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Microsecond Light-Induced Proton Transfer to Flavin in the Blue Light Sensor Plant Cryptochrome

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Abstract: Plant cryptochromes are blue light photoreceptors that regulate key responses in growth and daily rhythm of plants and might be involved in magnetoreception. They show structural homology to the DNA repair enzyme photolyase and bind flavin adenine dinucleotide as chromophore. Blue light absorption initiates the photoreduction from the oxidized dark state of flavin to the flavin neutral radical, which is the signaling state of the sensor. Previous time-resolved studies of the photoreduction process have been limited to observation of the decay of the radical in the millisecond time domain. We monitored faster, light-induced changes in absorption of an algal cryptochrome covering a spectral range of 375−750 nm with a streak camera setup. Electron transfer from tryptophan to flavin is completed before 100 ns under formation of the flavin anion radical. Proton transfer takes place with a time constant of 1.7 μs leading to the flavin neutral radical. Finally, the flavin radical and a tryptophan neutral radical decay with a time constant >200 μs in the millisecond and second time domain. The microsecond proton transfer has not been observed in animal cryptochromes from insects or photolyases. Furthermore, the strict separation in time of electron and proton transfer is novel in the field of flavin-containing photoreceptors. The reaction rate implies that the proton donor is not in hydrogen bonding distance to the flavin N5. Potential candidates for the proton donor and the involvement of the tryptophan triad are discussed.

Introduction

Cryptochromes are an important class of flavoproteins that serve as sensory blue light receptors.[1] They are setting the biological clock in insects[2] and in plants.[3] Furthermore they are involved in general development, i.e., photomorphogenesis, and several other responses in plants.[4] Cryptochromes have been shown to confer magnetosensitivity to fruit flies[5] and are plausible candidates for the magnetoreceptor for orientation of migratory birds in the earth's magnetic field.[6,7] Mammalian cryptochromes play a central but light-independent role in the biological clock.[8] A sensory role of human cryptochromes has been under debate and has been fueled by observation of a photoreaction in transgenic flies.[9]

Cryptochromes contain a ∼500 amino acid photolyase homology region (PHR) but lack the DNA repair activity of photolyases. Additionally, a C-terminal domain of varying length is present that acts in plant cryptochromes as a signal transducer.[10] This domain is lacking in a class of cryptochromes referred to as DASH.[11] DASH cryptochromes have the ability to repair single-stranded DNA[12] but have no sensory function, which groups them in the family of photolyases. The chromophore of animal and plant cryptochromes is a flavin adenine dinucleotide (FAD) in its oxidized state, which is noncovalently bound to the PHR domain.[13−15] A second putative antenna chromophore,[16] methenyl tetrahydrofolate (MTHF), has been identified in small amounts in plant and animal cryptochromes.[14,17,18]

In animal cryptochromes from insects, excitation leads to formation of a flavin anion radical $FAD^{-\bullet}$ from the oxidized

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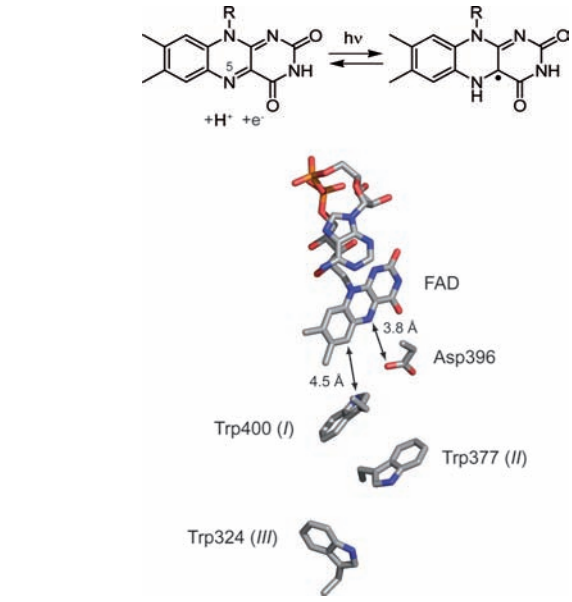

Figure 1. In plant cryptochromes, light absorption by flavin adenine dinucleotide (FAD) leads to formation of the neutral radical from the oxidized state. The tryptophan triad, Trp(*I*) to Trp(*III*), is involved in electron transfer to the excited flavin. The proton is presumably donated by an aspartic acid, such as Asp396 close to the flavin. The numbering of *Arabidopsis* cryptochrome 1 is used (PDB entry 1U3C).

