Chemical Magnetoreception: Bird Cryptochrome 1a Is Excited by Blue Light and Forms Long-Lived Radical-Pairs

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INTRODUCTION

Each year millions of migratory birds travel thousands of kilometres between their breeding and wintering grounds. A magnetic compass sense has been shown to play a key role in enabling them to find their way [1–3]. But how do migratory birds detect the reference compass direction provided by the Earth’s magnetic field? This remains one of the most fascinating unresolved mysteries of sensory biology. From behavioural experiments, we know that the ability of night-migrating birds to perform magnetic compass orientation is affected by the wavelengths of the ambient light [4,5] and that pinealectomised birds [6] are still able to use their magnetic compass to orient in the laboratory. This led to the suggestion that photochemical reactions in the birds’ eyes could produce spin-correlated radical pairs as the primary sensor in a magnetic field-dependent signalling cascade [7,8], based on the fact that the recombination yields of such radical pair reactions are sensitive to external static magnetic fields [9,10].

Spin-correlated radical pairs are typically the result of a photochemical reaction (e.g. electron transfer) that creates radicals from a molecular precursor that is in either an electronic singlet (S) or triplet (T) state. Under the influence of intramolecular electron-nuclear hyperfine interactions, the radical pair can oscillate between the S and T states, a process known as S-T interconversion or S-S mixing. External static magnetic fields can affect the extent and frequency of this process and hence alter the yields of the respective reaction products formed from the S and T radical pairs [10,11]. In order for a magnetic field to have a substantial effect on the S-T mixing process, the radical pair needs to be sufficiently long-lived (for an Earth-strength magnetic field) [12]. Despite encouraging theoretical considerations [8] and supporting calculations [12,13], there is no experimental proof as yet that the recombination yields of such radical pair reactions are sensitive to the orientation of the pair with respect to the external magnetic field (or indeed that any radical pair reaction responds significantly to a field as weak as the Earth’s (~50 μT)). This directional information is of course of pivotal importance in the discussion of magnetoreception in migratory birds.

Magnetic fields oscillating with frequencies in the MHz range are also known to affect the S-T interconversion in spin-correlated radical pairs, even in the absence of a static external magnetic field [8,14–16]. The hypothesis that the avian magnetic compass is based on a radical pair mechanism is strongly supported by the observation that magnetic compass orientation of migratory birds can indeed be disturbed by weak radio-frequency magnetic fields with frequencies in the MHz range [17,18]. However, for this radical-pair hypothesis to be realistic it must be shown that molecules with the required biophysical characteristics actually exist in the eyes of migratory birds. Cryptochromes [19], a class of proteins that exhibit a close homology to photolyases (flavoproteins involved in the repair of UV light-induced DNA damage), were first proposed by Ritz et al. [8] as the host molecules for the crucial radical pair cofactors that putatively act as a primary magnetoreceptor. It has recently been demonstrated, using UV/visible and EPR (electron paramagnetic resonance) spectroscopies [20–22], that photolyases are able to form long-lived radical-pairs including a flavin radical and a radical

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derived from a redox-active amino acid. So far it has been shown that cryptochromes exist in the eyes of migratory birds [23,24], and that at least some cryptochrome-containing cells within the retina are active at night when the birds perform magnetic orientation in the laboratory [23]. Furthermore, a distinct part of the forebrain which primarily processes input from the eye is highly active at night in night-migratory garden warblers (Sylvia borin) and European robins (*Erithacus rubecula*) [25,26]. All these findings are consistent with the hypothesis that magnetic compass detection in migratory birds takes place in the eye and that cryptochromes could be a primary magnetoreceptors. Despite this mounting body of indirect evidence, it has still be to demonstrated convincingly that cryptochrome is directly involved in the avian magnetic compass sense. For instance, several fundamental biophysical characteristics including the absorption spectrum and the formation of long-lived radical pairs have not been experimentally validated for any vertebrate cryptochrome [27]. The aim of the present study is to experimentally explore the biophysical properties of cryptochromes from migratory garden warblers.

**MATERIALS AND METHODS**

**Cloning**

For detection and cloning of garden warbler (gw) Cry1 transcripts, total RNA was extracted from the retina of a garden warbler performing magnetic orientation behaviour in an orientation cage [28] in the laboratory during the migratory season as described in [23]. The retina was collected immediately after the bird had shown at least 1 h of consistent migratory-restlessness behavior. cDNA synthesis was performed from 450 ng of RNA with Revert Aid H’First-Strand cDNA synthesis kit (MBI Fermentas) according to the manufacturer’s protocol.

