

Exploring the photochemistry and photosignalling mechanisms in cryptochromes and related model systems



Katherine Buckton
New College
University of Oxford

A thesis submitted for the degree of
Doctor of Philosophy
Trinity 2019

This thesis is dedicated to those lost along the way:
To Grandad, Grandma, Grandpa and Mummo,
I hope I've made you proud.

Acknowledgements

There are so many people without whom I wouldn't have made it through the past four years. Thank you, Stuart, for all your support and advice throughout my DPhil, I honestly couldn't have asked for a better supervisor. Thank you for keeping me on track when I'd lost my way, for making time for me and for making the group such a great place to work. It has been a privilege to be a part of the Mackenzie group and I'll always look back on these times warmly. Chris, thanks for providing me with such a wonderful time at Oxford, from the first tutorial right the way through my DPhil. Thank you for encouraging me to have confidence in myself when I didn't, and for supporting me through some of the hardest times of this DPhil. Your advice, both academic and personal, has helped me more than you probably realise. Peter, thank you for your truly invaluable knowledge, thoughtful insights, and for all the amazing opportunities that you have offered me over my DPhil. I feel very lucky to have had such brilliant supervisors.

I am forever indebted to the incredible people I have collaborated with over this DPhil. To Erik Schleicher, Ryan Rodriguez and everyone else I had the pleasure of meeting in Freiburg, thank you for making me feel so welcome, for imparting so much wisdom on cells and proteins, and for providing the proteins for a large number of the experiments in this thesis. A massive thank you to Justin Benesch and Shane Chandler for the introduction to HDX and for advancing this project in such a brilliant way. Specific thanks to Shane for spending time teaching me the experiment and for all his hard work throughout our project. Thanks to Victoire for the collaboration and her amazing work on the fluorescence experiment, and to Sabine, for the help and advice redesigning the fluorescence experiment. A big thank you also to Kevin for his knowledge on pretty much everything going on in the lab, and for his kindness and continued support throughout the duration of my DPhil.

Emrys and Lauren, thank you for introducing me to the world research during my Part 2 and making it enjoyable enough that I decided to stay for a DPhil. Emrys, thank you for your introduction to fluorescence and 23-19, and for your patience and help teaching me the ropes; Lauren, thank you not only for supervising me in my Part 2 but also for your continued support during this DPhil with the thioinine work.

Tilo, thanks for being the best lab and (bird) office companion I could've asked for (it has been lonely without you for the final year! Our lemon tree suffered!).

Alice, thanks for all the great times in the office and at tea, and for all the crazy hat making ventures we have been on! Dean, Ethan, Jochen, Jess, Marcin and all the other members of the Mackenzie/Timmel group, past and present, this experience would not have been the same without you. Thank you for your dedication to tea breaks and for making the office such a lovely place to work.

Gareth, I don't think I could have got through the last year without you. Thank you for bringing me endless cups of tea, for making me laugh when I was at my lowest points and for keeping me sane throughout this journey.

Thanks Al for the surprise packages of tea and flying saucers to help me through late night writing sessions. You're amazing and I couldn't ask for a better brother. Mum and Dad, thanks for all the love and support you've given me over the years, and for providing me with such amazing opportunities. Dad, thanks for bouncing ideas around with me when I was stuck for inspiration and for keeping me motivated, and thanks Mum for providing so many levels of encouragement and support (ranging from emotional to the TD variety). I love you all so much and could not have done this without you.

Abstract

The ability of migratory birds to sense and use the Earth's magnetic field during migration is hypothesised to involve cryptochrome proteins located in their retinas. It is proposed that photo-initiated reactions within these proteins generate radical pairs (RPs) as transient intermediates, which have product yields that are sensitive to the magnitude and direction of the external magnetic field, and ultimately allow birds to visualize the geomagnetic field. Cryptochromes are currently the only candidate chemical compass magnetoreceptor for this radical pair mechanism (RPM)-based hypothesis (Chapter 1) and thus it is important to take steps towards more fully understanding the photochemistry and photosignalling mechanisms occurring.

This thesis has two main themes. Firstly, a highly sensitive fluorescence-based spectroscopic technique has been developed to further increase sensitivity and make it more suitable for studying precious biological samples. Whilst many (absorption-based) spectroscopic techniques have investigated magnetic field effects (MFEs) on isolated cryptochrome proteins, these experiments have typically been performed *in vitro* and thus are still very far removed from biological conditions. This thesis describes the first attempts towards studying cryptochromes in living cells and thus observing an MFE *in vivo* (Chapter 2). A very small, negative MFE is observed, however this was attributed to the RP chemistry occurring in the growing medium rather than the cryptochromes. The exquisite sensitivity of the experiment is still highlighted, however, and the re-designed fluorescence technique is further used to explore the photochemistry of RPs in restricted environments (micelles) to investigate how altering the RP environment can affect the photochemistry of a system (Chapter 3). A negative MFE is observed in a thionine/DABCO system which is vastly increased when the system is constricted to an SDS micelle. The relationship between quencher concentration and MFE was shown to be biphasic, with dynamic quenching changing to static quenching with increasing DABCO concentration, and the importance of the micelle environment was demonstrated as altering the size or charge of the micelle affected the MFE.

Secondly, the relationship between the photochemistry and signal transduction mechanism in cryptochromes has been explored. Currently, very little is known about the photosignalling pathway in cryptochromes (specifically animal cryptochromes), however one widely held model proposes the involvement of a light-induced conformational change which initiates the signalling cascade. By using a

technique known as Hydrogen Deuterium Exchange Mass Spectrometry (HDX-MS), a blue-light induced conformational change has been observed on *DmCry* WT and been localised to a region of the protein known as the C-terminal tail (CTT). Further, by comparing the behaviour of *DmCry* WT with that of its mutants, *DmCry* W342F and W394F, in which the electron transfer (ET) chain is interrupted at the third and fourth tryptophan position respectively (by replacing the tryptophan with a redox inert phenylalanine), altering the photochemistry has been shown to have a direct effect on the magnitude of the conformational change, highlighting the importance of these tryptophans for the signalling state of the protein (Chapter 4).

Contents

List of Abbreviations	xiii
1 Introduction	1
1.1 Magnetoreception	2
1.1.1 Magnetite hypothesis	2
1.1.2 Radical Pair Mechanism (RPM) hypothesis	3
1.1.3 Evidence for a magnetic sense in birds	4
1.2 Cryptochromes	6
1.3 Photosignalling in cryptochromes	11
1.4 Spin Chemistry	12
1.4.1 Zeeman Interaction	13
1.5 Coupled spins: Radical Pairs	14
1.5.1 Exchange interaction	16
1.5.2 Dipolar interaction	18
1.5.3 Hyperfine interaction	18
1.6 Singlet-Triplet Mixing	20
1.6.1 Δg mechanism	20
1.6.2 Hyperfine mechanism	21
1.6.3 Relaxation mechanism	24
1.7 Radical Pair Mechanism	25
1.8 Magnetically Altered Reaction Yield (MARY)	27
1.9 Scope of thesis	29
2 Prompt Fluorescence MARY: Re-design and Initial Application	31
2.1 Introduction	31
2.2 Fluorescence and MFEs	32
2.3 Basis of PF MARY	33
2.4 Former PF MARY setup and limitations	35
2.4.1 Collection Efficiency	36
2.4.2 Photodegradation	38
2.5 Modifications and Improvements	40
2.5.1 Collection Efficiency	40

2.5.2	Minimising volume	43
2.5.3	Other modifications/improvements	45
2.6	Allan Deviation	47
2.7	Measuring MFEs by Fluorescence	50
2.7.1	Materials	50
2.7.2	Data Interpretation	50
2.8	Early attempts at observing an MFE on <i>DmCry</i> in <i>in vivo</i>	54
2.8.1	Difficulties of working with <i>DmCry</i> in Sf9 cells	55
2.8.2	Materials	56
2.8.3	Experimental	57
2.8.4	Results and Discussion	58
2.9	Conclusions and Future Work	62
3	Prompt Fluorescence MARY: Photochemistry of RP reactions in micelles	65
3.1	Introduction to Micelles	66
3.1.1	Structure of micelles	66
3.1.2	Hydrophobic Effect	69
3.2	Materials	71
3.3	Experimental	71
3.4	Thionine/DABCO/SDS: LFE and effect of a micelle	73
3.4.1	Photochemical scheme	73
3.4.2	MARY plots	76
3.5	Photoexcitation power dependence	80
3.6	DABCO concentration dependence	81
3.7	Effect of micelle size and charge	84
3.7.1	Micelle Charge	84
3.7.2	Micelle Size: SDS <i>vs.</i> SDeS <i>vs.</i> SOS	87
3.8	Thionine/aniline/SDS	88
3.8.1	Heavy atom effect on the thionine/aniline/SDS MARY curve	90
3.9	Conclusions	91
4	Photosignalling in <i>DmCry</i>	95
4.1	Introduction	96
4.2	Partial Proteolysis	98
4.2.1	Experimental	98
4.2.2	Preliminary results	99
4.3	Hydrogen Deuterium Exchange Mass Spectrometry	102
4.4	Experimental	104
4.4.1	Materials	104

4.4.2	Hydrogen-Deuterium Exchange Mass Spectrometry	104
4.4.3	Data Analysis	108
4.5	Results	108
4.5.1	Dark state dynamics of <i>DmCry</i> WT	108
4.5.2	Blue-light induced conformation changes in <i>DmCry</i>	110
4.5.3	<i>DmCry</i> W342F and <i>DmCry</i> W394F	116
4.6	Conclusions	121
4.6.1	Future work: searching for MFEs	121
5	Conclusions	125
	References	129

List of Abbreviations

CCD	Charge coupled device
CEAS	Cavity enhanced absorption spectroscopy
CMC	Critical micelle concentration
CRDS	Cavity ring-down spectroscopy
CTT	C-terminal tail
DABCO (or D)	1,4-diazabicyclo[2.2.2]octane
<i>DmCry</i>	<i>Drosophila melanogaster</i> cryptochrome
ED	Electron donor
ET	Electron transfer
F	Phenylalanine
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
HDX-MS	Hydrogen-deuterium exchange coupled with mass spectrometry
HEWL	Hen egg white lysozyme
ISC	Intersystem crossing
LED	Light emitting diode
LFE	Low field effect
LOVE	Light Oxygen Voltage
MARY	Magnetically altered reaction yield
MFE	Magnetic field effect
N_{agg}	Aggregation number
PC	Personal computer
PF	Prompt fluorescence
RF	Riboflavin
RP	Radical pair

RPM		Radical pair mechanism
SCRIP		Spin correlated radical pair
SD		Standard deviation
SDS		Sodium dodecyl sulfate
SDeS		Sodium decyl sulfate
SNR		Signal-to-noise ratio
SOS		Sodium octyl sulfate
ST		Singlet-triplet
TA		Transient absorption
TH		Thionine
W		Tryptophan
WT		Wild type

1

Introduction

The ability of some animals, in particular migratory birds, to extract directional information from the Earth's magnetic field and utilise this for navigation has long been of interest to the scientific community [1–3]. This phenomenon, known as magnetoreception, has been observed in over 50 different species, however the exact biophysical mechanisms that underpin it are still not fully understood [4–6].

One of the current leading hypotheses proposed to explain animal magnetoreception is based on cryptochrome proteins, and suggests that reactions occurring in these cryptochromes, involving radical pairs (RPs) as transient intermediates, may act as the primary magnetic sensor [7, 8]. This theory, based on the Radical Pair Mechanism (RPM), provides a plausible explanation of how extremely weak magnetic fields, even those on the order of magnitude of that of the Earth ($\sim 50 \mu\text{T}$), can have a measurable effect on the reaction yields of chemical reactions.

The following few sections of this introduction explain the background of magnetic sensing, the proposed involvement of cryptochrome and the suggested mechanism of signal transduction in these proteins. The rest of the chapter is devoted an explanation of the fundamental chemistry underlying the photochemical mechanisms believed to occur in systems such as cryptochrome, introducing concepts such as spin, the RPM and the mechanisms of singlet-triplet (ST) mixing, provid-

ing a basis for understanding the photochemistry and photosignalling processes discussed in this thesis.

1.1 Magnetoreception

The suggestion that migratory birds may use the Earth’s magnetic field for navigation (amongst other factors such as the sun, stars and olfactory cues) can be dated back to as early as the 19th century [1]. It was not, however, until a behavioural study on European robins (*Erithacus rubecula*) by Wiltschko and Merkel in 1966 that the first convincing evidence that birds do indeed have a magnetic compass sense came to light [2]. Since then, many other birds and animal species have been shown to have a similar geomagnetic compass, which relies on the inclination rather than the polarity of the field, and there has been much research attempting to characterise the methods by which this compass works [3, 9–15].

Currently there are two leading hypotheses proposed to explain the mechanism of magnetoreception: i) the magnetite (Fe_3O_4) hypothesis [16, 17] and ii) the RPM [7, 18]. The former relies on clusters of ferromagnetic particles (magnetite) to be the principle sensory molecule; the latter suggests the involvement of magnetically sensitive photo-induced RPs.

1.1.1 Magnetite hypothesis

The magnetite hypothesis proposes crystals of magnetite, found in the beak of certain species of birds, interact with the Earth’s magnetic field. Larger particles of magnetite (~ 50 nm) possess magnetic moments sufficiently large that they can align with the Earth’s ~ 50 μT magnetic field. Smaller particles (~ 20 nm), by contrast, are superparamagnetic and are known to develop a magnetic moment within a magnetic field and may attract or repel one another depending on the orientation of the external field [16, 19, 20]. Both cases result in the movement of magnetite crystals which may be detected by mechanoreceptors or by acting to open or close ion channels, for example. In short, the Earth’s magnetic field

is proposed to trigger a deformation of the magnetite crystals which brings about a neuronal response [16, 21, 22].

On the face of it, this explanation is appealing as magnetite crystals align themselves with the external magnetic field just like the needle of a compass. However, evidence for the magnetite hypothesis stemmed from the discovery of iron-rich compounds in the beaks of birds, but it has now been suggested that these iron-rich clusters are macrophages rather than magnetically-sensitive neurons [23]. Furthermore, the magnetite theory proposes that, following detection in the upper beak, transmission of the magnetic signal goes via the opthalmic branch of the trigeminal nerve to the brain [21, 24, 25]. However, Mouritsen *et al.* have shown that the magnetic sense of European robins is unaffected when the trigeminal nerve is cut, suggesting that this mechanism may not be as valid as it first appears [26]. More significantly, however, the magnetic compass has been shown to be light dependent (as discussed in Section 1.1.3) which is not easily explainable by the magnetite hypothesis.

1.1.2 Radical Pair Mechanism (RPM) hypothesis

The second hypothesis is based on the RPM and proposes reactions involving spin-correlated radical pairs (SCRPs) to be the principal basis of the geomagnetic compass. In such systems, the reaction rates and/or product yields of reactions involving SCRPs are modulated by an external field through variation of the ST mixing efficiency.

This RPM-based hypothesis dates back to 1978 when Schulten proposed that a chemical reaction could hypothetically yield directional information on the Earth's magnetic field and thus form the basis of the magnetic compass in magnetoreception [27]. In this seminal paper, Schulten laid out the prerequisite characteristics that any sensory molecule need possess in order to be involved in magnetoreception. The paper suggested that the photochemical process occurring in birds could involve RPs as intermediates and that the sensory molecule would be sensitive to the intensity and, crucially for a compass, the direction of the applied magnetic field. At the

time of publication the RPM (see Section 1.7) was a relatively new concept and the paper did not attract serious interest until many years later.

1.1.3 Evidence for a magnetic sense in birds

The Emlen funnel was invented in the 1960s to monitor the migratory orientation of birds, and since then has been used to provide much evidence in support of the RPM hypothesis [28]. The setup is shown in Figure 1.1A and is simple, yet effective: birds are placed in a cone lined with scratch-sensitive paper and their responses to applied magnetic fields are recorded by the position and number of scratches on the paper.

Figure 1.1B shows the results from a set of experiments performed by Zapka *et al.* [26] where European robins were studied in Emlen funnels in the natural magnetic field (NMF) and in a magnetic field with geomagnetic north turned horizontally 120° anticlockwise (CMF). Birds were captured during the autumn migratory season and tested the following spring migratory season. Each filled circle in Figure 1.1B represents the mean orientation of an individual bird for the given field conditions. The average orientation for the birds clearly correlate with the magnetic field direction: the birds show oriented magnetic behaviour, providing evidence for a magnetic compass sense. Emlen funnel experiments have been performed on a number of other independent occasions, and results have consistently shown a statistically significant correlation between the birds' orientation and the direction of the applied magnetic field [2, 29, 30].

In 1996, Wiltschko and Wiltschko used this technique to show migratory birds to have an inclination compass (just as Schulten had hypothesised) whereby they can distinguish the axis rather than the polarity of magnetic field lines (*i.e.* they cannot differentiate between north and south, but only between pole-wards and equator-wards) [3].

Further, it was shown that the ability of birds to orientate using the Earth's magnetic field was dependent on the wavelength of ambient light. Studies on the European robin showed that under blue (443 nm) and green (565 nm) light the birds were able to magnetically orientate; under red (633 nm) or yellow (590

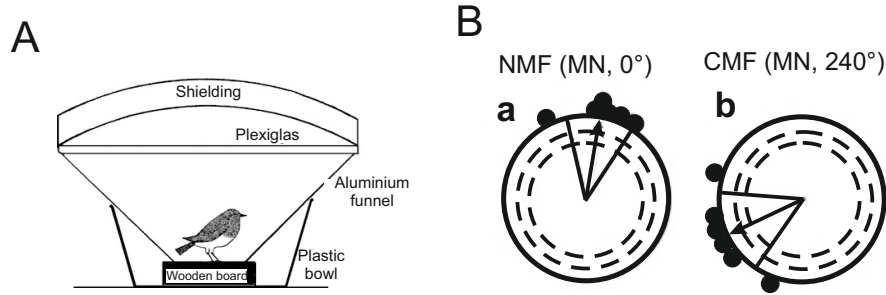


Figure 1.1: **A** Diagram depicting the side view of the original Emlen funnel. A bird is placed in the centre of the funnel on top of an ink pad on the bottom of the cage. The walls of the cage are covered with white blotting paper and so a bird jumping on the walls leaves ink-marks (or alternatively scratch-sensitive paper can be used). This figure is reproduced from Wiltschko *et al.* [31]. **B** The orientation of European robins (*Erithacus rubecula*) studied in Emlen funnels under a) natural magnetic field conditions (NMF), and b) in a magnetic field rotated 240° clockwise (CMF). Each circle represents the mean orientation of a bird over 9 separate experiments, and the arrows represent the group mean vectors. The cones flanking this vector mark the 95% confidence interval for the group mean direction. A clear magnetic-orientation is observed. This figure is reproduced with permission from Zapka *et al.* [26].

nm) light, however, they appeared disorientated and showed no signs of alignment with the geomagnetic field [32–34]. Crucially, this means that the mechanism for magnetoreception is not only light-dependent (suggesting the mechanism may operate in the eye) but also requires a certain wavelength of light (*i.e.* needs energy above a certain threshold) in order to work.

The requirement for the magnetic compass to be inclination-dependent and light-dependent provides substantial support for the RPM hypothesis over the magnetite hypothesis. Although the magnetite and RPM hypotheses are not mutually exclusive (indeed, it is likely that animals may have evolved to exploit different mechanisms to detect different components of the Earth’s field), if magnetite crystals do exist in migratory birds then it is more likely that they are related to the magnetic map rather than the magnetic compass sense [35].

Birds are believed to have both a magnetic map and compass sense which are likely to have distinct mechanisms and thus different properties. For example, whilst

the map sense will have no need to be sensitive to the intensity of the magnetic field, the compass sense will. The magnetic map sense is believed to be associated with the opthalmic branch of the trigeminal nerve, V1, and it is hypothesised that iron-mineral-based compounds may form the basis of the magnetic sensor. Therefore, whilst a purely-magnetite based hypothesis is no longer deemed feasible, it is possible that magnetite particles may be the basis of the map sense and work in synergy with the RPM-based magnetic compass sense [35].

The above findings in relation to the magnetic-compass sense, however, show the compass to be inclination- and light-dependent, which led to the proposition by Ritz *et al.* that the blue-light photoreceptor molecule cryptochrome could be the primary sensory molecule involved in the magnetic compass in birds [7].

1.2 Cryptochromes

Cryptochromes are blue-light photosensory molecules that belong to a family of flavoproteins including photolyases, Light Oxygen Voltage (LOV) domains and Blue-Light sensor Using FAD (BLUF) proteins [36, 37]. All these proteins contain a flavin cofactor and regulate biological functions in response to blue light.

Flavins play important roles in biology due to their ability to adopt multiple redox states (reduced, semiquinone, oxidised) under physiological conditions [38]. As shown in Figure 1.2, all flavins share an isoalloxazine ring but differ by the identity of the ribityl side chain. The nature of the ribityl side chain/R-group determines the hydrophobicity and reactivity of the compound.

Figure 1.3A shows the X-Ray crystal structure for *Drosophila melanogaster* cryptochrome, *DmCry*, which is cryptochrome from fruit fly. Cryptochromes are flavoproteins which bind a flavin adenine dinucleotide (FAD) cofactor (Figure 1.2C) shown in yellow in Figure 1.3A, and they have high sequence homology with photolyases [39]. Indeed, it is widely acknowledged that cryptochromes are evolutionary descendants of the photolyase family [40, 41]. The main difference between the two is functional: whereas photolyases are involved in the repair of photo-damaged DNA, cryptochromes have historically been linked to regulation

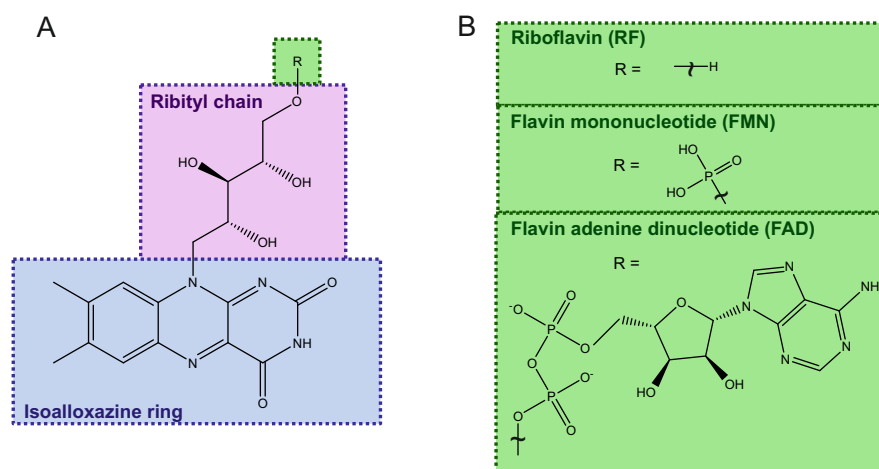


Figure 1.2: **A** General structure of a flavin, made up of an isoalloxazine ring (blue), a ribityl chain (purple) and an R group (green). **B** Structures of the R groups for riboflavin (top), flavin mononucleotide (middle) and flavin adenine dinucleotide (bottom).

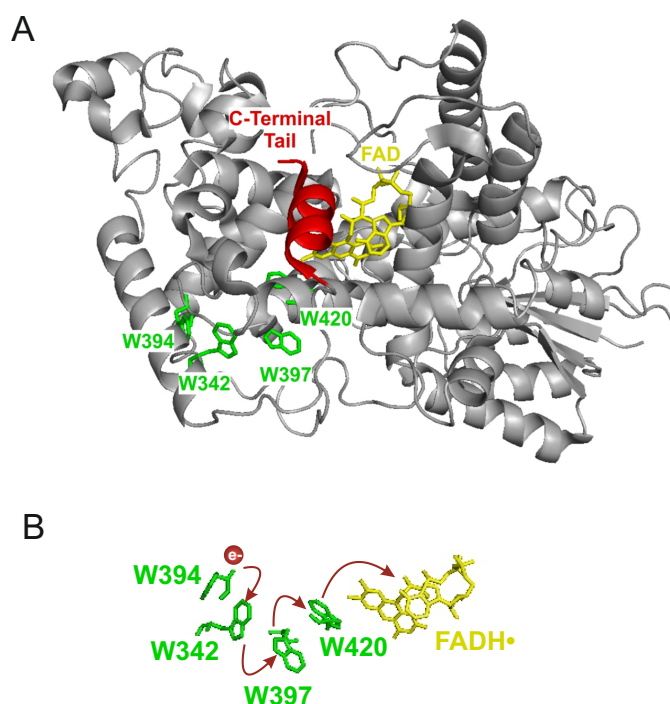


Figure 1.3: **A** *Drosophila melanogaster* cryptochrome (*DmCry*) X-ray crystal structure (PDB: 4GU5), highlighting the position of FAD (yellow), tryptophan residues (green) and the C-terminal tail (CTT) (red). It is hypothesised that a change in oxidation state of FAD leads to a conformational change centred at the CTT, increasing interactions with downstream signalling partners and inducing a signal cascade (see Section 1.3). **B** Blue light exposure results in FAD being reduced with the neighbouring tryptophan residues acting as a source of electrons to form a spin correlated radical pair (SCR) between the FAD and the fourth tryptophan (W394). W394 is close to the exterior of the protein near the α -22 helix. The RP is separated by ~ 2 nm between FAD and W394.