state FAD(ox).[14] EPR spectroscopy on whole cells provided evidence that this process takes place in vivo.[9] It is currently under debate whether the anion radical represents the signaling state[14] or the excited state of the anion radical acts as electron donor to the outside.[19,20] The formation of $FAD^{-\bullet}$ takes place within 1 ps, as has been demonstrated by ultrafast fluorescence and absorption spectroscopy.[20,21] The decay of the radical proceeds under aerobic conditions on the time scale of minutes.[14] Structural data on any member of the animal cryptochrome family are not available. Some insight has been gained by solving crystal structures of homologous (6−4) photolyases.[22,23]

Plant cryptochromes are clearly distinct from animal cryptochromes in amino acid sequence and photoreaction. It has been demonstrated for plant cryptochromes from *Arabidopsis thaliana* (*At*CRY1 and *At*CRY2)[15,24] and from the green alga *Chlamydomonas reinhardtii*[17] that blue light leads to formation of a flavin neutral radical $FADH^{\bullet}$ from FAD(ox) (Figure 1). $FADH^{\bullet}$ has been shown to represent the signaling state in plant cryptochromes.[24,25] A nearby aspartic acid with a distance of 3.8 Å has been proposed to be the proton donor to the flavin based on results from Fourier transform infrared spectroscopy.[26] Transient absorption spectroscopy on *At*CRY1 revealed that formation of $FADH^{\bullet}$ is accompanied by a tryptophan neutral radical, which decays within milliseconds into a tyrosine radical.[27] The flavin radical is formed with a low quantum yield of ~2% and decays with half-lives of 5 ms and >100 ms back into the oxidized state. The latter half-life might be related to the 200 s decay observed in algal cryptochrome.[17] Binding of adenosine triphosphate leads to a strong stabilization of the radical state for hours.[17]

A crystal structure of the PHR domain of *At*CRY1 has been solved. It shows a similar fold to that of *E. coli* DNA photolyase.[28] The homology includes conservation of a tryptophan triad that acts as a molecular wire in the photoreduction of photolyase. The triad transfers an electron to the excited $FADH^{\bullet}$ forming the fully reduced $FADH^{-}$.[29] This tryptophan triad has been demonstrated to be involved in the formation of the signaling state in *At*CRY1,[30] at least under low light conditions.[9] The tryptophans might play a role as a fast electron donor to the excited FAD(ox) in plant cryptochromes (Figure 1).

We have investigated the light-sensitive PHR domain of the algal cryptochrome *Chlamydomonas* photolyase homologue 1 (CPH1) to gain first insight into the formation process of $FADH^{\bullet}$ in plant cryptochromes. CPH1-PHR is closely related to the higher plant *At*CRY1-PHR with a 49% identity in sequence. Nanosecond time-resolved absorption spectroscopy was applied using a streak camera for detection.[31] Excitation led to formation of a transient species, which decayed with a time constant of 1.7 μs. This species is assigned to a flavin anion radical $FAD^{-\bullet}$, which is converted to $FADH^{\bullet}$ by microsecond proton transfer.

Materials and Methods

Sample Preparation. The *Chlamydomonas* photolyase homologue 1 (CPH1)-PHR domain (aa 1−504) from *Chlamydomonas reinhardtii* was expressed with an N-terminal His-tag and a C-terminal Strep-tag and purified as described previously.[17] Protein was obtained in 50 mM sodium phosphate, pH 7.5, 100 mM NaCl, and 20% glycerol. The sample did not contain MTHF. The concentration of the sample was adjusted to an optical density of 0.5 at 450 nm (Figure S1, Supporting Information).

Time-Resolved UV/Vis Absorption Measurements. About 600 μL of the sample was placed in a quartz cuvette with 2 mm optical path length for excitation and 10 mm for probe light. The sample was excited at 355 nm by the third harmonic of a Nd:YAG laser (Surelite II, Continuum) with a pulse width of 8−10 ns. The excitation light was focused into the sample with a cylindrical lens (f = 150 mm), and the pulse energy was adjusted to 5−10 mJ/pulse at the sample. The solution was moved by a magnetic stirrer to minimize multiple excitations of the same sample molecules by successive laser pulses. A mechanical shutter was used to select excitation pulses from the 10 Hz pulse train of the laser.

The setup of the transient absorption spectrometer with streak camera will be described in detail elsewhere. In short, a pulsed 150 W Xe lamp (MSP-05, Müller Elektronik-Optik) was used as probe light and refocused three times by a series of toric mirrors.

