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Response of the Sensory Animal-like Cryptochrome aCRY to Blue and Red Light As Revealed by Infrared Difference Spectroscopy

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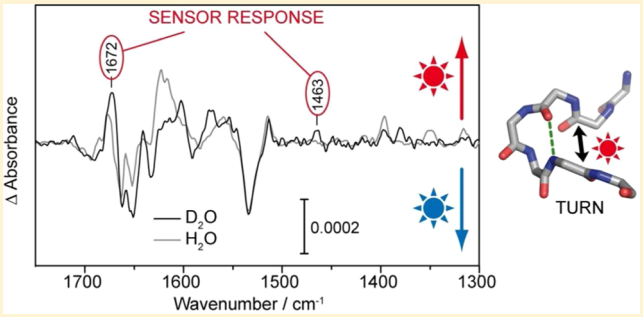
Supporting Information

ABSTRACT: Cryptochromes act as blue light sensors in plants, insects, fungi, and bacteria. Recently, an animal-like cryptochrome (aCRY) was identified in the green alga *Chlamydomonas reinhardtii* by which gene expression is altered in response to not only blue light but also yellow and red light. This unique response of a flavoprotein *in vivo* has been attributed to the fact that the neutral radical of the flavin chromophore acts as dark form of the sensor, which absorbs in almost the entire visible spectral range (<680 nm). Here, we investigated light-induced processes in the protein moiety of full-length aCRY by UV−vis and Fourier transform infrared spectroscopy. Findings are compared to published results on the homologous (6-4) photolyases, DNA repair enzymes. The oxidized state of aCRY is converted to the neutral radical by blue light. The recovery is strongly dependent on pH and might be catalyzed by a conserved histidine of the (6-4)/clock cluster. The decay is independent of oxygen concentration in contrast to that of other cryptochromes and (6-4) photolyases. This blue light reaction of the oxidized flavin is not accompanied by any detectable changes in secondary structure, in agreement with a role *in vivo* of an unphysiological preactivation. In contrast, the conversion by red light of the neutral radical to the anionic fully reduced state proceeds with conformational changes in turn elements, which most probably constitute a part of the signaling process. These changes have not been detected in the corresponding transition of (6-4) photolyase, which points to a decisive difference between the sensor and the enzyme.



Several of the major sensory light receptors in nature bind riboflavin derivatives as chromophores. One large and diverse family of flavin-containing light receptors comprises the cryptochromes (CRY), which act as blue light sensors in insects (animal type I CRY), plants, fungi, and bacteria.[1] Furthermore, they act within the central part of the oscillator of the biological clock in mammals[2] (animal type II CRY) and as magneto-receptors in flies.[3] They are highly homologous to photolyases, which use light to repair UV-induced DNA lesions. Depending on the type of lesion, photolyases repair either DNA strands containing cyclobutane pyrimidine dimers (CPD)[4] or (6-4) photoproducts.[5] CPD photolyases are homologous to plant cryptochromes, whereas (6-4) photolyases are similar in sequence to animal type II CRY. As a separate subfamily, DASH cryptochromes have been shown to repair single-stranded DNA and loop-structured double-stranded DNA *in vitro*.[6,7] All members of the cryptochrome/photolyase family bind flavin adenine dinucleotide (FAD) as a chromophore in the photolyase homology region (PHR), which comprises ∼500 amino acids. Cryptochromes and (6-4) photolyases additionally contain a C-terminal extension of variable length and sequence.

Recent findings have led to a revision of two paradigms in the cryptochrome field. First, the cryptochrome/photolyase family 1 (CPF1) proteins in the diatom *Phaeodactylum tricornutum*[8] and the microalga *Ostreococcus tauri*[9] have been shown to act both as light sensors and as DNA repair enzymes, which is in disagreement with the classical definition of cryptochromes being photolyase-like proteins without a repair function.[10] Second, the animal-like cryptochrome (aCRY) in the green alga *Chlamydomonas reinhardtii* has been shown to significantly alter gene expression in response to not only blue but also red and yellow light *in vivo*.[11] This finding is striking as aCRY is the first known flavoprotein that is driven to its signaling state by light outside the blue and UV spectral range. Its ability to respond to blue light and other light qualities was attributed to a stabilization of the neutral radical state of flavin as the dark form of the sensor *in vivo* (Figure 1). Evidence of this assignment comes from the agreement between the absorption spectrum of the FAD neutral radical in aCRY and the response recorded *in vivo*, both with maxima at ∼460, ∼590, and ∼635 nm and with a minimum at ∼700 nm. The possibility of such a red light-driven photochemistry of flavoproteins has been suggested before.[12] However, this role was not anticipated from

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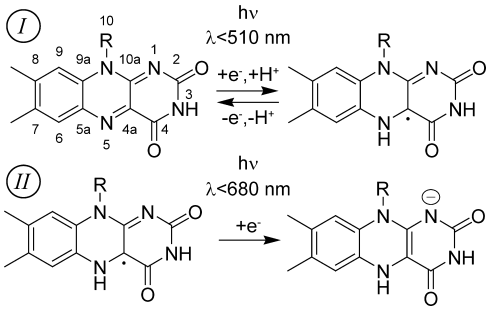

Figure 1. Photoreaction sequence of aCRY *in vitro*. (I) Oxidized flavin is reversibly converted by UV/blue light into the flavin neutral radical. (II) Further illumination of the radical with λ < 680 nm UV/visible light produces the fully reduced state of flavin. A selective conversion is achieved by λ > 510 nm light, such as red light. *In vivo*, the neutral radical has been assigned to the dark form and the fully reduced state to the signaling state of aCRY (step II).

the primary sequence of aCRY, where it groups together with animal type II CRY, (6-4) photolyases, and CPF1 proteins.[11]

Here, we followed light-induced changes in the protein moiety of aCRY by employing Fourier transform infrared (FTIR) difference spectroscopy. This technique isolates changes in vibrations of the chromophore, amino acid side chains, and the protein backbone from a vast background of unaltered signals. It has been successfully applied to identify changes in protonation states and hydrogen bonding of side chains with molecular sensitivity as well as changes in specific secondary structure elements of proteins. The latter information has been gained via the coupling of amide carbonyl vibrations of the backbone, the amide I vibration, at 1615−1695 cm^{-1}.[13] Additional unspecific evidence of changes in secondary structure can be obtained from the amide II vibration of the backbone at ∼1550 cm^{-1}.[13]

