Light-activated Cryptochrome Reacts with Molecular Oxygen to Form a Flavin–Superoxide Radical Pair Consistent with Magnetoreception

Received for publication, February 8, 2011, and in revised form, March 30, 2011 Published, JBC Papers in Press, April 5, 2011, DOI 10.1074/jbc.M111.228940

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Cryptochromes are flavin-based photoreceptors occurring throughout the biological kingdom, which regulate growth and development in plants and are involved in the entrainment of circadian rhythms of both plants and animals. A number of recent theoretical works suggest that cryptochromes might also be the receptors responsible for the sensing of the magnetic field of the earth (e.g. in insects, migratory birds, or migratory fish). Cryptochromes undergo forward light-induced reactions involving electron transfer to excited state flavin to generate radical intermediates, which correlate with biological activity. Here, we give evidence of a mechanism for the reverse reaction, namely dark reoxidation of protein-bound flavin in Arabidopsis thaliana cryptochrome (AtCRY1) by molecular oxygen that involves formation of a spin-correlated FADH•−superoxide radical pair. Formation of analogous radical pairs in animal cryptochromes might enable them to function as magnetoreceptors.

Cryptochromes are blue light-absorbing photoreceptors found throughout the biological kingdom, ranging from microbes to plants, animals, and humans (1–3). They are evolutionarily derived from photolyases, DNA repair enzymes using violet/blue light to repair damage (cyclobutane pyrimidine dimers) caused by exposure of DNA to UV light. Cryptochromes and photolyases exhibit a high degree of structural homology and bind the same flavin adenine dinucleotide (FAD) and folate light-absorbing cofactors; however, they differ very much in their function in biological systems. Cryptochromes have lost the ability to repair DNA, but they play a key role in controlling the growth and development in plants and in the circadian clock in plants and animals (4). Furthermore, recent evidence suggests that cryptochromes are likely to function as light-dependent magnetic field sensors, used for example by insects or migratory birds and fish for directional responses (5–7).

Although photolyases and the mechanism of their action have been subject to extensive studies in the last five decades (3, 9), the photochemistry and the signaling pathways of cryptochromes are just beginning to become known. Recent attention has focused primarily on the pathway of flavin photoreduction as a possible mechanism of photoreceptor activation (10–14). In the case of both plant and insect cryptochromes, studies with isolated proteins have shown that these can be photoreduced from flavin in the oxidized state to the radical state by light in the presence of reducing agent. This is in marked contrast to photolyases, where photoreduction results in formation of the fully reduced (FADH−) form, which is active in DNA repair (11). Photoreduction of flavin correlates with biological activity, as shown by action spectra that demonstrate typical 450-nm peak and shoulders indicative of oxidized flavin with almost no activity above 500 nm (13, 15). Furthermore, both plant and animal cryptochromes show reduced biological activity upon co-illumination with green light, which selectively depletes the flavin radical pool. Studies performed on cryptochromes within living insect cells (referred to as “in vivo” studies) have confirmed the FADox to radical interconversion for both plant and animal type cryptochromes. Finally, the difference in the FADox–FADH+ redox potential in photolyases and AtCRY1 (16) supports a greater than 90% oxidized flavin state in AtCRY1 in plant cells, consistent with this mechanism and in marked contrast to photolyases, which are reduced in vivo. The deduced photocycle for cryptochromes is therefore that the oxidized flavin state accumulates in the dark and is the resting state of the molecule. Illumination leads to accumulation of primarily the radical (signaling) state with presumable conformational change allowing interaction with the signaling partner. Return to darkness leads to re-accumulation of the inactive (oxidized) resting state.

In plant cryptochromes, photoreduction to FADH•− in vitro does not occur readily (hence accumulation of radical state flavin). It is only possible in AtCRY at very high light intensity, in the presence of a strong reducing agent (e.g. dithiothreitol (DTT)) and an oxygen-free environment (to prevent reoxidation, which is a competing reaction). To date, the main focus on the photocycle of cryptochromes has been on the light-induced forward reaction of reduction of FADox to FADH+, including subsecond processes that occur immediately after the absorption of a photon by oxidized flavin (10, 17–19). By contrast,