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First, it was focused on a mechanical shutter to block the continuous light from the Xe lamp, then on the sample cell, and finally on the entrance slit of an imaging spectrograph (Bruker, grating 100 grooves/mm) in front of the streak camera. To suppress scattered light from the 355 nm laser pulse, a long pass filter with a cut off at 375 nm (Schott WG 375) was placed in front of the spectrograph. The spectrograph disperses the incoming light horizontally before it is focused vertically on the entrance slit of the streak camera. The streak camera (C7700, Hamamatsu Photonics) converts the temporal information into vertically dispersed electrons, thereby producing two-dimensional images of the intensity distribution of the probe white light. Each transient absorption data set was calculated from taking four images in succession with a frequency of 0.5 Hz: an intensity image without laser, an image without any incoming light, an image with laser excitation, and again an image without any incoming light. Results on CPH1 represent the average of 100 individual measurement sequences with time windows of 20 and 200 μs and a time resolution of 100 ns and 1 μs, respectively. A screenshot of raw data in the 20 μs window is presented in Figure S2 (Supporting Information).

For comparison, flavin mononucleotide (FMN) was dissolved at 40 μM in 10 mM phosphate buffer pH 8 containing 10 mM NaCl, resulting in an optical density of 0.5 at 450 nm. Tryptophan and cysteine were added to final concentrations of 25 and 165 mM, respectively. 100 time-resolved measurements with a time window of 20 μs were averaged.

Data Analysis. Data were analyzed with homemade software to obtain decay associated difference spectra (DADS). This analysis corresponds to the least-squares fit (eq 1):

$$|\Delta A(t,\lambda) - \sum_{k}^{N} f_k(t)B_k(\lambda)|^2 = \min \qquad (1)$$

where $\Delta A(t,\lambda)$ is the measured data matrix, the $f_k(t)$ are exponential functions convoluted with a Gaussian function as the apparatus response function $g(t)$ (eq 2)

$$f_k(t) = g(t) \otimes \exp(-t/t_k) \qquad (2)$$

and the $B_k(\lambda)$ are the DADS. Hence, each DADS is associated with a particular decay time t_k. This procedure does not assume a specific kinetic model but is compatible with all models that involve only unimolecular reaction steps. The DADS are a unique result of the experiment. Species spectra derived from them depend on mechanistic assumptions, e.g., a linear decay sequence or models involving parallel reactions and branching.

For presentation in Figures 2A and S4, the noise level of the measured spectra was reduced by filtering with singular value decomposition (SVD)[32] using the MATLAB software (The MathWorks, Natick, MA). This method separates the data matrix into time-dependent and spectral components of descending significance. The standard deviation between the reconstructed matrix and the data set was calculated for determination of the number of significant SVD components. In both data sets, two components were significant for reconstruction.

Fitting of reference spectra to the measured difference spectra was performed by applying a least-squares algorithm without any constraints. A linear combination of reference spectra was formed to calculate the model spectrum, and the coefficients were optimized. Reference difference spectra of $FAD^{-\bullet}$[14] and $FADH^{\bullet}$[17] minus FAD(ox) had been recorded on setups with higher spectral resolution than that of the streak camera setup. Therefore, the spectra were convoluted with a Gaussian function with a full width at half-maximum of 10 nm. The reference spectrum of the tryptophan neutral radical acquired in this study was smoothed before processing.

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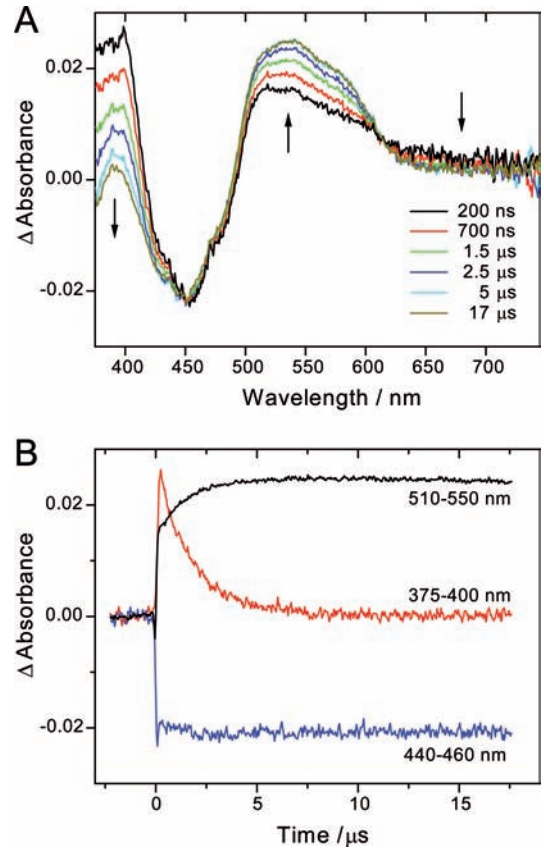

Figure 2. (A) Difference absorption spectra at indicated times after excitation of CPH1-PHR by a nanosecond laser pulse. The constant difference absorption at about 450 nm is attributed to the bleach of oxidized FAD. A decay is evident below 415 nm and above 610 nm, whereas absorption rises at 500−610 nm. Noise was filtered by singular value decomposition. (B) Kinetic traces extracted from indicated spectral ranges depict a process which takes place within few microseconds. An additional fast contribution is visible which is not resolved in this time window.

