Articles

Blue-Light-Induced Changes in Arabidopsis Cryptochrome 1 Probed by FTIR Difference Spectroscopy†

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ABSTRACT: Cryptochromes are blue-light photoreceptors that regulate a variety of responses in animals and plants, including circadian entrainment in *Drosophila* and photomorphogenesis in *Arabidopsis*. They comprise a photolyase homology region (PHR) of about 500 amino acids and a C-terminal extension of varying length. In the PHR domain, flavin adenine dinucleotide (FAD) is noncovalently bound. The presence of a second chromophore, such as methenyltetrahydrofolate, in animal and plant cryptochromes is still under debate. *Arabidopsis* cryptochrome 1 (CRY1) has been intensively studied with regard to function and interaction of the protein in vivo and in vitro. However, little is known about the pathway from light absorption to signal transduction on the molecular level. We investigated the full-length CRY1 protein by Fourier transform infrared (FTIR) and UV/vis difference spectroscopy. Starting from the fully oxidized state of the chromophore FAD, a neutral flavoprotein radical is formed upon illumination in the absence of any exogenous electron donor. A preliminary assignment of the chromophore bands is presented. The FTIR difference spectrum reveals only moderate changes in secondary structure of the apoprotein in response to the photoreduction of the chromophore. Deprotonation of an aspartic or glutamic acid, probably D396, accompanies radical formation, as is deduced from the negative band at 1734 cm^{-1} in D_2O. The main positive band at 1524 cm^{-1} in the FTIR spectrum shows a strong shift to lower frequencies as compared to other flavoproteins. Together with the unusual blue-shift of the absorption in the visible range to 595 nm, this clearly distinguishes the radical form of CRY1 from those of structurally highly homologous DNA photolyases. As a consequence, the direct comparison of cryptochrome to photolyase in terms of photoreactivity and mechanism has to be made with caution.

Cryptochromes are blue-light photoreceptors found in all kingdoms of life (*1*). Plants use cryptochrome to adapt to their environment via several photomorphogenetic responses. Cryptochrome sets the daily rhythm in both insects (e.g., *Drosophila*) and plants by regulating the circadian clock. In mammals, including humans, cryptochromes constitute a light-independent integral part of the circadian clock but are possibly also involved in the circadian photoentrainment (*2*). Cryptochromes contain an N-terminal photolyase homology region (PHR)[1] of about 500 amino acids (aa). The length of the C-terminal extension varies strongly. In general, plant-type cryptochromes contain a longer, species-specific C-terminal domain (>100 aa) than animal cryptochromes. The C-terminal domain is completely missing in a third class, named cryptochrome DASH (*3*), which is found mostly in bacteria but also in *Arabidopsis* (CRY3) (*4*), *Neurospora crassa*, and aquatic vertebrates (*5*). In the PHR domain of all cryptochromes, flavin adenine dinucleotide (FAD) is noncovalently bound as a chromophore (*3*, *6*, *7*). The presence of a second chromophore in cryptochromes is still under debate (*3*, *8*). In stoichiometric amounts, 5,10-methenyltetrahydrofolate (MTHF) has been shown to associate with members of the cryptochrome DASH family (*5*, *8*, *9*), where it probably acts as a photoantenna (*10*).

Two plant-type cryptochromes are present in *Arabidopsis*, CRY1 and CRY2, that regulate light responses such as inhibition of hypocotyl elongation and flowering time,

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; FAD, flavin adenine dinucleotide; FTIR, Fourier transform infrared; LOV, light-, oxygen-, and voltage-sensitive; MTHF, 5,10-methenyltetrahydrofolate; PHR, photolyase homology region.

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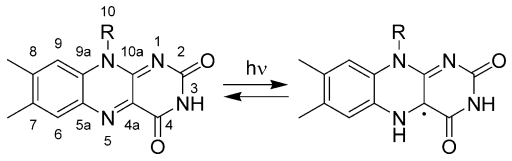

FIGURE 1: Scheme of the conversion from an oxidized flavin to a neutral flavin radical in *Arabidopsis* cryptochrome 1 upon blue-light illumination.

respectively (*11*). After heterologous expression in insect cells, the chromophore FAD in CRY1 is present in its oxidized state (*7*). Action spectra indicate that this is the native redox state of the chromophore (*12*). There is some evidence from expression in *Escherichia coli* that MTHF is present as a second chromophore in CRY1 (*13*). In most preparations, however, CRY1 is isolated devoid of a second chromophore. The structure of the PHR domain of CRY1 devoid of MTHF has been solved by X-ray crystallography to a resolution of 2.6 Å, revealing high structural similarity to photolyase (*14*).

