Magnetic field effects in flavoproteins and related systems


Within the framework of the radical pair mechanism, magnetic fields may alter the rate and yields of chemical reactions involving spin-correlated radical pairs as intermediates. Such effects have been studied in detail in a variety of chemical systems both experimentally and theoretically. In recent years, there has been growing interest in whether such magnetic field effects (MFEs) also occur in biological systems, a question driven most notably by the increasing body of evidence for the involvement of such effects in the magnetic compass sense of animals. The blue-light photoreceptor cryptochrome is placed at the centre of this debate and photoexcitation of its bound flavin cofactor has indeed been shown to result in the formation of radical pairs. Here, we review studies of MFEs on free flavins in model systems as well as in blue-light photoreceptor proteins and discuss the properties that are crucial in determining the magnetosensitivity of these systems.

1. Introduction

For many years, magnetic field effects (MFEs) in chemical systems have been investigated [1–3], allowing the theory of the radical pair mechanism which underlies the majority of these effects to become well established, yet our understanding of such magnetic effects in biological systems is still extremely limited [4,5]. However, the interest in this field of research is significant and mainly driven by two factors (i) the potential implications for human health if biological systems can indeed be affected by applied magnetic fields [6–9] and (ii) our fascination with the ability of many animals to exploit magnetic fields, most notably in the form of a magnetic compass sense [10]. In particular, research into avian magnetoreception has stimulated great interest in the past decade. Since the proposal by Ritz et al. [11] that the biological receptor may be the photoreceptor cryptochrome, significant effort has been expended in collecting spectroscopic evidence for MFEs in the cryptochrome family of proteins. In this review, we briefly discuss the evidence for the involvement of cryptochromes in biological magnetoreception, introduce the key mechanism by which MFEs commonly arise, explore the evidence for flavin-based MFEs in biologically relevant model systems and highlight recent key results in the in vitro investigation of cryptochrome proteins leading to detection of intramolecular MFEs.

1.1. Cryptochromes and magnetoreception in biology

It has now been established that cryptochrome and photolyase proteins possess the necessary physical properties to respond to magnetic fields and to transduce this physical signal into a chemical signal that could be integrated into conventional
cell signalling mechanisms [12]. If such field response were indeed to be exploited in nature, then it would be a rare example of a quantum effect being used in biology, and thus this area has attracted interest from the wider scientific community [13–15].

Many organisms, most notably migratory birds, are known to use magnetic information when choosing the direction of their migration. While there is evidence that magnetite might be at the heart of magnetoreception [16], other studies (particularly, involving passerine birds) prove the mechanism to be strongly light dependent [17,18] as well as being affected by radio waves applied at the resonance frequency of an electron in the Earth’s static field [19,20]. As radical pairs are typically formed following photoexcitation and show just such radio-frequency sensitivity [21–23], these findings strongly support the hypothesis of a radical-pair-based magnetocompass as originally suggested by Schulten et al. [24]. Following its discovery in the plant Arabidopsis thaliana in 1993 [25], the blue-light photoreceptor protein cryptochrome has appeared as the prime biological magnetoreceptor candidate in vivo. Recently, a laboratory response to magnetic fields has been shown in fruitfly (Drosophila melanogaster) [26–28], facilitating a detailed genetic dissection of the molecular identities and pathways involved. More recent developments and detailed analysis of the current literature of this field can be found in the references [12,16,29–34].

1.2. The radical pair mechanism

Radical pairs are typically formed by electron transfer or photolytic bond cleavage from a molecular precursor. Their properties and recombination characteristics are dominated by the radicals’ unpaired electrons which, like all electrons, possess an intrinsic spin angular momentum (quantum number \( s = \frac{1}{2} \)). This property gives rise to two states, known as spin up and spin down, labelled by the magnetic quantum numbers \( m_s = \pm \frac{1}{2} \) (formally, \( m_s \) specifies the projection of the spin angular momentum onto a fixed axis). Spin-correlated radical pairs (SCRPs) are generated from their molecular precursors under conservation of total spin angular momentum so that a singlet (or triplet) molecular precursor leads to a singlet (or triplet) born radical pair in which the two spins are aligned antiparallel (or parallel). The singlet state (labelled S) is characterized by a total spin quantum number \( S = 0 \) and has no overall spin angular momentum. As a result, the energy of the singlet state is independent of any applied magnetic fields. By contrast, the three triplet states \( T_{0}, T_{+}, \) and \( T_{-} \) are defined by a total spin \( S = 1 \) (with resulting spin angular momentum \( 2\hbar \)) and spin projection numbers \( M_S = 0, +1 \) and \( -1 \), respectively. In the presence of an applied magnetic field, the energy of the \( T_{0} \) state (\( M_S = 0 \)) is therefore also field independent, whereas the energies of the \( T_{+} \) and \( T_{-} \) states are shifted by the Zeeman interaction according to \( E_{Zeeman} = +g\mu_B B_0 M_S \), where \( g \) is the \( g \)-value for the electron and depends on the structure of the radical, \( \mu_B \) is the Bohr magneton and \( B_0 \) is the strength of the external magnetic field (figure 1a). In the absence of any dipolar interactions (e.g. for radical pairs in solution) the zero field energies of the singlet and triplet manifolds are separated by the exchange interaction \( J \), depicted as negative in figure 1a, which is pertinent in later discussions in this review.

In the absence of an external magnetic field, interconversion between the singlet and triplet states of the radical pair is driven by internal electron–nuclear hyperfine interactions. However, the progressive energetic isolation of the \( T_{+} \) and \( T_{-} \) states from the \( S/T_0 \) manifold with increasing magnetic field results in an ever less efficient interconversion between singlet and triplet states. This, in turn, may result in different yields of singlet and triplet products (if they are distinct; figure 1b) or different kinetics (if the recombination from singlet and triplet radical pairs occurs at different rates). Crucially, even though the interaction energy of the electron spins with the magnetic field is significantly less than the thermal energy \( k_BT \), the non-equilibrium state of the SCRP allows a kinetic effect if subsequent radical reactions are spin selective. Other mechanisms inducing MFEs on chemical reactions do exist [1,35] but are not the topic of this review.