Results

Light-induced processes in plant cryptochromes on a time scale faster than milliseconds have not been investigated before. The light-sensitive domain of *Chlamydomonas reinhardtii* cryptochrome, CPH1-PHR, was excited with a nanosecond laser at 355 nm, and the evolution of spectra was recorded with a streak camera setup. This approach allows for simultaneous probing of temporal and spectral information. Difference absorption spectra were extracted from a measurement with a duration of 20 μs (Figure 2A). The negative band at around 450 nm is assigned to bleaching of FAD(ox) in the dark state. It remained essentially unchanged within the 20 μs. Positive difference bands were detected at 375−415 nm and at 500−700 nm. Within several microseconds, difference absorbance below 415 nm and above 610 nm decreased, whereas difference absorbance at 500−610 nm increased. This development is illustrated by the kinetic traces displayed in Figure 2B. At early times, a further process is contributing that is too fast to be resolved in this time window.

To identify the underlying processes, we performed a global analysis using a sum of three exponential functions. The first component comprises all processes with a time constant <150 ns, e.g., fluorescence, ultrafast ground state recovery, or fast triplet state quenching. These processes were not resolved on this time scale and were therefore not further considered in the analysis. The second component exhibits a decay with a time

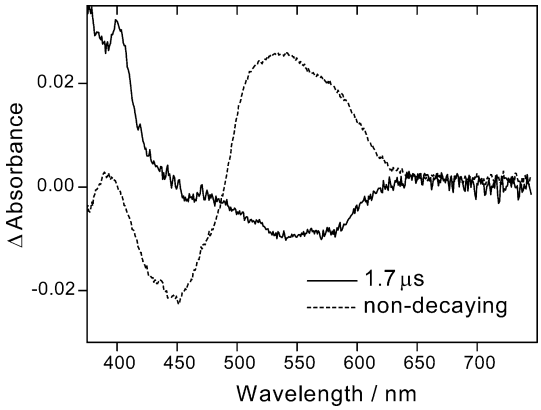

Figure 3. Global analysis of the experimental data yields two relevant components that are represented by their decay associated difference spectra (DADS). The first DADS decays with a time constant of 1.7 μs, whereas the second DADS represents a nondecaying component in the experimental time window of 20 μs.

constant of 1.7 μs. Its decay associated difference spectrum (DADS) comprises all net changes in absorbance with this time constant and is characterized by a positive band at 400 nm and negative bands at 540 and 580 nm. The third component is nondecaying within this time window (rate constant $k = 0$ fixed) (Figure 3).

The presence of only two relevant components allows for application of a simple kinetic scheme. The sum of both DADS represents the initial situation before the microsecond process (Figure 4A). The DADS of the nondecaying component represents the final state after the reaction (Figure 4B). The initial spectrum is characterized by a prominent absorption peak at 400 nm and a weak but distinct absorption at 610−745 nm. Both bands are clearly discernible in the 200 ns spectrum and decay in the microsecond process (Figure 2A). They are unique characteristics of flavin anion radicals in flavoproteins,[33] as exemplified by the difference spectrum of $FAD^{-\bullet}$ minus FAD(ox) of the animal cryptochrome dCRY from *Drosophila* (Figures 4A and S3).[14] The anion radical of free flavin in aqueous solution does not show an absorption maximum at 400 nm.[34] Another species with a characteristic but more narrow absorption band at around 400 nm would be a tyrosine radical. It has been shown to be formed in *At*CRY1 in later steps of the reaction on the millisecond time scale.[27] However, the tyrosine radical in *At*CRY1 absorbs rather at 405−410 nm than at 400 nm, in agreement with spectra in solution (Figure S3, Supporting Information).[35,36] Moreover, the formation of any amino acid or flavin species other than $FAD^{-\bullet}$ cannot fully account for the concomitant, strong bleach at 450 nm. Additional contributions to the initial spectrum at <415 nm and at 500−600 nm are discussed within the framework of possible reaction schemes (see the Discussion). The spectral features of the initial spectrum therefore point to the formation of an anion radical from the oxidized state, which is further supported by the following analysis of the microsecond process.