Light induces rapid phosphorylation of CRY1 (*15*, *16*) and probably changes its interaction with the E3 ubiquitin ligase COP1 (*17*, *18*). Replacement of the N-terminal PHR domain by β-glucuronidase in transgenic plants leads to a constitutively active phenotype, indicating that the signal is mediated through the C-terminal domain (*19*). From the influence of light on the rate of proteolytic digestion, it was concluded that a small region of the CRY1 C-terminal domain undergoes conformational changes upon illumination (*20*). Little is known about the signaling pathway from light absorption to phosphorylation and/or secondary structure changes in CRY1. It has been demonstrated by transient absorption spectroscopy that light induces formation of a neutral flavin radical, which decays on a millisecond time scale (*21*) (Figure 1). The detection of tryptophan and tyrosine radicals during this process led to a proposal for a photocycle in analogy to photoactivation in photolyases, with the difference that photolyase activation starts from the radical state of the chromophore and results in the formation of reduced FAD (*22*). In support of this proposal, substitution of two tryptophan residues in CRY1, W324 and W400, resulted in a reduction of light-induced autophosphorylation in vitro and of photoreceptor function in vivo (*23*).

Vibrational spectroscopy allows one to identify processes taking place at the chromophore, alterations of single amino acid side chains, and conformational changes of the apoprotein with molecular sensitivity. It has been already successfully applied to unravel photoinduced mechanisms in other flavin-containing blue-light receptors, such as phototropin (*24*, *25*) and AppA (*26*,*27*). In the present study, we investigate for the first time the putative signaling state of the full-length cryptochrome 1 from *Arabidopsis* by FTIR and UV/vis difference spectroscopy. Spectral changes upon blue-light illumination are unambiguously assigned to the formation of a neutral flavin radical. It is shown that, despite the high structural homology between *E. coli* DNA photolyase and *Arabidopsis* cryptochrome 1, both the vibrational signature and the visible absorption of the flavin radical differ significantly. The implications of this finding on the mechanism of CRY1 action are discussed.

MATERIALS AND METHODS

Sample Preparation. Arabidopsis thaliana cryptochrome 1 was isolated from a recombinant baculovirus expression system as reported previously (*7*). The protein was transferred into 50 mM Tris buffer, pH 8, containing 500 mM NaCl by repeated ultrafiltration using an Amicon Ultra 4 filter device (Millipore) with 10 kDa cutoff at 4 °C. The final protein concentration was ca. 200 μM.

A concentrated solution of protein for spectroscopy was obtained by applying a droplet of 5 μL to a CaF_2 cuvette and reducing the water content under a stream of nitrogen gas. For a hydrated film, the cuvette was subsequently transferred to a homemade anaerobic cell, and the atmosphere was replaced several times by evacuation. The exchange to D_2O was performed by gentle drying of the protein solution under a stream of nitrogen gas and addition of 5 μL of D_2O. This process was repeated four times. Subsequently, the atmosphere was exchanged several times to argon in the anaerobic cell. Cuvettes were sealed by a second CaF_2 window without a spacer. The optical path length of the sandwich cuvette was 5−15 μm.

FTIR and UV/Vis Spectroscopy. FTIR spectroscopy was performed on a Bruker IFS 66v spectrometer with a spectral resolution of 2 cm^{-1}. The temperature of the sample was adjusted to 4 °C by a circulating water bath. Absorbance spectra were recorded across the whole mid-infrared range. For difference spectroscopy, a broadband interference filter (OCLI) in front of the MCT detector improved the signal-to-noise ratio by restricting the recording range to 2000−900 cm^{-1}. Moreover, the filter efficiently blocked stray light. An LED (Luxeon Star, Lumileds) with an emission maximum at 445 nm (14 nm full width at half-maximum) provided 40 mW/cm^2 of blue light at the sample. For the concentrated solution and the deuterated film, difference spectra of 1024 scans were recorded under and after illumination with respect to the dark state. For the hydrated film, eight difference spectra of 1024 scans each were averaged after dark periods of 45 min.

UV/vis absorption spectra of the protein films were recorded using a Shimadzu UV 2401 spectrometer with a spectral bandwidth of 2 nm. Temperature and illumination conditions were identical to those in the FTIR experiments. Photoreduction of CRY1 in solution was performed in an anaerobic cuvette in the presence of 10 mM 2-mercaptoethanol and 10% glycerol after directing a stream of argon onto the solution for 30 min.