* This work was supported by National Science Foundation Grant 0848311 (to M. A.), a grant from Human Frontiers (to M. A. and T. Ritz), and a grant from the Agence Nationale de la Recherche (to M. A., K. Brettel, and F. Rouyer). Contribution no. 4792. 1 To whom correspondence should be addressed: Université Paris VI, Casier 156, 4 Place Jussieu, 75005 Paris, France. Fax: 33-144272916; E-mail: margaret.ahmad@upmc.fr.
2 The abbreviations used are: CRY, cryptochrome; AtCRY1, Arabidopsis thaliana cryptochrome; DmCRY, Drosophila melanogaster cryptochrome.
Cryptochrome Reoxidation

little or no attention has been paid to the back reoxidation reaction from FADH\(^+\) and FADH\(^-\) to restore oxidized flavin (FAD\(_{ox}\)) and thereby reset the system during the dark interval. Nevertheless, both the amplitude of radical accumulation in response to light and its lifetime in the dark following the end of illumination are determined by the equilibrium reached by these forward (reducing) reactions with their competing back (reoxidation) reactions. Hence, cryptochrome flavin reoxidation is of critical importance for lifetime and stability of the radical and therefore for amplitude of the biological response.

Here, we report for the first time on the kinetics and possible mechanism of oxidation of FADH\(^+\) and FADH\(^-\) by molecular oxygen in cryptochrome, involving a formation of superoxide/ hydroperoxide radicals and, eventually, of hydrogen peroxide. We show that the reaction is dependent on molecular oxygen concentration, that it occurs on a relatively slow time scale (minutes), and that the reaction from FADH\(^+\) to FAD\(_{ox}\) occurs by a distinct mechanism depending on whether the FADH\(^+\) radical is formed by oxidation of FADH\(^-\) or by reduction of FAD\(_{ox}\). In particular, superoxide appears to form a radical pair with flavin (FADH\(^+\)) when starting from a fully reduced flavin, FADH\(^-\). These observations suggest a potential mechanism for magnetoreception via radical pairs formed during the reoxidation reaction of cryptochromes.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—AtCRY1 protein was prepared by a well established procedure described elsewhere (20) with the only exception that 50 mM phosphate buffer was used instead of 50 mM Tris-HCl buffer (same pH, 7.5) for the purification procedures.

Spectroscopic Studies—Spontaneous dark reoxidation of AtCRY1 (final concentration c \(\sim 10 \mu M\), 2 mM DTT) was monitored by Uvikon 930 UV-visible spectrophotometer (Kontron Instruments) with an UV/Vis 900 Lite graphical interface (DuSoTec GmbH) and equipped with a cooled cuvette holder. Fresh AtCRY1 samples (in 0.5 M NaCl, 50 mM phosphate buffer, pH 7.5) were rid of oxygen by nitrogen purging for 2 h (on ice), and the flavin chromophore was subsequently photoreduced by blue LED (30 min, on ice, 465 nm, light intensity of \(\sim 1,500 \mu\)mol m\(^{-2}\) s\(^{-1}\)). After this treatment, virtually no oxidized flavin remained, and the samples contained a mix of reduced and 10–30% semireduced flavin (complete reduction to 100% FAD\(^-\) could not be achieved even at this blue light irradiance). When their UV-visible spectra had not changed within 15 min, the samples were considered oxygen-free. Defined volumes of air-saturated buffer (0.5 M NaCl, 50 mM phosphate buffer, pH 7.5, 20 °C) were then injected into the cuvette through a septum, and the spontaneous reoxidation of FAD\(^-\) and FADH\(^+\) to FAD\(_{ox}\) was monitored (while kept at 12 °C) until the complete recovery of the oxidized flavin.

Global Analysis—The resulting spectral sets were analyzed using SPECFIT, a global analysis system with singular value decomposition and non-linear regression modeling by the Levenberg-Marquardt method (21). For this analysis, kinetic difference spectra were generated by subtracting the time-zero spectrum from all subsequent spectra, and the resulting spectra were corrected for any small absorbance drift by setting the absorbance at 660 nm (where no form of flavin absorbs) to 0.

Oxygen Electrode Experiments—Oxygen consumption and evolution measurements were carried out using the Oxygraph system from Hansatech Instruments Ltd. (a cooled black acrylic chamber with a Clark type oxygen electrode disc, integral stirrer, digital output system with on-screen display of traces). The purified AtCRY1 samples (c \(\sim 10 \mu M\), 0.5 M NaCl, 50 mM phosphate buffer, pH 7.5) were rid of imidazole and glycerol using P-30 Micro Bio-Spin® chromatography columns right before the experiment. DTT (10 mM) was added as a reducing agent, and 2 ml of the samples were pipetted into the cylindrical oxygen electrode chamber (stirred and cooled to 12 °C). The chamber was left open until the oxygen concentration equilibrated. After closing the lid (so that there was no longer an air layer above the sample), the linear consumption of oxygen by the electrode itself (which is a principle of its operation) was monitored for about 2 min. The obtained slope was subtracted from the O\(_2\) consumption trace at the end of the experiment so that the trace reflected only the consumption of oxygen by reoxidation of the previously photoreduced forms of cryptochrome.