Under certain circumstances, an additional low field effect (LFE) [3] can also occur owing to changes in the quantum mechanical selection rules between zero and weak applied magnetic fields. This results in a response of opposite phase at low fields owing to an initial increase in singlet–triplet (ST) mixing when a weak magnetic field is applied (figure 1b; [36]). Effects of fields as weak as that of the Earth (approx. 50 \( \mu \)T) have indeed been observed in a biradical based on a molecular triad, a system which was also shown to be sensitive (at somewhat higher fields) to the direction of the applied magnetic field, providing a proof-of-principle that the radical pair mechanism could lie at the heart of the magnetic compass sense [37].

2. Magnetic field effect in flavins

2.1. Introduction to flavins

Flavins play a fundamental role in biology. The different redox states of flavin (oxidized, semiquinone and reduced) confer catalytic function in a variety of one-electron and two-electron processes [38]. Blue-light photoreceptor flavoproteins perform a wide range of functions [39,40] including phototropism by phototropin, transcriptional regulation by BLUF proteins in bacteria, light-induced DNA repair by photolyase, entrainment of the circadian clock in D. melanogaster by cryptochrome [41] and a proposed role for cryptochrome in magnetoreception [11].

All flavin derivatives possess an isoalloxazine component and differ in the nature of their ribityl chains (figure 2). The hydrophilicity of the flavin increases from riboflavin...
tetrabutyrate (RFTB) to riboflavin (RF) to flavin mononucleotide (FMN). Also shown is flavin adenine dinucleotide (FAD) which is closely related to FMN and possesses an additional adenine moiety. This allows FAD to support intramolecular radical pair formation (see below).

### 2.2. Photochemistry of flavins

Upon photoexcitation, flavins such as FMN, RFTB and RF populate the singlet excited state and, when unbound, typically undergo rapid intersystem crossing (ISC) to the triplet state on the timescale of a few nanoseconds (figure 3) [42]. The singlet state is short-lived owing to both ISC and competing radiative and non-radiative processes returning the flavin to its ground state; however, the longer-lived flavin triplet state is susceptible to intramolecular electron transfer from donor species (such as tryptophan, tyrosine and other reducing species) [43]. This results in creation of a SCRP which, by spin conservation, is formed in the triplet state \( ^3[F^- + D^+ + \cdots] \), where \( F \) and \( D \) represent the flavin and electron donor, respectively.

Over time, coherent ST mixing allows evolution of the triplet-born radical pair into the singlet state. However, in the presence of an applied magnetic field, energetic isolation of the \( T_\pm \) states from the \( S/T_0 \) manifold slows the hyperfine-driven conversion to the singlet state. As recombination of the radical pair occurs exclusively from the singlet state (the triplet state being unreactive), an increased magnetic field enhances the concentration of radicals and consequently reduces the population of ground state flavin. MFEs should hence be detectable on both radical and ground state concentrations. Finally, recombination and ST interconversion compete at all times with spin state unselective Brownian motion of the geminately born radicals, which leads, eventually, to their physical separation. As a consequence, free radicals of much longer lifetime than their geminate counterparts are formed (figure 3; [44–47]).

In a similar reaction, photoexcited FAD forms an intramolecular triplet SCRP in which the adenine moiety of triplet FAD donates an electron to the isoalloxazine ring [48,49]. Through photochemically induced dynamic nuclear polarization (photo-CIDNP) NMR, Kaptein and colleagues [50,51] demonstrated that the radical pair is produced by photoexcitation in the pH range 1.5–4.0. The absence of any photo-CIDNP signals at higher \( \mathrm{pH} \) was attributed to a strong (inter-radical) exchange interaction owing to stacking of the flavin and adenine moieties in the ground state conformation which is inhibited by protonation of the adenine at low \( \mathrm{pH} \) [50,51].

### 2.3. Detection of flavin magnetic field effects

MFEs on flavin-based systems have traditionally been measured using transient absorption (TA) spectroscopy [44–46,48,49]. In TA, the sample of interest is photoexcited by a laser pulse and the change in absorbance of the sample \( \Delta A \), with respect to its ground state, is recorded as a function of time. A Xe arc lamp is conventionally used to provide a broadband probe beam, which, in combination with a suitable monochromator and detector, allows TA data to be recorded at various wavelengths. As sample absorbance is related to the concentration of each species by the Beer–Lambert law, measurement of the change in absorbance in the presence and absence of an external magnetic field is a direct probe of the MFE \( \Delta A(B_0) = A(B_0) - A(0) \), where \( B_0 \) is the applied static magnetic field. To allow comparison of the size of the effect between different systems or samples, the MFE is often expressed as a percentage value which is obtained by dividing the subtraction data by the TA signal in the absence of magnetic field, according to \( 100\% \times \Delta A(B_0)/A(0) \).

The ground state absorption of flavins displays two broad bands centred at 445 nm (\( S_0 \rightarrow S_1, \pi \rightarrow \pi^* \)) and...
375 nm (S₀ → S₂, π → π*) (figure 4; [52,53]). For convenience, the third harmonic of the commonly available Nd:YAG laser (λ = 355 nm) is typically used to generate the excited singlet flavin which may subsequently form a radical pair with a suitable electron donor [44–46,48,49]. When working with cryptochrome and photolyase protein samples, it is important to minimize UV-induced photodegradation, hence care is taken to match the first absorption band more closely using a Nd:YAG pumped dye laser (λ ≈ 460 nm) for photoexcitation [54,55].

2.4. The effect of magnetic fields on flavin adenine dinucleotide

MFEs on FAD in solution have been studied by TA [48,49]. Following laser excitation, two components exhibiting different kinetics can be observed: one which is short-lived (λmax = 680 nm, τ = 0.7 μs at pH = 2.3) corresponding to the absorption of the cation of the flavin triplet excited state, and a second longer-lived component (λmax = 510 nm, τ = 50 μs at pH = 2.3) which is less easily assigned. The neutral flavin radical FADH⁺ is expected to give a broad absorption band with maxima at 502 and 580 nm (figure 4), but this could not be resolved beneath the broad (500–730 nm) triplet absorption band (not shown). The absorption at 580 nm, which monitors both flavin radical and triplet, is increased in the presence of an external magnetic field (figure 5i). This indicates that the radical pair is produced from a triplet precursor molecule, assuming that back-electron transfer to the ground state takes place exclusively from the singlet radical pair. The subtraction profile, ΔΔA(B₀), is dependent only on those species whose concentrations are magnetically sensitive. The pH-dependent changes in the time profiles of ΔΔA(0.2 T) shown in figure 5b cannot therefore be explained by a conformation change in the FAD ground state alone. This suggests that the dynamics of the FAD excited state and radical pair themselves are modulated by pH.