RESULTS

UV/Vis Spectroscopy. The absorption spectrum of cryptochrome 1 from *A. thaliana* was recorded on a hydrated film. It shows two absorption bands centered at 444 and 370 nm, which both exhibit vibrational fine structure (Figure 2A). These are typical features of a flavoprotein housing an oxidized flavin as chromophore. There is no indication for the presence of a second chromophore, such as MTHF, which would strongly absorb with a maximum in the range of 380−410 nm and an extinction coefficient of 20−25 mM^{-1} cm^{-1} (*28*). For comparison, FAD in water has an extinction coefficient of 11.3 mM^{-1} cm^{-1} at 450 nm (*29*). Illumination by blue light induced significant changes in the spectrum of CRY1 (Figure 2B). The difference spectra after 120 and 240

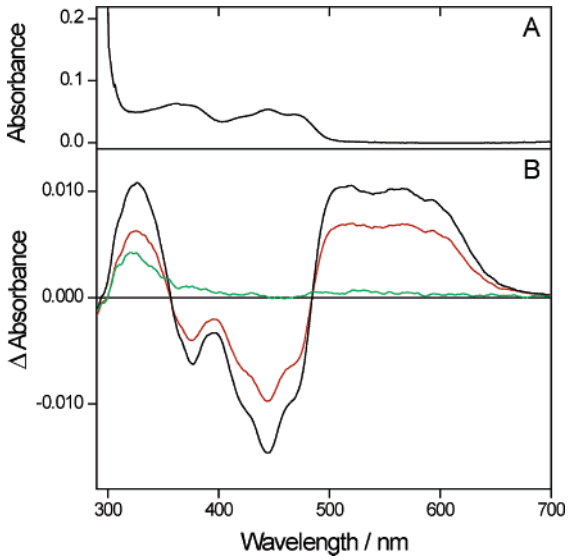

FIGURE 2: (A) UV/vis absorption spectrum of a hydrated film of CRY1 exhibiting the typical features of an oxidized flavoprotein. (B) Sequence of absorption difference spectra recorded after 120 (red) and 240 s (black) of blue-light illumination, showing the formation of a neutral flavoprotein radical. After 75 min in darkness (green), difference bands of the flavin completely decayed, leaving a residual absorption at 323 nm and between 490 and 580 nm.

s of illumination exhibit positive bands at 595, 570, 515, and 325 nm. The band positions are in agreement with previous spectra of the neutral flavoprotein radical of CRY1 (*7*, *21*). Negative bands at 444 and 375 nm are caused by the light-induced depletion of the oxidized flavin. Two isosbestic points at 485 and 355 nm accompany the process, indicating that reduced flavin or side products are not formed in the prolonged photoreaction to the radical.

After 75 min in the darkness, most difference bands had completely decayed, including all negative bands. The process is therefore fully reversible with respect to the flavin chromophore. In particular, a photoinduced degradation of the FAD was not observed, which would have resulted in a residual negative absorbance at 450 nm (*30*). Instead, a positive band at 323 nm and a weak, broad absorption between 490 and 580 nm were still present after the decay. The only aromatic compounds in the film besides FAD which might give rise to this absorption are tryptophan, tyrosine, and phenylalanine in their radical state. However, tyrosine radicals show an absorption at 405 nm (*31*), and phenylalanine radicals do not absorb at >450 nm (*32*). The spectral features point to the presence of a neutral tryptophan radical, which exhibits two bands at 325 and 510 nm in aqueous solution (*33*). It is noted that the ratio of the absorbance at 323 nm to that at 510 nm is higher than in the model study in water. It has been proposed for CRY1 that a tryptophan radical is formed by electron transfer to the excited FAD (*21*). However, this radical was detected as a transient species in a photocycle, decaying within milliseconds into a tyrosine radical. Stable neutral tryptophan radicals are not common in enzymes but have been observed in mutants of *Pseudomonas aeruginosa* azurin (*34*) and mouse ribonucleotide reductase (*35*). To clarify if the species causing the residual absorption is present simultaneously with the flavin radical or if it is formed during the decay of the latter, CRY1 was photoreduced in anaerobic solution in the presence of the exogenous electron donor 2-mercaptoethanol (Figure 3). The

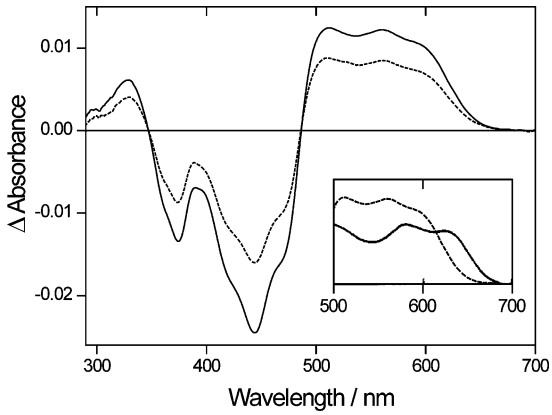

FIGURE 3: Illumination of CRY1 with blue light in anaerobic solution in the presence of the exogenous electron donor 2-mercaptoethanol. Difference spectra were recorded after 20 (dotted line) and 50 s (solid line) of exposure to light. The inset illustrates the strong blue-shift of the absorption maxima of the CRY1 radical (dashed line) as compared to *E. coli* DNA photolyase (solid line) (taken from ref *60*).

difference spectra differ from those obtained for the film by a significantly reduced absorbance at 325 nm and a small shift of the isosbestic point from 355 to 347 nm. This result points to a concomitant presence of flavin and tryptophan radical in the film after illumination.