The spectrum at 17 μs (Figure 2A) and the final spectrum (Figure 4B) do not show any feature of a flavoprotein anion

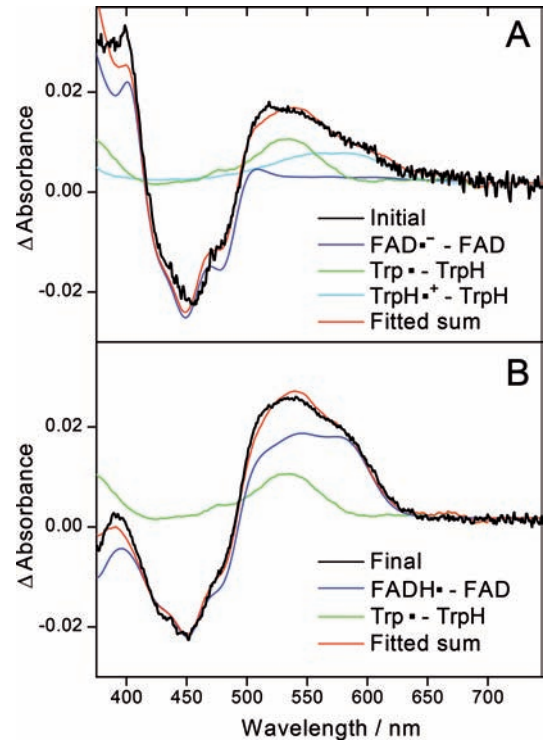

Figure 4. (A) The initial spectrum of CPH1-PHR before the microsecond process shows typical contributions of a flavin anion radical with a band at 400 nm and a broad absorption >600 nm. For direct comparison, the difference spectrum of $FAD^{-\bullet}$ minus FAD(ox) of the *Drosophila* cryptochrome was included (taken from ref 14). From spectral fitting it follows that two more species, $TrpH^{+\bullet}$ (taken from ref 39) and $Trp^{\bullet}$, need to be included to account for the observed differences at 500−600 nm. (*B*) The final state after the microsecond process was analyzed in a similar way. The difference spectrum of the formation of $FADH^{\bullet}$ from FAD(ox) in CPH1-PHR (taken from ref 17) together with that of $Trp^{\bullet}$ minus TrpH fully account for the observed difference absorption. The 1.7-μs process is assigned to protonation of the flavin anion radical.

radical. Instead, the spectra exhibit similarities to that of $FADH^{\bullet}$ minus FAD(ox) in CPH1 on the minute time scale (taken from ref 17) (Figure 4B). The formation of the new flavin species is especially evident at 580 nm, where the absorbance rises strongly by a factor of 1.7 as compared to the initial spectrum. The difference spectrum with its characteristic maxima at 540 and 580 nm is superimposed by an additional contribution with an absorption at 500−580 nm and below 410 nm. In agreement with previous difference spectra of *At*CRY1 on the millisecond time scale,[27] this species is identified as a tryptophan radical. Its spectrum is similar to that of tryptophan radical in water.[37−39]

As further support for these assignments, reference spectra in solution at pH 8 were generated by exciting oxidized flavin mononucleotide (FMN) at 355 nm in the presence of tryptophan. The resulting difference spectrum remained unchanged at 1−18 μs and was averaged (Figure 5). It reveals a high similarity to that of CPH1 after the microsecond process. The spectrum is assigned to show concomitant formation of a tryptophan radical and a flavin neutral radical.[40] It should be noted that the final spectrum of CPH1 differs by exhibiting typical fine-structure

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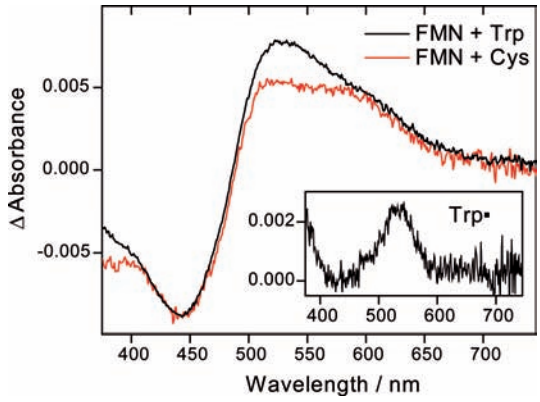

Figure 5. Photoreaction of a solution of flavin mononucleotide (FMN) with tryptophan or cysteine in the absence of apoprotein. Spectra remained constant at 1−18 μs after excitation and were averaged. The reaction with tryptophan leads to formation of a flavin neutral radical and a tryptophan radical. With cysteine, only the flavin radical is evident in this spectral range. The absorption difference spectrum of Trp• minus TrpH (inset) was obtained as the difference of the two transient spectra scaled to the same amplitude at 450 nm (i.e., same bleach of FMN).

