Section

Contributions to the spectra from changes in the water absorption. Thermal effects caused by the laser excitation of the sample may lead to changes in the water absorption at around 1650 cm\(^{-1}\). To investigate these effects on the water absorption, ferricyanide was used as a nonreactive chromophore. A droplet of 2 µL of a 100 mM ferricyanide solution in water was placed on a BaF\(_2\) window and then sealed. The step-scan experiments on ferricyanide were conducted analogously to those on CPH1-PHR, but with the following differences: The excitation rate was increased to 1 Hz and 8 coadditions were averaged for each step-scan experiment. 248 coadditions were selected and averaged.

The step-scan data set of ferricyanide was decomposed via singular value decomposition (SVD) into three significant components, from which a noise-reduced data matrix was reconstructed. Additionally, the reconstructed spectra were smoothed using a moving average algorithm. The resulting spectra were in principal agreement with those of different species obtained previously in temperature-dependent experiments on the water absorption.\(^1,2\) The spectra were used to subtract the water absorption difference band from the step-scan and rapid-scan spectra of CPH1-PHR.
**Synchronization of rapid-scan experiments.** TTL pulses from the spectrometer and the laser were integrated in a home-built device to achieve synchronization. It was demonstrated by a home-written simulation program in *Matlab 7.7.0* (The Mathworks) that after an initial overlap of the pulses, both pulses will overlap again after a few seconds. The delay times were chosen so that the laser pulse was emitted within the ~23-ms dead time of the spectrometer, which occurs after each forward-backward movement of the mirror, to further increase the time resolution. The first measurement started (8.6 ± 0.8) ms after the trigger pulse, which was in very good agreement with the simulation ((8.7 ± 1.0) ms).

**Non-linear fit using the kinetic model with back reactions.** In the model of a spectroscopic difference experiment, the introduction of back reactions only affects the amplitudes of the resulting SADS. The shape of the SADS is not altered, because the back reaction leads to the dark state without any contribution to the signal. Analytically, the extended kinetic model with back reactions as depicted in Scheme 1 can be represented by the following differential equations:

\[
\begin{align*}
\frac{d[I_1']}{dt} &= -k_{1'}[I_1'] \\
\frac{d[I_1]}{dt} &= k_{1'}[I_1'] - (k_{f,1} + k_{b,1})[I_1] \\
\frac{d[I_2]}{dt} &= k_{f,1}[I_1] - (k_{f,2} + k_{b,2})[I_2] \\
\frac{d[I_3]}{dt} &= k_{f,2}[I_2] - k_3[I_3]
\end{align*}
\]

These equations were solved for \([I_1'], [I_1], [I_2],\) and \([I_3]\) using the computer algebra system *Maxima 14.12.1* ([http://maxima.sourceforge.net](http://maxima.sourceforge.net)) with initial conditions at \(t = 0\) of \([I_1'](0) = 1\) and \([I_3](0) = 0.\) A back reaction for \([I_1']\) was not included in the model because \([I_1']\) is considered to represent a heat artifact (Figure S3A).

\[
[I_1'] = \exp(-k_{1'}*t)
\]

\[
[I_1] = [k_{1'}/(k_{f,1}+k_{b,1}-k_{1'})] * \left[ \exp(-k_{1'}*t) - \exp\left(-\left(k_{f,1}+k_{b,1}\right)*t\right) \right]
\]

\[
[I_2] = [k_{1'}*k_{f,2}*\exp\left(-\left(k_{f,2}+k_{b,2}\right)*t\right)] / \left[ k_{b,2}^2 + 2*k_{b,2} - k_{f,2} - k_{1'} \right]
+ \left[ k_{1'}*k_{f,2}*\exp\left(-\left(k_{f,2}+k_{b,2}\right)*t\right) \right] / \left[ k_{f,2} + k_{b,2} - k_{1'} \right]
- [k_{1'}*k_{f,1}*\exp\left(-\left(k_{f,1}+k_{b,1}\right)*t\right) / \left[ (k_{f,1}+k_{b,1}-k_{1'}) * k_{b,1}^2 + k_{b,1} - k_{1'} \right]
- [k_{f,1}+k_{1'}] * k_{b,1} + (k_{f,1}+k_{b,1}-k_{1'}) * k_{f,2} + k_{b,1} + k_{1'}]
+ \left[ k_{1'}*k_{f,1}*\exp\left(-k_{1'}*t\right) \right] / \left[ (k_{b,1}+k_{f,1}-k_{1'}) * k_{b,2} + (k_{f,2}-k_{1'}) * k_{b,1} + (k_{f,1}-k_{1'}) * k_{f,2} - k_{1'}\right]
\]