To date, FTIR experiments with sensory cryptochromes have been performed only on the activation of the plant cryptochromes *Chlamydomonas* photolyase homologue 1 (CPH1-PHR) from *C. reinhardtii*[14] and CRY1 from *Arabidopsis thaliana* (*At*CRY1)[15] by photoreduction of the oxidized state to the neutral radical state. These experiments revealed the deprotonation of an aspartic acid upon formation of the neutral radical and a secondary structural change in turn elements leading to a prominent signal at 1671 cm^{-1}. Complementary information is available from the studies on the homologous DNA repair enzymes DASH cryptochrome,[14,16] CPD,[17,18] and (6-4) photolyase,[19,20] in which additionally transitions to the anionic radical and fully reduced states of FAD have been characterized in detail.

We have investigated the response of aCRY carrying FAD in its oxidized and neutral radical states to blue and red light, respectively. The direct comparison with the recent study by FTIR spectroscopy on these transitions in the homologous *Xenopus laevis* (6-4) photolyase[19] allowed us to determine differences in the response of the light sensor as opposed to that of the DNA repair enzyme. Furthermore, the comparison of aCRY to the plant cryptochrome CPH1 from *C. reinhardtii* reveals fundamental differences and few similarities in the response of these two sensors despite the fact that both are cryptochromes in the same organism.

MATERIALS AND METHODS

Expression and Purification. Heterologous expression in *Escherichia coli* and purification of aCRY were conducted following published procedures.[11] Finally, aCRY was obtained in a 50 mM sodium phosphate buffer (pH 7.8), 100 mM NaCl, and 20% (v/v) glycerol.

UV−Vis Difference Spectra and Kinetic Experiments. UV−vis difference spectra were recorded in the presence and absence of 10 mM dithiothreitol (DTT) using a UV-2450 spectrometer (Shimadzu). The sample was consecutively illuminated for 5 s by a 455 nm LED (Luxeon Star, Lumileds) with an intensity of 10 mW/cm^2 at the sample and a full width at half-maximum (fwhm) of 20 nm and then for 20 s with a 636 nm LED (Luxeon Star, Lumileds) with an intensity of 34 mW/cm^2 and a fwhm of 25 nm.

The recovery of the oxidized FAD was recorded at 447 nm after illumination with blue light for 1 s with a 455 nm LED (Luxeon Star, Lumileds) with an intensity of 16 mW/cm^2 and a fwhm of 20 nm. The illumination time was controlled by a delay generator (Stanford Research Systems, DLG 645). The anaerobic sample was prepared by passing a stream of argon over the solution for 30 min.

To obtain different pH values, the buffer was exchanged via ultrafiltration (Vivaspin 500, Sartorius) to 50 mM sodium phosphate buffers, 100 mM NaCl, and 20% (v/v) glycerol with a pH in the range from 4 to 8.5. Below pH 6.0, the protein aggregated, which prevented further analysis. All kinetic experiments were conducted at 20 °C.

FTIR Experiments. The samples were concentrated to an OD_{447} of >5 by ultrafiltration using Vivaspin 500 filter devices (Sartorius, 50 kDa molecular weight cutoff). During centrifugation at 15000*g*, the protein was washed three times with 20 mM sodium phosphate buffer (pH 7.8), 100 mM NaCl, and 1% (v/v) glycerol. For experiments with deuterated samples, the sodium phosphate buffer was prepared with D_2O and all steps were conducted under a nitrogen atmosphere.

A 2 μL droplet of the sample solution was applied to a BaF_2 window (20 mm diameter) with some grease around the rim. It was kept at 20 °C and atmospheric pressure for 2 min to gently reduce the water content. The deuterated samples were prepared under an argon atmosphere in an anaerobic cell at 700 mbar for 30 s. The samples were sealed with a second BaF_2 window. The samples in D_2O were kept at room temperature for at least 36 h to allow for H−D exchange. Full hydration of the samples was checked by the absorbance ratio of amide I with water to amide II bands in the absorption spectra. An appropriate hydration of the sample is essential to ensure that the full extent of changes in secondary structure of the protein is detected.[21]

IR experiments were performed on a Bruker IFS 66s spectrometer. The difference spectra were obtained with 2 cm^{-1} resolution with an MCT detector and a long wave pass filter (OCLI) cutting off infrared light above 2256 cm^{-1}. The experiments were performed at room temperature. The blue light response of aCRY was induced by illumination for 8 s with an LED (Luxeon Star, Lumileds) with an emission maximum at 455 nm (fwhm of 20 nm) equipped with a diffusion disk and yielding an intensity of 24 mW/cm^2 at the sample. For a representative series of difference spectra, 1024 and 2048 scans were averaged in H_2O and D_2O, respectively. Red light illumination was conducted for 40 s with an LED emitting at 636 nm with an intensity of 28 mW/cm^2 at the sample (fwhm

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of 25 nm); 3072 and 2048 scans were averaged in H_2O and D_2O, respectively.

Quantum Chemical Calculations. Normal modes of lumiflavin in its oxidized and neutral radical state were calculated after geometry optimization using density functional theory (DFT) with B3LYP functional and a 6-311+G(2d,p) basis set, as implemented in *Gaussian03W*.[22] Four water molecules were included to model interactions with the environment (Figures S1 and S2 of the Supporting Information). D_2O exchange was achieved by replacing the N_3 hydrogen with deuterium. A single scaling factor of 0.975 was applied to all frequencies. The line spectrum was broadened with Lorentzians with a full width at half-maximum of 14 cm^{-1} as determined for homogeneous broadening of bands of flavin in water.[23] Potential energy distributions (PEDs) for the normal modes were calculated with a home-written algorithm in *Matlab* (The Mathworks, Natick, MA) from the internal force constants and amplitudes delivered by *Gaussian 03W*. Modes with a >80% contribution from water molecules were not included in the further analysis of the flavin normal modes.[24]

Structural Modeling. MODELER 9v7 was used to generate a model structure of the aCRY−PHR domain using the crystal structure of *Arabidopsis* (6-4) photolyase as a template (Protein Data Bank entry 3FY4).[25] The primary sequences of the two domains are 54% identical. FAD was included for optimization.