The samples were exposed to three light/dark cycles (2 min of blue light: 465 nm, light intensity \(\sim 100 \mu\)mol m\(^{-2}\) s\(^{-1}\), 8 min of dark). The irradiation by a blue LED took place through a transparent window on the side of the chamber. After the last cycle, 10 µl of buffer (0.5 M NaCl, 50 mM phosphate buffer, pH 7.5) were added (to see the effect of the addition of a small volume of liquid possibly having a slightly different O\(_2\) concentration) followed by 10 µl of catalase (c = 1 mg/ml) in the same buffer.

RESULTS

The cryptochrome photoreduction reaction has been correlated with biological activity in numerous studies and shown to occur both in vitro and in vivo (11–13). However, the spontaneous back reaction (reconstitution of the oxidized flavin), which resets the system to the resting state in the dark, has as yet been poorly characterized. As a means to show kinetics of dark reoxidation of the FAD chromophore in AtCRY1 cryptochrome and to determine the dependence of the rate of this reaction on oxygen concentration, we first performed a series of photoreduction experiments at different oxygen concentrations. No reoxidation was observed in the absence of molecular oxygen, indicating that oxygen is necessary and sufficient for the reaction to occur.

Spectroscopic Studies of CRY Reoxidation Dependence on Oxygen Concentration—Spectroscopic studies give evidence of complex reoxidation kinetics, which involve different mechanisms depending on whether the FADH\(^+\) radical is formed by oxidation of FADH\(^-\) or by reduction of FAD\(_{ox}\). Burney et al. (20) have reported on a mono-exponential reoxidation of FAD\(_{ox}\) to FAD\(_{ox}\) in an air-saturated solution with a time constant of 2.6 min (at 12 °C, 101.3 kPa, c(O\(_2\))\(_{sat}\) ~320 µM). This is consistent with our control experiment (reoxidation of FADH\(^+\) generated by irradiation of FAD\(_{ox}\) in an air-saturated solution at low light intensities around 100 µmol m\(^{-2}\) s\(^{-1}\) and a mild reducing agent, 10 mM β-mercaptoethanol, Fig. 1), but it is in a strong contrast with the overall rate observed for the FAD\(_{ox}\)
recovery from the mixture of FADH\(^{+}\) (10–30%) and FADH\(^{-}\) (70–90%; Fig. 2), generated by 30-min blue light illumination (intensity \(1,500 \mu\text{mol m}^{-2} \text{s}^{-1}\)) under strong reducing conditions (2 mM DTT), in an anaerobic cuvette (Fig. 3). This rate was observed to be more than two times faster than the FADH\(^{+}\) to FAD\(_{\text{ox}}\) transition in Fig. 1, although the concentration of oxygen after its addition was 250 \(\mu\text{M}\) (i.e. below the oxygen concentration level for air-saturated solutions at the given temperature and pressure: 12 °C, 101.3 kPa; see Table 1 and Fig. 4).

This was indeed astonishing as one would intuitively expect a slower rate (because the radical must first be generated from FADH\(^{-}\), the prevailing form in our samples) or about the same rate (if the first step, FADH\(^{-}\) to FADH\(^{+}\), was much faster than the second reoxidation step, FADH\(^{+}\) to FAD\(_{\text{ox}}\), in which case a significant accumulation of the intermediate FADH\(^{+}\) would have to occur). Because the FADH\(^{+}\) intermediate did not accumulate, it follows that the first oxidation step FADH\(^{+}\) to FADH\(^{-}\) is much slower than the second step: FADH\(^{-}\) to FAD\(_{\text{ox}}\). At the same time, the two-step reaction FADH\(^{+}\) to FAD\(_{\text{ox}}\) was found to be faster than the single-step reaction FADH\(^{+}\) + \(\text{O}_2\) to FAD\(_{\text{ox}}\), where FADH\(^{+}\) was generated by photoreduction of FAD\(_{\text{ox}}\) implying that oxygen cannot be the actual oxidizing agent in the second step of the reaction FADH\(^{+}\) to FAD\(_{\text{ox}}\), as the rate of FAD\(_{\text{ox}}\) recovery should either remain the same as in Fig. 1 or slow down.