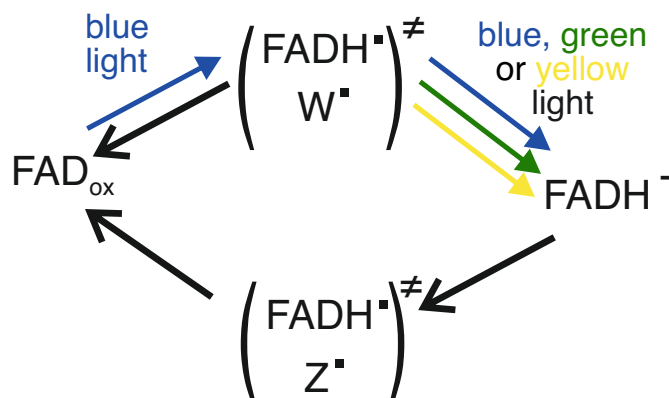


Figure 1.4: Full redox cycle of FAD. Black arrows indicate light-independent processes; coloured arrows indicate light-dependent processes. Upon exposure to blue light FAD_{ox} is reduced to $\text{FADH}^\bullet$ and the $[\text{FADH}^\bullet \text{ W}^\bullet]$ RP is formed. This may either be reduced again to form the fully reduced FADH^- which then re-oxidises back to FAD_{ox} in a light-independent reaction, or it may re-oxidise back to FAD_{ox} directly. This reoxidation is also light-independent.

of the circadian clock. More recently, however, cryptochromes have also been shown to exhibit magnetic field dependent responses (albeit under non-physiological conditions) [42] which, combined with the fact that they are present in the retinæ of migratory birds (specifically, in the double cones [43]) and are actively expressed during the migration season [44] suggests that cryptochrome is a serious candidate for the primary magnetoreceptor in the RPM hypothesis.

As shown for *DmCry* WT in Figure 1.3B, upon blue-light photoexcitation an electron cascade occurs along the neighbouring tryptophan residues (green) to the flavin (yellow) to form a flavin/tryptophan RP. In *DmCry* the ET chain comprises a tetrad of tryptophans (W420, W397, W342, W394). The photochemical cycle is discussed in detail in 1.6.3 and the importance of this tetrad to the photosignalling pathway is explored in Chapter 4.

Figure 1.4 shows the photoreduction/oxidation cycle of FAD, with coloured arrows depicting light-dependent processes and black arrows indicating processes that happen light-independently. In the dark, FAD exists in the oxidised form, FAD_{ox} . FAD_{ox} is reduced in the presence of blue light with the neighbouring tryptophan residues acting as the source of electrons (as discussed above). This

forms a flavin semiquinone and oxidised tryptophan RP, $[\text{FADH}^\cdot \text{W}^\cdot]$. Under blue, green or yellow light, $\text{FADH}^\cdot$ can be reduced further to form the fully reduced FADH^- ; however, it may also be reoxidised back to FAD_{ox} in a light-independent process. Similarly, FADH^- can also be reoxidised back to FAD_{ox} in another light-independent process [45]. During this reoxidation a second RP is involved, $[\text{FADH}^\cdot \text{Z}^\cdot]$, where $\text{Z}^\cdot$ represents a currently unknown RP partner for $\text{FADH}^\cdot$. It is hypothesised that $\text{Z}^\cdot$ may be a superoxide radical, $\text{O}_2^\cdot$, which arises from the reaction of molecular oxygen oxidising $\text{FADH}^\cdot$ back to FAD_{ox} [46] and it has been suggested that ascorbic acid could be the source of $\text{Z}^\cdot$ [47].

Whilst there is a growing body of evidence to suggest that magnetoreception relies on photochemical reactions occurring in cryptochromes, there is a lack of evidence confirming that this is specifically a result of the $[\text{FADH}^\cdot \text{W}^\cdot]$ RP *in vivo*. Although it is widely held that $[\text{FADH}^\cdot \text{W}^\cdot]$ is responsible for magnetic sensing, the redox cycle of FAD in Figure 1.4 illustrates how other RPs could be responsible for magnetic sensing in cryptochromes, notably $[\text{FADH}^\cdot \text{Z}^\cdot]$ [47]. Unlike $[\text{FADH}^\cdot \text{W}^\cdot]$, which is singlet born in cryptochrome by virtue of rapid ET, these other RPs could hypothetically be triplet born. Moreover, it is known that animals are able to magnetically orientate during the dark, however the photochemistry $[\text{FADH}^\cdot \text{W}^\cdot]$ is light-dependent; $[\text{FADH}^\cdot \text{Z}^\cdot]$ on the other hand could theoretically operate in the dark as its formation is light-independent [45, 47]. If this alternative RP, $[\text{FADH}^\cdot \text{Z}^\cdot]$, was magnetically sensitive then this could be the magnetic sensor in animal magnetoreception and explain how animals are able magnetically orient in the dark. However, there are a number of issues with the suitability of the $[\text{FADH}^\cdot \text{Z}^\cdot]$ RP for a magnetic sensor. For example, although $\text{Z}^\cdot$ has been hypothesised to be superoxide, it is still not known for certain what this species is. Currently only superoxide, $\text{O}_2^\cdot$, and dioxygen, O_2 have been suggested as candidates for Z , but if Z was indeed a superoxide radical then issues arise with extremely fast electron spin relaxation due to strong spin-orbit coupling. Similarly, if Z was dioxygen then similar issues arise due to strong zero-field splitting [47].

Cryptochrome involvement in magnetoreception

Much evidence for the involvement of cryptochrome in animal magnetoreception has arisen through studies on *Drosophila*. Behavioural assays on *Drosophila* have shown that their responses to magnetic fields in a binary choice maze are dependent on cryptochrome: cryptochrome-deficient flies do not exhibit responses to magnetic fields whereas wild type (WT) flies do [48]¹. In addition, electrophysiological experiments have shown that magnetic fields are able to modulate blue-light neuronal activation in *Drosophila* [49]; cryptochrome is known to regulate neuronal activity in flies (blue-light activation causes depolarisation and activation of *Drosophila* neurones [50]) and application of a magnetic field significantly increases this cryptochrome-dependent effect. Moreover, both the aforementioned results saw the MFE abolished when the C-Terminal Tail (CTT) is removed, but the MFE persists when the fourth tryptophan in the ET chain is replaced with a redox inert residue. The latter results are important when considering the photosignalling pathway in cryptochromes, as discussed below in Section 1.3. The above results provide direct evidence that magnetoreception in animals is dependent on the presence of cryptochrome. However, despite cryptochrome being a promising candidate for the primary sensory molecule in the RPM hypothesis [40], much of the evidence supporting its involvement is incomplete and/or unsatisfactory [51–53]. For example, although magnetic field dependent responses have recently been recorded in cryptochrome samples *in vitro*, these experiments have not yet demonstrated an anisotropic response, which is crucial for a magnetic compass sense, and further have not been carried out in *in vivo* in conditions which match a cellular environment [6, 42]. Chapter 2 introduces first attempts to observe an MFE on cryptochromes *in vivo*.

¹These results should be considered with caution, however, as recent unpublished data from Peter Hore’s group (Marco Bassetto) suggest that a response to a magnetic field may not exist in *Drosophila* afterall.

1.3 Photosignalling in cryptochromes

Over the past few years the photochemistry occurring in cryptochromes has become more fully understood [42, 53, 54]. However, what is less clear is how a magnetically induced change in the quantum yield of radicals in cryptochrome could eventually result in neuronal signals.

Photolyases are known to have a signalling method involving light-induced cyclic electron transfer (ET). Photoinduced cyclic ET from the FADH^- cofactor to a pyrimidine photodimer repairs the damaged DNA and also regenerates FADH^- for new rounds of catalysis [39, 55]. Although cryptochromes are closely related to photolyases, there is minimal evidence to suggest that they undergo a similar photosignalling process.

In contrast to photolyases, LOV and BLUF domain proteins initiate the signal transduction pathway via a light-induced conformational change. The lack of compelling evidence to suggest photosignalling in cryptochromes involves a cyclic redox reaction lead to the proposition that the photosignalling mechanism in cryptochromes may be more similar to other flavoproteins, i.e. BLUF and LOV domains, than photolyases.

In plants, the blue-light signalling functions of cryptochromes have become reasonably well understood, and photosignalling in *Arabidopsis thaliana* cryptochrome (*AtCry*) has been shown to be initiated in a similar way to BLUF and LOV domains [56, 57]. The photosignalling mechanism is induced by a light-induced conformational change (brought about by the photo-reduction of FAD) which alters the ability of the protein to bind to other proteins. By analogy to plant cryptochromes, it is presumed that photosignalling in animal cryptochromes, such as *DmCry*, may also involve a similar conformational change.

More specifically, it is presumed that this conformational change occurs at region of the protein called the C-terminal tail (CTT), which is shown in red in Figure 1.3A. Changing the oxidation state of the FAD is thought to lead to the CTT becoming more exposed, enabling the protein to interact with downstream signalling partners. This, in turn, is imagined to lead to a biochemical signalling

and amplification cascade and hence changes in the rate and/or relative timing of action potentials and/or the rate of release of neurotransmitters. An investigation of the hypothesis that a conformational change initiates photosignalling and how the photochemistry occurring in *DmCry* is linked to the signal transduction pathway is presented in Chapter 4.

1.4 Spin Chemistry

In order to understand the mechanisms behind the photochemistry occurring in cryptochromes and related RP systems, it is essential to consider spin. Spin is a type of intrinsic angular momentum of a particle; it is a purely quantum mechanical phenomenon with no classical analogue. The magnitude of the spin angular momentum vector of a particle, $\vec{s}$, is quantised according to:

$$|\vec{s}| = \hbar\sqrt{s(s+1)}, \quad (1.1)$$

where $\hbar$ is the reduced Planck's constant and s is the spin quantum number.

Particles may have half-integer spins (*e.g.* electrons, protons, neutrons, with $s = \frac{1}{2}$), in which case they are known as fermions, or integer spins (*e.g.* photons, with $s = 1$), and are known as bosons. Consequently, electrons have $2s+1 = 2$ possible spin projection quantum numbers. The values of the spin angular momentum projection quantum number, m_s , are determined by the Clebsch-Gordon series:

$$m_s = s, s-1, \dots, -s. \quad (1.2)$$

The projection of $\vec{s}$ onto a fixed arbitrary axis (the z -axis) is quantised by:

$$s_z = m_s \hbar \quad (1.3)$$

For an electron $m_s = \pm\frac{1}{2}$, and therefore the electron may adopt two spin states: either $m_s = +\frac{1}{2}$ (α or 'spin up'), or $m_s = -\frac{1}{2}$ (β or 'spin down').

As electrons are charged particles, the precession of their angular momentum gives rise to an associated spin magnetic moment, $\vec{\mu}_s$, which is directly related to $\vec{s}$ by:

$$\vec{\mu}_s = -\frac{g_e \mu_B \vec{s}}{\hbar}. \quad (1.4)$$

The value $g_e = 2.002319304$ is the dimensionless g -value for a free electron. μ_B is the Bohr magneton, the value of which is given by:

$$\mu_B = -\gamma_e \hbar = \frac{e \hbar}{2m_e}, \quad (1.5)$$

where e is the elementary charge of the electron, m_e the mass of the electron, and γ_e is the gyromagnetic ratio. μ_B is a proportionality constant relating the magnetic moment of a particle to its spin angular momentum.

1.4.1 Zeeman Interaction

In the absence of an external field, the states corresponding to different m_s values are degenerate. However, when placed in a magnetic field these states are split in energy by the Zeeman interaction as the applied field interacts with the electron's spin magnetic moment. In order to understand this phenomenon more fully, consider a classical magnetic moment, $\vec{\mu}$, in a magnetic field, $\vec{B}$. The interaction energy, E , is given by:

$$E = -\vec{\mu} \cdot \vec{B}. \quad (1.6)$$

The Hamiltonian describing the interaction of the spin angular momentum of an electron with the external magnetic field can equivalently be defined as:

$$\hat{H}_s = -\vec{\mu}_s \cdot \vec{B} \quad (1.7)$$

where $\vec{\mu}_s$ is the quantum mechanical operator for the spin magnetic moment.

Using equations 1.4 and 1.5, this Hamiltonian can be rewritten:

$$\hat{H}_s = \frac{g_e \mu_B \vec{s}}{\hbar} \cdot \vec{B} = -g_e \gamma_e \vec{s} \cdot \vec{B}, \quad (1.8)$$

which is simplified further when the field, $\vec{B}$, is quantised along an arbitrary axis, which is defined as the z -axis. In this case the Hamiltonian for the system reduces to:

$$\hat{H}_z = -\hat{\mu}_z \cdot B_0 = -g_e \gamma_e \hat{s}_z B_0, \quad (1.9)$$

where $\hat{H}_z$ is the Hamiltonian for the projection of the field onto the z -axis, $\hat{s}_z$ is the operator for the z -component of the spin angular momentum and B_0 is the magnitude of $\vec{B}$. As the eigenvalues of $\hat{s}_z$ are known ($m_s \hbar$), it follows that the energies of the system are:

$$E_z = -g_e \gamma_e m_s \hbar B_0 = g_e \mu_B m_s B_0. \quad (1.10)$$

Upon application of a magnetic field, the different m_s states are split in energy as illustrated in Figure 1.5. Following equation 1.10, the Zeeman energies of the spin states α ($m_s = +\frac{1}{2}$) and β ($m_s = -\frac{1}{2}$) are given by:

$$E_\alpha = +\frac{1}{2} g_e \gamma_e \mu_B B_0, \quad (1.11)$$

$$E_\beta = -\frac{1}{2} g_e \gamma_e \mu_B B_0. \quad (1.12)$$

The energy difference between the two m_s states, ΔE , is known as the Zeeman splitting:

$$\Delta E = g_e \mu_B B_0. \quad (1.13)$$

1.5 Coupled spins: Radical Pairs

A RP comprises two electrons spins and may be correlated or uncorrelated. A spin correlated RP refers to two radicals whose spins are mutually orientated and related in such a way that the quantum state of each radical can not be described independently; if one radical does a spin flip, the other one must do one also

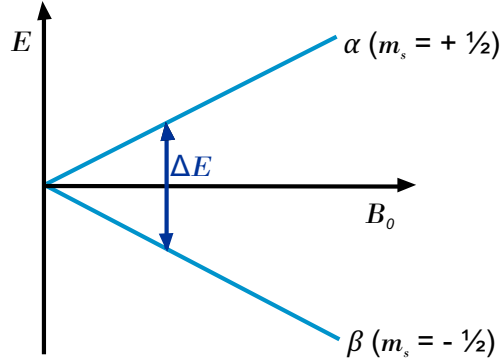


Figure 1.5: Zeeman splitting of a single electron, with the difference in energy of the two states (α and β) being the Zeeman energy and given by $\Delta E = g_e \mu_B B_0$.

in order to maintain the RP as a whole in the same state. This is common for photochemically generated RPs in close contact, like those under consideration in this thesis. They may be formed from decomposition of a molecular precursor, for example. An uncorrelated RP, on the otherhand, has its spins randomly orientated and may be formed from chance encounters of free radicals in the bulk solution: these are denoted "F-pairs", or "freely diffusing pairs". F-pairs are initially uncorrelated but may become spin correlated over time by the exchange parameter $J(r)$ [58].

Singlet or triplet states of SCRPs are formed from mutually parallel or anti-parallel orientations of electron spins. For an isolated spin $s = \frac{1}{2}$ there are two possible states, corresponding to $m_s = \pm \frac{1}{2}$. However, for a two electron system, such as in a RP, with two spins, the total spin angular momentum vector, $\vec{S}$, is given by the vector sum of the two individual spins:

$$\vec{S} = \vec{s}_1 + \vec{s}_2 \quad (1.14)$$

where $\vec{s}_1$ and $\vec{s}_2$ are the spin angular momenta for electron 1 and 2. The subsequent possible values of the total spin angular momentum quantum number, S , are given by the Clebsch-Gordon series:

$$S = (s_1 + s_2), (s_1 + s_2 - 1), \dots, |s_1 - s_2|. \quad (1.15)$$

Table 1.1: Possible spin states of a RP with coupled spins, $|S, M_s\rangle$. There is one singlet state ($S = 0$) and three triplet states ($S = 1$).

	Spin state	$ S, M_s\rangle$
singlet	$ S\rangle$	$ 0, 0\rangle$
triplet	$ T_0\rangle$	$ 1, 0\rangle$
triplet	$ T_+\rangle$	$ 1, +1\rangle$
triplet	$ T_-\rangle$	$ 1, -1\rangle$

As $s_1 = s_2 = \frac{1}{2}$, there are two possible values of S : $S = 0$ (antiparallel spins) or $S = 1$ (parallel spins), corresponding to the singlet (S) and triplet (T) states, respectively.

The total spin projection quantum number, M_s , for $S = 0$ is $M_s = 0$ which corresponds to the singlet state and is denoted S, and for $S = 1$, $M_s = -1, 0, +1$, corresponding to the three triplet states denoted T_- , T_0 , T_+ , respectively. Thus, in the absence of coupling to any other nuclei, we have four possible states that the RP can form, as summarised in Table 1.1.

In the same way to how the two m_s states ($m_s = \pm\frac{1}{2}$) in a single electron have their degeneracy lifted upon exposure to a magnetic field, when a pair of spins is placed in a magnetic field, the T_+ and T_- triplet states ($M_s \neq 0$) are split in energy according to the Zeeman effect (see Figure 1.6), with the energy of each M_s state given by:

$$E_{zeeman} = g\mu_B B_0 M_s \quad (1.16)$$

The T_0 and S states both have $M_s = 0$ and consequently $E_{zeeman} = 0$. T_0 and S are unaffected by the external field as they have no component of spin angular momentum in the direction of the applied field and thus there is no interaction.

1.5.1 Exchange interaction

In Figure 1.6 the singlet and triplet states are not degenerate even at zero field. This is a result of a quantum mechanical phenomenon known as the exchange interaction. The exchange interaction arises as a result of the Pauli Exclusion Principle, whereby the overlapping of the wavefunctions of two unpaired electrons

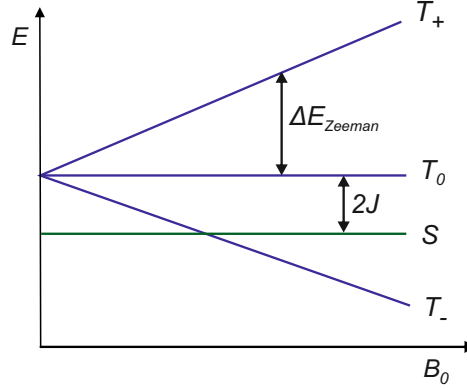


Figure 1.6: Zeeman splitting of a pair of electrons, including the effects of exchange energy.

in the RP results in the degeneracy of singlet and triplet RPs being lifted even when $B_0 = 0$. The Hamiltonian for this interaction, and the respective triplet (E_{ex}^T) and singlet (E_{ex}^S) energies, are given by:

$$\hat{H}_{ex} = -2J(r)\hat{s}_1 \cdot \hat{s}_2 \quad (1.17)$$

$$E_{ex}^T = -\frac{3}{2}J(r) \quad (1.18)$$

$$E_{ex}^S = +\frac{1}{2}J(r) \quad (1.19)$$

where $\hat{s}_1$ and $\hat{s}_2$ are the spin angular momentum operators for electrons 1 and 2, respectively, and $J(r)$ is the exchange interaction constant. The exchange interaction is strongly distance dependent owing to $J(r)$ being a function of the separation, r , between the electrons in the RP. $J(r)$ decreases exponentially with increasing r and at distances $r > 1$ nm the exchange interaction is taken to be negligible and the S and T states are approximately degenerate [59, 60].

In the strong exchange region, the S and T states are eigenstates of $\hat{H}_{ex}$. Radicals must diffuse apart in order for spin mixing to occur and for spin evolution of the RP to begin and spin states to interconvert: evolution of the RP only begins once the radicals have separated to a distance where the exchange interaction and hyperfine and Zeeman interactions are comparable. For solutions with a viscosity similar to water, separation of radicals to distances greater than 1 nm after the formation

of the RP typically occurs on a timescale of 100s of picoseconds [60]. However, increasing the solvent viscosity or restricting the diffusion of the RP, for example by constricting the RP to a micelle, may cause the exchange interaction to play a larger role. Consequently for the RP systems studied in micelles in Chapter 3 the exchange interaction may become more significant. Other RPs studied in this thesis, however, rapidly reach values of r greater than 1 nm and thus the exchange interaction can be ignored and the S and T states are degenerate in the absence of a magnetic field.

1.5.2 Dipolar interaction

The dipolar interaction concerns the interaction between electron spins in a RP. It describes the interactions between the magnetic dipoles of unpaired electrons in a RP. The dipolar interaction is anisotropic, and has the following Hamiltonian:

$$\hat{H}_{dip} = \hat{s} \cdot D \cdot \hat{s}, \quad (1.20)$$

where D is the dipolar tensor and $\hat{s}$ is the vector sum of the two individual electron spin operators, $\hat{s} = \hat{s}_1 + \hat{s}_2$. The interaction is once again distance dependent (being proportional to $\frac{1}{r^3}$) but drops off less rapidly with distance than the exchange interaction. Consequently, at larger RP distances, the dipolar interaction becomes more predominant than the exchange interaction.

However, as the dipolar interaction is anisotropic, it can be assumed to average to zero for molecules tumbling in solution due to fast molecular motion and therefore, as all the systems studied in this thesis are in aqueous solution, this interaction can be ignored.

1.5.3 Hyperfine interaction

Whereas the exchange and dipolar interactions describe the interactions between electron spins within a RP, the hyperfine interaction considers interactions of electrons in the RP with neighbouring magnetic nuclei (those that possess a nuclear spin angular momentum, $\vec{I} \neq 0$).

The hyperfine interaction has two magnetic² components: anisotropic dipole-dipole interactions and the isotropic Fermi contact interaction. The dipole-dipole interaction is analogous to the electron-electron dipolar interaction discussed in Section 1.4.2. As above, the interaction is anisotropic and so can be ignored for molecules in solution, where rapid tumbling causes the interactions to average to zero.

The second hyperfine component is the Fermi contact interaction. At electron-nuclear distances comparable to the nuclear radius the anisotropic dipolar interaction between the electron and the nucleus is replaced by an isotropic interaction known as the Fermi contact interaction. This interaction persists in solution and can modify the effective field experienced by the electron. The magnetic hyperfine interaction acts to produce a local contribution, B_{hf} , to the magnetic field, B , to produce an effective magnetic field, B_{eff} , experienced by the electron:

$$B_{eff} = B + B_{hf}. \quad (1.21)$$

B_{hf} , can be calculated from the nuclear magnetic quantum number, $m_I (= I, I - 1, \dots, -I)$, and the isotropic hyperfine coupling constant, a , according to:

$$B_{hf} = m_I a. \quad (1.22)$$

This extra contribution to the field results in spins precessing at different Larmor frequencies and forms the basis of the hyperfine spin-mixing mechanism (see Section 1.6.2).

The Fermi contact component of the hyperfine mechanism operates when there is a finite spin density of the electron at a neighbouring nucleus. The strength of this interaction depends on the probability density of the unpaired electron in an s -orbital (the only orbital to have electron density at the nucleus). Consequently this spin-coupling mechanism depends on the amount of ‘ s -character’ of the neighbouring

²Technically, the hyperfine interaction contains both magnetic and electronic components, however the electronic component occurs with magnetic nuclei containing a quadrupolar electric moment and thus scales with $\frac{1}{r^5}$. This distance separation makes it negligible at typical electron-nuclear separations in RPs, therefore only the magnetic component of the hyperfine interaction is relevant here and considered in this section.