Additional information was obtained from the wavelength-resolved difference spectra (averaged across time; data not shown). Such MFE action spectra for the FAD system were recorded as a function of pH and compared with reference absorption spectra of a flavin radical and triplet obtained after photoexcitation of FMN in the presence and absence of a triplet quencher [49]. Using this approach, the MFE action spectrum of photoilluminated FAD solution at pH = 3.9 was found to have a broad band around 550–600 nm, assigned to the neutral flavin radical (FADH⁺). At pH = 2.3, the action spectrum has an extra peak around λ = 650 nm, which is assigned to the cationic form of the flavin triplet excited state (³FADH⁺). At intermediate pH, there is a continuous change in the spectral profile, which is successfully analyzed as a linear combination of the flavin neutral radical and triplet excited states. The pH-dependence of the ³FADH⁺ component in the MFE action spectrum indicates both that the triplet is reformed from the radical pair and that this process is promoted by protonation. This unusual pathway is consistent with the shift and narrowing of the subtraction time profiles with increasing pH, as seen in figure 5b. This study highlights how MFEs can be utilized to investigate kinetics not accessible by optical spectroscopy at zero field.

2.5. Magnetic field effects in micelles

The effect of confinement on intermolecular MFEs between different flavins and indole rings has been measured using detergent micelles and the results have been related to the variation in the hydrophobic properties of the flavins [44]. Micelles restrict the diffusion of the radicals in solution and arguably provide a more biologically relevant environment than free solution [56]. Moreover, the MFE simplifies the kinetic analysis compared with conventional TA by allowing selective observation of radical species, whose absorption spectra would otherwise overlap with that of the triplet state.

Before discussing the effect of an applied magnetic field, it is helpful to determine the location of the reacting species within the micelle: the isoalloxazine being hydrophobic but the ribityl chains being more hydrophilic in nature [44]. The authors show that the time profiles for the decrease of the RF triplet excited state population and increase of the MFE on the radical concentration are comparable; this suggests that there is dynamic quenching of the RF triplet excited
state by the indole moiety. A Stern–Volmer analysis of this quenching in sodium dodecyl sulfate (SDS) micellar solutions gave values of $k_q = 2.9 \times 10^{9} \text{M}^{-1} \text{s}^{-1}$ (tryptophan) and $2.6 \times 10^{9} \text{M}^{-1} \text{s}^{-1}$ (indole), respectively. These values are similar to that obtained in water ($2.2 \times 10^{9} \text{M}^{-1} \text{s}^{-1}$), indicating that for RF the radical pairs are formed close to the interface between the amphiphilic headgroups and solvent molecules rather than being buried deep within the hydrocarbon core of the micelles.

SCRPs were generated between a number of different flavins (RF, FMN or RFTB) and either indole or its derivative tryptophan at different concentrations of the detergent SDS, and the MFE of a 0.2 T field recorded (figure 6). The MFE of RF with both tryptophan and indole was independent of [SDS] below the critical micelle concentration ([SDS] = 8.1 mM [57]) and then rose to a saturation value for [SDS] greater than 30 mM. This indicates that the photoinduced electron transfer from tryptophan/indole to RF takes place greater than 30 mM owing to insolubility in [SDS]. This result is consistent with the expected tendency for the hydrophobic flavin to situate near the core of the micelle where it may be quenched by electron donation from the phenyl ether group to form a radical pair.

The authors also studied radical pair formation between flavins and the aromatic part (phenyl ether group) of the surfactant Triton X-100 (figure 7a [45]). The Triton X-100 system produces non-ionic micelles and benefits from the huge advantage that the location of the electron donor, and hence the initial radical pair location within the micelle are known. Using TA measurements, a decrease in the lifetime of the flavin triplet excited state with increasing hydrophobicity was observed: FMN, $\tau = 2.0 \mu s$; RF, $\tau = 1.5 \mu s$; RF tetracacetate (RFTA), $\tau = 0.9 \mu s$; RF tetrapropyrate (RFTP), $\tau = 0.5 \mu s$; and RFTB, $\tau = 0.4 \mu s$. This is due to an increasing tendency for the hydrophobic flavin to situate near the core of the micelle where it may be quenched by electron donation from the phenyl ether group to form a radical pair.

MFE action spectra for the flavin/Triton systems were recorded to follow the kinetics of the radical species while avoiding spectral overlap from the magnetically insensitive triplet excited state, whose strong absorption dominates conventional zero-field TA spectra. Protonation of the flavin radical anion to the neutral form $(F^+ + H^+ \rightarrow FH^+, pK_a \approx 8)$ could be monitored by following the decrease in radical anion absorption at $\lambda = 390 \text{ nm}$. Analysis of growth of the corresponding neutral radical band at 600 nm was not possible for all systems owing to a strong dependence of this band on changes in the polarity of the radical environment as determined by its location within the micelle. If protonation is carried out by water molecules, its rate should reflect the dynamic motion of the anion radical away from the hydrophobic micellar core. The MFE subtraction profile ($\Delta A(0.2 \text{ T})$) was recorded for RFTA at 390 nm for a number of values of pH (figure 7b). The extinction coefficient of $F^+$ at this wavelength is greater than that of ground state $\text{F}$ (figure 4) so an increased radical anion concentration gives a positive signal, whereas $\text{FH}^+$ has a lower extinction coefficient than $\text{F}$ resulting in a negative signal as the protonation reactions progress. The subtraction profiles were analysed, and the protonation rate constants were determined to be $k_p = 4.9 \pm 0.5 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$ for RF and $k_p = 3.1 \pm 0.1 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$ for RFTA (figure 7c). Again, these values reflect the hydrophobicity of the flavins: the more hydrophobic RFTA is associated with a lower protonation rate constant than the RF.

The overall result is that after photoexcitation, the triplet excited state of hydrophobic flavins is quenched rapidly by electron transfer, but the anion radical is then only slowly protonated. MFE action spectra for RFTA at pH = 6 confirm that although for hydrophilic flavins, the triplet is quenched more slowly, the electron transfer precedes the rapid protonation step.

### 2.6. Intermolecular magnetic field effects involving proteins

Apart from the studies in photosynthetic reaction centres [59–61], there have been relatively few observations of MFEs on radical pairs involving proteins. Importantly, however,
radical pairs formed with surface-exposed protein residues are commonly exploited to give selective NMR signal enhancements providing information on protein binding and folding in photo-CIDNP experiments [62,63]. Perhaps, the first study exploiting just such protein-surface-derived radical pairs in MFE studies was that by Mohtat et al. [64], who demonstrated MFEs on bovine serum albumin (BSA), human serum albumin and calf thymus DNA albeit with radical partners of limited biological relevance (namely, the ketyl radical formed from photoreduction of benzophenone derivatives). This early work highlighted the importance of Coulombic effects between the protein and radical precursor.