Hot start PCR amplification reactions were performed in 25 µl total reaction volume containing 1 µl of the cDNA synthesis reaction as template, 1× reaction buffer (SAWADY/Amersham-Pharmacia, depending on the DNA polymerase used), 0.25 mM dNTPs (Pharmacia Biotech), of each primer 1.25 pmol/µl PCR volume, 1 unit of *Pso*I polymerase (SAWADY) or 2 units of *Taq*polymerase (Amersham-Pharmacia). The PCR reaction was carried out in a MWG Primus25 thermocycler. The temperature profile for all hot-start PCR reactions was as follows: first denaturation 3 min 94°C; 45 cycles of 45 s at 94°C, 45 s annealing at 54°C and 40 s extension at 72°C (the duration of extension phase depended on the expected size of the amplified fragment and the enzyme used; the extension step comprised 1 min/kb DNA for amplification with *Taq*polymerase and 2 min/kb DNA for *Pso*I polymerase); the last cycle was extended to 5 min at 72°C to ensure completion of the final extension. The full length sequence of gwCry1a (GenBank accession no. AJ632120) was reconstructed as described in Mouritsen et al. [23].

To probe for potential influences of the C-terminal extension of gwCry1a in the reaction process, we additionally expressed the photolyase homology region (510 aa) of gwCry1a (Cry1-PHR). The PHR lies within the N-terminal domain, which is identical for both gwCry1a and gwCry1b (GenBank accession no. DQ833738), is similar to photolyases and highly conserved in all members of the photolyase/cryptochrome superfamily [29,30]. For expression of gwCry1-PHR the N-terminal 510 amino acids were chosen based on the structural analysis of A. thaliana Cry1-PHR [29] and the lack of homology between garden warbler and A. thaliana Cry1 beyond amino acid residue 510. A C-terminal fragment for gwCry1-PHR was PCR-amplified from a cloned gwCry1a template with the primer pair: DSP primer (p741), 5'-TAGTTCCTTGTGTCGACGGTTT-3'; USP1 primer (p742), 5'-AGCAAGCGGCCTCTCTTTATCAA GTTGCAAGAGACCCAGTCCT-3'. The amplified fragment (320 bp) was digested with *Bst*XI/*Nde*I (resulting in a 107 bp fragment) and cloned with 1406 bp N-terminal fragment isolated from a full-length gwCry1a plasmid by *Nde*I/*partial Bst*XI digest of the full length gwCry1a plasmid.

Coding regions for gwCry1a and gwCry1-PHR proteins were subcloned into a pVL1392-derived baculovirus transfervector to allow for subsequent recombinant expression of the target proteins in a S9/baculovirus expression system. For selective purification by means of immobilised metal affinity chromatography (IMAC) matrices, we expressed the genes as fusions with a vector-encoded N-terminal *His6*-tag. The full length sequence of gwCry1a (1866 bp) was subcloned into the polylinker region of the pDIA92B-His transfervector by *Nde*I/*NalI* digest. The PHR region of gwCry1 (1536 bp) was subcloned into the polylinker region via *NalI*/Nde*I digest. Recombinant baculoviruses were generated by cotransfection of transfer vectors and baculoviral genomic DNA and clonal isolation of gwCry1 protein-expressing baculoviruses according to standard methods [31,32].

**Recombinant expression**

To characterise the molecular properties of gwCry1a and gwCry1-PHR proteins, we expressed the proteins in S9 cells transfected with the corresponding recombinant baculovirus. Infected cells were incubated in ExCell420 culture medium (JRH) supplemented with 25 µM each of FAD and FMN (Sigma) in spinner flask cultures (0.6 l culture volume) for 66 h (±4 h) at 27°C. Cell pellets were harvested, washed in PBS and quick-frozen in liquid nitrogen.

**Purification**

gwCry1a (628 aa; ~62 kDa) and gwCry1-PHR (510 aa; ~56 kDa) were expressed as soluble fusion proteins and purified to near homogeneity by immobilized metal affinity chromatography (IMAC) in 20 mM HEPES buffer (pH 7.4) containing 500 mM NaCl and 50 µM FAD. Recombinant gwCry1a was bound to the Chelating Sepharose matrix and unspecifically bound contaminants were washed away with 20 mM HEPES buffer containing 500 mM NaCl and 50 µM FAD (pH 7.4). The column-bound protein was denatured by a linear gradient (10 column volumes) of 0 M to 6 M urea in 20 mM HEPES buffer containing 50 µM FAD, renatured by a “reversed” linear gradient from 6 M to 0 M urea in 20 mM HEPES 50 µM FAD buffer, and finally eluted by increasing imidazole concentration. Samples were concentrated in Vivaspin 2000 (Amersham Bioscience). Samples were stored in 20% glycerol at a final protein concentration of 15–25 µM, shock frozen in liquid nitrogen and stored at ~80°C.