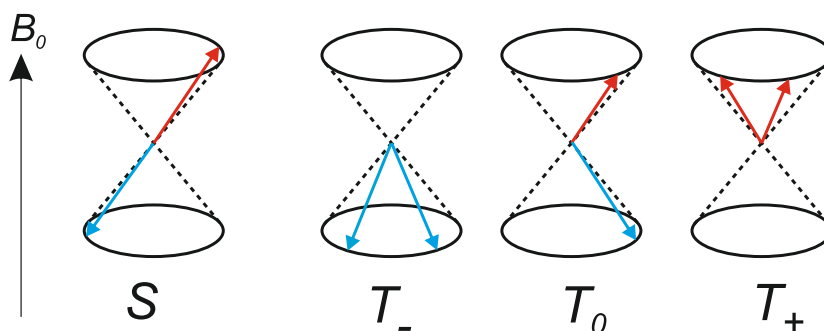


Figure 1.7: Singlet (S) and triplet (T_- , T_0 , T_+) spin states of a SCRPs under a magnetic field, B_0 .

nucleus. The hyperfine interaction causes additional splittings of the electron spin states and is the major source of the magneto-sensitive behaviour investigated here.

1.6 Singlet-Triplet Mixing

Singlet-triplet mixing (ST mixing) describes the processes by which S and T states (depicted in Figure 1.7) may interconvert in a RP. There are three distinct mechanisms by which ST mixing can occur: the hyperfine mechanism, the Δg mechanism and relaxation.

In order for a magnetic field to have an effect on a chemical reaction involving SCRPs it is essential that the RPs are able to undergo ST mixing: the S and T states must be able to interconvert so that their populations can be affected by the Zeeman splitting in the presence of a magnetic field. If the spin states were stationary (*i.e.* at high exchange and dipolar interactions), then application of a magnetic field, resulting in the splitting of energy levels (via the Zeeman effect - see Section 1.4.1), would have no effect on the populations of the S or T states and thus no effect on the outcome of the reaction. Reaction pathways and rates would remain as before, unaffected, leading to no MFE.

1.6.1 Δg mechanism

The Larmor frequency of an electron is given by:

$$\omega_i = \frac{g_i \mu_B B_0}{\hbar}. \quad (1.23)$$

With the Δg mechanism, ST mixing is brought about by a difference in g -values of the two electrons in the RP, where the g -value is a proportionality constant that characterises the magnetic moment and gyromagnetic ratio of a particle.

As shown in equation 1.23 the value of g directly affects the Larmor frequency of an electron spin in a magnetic field and thus if the two electrons have different g -values they will precess at different rates. The difference in precession frequencies is expressed as:

$$\Delta\omega = \omega_1 - \omega_2 = \frac{\Delta g \mu_B B_0}{\hbar}. \quad (1.24)$$

This, in turn, means the relative phases of the two electron spins in the RP are altered which can bring about mixing between the S and T_0 states because they differ only by a π phase shift (and no spin flip), as depicted in Figure 1.7. For systems studied here no appreciable ST mixing occurs via the Δg mechanism as we consider carbon-centred RPs in which the value of Δg can be approximated to 0.001. Assuming a RP lifetime of $\tau \approx 10^{-8} \text{ s}$, magnetic fields of the order of 10^4 mT , or greater, would need to be employed to ensure appreciable ST mixing occurs within the lifetime of the RP (*i.e.* $\Delta\omega\tau \geq 1$), which are much larger than those used in this thesis.

1.6.2 Hyperfine mechanism

The hyperfine mechanism describes how the Larmor frequency of an electron in a RP is affected by the local magnetic field experienced by that electron. The total magnetic field experienced by an electron is directly affected by its hyperfine coupling to neighbouring nuclei, as shown in equation 1.21 and discussed in Section 1.5.3. If the two unpaired electrons comprising a RP are in different hyperfine coupling environments they will precess at different frequencies causing the spins to lose phase coherence, bringing about ST mixing.

This can be understood by considering a RP in which one of the electron spins, s_1 , is coupled via an isotropic hyperfine coupling (see Section 1.5.3) to a nearby nucleus (with $I = \frac{1}{2}$) but the other electron spin, s_2 , is not. For applied external fields that are significantly larger than the field generated from interaction of the magnetic nucleus with s_1 , the magnetic moment of the nucleus and the magnetic moment of s_1 are both quantised along the field axis. This results in the magnetic moment of the nucleus modifying the local magnetic field, B_{eff} , that s_1 experiences, acting either to increase or decrease B_0 depending on the relative orientation of the nuclear spin. From equation 1.21, the local magnetic field, B_{eff} , experienced by s_1 is given by:

$$B_{eff} = B \pm \frac{a}{2}, \quad (1.25)$$

where a is the isotropic hyperfine coupling constant.

Consequently, the Larmor frequency for s_1 differs from the unperturbed Larmor frequency experienced by the non-coupled spin, s_2 :

$$\omega_1 = \frac{g_1 \mu_B B_0}{\hbar} \pm \frac{a}{2} \quad (1.26)$$

$$\omega_2 = \frac{g_2 \mu_B B_0}{\hbar} \quad (1.27)$$

In short, each spin is in a different hyperfine coupling environment and so experiences a different local magnetic field according to equation 1.25. Consequently the spins will precess at a different rate, which results in a change in the relative phase between the two. A vector model of the possible spin states in a SCRP is depicted in Figure 1.7. The only difference between the S and T_0 states is the relative phase of the two electron spins (π phase difference). Therefore, changes in the relative phases of the spins, brought about through variations in their respective Larmor frequencies as described above, can convert S into T_0 , however not into T_+ or T_- as this would not only require a change in precessional frequencies but also a spin flip. This type of ST mixing gives rise to a ‘normal’ or ‘high field’ MFE.

The above situation arises when the external field is large, $B \gg a$, however, if the magnetic field is weak, and so $B \leq a$, then the spins are no longer quantised

along the field axis but instead align with the resultant of the external and hyperfine fields. In this situation, more extensive spin mixing is possible than in the high field case. If, as before, s_1 is the only electron spin hyperfine coupled to a nuclear spin, then its projection onto the external field axis can change in time while the projection of s_2 remains constant. This effectively leads to a spin-flip transition, and so interconversion between S and all of T_+, T_- and T_0 is possible.

Low Field Effects

At external field strengths that are much weaker than the average hyperfine coupling in the RP, a low-field effect (LFE) may be observed, whereby an initial increase in the ST mixing efficiency is observed as the field increases from zero before the Zeeman splitting becomes dominant.

This arises due to partial relaxation of the angular momentum selection rules governing transitions between states upon moving from zero to weak field. The total spin angular momentum, F , of a system is given by the addition of the electron spin angular momentum, S , and nuclear spin angular momentum, I , as below:

$$F = S + I. \quad (1.28)$$

As with all spin angular momenta, M_F , the projection of the total spin angular momentum onto the static field axis, is given by $F, F - 1, \dots, -F$.

In zero-field, *i.e.* when $B_0 = 0$, the selection rules state $\Delta F = 0$ and $\Delta M_F = 0$. However, for $B_0 \neq 0$, only $\Delta M_F = 0$ must be obeyed and thus the number of allowed transitions increases. Consequently, the efficiency of ST mixing is increased, until the Zeeman effect begins to dominate (the ‘high-field’ region).

The LFE observed depends on the competition between the initial relaxation of the selection rules and the rate of the Zeeman effect becoming dominant. In order for a significant LFE to be observed it has been suggested that the RP should exhibit characteristics such as a weak exchange interaction compared to the hyperfine coupling, a long geminate pair lifetime (but not so long that relaxation mechanisms become dominant) and a slow relaxation rate [59, 61]. LFEs are of

huge importance for magnetoreception as they lead to an increased sensitivity to low fields comparable to that of the Earth. Photochemical systems involving LFEs are explored in Chapter 3.

1.6.3 Relaxation mechanism

Relaxation of spins in an external field brings about incoherent ST interconversion, destroying the initial spin polarisation created upon creation of the RP. The relaxation mechanism drives incoherent ST mixing *via* both spin-spin and spin-lattice relaxation.

Spin-spin relaxation allows mixing between the S and T_0 states by acting to destroy phase coherences between two electron spins. The result of spin-spin relaxation is very similar to that of the Δg and hyperfine mechanisms: altering Larmor frequencies to destroy phase coherence results in spins precessing at different rates and brings about conversions between S and T_0 (but not between S and $T_{\pm}$ as this would require a ‘spin-flip’ - see Section 1.6.1). However, in contrast with these coherent mechanisms, $S - T_0$ mixing resulting from spin-spin relaxation is randomised.

Spin-lattice relaxation, however, allows mixing of the S state with the $T_{\pm}$ states and is caused by application of fluctuating magnetic fields which, if oscillating at the ‘correct frequency’ (the Larmor frequency), stimulate spins to randomly flip between spin states, returning the system to an equilibrium spin polarization. This frequency occurs when one of the component frequencies of the external field matches the energy separation of the S/T_0 and the $T_{\pm}$ states that is created by the Zeeman interactions.

Relaxation mechanisms can act to obscure effects of coherent spin mixing and thus reducing the MFE observed. However, for most organic radicals, relaxation rates are usually much longer than typical geminate pair lifetimes (typically 10-100 ns), and so can be ignored. When dealing with biological systems such as cryptochromes, however, geminate RP lifetimes can be longer, and so relaxation can cause significant challenges in the measurement of MFEs.

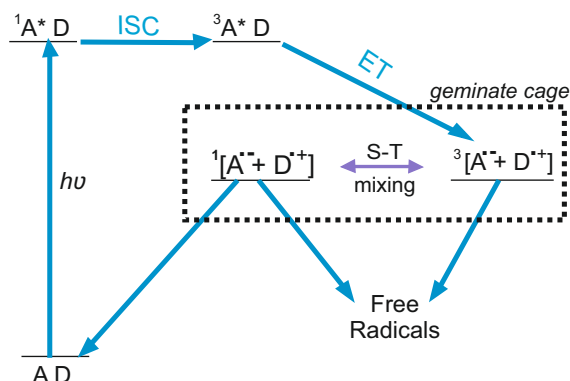


Figure 1.8: Simplified photochemical scheme for a triplet-born radical pair formed from donor, D, and acceptor, A. ISC = intersystem crossing; ET = electron transfer.

1.7 Radical Pair Mechanism

The radical pair mechanism (RPM) is integral to the mechanism of avian magnetoreception investigated in this thesis. The RPM provides an explanation of how a magnetic field, even as weak as that of the Earth's, may affect the rate of ST mixing in systems containing SCRPs and thus perturb the product yield and/or recombination kinetics in such reactions.

Consider Figure 1.8, which depicts a simplified photochemical scheme for a triplet-born RP system. Upon photoexcitation, the ground state photoreceptor molecule, A, is excited to the first excited singlet state, $^1A^*$ where it then undergoes rapid intersystem crossing (ISC) to the triplet state, $^3A^*$. From here, $^3A^*$ accepts an electron from a donor molecule, D, to form a RP with correlated spins, $^3[A^{\bullet-} D^{\bullet+}]$. The RP is formed under conservation of total spin angular momentum and so the triplet SCRP is formed. Initially the radicals are formed within a geminate cage; however, in time they may diffuse apart to form free radicals. If two free radicals encounter they may combine to form an F-pair.

Due to the proximity of the radicals in the geminate pairs the chance of re-encounter is high. Recombination to the ground state, however, is state selective: it is spin-forbidden from the triplet RP state and so only possible via the singlet

RP state. Consequently, unless there is a mechanism by which the triplet and singlet SCRPs can interconvert, the initially formed triplet RP will eventually just diffuse apart, out of the geminate radical cage, where the radicals may then undergo further reactions to give a variety of products.

Singlet and triplet states may interconvert via ST mixing, as discussed in detail in Section 1.6. The rate of ST mixing is magnetically sensitive as a result of interplay of the Zeeman and hyperfine interactions. In zero field, all states ($S_0, T_0, T_{\pm}$) are degenerate in absence of $J(r)$. However, as the applied field increases the $T_{\pm}$ states become energetically isolated, meaning interconversion driven by hyperfine interactions between the different states becomes less energetically favourable and ST mixing slows. Changing the efficiency of ST mixing directly affects the ratio of singlet:triplet RPs in the geminate cage and thus affects the product ratio of the reactions owing to the fact that the singlet and triplet RPs have different fates. For the reaction scheme considered above, in which the SCRPs are triplet born, application of a magnetic field traps the system in the triplet state, thereby increasing the yield of triplet-born free radicals and reducing the concentration of the singlet recombination product. This is a normal magnetic field effect (MFE) and occurs when the applied field is considerably larger than the hyperfine interaction (Section 1.6.2).

The RPM provides an explanation as to how a magnetic field as weak as that of the Earth's ($\sim 50\mu\text{T}$) can have an effect on the outcome of a chemical reaction. Although thermodynamically this initially appears highly implausible, considering the interaction energy of a single molecule with an Earth-strength magnetic field is far weaker than the thermal energy $k_B T$ (over a million times so), within the framework of the RPM this can be explained. Within the RPM, a photon of light provides sufficient energy to cause the system to be generated in a state far from equilibrium. Consequently, as the system has already been brought into a highly nonequilibrium state, when the field interacts with it only small interactions are required to have pronounced effects on the system. Therefore even very weak magnetic fields can have an effect on the outcome of the reaction.

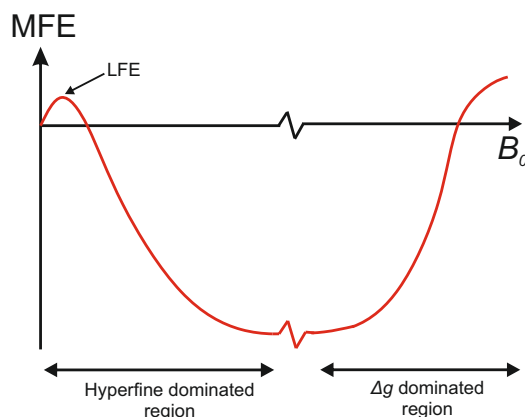


Figure 1.9: Schematic MARY curve for a triplet-born SCRP detected via fluorescence of the first excited singlet state $^1A^*$, with the hyperfine and Δg dominated regions indicated. In this thesis only the LFE and hyperfine region is of relevance as the Δg mechanism only becomes prevalent at higher field strengths.

Nonetheless, MFEs resulting from the RPM are still predicted to be very small. It is likely that biological amplification mechanisms are necessary in order for the small MFEs on a molecular level to manifest in a behavioural response. Experimentally (assuming no amplification) exquisitely sensitive techniques are required to observe these small MFEs. Chapter 2 introduces one such technique, redesigning an already highly sensitive technique which uses the fluorescence of the system to indirectly monitor MFEs.

1.8 Magnetically Altered Reaction Yield (MARY)

Magnetic fields can alter the outcomes of chemical reactions as explained by the ST mixing mechanisms discussed in the previous sections. The effect that a magnetic field can have on a reaction yield can be depicted as a Magnetically Altered Reaction Yield (MARY), where the percentage magnetic field effect (MFE) can be plotted as a function of magnetic field strength (B_0). A typical schematic MARY curve for a triplet-born RP system is shown in Figure 1.9.

The method for calculating the MFE for a system varies depending on the detection method used. For fluorescence-based MFEs (as studied in this thesis), the MFE is calculated by measuring the change in the intensity of fluorescence

upon application of a magnetic field, ΔIF ($= IF_{on} - IF_{off}$), in relation to the fluorescence intensity with no magnetic field, IF_{off} as follows:

$$MFE = \frac{\Delta IF}{IF_{off}} \cdot 100\% \quad (1.29)$$

A more detailed, step-by-step discussion of MFE calculation for fluorescence-based detection techniques can be found in Chapter 2.

The MARY schematic shown in Figure 1.9 is appropriate for the intermolecular model systems studied in this thesis, as these are triplet born RP systems by virtue of rapid intersystem crossing (ISC). Cryptochromes have highly efficient electron transfers (ET), which out-competes the rate of ISC meaning that they form predominantly singlet-born RPs. When envisaging a MARY curve for a singlet-born RP system such as cryptochromes, the schematic in Figure 1.9 is simply inverted.

The MARY schematic is split into different regimes: the hyperfine dominated region (including the LFE region) and the Δg dominated region. The behaviour of each region is dictated by the mechanism of ST mixing.

Initially, at low field strengths, there is a relaxation of selection rules (Section 1.6.2) which results in an initial increase in the efficiency of ST mixing. Consequently, a higher concentration of the singlet state RP is observed compared to the zero field case, which increases the chance of spin-allowed recombination to the ground state. An increase in ground state concentration leads to a positive MFE being observed.

As the field strength begins to increase, it will start to exceed the average hyperfine coupling. At this point, the Zeeman effect begins to dominate causing the $T_{\pm}$ states to split from the T_0 and S states (Section 1.6.2), reducing the efficiency of ST mixing and decreasing the MFE. These effects result in the LFE region on the schematic.

Increasing the magnetic field strength further causes the $T_{\pm}$ states to continue to split from the T_0 and S states, further decreasing the efficiency of ST mixing. RPs become trapped in the $T_{\pm}$ states, reducing the concentration of molecules returning to the ground state via the spin allowed transition from the singlet RP. This section is known as the ‘normal MFE’ region. As the $T_{\pm}$ states continue

to split from the S and T_0 states, S- T_0 mixing becomes the only interconversion allowed, the rate of which is determined by the field-independent hyperfine coupling constant. Consequently, the MARY curve eventually plateaus.

At even higher fields, the rate of S- T_0 interconversion increases as the field-dependent g-mechanism starts to dominate. This causes the overall rate of spin mixing to increase, increasing the concentration of the ground state and thus bringing about a positive MFE. However, this mechanism is not relevant for field strengths used in this thesis.

1.9 Scope of thesis

This thesis builds on the theory presented in this chapter to explore both the photochemistry of cryptochromes and micelle-based RP systems, and the subsequent photosignalling processes in cryptochromes.

To investigate photochemical mechanisms, a novel fluorescence-based detection technique is redesigned to increase sensitivity and improve its suitability to detect very small MFEs on precious biological samples, specifically cryptochromes *in vivo*. The redesigned technique is characterised and the photochemistry of RPs in micelles is explored to investigate how factors such as the environment of the SCRPs affects MFEs.

A highly quantitative technique known as Hydrogen-Deuterium Exchange Mass Spectrometry (HDX-MS) is then employed to explore the photosignalling mechanisms in cryptochrome, of which little is currently understood. How the photochemistry occurring in cryptochromes is linked to the signal transduction process is explored by investigating if any light-induced conformational change occurs in these proteins, with the nature of the HDX-MS technique allowing localisation of this conformational change to a near peptide level.

2

Prompt Fluorescence MARY: Re-design and Initial Application

2.1 Introduction

Studying the influence of magnetic fields as weak as that of the Earth's ($50 \mu\text{T}$) on biological samples requires exquisitely sensitive spectroscopic techniques. To date, MFEs in biological systems have been recorded using a variety of optical spectroscopic methods, the majority of which have been based on direct absorption.

Transient Absorption spectroscopy (TA) has traditionally been a popular choice of detection method owing to its simplicity and near universal applicability [62]. However, in its most common, single-pass implementation, TA is limited in its sensitivity by the need to detect small changes in a typically high background level. Improved sensitivity in measuring MFEs has been achieved through the development of multi-pass absorption techniques, such as Cavity Ring-Down Spectroscopy (CRDS) and Cavity Enhanced Absorption Spectroscopy (CEAS), which increases the effective pathlength of the sample [54, 63–65]. However, even higher sensitivity can be gained through fluorescence-based detection methods, in part because these can represent background free techniques.

Until recently, reports on fluorescence detected MFEs have been limited to systems that form fluorescent species directly from the geminate pair. However,

recent developments have shown that steady-state fluorescence from the first excited singlet state ('prompt fluorescence' (PF)) can be exploited to allow indirect measurement of MFEs [66]. This vastly increases the number of systems that can be studied by fluorescence.

This chapter describes changes to an existing highly-sensitive PF MARY experiment, designed to increase sensitivity and make it more suitable for studying precious biological samples. To date, only model systems of cryptochromes have been studied by PF MARY, and whilst other absorption-based techniques have observed MFEs on cryptochromes *in vitro*, these systems are still fairly far removed from biological conditions [54, 66]. Observing an MFE on cryptochromes *in vivo*, by studying cryptochrome overexpressed in insect cells, would be closer to biological conditions than any other system studied to date. In order to do this, however, a highly sensitive experiment able to accommodate this specific biological sample is required, which is where the re-designed PF MARY experiment would be ideal.

This chapter introduces the concept of PF-MARY, discusses the limitations of the former setup and the improvements made in order to increase the sensitivity of the experiment. The chapter goes on to present early attempts to observe an MFE on *DmCry* in *Spodoptera frugiperda* 9, *Sf9*, cells (ovarian insect cells) which were done in collaboration with Victoire Dejean, and provide suggestions on how to improve on this in future.

2.2 Fluorescence and MFEs

Fluorescence has long been a popular choice of detection method due to its highly sensitive nature. As such, many fields exploit fluorescence as a detection probe and it is used in processes ranging from medical imaging and diagnostics, to conformational analyses, to monitoring MFEs in photochemical systems [67].

Historically, the use of fluorescence to monitor MFEs has been restricted to monitoring the 'delayed fluorescence' of a system. For example, when recombination from the singlet geminate RP produces a fluorescing exciplex, such as in the pyrene

and N,N-dimethylaniline/dicyanobenzene system [68], or when geminate RPs are formed from radiolytically excited aromatic hydrocarbons in organic solvents, such as fluorene/anthracene in squalane/cyclohexane, and recombine to give fluorescent excited states [69–71]. Any ‘non-delayed’ fluorescence experiments have involved a two-colour approach whereby a second excitation source induces fluorescence in the reaction products, such as the setup used to study the photochemistry of anthraquinone in micellar solutions [72].

However, in this thesis, a novel type of fluorescence measurement is used in which the prompt fluorescence (PF) of the photoexcited molecular precursor is exploited to allow an indirect measurement of MFEs. This measurement technique, known as PF MARY, has allowed MFEs to be recorded on a much wider range of samples as the requirement of the fluorescence to occur as a direct result of recombination of the geminate pair is lifted. Any sample which exhibits prompt fluorescence may be studied by this method.

To date, MFEs on model systems of cryptochromes have been observed by this technique, both as intermolecular model systems, *e.g.* free flavin and an electron donor in aqueous solution, and as intramolecular model systems, *i.e.* protein-based model systems of cryptochromes known as maquettes [66, 73].

2.3 Basis of PF MARY

As discussed in the introduction (Chapter 1), upon exposure to blue-light, flavin is photoexcited to the first excited singlet state. Figure 2.1 shows a simplified photochemical scheme for system comprising a fluorophore such as flavin, F, and an electron donor, D, highlighting the pathways for a both singlet-born and triplet-born SCRP. Here, the fluorophore, F, is photoexcited to the first excited singlet state $^1F^*$. From here, the system may either:

(i) undergo ET to form a singlet SCRP: $^1F^*D \rightarrow ^1[F^{\cdot-} + D^{\cdot+}]$,

(ii) undergo ISC to the triplet state, followed by ET to form a triplet SCRP:
 $^1F^*D \rightarrow ^3F^*D \rightarrow ^3[F^{\cdot-} + D^{\cdot+}]$,

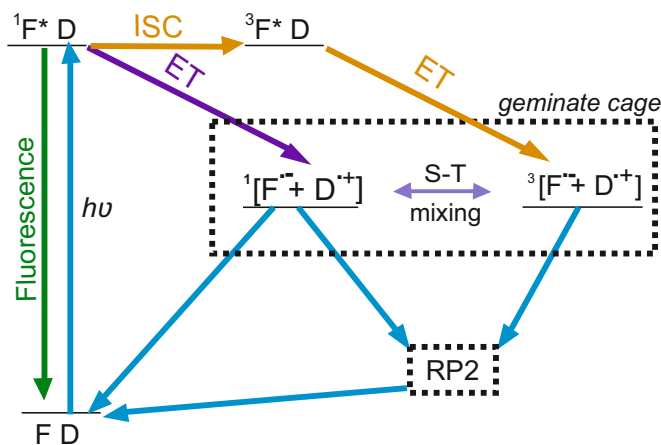


Figure 2.1: Simplified photochemical scheme for a system formed of a fluorophore, such as flavin, F, and an electron donor, D. Upon photoexcitation to the first excited singlet state the system may either: i) undergo ET to form a singlet-born SCRPP (purple), ii) undergo ISC to form the triplet excited state and then undergo ET to form a triplet-born SCRPP (orange), or iii) fluoresce back to the ground state (green).