Photo-CIDNP showed intermolecular radical pair formation between RF and residues Trp62 and Trp123, in the native state of hen-egg-white lysozyme (HEWL) [65]. Subsequent MFE studies of FMN with HEWL showed that a 0.25 T static field increased the radical yield by 13%, again indicating triplet SCRP formation (with radical recombination occurring from the singlet state only) [46]. The field effect in this FMN/HEWL system is much higher than the field-induced 2% increase in radical yield observed for the analogous mixture of FMN and tryptophan (as a free amino acid), suggesting that the lifetime of the geminate radical pair is longer in the flavin/protein system. The authors rationalize this in terms of slower diffusion for the protein compared with the small free amino acid, resulting in a larger re-encounter probability of the separated radicals for the flavin/protein systems.

The MFE between FMN and HEWL was also shown to decrease with increasing salt concentration, although this was not observed for RF/HEWL (figure 8). This is rationalized in terms of the attractive Coulombic interaction between the negatively charged phosphate group of FMN (the pK_a of the first titratable phosphate is 0.7 [66]) and the positively charged HEWL (isoelectric point, pi = 11.4). Increase in ionic strength of the solution results in charge screening and rapidly decreases the MFE to that observed for the uncharged RF analogue (7%). The Coulombic interaction is also responsible for additional static quenching observed for the flavin triplet excited state (λ = 690 nm), and it was concluded that the attractive Coulombic interaction affects the dynamics of both the ground state and the radical pair.

Coulombic effects were explored further by replacing the flavin chromophore with anthraquinone-2,6-disulfonate (AQDS^-2) [47]. The photochemistry of AQDS^-2 with HEWL is similar to that with flavin; the chromophore forms the singlet excited state 1AQDS^-2* upon photoexcitation and consequently undergoes fast ISC to triplet 3AQDS^-2*. This species is susceptible to rapid electron transfer from a nearby tryptophan to form the triplet SCRP, [3AQDS^-2* + Trp]*. Owing to the double negative charge of AQDS^-2 in solution, a larger Coulombic interaction was expected for AQDS^-2*/HEWL than FMN/HEWL in both the ground state and radical pair. This was confirmed experimentally as MFEs of 22% and 7% at 0.04 T were observed on the radical yields for the AQDS^-2*/HEWL and FMN/HEWL systems, respectively. Examining the effect on
the MFE of varying the concentration of added salt for AQDS\(^2^+\)/HEWL and AQDS\(^2^+\)/BSA systems also identified differences in the AQDS\(^2^+\) binding site. 

Although suitable for MFE detection in model chemical systems, the TA method described above is less applicable to biological samples in which signals are typically weak owing to low concentration and small sample volume. Moreover, the intense photoexcitation pulses used frequently result in rapid photodegradation of protein samples. These shortcomings motivated the application of cavity enhanced spectroscopic techniques to MFE detection resulting in significant sensitivity gains compared with the single-pass TA experiment without increasing sample volume [67,68]. Using the FMN/HEWL and AQDS\(^2^+\)/HEWL systems discussed above, MFE detection by cavity ring-down spectroscopy (CRDS) was demonstrated, and a marked improvement over conventional TA detection observed [67]. By enclosing the sample within a high-finesse optical cavity and injecting a pulse from a probe laser, the concentration of the absorbing species can be determined from a measurement of the characteristic decay (the ring-down time) of light within the cavity. The effective increase in optical path length obtained from multiple passes of the probe light through the sample permits the use of reduced sample concentrations. To obtain a concentration versus time profile, CRDS is used as the detection pulse in a pump-probe experiment. In a recent proof-of-principle study, this technique enabled detection of an MFE in the FMN/HEWL system at concentrations below the detection limit for the TA technique (figure 9).

3. Cryptochrome family proteins

The work described in this review so far has focused on MFEs detected in radical pairs involving flavins or similar photoactive substances in chemical and biological model systems only. Although some significant insights into crucial factors, such as confinement and relaxation, can be gained from these investigations, the study of the cryptochrome proteins themselves is the ultimate goal if questions as to the ability of these molecules to act as magnetoreceptors are to be addressed.

Cryptochromes are a divergent and ancient protein family occurring in all three kingdoms of life. With their siblings, the photolyases, they share a common three-dimensional fold, sequence homology and the redox-active FAD cofactor in an unusual U-shaped conformation (figure 10). Many DNA photolyases also contain a second cofactor which is often either methenyl-tetrahydrofolate or 8-hydroxydeazaflavin. Photolyases repair specific UV-induced DNA damages by a light-driven process most probably involving radical pair intermediates between FAD and DNA. However, cryptochromes serve many different functions: controlling plant growth and development; entraining the circadian clock in insects; being the core component of the circadian clock in mammals; and their speculated role in the magnetoreception of migratory birds and other animals [69].

3.1. Structure of cryptochrome proteins

In structural terms, both cryptochromes and photolyases are single domain proteins sharing a highly conserved photolyase homology region (PHR domain), consisting mainly of \(\alpha\)-helices, with a single region of \(\beta\)-sheets towards the N-terminus of the protein. FAD is buried within the protein structure and, in DNA photolyases, the isoalloxazine ring of the FAD is known to be involved in electron transport to and from damaged DNA in the nearby DNA-binding pocket [70–72]. In contrast to DNA photolyases which consist of a single PHR domain, cryptochromes possess additional C-terminal (and occasionally N-terminal) extensions which are involved in interactions with protein binding partners and with the function of cryptochromes in the cell. While structures of PHR domains have been known for over 15 years, it is only in the last 2 years that X-ray evidence on C-terminal conformations has emerged [73–75].