FTIR Difference Spectroscopy. To follow the light-induced processes in CRY1, a concentrated solution was illuminated by blue light for 90 s. Difference spectra with respect to the dark state were recorded under continuous illumination and after illumination but did not differ significantly. In Figure 4A, the difference spectrum after illumination is presented. The significance of the observed changes is demonstrated by comparison with a difference spectrum without illumination (dotted line). Due to the high water content of the sample, the absorbance in the region >1550 cm^{-1} was strongly fluctuating, which prevents any interpretation of vibrational changes in this region. Therefore, a hydrated film of CRY1 was investigated following the same procedure (Figure 4B). As can be seen, the positions of the difference bands at different hydration levels are identical with minor changes in relative intensities. Further drying of the film was not carried out because the protein is only stable in the presence of 500 mM salt and at a high hydration level.

Blue light induced a depletion of the dark state, represented by negative bands, and caused the formation of a new species, characterized by the positive features (Figure 4B). The difference signals did not decay on the time scale of minutes. Most of the negative bands form a pattern that is straightforwardly assigned to vibrational modes of the depleted oxidized flavin chromophore. As a reference, the LOV1 domain of the blue-light receptor Phot from *Chlamydomonas reinhardtii* was chosen (*24*). Characteristic bands of flavin at 1578, 1509, 1405, 1349, 1253, 1227, and 1179 cm^{-1} show shifts of <5 cm^{-1} in the direct comparison (Table 1). Only the band at 1543 cm^{-1} is shifted by 9 cm^{-1}, which will be analyzed in detail in the Discussion section. For FAD in solution, characteristic bands at 1580 and 1548 cm^{-1} have been reported (*36*), which is distinctively higher in frequency than in CRY1 (1578 and 1543 cm^{-1}). Therefore, significant contributions from free FAD to the difference spectrum of CRY1 can be ruled out.

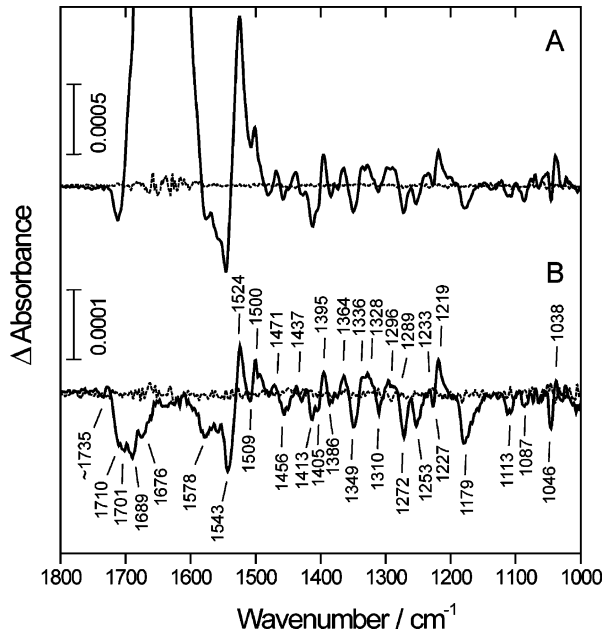

FIGURE 4: FTIR difference spectra of CRY1 with respect to the dark state recorded in the dark (dotted line) and after illumination with blue light (solid line). For comparison, spectra of a concentrated solution (A) and of a hydrated film (B) are shown. Negative bands correspond to the dark state of CRY1, incorporating an oxidized FAD. Positive bands show the light-induced state of CRY1, comprising the vibrational signature of a neutral flavin radical. Bands are obscured by the strong water absorption in the regions >1550 (A) and 1660−1590 cm^{-1} (B), respectively.

Table 1: Negative FTIR Difference Bands for CRY1 after Blue-Light Illumination in Comparison to Those of Flavin in *C. reinhardtii* Phot-LOV1 (*24*)

A.t. CRY1 in H_2O	*A.t.* CRY1 in D_2O	*C.r.* Phot-LOV1	assignment[a,b]
1710	1705	1712	C_4=O
	1638	1637	band I
1578	1577	1583	band II
1543	1544	1552	band III
1509	1512	1506	band IV
1405	1406	1404	band VI
1349	1348	1352	band VII
1253		1248	band X
1227	1229	1223	band XI
1179	1173	1178	band XII

[a] The assignment is based on published data (Ataka et al. (*24*) and references therein). [b] The band notation was introduced by Bowman and Spiro (*47*).