\]
As the resulting equations for I₁ and I₂ exclusively contain exponential terms with the apparent rate constants k₁ = k₁' + k₁, and k₂ = k₂' + k₂, respectively, the FT-IR data could be fitted to the new model using the rate constants k₁', k₁, k₂, and k₃ obtained from the original multiexponential global fit with sequential reactions without back reactions. The equations were implemented in Matlab, and k₁ and k₂ were fitted to obtain a similar scaling of the SADS of I₂ and I₃ to I₁ as in the scaling based on marker bands in the SADS from the multiexponential global fit (Figure S3B). The non-linear fit was performed using SolvOpt. The SADS were calculated via matrix division according to \( D = A C^* \) as described in the Experimental Section.

**Reference spectra in the UV/vis.** A reference spectrum of FADH’ minus FAD(ox) in CPH1-PHR was obtained by adding β-mercaptoethanol (Serva) to the protein solution to a final concentration of 10 mM. The presence of reductant led to a quenching of contributions by TyrO•³ and Trp’. A total volume of 3 mL was used with an absorbance at 450 nm of 0.4. Spectra were recorded at 5 ms (4.95–5.05 ms) after laser excitation with an energy density of 5 mJ/cm². 135 experiments were averaged, in each of which the sample was excited 15 times.

A reference spectrum of TyrO’ minus TyrOH in water was obtained by recording spectra of 3-mL samples of 2 mM L-tyrosine (98%; Sigma) in presence of 30 µM FAD as photooxidant in 50 mM sodium phosphate buffer, 10 mM NaCl, pH 8. To isolate the contributions of TyrO’ to the
difference spectra, FAD was photoreduced under the same conditions in absence of tyrosine but in presence of 1.7 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA; ≥99%; Carl Roth). The product of the oxidation of EDTA does not absorb in the UV/vis spectral range investigated. Difference spectra were recorded at 25 µs (15–35 µs) and 30 µs (20–40 µs) in presence of tyrosine and EDTA, respectively, after laser excitation with an energy density of 5 mJ/cm². 225 experiments were averaged, in each of which the sample was excited 15 times with a repetition rate of (1.6 s)⁻¹. The difference spectrum of TyrO’ minus TyrOH was finally generated by subtracting the spectrum recorded in presence of tyrosine from that recorded in its absence.
Figure S1. The partial conversion of the flavin anion radical to the neutral radical in CPH1-PHR was simulated to analyze the time-resolved spectrum at 500 ns (Figure 2A). Scaled steady-state spectra of the mutant D396C and of the wild type were taken as the limiting values for 0% and 100% conversion. An equal absorbance of the prominent marker bands at 1626 and 1641 cm$^{-1}$ was reached at a theoretical conversion of 70% of the anion radical to the neutral radical. A similar relative absorbance was detected with a small shift in frequency to 1621 and 1641 cm$^{-1}$ in the 500 ns-spectrum (Figure 2A). A conversion of 70% at 500 ns is reached in a monoexponential decay with $\tau \approx 400$ ns, which is therefore considered to represent the lower limiting value for the conversion in wild-type CPH1-PHR. Higher values for the time constant would better explain the prominent contribution of the anion radical to the 500 ns-spectrum at 1518 cm$^{-1}$, which is not found in the simulation at 70% conversion. This discrepancy might be rationalized by different contributions of amide I vibrations to the steady-state and time-resolved experiments, respectively.
Figure S2. Concentration profiles of the four intermediates I₁', I₁, I₂, and I₃ from the global fit of the time-resolved FT-IR data set of CPH1-PHR using a model of sequential first-order reactions. The points represent the experimental data, the straight lines represent the fitted concentration profiles. Larger deviations are observed only for I₃ due to the low signal in the spectra at late time points in the rapid-scan experiment ($\Delta A_{1547} \approx 2 \times 10^{-5}$ at 500 ms, corresponding to ~11% of the $\Delta A$ value at 3.5 µs). As a consequence of this overall low amplitude of I₃, even large variations in its concentration do not affect the linearly combined SADS of the other intermediates to a significant extent. The low amplitudes also lead to stronger deviations of the profiles from the model at time points later than 5 ms.
Figure S3. SADS from the global fit of the time-resolved FT-IR data set of CPH1-PHR using a model of sequential first-order reactions. (A) The SADS of the intermediates I₁’ and I₁ are very similar, but I₁’ exhibits broad negative contributions at around 1600 and 1700 cm⁻¹ at the edges of the water absorption. The bandwidth of this contribution is too high to suggest an assignment to specific molecular modes of the protein, which may indicate that I₁’ contains some heat artifact caused by the laser irradiation. (B) The SADS of I₁, I₂, and I₃ were scaled to the band at 1733 cm⁻¹ for better comparison because of its isolated spectral position. The scaling is verified by a constant absorbance of the band of oxidized flavin at 1354 cm⁻¹ in I₁ and I₂.
Figure S4. Concentration profiles of the four intermediates \( I_1', I_1, I_2, \) and \( I_3 \) from the global fit of the time-resolved FT-IR data set of CPH1-PHR using a model of sequential first-order reactions with additional back reactions from \( I_1 \) and \( I_2 \) to the dark state (Experimental Section, Supporting Information). The points represent the experimental data, the straight lines represent the fitted concentration profiles. The recovery of the dark state is indicated by a black line.