in the difference bands at 430, 475, and 580 nm, which provide evidence for the binding of the FAD to the protein (Figure 4B). The contribution of the tryptophan radical to the difference spectrum was isolated by recording spectra of the photoreaction of FMN with cysteine (Figure 5). These spectra only show absorption of flavin in this spectral region. The isolated difference spectrum is assigned to a tryptophan neutral radical Trp• due to its absorption maximum at about 520 nm (Figure 5, inset).[38,39] The spectrum was then available as a reference for fitting to the final spectrum using a least-squares algorithm (Figure 4B). The agreement between the fitted sum of tryptophan and flavin neutral radicals and the measured final spectrum is very high considering the fact that the tryptophan reference was obtained not in a protein environment but in solution. In conclusion, the final spectrum reflects formation of FADH• and of a tryptophan neutral radical, tentatively that of the distal Trp(*III*). This assignment is in agreement with those previously done in *At*CRY1[27] and photolyase.[29]

The processes after 20 μs in CPH1 were investigated by recording spectra in a 200 μs window. Extracted difference spectra and kinetics show a partial decay in the spectral region of 500−600 nm (Figure S4, Supporting Information). The bleach of FAD(ox) at about 450 nm is reduced to a minor extent by about 10%. This development points to a process in the late microsecond region that is dominated by tryptophan radical decay but contains additional contributions of a flavin radical decay. These processes have been fully resolved in *At*CRY1, where the tryptophan radical decay takes place with a half-life of 1 ms and the flavin radical decay with 5 ms.[27] The complete decay process of CPH1 to the dark state was not monitored in this study. It has been shown that the flavin radical in CPH1 fully recovers to the oxidized state with $\tau = 200$ s under aerobic conditions.[17]

Discussion

We have detected a microsecond light-induced process in the plant cryptochrome CPH1. After excitation of the oxidized FAD, the difference spectrum at 200 ns shows typical bands of a flavin anion radical. These bands decay with a time constant of 1.7 μs while simultaneously bands build up that are assigned to formation of a flavoprotein neutral radical. During the whole

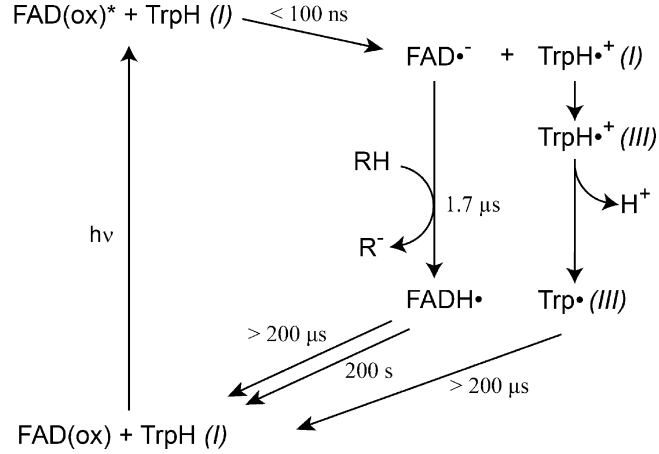

Figure 6. Schematic overview of the processes after light excitation of FAD(ox) in the plant cryptochrome CPH1-PHR. The flavin anion radical $FAD^{-\bullet}$ is formed by electron transfer from the proximal tryptophan Trp(*I*) within 100 ns. Proton transfer to $FAD^{-\bullet}$ occurs with a time constant of 1.7 μs donated by a residue R, most likely an aspartic acid. Meanwhile, the distal tryptophan neutral radical Trp•(*III*) is formed by fast electron transfer from the proximal tryptophan and subsequent proton release, probably to the bulk solution. The flavin and tryptophan neutral radicals decay with time constants of >200 μs into the dark form.

time, the loss of oxidized FAD remains constant. We therefore identify the microsecond process as proton transfer to the flavin chromophore (Figure 6).

Previous Observation of Flavin Anion Radicals. Flavin anion radicals are formed by electron transfer in the initial step of the reaction of free flavin with tryptophan, as has been shown after a long-lasting debate.[41,42] This reaction takes place from the long-lived triplet excited state[40,43] due to the necessity of diffusion for an encounter. $FAD^{-\bullet}$ is furthermore commonly observed in flavoproteins after photoreduction under anaerobic conditions in presence of external electron donors.[33] We studied the photoreaction of CPH1 in absence of any external electron donor to be able to differentiate the intrinsic reaction of the protein from this common and nonphysiological photoreduction. Similarly, the animal cryptochrome dCRY forms $FAD^{-\bullet}$ in the absence of electron donors.[14] In dCRY, $FAD^{-\bullet}$ is stable for minutes in the presence of oxygen. It should be noted that its protonation state is unaffected by external buffer pH.