(iii) fluoresce back to the ground state: ${}^1\text{F}^*\text{D} \rightarrow \text{F} + h\nu$.

The above three scenarios correspond to the pathways highlighted in purple, orange and green, respectively, in Figure 2.1. The pathway taken, and thus the nature of the SCRPP formed, depends on the system being studied: intermolecular systems (such as free flavin and an electron donor) are triplet-born by virtue of rapid intersystem crossing (ISC) outcompeting the rate of ET; cryptochromes, however, are predominantly singlet-born as ET outcompetes the rate of ISC.

Under continuous illumination, the S_1 - S_0 fluorescence intensity provides a measure of the ground state population. Any process that alters the concentration of the ground state will, consequently, alter the intensity of fluorescence. As ST mixing alters the likelihood of recombination of the SCRPP, any field effect on the rate of ST mixing will be translated to the concentration of ground state molecules and be expressed in the intensity of fluorescence. As a result, MFEs can be measured indirectly via the field dependence of the prompt fluorescence, allowing

the study of systems containing flavin-based RPs, which do not recombine to a fluorescent product.

For triplet-born RPs, such as intermolecular model systems of cryptochromes such as flavin and an electron donor in solution, a negative MFE is observed by fluorescence. Application of an external field, with a magnitude exceeding that of the hyperfine coupling, results in separation of the T_+ and T_- sub-levels from the S and T_0 states. This causes the geminate radicals to get trapped in the T_+ and T_- states, which results in an increase in steady state radical concentration and a decrease in the ground state concentration. Consequently, a reduction in fluorescence intensity is observed, as a negative MFE. MFEs are probed indirectly through monitoring the changes in fluorescence of the system in the presence and absence of a magnetic field.

2.4 Former PF MARY setup and limitations

The PF MARY experiment has been successful in observing MFEs on flavin-based biological systems [66, 73]. However, the setup was developed rapidly and was never fully optimised. Upon inspection, it became apparent that many aspects of the setup were not ideal and needed improving if the setup was ever to be used to study biological samples, specifically those in a cellular environment.

The former PF MARY experimental setup is shown in Figure 2.2. Samples were held in a Quartz SUPRASIL® sample cell with a pathlength of 1 mm and volume of 200 μ L. The sample cell was positioned between two Helmholtz coils which provided static magnetic field strengths of 0 mT to +12 mT. The field was stepped between the two extremes in a random order, reaching a desired field value within 5 ms. A delay time, t_d , was introduced between switching the field strength and taking a measurement to ensure that the field strength had reached the desired value and plateaued during the integration time. t_d was set to at least 12 ms in order to be sufficiently greater than the coils' and the CCD camera's response times.

For flavin-based systems, a 405 nm or 450 nm diode laser (Power Technology) was used to continuously photoexcite the sample, allowing the system to reach a steady state equilibrium. Fluorescence from the sample, by nature, is isotropic

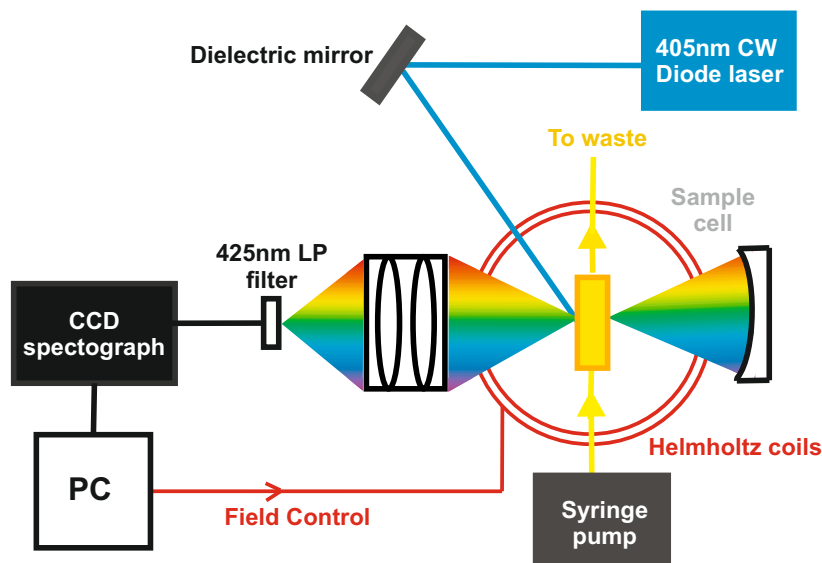


Figure 2.2: Former fluorescence setup used for PF MARY experiments. The sample was flown to waste using a syringe pump. A diode laser was used to photoexcite the sample and the subsequent fluorescence was collected by an optical fibre using an achromatic doublet pair (with a back mirror used to enhance the collection efficiency). The sample cell was positioned between two Helmholtz coils and the sample's response to magnetic fields was recorded. See text for a full explanation the setup.

and was collected by an optical fibre using an achromatic doublet pair (ADP). However, by the nature of this experimental design, only the fluorescence emitted perpendicularly to the sample cell could be collected. Furthermore, due to physical restrictions of the setup and the requirement for the ADP and back mirror to be 1 focal length away from the sample, the ADP could be positioned at closest ~ 30 mm from the cell resulting in low collection efficiency.

2.4.1 Collection Efficiency

The collection efficiency of the former setup can be calculated/estimated as follows. Assuming fluorescence is isotropic, the fluorescence of the sample can be considered to occupy a sphere radiating from the centre of the sample, as depicted in 2.3. The collection efficiency of the setup is determined by the distance of the achromatic lens from the sample, r , which will be the radius of the fluorescence sphere. The fraction of the fluorescence collected is also dependent on the diameter of the lens

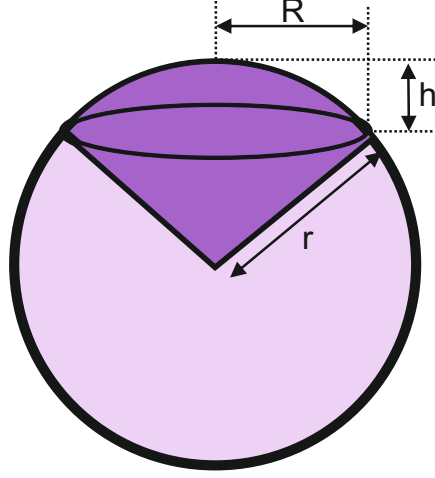


Figure 2.3: Illustration of the fluorescence sphere radiating from the centre of the sample. The spherical cone representing the fluorescence that is collected by the detector is also highlighted (dark purple). The fraction of fluorescence collected, *i.e.* the size of the spherical cone, is dependent on the radius of the fluorescence sphere, r , (determined by the distance of the sample from the ADP), the radius of the lens of the ADP, R , and the height of the spherical cap, h .

of the ADP, $d = 2R$. The collection efficiency will be the volume of the segment of the fluorescence sphere that is collected divided by the total volume of this sphere.

The volume, V , of the whole fluorescence sphere is given by,

$$V = \frac{4\pi r^3}{3}, \quad (2.1)$$

where r is the radius of the sphere *i.e.* the distance between the centre of the fluorescence (the sample) and the ADP lens. The volume of the collected segment, $V_{segment}$ is,

$$V_{segment} = 2\pi r^2 \frac{h}{3}, \quad (2.2)$$

where h is the height of the cap of the fluorescence sphere, as indicated in Figure 2.3. Consequently, the collection efficiency of the setup, C_{eff} , is found by,

$$C_{eff} = \frac{V_{segment}}{V} = \frac{\frac{h}{2}}{r} = \frac{hr}{2}, \quad (2.3)$$

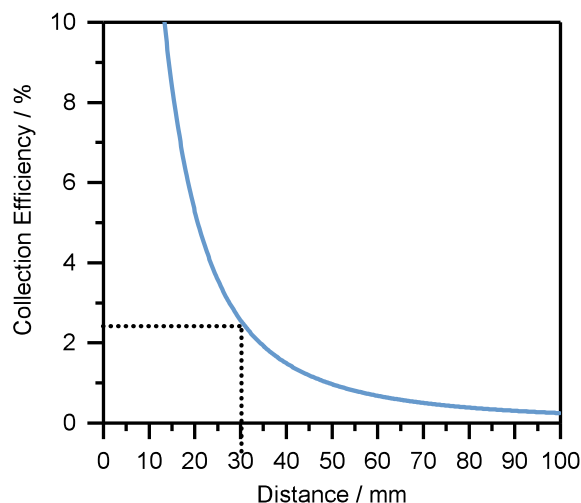


Figure 2.4: Percentage collection efficiency of the former fluorescence setup as a function of distance of the ACP lens from the sample. Calculated according to equation 2.3 for a lens diameter of 20 mm.

The fluorescence collection efficiency (as a percentage) as a function of the distance of the achromatic doublet pair from the centre of fluorescence is shown in Figure 2.4 for $d = 20$ mm. This highlights just how rapidly collection efficiency drops with distance: at 30 mm away the collection efficiency is already $< 3\%$. Although a concave mirror collects the back-scattered fluorescence in an attempt to increase the collection efficiency, at most this only increases collection efficiency by a factor of 2. Therefore the overall collection efficiency was at best $< 6\%$, which is very poor.

2.4.2 Photodegradation

Flavin-based samples are susceptible to photodegradation. Figure 2.5 depicts the absorption spectra for a fresh sample of $10 \mu\text{M}$ RF (light blue), and the same sample after having been illuminated with a 405 nm diode laser whilst being recirculated (purple, dashed) or whilst static (dark blue). A typical flavin absorption spectrum is observed for all three cases, with two distinctive broad peaks at 445 nm and 375 nm which correspond to the $S_0 \rightarrow S_1(\pi - \pi^*)$ and $S_0 \rightarrow S_2(n - \pi^*)$ transitions of the isoalloxazine group respectively (see Section 1.2) [74, 75].

As shown in Figure 2.5, the quality of the samples severely degrades when

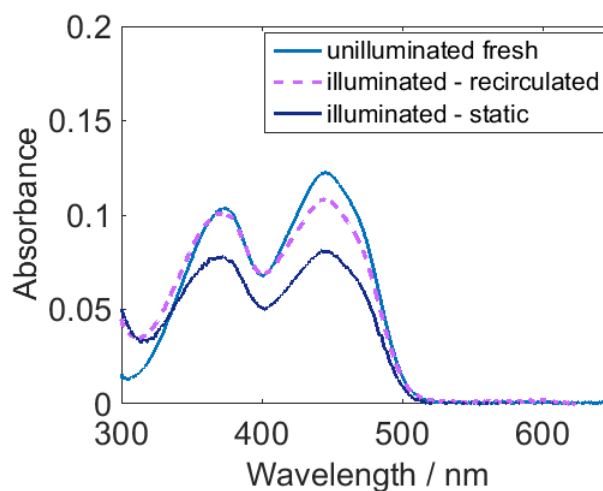


Figure 2.5: UV-Vis spectra of a sample of 10 μM RF (aq) when fresh (light blue) and then after having been photoexcited with 405 nm diode laser at 230 mW for 1 hour i) whilst recirculated (purple dashed), and ii) whilst static (dark blue). A reduction in absorbance of the peak between 400-500 nm and relative increase in the peak around 370 nm indicates the formation of photodegradation products such as lumichrome. The extent of photodegradation is clearly worse for the static sample as opposed to when it is recirculated.

left static during an experiment compared to when the samples are flowed. Upon exposure to light, flavin can degrade into photoproducts such as lumichrome and lumiflavin. This is seen more notably when the sample is static, as indicated by a decrease in sample absorbance at the peak around 400-500 nm and a relative increase in magnitude of the peak around 375 nm, indicative of the formation of lumichrome.

In order to reduce these effects in the former PF MARY setup samples were flowed using a syringe pump with the flow rate typically set at 0.25 ml/min to ensure the cell was fully replenished and new molecules were photoexcited with each successive measurement. As recirculation with a syringe pump is not possible this inherently required tens of millilitres of sample per experiment. Although this worked well with model systems, which are cheap and thus can be made in large quantities and flown to waste, problems arise when working with precious biological samples which are more difficult to acquire and not so disposable. Any previous attempts to study biological samples, such as cryptochromes, have thus had to be done on static samples introducing issues with photodegradation. Ideally

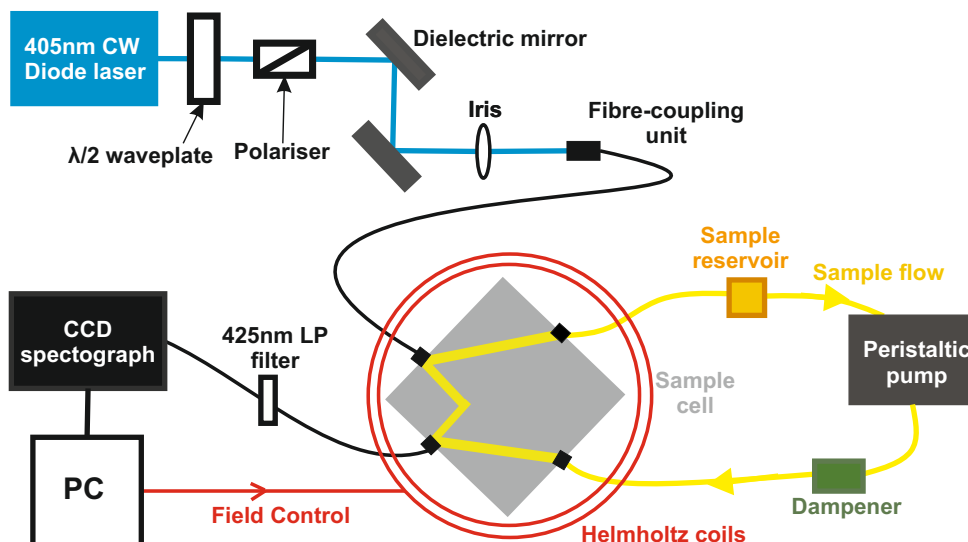


Figure 2.6: The re-designed setup used for PF MARY experiments. The ‘through-space’ design has been replaced with fibres to couple the laser light into the cell directly, and a $\lambda/2$ waveplate and polariser have been introduced to provide a convenient way of changing the photoexcitation power.

a way of flowing and recirculating small volumes of biological samples is needed to avoid issues with photodegradation and sample waste.

2.5 Modifications and Improvements

Clearly, the two main issues that need to be addressed are the low collection efficiency, which impacts the sensitivity of the experiment, and the large volume of sample required for each experiment in order to have an acceptable signal. In order to maximize the collection efficiency, increase sensitivity, and reduce the overall volume of the experiment several modifications have been made to the above setup, as shown in Figure 2.6.

2.5.1 Collection Efficiency

One of the main reasons why the collection efficiency was so poor in the former setup was because the fluorescence was collected through space by an ADP, positioned an unavoidably large distance away from the sample (the ADP has to be at one

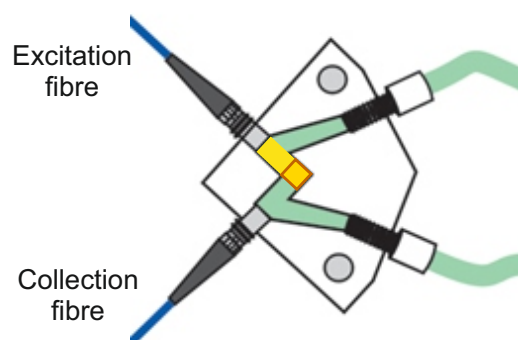


Figure 2.7: Diagram of the new fluorescence cell (Ocean Optics, FIA-SMA-FL-ULT), highlighting the area of sample that is photoexcited (yellow) and the detectable portion of sample (orange box). The laser light is coupled to the cell through the excitation fibre and the subsequent fluorescence collected by the collection fibre. The design of the cell allows the fluorescence to be collected from the cell directly using optical fibres rather than having to collect the fluorescence through space.

focal length from the sample and was further limited in its position by steric hindrance from the Helmholtz coils).

To mitigate this problem a new sample cell (Ocean Optics, FIA-SMA-FL-ULT) was employed (Figure 2.7) which allows the fluorescence to be collected by an optical fibre from the cell directly, without the need to couple through space. This abolishes the need for an ADP in the setup all together.

The excitation laser light is coupled to the cell through an optical fibre ('excitation fibre'), which excites a small amount of sample, highlighted in yellow in Figure 2.7. The detectable volume is highlighted in an orange box, and the subsequent fluorescence from here is collected by another optical fibre at right angles to the first ('collection fibre'). Having the excitation and collection fibres at right angles minimises the amount of laser light reaching and saturating the detector. A 420 nm / 475 nm LP filter, however, is still incorporated just before the detector to cut out any remaining 405 nm / 450 nm laser light. A lens was also introduced before the detection fibre in order to focus the fluorescence into the fibre and

maximise the amount of fluorescence collected.

The specific arrangement shown above (Figure 2.7) was chosen in order to minimise the amount of sample being irradiated/photodegraded unnecessarily before collection. If the excitation fibre and the collection fibre were swapped then the sample would be irradiated in a region of the cell where the fluorescence is undetectable. This would mean unnecessarily photodegrading the sample before it was able to be detected.

Estimating New Collection Efficiency

An estimate of the collection efficiency of the new cell can be calculated by considering the fluorescence of a point in the centre of the detectable volume of the cell, which has a volume of 2 x 2 x 2 mm. The general geometry of the cell can be thought of as being similar to that considered for the former setup. The fluorescence is still isotropic and can be considered to be a sphere radiating from the centre of this 2 x 2 x 2 mm volume, but instead of having a 20 mm diameter lens at a distance of 30 mm from the sample, we now have a 2 mm diameter lens at 1 mm distance.

If it is presumed that every photon bouncing off the walls in the right angle will reach the detector, then by implementing these changes the collection efficiency can be calculated once again using equation 2.3 and shows a near 5-fold increase from <6% to 30% (taking into account the back mirror/wall in both cases). This is a vast improvement to an already extremely sensitive experiment and will prove invaluable when measuring biological samples where MFEs are predicted to be low and so any extra sensitivity will be extremely helpful.

Other changes resulting from the new flow cell

As well as addressing the issue of collection efficiency, the new cell has a reduced volume of 50 μL so is ideal for precious samples where volume needs to be kept at a minimum. Biological samples, such as cryptochromes, are not only more expensive than model systems, but are also more difficult, labour-intensive and time-consuming to make and so are available in much lower volumes and concentrations.

Consequently these samples are precious and any method to reduce the volume required for an experiment will prove to be useful.

Furthermore, as the sample cell is made out of an inert man-made polymer, ULTEM, it is not magnetic and so will not interfere with magnetic field experiments, nor will it contribute to any undesirable chemistry with the samples of interest.

Unfortunately, as the cell's 'W'-shape is quite constraining and the flow path narrow, any bubble that passes through the cell has the potential get stuck in the detection path, which makes the signal drop dramatically. In an attempt to mitigate this problem a homemade 'bubble trap' was employed in the form of a piece of large tubing attached to the beginning of the flow system. The tubing works to prevent bubbles forming in the first place and was found to be an effective bubble trap by preventing bubble formation and ensuring a stable flow.

2.5.2 Minimising volume

As discussed above, the volume of the former setup was inherently large. In order to achieve satisfactory SNR and have signal that can be averaged over the course of the experiment it is important that photodegradation is minimised. For the photosensitive samples of interest in this thesis this requires the sample to be flowed, however this was previously only possible by using a syringe pump. Consequently tens of millilitres of sample were required which is not appropriate when moving towards looking at precious biological samples.

Two main improvements were made to the setup to help minimise the total volume: the first was by choosing a new sample cell that not only increased fluorescence collection efficiency but also had a low volume (discussed above), and the second was by investing in a new peristaltic pump to allow sample recirculation.

Choice of pump

Although using a syringe pump is a convenient way to flow samples relatively smoothly, it does not provide the possibility of recirculating the sample as other pumps do, such as peristaltic and diaphragm pumps.

Diaphragm pumps come in many varieties, but the basic overriding mechanistic principle involves diaphragms systematically moving up and down to force the sample to flow. The diaphragms are contained within a chamber with valves controlling the flow direction and preventing back flow. When a diaphragm moves up, the pressure in that area decreases causing the sample to flow into the chamber; when the diaphragm moves down the sample is displaced out of the pump chamber. The flow rate can be determined by changing the frequency of the diaphragm oscillations and/or the height (voltage) of the diaphragm displacements.

Peristaltic pumps on the other hand work by having flexible tubing wrapped around a number of rollers which systematically squeeze the tubing, creating peristaltic pressure and forcing the sample to flow in a uniform direction. The alternate compression and relaxation of the tubing draws the sample through the tubing.

The schematic in Figure 2.8 outlines the mechanism for how peristaltic pumps operate. Here the most basic peristaltic pump with only one roller is considered for simplicity. The roller rotates, pressing up against, and completely compressing, the tubing in order to create a seal. As the roller moves around and releases the tubing, a vacuum is formed which pulls the sample up and around the tubing. This process repeats as the roller continues to rotate, and the flow rate can be determined by altering the rotation speed of the roller(s).

Although both pumps are efficient at recirculating the sample, the peristaltic pump was favoured over the diaphragm pump due to the noise of each of the instruments. Standard Deviation studies showed that under similar conditions, the diaphragm pump had a higher standard deviation than the peristaltic pump (48 compared to 28) which results in a worse detection limit.

However, although the peristaltic pump is preferable to the diaphragm pump, no pump is completely noise-free and peristaltic pumps do inherently still have some noise issues due to the pulsations of the pump mechanism. Whilst this noise is hard to eradicate completely, increasing the number of rollers typically reduces pulsations and smooths out the flow. The peristaltic pump chosen (Elemental Scientific, MP2-S Precision Stand Alone Pump) has 12 closely spaced rollers precisely for this reason.

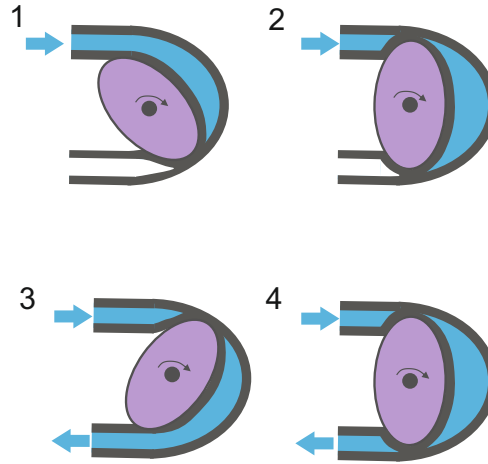


Figure 2.8: Schematic of how a peristaltic pump operates. The roller (purple) rotates to compress the tubing and draws the sample (blue) through using peristaltic pressure.

Moreover, it attempts to improve signal stability even further by adopting a roller configuration that minimises the gaps between each roller in order to increase the number of pressure points on the tubing and reduce pulsations.

Unfortunately, despite best attempts to smooth out pulsations with this peristaltic pump, the signal still had unacceptable noise. To address this issue, a large, soft piece of tubing was introduced between the pump and the cell to act as a dampener. The dampener acts to attenuate the high-frequency pulses generated by the pump and ensure a smooth flow as shown in Figure 2.9. This vastly reduced the noise generated and provided a smoother signal to average over.

2.5.3 Other modifications/improvements

In addition to addressing the main flaws (sensitivity and high volume) of the pre-existing setup, other improvements were made in order to make the experiment more user-friendly.