Several positive bands appeared upon illumination, with the most prominent band being located at 1524 cm^{-1}. Together with the bands at 1395, 1328, 1296, and 1219 cm^{-1}, this band pattern resembles the resonance Raman spectrum of *E. coli* DNA photolyase (*37*), where the flavin chromophore is in the neutral radical state. The shifts between the band positions of the two different proteins are <5 cm^{-1}. Therefore, the photoproduct of CRY1 can be identified as a neutral flavoprotein radical, in agreement with the conclusion drawn from UV/vis spectroscopy on the same protein film (Figure 2B). The similarity of the band frequencies reflects the fact that the crystal structures of the CRY1 PHR domain from *A. thaliana* and DNA photolyase from *E. coli* (*38*) are very similar, with a root-mean-square deviation of the C_α atoms of only 1.3 Å (*14*). From the spectral comparison, a preliminary assignment of the chromophore bands was achieved (Table 2). Many other positive difference bands in the CRY1 spectrum do not have a counterpart in the resonance Raman spectrum of DNA photolyase. They may represent vibrations from the apoprotein that are not enhanced in the resonance Raman approach, which is selective only for the chromophore vibrations.

Table 2: Marker Bands of the Neutral Flavin Radical: Comparison of Positive FTIR Difference Bands in CRY1 after Blue-Light Illumination to Resonance Raman Bands of *E. coli* DNA Photolyase (*37*)

A.t. CRY1 in H_2O	*A.t.* CRY1 in D_2O	*E. coli* DNA photolyase	assignment to contributions from[a]
	1609	1606	ν(C(4a)−C(10a))[c] + ν(C(5a)−C(9a))
	1596	1594[b]	ν(C−N(5)) + δ(N(5)−H) + ν(C−C)
1524	1525	1528	ν(C−N(5)) + ν(C−C)
1395	1393	1391	ν(C−N(10))
1328		1332	
1296		1301	ν(C−N(5))
1219		1220	ν(C−N(1)) + ν(C−N(3))

[a] According to Murgida et al. (*37*) and references therein. [b] Only visible in D_2O. [c] A different assignment to a C=C stretching mode is discussed by Schelvis et al. (*50*).

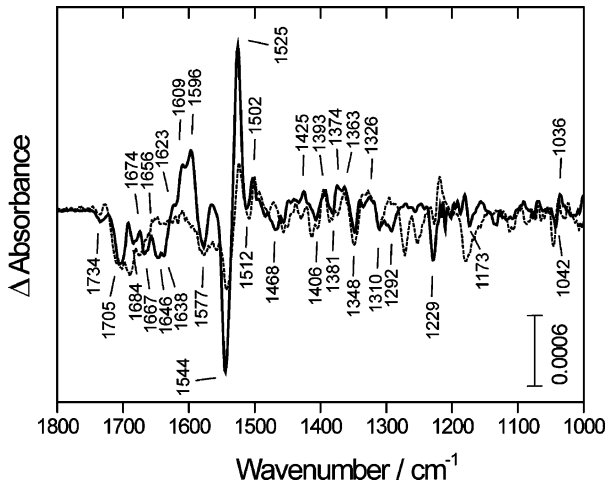

FIGURE 5: FTIR difference spectrum of CRY1 in D_2O after illumination with blue light. Band frequencies are indicated. The negative band at 1734 cm^{-1} points to the deprotonation of an aspartic or glutamic acid upon illumination. The difference spectrum of CRY1 in H_2O (dotted line) is replotted from Figure 4B for comparison.

The light-induced difference spectrum of CRY1 in D_2O is depicted in Figure 5. The direct comparison with the spectrum in H_2O shows that the overall shape of the band pattern in the region of 1600−1300 cm^{-1} did not change much upon deuteration. This agrees with Raman studies on other flavoproteins, which showed that most of the chromophore bands are insensitive to deuteration. This applies to the negative bands of the oxidized flavin (*39*) as well as to the positive bands of the neutral flavin radical (*37*) and reflects the fact that the hydrogen at the N_3 position is the only exchangeable proton in the isoalloxazine ring. Difference bands are visible in the region of 1660−1590 cm^{-1} that were obscured in the experiments in H_2O by the strong water absorption. Some contributions to this region are due to chromophore vibrations: The positive bands at 1596 and 1609 cm^{-1} can be assigned to flavin modes of the radical

state by comparison to the Raman spectrum of DNA photolyase in D_2O (*37*) (Table 2). The negative band at 1638 cm^{-1} is assigned to the C═C stretching vibration of the benzene ring of flavin, commonly denoted as band I (Table 1). The C_2═O stretching vibration of oxidized FAD in D_2O is found at 1656 cm^{-1} (*36*) but cannot yet be assigned for CRY1 on the basis of our data. A strong and dominating positive band of a carbonyl vibration of the flavin radical was observed around 1652 cm^{-1} in studies on riboflavin tetraacetate (*40*). This band might be overcompensated in CRY1 by a negative amide I band of the protein backbone.