**Global Analysis of Cryptochrome Reoxidation**—To further resolve the question of reaction intermediates and mechanism of oxidation in the two cases (FADH\(^{+}\) to FAD\(_{\text{ox}}\) and FADH\(^{-}\) to FAD\(_{\text{ox}}\)), global analysis was performed with the spectra in Fig. 3. As expected, using the intuitive model A(FADH\(^{+}\)) \(\rightarrow\) B(FADH\(^{-}\)) \(\rightarrow\) C(FAD\(_{\text{ox}}\)) (with non-zero concentrations of A and B at time 0) did not yield a satisfactory fit (the rate constants could not be calculated) as the concentration of the intermediate component B did not rise at any point of the experiment (implying that the oxidation of FADH\(^{+}\) to FAD\(_{\text{ox}}\) proceeds at a much faster rate, at least by one order of magnitude, than the oxidation of FADH\(^{-}\) to FAD\(_{\text{ox}}\))

\[ \text{A(FADH}^{+}\text{)} \rightarrow \text{B(FADH}^{-}\text{)} \rightarrow \text{C(FAD}^{\text{ox}}\text{)} \]

For systems A \(\rightarrow\) \(k_1\) \(\rightarrow\) B \(\rightarrow\) \(k_2\) \(\rightarrow\) C, where the rate of the second reaction step \(k_2\) is much faster than the first one \(k_1\), it is very difficult and often even impossible to calculate the
redox kinetic behavior of flavin in AtCRY1 described above, we postulate the following reaction schemes.

Oxidation of the FADH\(^{+}\) radical by molecular O\(_2\):

\[
O_2 + FADH^{+} \rightarrow FAD_{\text{ox}} + O_2^{+} + H^+ 
\]

\[
H^+ + O_2^{+} \rightleftharpoons HO_2^* \quad (pK = 4.8)
\]

\[
HO_2^{*} + O_2^{+} + H^+ \rightarrow H_2O_2 + O_2
\]

(or 2 HO\(_2^{*}\) \(\rightarrow\) H\(_2\)O\(_2\) + O\(_2\))

**SCHEME 1**

Oxidation of the fully reduced flavin FADH\(^{-}\) by molecular O\(_2\):

\[
O_2 + FADH^- \rightarrow FADH^+ + O_2^{+}
\]

\[
H^+ + O_2^{+} \rightleftharpoons HO_2^*
\]

\[
FADH^+ + HO_2^* \rightarrow FAD_{\text{ox}} + H_2O_2
\]

**SCHEME 2**

The first oxidation step in Scheme 2 yields a pair of radicals: FADH\(^{+}\) and O\(_2\)\(^{+}\) anion radical. O\(_2\)\(^{+}\) is in equilibrium with its protonated form, the hydroperoxy radical HO\(_2\)\(^{+}\) (23–25), which is a strong oxidizing agent. In the last step, the hydroperoxy radical oxidizes FADH\(^{+}\) to FAD\(_{\text{ox}}\) and yields hydrogen peroxide as a byproduct. It is of note that in order for such a reaction to take place, the diffusion of the radicals has to be significantly limited (one does not observe such a reaction for a free riboflavin where the radicals get separated by diffusion and react with other partners present in the environment (22)). It indeed seems that the flavin-binding pocket in AtCRY1 can efficiently trap the superoxide/hydroperoxide radicals and thus make the reaction of HO\(_2\) with FADH\(^{+}\) possible. The fact that HO\(_2\) is trapped in the vicinity of the flavin radical (its effective concentration is thus very high) and that it is a much better oxidizing agent than molecular oxygen (26) (which, on top, is a triplet in the ground state) accounts for the observed phenomenon of accelerated FADH\(^{+}\) reoxidation.

**Oxygen Electrode Experiments**—To determine the possible intermediates of the reoxidation reaction, oxygen electrode experiments were performed with AtCRY1 samples in 50 mM phosphate buffer, 10 mM DTT, and 465-nm blue light. Given the experimental conditions (relatively low light intensity of 100 \(\mu\)mol m\(^{-2}\) s\(^{-1}\), air-saturated solution), the FAD chromophore in AtCRY1 was photoreduced only to the radical species (FAD\(^{+}\)), which was then re-oxidized back to FAD by molecular oxygen present in the solution following the classic reaction mechanism (22) involving the formation of superoxide.

\[
O_2 + FADH^+ \rightarrow FAD + O_2^{+} + H^+
\]

**REACTION 1**

The fate of superoxide in aqueous solutions is reported (23) to be given by this reaction scheme.