An achromatic $\lambda/2$ wave plate (HR 400-750) and a polariser have been introduced in order to conveniently and gradually adjust the intensity of the excitation source. Previously any adjustments to laser power had to be done using neutral density (ND) filters, which not only gives less control over what powers can be chosen but

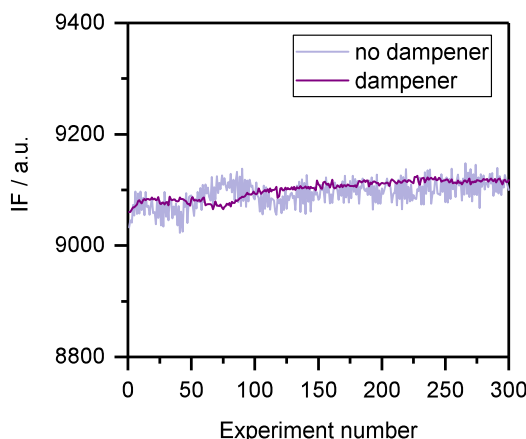


Figure 2.9: Fluorescence intensity (IF) as a function of experiment number for a sample of $20\ \mu\text{M}$ RF / $1\ \text{mM}$ W both with (dark purple) and without (light purple) a dampener present. The presence of a dampener clearly acts to smooth out the flow and provide a more steady fluorescence intensity over time.

also are not facile or quick to use. The $\lambda/2$ wave plate and polariser provide a way to easily and quickly reduce the laser intensity to a wide range of low (and high) powers. This will be particularly useful when measuring biological samples where laser power is important: if cryptochromes are illuminated with too high a power they will denature, rendering the sample unusable.

As before, a spectrograph coupled to the optical fibre separates the component wavelengths onto a CCD camera, however the CCD camera itself has changed from Andor Technology to Avantes. Replacing the Andor Technology CCD camera with an Avantes one was undesirable as the Andor CCD camera was far superior in quality to the Avantes and provided a much higher spectral resolution. However, this change was necessary as the CCD camera needed replacing and, even though the spectral resolution was far superior, the additional capabilities offered by the Andor CCD camera were superfluous for the requirements of the PF MARY experiment and the Andor CCD camera was prohibitively expensive. Consequently, an Avantes CCD camera was purchased as a replacement, however this resulted in a decrease in the spectral resolution and quality of the data collected. Despite this, the other improvements to the PF MARY data have still managed to improve the

signal quality, as demonstrated in Section 2.7.2, but some loss of resolution is observed in collected spectra.

The spectrograph slit width and CCD camera exposure time are still optimised for each experiment to maximise signal and use the full dynamic range of the detector. A 405 nm or 450 nm diode laser (Power Technology) is used when studying flavin-based systems, and continuously photoexcites the sample with a 420 nm or 475 nm long-pass filter, respectively, employed to reduce the amount of scattered laser light reaching the CCD camera.

2.6 Allan Deviation

The standard deviation of a series of measurements is a good measure of the quality of a dataset. The standard deviation measures the dispersion of the dataset from the mean, giving an indication of how spread out the data are. The standard deviation, SD or σ_x , of a series of N measurements is given by:

$$\sigma_x = \sqrt{\frac{1}{N-1} \sum_{i=1}^N (x_i - \bar{x})^2}, \quad (2.4)$$

where x is any given value of the dataset. Taking an increased number of measurements should lead to a more accurate representation of the true value. The standard error of the mean, SEM or $\sigma_{\bar{x}}$, accounts for this and indicates how an increased sample size provides better representation of the population of the mean.

The SEM of a series of N measurements measures how the data deviate from the mean value, *i.e.* how close the sample mean is to the actual population mean, and is given by:

$$\sigma_{\bar{x}} = \frac{\sigma_x}{\sqrt{N}}, \quad (2.5)$$

From equation 2.5, it would be expected that taking an infinite number of measurements would decrease the uncertainty and lead to a more true value, as the denominator tends closer and closer to zero. Indeed, for a system with purely random errors this would be true. However, in reality systematic error is present

and unavoidable, and at some point systematic noise will start affecting signal stability and will lower the quality of the data, making further averaging futile.

Allan Deviation, σ_A , balances both the random noise and systematic noise in a series of measurements, making it possible to identify a ‘sweet spot’ where the contribution from each type of noise is minimised as much as possible. From this, one can deduce the optimum parameters for an experiment, where there are enough averages to account for the white noise but not too many so to avoid the systematic noise regime.

Consider a series of N measurements. This set of measurements can be split into L subgroups, each containing k individual measurements, so that:

$$L = \frac{N}{k}. \quad (2.6)$$

For the subsequent studies N was chosen to be 5000. Therefore if $k = 2$, for example, there would be 2500 subgroups, each containing 2 measurements averaged together; if $k = 5$, there would be 1000 subgroups containing the average of 5 subsequent measurements, and so on for all integer values of k . The standard deviation, σ_{SD} for each value of k can be calculated according to:

$$\sigma_{SD}(k) = \sqrt{\frac{1}{(L-1))} \sum_{i=1}^L (x_i - \bar{x})^2}, \quad (2.7)$$

and the Allan deviation, σ_A , can be calculated by:

$$\sigma_A(k) = \sqrt{\frac{1}{2(L-1))} \sum_{i=1}^{L-1} (x_{i+1} - x_i)^2} \quad (2.8)$$

A plot of k vs. σ_A allows the optimum integration time to be found by reading off the minimum value.

Figure 2.10 shows a log-log plot of k vs. the Allan and standard deviations for a sample of 10 μ M RF flowed through the new fluorescence setup using both: a) a syringe pump, and b) a peristaltic pump. For these measurements the CCD camera was set at a short integration time of 5 ms for each measurement.

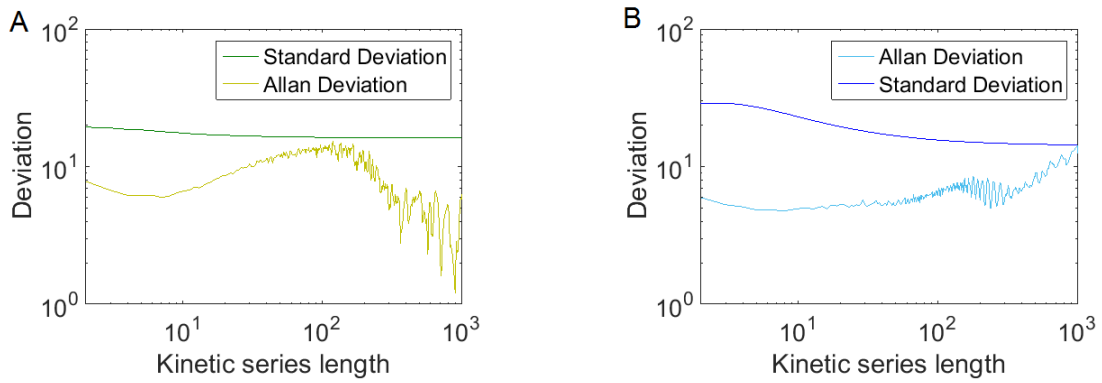


Figure 2.10: A log/log plot of the Standard deviation (dark green/dark blue) and Allan Deviation (light green/light blue) for a sample of $10 \mu\text{M}$ RF flowed at a rate of 0.25 ml/min with A) a syringe pump (left) and B) a peristaltic pump (right). In both cases a 450 nm laser at a power of 40 mW was used. The optimal for both is at a kinetic series length of ~ 8 , corresponding to an exposure time of 40 ms . The standard deviation at this exposure time is 18 ms for the syringe pump and 24 ms for the peristaltic.

The SD for both pumps is relatively consistent with increasing kinetic series length, only slightly dropping at early times due to the initial increase in size of the dataset. After this, the SDs plateau, as would be expected considering that the spread of the dataset should not be dependent on the number of averages (this is not true for the SEM).

The Allan deviations, however, show a sharper initial drop, indicating that there is a slight improvement/reduction in noise at the beginning (where random noise *e.g.* white noise dominates) before long-term drift starts to dominate at later times. The fact that the minimum is less pronounced in Figure 2.10B (peristaltic pump) suggests that this setup is slightly more strongly dominated by long-term drift.

When run at similar flow rates, the Allan deviation for both the peristaltic pump and syringe pump gives rise to similar minima of $k = 8$, corresponding to an optimum integration/exposure time of 40 ms ($8 \times 5 \text{ ms}$). After this point the systematic noise begins to dominate leading to an increase in deviation, and so after this point there is no benefit in averaging further. Any further averaging will not increase the precision of the dataset. Figure 2.10A seems to suggest another minima at a higher kinetic series length of $k = 1000$, but this corresponds to an exposure time of 5 s which would lead to unacceptably long experiment times.

Where possible, experiments were run using the optimum exposure time of 40 ms, however, occasionally when the fluorescence quantum yield was high this led to the CCD camera saturating and so was not feasible. In these instances slightly lower exposure times had to be chosen or, if possible, lower laser powers were used instead. Furthermore, in the case of biological samples where fluorescence quantum yields are low, higher exposure times were required in order to have enough light reaching the CCD to give an acceptable signal.

Although the Allan deviation studies are similar for both pumps, the standard deviation for the syringe pump (at the optimal exposure time of 40 ms) is 18 ms compared to 24 ms for the peristaltic pump. For this reason, the syringe pump was always favoured wherever possible, *i.e.* when the sample was not precious and could afford not to be recirculated, in order to give the best possible quality of data.

2.7 Measuring MFEs by Fluorescence

In order to calculate MFEs using the PF MARY experiment described above, the properties/spectra discussed below need to be considered. The model system of riboflavin (RF) and tryptophan (W) is used as a demonstration.

2.7.1 Materials

RF and W were purchased from Sigma Aldrich and used without further purification. Samples were made up in ultrapure Milli-Q water ($18\text{ M}\Omega\cdot\text{cm}$) to concentrations of $20\text{ }\mu\text{M}$ RF and 1 mM W. At all concentrations used it is assumed that W is in large excess.

2.7.2 Data Interpretation

Figure 2.11 depicts a typical fluorescence emission profile of a system of $20\text{ }\mu\text{M}$ RF / 1 mM W in the absence of a magnetic field; Figure 2.12 shows the same emission spectrum overlaid with the emission profile of the sample in the presence of a static magnetic field, $B_0 = 12\text{ mT}$. The effect of applying an external field is

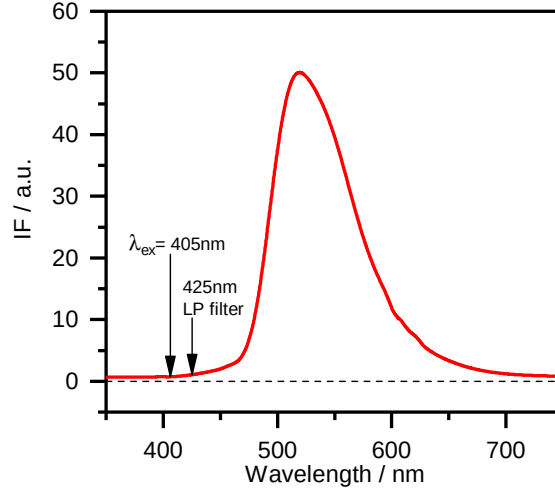


Figure 2.11: Fluorescence intensity, IF, profile for an aqueous solution of 20 μ M RF / 1 mM W in the absence of an external magnetic field ($B_0 = 0$ mT), flowed at a rate of 0.25 ml/min and continuously photoexcited at 405 nm, with a 425 nm longpass filter employed to minimise scattered laser light reaching the CCD camera. A minor feature at 470 nm is expected to be observed due to the Raman scattering of the 405 nm laser light by water, however the loss of spectral resolution with the Avantes CCD means this detail is not observable.

clearly illustrated in the reduction of fluorescence intensity, which is expected for photochemical systems with triplet born RPs (see Section 2.3).

The fluorescence intensity subtraction profile, ΔIF , is shown in Figure 2.13 and is the calculated difference in the fluorescence intensities for field on (IF_{on} , $B_0 = 12$ mT) and field off (IF_{off} , $B_0 = 0$ mT) according to:

$$\Delta IF = IF_{on} - IF_{off}. \quad (2.9)$$

This ΔIF plot maps directly onto the inverted fluorescence emission spectrum (Figure 2.11) indicating that the field-dependent fluorescence is definitely from $^1F^*$. The MFE can therefore be calculated using the equation:

$$MFE = \frac{IF_{on} - IF_{off}}{IF_{off}} = \frac{\Delta IF}{IF_{off}}. \quad (2.10)$$

It should be noted that MFEs calculated from fluorescence experiments using the above equation are not directly comparable to the MFEs obtained by the

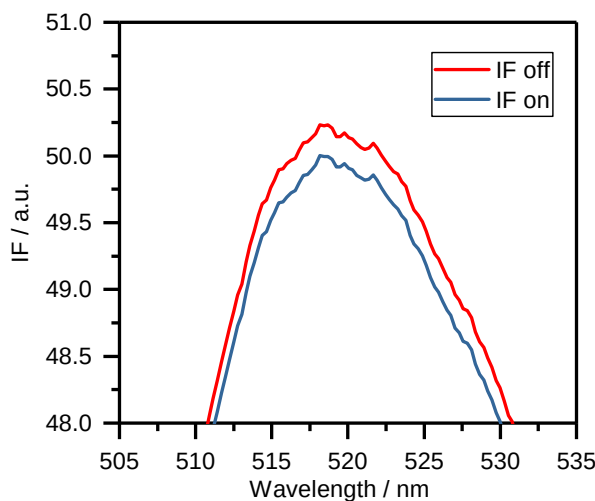


Figure 2.12: Fluorescence intensity profile for 20 μM RF / 1 mM W in the absence (red line) of an external magnetic field, IF_{off} ($B_0 = 0$ mT), and presence (blue line) of an external field, IF_{on} ($B_0 = 12$ mT). Data are smoothed using a 10 nm moving average filter. An unambiguous negative MFE is observed.

traditional absorption-based techniques. Absorption-based techniques monitor the radicals directly, and so although these methods are dealing with a small change, the small change is with respect to a much smaller number/proportion of molecules. This results in larger MFEs being observed. With fluorescence, however, MFEs are monitored indirectly and are detected on top of a large background fluorescence (IF_{off}). Consequently, the denominator in equation 2.10 is typically large, and MFEs observed by fluorescence are normally significantly smaller than those observed using absorption-based methods. Fluorescence techniques are able to observe these small changes, however, due to their high sensitivity.

Subsequently, after calculating MFEs, the Magnetically Altered Reaction Yield (MARY) curve can then be obtained via plotting MFE as a function of field strength (Section 1.8). Figure 2.14 shows a MARY plot for 20 μM RF / 1 mM W obtained on both the former fluorescence setup (orange) and the new setup (purple). The reduction in noise in the new fluorescence setup is apparent in the increased smoothness of the MARY curve.

The slight difference in magnitude of the MFEs results from the difference of laser power in the two experiments. The new setup involves focussing the laser

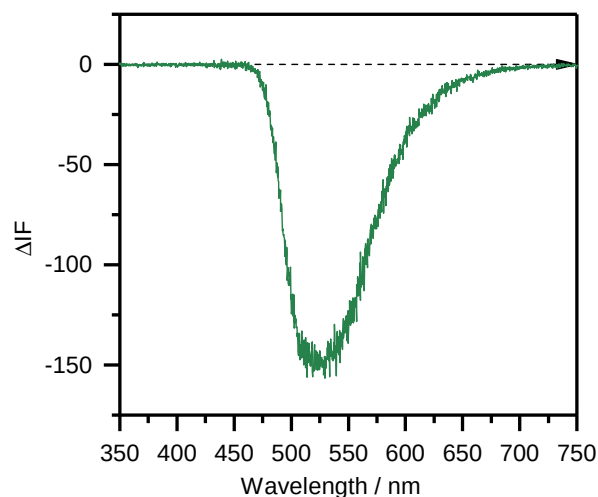


Figure 2.13: Fluorescence intensity subtraction profile, ΔIF ($\Delta IF = IF_{on}(B_0 = 14 \text{ mT}) - IF_{off}(B_0 = 0 \text{ mT})$), at maximum field strength ($B_0 = 14 \text{ mT}$) for a sample of $20 \mu\text{M}$ RF / 1 mM W.

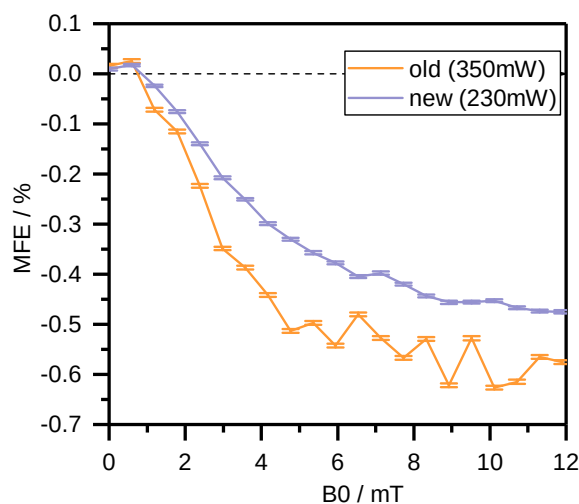


Figure 2.14: MARY plot for a sample of $20 \mu\text{M}$ RF / 1 mM W in both the former fluorescence setup (orange) and the re-designed fluorescence experiment (purple). Error bars represent the standard error of the mean (SEM) of 500 experiments. Both plots show a negative MFE indicative of a triplet born RP species. The difference in absolute magnitudes of the two curves arises from the change in photoexcitation power used in the two experiments.

light into a fibre which directs the light into the sample cell. In optical setups such as these, the change of refraction index upon going from air to fibre results in some of the laser energy being reflected backwards. This is known as coupling loss. Therefore, although both experiments use the same 405 nm diode laser operating at the same power from the laser source, the sample itself sees a lower laser power in the new setup as a result of these coupling losses.

A reduction in laser power results in a decrease in MFE for intermolecular RP systems as a result of fewer molecules being photoexcited and participating in the photocycle. Photoexcitation power dependence is discussed in more detail when considering the photochemistry of thionine in Chapter 3.

A small LFE is observed for the RF/W system which implies that the RP formed is long lived enough to have an effect on the spin dynamics of this system. LFEs are also observed in the thionine systems studied in the following chapter (Chapter 3) and are discussed in detail there.

2.8 Early attempts at observing an MFE on *DmCry* in *in vivo*

MFEs have already been observed on a wide range of cryptochrome-related systems: from model systems of free flavin and an electron donor, to protein-based model systems of cryptochromes, to cryptochromes themselves (all in aqueous solution) [42, 54, 73]. Ultimately the aim is to get even closer to natural biological conditions, yet even with pure cryptochrome samples the protein is exposed to aqueous solution, hardly similar to the environment it would experience in the environment of a cell *in vivo*.

Spodoptera frugiperda, Sf9, cells are cells from the ovaries of a fall armyworm and are commonly used as an environment to express protein (as an insect cell alternative to expressing protein in bacterial cells, such as *E. Coli*) [76, 77]. Sf9 cells can be grown and cultivated to overexpress cryptochromes such as *DmCry*. This offers the potential to study *DmCry in vivo* and thus study the photochemistry of a system closer to natural biological conditions than has ever been studied previously.

Whilst absorption based techniques have admittedly observed MFEs on cryptochromes *in vitro*, the increased sensitivity offered by the new PF MARY experiment combined with the complicated requirements of working with living cells suggests fluorescence-detection to be the best chance of observing an MFE *in vivo*.

2.8.1 Difficulties of working with *DmCry* in *Sf9* cells

Unsurprisingly working with proteins and living cells comes with complications. Due to the time and complexity required to grow a batch of *Sf9* cells and successfully infect them with *DmCry*, these samples are only available sparingly and in low volumes and concentrations. Moreover, an infection rate of 100% is unheard of: in reality only around 50-70% of the *Sf9* cells in solution will be infected with *DmCry*. Consequently the sample will not only have a low concentration of *Sf9* cells, but not all the *Sf9* cells will be infected with *DmCry*.

Even with cells that are infected with *DmCry* issues arise due to very small quantum yields of fluorescence. The fluorescence quantum yield of *DmCry*, at 6.8×10^{-4} [78], is many orders of magnitude lower than that of RF, (0.28 [79]). Low fluorescence quantum yields result in weaker signals which adds to the complexity of observing an MFE on this sample; looking for a small change in an already very small signal is quite a challenge.

In addition to not having the most ideal photochemistry, there are obstacles to overcome in the practicalities of working with *Sf9* cells themselves. Once the *Sf9* cells are infected with *DmCry* the cells slowly begin to die. Upon cell death, known as apoptosis, the cell walls will break releasing the contents of the cell, including *DmCry*, into the surrounding solution. From this point onwards, not all the *DmCry* remains in a cellular environment and so it is not possible to be sure that any observed MFE is coming from the *DmCry* in solution or *in vivo*. Consequently, it is essential to start the experiment quickly and, more than that, the experiment itself needs to not take too long. This makes the re-designed fluorescence experiment ideal as the increased sensitivity reduces the experiment

time, and its simple setup does not require any complicated alignment meaning experiments can be setup and started quickly.

Sf9 cells are cultivated and grown in a nutrient-rich medium, and cell death is often prompted by bacterial infection from bacteria growing in this medium. The medium contains a variety of vitamins and nutrients that provide ideal growing conditions for the *Sf9* cells, however unfortunately these conditions are also ideal for bacterial growth. Whilst all cell cultures are grown in sterile conditions, it is not possible to maintain these conditions during the experiment stage. Instead, care has to be taken throughout the experiment to work in semi-sterile conditions in order to prevent the medium becoming infected by bacteria. Cold working conditions also act to slow bacterial growth in an attempt to slow cell death arising from bacterial infections and so cooling the sample and experimental apparatus to 5-10°C can be helpful.

DmCry Sf9 samples are precious, rapidly degrade and have exquisitely low fluorescence quantum yields, therefore a technique is needed that is highly sensitive, gentle on the sample and requires low sample volumes to allow the measurement of very small MFEs in the short window of time before the cells die. The newly improved fluorescence experiment fits these requirements better than absorption based techniques and should thus provide the best chance for observing MFEs on *DmCry* in *Sf9* cells.

2.8.2 Materials

In order to carry out these experiments, I spent time in Freiburg learning how to handle cryptochromes in living cells, monitor cell health and work in semi-sterile conditions. All *Sf9* cells were cultivated and infected with *DmCry* in Freiburg. *Sf9* cells were grown in ‘Insect Xpress’ medium (Lonza Bioscience) under sterile conditions.

Cells were transfected in Freiburg before being shipped to Oxford on ice. Tests were carried out in Freiburg to simulate shipping conditions (for periods of time similar to the length of time it takes to ship the sample to Oxford) to confirm

that no substantial cell death occurs during shipping. Whilst cell death was not substantial during these tests, unsurprisingly some cell death did occur (as cells begin to die as soon as they are transfected). In the future there may be merit in transfecting the cells more locally so that experiments can be carried out as close to transfection as possible. This would hopefully minimise issues with cell death for the experiment, and provide more confidence that the MFEs observed are from cryptochromes *in vivo* and not from cryptochrome free in solution after leaking into solution after apoptosis occurs.

Upon arrival in Oxford, samples were centrifuged at a rate of 300 rpm for 5 minutes, after which the supernatant was removed and the cells were re-suspended in fresh Insect Xpress medium. This process ensured any free FAD/*DmCry* was removed from the solution, whilst also providing an opportunity to slightly concentrate the sample.

Two variations of *DmCry* were used: the *DmCry* ‘wild type’ (*DmCry* WT), and a mutant, *DmCry* W4 (also known as *DmCry* W394F). *DmCry* WT has the same protein sequence as that found in *DmCry* in nature. *DmCry* W4 is identical to *DmCry* WT except the fourth tryptophan (W394) in the ET chain is replaced by a redox inert phenylalanine. Other cavity experiments have observed MFEs on *DmCry* W4 orders of magnitude larger than that of *DmCry* WT [80]. Consequently, the *DmCry* W4 in *Sf9* cells were cultivated not only to act as a comparison to *DmCry* WT but also to provide the best chance of observing an MFE *in vivo*.

2.8.3 Experimental

The redesigned PF MARY arrangement introduced above was used to monitor MFEs (Figure 2.6), with the peristaltic pump favoured over the syringe pump to allow recirculation of the precious sample protein samples.¹ A 450 nm diode laser with a 475 nm LP filter was employed to best correspond with the absorption peak of the FAD in *DmCry*.

¹A slight variation for experiments on *DmCry* W4 was that the CCD camera and peristaltic pumps had been upgraded to a higher quality (Andor Technology and CorSolutions PeriWave pump), with the coding for the new CCD camera written by Victoire Dejean.