RESULTS

Oxygen and pH Dependence of Recovery after Illumination with Blue Light. Oxygen has a major negative influence on the stability of the FAD radical in animal cryptochrome type I from *Drosophila* (dCRY)[26] and the plant cryptochromes CPH1-PHR[27] and *At*CRY1.[28] These studies revealed an acceleration of the back-reaction from the neutral and anionic radicals to the oxidized FAD by oxygen. For *At*CRY1, a linear dependency of the recovery lifetimes on the oxygen content has been shown.[28] Accordingly, the influence of oxygen was tested on aCRY. The sample was treated with argon for 30 min. Afterward, the sample was illuminated for 1 s with blue light and the absorbance was followed at 447 nm (Figure 2). The analysis showed a monoexponential kinetics with a lifetime of ~450 s, very similar to that in the presence of oxygen.[11] The lacking influence of oxygen implies a high stability of the radical state of FAD in aCRY as opposed to that of animal type I cryptochromes, plant cryptochromes, and (6-4) photolyases.[29] Furthermore, it points to a different electron acceptor for the recovery of the oxidized FAD in aCRY.

To investigate if a specific amino acid acts as proton acceptor in the recovery of the oxidized FAD after illumination, kinetic experiments were conducted at pH values ranging from 6.0 to 8.5. The pH interval was limited by the stability of the protein that aggregated at pH <6.0. At pH 6.0, the lifetime of the FAD radical was ~3500 s. With an increase in pH, the lifetime was reduced to 1340 s (pH 6.9) and 457 s (pH 7.8). Measuring kinetics at pH 8.5 revealed a much faster decay of the neutral radical with a lifetime of 63 s. The lifetimes were plotted against the pH values and showed a clear correlation (Figure 3). Thus, the pH value has a strong influence on the stability of the FAD neutral radical.

Red Light Illumination of the FAD Neutral Radical. An established procedure[11] was applied for the investigation of the selective photoreaction of the neutral radical upon illumination with red light. After production of the radical by a 5 s illumination with blue light, the sample was exposed to red light for 20 s. In the presence of 10 mM DTT as a reductant, the UV−vis difference spectrum shows the formation of an almost featureless product by red light illumination (Figure 4a), which

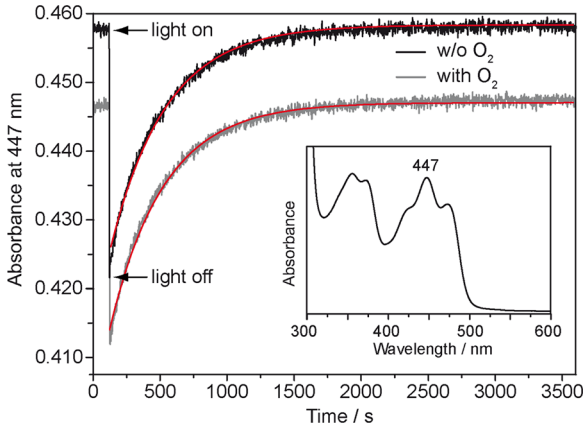

Figure 2. Effect of oxygen on the recovery after blue light illumination. The UV−vis absorbance was recorded at the absorption maximum of the oxidized state of aCRY (see the inset). The sample was illuminated with blue light for 1 s under anaerobic conditions, and the recovery was monitored (black). The monoexponential fit yielded a lifetime of 450 s (red). For comparison, a trace under aerobic conditions is shown with an identical lifetime as taken from ref 11.

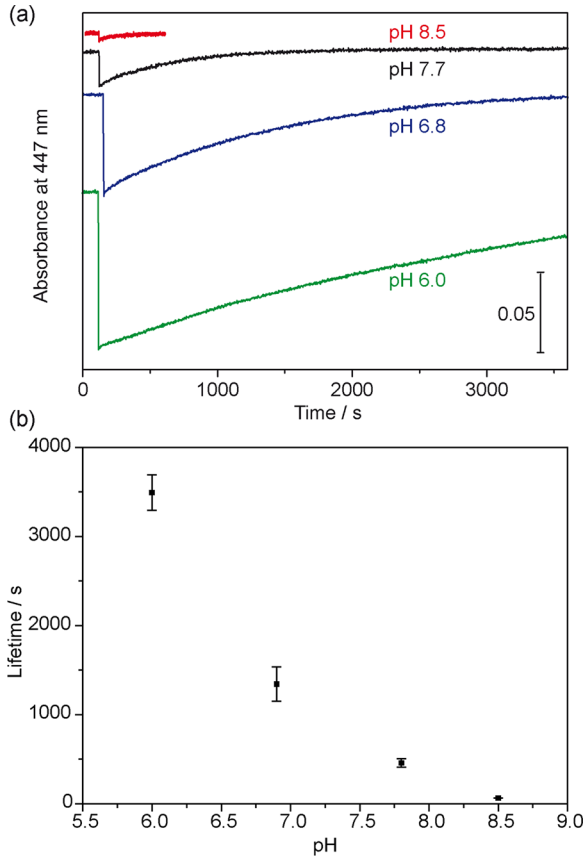

Figure 3. Effect of pH on the recovery after blue light illumination. (a) Representative kinetic traces of the recovery of the oxidized state of aCRY after blue light illumination at different pH values. (b) Resulting lifetimes from a monoexponential fit and their standard deviations from several experiments.