The two structural elements important for the photochemistry of both, DNA photolyases and cryptochromes, are the FAD cofactor and a chain of three conserved tryptophans (termed Trp\(_\alpha\), Trp\(_\beta\) and Trp\(_\gamma\) for proximal, medial and terminal Trp with respect to the FAD) connecting the flavin cofactor with the protein surface. This ‘Trp triad’ is believed to form an electron transport chain from Trp\(_\gamma\) to the isoalloxazine ring. With their average spacing of approximately 5 Å, these Trps are suitable for stepwise ultrafast electron transport, which, for the photolyase from *Escherichia coli*, has been demonstrated as complete within 30 ps [76]. It is these Trps that are most likely involved in forming the radical pairs at the heart of the proposed magnetic compass (see §§4 and 5 below). This canonical tryptophan chain is neither present in Class II CPD photolyases nor on the newly discovered group of photolyases and cryptochromes containing an FeS cluster [77], but functionally equivalent chains of three and two alternative tryptophans, respectively, are found leading from the opposite face of the FAD to the protein surface (figure 10b; [78–80]).

Although in terms of their overall structure different PHR domains are very similar to one another, there are subtle differences in the positioning of individual elements of secondary structure and of some loops. These variations appear to cluster with different groups of structures and are presumably relevant to biological function (although this relationship does not appear to have been studied in detail). A fuller comparison of the structure of different PHR domains can be found in our recent review on signalling and mechanism in cryptochrome magnetoreception [34].

A further residue in the FAD-binding pocket which appears to be important in cryptochrome and photolyase function is found 3.5 Å from the N5 nitrogen of the
isoalloxazine ring, towards the top of helix $\alpha_{16}$ (figure 10c, helical numbering from ref. [34]). In most cryptochromes and photolyases this residue is an asparagine; however, the asparagine is replaced by a cysteine in insect cryptochromes and by an aspartic acid in *Arabidopsis* CRY1 (*AtCry1*). In *AtCry1*, the aspartic acid has been shown to be able to act as a proton donor to the N5 of the FAD isoalloxazine ring [81,82].

3.2. Photochemistry in cryptochromes

The photochemistry in cryptochromes has been studied in nowhere near as much in detail as that of photolyases [83,84], although it is now pretty well established that in *vivo* the photoactive form of most (if not all) cryptochromes is the oxidized flavin (FAD$^{\text{ox}}$) [85–87] in contrast with photolyase where it is the fully reduced flavin [70,88].

![Figure 9](http://rsfs.royalsocietypublishing.org/)

**Figure 9.** (a) and (c): decay-time profiles of a photoexcited aqueous solution of 10μM FMN and 0.5 mM HEWL, measured by (a) CRDS (0.75 ml pump pulse energy, 1 mm path length) and (c) TA (1 ml pump pulse energy, 10 mm path length), in the presence and absence of an applied magnetic field (52 mT for CRDS and 30 mT for TA). (b) and (d): subtraction profiles $\Delta \Delta A(B_0) - \Delta \Delta A(0)$, determined from decay profiles (a) and (c), respectively. Note the breaks in the vertical axes and the difference in $\Delta \Delta A(t)$ between CRDS and TA of roughly two orders of magnitude. All data points are 2000-shot averages. Adapted with permission from [67]. Copyright © 2011, American Chemical Society.

![Figure 10](http://rsfs.royalsocietypublishing.org/)

**Figure 10.** The tryptophan triad in cryptochrome structures. (a) PHR domain of *Drosophila* cryptochrome (grey-blue) with C-terminal extension (dark pink) (b) class II CPD photolyase (rice) with non-canonical Trp triad. (c,d) Cry-DASH from *Synechocystis* (c) environment of the isoalloxazine ring of FAD—Asn392 on helix $\alpha_{16}$ interacts with N5 of the isoalloxazine ring (dashed line—the top of helix $\alpha_{15}$ has been omitted for clarity); (d) close-up on canonical tryptophan triad and alternative terminal tryptophan, TrpC (bright pink), in *Synechocystis*. FAD (light pink), tryptophan triad (orange) and Asn392 (yellow; (c,d) only) shown in stick representation. The orientation of cryptochromes is identical in (a) and (b) to emphasize the difference between canonical and class II CPD photolyase triads. Figures drawn from PDBs 4GU5, 3UMV and 1NP7.
The primary photoreaction of photolyases is the repair of UV-damaged DNA by transferring an electron from the blue-light excited flavin cofactor in its fully reduced form to the DNA, transiently forming a radical pair between FAD and DNA. Additionally, these proteins show a second photoreaction, termed photoinactivation, in case of the flavin cofactor not being in its catalytically competent, fully reduced form. Here, upon excitation of the flavin cofactor with blue light, an electron is abstracted from the proximal Trp of the Trp triad (Trp$_d$) followed by a rapid charge separation along the chain of Trps to Trp$_C$. As cryptochromes do not exhibit DNA repair activity but share the electron transfer pathway with the photolyases, by analogy, it is assumed that this second photoreaction of photolyases is the primary one in cryptochromes except starting here from the fully oxidized flavin.

Blue-light photoexcitation of FAD$^{**}$ produces the singlet excited state, $^1$FAD*, followed by sequential electron transfer along the Trp triad resulting in a flavosemiquinone radical, FAD$^+$ or FADH$^+$ after protonation, together with a radical derived from the terminal residue of the Trp triad. It is noteworthy that the close vicinity of the proximal tryptophan to the flavin means that $^1$FAD* is quenched prior to ISC of the flavin excited state, resulting in formation of the radical pair in an initial singlet state in contrast to the free flavin systems discussed above. Any MFE on the coherent spin mixing between the spin states of the radical pair by the hyperfine mechanism therefore directly affects the proposed flavin-based semiquinone signalling state speculated to govern magnetoreception [11].

4. Evidence for formation of radical pairs in cryptochromes

In order for cryptochromes to act as magnetosensors, they have to fulfil three necessary (but not sufficient) requirements: (i) the formation of SCRPs which are (ii) both sufficiently long-lived to undergo magnetic-field-dependent ST mixing (the radical lifetime for a 50 $\mu$T field should exceed 1 $\mu$s) and (iii) exhibit spin-dependent reactivity. We will treat these requirements in turn, highlighting both the recently observed MFEs on certain cryptochrome and photolyase proteins and the supporting evidence provided by other techniques, for example time-resolved electron paramagnetic resonance (TREPR) spectroscopy.