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**Table 1**

Calculated pseudo-first order rate constants for parallel oxidations FADH3 → FADox (k\(_{\text{obs,1}}\)) and FADH3 → FADox (k\(_{\text{obs,2}}\)) and the corresponding lifetimes \(\tau\) (time constants) of the individual reduced species at given oxygen concentrations

| c(O\(_2\)) [\(\mu\)] | \(k_{\text{obs,1}}\) [s\(^{-1}\)] | \(k_{\text{obs,2}}\) [s\(^{-1}\)] | \(\tau\) (FADH\(^{+}\)) [s] | \(\tau\) (FADH\(^{-}\)) [s] |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| 6               | (5.1 ± 1.2) \times 10\(^{-4}\) | (1.4 ± 0.6) \times 10\(^{-4}\) | 1,980           | 7,050           |
| 80              | (4.5 ± 0.5) \times 10\(^{-3}\) | (8.0 ± 0.7) \times 10\(^{-3}\) | 224             | 1,250           |
| 160             | (8.0 ± 1.0) \times 10\(^{-3}\) | (1.7 ± 0.9) \times 10\(^{-3}\) | 125             | 604             |
| 250             | (1.1 ± 0.3) \times 10\(^{-2}\) | (4.6 ± 0.8) \times 10\(^{-2}\) | 91              | 218             |
| 320*            | (6.4 ± 0.0) \times 10\(^{-3}\) | (10 ± 10) \times 10\(^{-3}\) | 156             | 156             |

* Reoxidation of the FADH\(^{+}\) radical in an air-saturated aqueous solution (Ref. 20), confirmed in an independent experiment (Fig. 1). \(k_{\text{obs,1}}\) could not be measured or calculated because relevant concentrations of FADH\(^{+}\) can only be generated in oxygen-poor/oxygen-free solutions.

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**Figure 4.** Calculated pseudo-first order rates of parallel reactions FADH3 → FADox and FADH3 → FADox (model A → C, B → C) plotted against oxygen concentration. The last point in the FADH3 set (320 \(\mu\)M O\(_2\)) is a rate constant of FADH3 reoxidation in an air-saturated AtCRY1 solution (20). B (FADH3) is only the radical formed by reducing FADox, which is present in the solution before the addition of O\(_2\). FADH3 generated by oxidation of FADH3 is neglected because its concentration at any point of the reaction was shown to be negligible (its oxidation proceeds much faster rate).

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The value of \(k_2\) from an experimental spectral data set but, on the other hand, the "steady-state approximation" d[C]/dt = \(k_1\)[A] can be used. The applied model of two independent processes A → C (rate constant \(k_{\text{obs,1}}\)) and B → C (rate constant \(k_{\text{obs,2}}\)), where A stands for FADH\(^{+}\) and B stands for FADH\(^{+}\) present in the solution at \(t = 0\) s (i.e. already before the addition of oxygen), did indeed yield time-independent species spectra, which are in a very good agreement with known spectra of oxidized and semi-reduced flavin in AtCRY1, and also resulted in a very good fit along all wavelengths. This justifies the steady-state approximation and the choice of the fitting reaction model. The results of the global analysis are summarized in Table 1 and Fig. 4.

The data analysis clearly shows that there is a substantial difference in the rate of FADH3 oxidation depending on whether it was formed by oxidation of FADH\(^{+}\) or by (photo-)reduction of FADox (other conditions being the same). This indicates that the oxidizing agent in the two cases is not the same. Based on the known reactions of free riboflavin (22), the fate of superoxide in aqueous solutions (23), and the observed
The equilibrium above implies the lifetime of superoxide increases by an order of magnitude per pH unit above pH 5. Knowing that the half-life of superoxide at pH 11 (aqueous solution) is \(15\) s (24) gives us a rough estimate of the superoxide lifetime on the order of a few milliseconds at pH 7.5 (consistent with Ref. 25).

Superoxide thus cannot be detected directly by adding superoxide dismutase to the solution after irradiation. Indeed, the addition of superoxide dismutase solution in a control experiment had virtually no effect (i.e. the same effect as an addition of pure buffer).