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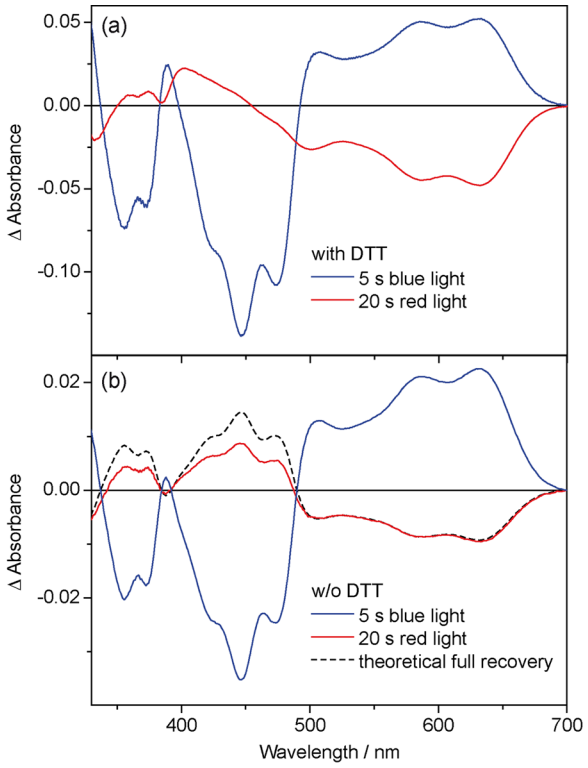

Figure 4. Illumination of the FAD neutral radical in aCRY with red light. Prior to illumination, the radical was generated with its characteristic absorption maxima at 585 and 633 nm by conversion of the oxidized FAD with blue light. (a) UV−vis difference spectra were recorded in presence of the reductant DTT. Red light illumination produced an almost featureless product, which is assigned to the fully reduced state of FAD. (b) The difference spectrum in the absence of any reductant shows the simultaneous formation of the fully reduced state and the oxidized state of FAD by red light illumination. The presence of the fully reduced state is deduced from the fact that a full thermal recovery to the oxidized state would have led to a much more pronounced absorption (---).

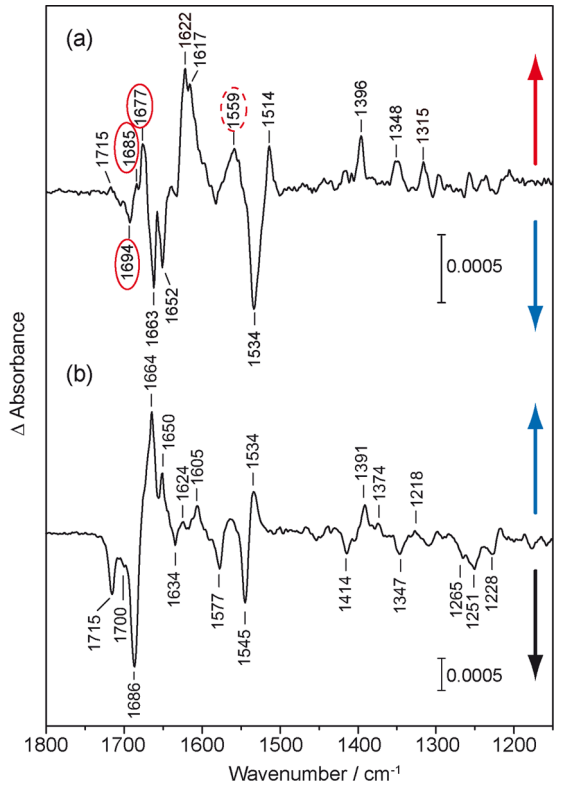

Figure 5. Light-induced infrared difference spectra in H_2O. (a) The sample was preilluminated with blue light and then illuminated with red light to selectively convert the flavin neutral radical into the fully reduced state. (b) Prior to this, oxidized flavin of the dark form was illuminated with blue light to produce the flavin neutral radical. Contributions that were identified in the analysis as originating from the protein moiety are encircled in red.

has been assigned to the fully reduced state of FAD.[11] In the absence of any reductants, the radical state is less stable and the oxidized state is formed during the 100 s recording time of the UV−vis difference spectrum (Figure 4b). However, this thermal recovery accounts for only ∼60% of the conversion of the radical state. This value is calculated from the ratio between a theoretical full recovery (Figure 4b) and the recorded absorbance at 454 nm, which represents an isosbestic point of the radical-to-reduced transition. The other ∼40% of the conversion led to a featureless species, which is again attributed to the fully reduced state formed by red light illumination. It should be noted that this assignment is merely based on the exclusion of other flavin species but does not represent direct evidence of the presence of the fully reduced state because of a lack of marker bands.

FTIR Experiments Examining the Blue Light-Induced Radical Formation. Light-minus-dark difference spectra in the mid-infrared region were taken after illumination with blue light at 455 nm for 8 s (Figure 5b). Only bands of those vibrational modes that change upon illumination are visible. Subsequent illumination with red light led to a further conversion (Figure 5a), which will be explored in a separate section below. The negative bands in the blue light-induced difference spectrum represent the dark state (Figure 5b). The band pattern originates from the oxidized state of FAD in agreement with *in vitro* UV−vis experiments[11] and with reference spectra of free FAD in water[23] as well as spectra of plant cryptochromes.[14,15] All bands were assigned by comparison with quantum chemical calculations using DFT on lumiflavin in a model environment (Table S1 of the Supporting Information). Experimental and theoretical bands agreed very well with a deviation of ≤2% in frequency (Figure 6c).

In contrast to the spectra of plant cryptochromes, a band at ∼1733 cm^{-1} is absent in the spectrum of aCRY.[14,15] This spectral region is typical for vibrations of the side chains of aspartic and glutamic acid. An aspartic acid close to flavin was proposed to act as the proton donor for neutral radical formation of FAD in plant cryptochromes.[14,15] The lack of signals in this region excludes aspartic or glutamic acids as a proton donor in aCRY.