Whereas pairs of radicals can readily be detected and identified by means of optical spectroscopy, the spin correlation necessary for the radical-pair-based magnetic compass manifests itself only in other types of spectroscopy, namely TREPR and direct measurements of MFEs. Radical pairs formed in spin-correlated states are known to exhibit in their TREPR spectra so-called antiphase doublets in which signals are observed in both emission and absorption [89–91]. This antiphase pattern is a direct consequence of the spin-polarized state (non-Boltzmann distribution) in which the radicals are formed following photoexcitation of their precursor. The spectral shape is determined by the magnetic interactions within the radical pair (both inter-radical and hyperfine) as well as with the applied magnetic field. Any EPR signal that appears in such an antiphase structure is hence the first indication for the presence of a radical pair which might exhibit a MFE. Furthermore, TREPR spectra might be exploited, using spectral simulations, to obtain information on the initial spin state of the radical pair. Finally, as EPR detects the magnetization associated only with unpaired electron spins, closed shell molecules will not contribute to the signal rendering TREPR a (near) zero-background technique.

First, spin-polarized signals in flash photolysis subjected samples of E. coli CPD photolyase (EcPL) were detected by time-resolved EPR by Kim et al. and Esslemacher et al. in 1992 and erroneously assigned to a single Trp cation radical [92,93]. Later analysis by Gindt et al. [94] demonstrated that the signal arises from the fully oxidized flavin FAD$^{**}$ undergoing the above-named electron transfer to form the semiquinone and the Trp cation radical. Weber et al. [95] also photoexcited the fully oxidized state of the flavin cofactor in Xenopus laevis (6–4) photolyase in time-resolved EPR experiments to demonstrate the formation of a radical pair. In contrast to CPD photolyase, the antiphase EPR signal could be analyzed to show that the final electron donor (and hence radical pair partner) is a tyrosine rather than a tryptophan radical.

Biskup and colleagues reported extensive TREPR investigations on the X. laevis Cry-DASH (XICry-DASH) proteins, which are available as stable, recombinantly expressed proteins [96–98]. These experiments were carried out at 274 K, with photoexcitation of the protein containing fully oxidized FAD (the physiologically relevant dark state in plant and animal cryptochromes) at 460 nm. The spectral shape (figure 11) unequivocally assigns the photoproduct as a radical pair rather than flavin triplet (which would produce a much broader signal [99]). Although only able to provide a lower bound on the lifetime, the time evolution of the TREPR signal indicates that the radical pair state of XICry-DASH persists and remains spin polarized for at least 6 $\mu$s, exceeding the minimum lifetime requirement for a MFE arising from the Earth’s weak magnetic field.

The TREPR spectra detected in the XICry-DASH system closely resembled those obtained from photoreduction of FAD in EcPL that were assigned to the [FADH$^+$ + W306$^*$] radical pair, where W306 is the terminal Trp residue (Trp$_C$) of the Trp triad introduced above. On the basis of the high sequence conservation in PHR domains, the radical pair in XICry-DASH was assigned to [FADH$^+$ + W324$^*$], where W324 is Trp$_C$ in this protein and the conclusion supported by mutagenesis studies [97]. It might seem tempting to assume that all members of the cryptochrome family possessing this conserved Trp triad will form radical pairs between Trp$_C$ and the FAD cofactor. However, that this is not generally the case shall be illustrated...
with the example of the Cry-DASH from the cyanobacterium \textit{Synechocystis} \textit{sp.}, in which the W320F mutation replaces the TrpC residue \cite{100}. This mutated photoexcited sample showed a TREPR signal almost identical to the wild-type protein, hence TrpC cannot be an integral part of the electron transfer chain in this case. Further mutant studies identified residue W375 (hereafter denoted TrpC') as the terminal electron donor and TrpB as a crucial intermediate in the electron transfer pathway (figure 12). While it is not unexpected that TrpB knockout (W375F) removes the observable radical pair signal, it is surprising that the TrpC' knockout (W375F) has the same effect. This indicates that TrpC', rather than TrpC, takes on the role of ultimate electron donor despite TrpC' being further apart from TrpB than TrpC from TrpB. Additionally a [FAD$^-$ + TrpC'] radical pair will not be stabilized in this protein \cite{100}. Further details of the variable electron transfer pathways in cryptochrome proteins have been elucidated and are discussed elsewhere \cite{96}.

Although Ahmad, Bittl and co-workers \cite{87} conducted continuous-wave (cw) EPR of photoexcited FAD$^{\cdot\cdot}$ in human and insect cryptochrome under steady-state illumination conditions, and hence could not detect any SCRPs, they were able to observe radical signals. Further investigations using electron nuclear double resonance let the authors conclude that the radical observed can be assigned to FAD$^*$ in contrast to the situation in plant cryptochromes in which the neutral flavin radical is accumulated on illumination \cite{85,86,101,102}.

The radicals formed by intramolecular electron transfer within the cryptochromes exhibit characteristic, if overlapping, absorption bands (figure 4). These have been used extensively to confirm the formation of radicals following photoexcitation of the cryptochromes although no information can be gained regarding the spin correlation within the pair of radicals. Of particular value, however, are flash-photolysis-based studies which allow a quantification of the radical kinetics. The discussion of the light-induced electron transfer in AtCry1 using flash photolysis studies by Giovanni et al. \cite{101} serves as an example of the powerful capabilities of this technique. In their work, the authors were able to show that photoexcitation of FAD$^{\cdot\cdot}$ in AtCry1 results in the formation of FADH$^*$ and Trp$^*$ radicals, followed by an electron transfer from a tyrosine to the Trp$^*$ radical and subsequent back-electron transfer from the FADH$^*$ to the tyrosyl radical.

A TA spectrometer using a streak camera was used by Kottke and co-workers \cite{103} to obtain microsecond time resolution on the light-induced processes of a plant cryptochrome (\textit{Chlamydomonas reinhardtii} CPH1-PHR). A global analysis of their time and wavelength-resolved data allowed the authors to conclude that photoinduced electron transfer from tryptophan to FAD$^{\cdot\cdot}$ occurs in less than 100 ns leading to the formation of the flavin anion radical with a time constant of 1.7 $\mu$s. The latter then undergoes protonation to FADH$^*$ and finally both radicals decay with a time constant of greater than 200 $\mu$s.

It has proved particularly difficult to express cryptochrome from migratory birds in sufficient yield, concentration and purity to allow many detailed analyses by EPR or most other spectroscopic techniques. Nevertheless, using an insect cell (S9) expression system sufficient garden warbler cryptochrome (\textit{Sylvia borin}, both full-length CRY1a and the PHR domain of CRY1a alone) was obtained to allow TA measurements \cite{104}. Excitation at 355 nm generated a transient species with an absorption peak at 510 nm, attributed to a flavin radical with a lifetime of the order of tens of milliseconds. Yet, as in all zero-field optical spectroscopy, no information on the vital spin correlation of the radical pairs can be obtained which emphasizes again the need to perform a field effect study on these proteins—the only technique which can conclusively test for magnetosensitivity of any observed radicals.