Nevertheless, if the reaction scheme above is correct and hydrogen peroxide is formed in the course of the reaction, the addition of catalase (enzyme converting \(\text{H}_2\text{O}_2\) to \(\text{O}_2\)) subsequent to irradiation is predicted to recover 50% of the oxygen consumed during the course of the experiment as the catalytical disproportionation of \(\text{H}_2\text{O}_2\) can described by the following simple reaction

\[
\text{H}^+ + \text{O}_2^- \rightleftharpoons \text{HO}_2^- \quad (pK = 4.8)
\]

\[
\text{HO}_2^- + \text{O}_2^- + \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

\[
\text{(or } 2 \text{HO}_2^- \rightarrow \text{H}_2\text{O}_2 + \text{O}_2)\]

SCHEME 3

The increase in oxygen concentration after the addition of catalase proves that hydrogen peroxide is formed in the course of the redox photocycle. The recovered oxygen amounts only to 50% of the consumed oxygen as hydrogen peroxide disproportionates to oxygen and water according to the following reaction scheme: \(2\text{H}_2\text{O}_2 + \text{catalase} \rightarrow 2\text{H}_2\text{O} + 1\text{O}_2\), i.e. two molecules of hydrogen peroxide give rise to one molecule of oxygen.

To test this hypothesis, catalase was added to the oxygen electrode experiments subsequent to illumination. Indeed, the experimental observations were in a perfect agreement with the predicted outcome; about a half of the previously consumed oxygen was restored after the addition of catalase (Fig. 5).

In conclusion, the illumination of cryptochrome results in a continuous consumption of oxygen, consistent with continuous cycling of cryptochrome between the radical and oxidized state during the "small" photocycle (i.e. \(\text{FAD}_{\text{ox}}\) to \(\text{FADH}^+\) and back). Scheme 1 implies that half an \(\text{O}_2\) molecule is consumed per one small photocycle: \(\text{FAD}_{\text{ox}} \rightarrow \text{FADH}^+ \rightarrow \text{FAD}_{\text{ox}}\).

The "complete" photocycle \(\text{FAD}_{\text{ox}} \rightarrow \text{FADH}^+ \rightarrow \text{FADH}^- \rightarrow \text{FADH} \rightarrow \text{FAD}_{\text{ox}}\) results in consumption of one whole \(\text{O}_2\) molecule (see Scheme 2). A formation of hydrogen peroxide byproduct is common to both photocycles (Fig. 6), but the partners for the reaction \(\text{FADH}^- \rightarrow \text{FAD}_{\text{ox}}\) in the two photocycles are not the same oxidizing agents. In the small cycle, \(\text{FADH}^+\) is oxidized by molecular oxygen with a rate of about \(20\) \(\text{m}^{-1}\) \(\text{s}^{-1}\). In the complete cycle, molecular oxygen acts as the oxidizing species only in the first step (\(\text{FADH}^- \rightarrow \text{FAD}_{\text{ox}}\), rate \(\sim 50\) \(\text{m}^{-1}\) \(\text{s}^{-1}\)), and the resulting radicals, \(\text{FADH}^+\) and the superoxide/hydroperoxide, react with each other at a much faster rate (due to high efficient concentrations of the radicals in the flavin-binding pocket), which could not be resolved because this step is preceded by a slow process and the intermediate concentration is too low to allow the calculation of the second rate constant.

**DISCUSSION**

In this work, the mechanism of cryptochrome reoxidation is investigated in isolated proteins under differing oxygen concentrations. Contrary to expectations, flavin reoxidation is a relatively slow process (rates of \(\sim 20\) \(\text{m}^{-1}\) \(\text{s}^{-1}\) for reoxidation of \(\text{FADH}^+\) and \(\sim 50\) \(\text{m}^{-1}\) \(\text{s}^{-1}\) for \(\text{FADH}^-\)) taking several minutes even in air-saturated solutions. The rate of reoxidation is therefore not controlled by the diffusion rate of oxygen in solution.