Difference bands in the amide I range of 1695−1615 cm^{-1} indicate conformational changes of the apoprotein. In CRY1, this spectral region contains several pronounced bands at 1684(−), 1674(+), 1667(−), 1656(+), 1646(−), and 1623(+) cm^{-1} (Figure 5). The multitude and position of the bands are a strong indication for the occurrence of moderate conformational changes in CRY1 upon illumination. It is noted that some contributions from amino acid side-chain vibrations, such as glutamine or asparagine, cannot be ruled out. Further studies are currently in progress to specifically assign the bands.

A characteristic vibrational band of flavoproteins in the oxidized state is the C_4═O stretching vibration of the chromophore, which is usually observed at 1723−1687 cm^{-1} (*41*). A low frequency of the band implies a strong hydrogen bonding. The crystal structure of the CRY1 PHR domain reveals a single, weak hydrogen bond of C_4═O to the peptide nitrogen of D392, with a distance of 3.2 Å (*14*). Therefore, we assign the band at ∼1710 cm^{-1} to the flavin C_4═O vibration of the CRY1 dark state. The observed weak isotope shift in D_2O to 1705 cm^{-1} is in agreement with this assignment, as in other flavoproteins, and for free FAD shifts of 4−13 cm^{-1} have been determined (*26*, *36*, *42*).

In the hydrated and the deuterated films of CRY1, a negative band is present at the high frequency of ∼1735 and 1734 cm^{-1}, respectively. In this spectral region, only C═O stretching modes from carboxylic acids contribute, which implies a deprotonation of an aspartic or glutamic acid upon illumination of cryptochrome 1. The isotope effect is difficult to quantify, because the position of the band in the hydrated film cannot be precisely determined. The intensity of this signal before averaging over eight measurements is close to the noise level. Shifts of 6−7 cm^{-1} have been reported in phytochrome and rhodopsin for a similar frequency of the carbonyl mode (*43*, *44*). An unusually small shift in CRY1 might be due to a solvent-inaccessible position of the residue.

DISCUSSION

We have demonstrated that illumination with blue light leads to the formation of a neutral flavoprotein radical in full-length cryptochrome 1 from *Arabidopsis*. The light-induced state was characterized using UV/vis and FTIR difference spectroscopy on a well-hydrated film of CRY1. In a previous study using laser flash photolysis, it was concluded that, upon reduction of the flavin by light, a tryptophan radical is formed which decays into a tyrosine radical (*21*). The recovery of the dark state proceeded with half-lives of 1, 5, and >100 ms. In the protein film, an accumulation of the flavin radical state with the unresolved half-life of >100 ms might have occurred under continuous illumination, as it cannot be deduced from the time-resolved data that the process is completely reversible. During the accumulation and after the complete decay of the flavin radical in the film, an additional species is detected, the spectral properties of which resemble those of a tryptophan radical (Figures 2B and 3). This might be interpreted as the stabilization of an intermediate state in the proposed CRY1 photocycle under our experimental conditions. The discrepancy in the radical stabilities might be caused by exposing the concentrated protein in a closed compartment to high light intensity. The long illumination times were necessary for the concomitant FTIR measurements to drive sufficient molecules into the light-induced state. This is mandatory to obtain a reasonable signal-to-noise ratio, taking into account the large size of the protein of 75 kDa and the low quantum yield of ∼2% of the photoreduction to the radical in CRY1 (*21*). In contrast to previous studies (*7*, *21*), no exogenous electron donor such as ethylenediaminetetraacetic acid (EDTA) or 2-mercaptoethanol was added to stabilize the radical. Furthermore, the presence of oxygen was not thoroughly excluded during preparation. This clearly distinguishes our procedure from the common anaerobic photoreduction of most flavoproteins to the anionic or neutral radical. Only in the LOV2-C450A mutant of the blue-light receptor phototropin, addition of an exogenous donor was not necessary for formation of the radical (*45*). The authors used this finding to propose a radical mechanism for LOV domain photoreactions in general. We analogously conclude from our observations that the demonstrated photoreaction of CRY1 is an intrinsic property of the protein.