The quality of the purified proteins was analysed by SDS-PAGE and Western blot analyses using standard procedures. On PVDF membrane (Pall) Western blots, gwCry1a protein was probed by goat polyclonal antibody z(anti)-Cry1 (A-20) sc-3933 (Santa Cruz Biotechnology), and gwCry1-PHR was detected by a commercially available mouse monoclonal z-His tag antibody (PentaHis antibody, Qiagen); the primary antibodies were detected by appropriate alkaline phosphatase-conjugated secondary antibodies and nitroblue tetrazolium/bromo-chloro-indoxyl phosphate staining on the membrane.

Samples were concentrated by ultrafiltration through 30 kDa microconcentrators (Millipore, Billerica, MA, USA) at 4°C. The flowthrough was periodically monitored spectroscopically: concentrations of free flavin were below the detection limit of 1 µM.
UV/visible spectral analysis

Purified *gwCry1a* and *gwCry1-PHR*, both containing FAD in its fully oxidised redox state, were supplemented with 5 mM DTT, deoxygenated and illuminated at 290 K with blue light (Halolux 30HL, Streppel, Wermelskirchen-Tente, Germany) selected with a 420–470 nm band filter (Schott, Mainz). Oxidation was performed under aerobic conditions in the dark. The concentration of protein-bound flavin was estimated based on its absorbance at 450 nm ($e_{450} = 1.3 \times 10^4$ M$^{-1}$ cm$^{-1}$). The concentrations of *gwCry1-PHR* and *gwCry1a* holoproteins, with FAD bound, were 7 μM and 4 μM respectively.

Transient absorption experiments

Solutions of proteins (concentrations in the range 15–25 μM) in 20 mM HEPES buffer and 20% glycerol at 270 K were photoexcited at 355 nm using a Nd-YAG laser (5 ns, 5 mJ/pulse). The monitoring beam was provided by a tungsten lamp (150 W). Light from the sample was fed to a monochromator (Newport model 77250) before entering a photomultiplier tube (Hamamatsu R-928). The time-resolution of the measurement was 20 μs. The signals presented in Figure 1 are averages of 2 measurements.

NMR spectroscopy

600 MHz 1H NMR spectra of the ~20 μM *gwCry1a* samples used for the transient absorption experiments were recorded on a Varian Inova 600 (14.1 T) NMR spectrometer to confirm the absence of significant amounts of free flavin (see Supporting Information (Text S1, Fig. S1)).

Animal husbandry as well as all experiments and animal procedures were approved by the Animal Care and Use Committees of the Bezirksregierung Weser-Ems (Oldenburg, Germany).

RESULTS

Garden warbler cryptochrome (gwCry1a) absorbs blue light

To determine the visible absorption spectra of garden warbler cryptochromes, we recombinantly expressed and purified *Cry1a* and the photolyase homology region of the migratory warbler (shown for *gwCry1a* in Fig. 2). The absorption spectra of purified *gwCry1a* and *gwCry1-PHR* proteins are shown in Fig. 3. FAD (flavin adenine dinucleotide) cofactors show characteristic optical absorption properties in all three of their biologically relevant redox states: fully reduced (FADH$^-$ or FADH$_2$), semiquinone radical (FAD$^*$ or FADH), and fully oxidised (FAD$_{ox}$). Both cryptochrome constructs were isolated in a yellow form characteristic of a flavoprotein with a fully oxidised cofactor, FAD$_{ox}$. *gwCry1a* and *gwCry1-PHR* with bound FAD$_{ox}$ both have absorption maxima at 450 and 366 nm representing respectively the $S_0$$\rightarrow$$S_1$ and $S_0$$\rightarrow$$S_2$ transitions of the flavin cofactor. Members of the photolyase family often bind a pterin or deazaflavin in addition to the FAD [for reviews see 30,33,34]; there was no evidence of a second chromophore in either of the samples. The spectra resemble those of other flavoproteins [35] except that the vibrational structure of the 450 nm band is not pronounced; we return to this point below.