At 1715 and 1686 cm^{-1}, bands are assigned to the C_4O and C_2O carbonyl vibrations of the flavin chromophore, respectively. Additionally, a third weak band is detected at 1700 cm^{-1}, which most probably originates from an additional carbonyl vibration as has been shown for the light, oxygen, voltage (LOV) domain of phototropin1 from *Avena sativa*.[30]

The positive bands of the light-induced difference spectrum of aCRY represent the state after the photoreaction. The bands at 1664, 1605, 1534, and 1391 cm^{-1} are identified as typical marker bands for the flavin neutral radical in proteins.[14,31] Any indications of the generation of a fully reduced state with a marker band at ∼1515 cm^{-1}[32] are missing, which demonstrates

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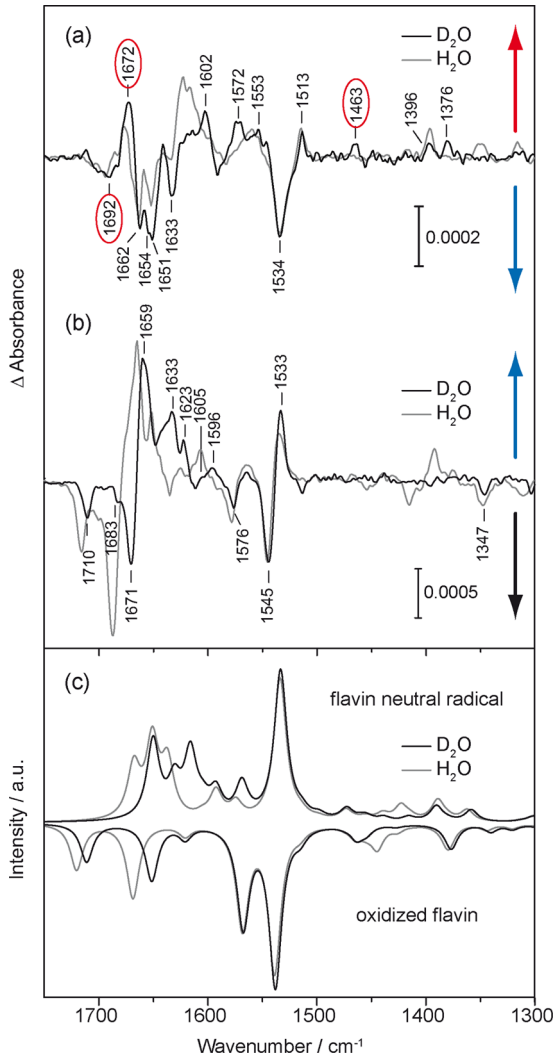

Figure 6. Light-induced infrared difference spectra in D_2O (black). Scaled difference spectra in H_2O are included to illustrate the shift by isotope exchange (gray). (a) The sample was preilluminated with blue light and then illuminated with red light to selectively convert the flavin neutral radical to the fully reduced state. (b) Prior to this, oxidized flavin of the dark form was illuminated with blue light to produce the flavin neutral radical. Contributions that were identified in the analysis as originating from the protein moiety are encircled in red. (c) Results from quantum chemical calculations with lumiflavin with four water molecules are depicted in its oxidized state (negative sign) and neutral radical state (positive sign). Lines were broadened with Lorentzians.

the selective conversion of the oxidized state to the neutral radical state of flavin under these conditions. In contrast, irradiation of the homologous *X. laevis* (6-4) photolyase led to the production of a mixture of radical and fully reduced states.[19] All bands of the neutral radical were assigned by comparison with quantum chemical calculations on lumiflavin (Table S1 of the Supporting Information). Again, experimental and theoretical bands agreed in frequency with a deviation of ≤2%. Intensities are not as well represented as in the case of the oxidized state (Figure 6c). Deviations can be attributed to the simplified model, which replaces the protein environment with four water molecules. Frequencies and assignments are in overall good agreement with previous DFT calculations on the Raman spectrum below 1610 cm^{-1} of the lumiflavin neutral radical in vacuum.[33]

By far the most prominent difference band shows up at 1664(+)/1686(−) cm^{-1}. The negative band is much more pronounced than in spectra of free FAD, CPH1, and *At*CRY1, where band III of flavin at 1547, 1543, and 1550 cm^{-1}, respectively, is the most prominent band in this spectral region.[14,15,23] For aCRY, in contrast, the band at 1686 cm^{-1} shows twice the intensity of the band at 1545 cm^{-1}. This difference raises the question of whether this band originates from changes in secondary structure or from amino acid side chains. To address this question, we performed isotope labeling by exchanging labile protons in D_2O.

Assignment of Blue Light-Induced Difference Bands by Isotope Exchange in D_2O. After exchange of H_2O for D_2O in the buffer, the sample was kept in the dark for 36 h at room temperature to allow for H−D exchange in the protein. Difference spectra were recorded after illumination for 8 s as for the sample in H_2O (Figure 6b). The observed, strong shifts can be explained by a deuteration of the flavin at N_3, which is the only exchangeable position of the chromophore.

The negative band at 1710 cm^{-1} in H_2O was downshifted by 5 cm^{-1}. The extent of the shift is in good agreement with the assignment to a C_4O stretching vibration, as demonstrated by quantum chemical calculations with a theoretical shift of 9 cm^{-1} (Figure 6c; Table S1 of the Supporting Information). The most prominent negative band at 1686 cm^{-1} in H_2O shows a large downshift of 15 cm^{-1}, in agreement with a theoretical shift of the C_2O vibration of 18 cm^{-1}. The strong and complete shift of the band demonstrates the success of the H−D exchange. Most importantly, it can be deduced that this band in D_2O originates exclusively from the flavin chromophore. Vibrations from the protein backbone cause the amide I band between 1615 and 1695 cm^{-1}; however, amide I bands shift by only <10 cm^{-1} in D_2O, whereas contributions from side chains such as glutamine or asparagine would lead to an overly large shift of >30 cm^{-1}.[13]

The weak band at 1700 cm^{-1} is shifted to a similarly large extent to 1683 cm^{-1}. This fact indicates that the band represents another C_2O vibration in contrast to the assignment to C_4O in *A. sativa* phototropin1-LOV2.[30] There, bands at 1714 and 1694 cm^{-1} were assigned to C_4O with different numbers of hydrogen bonds. Most other negative bands do not show larger shifts as they originate from CN and CC stretching vibrations. An exception is the band at 1414 cm^{-1}, which cannot be assigned anymore in D_2O, in agreement with the coupling to an N_3H bending mode (Table S1 of the Supporting Information).