5. Magnetic field effects in flavoproteins

5.1. Measurement of magnetic field effects in cryptochrome family proteins

To date, MFEs have been measured in two members of the cryptochrome family of proteins: EcPL \cite{54,55} and AtCry1 \cite{55}. In both cases, the experiments were performed by TA using approximately 250 $\mu$l volumes of protein, at 250 K, in a glycerol–water mixture (60% and 50% v/v for AtCry1 and EcPL, respectively) and following photoexcitation at 460 nm. As in the TA measurements on garden warbler cryptochrome discussed above, it was possible to identify formation of FAD and Trp radicals from characteristic optical absorption bands at less than 420 nm and greater than 500 nm and to identify depletion of the FAD ground state at 460 nm (figures 13a,b) following photoexcitation. The main differences between the two spectra are (i) the rapid decay of the absorption signal below 420 nm for AtCry1 and (ii) the pronounced time dependence of the absorption profile in the range 500–650 nm for EcPL. The former is most likely owing to the protonation of FAD$^{\cdot\cdot}$ to FADH$^*$ in agreement with Kottke’s observation of flavin radical

\[ \text{FAD - TrpA - TrpB - TrpC} \]

\[ \text{FAD - TrpA - TrpB - TrpC'} \]

\[ \text{FAD - TrpA - TrpB - TrpC} \]
protonation in the cryptochrome from *C. reinhardtii* [103]. The change in the signal shape found for *EcPL* in the range 500–650 nm is ascribed to deprotonation of the TrpH$^{+}$ radical to form the neutral tryptophan radical. Both processes, the deprotonation of the TrpH$^{+}$ radical in *EcPL* and the protonation of FAD$^{+}$ in AtCry1 compete with recombination of the initially formed radical pair [FAD$^{+}$ + TrpH$^{+}$], henceforth referred to as RP1, and are complete in approximately 10 µs.

Furthermore, the difference of the full-absorption spectra recorded in the presence and absence of a 28 mT magnetic field (figures 13c, d) demonstrate that in both proteins, the magnetic field enhances the recovery of the FAD ground state and reduces the transient yield of both radicals providing conclusive proof that the radical pair is formed in an initial singlet state. The MFE spectra for the two proteins are strikingly similar, although the signal at less than 420 nm shows an additional decay in the AtCry1 case, consistent with the protonation of the radical anion FAD$^{+}$ to the neutral radical FADH$^{-}$.

Finally, the magnetic sensitivity of both photoexcited proteins is clearly displayed in figure 14, which depicts the TA kinetics recorded for AtCry1 (figure 14a) and *EcPL* (figure 14c) at 510 nm (where all involved radical species have significant absorptions) in the absence (blue) and presence (red) of a 28 mT field. Again, the data for the two proteins are broadly similar showing a rapid rise in absorption followed by a biexponential decay. The initial rapid drop in absorption can be ascribed to the transformation of the initial formed radical pair, to [FADH$^{+}$ + Trp$^{+}$] for AtCry1 and [FAD$^{+}$ + Trp$^{+}$] for *EcPL*. These latter radical pairs shall henceforth be referred to as RP2. The drop in radical absorption in the presence of the magnetic field in both proteins reflects the decrease in radical concentration owing to the field-induced retardation of the ST mixing.

### 5.2. Photocycles and downstream signalling of *EcPL* and AtCry1

The observations detailed in the previous section have led to the proposed photoscheme in figure 15. Once formed in the singlet state, RP1 undergoes coherent spin evolution at a rate dependent on the applied magnetic field and has two possible fates: only from the singlet state, it may undergo a back-electron transfer such that the radical pair recombines to the ground state or from either spin state it may undergo a change in protonation state to form the long-lived RP2. Only the former process is spin selective but together the two competing pathways confer a magnetic field dependence on the lifetime of RP1 and yield of RP2.

Although the MFE can be observed as a reduction in radical yield during the slow decay of RP2, its magnitude remains seemingly constant throughout this time (figure 14), potentially providing a longer-lived readout to which any subsequent signalling pathway may respond. This is crucially important as the conformational changes likely to be needed to generate the biological signalling start at the state of the magnetoreception signalling pathway are unlikely to occur within the limited lifetime of RP1. This requirement for a long-lived state could not be met simply by a system in which the lifetime of the radical pair undergoing magnetically sensitive reaction was significantly longer: were the

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**Figure 13.** TA spectra and magnetic-field action spectra of AtCry1 (a,c) and *EcPL* (b,d). TA spectra, $\Delta A(0)$, of (a) AtCry1 and (b) *EcPL*. $\Delta A(0)$ is the difference between signals recorded with and without a 460 nm, 5 ns pump light pulse in the absence of an applied magnetic field. The spectra were integrated over 1 µs periods centred at the indicated times after the pump pulse. Each spectrum is the average of two transients. The laser repetition rate was kept low (0.05 Hz) to minimize protein photodegradation and allow for sample reoxidation. The 1.5 µs signal in (b) at 460 nm is distorted by a transient effect of the laser pulse on the photomultiplier detector. Experimental conditions: AtCry1, 250 K, 60% (v/v) glycerol/water solution; *EcPL*, 250 K, 50% (v/v) glycerol/water solution. (c,d) Magnetic-field action spectra of AtCry1 and *EcPL*, respectively, recorded under the same conditions as (a,b), presented as $\Delta A(28 \text{ mT}) - \Delta A(0)$. Two transients each were recorded in the absence and presence of a 28 mT magnetic field. At each wavelength, the double-difference kinetic time profiles were smoothed with a 2 µs boxcar function and the mean and standard deviation calculated over the indicated time intervals. At each wavelength, the mean ± s.d. is plotted. Adapted from [55]. Copyright © 2011, National Academy of Sciences.
 lifetime of RP1 to be extended, spin decoherence would occur prior to radical recombination thus eliminating the magnetic sensitivity.