Illumination of *gwCry1a* and *gwCry1-PHR* with blue light (420<λ<470 nm) attenuates both bands (Fig. 3). This behaviour is characteristic of the photolyase/cryptochrome superfamily [e.g. 36,37,38]. After illumination in the presence of dithiothreitol (DTT; as an exogenous electron donor), the fully (two-electron) reduced FADH$^-$ form of the flavin was formed (Fig. 3), exhibiting a broad absorption band around 360 nm. In contrast to other members of the photolyase/cryptochrome family, however, flavin radicals (exhibiting absorption maxima at around 380 and 620 nm) do not accumulate to a significant extent in *gwCry1a* and *gwCry-PHR*. Moreover, no significant radical formation is observed while...
Garden warbler cryptochrome forms long-lived radical-pairs

To test whether gwCry1a forms radical-pairs upon photoexcitation and to investigate whether they have sufficient lifetimes for the putative geomagnetic field effects to take place, we recorded transient absorption spectra for gwCry1a protein using laser flash photolysis at 355 nm. The identity of the transient photo-intermediates formed from gwCry1a was investigated via their time-resolved absorption characteristics (Fig. 1). Figure 1A shows the transient absorption spectra obtained for gwCry1a as a function of both the time delay between the laser flash and the 500 μs monitoring window (τdelay = 0.5, 3, 5, 9, 13 ms) and the wavelength of the monitoring light (horizontal axis). The spectra show that garden warbler cryptochrome forms long-lived radical pairs and that this radical-pair formation is strongly dependent on both wavelength and time, with a pronounced absorption peak around 510 nm at early times (τdelay = 0.5 ms). It is clear from a comparison of the five spectra, that this peak (blue dotted frame) has a shorter lifetime than the features at longer wavelengths (red dotted frame). Figure 1B shows this behaviour in a more quantitative fashion. Here, the absorption of the sample is detected as a function of time after the laser flash for the two wavelength regions (blue curve: 490–550 nm; red curve: 550–630 nm). The shorter wavelength decay can only be modelled satisfactorily as a double exponential decay with lifetimes of 4 ms and 14 ms, while the longer wavelength decay can be accurately modelled as a single exponential with a lifetime of 14 ms.

The absence of pronounced vibrational structure around 450 nm in Fig. 3 may, at first glance, suggest a free flavin molecule rather than a flavin cofactor bound to a photolyase or a cryptochrome. The protein purification protocol used (see Material and Methods and Supporting Information [Text S1, Fig. S1, Fig. S2]) makes it very unlikely that there were any significant amounts of free flavin left in our samples. Nevertheless, considering the form of the recorded spectra, it is important to demonstrate unequivocally that the transient absorption data (Fig. 1) cannot originate from radicals formed from free flavin molecules. We therefore performed a number of control experiments including NMR spectroscopy and transient absorption measurements on solutions of FAD and FMN (flavin mononucleotide) with either tryptophan or hen lysozyme, a ∼14 kDa protein containing two solvent-accessible tryptophan residues that are known to be reactive with photoexcited flavins [39,40]. As described in detail in the Supporting Information (Text S1, Fig. S1, Fig. S2), these control experiments include NMR spectra that show no detectable amount of free flavin in the samples; additional ultra-filtration steps after protein purification that yield no detectable amount of free flavin; transient absorption measure-
ments showing that free flavin results in signals that are one to two orders of magnitude weaker than the cryptochrome signals presented here and that free flavin radicals are about two orders of magnitude shorter lived than those observed for garden warbler cryptochrome under the same conditions. These control experiments unequivocally establish that the data presented here do indeed result from photo-induced intra-protein electron transfer between garden warbler cryptochrome and its bound flavin cofactor.

Discussion

We have experimentally verified several key assumptions crucial for the theoretical concept of light-mediated magnetoreception in birds [7,8]. We succeeded in expressing gwCry1a, which occurs in the retinae of magnetically orienting night-migratory garden warblers [23], and its highly conserved N-terminal PHR domain as holoproteins (with their flavin cofactor bound). The absorption spectra of flavoproteins are dependent on the redox status of the bound flavin cofactor, which in turn is influenced both by the apoprotein and by the redox environment [41]. gwCry1a and gwCry1-PHR purified from insect cells both contained the flavin cofactor in the fully oxidised state (Fig. 3), as was also found for isolated ACry1 from the plant Arabidopsis thaliana [36]. In isolated photolyases, however, the flavin cofactor is usually bound in a mixture of reduced and semiquinone states [e.g. 42,43]. The absorption spectrum of gwCry1a fits with the observations that songbirds, including garden warblers, are able to orient well using only their magnetic compass under blue and turquoise light, whereas they seem challenged doing this under yellow and red light [2,4,5]. The fact that songbirds are well-oriented under green light (565 nm) is not consistent with the absorption spectra of the fully oxidised form of garden warbler cryptochrome. However, a second longer-wavelength receptor could well be involved [e.g. 5,44,45].