The positive carbonyl bands of the neutral radical at 1664 and 1650 cm^{-1} show shifts very similar to those of the oxidized state with values of 5 and 17 cm^{-1}, respectively (Figure 6b). Accordingly, these two signals are assigned to represent C_4O and C_2O vibrations, respectively. Thereby, the carbonyl bands of the neutral radical of a flavoprotein can be specifically assigned for the first time with the help of the characteristic experimental shifts. In this case, the calculations are not sufficiently accurate for a direct support, as they show stronger shifts caused by mixed contributions from both CO vibrations to the respective signals (Figure 6c). It should be noted that the neutral radical can exchange at N_3 and additionally at N_5. The best agreement with the experiment was obtained with a single exchange at N_3. All other experimental bands can thereby be directly assigned to the flavin neutral radical (Table S1 of the Supporting Information). Contributions other than those from

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Table 1. Assignment of FTIR Difference Bands of aCRY in the Fully Reduced State[a]

aCRY in H_2O	aCRY in D_2O	*X. laevis* (6-4) photolyase[b] in H_2O	free FAD[c] in H_2O	glucose oxidase[c] in H_2O	assignment (aCRY)
1685			(1692)		Asn,[d] counterpart at 1694
1677	1672				turn elements, amide I
1622/1617	1614/1602	1625	1634/1600	1624/1598	flavin CO
1559	1572/1463	sim.[e]	1568	1566	flavin and amide II
1514	1513	1515	1520	1516	flavin
1396	1376	1397	1412	1410	flavin, δNH[f]
1348	(lost)	sim.[e]	sim.[e]	sim.[e]	flavin
1315	(lost)	sim.[e]		sim.[e]	

[a]Band positions are given in inverse centimeters. [b]From ref 19. [c]From ref 32. [d]From ref 16. [e]Band position similar but not given. [f]From ref 17.

flavin are not directly evident. The band at 1664 cm^{-1} shows a small shift of 5 cm^{-1}, in general agreement with contributions from the protein moiety. However, it does not exhibit any splitting or broadening, indicating contributions other than those from C_4O of flavin. The broad band at 1633 cm^{-1} can be attributed to flavin contributions, as shown by comparison to the calculations (Figure 6c). In summary, there is not any clear evidence of a response of the protein moiety to blue light.

FTIR Experiments Examining the Conversion upon Red Light Illumination. UV−vis spectra indicated that selective red light absorption by the radical state leads to formation of the fully reduced state (Figure 4),[11] which lacks characteristic spectroscopic features in this spectral range. IR spectroscopy experiments were performed to unambiguously assign the red light-induced photoproduct and to identify resulting responses of the protein moiety. To generate a sufficient amount of neutral radical, the sample was first illuminated with blue light for 8 s. A difference spectrum of neutral radical minus oxidized state was taken and compared for verification to the results of the previous set of experiments. Immediately afterward, the sample was illuminated with red light for 20 s and a difference spectrum was recorded (Figure 5a).

The negative bands are directly assigned to the neutral radical state by comparison with the corresponding positive bands in the blue light-generated spectrum (Figure 5b). The positive bands show only a very weak contribution of the oxidized state, which can be recognized at 1715 cm^{-1}. It is caused by the thermal decay of the radical state, which proceeds in the absence of reductants simultaneously to the photoreduction. The recovery of the oxidized state is therefore slower than in the UV−vis experiments (Figure 4b), which is attributed to differences in conditions such as protein concentration.

A weak negative band is found at 1694 cm^{-1}, which does not originate from the FAD neutral radical. In experiments with the DASH cryptochrome from *Arabidopsis* (*At*CRY3), a similar band was assigned to a signal of the protein moiety.[14] This assignment was refined by mutational studies on DASH cryptochrome from *Synechocystis* sp. PCC6803 (SCry-DASH), where an asparagine close to the FAD was exchanged with a cysteine.[16] The experiment led to the identification of a contribution by this residue at 1693(+)/1687(−) cm^{-1} in the difference spectrum. Accordingly, the band at 1694 cm^{-1} of aCRY and a weak counterpart at 1685 cm^{-1} are tentatively assigned to the change in hydrogen bonding of the side chain of the corresponding N395 in aCRY.

Positive bands in the red light-induced spectrum represent the anionic fully reduced state of FAD (Table 1). This state can unambiguously be assigned on the basis of the presence of marker bands at 1514 and 1396 cm^{-1}.[32] These bands have similarly been found for *At*CRY3,[14] SCry-DASH,[16] and (6-4) photolyase from *X. laevis*[19] upon photoreduction of the neutral radical state. Quantum chemical calculations are presently not available for the anionic fully reduced state of flavin in a model environment, which is caused by the complications arising from the presence of a negative charge.

In general, the difference spectrum of reduced state minus neutral radical state is identical to that of (6-4) photolyase from *X. laevis*,[19] taking into account a slightly higher spectral resolution. However, an additional positive band is found at 1677 cm^{-1} that is absent in the spectrum of (6-4) photolyase as well as in those of free FAD and glucose oxidase.[32] Therefore, it might be assigned to a contribution from the protein moiety, which needs to be further supported by experiments in D_2O.

Assignment of Red Light-Induced Difference Bands by Isotope Exchange in D_2O. The illumination of the sample in D_2O was identical to that of the experiment in H_2O with a consecutive illumination with blue and red light. The resulting spectrum shows a double pattern indicating that only a partial H−D exchange was achieved in this set of experiments (Figure 6a). The limited exchange leads to the presence of bands at the same position as in H_2O next to new bands resulting from the isotope shift (Figure 6a). This effect is evident for the carbonyl bands, where four negative bands are obtained instead of two. For the negative band at 1692 cm^{-1}, the small shift of 2 cm^{-1} and a constant intensity point to a buried residue of the protein moiety, in agreement with a tentative assignment to the N395 carbonyl vibration.