It is interesting to note that the proposed photoscheme is based upon generation of a SCRP in the singlet state. This is in contrast with the free flavin systems described above, which form intermolecular SCRP's in the triplet state after ISC from the directly formed excited flavin singlet. This difference in precursor spin state was first proven by the reduction in radical yield and ground state bleaching observed in the presence of an applied magnetic field [54,55] and later confirmed by TREPR data and their simulation [97,98,100]. This result helps to verify that the FAD cofactor in the expressed protein is correctly bound allowing the necessary environment for direct singlet radical pair formation on a timescale that competes effectively with ISC. Direct formation from the excited singlet state probably increases the quantum yield of the radical pair compared to a hypothetical situation proceeding via a triplet precursor; rapid electron transfer (1FAD* + TrpH+ → 1[FAD– + TrpH.]) will compete effectively with processes quenching the singlet excited state, for instance direct fluorescence, whereas competition from slow ISC to the triplet state is likely to be less effective.

The nature of the biological environment in which animal cryptochromes may be found in vivo is not well characterized, yet it is reasonable to speculate that in the case of avian cryptochrome, the protein may be found in an environment providing some degree of immobilization and alignment [11] (simulations have attempted to quantify the degree of disorder that can be tolerated [105,106]). The use of glycerol–water mixtures to reduce molecular motion (stabilize the protein against aggregation) for in vitro experiments therefore seems justified. Through varying the glycerol concentration and temperature, further information on how the environment influences the magnitude of the MFE was obtained. For EcPL, the MFE on the yield of RP2 (observed at 510 nm) was shown to vary linearly with the lifetime of RP1 (measured at 600 nm) [55]. This reflects the importance of the kinetics of the deprotonation of TrpH+ to Trp+, the spin-unselective conversion of RP1 to RP2 that competes with the spin-selective recombination of RP1. Assuming some evolutionary optimization of the protein environment will have occurred, it is therefore reasonable to imagine that the biological environment could be tailored to maximize any MFE.
5.3. The significance of inter-radical interactions

The discussion presented above has focused nearly exclusively on the interplay of Zeeman interaction and hyperfine coupling in determining the efficiency of interconversion between the singlet and triplet states. However, if the radicals are spatially close (less than 3 nm), the ST evolution of the pair is also affected by interactions of the two electron spins: the dipolar (through space) interaction, $D$, and the scalar interaction, $J$, which splits the triplet and singlet manifolds even at zero field (figure 1a). It has been shown that both interactions can significantly quench the magnitude of the LFE [107] especially for fields as weak as that of the Earth. Based on a point dipole approximation, the interspin distance between the FAD and TrpC radicals is computed to be as large as $D = -360\,\mu$T whereas simulations of TREP spectra of XICry-DASH have allowed the value of the scalar interaction to be determined as $J = 73\,\mu$T [98]. The magnitudes of the two interactions are therefore related by $D \approx -3J$, a condition under which quantum mechanical simulations have shown that the effects of exchange and dipolar interactions partially cancel. [108]. This could allow a significant effect of an approximately $50\,\mu$T magnetic field even with non-negligible radical–radical interactions.

5.4. Predicting the response to low magnetic fields

The majority of the discussion thus far has focused on the effects of relatively strong magnetic fields of up to $28\,\text{mT}$. Importantly, the data in figure 16 indicate the existence of an LFE even though the signal-to-noise does not allow for a full quantification of the effect. Theoretical analysis of the overall shape of the MFE does, however, offer some further insight into the likely effects of weaker fields. The shape of the field profiles is usually quantified by the so-called $B_{1/2}$ value, the field at which the MFE reaches half saturation [109]. For both EcPL and ATcry1, the experimental $B_{1/2}$ value exceeds the theoretically estimated one significantly unless an additional spin-decoherence process is taken into account. This is attributed to modulation of the electron exchange interaction in RP1 by rapid reversible electron hopping between TrpB and TrpC. As shown in the simulations in figure 16, slow ST dephasing (STD) is required for the evolution of significant LFEs. Given the TREPR evidence for an unexpected alternate electron transfer pathway in *Synechocystis* Cry-DASH [100], it may be that minor changes in the protein sequence of avian cryptochrome alter the electron transfer rates so as to reduce the STD rate thus conferring the predicted greater sensitivity to the Earth’s strength magnetic fields.

As with most MFE studies to date, the data discussed above were collected using conventional TA techniques, which, owing to their inherently low sensitivity, require forbiddingly large quantities and concentrations of these precious biological samples. A major disadvantage of the technique is the photoreduction of the sample necessitating long waiting times between flash photolysis shots to allow for sample reoxidation. By way of example, the data in reference [55] was painstakingly collected using 250 $\mu$L samples in a 10 mm optical path-length cell excited by a 1 mJ laser pulse at the low repetition rate of 0.05 Hz. Hence, to allow further investigations more sensitive methodologies are required. One such development is the application of CRDS to MFE studies as introduced in §2.6 [67]. In proof-of-principle demonstrations this allowed the MFE on EcPL to be measured.
using a 10 Hz repetition rate and only approximately 4 µl (effective volume) of sample in a 1 mm optical path-length cell (figure 17).

The CRDS technique is not without drawbacks; temporal information is obtainable only by repeated experiments utilizing different pump-probe delay times and spectral information is sacrificed through use of a monochromatic probe beam. The latter problem can be overcome by the related technique of cavity enhanced absorption spectroscopy (CEAS) in which continuous-wave probe and excitation beams are used. Although CEAS provides limited temporal information, it has been applied successfully to detect MFEs [68] and recent developments of a broadband variant offer scope for obtaining full spectral information [110]. For future studies of cryptochromes, it is likely that a combination of cavity-based techniques will be applied to allow investigation of a greater number of proteins with sample requirements more appropriate for biological species.

6. Conclusions and future directions
In this paper, we have reviewed MFEs measured on FAD alone, on flavins in solution with tryptophan, indole or proteins and in cryptochrome family proteins. Flavin-based radical pairs in solution are created in the initial triplet state, whereas those formed intramolecularly within cryptochrome family proteins are generated in an initial singlet state. We have highlighted very recent developments in the understanding of MFEs in cryptochrome proteins, particularly those which suggest how magnetically sensitive information can persist for long enough to be integrated into cellular processes. On the basis of the evidence currently available, it appears likely that most, if not all, members of the cryptochrome family are capable of forming the SCRPs necessary to exhibit magnetosensitivity. Owing to limited sample availability, it has not yet been possible to verify the existence of MFEs in avian cryptochromes, although evidence for radical formation has been obtained. Observation of reproducible field effects on radical pairs and their kinetics in particular in fields as weak as that of the Earth, thus depends on reliable protein synthesis and purification routes as well as the development of more sensitive spectroscopic techniques with recent advances in cavity-based spectroscopic techniques offering a viable alternative.

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