The coils were cooled to a temperature of -20°C , and the rest of the setup was cooled prior to use through the use of ice packs and dry ice in order to give an ambient temperature of $5\text{-}10^{\circ}\text{C}$. Although ice packs are an unideal method for controlling temperature as they melt over the course of the experiment resulting in temperature fluctuations, the temperature of the sample was measured prior to and following the experiment and remained within a range of $5\text{-}10^{\circ}\text{C}$. Lowering the temperature of the experiment is beneficial to working at room temperature as it keeps conditions more ideal for observing MFEs on the protein, and also slows bacterial growth in the medium in attempt to keep the *Sf9* cells alive for longer. Consequently the slightly crude technique of using ice packs was favoured over working at room temperature.

2.8.4 Results and Discussion

A PF MARY experiment was performed on both *DmCry* WT and *DmCry* W4, the results of which are shown in Figures 2.15 and 2.16, respectively. In order to maximise the chance of seeing an appreciable MFE a long delay time was chosen [81]. For both samples of *DmCry* a very small negative MFE is observed ($\sim -0.0055\%$ for *DmCry* WT and $\sim -0.004\%$ for *DmCry* W4, at a field strength $B_0=11\text{ mT}$). Despite large error bars, the majority of data points for both systems lie below zero suggesting a negative MFE is present, even if very small.

The fact that the MFE is negative for both *DmCry* WT and *DmCry* W4 is perhaps surprising as for an intramolecular RP system, such as cryptochrome, a positive MFE is expected to be observed by fluorescence. In intramolecular systems the rate of ET outcompetes the rate of ISC to form a the singlet-born SCRP, which results in a positive MFE by fluorescence (Section 2.3). However, it could be possible that the MFE observed here arises as a result of the $[\text{FADH}\cdot\text{Z}\cdot]$ RP, rather than the $[\text{FADH}\cdot\text{W}\cdot]$ one, which does not necessarily have to be singlet born. $[\text{FADH}\cdot\text{Z}\cdot]$ could be a triplet born RP which would explain the sign of the observed MFE.

However, a PF MARY experiment was also performed on solely the Insect Xpress medium *i.e.* without any *DmCry* or *Sf9* cells present, the results of which

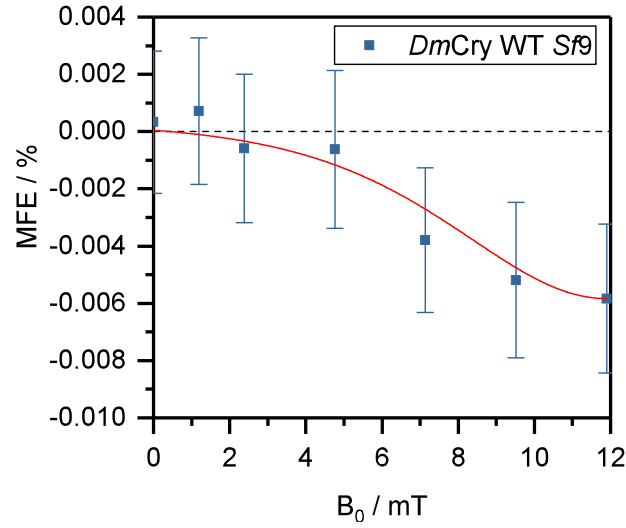


Figure 2.15: MARY curve of *DmCry* WT in *Sf9* cells in Insect Xpress medium, wavelength averaged over 500-600 nm. The sample was flowed at a rate of 0.25 ml/min, and a delay time (*i.e.* the time between switching the field and turning on the CCD camera) of 1 s was used. Error bars represent the SEM of 500 measurements. The red line is a polynomial fit added to guide the eye. A small negative MFE is observed.

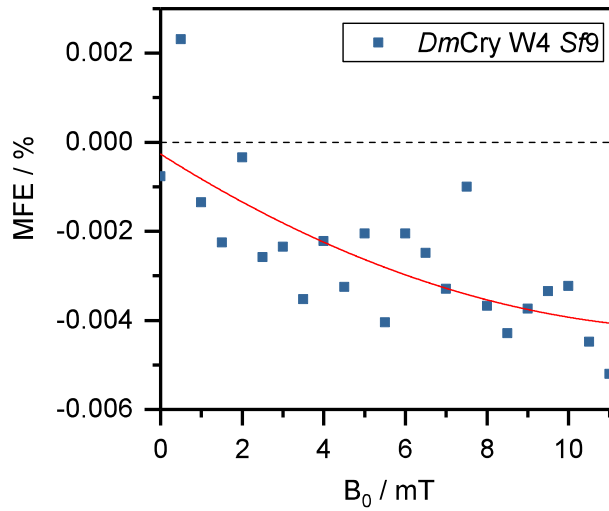


Figure 2.16: MARY curve of *DmCry* W4 in *Sf9* cells in Xpress medium, wavelength averaged over 500-600 nm. A delay time of 2 s was used in attempt to maximise the size of the MFE, and once again the red line is present to guide the eye. Error bars not included as this is not yet possible with the new analysis. Coding for the analysis was written by Victoire Dejean.

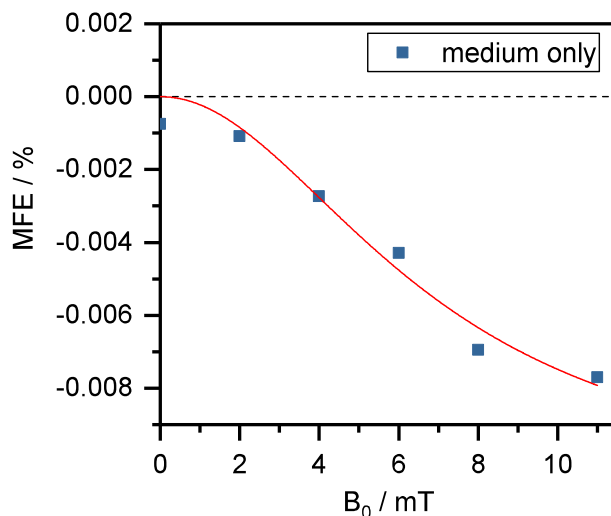


Figure 2.17: MARY curve of the Xpress medium only (no *DmCry* or *Sf9* cells present) as obtained by fluorescence with wavelength averaged over 500-600 nm. A delay time of 2 s was used, and the red line is a polynomial fit to guide the eye. Error bars not included as this is not yet possible with the new analysis. Once again, a small negative MFE is observed.

are shown in Figure 2.17. Unexpectedly, a small negative MFE was observed here too. Although the MFE was small, it was slightly larger in magnitude than that obtained for both the samples of *DmCry* WT and *DmCry* W4 in *Sf9* cells. The MFE for the medium only was $\sim -0.0075\%$ at $B_0 = 11$ mT, nearly twice as big as that for *DmCry* W4.

Despite the fact that the medium should not contain any flavin (the medium used in these experiments was never in contact with flavin or *DmCry*, just used as provided, and was never in a position to be contaminated with flavin), the medium fluoresces between 500-600 nm as shown in Figure 2.18. Figure 2.18 shows the normalised fluorescence spectra for samples of *DmCry* W4 in *Sf9* cells suspended in the Insect Xpress medium (light blue), uninfected *Sf9* cells suspended in the Insect Xpress medium (dark blue) and the Insect Xpress medium by itself (purple). All three samples have the same spectral shape which suggests that the same species is responsible for the fluorescence in each case. The shape of the peak centered around 500-600 nm is characteristic of the fluorescence spectrum of flavin. The medium and uninfected cells fluoresce with the same spectral shape as that of *DmCry* W4 which

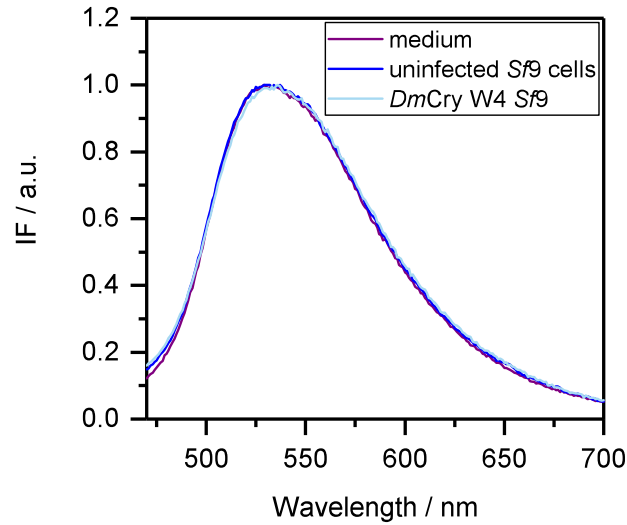


Figure 2.18: Normalised fluorescence spectra of *DmCry W4* in *Sf9* in Xpress medium, uninfected *Sf9* cells in Xpress medium and Xpress medium only. All spectra overlay with the same spectral shape, indicating that the fluorescence is coming from the same species.

suggests that flavin is present in both the *DmCry W4* sample (as would be expected due to the FAD), but also in the uninfected *Sf9* cells and in the medium alone.

Media used for cell growth typically contain a wide variety of essential nutrients, vitamins and amino acids required for growth. Unfortunately, for the specific Insect Xpress medium used, this happens to include vitamin B2 *i.e.* riboflavin (RF). Although the exact composition of the Insect Xpress medium is a trade secret, it would not be implausible for there to be an electron donor in the medium as well that could form a RP with the RF. A variety of amino acids and vitamins are able to act as ED, including ascorbic acid (vitamin C) which has known photochemistry with flavin partners [82]. Consequently the flavin and ED in solution could form a SCRP and give rise to an unwanted MFE.

If the MFE was from free RF and an ED in solution then this would explain the perhaps unexpected sign of the MFE observed. As previously stated, for cryptochromes a positive MFE would be expected (if the RP responsible for magnetic sensing is the FAD/W one) as the rate of ET is fast in intramolecular systems and outcompetes the rate of ISC. Consequently the singlet SCRP is formed initially and so upon application of a magnetic field fewer radicals become trapped in the T_+

and T_- states. This means more radicals can return to the ground state (from the singlet SCRP only), increasing the ground state concentration and the intensity of fluorescence resulting in a positive MFE. With intermolecular systems, however, the opposite is true: ET is slower and is outcompeted by ISC resulting in a triplet born RP being formed, and corresponding to a negative MFE observed by fluorescence.

As the MFEs observed for the *DmCry* samples were slightly smaller than the MFE observed from the medium itself, this could suggest that the *DmCry* samples are giving rise to a very small positive MFE, but it is being drowned out by the larger negative MFE from the free flavin/ED in solution. Consequently the unwanted photochemistry from the medium is obscuring any MFEs from the *DmCry Sf9* systems. These results highlight the high sensitivity of the PF MARY experiment, with the technique being able to distinguish very small negative MFEs on biological systems. However, in order to conclude if an MFE can be observed on *DmCry* in *Sf9* cells, these experiments need repeating in the absence of the Insect Xpress medium to eliminate any unwanted photochemistry from flavin and other chemicals in the medium. Moreover, as the Insect Xpress medium did not provide a negative control an experiment on water should be compared to the results above.

2.9 Conclusions and Future Work

Overall, in this chapter a highly sensitive fluorescence-based technique for detecting MFEs has been developed. The PF MARY technique, which already had high sensitivity, was redesigned in order to further increase sensitivity, and steps were taken to make the setup more appropriate for studying biological samples. The overall sample volume required for an experiment has been reduced from tens of millilitres to less than 1.5 ml, and a vast increase in sensitivity has been observed, with MARY curves for model systems of RF / W being much smoother and showing a reduction in SNR compared to those obtained with the previous setup. Moreover, a method for flowing and recirculating small volumes of sample has been introduced by employing a peristaltic pump, which eliminates the requirement to flow large volumes of samples to waste. All of these features are essential when

measuring precious biological samples, such as cryptochromes in *Sf9* cells, which are only available in low concentrations and volumes, and so require a highly sensitive, low volume experiment.

The first attempts to observe an MFE on cryptochrome *in vivo* have been made by studying *DmCry* in *Sf9* cells. Due to the exquisitely sensitive nature of the fluorescence technique, very small negative MFEs were recorded on experiments with *DmCry* WT and *DmCry* W4. It was concluded that these MFEs are likely to have arisen from free flavin in the medium (and an ED) and not from the *DmCry Sf9* system itself. However, these results highlight the capabilities of the experiment; the fact that the PF-MARY technique was able to detect such a small MFE demonstrates its high sensitivity.

In the future, it may be beneficial to cultivate the cells more locally rather than transfecting them in Freiburg and then shipping them to Oxford. As soon as cells are transfected it is a race against time to carry out experiments before cell death occurs (which could result in cryptochrome being free in solution as well as contained in the cells). Local transfection would allow experiments to be carried out much more rapidly after transfection and hopefully result in better quality experiments being performed.

If *DmCry* is going to be continued to be overexpressed in *Sf9* cells grown in Xpress medium, then the *Sf9* cells need to be removed from this solution prior to being used on the PF MARY experiment. This could be done by centrifuging the cell suspension at a low speed for several minutes, which will cause the cells to accumulate at the bottom of the Eppendorf and form a pellet. At this point the supernatant can be gently pipetted off in the same process as carried out in Section 2.8.2. The key difference being that the cells can then be re-suspended into a different buffer, rather than back into Insect Xpress medium. If necessary, this whole process can be repeated multiple times to ensure near all of the medium is removed. A balance must be struck, however, between exchanging as much of the medium as possible but also performing the experiments as efficiently and quickly as possible to avoid issues with *Sf9* cell death.

The difficulty with this process is that the Insect Xpress medium contains key nutrients which the *Sf9* cells need for growth. Care must be taken to choose either an appropriate buffer solution in which the *Sf9* cells can survive, or another medium that does not contain any flavin, ED or other chemicals that could contribute to undesirable photochemistry. However, if an appropriate buffer/medium is found then the redesigned PF MARY experiment has been shown to be highly sensitive and should be able to observe an MFE on the *DmCry Sf9* system in future.

Alternatively, PF MARY experiments could be done on *DmCry* overexpressed in bacterial cells, such as *Escherichia coli* (*E. coli*). Bacterial cells are much more straightforward to cultivate and the requirement to grow them in a medium, such as Insect Xpress medium, is eliminated. This would remove the complications of having RF and EDs present in the solution and would potentially result in a more straightforward experiment.

3

Prompt Fluorescence MARY: Photochemistry of RP reactions in micelles

In this Chapter the re-designed prompt fluorescence MARY (PF MARY) technique introduced in Chapter 2 is further characterised, performing proof-of-principle experiments whilst exploring the photochemistry occurring in systems of thionine and an electron donor (ED) in micelles. The chapter begins by providing an introduction to micelles, discussing the motivations behind using micelles to study photochemical reactions, and then goes on to introduce the chemical systems discussed in the rest of the chapter and the motivations behind the work performed. In order to carry out characterisation and proof of principle experiments, a system with a well understood photochemical scheme was chosen: thionine/1,4-diazabicyclo[2.2.2]octane (DABCO)/sodium dodecyl sulfate (SDS) (introduced in detail in Section 3.2). Following this, experimental parameters have been varied to explore how certain aspects of the micelle and/or RP affect the photochemistry of the system (*e.g.* how ED concentration or micelle size/charge affect the MFE), and the effect that different EDs with aniline has on the photochemistry and subsequent MFE was probed.

3.1 Introduction to Micelles

Micelles have long been employed as nanocontainers in studying reactions involving RPs. Owing to their small size (typically 1-10 nm), micelles can act to significantly restrict the diffusion of reactants, which can have dramatic effects on reaction rates and/or product yields [83–86]. Considering the RPM (Section 1.7), in order to observe significant MFEs it is often beneficial to extend the geminate lifetime of the RP (which is typically of the order of 10 ns for systems such as those studied in this thesis [87]). Micelles act to restrict diffusion of the SCRP, increasing the lifetime of the geminate pair and thus potentially leading to an increased magnitude of MFE. Changing the size or charge of a micelle can have a significant effect on the diffusion and/or the position of RPs in the micelle environment and thus have an effect on outcome of a reaction (see Section 3.7).

Micelles also have a biological relevance, given certain components of cells and other biological structures are based on the structure of a micelle or are formed of similar amphiphilic monomers (*e.g.* lipid bilayers, the cell membrane and vesicles) [88, 89].

3.1.1 Structure of micelles

Micelles are self-aggregating systems made up of surfactant monomers. The structure of an anionic micelle formed from sodium dodecyl sulfate (SDS) monomers is shown in Figure 3.1. Surfactants are amphiphilic molecules, meaning they possess both hydrophobic and hydrophilic groups, and adding a surfactant to water acts to lower the surface tension of the solution. Surfactants are categorised according to the type of polar head group which may either be charged (cationic or anionic), or uncharged. SDS monomers, as shown in Figure 3.1, are anionic surfactants owing to their negatively charged head group.

Micelles arrange themselves in an approximately spherical shape in aqueous solution. The polar head groups are hydrophilic and position themselves on the outside of the structure in contact with the aqueous solution, whilst the hydrophobic tails (long hydrocarbon chains) occupy the centre of the micelle from which water

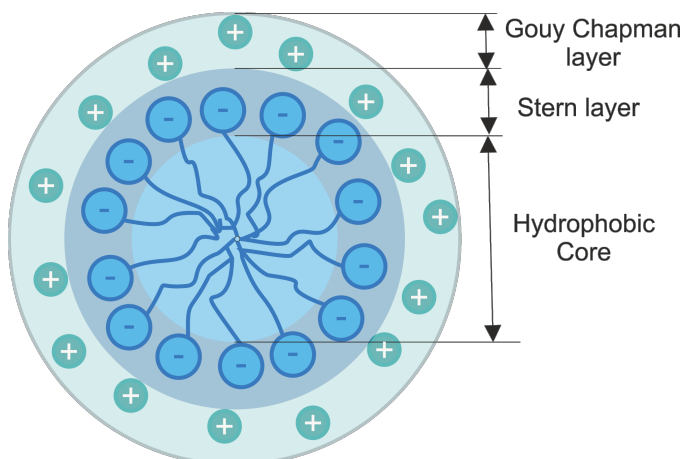


Figure 3.1: The structure of an anionic micelle (such as SDS), with the hydrophobic core, Stern layer and Gouy Chapman layer highlighted. Micelles are formed from surfactant monomers (dark blue) comprising a polar head group, positioned in the Stern layer, and a non-polar hydrocarbon tail, forming the hydrophobic core. The structure is not to scale (typically the core has a diameter of $\sim 10\text{-}30$ Å, the Stern layer is $\sim 1\text{-}8$ Å and the Gouy Chapman layer is $\sim 100\text{-}400$ Å).

molecules are excluded. The hydrocarbon tails form the hydrophobic core, and are more restricted in motion compared to a pure solution of a hydrocarbon of the same chain length, but are however still mobile [90]. The amphipathic nature of micelles causes self assembly in solution as a result of the hydrophobic effect which is discussed in detail in Section 3.1.2.

As seen in Figure 3.1, the hydrophilic head groups occupy a layer which is the closest water molecules get to the centre of the micelle. In this example of an SDS micelle, the head groups are anionic resulting in a negatively charged Stern layer. The Gouy-Chapman layer is a diffuse layer of counter ions, which in SDS micelles are Na^+ ions, which form a co-ordination sphere around the polar head groups. The thickness of this layer is dependent on the ionic strength of the solution but is typically $10\text{-}40$ Å.

Molecules may occupy a range of locations in a micelle, dependent on what environment (hydrophobic/anionic/cationic) it is best suited to. A more detailed discussion of this, in respect to thionine/DABCO/SDS system, is introduced in in Section 3.4.

Micelles have a number of important related physical parameters that can have

a significant effect on the diffusion and subsequent reaction of an encapsulated RP. A number of these effects are introduced below.

Critical micelle concentration (CMC)

The critical micelle concentration (CMC) is the surfactant concentration above which aggregation into a micelle occurs spontaneously. Adding a surfactant to water decreases the surface tension up to a certain point. At surfactant concentrations below the CMC, the surfactants exist as monomers in solution. After this point, however, the addition of any more surfactant molecules has no further effect on the surface tension, but results in the aggregation of monomers to form a micelle. The CMC is thus calculated by looking at the discontinuity of factors such as conductivity in the solution (*i.e.* the inflection point on a graph of conductivity *vs.* concentration).

Aggregation number (N_{agg})

The aggregation number, N_{agg} , of a micelle defines the number of surfactant monomers aggregating to form a single micelle. The value of N_{agg} and the size of the micelle are strongly linked, and N_{agg} typically falls into the range of 50-100 [91]. It is worth noting that the value of N_{agg} may vary dramatically not only between different surfactants but also for the same surfactant. External factors, such as temperature and ionic strength of the solvent, have a huge effect on micelle size and structure, and consequently on the aggregation number. Furthermore, the value of N_{agg} for a micelle is always an average: micelles are dynamic structures meaning that monomers are replaced with those from solution on a microsecond timescale [92] and the value of N_{agg} is not completely constant for any given species of surfactant.

The value of N_{agg} is determined by a balance of the hydrophobic effect and repulsions of the like-charged head groups. Clearly there has to be a large enough number of monomers in the micelle to effectively expel water from the centre. However, as the number of monomers increases so too does the repulsion between the polar head groups as they begin to be forced closer and closer together. At some point the energetically unfavourable repulsion between head groups becomes

strong enough that it outweighs the energetic gain of removing the hydrophobic tail from being in contact with water. This point determines the value of N_{agg} .

Krafft temperature

The Krafft temperature is the temperature below which micelles will not form. The origin of this lies in the requirement of the surfactant to be in liquid form in order to form a micelle: the surfactant monomers have to be mobile.

The Krafft temperature is determined both by the length of the hydrophobic tail and the nature of the hydrophilic head group. As surfactants must be liquid to form a micelle, increasing the length of the hydrocarbon chain raises the Krafft temperature as the melting point of the substance increases. Conversely, increasing the size of the head group can decrease the Krafft temperature if it acts to prevent the hydrophobic tails from being in close contact which can help prevent solidification.

3.1.2 Hydrophobic Effect

The hydrophobic effect relates to the tendency of nonpolar substances to aggregate together in aqueous solutions resulting in the expulsion of water from interacting surfaces, and is essential when considering the formation of micelles. It is a short-range interaction, describing the preference for nonpolar molecules to interact with each other rather than polar surfaces. The effect is a result of competition between enthalpic and entropic effects [93, 94].

Hydrophobic substances are defined as those that are readily soluble in nonpolar solvents but sparingly soluble in polar ones. An important distinction between a hydrophobic substance and one that is simply just sparingly soluble in all solvents, is that hydrophobic substances remain in a liquid form when expelled from water as they form solids with strong intermolecular cohesion. Although surfactant monomers are not hydrophobic in their entirety, they contain long hydrocarbon chains which are hydrophobic and experience the hydrophobic effect.

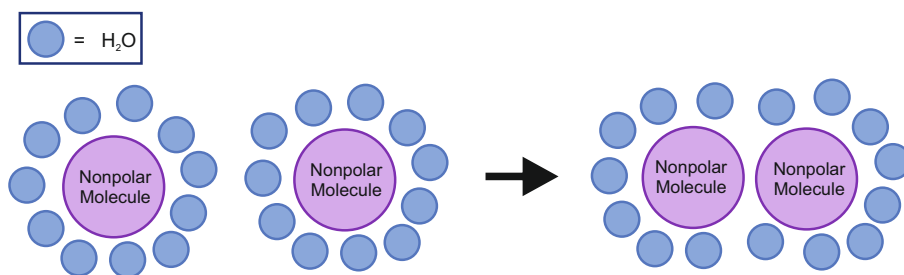


Figure 3.2: Simplified depiction of the effect of two nonpolar molecules associating together resulting in a decrease in total hydrophobic surface area and thus a release of water (H_2O) molecules. This is an entropically favourable process.

At room temperature, the enthalpy of mixing a hydrophobic solute with water is usually considered close to zero or slightly positive [95]. It is instead entropy rather than enthalpy that are the dominant contributions to the hydrophobic effect.

When a nonpolar/hydrophobic solute is exposed to water it is incapable of forming hydrogen bonds or any other electrostatic interaction with water molecules and so disrupts the water-water hydrogen bonding network. This drives the water molecules to re-orient in a way such as to minimise the disruption to the hydrogen bonding network as much as possible, resulting in an ordered cage of water molecules forming around the nonpolar surface. This process is enthalpically driven (through maximising the number of hydrogen bonds between water molecules) but is entropically unfavourable. The decrease in entropy from the ordering of water molecules into a cage outweighs the increase in entropy from mixing the water and solute molecules, resulting in an overall negative entropy of dissolution. With polar solutes, molecules are charged (or partially charged) and thus are able to interact with water molecules to form strong ion-dipole interactions. The unfavourable negative entropy from ordering of water molecules is outweighed by the favourable enthalpy of the water-solute interaction, making polar substances soluble in water.