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**FIGURE 5.** The consumption and evolution of oxygen in the \(\text{O}_2\) electrode chamber containing a solution of AtCRY1 during three light-dark cycles and a final addition of catalase (enzyme converting \(\text{H}_2\text{O}_2\) to \(\text{O}_2\)). The increase in oxygen concentration after the addition of catalase proves that hydrogen peroxide is formed in the course of the redox photocycle. The recovered oxygen amounts only to 50% of the consumed oxygen as hydrogen peroxide disproportionates to oxygen and water according to the following reaction scheme: \(2\text{H}_2\text{O}_2 + \text{catalase} \rightarrow 2\text{H}_2\text{O} + 1\text{O}_2\), i.e. two molecules of hydrogen peroxide give rise to one molecule of oxygen.
because mixing is complete within less than 2 s after adding oxygen, and indeed reoxidation occurs much more rapidly to flavins in solution (~250 m^{-1} s^{-1}) (27). It is therefore evident that the protein environment around the flavin-binding pocket is of critical importance for stabilizing the reduced forms of flavin and thereby prolonging the lifetime of the biologically active (signaling) state. Not surprisingly, the reaction rate is proportional to oxygen concentration, and under completely anaerobic conditions, flavin reoxidation does not occur at all. The observed slowdown in the reoxidation rate is possibly caused by residues in the flavin pocket sterically hindering direct access of oxygen to flavin and thereby an efficient electron transfer between the two partners. In sum, molecular oxygen is necessary and sufficient for the dark resetting reaction of cryptochrome, and the reaction rate is oxygen concentration-dependent.

Another unexpected finding emerging from this study is the observation that the rate of reoxidation from the fully reduced (FADH^-) flavin state of cryptochrome is significantly more rapid than that from the radical (FADH^-) state. It is concluded that this is due to production of superoxide in the course of the former reaction, which in turn oxidizes FADH^+ at an accelerated rate as compared with interaction of FADH^+ with molecular oxygen directly. It is further interesting that hydrogen peroxide is accumulated in the course of cryptochrome reoxidation reactions, whether originating from FADH^- or FADH^+ flavin states. Thus, under conditions of continuous blue illumination and especially at high light intensity, hydrogen peroxide can accumulate in concentrations high enough to cause significant protein degradation and/or partial unfolding. In this regard, it is interesting that in numerous instances, e.g. in Drosophila cryptochrome (DmCRY) and Arabidopsis AtCRY2, continuous illumination leads to protein degradation and target to the proteasome (13), consistent with peroxide accumulation. Given certain recent confusion in the literature regarding the redox state of cryptochromes in vivo due to conflicting reports on midpoint potential for different cell types and redox couples (16, 28), it should be emphasized that both plant and animal (Drosophila type I) cryptochromes occur with flavin in the oxidized state in darkness. There is no doubt that activation in plants involves excitation of the oxidized flavin due to the complete absence of biological activity upon illumination at wavelengths above 550 nm, which could be absorbed by the neutral radical but not the oxidized flavin. Similarly for Drosophila cryptochrome, the resting state of cryptochrome has been shown to be the oxidized form; this photoreaction occurs in living insect cells and has been documented by independent techniques (13). A suggestion that illumination of radical in DmCRY may lead to activation by an unknown mechanism (29, 30) is not in contradiction to these results as formation of the radical would still require photoreduction of oxidized flavin and be opposed by flavin re-oxidation. Therefore, an important effect of oxygen is implied in the photoreactions of both animal and plant cryptochromes, and conclusions from this study can be generalized to all cryptochromes that undergo a reoxidation of flavin to FAD^+ in darkness subsequent to illumination.

Implications for Biological Activity—Illumination of cryptochrome leads to significant radical formation, which has been correlated in numerous studies with biological activity. In the case of insect (Drosophila) cryptochromes, photoreduction to the fully reduced state has not been reported to occur in vitro (14) and occurs only at very high illumination under anaerobic conditions for AtCRY1 (11). However, there is apparently a more favorable redox environment for photoreduction in living cells (see discussion in Ref. 13), consistent with the suggestion that both radical (active) and a smaller proportion of fully reduced (inactive) flavin states of cryptochrome could be formed upon illumination in vivo.

As a result of the present work, it is evident that during cryptochrome illumination, there will be a simultaneous and competing reoxidation reaction, the rate of which is dependent on the concentration of molecular oxygen. This phenomenon is clearly shown by the oxygen electrode experiment demonstrating oxygen consumption upon illumination of cryptochrome in vitro (Fig. 5). The extent of radical accumulation in vivo, and consequently of cryptochrome biological activity, will therefore not be dependent solely on the rate of the light-activated flavin-reducing reaction but rather on a complex equilibrium reached between forward and reverse redox reactions of the flavin cofactor. In terms of cryptochrome function, this means that factors such as temperature (which co-determine the O_2 concentration in the cells), the actual free oxygen available in different living tissues, and the presence of reactive oxygen species or oxygen radicals that destabilize the reduced flavin should significantly alter the biological responsiveness of CRY to blue light.