Proton Donor to the Flavin: Aspartic Acid or Tryptophan Cation Radical. The most likely candidate for a proton donor to the flavin is an aspartic acid (residue R in Figure 6). Deprotonation of an aspartic acid has been observed in FT-IR experiments on *At*CRY1.[26] An infrared difference band for light-induced deprotonation of an aspartic acid was accordingly detected in CPH1 (T. K. and D. I., unpublished). It has been suggested for *At*CRY1 that Asp396 close to the chromophore is the proton donor. This aspartic acid is conserved in CPH1 as Asp393. The countercharge of the tryptophan cation radical $TrpH^{+\bullet}$ might be compensated by deprotonation to the solvent due to its low pK_a of 4.3.[38,39] In photolyase, deprotonation of the distal $TrpH^{+\bullet}$ to the bulk occurs with a time constant of 300 ns.[29]

Spectroscopically, the tryptophan cation radical $TrpH^{+\bullet}$ can be distinguished from the neutral radical Trp• by a red-shift of

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the absorption maximum from about 510 nm to 560/580 nm in water.[39] In our experiments, the initial spectrum before the microsecond protonation of flavin shows contributions at 500−600 nm. A fitting procedure was applied, which quantitatively reproduced the initial spectrum by including both tryptophan radical species and $FAD^{-\bullet}$ (Figure 4A). When $FADH^{\bullet}$ was additionally considered in the analysis, this did not lead to any change in the fitted sum. $FADH^{\bullet}$ does not contribute significantly to the initial spectrum, because this would lead to a strongly reduced sum of absorption at <415 nm (Figure S5, Supporting Information). It follows that a variation in the amount of $FAD^{-\bullet}$ is not possible, because this species has to account for the complete, concomitant bleach of FAD(ox) at 450 nm. Therefore, the ratio of $Trp^{\bullet}$ to $TrpH^{+\bullet}$ in the initial spectrum is determined to about 2:1 using the extinction coefficients in solution of 2.3 and 3.0 mM^{-1} cm^{-1}, respectively.[39] It can further be deduced that the integrated contribution of tryptophan radicals at 500−630 nm is higher in the initial than in the final spectrum (Figure 4). This absolute quantification, however, needs to be taken with caution, because ambiguities arise from applying reference spectra of $FAD^{-\bullet}$ from dCRY[14] and of $TrpH^{+\bullet}$ in solution.[39] Contributions of $FAD^{-\bullet}$ to the difference spectrum at 500−550 nm vary strongly between different proteins (Figure S3, Supporting Information), which might lower the contribution of $Trp^{\bullet}$. Additionally, possible shifts of the $TrpH^{+\bullet}$ absorption induced by the lower polarity of the environment of the protein further complicate the picture. These ambiguities furthermore explain the small deviation of the fitted sum from the initial spectrum at around 450 nm and <415 nm. Despite these ambiguities, a reasonable fit to the absorption at 500−600 nm requires to include both tryptophan radical species.

Another candidate for proton transfer to $FAD^{-\bullet}$ besides aspartic acid would be the tryptophan cation radical $TrpH^{+\bullet}$. From the above analysis, however, it follows that the formation of $Trp^{\bullet}$ takes place to a considerable extent before 200 ns. As a consequence, the deprotonation of $TrpH^{+\bullet}$ is mostly decoupled from the protonation of flavin observed with a time constant of 1.7 μs. The residual, concomitant deprotonation of $TrpH^{+\bullet}$ might point to an impact of flavin protonation on the pK_a value of $TrpH^{+\bullet}$. Another argument against a direct protonation of $FAD^{-\bullet}$ by $TrpH^{+\bullet}$ is that the radical pair has to be separated in space on an ultrafast time scale to avoid recombination. In photolyases, separation up to a distance of 15 Å proceeds in <30 ps.[44] This reaction in photolyase starts from $FADH^{\bullet}$ and not from FAD(ox) as in cryptochrome, which might influence the rate of radical pair generation and recombination. The electron transfer rates within the tryptophan triad of <30 ps, however, are likely to be comparable due to the structural homology. A putative back transfer of the proton in CPH1 from the distal $TrpH^{+\bullet}$ to the flavin in microseconds would require postulation of a yet unidentified proton transfer channel. The presumably complex kinetics of the $TrpH^{+\bullet}$ decay or conversion before 200 ns and in the microsecond range was not fully resolved in this study due to the limited time resolution, the low extinction coefficient of the radical, and the broad spectral overlap with the flavin conversion. This analysis needs to be performed after further approaches employing, e.g., time-resolved infrared spectroscopy.