The extent of the entropy decrease for nonpolar substances is dependent on the size and/or volume of the molecule: for small hydrophobic molecules the entropy decrease is approximately proportional to the volume of the molecule, whereas for larger molecules ($> 1 \text{ nm}^2$ surface area) it is proportional to nonpolar surface area [96].

Upon micelle formation, the water molecules become more disordered and the entropy of the system increases. As shown in Figure 3.2, the association of two nonpolar molecules decreases the overall nonpolar surface area, and so the amount of water-nonpolar interactions are decreased. Some of the water molecules that were involved in forming the cage around the hydrophobic substance are now released, which is entropically favourable, making aggregation of hydrophobic molecules an energetically favourable process.

The hydrophobic effect is still applicable when considering amphiphilic molecules such as surfactants despite them having a hydrophilic head-group as well as a hydrophobic tail [96]. The hydrophobic tails of the surfactant experience the same effect as above, with a gain in entropy observed upon micelle formation as the hydrophobic tails are separated from the water increasing the disorder of the system.

3.2 Materials

All reactants used in the following sections (thionine, 1,4-diazabicyclo[2.2.2]octane (DABCO), sodium dodecyl sulfate (SDS), sodium decyl sulfate (SDeS), sodium octyl sulfate (SOS), aniline, 4-chloro-aniline, 4-bromo-aniline and 4-iodo-aniline) were purchased from Sigma Aldrich and used without further purification. Samples were prepared using ultrapure Milli-Q water (18 M Ω ·cm) and made up to concentrations specified for each experiment.

3.3 Experimental

The re-designed PF MARY setup that was introduced in Chapter 2 was used for all experiments in this chapter, only slightly modified as shown in shown in Figure 3.3, in order to swap the 405/450 nm diode laser for a 532 nm diode laser (Torus) to better correspond to an appropriate excitation wavelength for thionine. Figure 3.4 depicts the absorption spectrum for a sample of 5 μ M thionine in aqueous solution, showing an intense peak centred around 600 nm but with a tail that extends below 500 nm, meaning there is still appreciable absorption at the excitation wavelength of 532 nm.

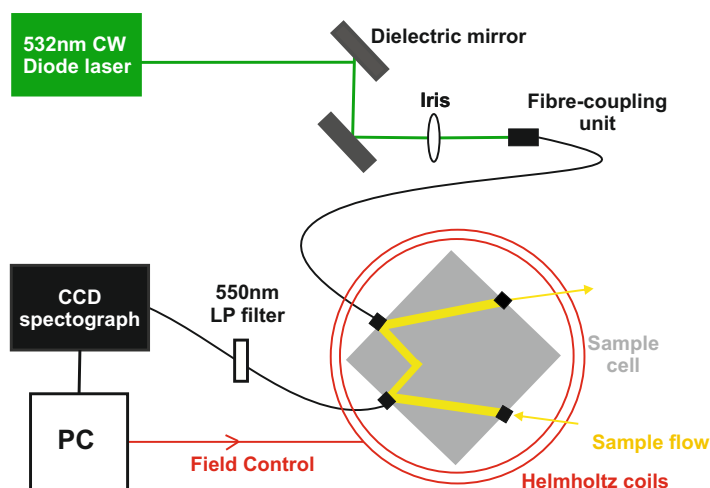


Figure 3.3: PF MARY setup used for thionine experiments. The setup is the same as that introduced in Chapter 2, with the exception of changing the wavelength of the laser to 532 nm to better suit the thionine systems of interest. This also means changing the LP filter to a 550 nm one in order to effectively cut out the 532 nm laser light from the CCD camera.

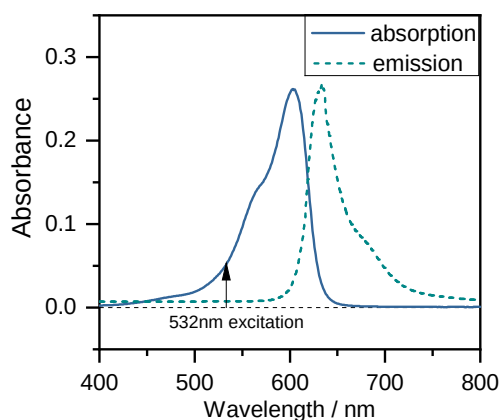


Figure 3.4: Absorption (blue, solid line) and emission (blue, dashed line) spectra of a sample of 5 μ M thionine in aqueous solution obtained from UV-Vis. The excitation wavelength used for the thionine experiments in this chapter (532 nm) is highlighted.

For all results presented in this chapter, a syringe pump was favoured over the peristaltic pump as thionine and all other chemicals used in the following sections are readily available and cheap to purchase and can be flowed to waste (unlike biological samples such as cryptochromes).

3.4 Thionine/DABCO/SDS: LFE and effect of a micelle

For the initial proof of principle experiments RPs were studied in a system that is well characterised. Thionine not only has a well understood photochemical chemical scheme with EDs such as 1,4-diazabicyclo[2.2.2]octane (DABCO), but also exists as a positively charged species (TH^+) at neutral pH and so is ideal for studying with negatively charge micelles such as SDS.

The structures of thionine, DABCO and SDS are shown in Figure 3.5a, b and c, respectively. Thionine is a cyclic heteroaromatic molecule belonging to the phenothiazine group of dyes, and shares some structural similarities to flavin (see Figure 1.2); the cyclic structure of thionine is similar to the isoalloxazine ring in flavin. DABCO is an organic compound which is neutrally charged at neutral pH. SDS is an anionic surfactant which self aggregates in water to form a micelle, as discussed in previous sections, and consists of a sulphate group making up the hydrophilic head, and a 12-carbon chain hydrophobic tail.

3.4.1 Photochemical scheme

The photochemical scheme for a system of thionine (TH) and DABCO (D) is shown in Figure 3.6. In a similar way to the flavin RP reactions discussed previously, upon photoexcitation by green light (rather than blue light as for flavins), the positively charged thionine ground state molecule, TH^+ , is photoexcited to the first excited singlet state, $^1\text{TH}^{+*}$. From here, the system undergoes rapid ISC to form the triplet state, $^3\text{TH}^{+*}$. ET from a DABCO molecule initially results in the formation of the triplet SCRP due to conservation of spin, but may undergo ST mixing to form the singlet SCRP as laid out in Section 1.6. The SCRP of interest for MFEs comprises

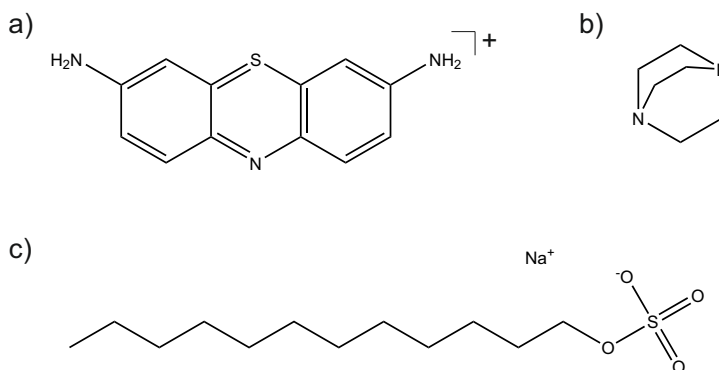


Figure 3.5: Chemical structure of a) thionine b) 1,4-diazabicyclo[2.2.2]octane (DABCO) and c) sodium dodecyl sulfate (SDS).

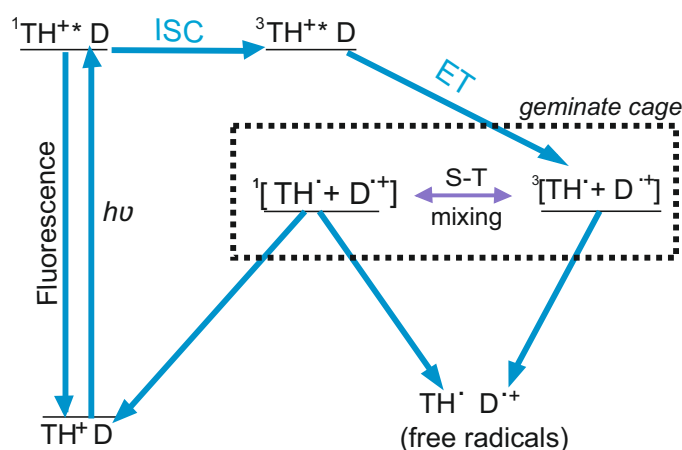


Figure 3.6: Simplified photochemical scheme of a system of thionine (TH) and DABCO (D), where ISC = intersystem crossing and ET = electron transfer.

a thionine semiquinone radical and DABCO cationic radical ($\text{TH}^\cdot + \text{D}^{\cdot+}$), in either an aqueous solution or in a micellar solution of SDS.

In solutions of SDS, molecules can occupy a variety of locations in the micelle, including the hydrophobic core, the Stern layer or the bulk solution. The exact location of where thionine and DABCO sit in an SDS micelle is not known for certain, however, by drawing comparisons between a similar system of DABCO/Xanthone (Xa), where the micelle positions are known, a sensible hypothesis can be drawn [97, 98].

With the Xa/DABCO system, at the moment of creation of the RP the

hydrophobic Xa is located in the core of the micelle, associating with the hydrophobic hydrocarbon tails. DABCO⁺, on the other hand, is created from its molecular precursor on the opposite side of the phase boundary just outside the micelle, as a result of the negative surface charge of the micelle, and subsequently ends up trapped in the negatively charged Stern layer. Xa sits in the hydrophobic core (surrounded by the negatively charged cage of head groups) from which it escapes very slowly. Consequently the radicals are effectively trapped and are kept in closer proximity to one another for longer than they would in free solution.

In a similar manner, in the thionine/DABCO system the DABCO⁺ radical is likely to end up trapped in the negatively charged Stern layer. Initially, TH⁺ will associate with the surface of the negatively charged micelle. However, upon formation of the neutral, slightly hydrophobic TH radical, it will migrate to the hydrophobic core of the micelle. Both radicals are found close to the Stern layer, held in closer proximity, and have a longer lifetime than RPs free in solution.

Considering the kinetics of the RP partners, experimental data show that the escape rate of ³TH⁺ from an SDS micelle to be 0.6 μ s, which only slightly decreases when the surfactant is changed to DTAC [97, 99]. Previous work by Goez et al., looking at the decay rates of the free radical ions, confirm that there is a relatively slow escape of the radicals from the SDS micelles [97]. Consequently, by comparing to the relative rates of the photochemical process highlighted in Figure 3.6, it is expected that the thionine/DABCO RP components will remain in their relative positions in the micelle for longer than the relative timescales of the aforementioned photochemical processes (ET etc.).

Considering the SDS micelles themselves, micelle aggregation in solution can be described by DLVO theory of colloidal interactions. DLVO theory states that the total potential energy of interactions between charged colloid particles, V_{tot} , such as SDS, is given by the sum of the attractive interactions, V_A , and the repulsive ones, V_R :

$$V_{tot} = V_A + V_R. \quad (3.1)$$

The value of V_R in equation 3.4.1 is dependent on the surface potential of the micelles, as well as relative the dielectric coefficient of the aqueous medium, the radius of the micelle, and the Debye screening length. The interaction energy between two micelles therefore varies with changes of these variables, and so factors such as the concentration of counter ions in solution will determine the interaction/aggregation of micelles in solution. If the value of V_{tot} is greater the kT then the micelle dispersion will be stable [100].

3.4.2 MARY plots

Figure 3.7 shows a MARY plot for the photoinduced RP comprising the photosensitiser thionine and the electron donor DABCO both in free solution (blue) or when constricted to SDS micelles (orange). For experiments with SDS, 10 mM SDS was used in order surpass the critical micelle concentration of 8.2 mM. This concentration leads to a micelle concentration of $\sim 150 \mu\text{M}$ given that $N_{agg} = 64$. 40 μM thionine and 0.2 M DABCO were used for all experiments.

A negative MFE by fluorescence is clearly observed in both systems, as expected for a triplet-born SCR system. Application of a magnetic field reduces the intensity of fluorescence as the Zeeman interaction causes energetic isolation of the $T_{\pm}$ states from the S and T_0 states. Thus, more of the system becomes trapped in the triplet RP state, reducing the ground state population (and thus the fluorescence intensity). This results in a negative ΔIF ($= IF_{on} - IF_{off}$), which translates to a negative MFE (equation 2.10).

Figure 3.7 also highlights the result of constricting the RP to a micelle such as SDS: an over 5 fold increase in MFE observed on the thionine/DABCO RP system in SDS (orange) compared to in free solution (blue). Confining the RP to a micelle restricts the diffusion of the RP partners and thus acts to increase the lifetime of the geminate pair, ultimately leading to an increase in the magnitude of the MFE. As discussed above, the thionine and DABCO radicals are likely to be found in the hydrophobic core (but close to the phase boundary as the TH^+ precursor is likely to associate with the negatively charged micelle surface) and Stern layer, respectively.

The micelle holds the RPs in closer proximity and so the chances of recombination to the ground state rather than diffusing apart to form free radicals are increased.

The data clearly suggest the presence of an LFE ($0 \leq B \leq 2$ mT), which is seen more clearly in Figure 3.8 where an initial MFE of the opposite sign to the ‘normal’ MFE is observed. Figure 3.8 shows a MARY plot for the same 40 μ M thionine / 0.2 M DABCO / 10 mM SDS chemical system, but with an increased number of data points at low field to probe the LFE region more thoroughly.

LFEs are of high importance when considering the RPM in regards to events such as magnetoreception as they offer increased sensitivity to weak magnetic fields, which are applicable to Earth strength fields, ~ 50 μ T. As discussed in the introduction (Section 1.6), the size of the LFE is strongly dependent on the RP lifetime, with the condition that $\tau^{-1} \ll a$ (where τ is the RP lifetime, and a the average hyperfine coupling of the RP) for the greatest effects to be seen. The discrepancy in the absolute magnitude of the MFEs between Figure 3.7 (orange) and Figure 3.8 is due to the difference in photoexcitation power used, an explanation of this effect can be found in Section 3.5.

$B_{\frac{1}{2}}$ values and the Weller formula

Whilst the effect of confining RPs to a micelle can easily be seen by the large increase in magnitude of the MFE, the shape/breadth of the MARY curve can also give information about certain aspects of the photochemical system. The shape of the MARY curve is individual to each RP species and its spin dynamics, and the $B_{\frac{1}{2}}$ value obtained from the MARY plot provides a parameter for comparing these MFE measurements.

The $B_{\frac{1}{2}}$ value is defined as the strength of the applied magnetic field, B_0 , at an MFE of exactly half that observed at saturation for field strengths that are greater than the local field induced by hyperfine interactions. In practice, the exact field strength corresponding to the saturated MFE value is hard to obtain and so a Lorentzian function can be fitted to the MARY plot and the half-width half-maximum (HWHM) value used to find $B_{\frac{1}{2}}$. Although a Lorentzian function

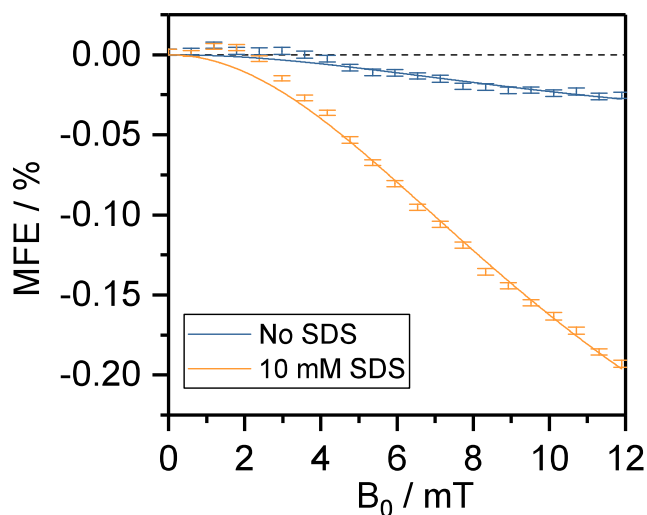


Figure 3.7: MARY plot for 40 μ M thionine / 0.2 M DABCO only (blue) and in the presence of 10 mM SDS (orange) measured by PF MARY. Wavelength averaged 600-700 nm, with a photoexcitation power of 70 mW. Uncertainty bars on the data points represent ± 1 standard error of the mean of 500 individual measurements. Lorentzian fits have been added to the data. The presence of SDS clearly acts to increase the magnitude of the MFE.

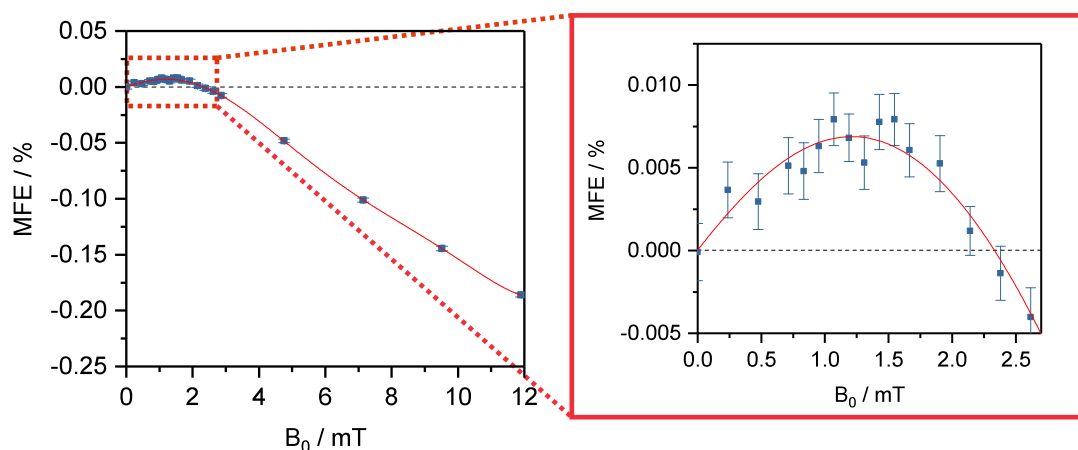


Figure 3.8: MARY plot for 40 μ M thionine / 0.2 M DABCO / 10 mM SDS, with a zoomed in panel showing the low field region. Wavelength averaged 600-700 nm and a photoexcitation power of 100 mW was used. Uncertainty bars on the data points represent ± 1 standard error of the mean of 500 individual measurements. A low field effect can clearly be observed.

provides a good fit for MARY curves in the majority of cases, the function is unable to account for the presence of LFEs and so the fit is less good on these occasions. However, even when an LFE is present, a Lorentzian fit still usually provides a reasonable fit to the data (excluding the LFE region).

The value of $B_{\frac{1}{2}}$ obtained from experiment, $B_{\frac{1}{2}}^{exp}$, can be compared to that predicted by the Weller formula, $B_{\frac{1}{2}}^{Weller}$ [101]:

$$B_{\frac{1}{2}}^{Weller} = \sqrt{3} \frac{<\alpha_F>^2 + <\alpha_D>^2}{<\alpha_F> + <\alpha_D>}, \quad (3.2)$$

where F and D are the two radicals comprising the RP and $<\alpha_X>$ is the effective hyperfine coupling constant for $X = F, D$, defined as:

$$<\alpha_X> = \sqrt{\frac{4}{3} \sum_i \alpha_{iX}^2 I_{iX} (I_{iX} + 1)} \quad (3.3)$$

where I_{iX} is the nuclear spin quantum number for nuclear spin, i , in radical X, interacting with a hyperfine coupling constant, α_{iX} .

Although the Weller formula provides a good estimate of $B_{\frac{1}{2}}$ it does not account for spin-dephasing or relaxation processes. Therefore, it typically fails if the radicals are subjected to fast spin relaxation, in which case a substantial broadening of the MFE graph is observed and a larger $B_{\frac{1}{2}}^{exp}$ is obtained.

For the case of the thionine/DABCO/SDS system presented in Figure 3.7, the $B_{\frac{1}{2}}$ value predicted by the Weller formula, calculated using the isotropic hyperfine coupling constants for each species [102, 103], is approximately 7 mT, whereas the experimentally determined $B_{\frac{1}{2}}$ value is around 12.5 mT. Even at 12 mT, the MARY curve shows no significant sign of saturation, which is typical for species confined to micelles where broadening of MARY curves is common.

The discrepancy in the calculated and predicted $B_{\frac{1}{2}}$ values suggests significant spin dephasing in the thionine/DABCO/SDS system, which may be either singlet-triplet (ST) or triplet-triplet (TT) dephasing [104]. ST dephasing involves the random modulation of the electron exchange interaction, brought about by re-encounter of the RP; whereas TT dephasing is associated with random modulations

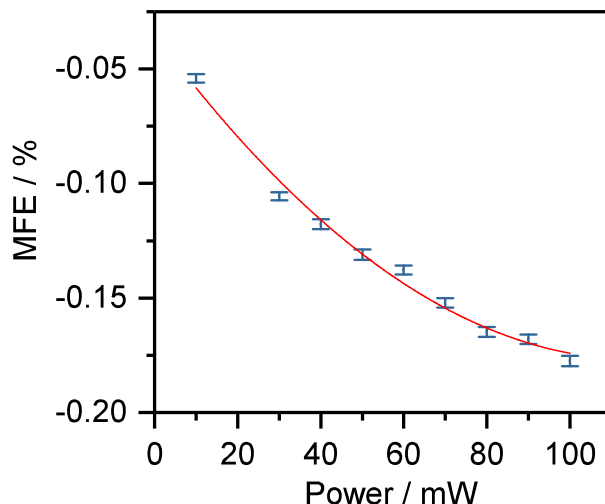


Figure 3.9: MARY plot for 40 μ M thionine / 0.2 M DABCO / 10 mM SDS for a range of excitation powers of a 532 nm laser between 10-100 mW. Wavelength averaged 600-700 nm. Uncertainty bars on the data points represent ± 1 standard error of the mean of 500 individual measurements and the red line is a polynomial (second degree) fit to the data. Increasing the laser power acts to increase the size of the MFE.

of anisotropic dipole-dipole interactions (*i.e.* the destruction of coherences by spin-lattice relaxation). TT dephasing can be from electron-electron dipolar interactions or from hyperfine interactions: the former is affected by rotations of the RP as a whole whereas the latter is affected by rotations of the individual radicals in the RP. As the extent of ST and TT dephasing depend on the probability of the RP partners re-encountering, confining the RPs to a micelle can vastly increase the rates of both of these processes and so consequently lead to a broadening of the MARY curve and an increased $B_{\frac{1}{2}}$ value.

3.5 Photoexcitation power dependence

Figure 3.9 illustrates the variation in the observed MFE for a system of 40 μ M thionine / 0.2 M DABCO / 10 mM SDS as a function of the power of the 532 nm diode laser in the range 10 mW - 100 mW. A smooth increase in the magnitude of the MFE is observed with increasing power, agreeing with the trend predicted for a RP system with intermolecular electron transfer. Increasing the photoexcitation power increases the rate of excitation of ground state molecules and consequently

increases the number of molecules involved in the photochemical cycle. This, in turn, results in an increase in MFE measured by fluorescence.

It should be noted that the MFEs observed by fluorescence-based techniques, such as PF MARY, are generally much smaller than those observed by absorption-based methods (TA, CRDS, CEAS etc.). In absorption-based detection methods, MFEs are observed by monitoring the radicals directly, and so even though the number of molecules in this part of the photochemical scheme may be low, the fractional change in the number of molecules can be large. Even if only small, a change against a small background results in a larger fractional difference than changes against a large background. With fluorescence, MFEs are monitored indirectly by observing small changes in fluorescence intensities against a large background fluorescence, and consequently these MFEs are often smaller than when detected using absorption-based techniques. It is not uncommon to observe MFEs by fluorescence that are of the order of a fraction of a percent.

The initial increase in the MFE is slightly more rapid for the low photoexcitation powers; however, at higher powers it would be expected that the dependence would become weaker as the reaction becomes limited by the rate of free radical recombination and will eventually plateau.