The observation that the fully reduced flavin FAD^- oxidizes more rapidly than the FAD^+ radical (signaling state) is consistent with the observed effects of green light on cryptochrome biological activity in co-illumination experiments (11–13). Green light would be expected to drive the photoreduc-
tion further toward FADH\(^-\), which then rapidly reoxidizes to the inactive FAD\(_{ox}\) form in keeping with the observed antagonistic effect of green light on cryptochrome biological activity.

**Spin-correlated Radical Pair as a Candidate for Magnetoreception**—Considerable recent discussion has centered around the possibility that cryptochromes may function as magnetoreceptors (31–34). Although as yet there is no hard evidence that cryptochrome itself is the magnetoreceptor, plausible mechanisms of cryptochrome-based magnetoreception involve light-induced formation of a spin-correlated radical pair that evolves between its singlet and triplet states and either forms different reaction products, depending on its spin multiplicity at the moment of the reaction, or favors suppresses the photophysical processes (such as fluorescence, phosphorescence, vibrational relaxation to the ground state, etc.), which compete with the photochemical reaction and affect the reaction yield. The singlet-triplet evolution and hence the products would be modulated by an external magnetic field. Candidates for the spin-correlated radical pair in cryptochromes proposed in the literature are (FADH\(^-\)–Trp\(^+\)) or the (FADH\(^-\)–Tyro\(^+\)) formed by intraprotein electron transfer upon excitation of FAD\(_{ox}\) to its singlet excited state (34). Nevertheless, a recent study (33) has shown that the Trp triad is not necessary for magnetosensitivity in *Drosophila*. It is therefore conceivable that instead of an amino acid radical, the superoxide radical O\(_2^-\) or even simply O\(_2\) (triplet in its ground state) could be the partner of FADH\(^-\) in the magnetosensitive reaction.

Because of spin conservation, the radical pair \((FADH⁻–O₂⁻)\) is originally formed in an overall triplet spin configuration

\[
{¹FADH⁻ + 3O₂ \rightarrow ¹(FADH⁻–O₂⁻)}
\]

\[
〈²(FADH⁻–O₂⁻) \rightleftharpoons ¹(FADH⁻–O₂⁻)⟩
\]

\[
+ H⁺
\]

\[
¹(FADH⁻–O₂⁻) \rightarrow ¹(FADₕox + H₂O₂)
\]

**Scheme 4**

The second oxidation step (formation of FAD\(_{ox}\) and H\(_2\)O\(_2\)), however, requires an overall singlet spin configuration and hence a spin-forbidden triplet-to-singlet transition (intersystem crossing) of the radical pair. This transition, indeed, has a potential to be modulated by external magnetic fields.

Formation of the FADH\(^-\)–O\(_2\)\(^-\) radical pair as one of the plausible mechanisms of cryptochrome-based magnetoreception has been proposed before (35–37); however, the authors did not favor this mechanism because of the difficulties associated with a complete reduction of the flavin cofactor *in vitro*. Nevertheless, it has been shown that certain isolated mutant insect cryptochromes undergo full photoreduction even under conditions that fail to reduce DmCRY *in vitro* (38). Furthermore, in the case of at least one cryptochrome species thought to be involved in magnetic orientation in birds, namely the bird CRY1a, fully reduced flavin is readily formed upon photoreduction even *in vitro* (8). Finally, *in vivo* studies in multiple organisms imply that the flavin radical can indeed be reduced further to FADH\(^-\) by light (confirmed for example by antagonistic effects of green and blue light on plant cryptochrome and DmCRY biological activity (11–13)). It thus appears likely that photoreduction *in vivo* occurs by more efficient mechanisms (13) and may involve reducing agents that are as yet unknown. The presented results may thus displace the current focus on CRY as a possible magnetoreceptor from the photoreduction to the dark reoxidation pathway.

**Acknowledgments**—We thank Dr. Anja Krieger-Liszay and Dr. Winfried Leibl (CEA Saclay, France) for providing us access to their oxygen electrode setup and Dr. Klaus Brettel (CEA Saclay) for fruitful discussions of the global data analysis and proofreading the manuscript. We further thank Jeffrey Steward (Penn State University) for help with protein purification.

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