For the fully reduced state, the prominent bands at 1622/1617 cm^{-1} seem to be partially shifted to 1602 cm^{-1}. The extent of the shift is in good agreement with an assignment to contributions from carbonyl vibrations of the fully reduced flavin (Table 1). The positive band at 1677 cm^{-1} is shifted to 1672 cm^{-1}, and its intensity strongly increases, this band becoming the most prominent positive band. The small shift supports an assignment to a contribution from the protein moiety. Its frequency and intensity are characteristic of a turn structural element, which typically absorbs at 1660−1690 cm^{-1}.[13,34] The increase can be explained by a downshift of a compensating negative band in the difference spectrum. A negative counterpart of this band might be present at 1662 cm^{-1}, because the latter band keeps its intensity after H−D exchange even though there is a partial shift to 1654 cm^{-1}. Additional evidence of changes in secondary structure upon photoreduction is found in the positive band newly arising at 1463 cm^{-1}. This spectral region is typical for contributions from the protein backbone in D_2O (amide II′ band).[13] Its counterpart in H_2O, the amide II band, is found at ∼1550 cm^{-1}.[13] In aCRY, the difference spectrum in H_2O indeed shows

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a positive band at 1559 cm^{-1} (Figure 5a), which most probably contains contributions from both the protein backbone and flavin (Table 1).

DISCUSSION

Factors Influencing the Stability of the Neutral Radical *in Vitro* and *in Vivo*. The surprising finding of a red light response by a flavin-containing sensor can be explained only by the absorption of the neutral radical state, which implies that the neutral radical is the stable dark form of aCRY *in vivo*. However, the situation *in vitro* is more complex, because the results described above show that the radical state of aCRY is not stable in the dark. Its decay proceeds independently of the presence of oxygen and in a physiological pH range with a short lifetime of several minutes. Previous studies indicate cytosolic pH values in *C. reinhardtii* of 7.1 and 7.4.[35,36] To explain this discrepancy, a binding partner might change the redox potential *in vivo*. For plant CRYs, binding of ATP has been shown to strongly stabilize the radical state by modulation of the redox potential of the flavin.[27,37] The presence of ATP, however, does not change the lifetime of the aCRY radical to a larger extent (Figure S3 of the Supporting Information). An alternative explanation would be a two-photon activation with a first, blue/UVA photon required for preactivation of the sensor. This model is, however, not in agreement with the *in vivo* experiments, in which the algal cells were not preilluminated with blue light before the red light treatment.[11] Additionally, the quantum yield of the blue light reaction of the oxidized state to the radical is <7% (Figure S4 of the Supporting Information), thereby being very inefficient for such a preactivation process. Finally, a strongly reducing cellular environment might increase the stability of the radical state, a hypothesis that requires further investigation.

The radical state as the dark form of the sensor has been postulated for the insect cryptochrome dCRY as well, based on proteolysis studies.[38] In contrast to aCRY, this reaction model does not lead to a red light-sensitive receptor, because the anion radical of FAD is formed in dCRY without considerable absorption at >530 nm.[26] Moreover, the fully reduced flavin in dCRY cannot be formed by absorption of the anion radical,[39] because of the inhibited proton transfer to the flavin. This reaction model with the anion radical as the dark form of dCRY has been challenged recently,[39] which would leave aCRY currently as the only candidate for a flavoprotein with a radical as the dark form *in vivo*.

A strong effect of pH on the stability of the radical state has been found in aCRY, which might point to residues with a central role in the mechanism of the decay. The recovery might be accelerated by deprotonation of the FAD neutral radical. Interestingly, a very similar pH dependency was found for the DNA repair efficiency of the (6-4) photolyase from *X. laevis*.[40] The repair efficiency was maximal at pH 8.5 and strongly decreased at pH 6.0. Two histidines, H354 and H358, were identified as being essential for DNA repair and were therefore suggested to act as acid/base catalysts in the repair mechanism. More specifically, H358 was shown by electron nuclear double-resonance spectroscopy to become deprotonated with a change in pH from 6 to 9.5, whereas H354 was insensitive to this change.[29] Because of the high level of sequence identity of 54% of aCRY with *X. laevis* (6-4) photolyase in the PHR domain, these histidines are conserved in the sequence of aCRY as H357 and H361 (Figure 7). These residues are part of the highly conserved (6-4)/clock cluster HHLARH close to FAD

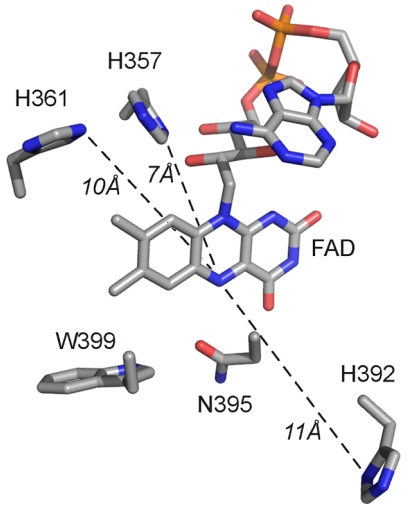

Figure 7. Position of histidine residues close to the FAD. Histidine residues within 12 Å of the N_5 position of FAD are shown in a model of aCRY derived from the crystal structure of *Arabidopsis* (6-4) photolyase.[25] These histidines are candidates for catalyzing the deprotonation and decay of the FAD neutral radical at high pH. H357 and H361 are part of the highly conserved (6-4)/clock cluster, whereas H392 of aCRY is conserved as a tryptophan in (6-4) photolyases.

and are located 7.2 and 9.8 Å, respectively, from the N_5 position of FAD in the crystal structure of *Arabidopsis* (6-4) photolyase.[25] Because of distance considerations, H392 of aCRY might alternatively be involved. It replaces a highly conserved tryptophan of (6-4) photolyases (W389 in *X. laevis* photolyase) and is found in a model of aCRY at a corresponding distance from N_5 of FAD of ~11 Å (Figure 7). It is conceivable that one of these histidines in aCRY becomes deprotonated at high pH because of its pK_a of 6.0 in solution. This histidine might abstract the N_5 proton from the FAD radical via a water and/or hydrogen bonding bridge and thereby accelerate the decay. The influence of these histidines on the decay will be studied in detail in the future, because point mutations at these residues might lead to a strong stabilization of the neutral radical as the dark form of aCRY. It should be noted that both the neutral radical and the fully reduced state were formed in the absence of any external electron donors. Intrinsic reductants seem to be present in aCRY as in (6-4) photolyases,[19] which remain to be identified.