Formation of the neutral flavin radical in CRY1 requires protonation of the flavin at N_5. The negative band at 1734 cm^{-1} in the CRY1 difference spectrum in D_2O points to the deprotonation of a glutamic or aspartic acid upon radical formation. Accordingly, four candidates for the role of a proton donor are located in the vicinity of the flavin: D392, D359, D390, and D396. The former two are not sufficiently close to the flavin N_5, with a distance to the side-chain oxygen of 7.2 and 7.1 Å, respectively (Figure 6). D390 forms a conserved salt bridge with R362, parallel to the isoalloxazine ring (*38*). A proton release from this site cannot cause the observed signal, as D390 is present in its deprotonated form. Therefore, we consider D396 to be the most likely candidate, with a distance of 3.8 Å to flavin N_5 (Figure 6). This interpretation is supported by the fact that the corresponding amino acid in *E. coli* DNA photolyase is an asparagine (N378), which stabilizes the protonated radical by a hydrogen bond to N_5 (*38*). Moreover, D396 is located between FAD and W400 (Figure 6), which has been shown to be an essential residue for radical formation in CRY1 (*23*). W400 might act as the primary electron donor in a sequence of three tryptophans (W400, W377, and W324), in analogy with the electron-transfer pathway for photoreduction in *E. coli* photolyase (*22*). Accordingly, the negative band at 1734 cm^{-1} in the CRY1 difference spectrum in D_2O might be assigned to the C═O stretching vibration of protonated D396 in the initial state (Figure 5). The presence of a protonated D396 in the dark requires an apolar environment, which is provided by M381, F384, W385, D392, S395, A397, and W400 in a 5 Å shell around D396. We propose that proton transfer from D396 to the flavin N_5 represents a crucial step in the photoactivation mechanism of CRY1.

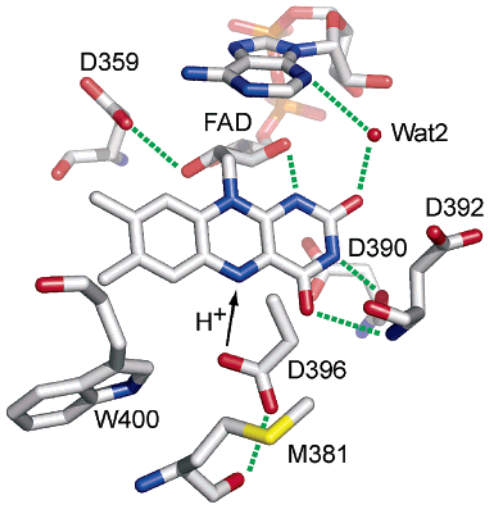

FIGURE 6: Chromophore pocket of *Arabidopsis* CRY1 obtained from the crystal structure of the PHR domain (*14*). Possible hydrogen bonds are depicted as green dotted lines. Upon illumination, D396 is proposed to donate the proton to the N_5-position of the oxidized flavin for formation of the neutral flavin radical, whereas W400 might act as the electron donor. Two other putative proton donors, D359 and D392, can be excluded due to their distance from flavin N_5. D390 is involved in a salt bridge in its deprotonated form.

The position of the major negative band at 1543 cm^{-1} is shifted to lower frequencies as compared to other flavoproteins and flavoprotein radicals (Figure 4B). Oxidized flavoproteins with a planar isoalloxazine ring, such as CRY1, generally show bands in the range of 1553−1544 cm^{-1} (*46*). This band has been assigned to the diazabuta-1,3-diene moiety of flavin (N_5═C_{4a}—C_{10a}═N_1), with a strong contribution from the C_{4a}—C_{10a} mode (*46*, *47*). The low frequency of 1543(−) cm^{-1} can be attributed to the lack of hydrogen bonding to the N_1 and N_5 positions of the oxidized flavin (*46*), which leads to a weakening of the C_{4a}—C_{10a} bond. This is in line with the crystal structure data of the PHR domain of CRY1 (*14*), where the only donor within H-bonding distance to one of these nitrogen atoms is the 3′ hydroxyl group of the riboflavin moiety.

The most prominent positive band in the difference spectrum is located at 1524 cm^{-1}, which is to our knowledge the lowest frequency of this radical mode determined in any flavoprotein so far. For flavoprotein radicals, frequencies of 1539−1532 cm^{-1} have been determined, with photolyase showing an exceptionally low frequency of 1528 cm^{-1} (*37*). Recent FTIR experiments on DNA photolyase show a higher position of the band of 1532 cm^{-1} (*48*). Therefore, the band at 1524(+) cm^{-1} of the CRY1 radical is downshifted by 4−8 cm^{-1} in comparison to the spectrum of DNA photolyase, despite the highly similar structure. This mode was analyzed to include distinct contributions from N_5—C stretching vibrations, which was concluded from a strong downshift to 1516 cm^{-1} for the N_5-methylated radical in water (*37*, *49*). The weakening of these N_5—C bonds may originate from the highly localized spin density on these atoms, as has been proposed for DNA photolyase (*50*) from electron paramagnetic resonance (EPR) experiments (*51*). The particularity of the electron density in the FAD radical in CRY1 is also reflected in the UV/vis difference spectra (Figures 2 and 3). The shoulder at 595 nm of the lowest energy transition is blue-shifted by 30 nm as compared to DNA photolyase (*52*) (Figure 3 inset) and is not typical for flavoprotein radicals, which absorb at $\lambda > 600$ nm (*53*). Studies on a 5-ethylated flavin radical revealed a blue-shift of the absorption upon changing the solvent from benzene over ethanol to water (*53*). Additionally, the shorter wavelength maximum of the doublet around 600 nm was getting more pronounced, which is also observed in the comparison of CRY1 to photolyase (Figure 3 inset). Therefore, the differences might be partly explained by a higher polarity of the binding pocket in CRY1 than in photolyase. Two asparagines close to the chromophore in *E. coli* DNA photolyase (D341 and D378) are replaced by aspartates or aspartic acids in CRY1 (D359 and D396). However, stronger molecular contributions to the spectral shift have yet to be revealed. Taken together, these results demonstrate the uniqueness of the spectroscopic properties of the CRY1 radical.