Using a laser flash photolysis experiment, we were able to prove that illumination of gwCry1a (at 355 nm) produces radical pair species with lifetimes of the order of milliseconds. This is a crucial finding as the evolution of a magnetic field effect relies on the transient absorption control experiments (see Supporting Information, Text S1, Fig. S1, Fig. S2) all indicate strongly that the flavin cofactor in the fully oxidised state (Fig. 3), as was also found for isolated ACry1 from the plant Arabidopsis thaliana [36]. In isolated photolyases, however, the flavin cofactor is usually bound in a mixture of reduced and semiquinone states [e.g. 42,43]. The absorption spectrum of gwCry1a fits with the observations that songbirds, including garden warblers, are able to orient well using only their magnetic compass under blue and turquoise light, whereas they seem challenged doing this under yellow and red light [2,4,5]. The fact that songbirds are well-oriented under green light (565 nm) is not consistent with the absorption spectra of the fully oxidised form of garden warbler cryptochrome. However, a second longer-wavelength receptor could well be involved [e.g. 5,44,45].

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concentration to perform these additional analyses. Furthermore, of radical pair formation [37]. So far, no animal cryptochrome has magnetic field-sensitivity of the radical pair lifetime; both addressed by time-resolved EPR and by measurements of the correlation has also to persist for at least

magnetic field effects. It is important to realise, however, that the result in lifetimes so similar to those measured by Giovani et al. [37]. By elimination, therefore, we are left with the conclusion that the flavin is bound in the correct site and with the correct orientation to undergo radical transfer reactions with aromatic amino acid sidechains in the protein. The absence of more pronounced vibrational structure in the absorption spectrum seems to be a characteristic of the holo-form of Cry1a, to formation of radical species that live about five orders of magnitude longer than the ~1 µs theoretically required to elicit significant Earth-strength magnetic field effects. It is important to realise, however, that the radical pairs do not only have to be long lived, but their spin-correlation has also to persist for at least ~1 µs. This issue can be addressed by time-resolved EPR and by measurements of the magnetic field-sensitivity of the radical pair lifetime; both experiments are challenging because of the low quantum yield of radical pair formation [37]. So far, no animal cryptochrome has been expressed and isolated as a holoprotein in sufficiently high concentration to perform these additional analyses. Furthermore, the cryptochromes have to be restricted in their motion in order for any potential magnetic field effect to encode directional information. In any case, our observation of very long-lived photoinduced radical pairs in a migratory bird cryptochrome considerably strengthens the hypothesis of cryptochromes as the primary magnetoreceptor molecules in the avian magnetic compass [8,49].

SUPPORTING INFORMATION

Text S1 NMR measurements and transient absorption control experiments. Found at: doi:10.1371/journal.pone.0001106.s001 (0.04 MB DOC)

Figure S1 Aromatic region of the 600 MHz 1H NMR spectrum of gutCRY1a protein. Aromatic region of the 600 MHz 1H NMR spectrum of the CRY1a sample used for the transient absorption experiments before (blue) and after (red) addition of 20 µM FAD. Note the additional signals from FAD in the latter spectrum and their absence in the former. Found at: doi:10.1371/journal.pone.0001106.s002 (3.63 MB TIF)

Figure S2 Transient absorption signals at 550 nm for garden warbler cryptochrome and equimolar mixtures of FAD with hen lysozyme. Transient absorption signals at 550 nm recorded for aqueous solutions of 20 µM cryptochrome (purple) and for equimolar mixtures (20, 40 and 80 µM) of FAD with hen lysozyme (blue, orange and red, respectively). The abscissa is time (µs); the ordinate is absorbance. Note the much stronger and much longer lived signal from flavin radicals in the case of the cryptochrome compared to that observed for reactions of free flavin with lysozyme. Found at: doi:10.1371/journal.pone.0001106.s003 (4.19 MB TIF)

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Author Contributions

Conceived and designed the experiments: HM, ML, TS, CT, PH. Performed the experiments: MI, KM, KH, ES. Analyzed the data: MI, KM, KH, ES, CT, PH. Wrote the paper: HM, ML, CT, PH.

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