Different Photoresponse of Plant and Animal Cryptochromes. Animal and plant cryptochromes undergo similar processes directly after excitation. In both protein families, the flavin anion radical has been detected. In animal cryptochromes, it is formed 1 ps after excitation[20] and decays in minutes.[14] In plant cryptochromes, the formation occurs before 100 ns and protonation of $FAD^{-\bullet}$ takes place within few microseconds (Figure 6). In plant and animal cryptochromes, the tryptophan triad is directly involved in photoreduction,[19,30] at least under low light conditions.[9] However, formation of the neutral radical $FADH^{\bullet}$ only occurs in plant cryptochromes. Therefore, the protonation step is the key difference between animal and plant cryptochromes. It further distinguishes cryptochromes and its homologous photolyases, because proton transfer has not been observed in the photoreduction of FAD(ox) in photolyases.[45] The fact that the proton transfer occurs only in plant cryptochromes speaks in favor of an aspartic acid as proton donor to the FAD (residue R in Figure 6). The aspartic acid denoted by Asp396 in *At*Cry1 is a distinctive feature of all plant cryptochromes and neither found in the sequences of animal cryptochromes nor photolyases.

Animal cryptochromes have been shown to act as magnetosensors.[5] For plant cryptochromes, this function is currently under debate.[46,47] Our results indicate that the radical pair sensitive to the magnetic field includes the flavin anion radical because both types of cryptochrome form this flavin species with a lifetime above the 100 ns limit imposed by theory.[7] In contrast, other physiological responses to plant cryptochromes have been shown to be initiated by the flavin neutral radical.[24,25] Further studies are therefore needed to resolve the role of the proton transfer.

Three Flavin-Containing Sensory Blue Light Receptors: Three Principles. The distinctive feature of the plant cryptochrome photoreaction is the separation in time of electron and proton transfer probably by as much as 5 orders of magnitude. This separation is unprecedented among flavin-containing blue light receptors. It is conceivable that generation of a charged aspartate in a hydrophobic pocket might initiate structural changes in the protein that are required for signaling. We therefore propose that this separation has a functional role for signaling and does not simply constitute a charge compensation process.

In the sensor of blue light using FAD (BLUF) domain, an ultrafast proton transfer has been detected with a time constant of 6 ps,[48] preceded by an electron transfer probably from a tyrosine residue. A glutamine hydrogen bonded to the flavin N5 has been postulated to donate the proton over a distance of ~3 Å. The microsecond time scale of proton transfer in CPH1 therefore speaks against the presence of a hydrogen-bonded proton to the flavin N5 before excitation in plant cryptochromes. In the crystal structure of the homologous *At*CRY1-PHR,[28] Asp396 is as close as 3.8 Å to the flavin N5. However, a hydrogen bond of the Asp396 to the far side of the flavin is detected making contact to the backbone carbonyl of Met381 with a distance of 2.7 Å.

In the light-, oxygen-, and voltage-sensitive (LOV) domain of phototropin, the photoreaction proceeds much slower than

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in cryptochrome due to the lower reactivity of cysteine as compared to tryptophan. A covalent adduct with a cysteine is formed from the triplet excited state of flavin within a few microseconds. The mechanism of formation of this adduct has not been completely resolved,[49] but it is evident that all processes such as electron, proton, or group transfer are tightly linked in time with the microsecond decay of the triplet state.[50]

Conclusions

Different suggestions have been made about the kinetics of flavin protonation in plant cryptochromes. On the one hand the transfer has been postulated to proceed on a slow time scale.[20] On the other hand, it has been speculated to even precede electron transfer on an ultrafast time scale.[51] In this study, we have clarified this issue by providing evidence for a microsecond proton transfer to the flavin anion radical. Thereby, the photoresponse of plant cryptochrome can be clearly distinguished from those of animal cryptochrome from insects and photolyase. Moreover, the isolated, microsecond proton transfer has to our knowledge no parallel in other flavin-containing proteins, which show a tight linkage in time of electron and proton transfer to the excited state lifetime.

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Supporting Information Available: Absorption spectrum of CPH1-PHR, sample image of streak camera, absorption difference spectra of flavoprotein anion radicals and tryptophan cation radicals, difference spectra in 200 μs time window, and spectral fitting of initial spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.

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