Figure S5. Time-resolved UV/vis difference spectra of CPH1-PHR at indicated time points after the laser flash.
Figure S6. Electrostatic surface potentials of the PHR domains of the plant cryptochromes AtCry1 and CPH1 (modeled), as well as that of the mouse cryptochrome mCry1. The labels “α/β” and “α” refer to the location of the corresponding subdomains of the PHR. The colors are ramped from $-4kT/e$ (red) to $+4kT/e$ (blue). A positively charged surface patch is found in the α/β subdomain for plant cryptochromes (arrow), but not for the mouse cryptochrome. The positively charged patch may function as an interaction site for the acidic regions of the CCT. The electrostatic surface potentials of the PHR from AtCry1$^5$ (PDB: 1U3C), CPH1, and mouse cryptochrome mCry1$^6$ (PDB: 1K03) were calculated using the programs APBS 1.4,$^7$ PDB2PQR 1.9.0,$^8$ and PyMOL APBS Tools 2.1.$^9$ The structure of CPH1-PHR (amino acids 7–493) was modeled using Swiss-Model$^{10}$ with the crystal structure of AtCry1-PHR$^5$ (PDB: 1U3D) as a template. The resulting three-dimensional structures were visualized in PyMOL 1.7.0.0.
Figure S7. Amino acid sequences of the CCTs of *AtCry1* (506–681) and CPH1 (505–1008). The acidic amino acids aspartate (D) and glutamate (E) are highlighted. The CCTs show a net excess of negative charges as indicated by the high number of acidic amino acids in the primary sequence.
Figure S8. Comparison of the steady-state FT-IR difference spectrum of CPH1-PHR in the absence of ferricyanide with the SADS of CRYβ (I2) and CRYγ (I3) from the global fit of the time-resolved FT-IR data set. The SADS have been scaled with respect to the 1545-cm⁻¹ band of the steady-state spectrum. (A) In the comparison with CRYβ (I2), the similar band intensities at 1693 and 1632 cm⁻¹ imply that the loss of β-sheet as the defining feature of CRYβ is still pronounced in the time range of minutes. Furthermore, the amide I bands at 1671/1668 cm⁻¹ are of nearly identical intensity, which indicates a similar kinetics of these conformational changes in turn elements and those in β-sheets. (B) In the comparison with CRYγ (I3), an agreement is found in the bands at 1733, 1662, 1643, 1531, and 1415 cm⁻¹. Additionally, the bands at 1625 and 1611 cm⁻¹ are very similar in frequency but not in intensity, probably because of their overlap with the negative 1632-cm⁻¹ contribution of CRYβ in this region. The light-induced steady-state difference spectra of CPH1-PHR were recorded as described previously.11 Protein samples were prepared as for the time-resolved experiments but without the addition of ferricyanide. Before and after illumination with an LED for 10-15 s, scans were recorded for ~2 min, and a total number of 17,920 scans were averaged. The resulting difference spectrum is in good agreement with a spectrum of CPH1-PHR obtained previously.11
References


(9) APBS plugin for PyMOL, version 2.1; University of Michigan: Ann Arbor, 2006