It has been claimed that the reduced state of flavin is the native state of cryptochromes in vivo (*10*). Moreover, oxidized and radical states were proposed to be artifacts from the preparation procedure (*10*), as has been shown for photolyase. This might be true for members of the cryptochrome DASH family, such as cryptochrome 1 from *Vibrio cholerae*. However, the action spectrum of hypocotyl growth inhibition mediated by *Arabidopsis* CRY1 clearly deviates from that of photolyase-mediated DNA repair. In the former case, the maximum activity was found at 390−480 nm (*12*), as opposed to 320−440 nm for *E. coli* DNA photolyase (*54*). For photolyase, the action spectrum matches the absorption spectrum of the enzyme, which is dominated by the contribution of the second chromophore MTHF. The reduced flavin present in photolyase does not absorb significantly in this spectral range. For CRY1, the action spectrum provides evidence for the presence of an oxidized flavin without strong contributions from MTHF. It resembles the absorption spectrum of CRY1 obtained in vitro after purification from insect cells (Figure 2A). It is noted that the absorption spectrum taken directly after cell lyses shows a single maximum at 420 nm (*12*), probably due to the presence of MTHF (*13*), and therefore deviates from the action spectrum. This discrepancy indicates that, in contrast to photolyases, the antenna pigment does not contribute significantly to the action spectrum in CRY1. This might be caused by unspecific binding and, consequently, unfavorable orientation of MTHF relative to flavin. The comparison to DNA photolyase shows that the binding pocket for MTHF is largely occupied with amino acid side chains in CRY1 (*14*) and that five out of six residues involved in binding of MTHF are not conserved in CRY1 (*38*). Thus, it is unlikely that the absence of MTHF in our preparation as well as in the previous work (*21*) fundamentally changed the light-dependent behavior of CRY1. From the present study, we conclude that photolyase and cryptochrome radical differ significantly in the electron distribution in the isoalloxazine ring. This complicates a direct comparison of cryptochrome with the photolyase system in terms of photoreactivity and mechanism. It is therefore not unlikely that the functional mechanism of CRY1 indeed starts from the oxidized state of FAD.

In a current model for CRY1 signal transduction, it is proposed that a light-induced conformational change of the protein effects the interaction of CRY1 with the binding partners COP1 and HY5, resulting in a degradation of the transcriptional regulator HY5 (*55*). From our FTIR data, only

moderate conformational changes accompany the formation of the radical (Figure 5). This is in line with a proteolytic digestion assay on CRY1, where it was concluded that only a relatively small region of the C-terminal extension rearranges in response to light (*20*). The extent of conformational change does not necessarily need to be dramatic, since the interaction of CRY1 with COP1 is only altered, not lost. Moreover, the biggest part of the C-terminal extension was predicted to be unstructured (*20*), in which case changes in conformation are difficult to detect by FTIR spectroscopy. Inside the PHR domain, the signal might be transferred from the chromophore to the C-terminal extension without strong structural disturbances. In the case of phototropin, it has been shown by X-ray crystallography that light-induced changes in conformation in the LOV domains themselves are small (*56*, *57*), whereas the activation of the kinase proceeds via unfolding of an α-helix downstream of LOV2 (*58*, *59*).

CONCLUSIONS

Arabidopsis CRY1 is currently the best model system available for studying processes of cryptochrome signal transduction. The function of the protein and its interaction with signaling partners have been intensively studied. The availability of time-resolved absorption spectra on the full-length protein as well as a crystal structure of the PHR domain facilitates interpretation of changes at the molecular level. On this basis, we were able to characterize the vibrational changes occurring in the full-length CRY1 upon illumination with blue light. The reversible formation of a neutral flavin radical by blue light is accompanied by changes in secondary structure of the apoprotein. Proton transfer from an aspartic or glutamic acid, probably D396, to the flavin is proposed to represent an important step in the photoactivation of CRY1. Major negative and positive difference bands were assigned by comparison to those of other flavoproteins. This analysis is novel for FTIR studies on flavoprotein radicals in general and will facilitate future work on the cryptochrome signaling process.

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SUPPORTING INFORMATION AVAILABLE

Infrared absorbance spectra of CRY1 showing the hydration level of the protein films and the exchange ratio to D_2O. This material is available free of charge via the Internet at http://pubs.acs.org.

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