Light-Induced Conformational Changes in aCRY. Clear-cut transitions from the oxidized to the neutral radical and further from the neutral radical to the anionic fully reduced state of aCRY have been characterized in the FTIR difference spectra by applying the appropriate illumination conditions. The reaction induced by blue light of the oxidized state did not reveal any definite contributions from the protein moiety, which is in agreement with a passive role as a preactivation process. This role is somehow comparable to that of the photoreduction of CPD photolyases to the fully reduced state, which is not strictly required for activity of the enzyme *in vivo*.[41] The response of aCRY to blue light as demonstrated *in vivo*[11] is therefore attributed to the absorption of the neutral radical in this spectral range.

Two findings provide evidence of changes in turn structure upon illumination of the neutral radical state. First, a characteristic and prominent band was detected at 1677/1672

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cm^{-1} (H_2O/D_2O), and second, a contribution from amide II′ modes was found at 1463 cm^{-1} in D_2O. The high intensity of the former band in D_2O argues against a contribution from a side chain such as the asparagine previously identified for the oxidized state of *S*Cry-DASH at 1687 cm^{-1}.[16] Most importantly, this band is missing in the homologous *X. laevis* (6-4) photolyase,[19] which constitutes the only clear difference between the red light response of the DNA repair enzyme and the sensor aCRY.

Other conformational rearrangements have been identified before for CPD and (6-4) photolyases.[18−20,42] However, these rearrangements were observed either in the context of DNA binding, repair, and dissociation or in the formation reactions of the radical states. The high degree of sequence homology between aCRY and *X. laevis* (6-4) photolyase in the PHR domain might point to a structural change in the C-terminal extension of aCRY, which is ∼70 amino acids longer than that of the photolyase. For the extension of *At*CRY1, light-induced conformational changes[43] and a transient rearrangement with a time constant of 0.4 s[44] have already been demonstrated. These changes possibly disrupt the inhibitory interaction with the PHR domain, because it has been shown that this extension alone is sufficient to mediate signal transduction *in vivo*.[45] Currently, the only crystal structures of a long cryptochrome C-terminal extension are available of dCRY from insects, where it folds back to a groove close to the flavin binding pocket.[46,47] Additional structural responses might exist in aCRY that are of a more transient nature. Time-resolved infrared spectroscopy will be applied in the future to address this question.

A similar prominent band at 1671 cm^{-1} has been identified and assigned to a change in turn structure in the plant cryptochrome CPH1-PHR (Figure 8).[14] It should be noted that

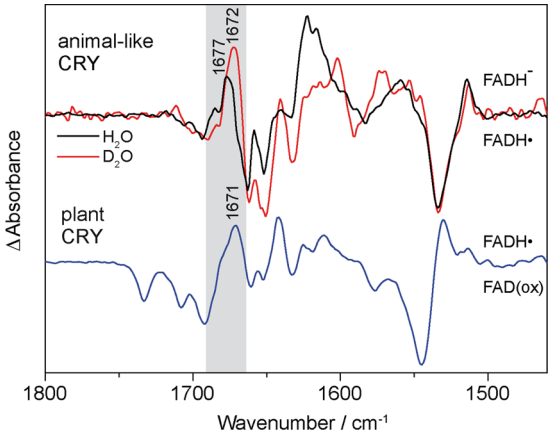

Figure 8. Comparison of responses of aCRY and plant cryptochrome. Infrared difference spectra for the transition from the dark form to the signaling state of a plant cryptochrome (CPH1-PHR, blue)[14] and aCRY (H_2O, black; D_2O, red) are shown. Both sensors exhibit a change in turn structural elements highlighted in gray. All other signals differ, because different redox states of flavin are involved, i.e., conversion of the oxidized state to the neutral radical state and conversion of the neutral radical state to the fully reduced state.

the latter response is detected in the step of the plant CRY from the oxidized state to the neutral radical state, which has been assigned to the formation of the signaling state.[48,49] In the future, it will be necessary to clarify whether common signaling pathways via turn structural changes might exist between plant cryptochromes and aCRY despite their otherwise strong differences in properties and mechanism. In this context, it is interesting to note that in spectra of DASH cryptochromes a signal in this spectral range is found as well, albeit smaller in extent than the signal of aCRY in D_2O. It has been detected in *At*CRY3 at 1681 cm^{-1} and accompanies the formation of the reduced state from the neutral radical.[14] For *S*Cry-DASH, it is unclear whether the signal at 1675 cm^{-1} is produced upon full reduction or formation of the radical from the oxidized state.[16] As the role *in vivo* of this family of proteins remains largely unclear, a similarity can be only suggested.

In summary, this study shows by a detailed characterization of the sensor aCRY that there are structural differences in the response of the sensor as compared to a (6-4) photolyase. Infrared spectroscopy is sufficiently sensitive to isolate these differences in mechanism, although both systems are highly homologous. These differences in response between the sensor and enzyme have not been anticipated. The downstream signaling of aCRY might as well have evolved without altering the response of a (6-4) photolyase to photoreduction, for example, via reduction of a substrate in the DNA-binding cavity. In contrast, the oxygen insensitivity of the aCRY neutral radical state points to further differences to (6-4) photolyases on a single-residue level, which are connected to proton and electron donors in aCRY. Further studies will be necessary to determine the specific role of these residues as well as of the C-terminus in the signaling process.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Quantum chemical calculations on oxidized lumiflavin and its neutral radical state, assignment of the FTIR difference bands in the reaction from the oxidized state to the neutral radical state of FAD, influence of ATP on the radical decay, and quantum yield of the blue light-induced photoreaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

aCRY, animal-like cryptochrome; *At*CRY1, cryptochrome 1 from *A. thaliana*; *At*CRY3, DASH cryptochrome from *A. thaliana*; CPD, cyclobutane pyrimidine dimer; CPF1, cryptochrome/photolyase family 1; CPH1, *Chlamydomonas* photolyase homologue 1; dCRY, cryptochrome from *Drosophila melanogaster*; DFT, density functional theory; DTT, dithiothreitol; FTIR, Fourier transform infrared; PHR, photolyase

homology region; *S*Cry-DASH, DASH cryptochrome from *Synechocystis* sp. PCC6803.

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