3.6 DABCO concentration dependence

The dependence of the MFE on the concentration of the quencher, DABCO, was measured, for the same 40 μ M thionine/10 mM SDS system. As illustrated in Figure 3.10, a marked initial increase in magnitude of the MFE is observed with increasing DABCO concentration; however, as the concentration continues to increase the percentage MFE decreases.

The initial trend of the MFE increasing with DABCO concentration suggests that quenching of $^3\text{TH}^*$ is predominantly dynamic at low DABCO concentrations. DABCO in aqueous solution dynamically quenches $^3\text{TH}^*$ to produce the spin correlated RP, and so an increase in DABCO concentration naturally acts to increase the concentration of SCRPs and thus increase the magnitude of the MFE.

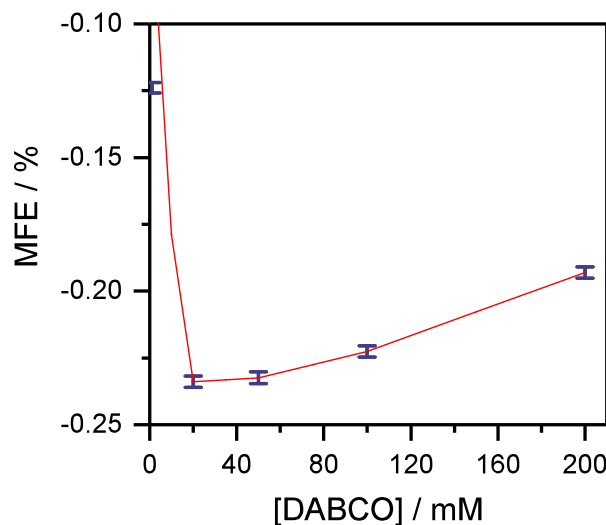


Figure 3.10: MARY plot for 40 μ M thionine / 10 mM SDS with a range of DABCO concentrations (1 - 200 mM). Wavelength averaged 600 - 700 nm. Uncertainty bars on the data points represent ± 1 standard error of the mean of 500 individual measurements. Increasing the DABCO concentration initially acts to increase the size of the MFE, but increased concentrations reduce the MFE.

As the concentration of DABCO increases, however, the magnitude of the MFE begins to decrease again. This can be accounted for by static quenching of thionine by DABCO stabilised in SDS micelles. In this case, the only possible outcome is regeneration of the starting materials and so a smaller fraction of the system goes on to form SCRPs and participate in the magnetically sensitive section of the photochemical scheme. Instead, the molecules return to the ground state with no MFE. With fewer molecules forming SCRPs and thus ending up in the magnetically sensitive cycle, a reduction in the magnitude of MFE is observed.

DABCO is a hydrophilic molecule and so, as previously discussed, is mainly found on the outside of the SDS micelle, on the opposite side of the phase boundary than the thionine in the hydrophobic core. However, a small proportion of DABCO is expected to be found within the micelle itself. Therefore, at high DABCO concentrations some SDS micelles will contain both DABCO and thionine as DABCO is solubilised by SDS, and so DABCO is able to statically quench $^3\text{TH}^*$. In order for an MFE to be observed, the component radicals of the RP must be able to diffuse apart before recombining. Without this, when the geminate pair is created

the two radicals are so close to one another that their mutual exchange interaction locks the pair into its initial spin state. Only when the radicals have separated to a point where the exchange has sufficiently decreased so that it is comparable to the hyperfine and Zeeman interactions can effective ST mixing begin. In order to observe an MFE, the radicals must then diffuse back together and re-encounter in order to be able to recombine (but only from the singlet state). In the hydrophobic core of a micelle this separation and re-encounter is prevented by the Coulombic attraction between the radical ions. Consequently, for micelles containing both thionine and DABCO, no ST mixing is possible and the only possible outcome is regeneration of the ground state products with no MFE generated. At high concentrations of DABCO, increasing DABCO concentration increases the likelihood of the interior of a micelle containing both thionine and DABCO, which decreases the proportion of the system that passes through the magnetically sensitive portion of the photochemical scheme and thus decreases the MFE.

Stern-Volmer analyses may be used to confirm that the quenching occurring at low [DABCO] is dynamic. The Stern-Volmer equation is given by:

$$\frac{I_f^0}{I_f} = 1 + K_{SV}[Q], \quad (3.4)$$

where I_f^0 is the fluorescence intensity in the absence of a quencher, I_f is the fluorescence intensity in the presence of a quencher, K_{SV} is the Stern-Volmer quenching constant ($= k_q\tau^0$ where k_q is the quenching rate constant and τ^0 is the fluorescence lifetime) and $[Q]$ is the quencher concentration. If the quenching is purely dynamic then a straight line, with a gradient equal to K_{SV} and a y-intercept of 1, would be expected when plotting $\frac{I_f^0}{I_f}$ against $[Q]$.

However, if there is purely static quenching, equation 3.6 becomes:

$$\frac{I_f^0}{I_f} = 1 + K[Q], \quad (3.5)$$

where K is the equilibrium constant for the formation of the 'dark' complex from the thionine/DABCO. Therefore, a similarly linear plot would be expected

when plotting $\frac{I_0^f}{I_f}$ against $[Q]$, with a gradient equal to K and y-intercept of 1. Hypothetically the two cases could be distinguished by comparing the gradients (dynamic quenching gradient = K_{SV} ; static quenching gradient = K), however, in reality, without knowing the values of K and K_{SV} these cases would be hard to distinguish from one another.

Alternatively, fluorescence lifetime measurements could be used to distinguish between static and dynamic quenching. Fluorescence lifetimes are not affected by the presence of a 'dark' complex, and so by comparing the fluorescence lifetimes at high and low [DABCO] the mechanism of quenching could be determined. If it is indeed static quenching that is occurring at high [DABCO] then it would be expected that there would be no effect on the fluorescence lifetime. Moreover, static quenching results in the formation of a 'dark' complex which does not fluoresce and should result in a shift in the absorption spectrum of the fluorophore, thionine. The existence of any such shifts at high [DABCO] would be evidence of static quenching occurring.

3.7 Effect of micelle size and charge

Confining RPs to a micelle clearly increases the magnitude of the observed MFE, as shown in the previous sections. The next section investigates if it is possible to alter the spin dynamics further by adjusting the environment of the micelle, either through changing the micelle charge or size.

3.7.1 Micelle Charge

SDS forms negatively charged micelles as the head group of the surfactant is an anionic sulfate group, which leads the the surface of the micelle having a negative charge upon formation. Dodecyltrimethylammonium chloride (DTAC) comprises the same hydrophobic 12-carbon chain as SDS but has a cationic tertiary ammonium compound as the polar head group as shown in Figure 3.11a. Consequently, DTAC forms positively charged/cationic micelles which are of a similar size to SDS, making it is a good system for a comparison of the effects of altering micelle charge.

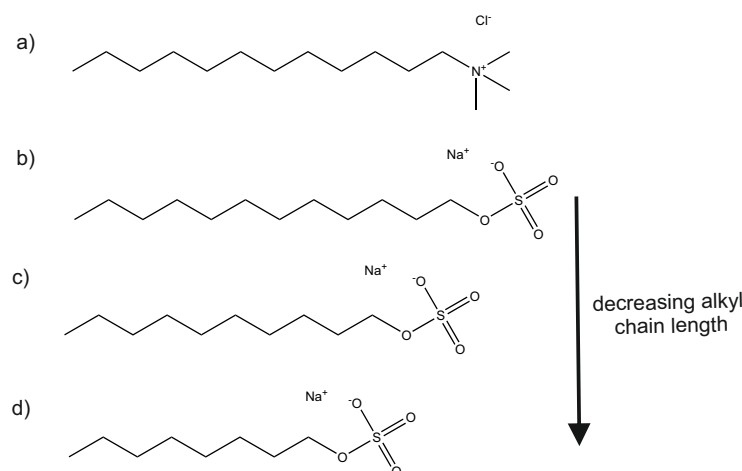


Figure 3.11: Chemical structure of a) DTAC b) SDS c) SDeS and d) SOS. The length of the alkyl chain decreases from 12 to 10 to 8 carbons going from SDS to SDeS to SOS.

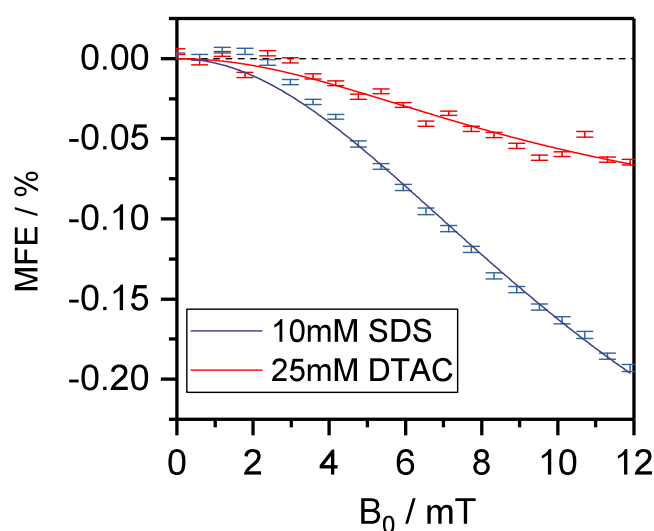


Figure 3.12: MARY plot for 40 μM thionine / 0.2 M DABCO with 10 mM SDS (blue) or 25 mM DTAC (red). The MARY curve for 25 mM DTAC has been scaled by a factor of 5 in order to more clearly observe the shape of the curve. Wavelength averaged 600-700 nm. Uncertainty bars on the data points represent ± 1 standard error of the mean (SEM) of 500 individual measurements, and solid lines represent Lorentzian fits of the data points. Changing the charge of the micelle from negative (SDS) to positive (DTAC) is clearly observed by a reduction in size of MFE.

Figure 3.12 shows a MARY plot for a system of 40 μM thionine / 0.2 M DABCO with either 10 mM SDS (blue) or 25 mM DTAC (red). Both data sets have been fitted with a Lorentzian function, and the DTAC MARY plot has been scaled by a factor of 5 in order to be able to more clearly observe the shape of the curve. The concentrations of the surfactants were chosen in order to ensure they exceed their respective CMCs of 8.2 mM and 21.3 mM [105] to ensure that micelle formation occurred. The magnitude of the percentage MFE decreases by at least a factor of 20 when the anionic micelle environment is changed to a cationic one.

As the length and hydrophobicity of the surfactant tail is unaltered between SDS and DTAC (in both cases the tail consists of a 12-carbon-length hydrocarbon), the hydrophobic environment is effectively unchanged between the two. The surface charge of the micelle and charge of the Stern layer, however, changes from negative in SDS to positive in DTAC due to the change from an anionic to cationic head group respectively. Consequently, the positions of the thionine and DABCO radicals in the micelle are altered due to the change of head group.

Whereas with SDS micelles the TH^+ molecule associates with the negatively charged micelle surface upon its creation, with DTAC the surface charge is positive and so repels the like-charged TH^+ molecule. It is more likely that the TH^+ is found in the Gouy Chapman layer or in the bulk solution where the environment is less electrostatically unfavourable. As the hydrophobic core remains the same, upon formation of the hydrophobic TH radical the radical will likely then associate with the hydrophobic core of the micelle.

Similarly, upon formation of the DABCO^+ radical from its molecule precursor, the radical will be repelled by the Stern layer/surface of the micelle and so once again will be more likely to be found in the bulk solution. Consequently, upon formation of the radicals, the TH radical is trapped in the hydrophobic micelle core whereas the DABCO radical is expelled into the bulk solution, and so the radicals are not held in close proximity to one another. The DTAC micelle does not efficiently act to keep the radicals together and so is not effective at increasing the lifetime of the geminate pair.

This results in the magnitude of the MFE being far lower with DTAC than with SDS micelles, as shown in Figure 3.12. The charge of the micelle has a significant effect on the positions of the reactants within with micelle which alters the closeness of the radicals to each other and consequently lowers the magnitude of the MFE.

3.7.2 Micelle Size: SDS *vs.* SDeS *vs.* SOS

As well as altering the charge of the surfactant, the size of the micelle can also be modified to see how this effects RP reactions. One method for doing this is by changing the alkyl chain length: SDS is closely related to sodium decyl sulfate (SDeS) and sodium octyl sulfate (SOS), with all three surfactants possessing the same sulfate head group, but differing in the number of carbons that make up the hydrocarbon tail. Figure 3.11 illustrates these differences, showing the decrease in hydrophobic tail length from 12 to 10 to 8 carbons going from SDS to SDeS to SOS, respectively.

Lengthening the hydrocarbon tail of the monomer, whilst keeping the head group the same, is expected to increase the diameter of the micelle. As previously discussed, the value of N_{agg} and the size of the micelle are strongly linked, and the value of N_{agg} is determined by a competition between the hydrophobic effect and repulsions of like-charged head groups. Although repulsions between head groups are unchanged between SDS, SDeS and SOS (as they all comprise the same sulfate head group), the hydrophobic effect will be vary according to the length of the hydrocarbon tail. Longer hydrocarbon tails increase the hydrophobic portion of the surfactant and consequently the hydrophobic effect becomes more significant: energetically it is more favourable to remove a 12-carbon hydrophobic molecule from water than it is to remove a 8-carbon one (see Section 3.1.2). The tendency to form micelles is increased with longer length of the hydrophobic tail, and this is reflected in the smaller CMC for SDS (8.2 mM) than for SDeS (30 mM) and SOS (134 mM) [106]. The hydrophobic effect is expected to be larger in SDS, and so the point at which energetic gain of removing the hydrophobic tails from water becomes outweighed by the energetically unfavourable repulsion between head groups will be increased, resulting in a larger N_{agg} value and so larger micelle diameter/size.

Figure 3.13 shows small decrease in size of both the MFE with increasing alkyl chain size for a solution of 40 μM thionine / 0.2 M DABCO. The difference in concentrations of the surfactants used in each experiment is to ensure the CMC is surpassed and micelles are formed (CMC = 8.2 mM, 30 mM and 134 mM for SDS, SDeD and SOS, respectively [106]). The different surfactant concentrations required for each experiment may also have an effect on the viscosity of the solution, which in turn may impact the MFE observed. Increased viscosity can result in a larger MFE being observed as the lifetime of the geminate pair is increased.

Increasing the size of the alkyl chain results in both a slightly smaller MFE and LFE being observed, which may be explained from the marginal increase in micelle diameter that is expected to accompany an increased hydrophobic tail length. An increase in micelle size means an increased number of radicals are able to associate within a single micelle, possibly leading to an increase in quenching efficiency which acts to slightly reduce the magnitude of the MFE. The LFE depends strongly on the RP lifetime (see Section 1.6.2) and decreases with increasing alkyl chain length, which may be accounted for by the RP lifetime becoming shorter as the size of the micelle increases.

The observed effect for both MFE and LFE is only small because altering the alkyl chain length by two or four carbons is only expected to have a marginal effect on the size of the micelle. However, the fact that the PF MARY experiment is able to distinguish between such a change shows once again just how sensitive the re-designed experiment is.

3.8 Thionine/aniline/SDS

The structure of aniline and its 4-halo-substituted variants are shown in Figure 3.14. Unlike DABCO, aniline is an aromatic amine comprising a single aromatic ring with a amine moiety. When the X substituent shown in Figure 3.14 is hydrogen, then the structure shown is aniline, but X may also be a range of halogens (Cl, Br, I) as indicated.

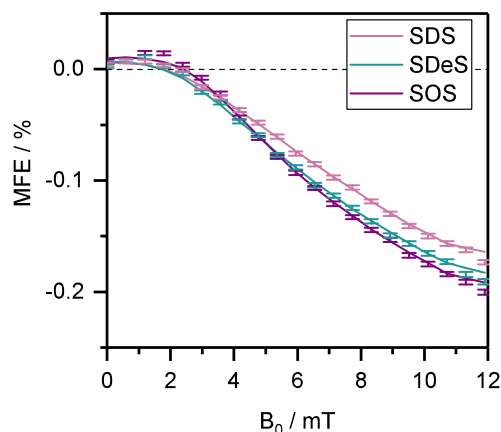


Figure 3.13: MARY plot for 40 μM thionine / 0.2 M DABCO with 10 mM SDS (pink), 35 mM SDeS (blue), or 140 mM SOS (purple). Concentrations of surfactant were chosen to be above the critical micelle concentration in each case. Wavelength averaged 600-700 nm. Uncertainty bars on the data points represent ± 1 standard error of the mean of 500 individual measurements, and a Lorentzian function has been fitted to each data set. Changing the size of the micelle from largest (SDS) to smallest (SOS) potentially acts to marginally increase the size of the MFE.

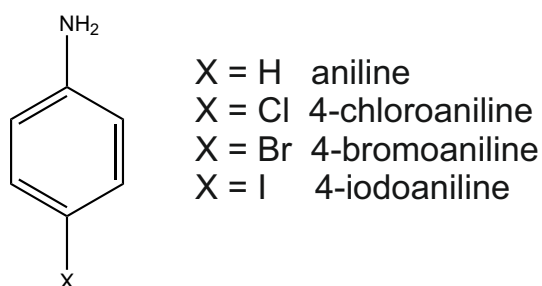


Figure 3.14: Chemical structure of aniline (when $\text{X}=\text{H}$) and 4-halo-aniline (when $\text{X}=\text{Cl}/\text{Br}/\text{I}$).

Thionine is known to form exciplexes via π -stacking with anilines, which was not possible in the previously studied systems with DABCO as the ED. This is significant as exciplex formation could lead to MFEs by the triplet mechanism as discussed below.

The photochemical scheme for the thionine/aniline system is largely similar to that of thionine/DABCO depicted in Figure 3.6, with the exception of additional photochemistry arising from the triplet exciplexes in the thionine/aniline case. As before, upon photoexcitation to the first excited singlet state, thionine undergoes

rapid ISC to produce the excited triplet state. However, unlike the thionine/DABCO system, from here a triplet exciplex is formed as a result of thionine π -stacking with anilines. The triplet exciplex then decays via a choice of two competing pathways: either ISC to the ground state ($T_1 \rightarrow S_0$) or dissociation into radicals. Although magnetic fields do not directly affect this process, MFEs can arise from recombination of the geminate RPs formed from dissociation of the triplet exciplex. Consequently, any process which alters the ratio of the exciplex undergoing ISC back to the ground state *vs.* the exciplex dissociating into radicals can alter the MFE observed.

The rate of ISC is strongly dependent on the rate of spin-orbit coupling, being directly proportional to Z^4 (in a hydrogenic molecule), where Z is the atomic number. Consequently, if the electron donor (aniline) contained a heavy atom substituent then the deactivation of the triplet exciplex to the singlet groundstate via ISC would become increasingly efficient with increasing SOC. As this process competes with the dissociation of the exciplex into radicals then this could act to lower the magnitude of the observed MFE.

3.8.1 Heavy atom effect on the thionine/aniline/SDS MARY curve

Here, the effect of magnetic fields on a system of thionine/aniline/SDS is investigated, and the effect of adding a heavy atom substituent to the aniline is probed in order to provide insight into the reaction mechanism and how SOC affects the observed MFE in the ways predicted above.

Figure 3.15 depicts the MARY plot for 40 μ M thionine / 10 mM SDS with 5 mM aniline (green) and a range of 4-haloanilines: 4-chloroaniline (yellow), 4-bromoaniline (red) and 4-iodoaniline (blue). As predicted from the discussion above, as Z (and thus the spin orbit coupling constant) increases from unsubstituted aniline to 4-iodo-aniline, the size of the MFE is reduced.

Increased SOC leads to faster quenching of T_1 thionine to S_0 by reverse ISC. As the rate of ISC increases, this process starts to become more efficient and more strongly competes with the rate of dissociation of the exciplex into radicals. In fact,

the rate of ISC is so fast with 4-iodoaniline that the MFE is effectively abolished as ISC back to the ground state outcompetes the rate of exciplex dissociation to form RPs. If fewer exciplexes are going on to form SCRPs then fewer molecules are involved in the magnetically sensitive portion of the photochemical system. The proportion of molecules undergoing ISC begins to swamp those exploring the magnetically sensitive part of the scheme and thus the observed MFE decreases.

Whereas an LFE was observed in the previously studied thionine systems in this Chapter, no LFE is present for any of the thionine/aniline systems studied in this section. Generally, a lack of LFE either suggests that the RP formed is too short-lived for the low magnetic field to have any significant effect on the spin dynamics of the system or the hyperfine couplings of one or both of the RP partners are larger (which would remove the long-term spin correlation necessary for the development of a substantial MFE). Considering the RP is formed in the environment of a micelle which acts to extend the lifetime of the RP, it is likely that the latter reason is more likely. However, the hyperfine coupling constants reported for aniline in Ref. [107] are not particularly large, which might suggest the former hypothesis to be more likely.

3.9 Conclusions

Using the model system of thionine/DABCO, this Chapter has demonstrated the effect of encapsulating a RP in an SDS micelle in increasing the lifetime of the geminate pair and thus increasing the magnitude of the MFE. A negative MFE was observed on thionine/DABCO confirming a triplet-born RP, which was increased by over a factor of 5 upon addition of SDS. Characterisation experiments investigating the effects of photoexcitation power showed an increase in MFE with increasing power, which is attributed to a greater number of molecules involved in the photochemical cycle. The relationship between MFE and quencher concentration was shown to be biphasic. It was proposed that initially an increased quencher concentration acts to increase the MFE as dynamic quenching dominates, but at higher concentrations the MFE begins to decrease again due to static quenching.

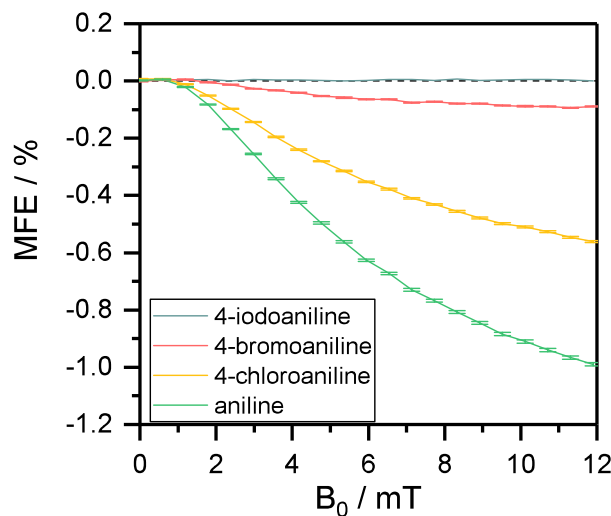


Figure 3.15: MARY plot of 40 μ M thionine / 10 mM SDS with 4-iodo-aniline (blue), 4-bromo-aniline (red), 4-chloro-aniline (yellow) or unsubstituted aniline (green). Decreasing the size of the halogen substituent acts to increase the size of the MFE. Wavelength is averaged 600-700 nm, and the uncertainty bars on the data represent ± 1 standard error of the mean of 500 individual measurements.

Factors affecting the micelle environment have also been investigated, with experiments with cationic DTAC - a surfactant that is largely similar to SDS in many ways but with a positive head group rather than a negative one - compared to those with SDS, showing just how significant the charge of the micelle is as the MFE of thionine and DABCO in DTAC is over 20 times smaller than in SDS. The charge of the micelle dictates the positions of the reactants/radicals in the micelle and thus affects the proximity of the radicals to one another and the lifetime of the geminate RP and subsequent MFE. Furthermore, experiments on thionine and DABCO investigating the effect of the size of the surfactant monomer (which has a knock on effect on the size of the micelle) have shown a small decrease in MFE for increasing alkyl chain length (increased micelle size), which is accounted to an increased numbers of molecules being able to occupy the hydrophobic core in larger micelles, leading to increased quenching and a smaller MFE. It can be concluded that micelle charge and size have an effect on the MFE observed, which may be relevant when considering biological systems. Although still far removed from biology, RPs in SDS micelles are more similar to the cellular environment that

cryptochromes are in than RPs in free solution. As thionine has some structural similarities to flavin, it would not be surprising if factors such as micelle charge/size affect the photochemistry occurring in cryptochromes in a similar manner.

Finally, the photochemical system of thionine/aniline in SDS micelles was investigated. Here, the photochemical reaction scheme is slightly different to those previously studied, due to the ability of thionine to undergo π -stacking with aniline. MFEs arise from the triplet mechanism which is dominated by the rate of SOC rather than hyperfine coupling, Zeeman interactions and exchange coupling. The MFE observed was shown to decrease as SOC becomes more efficient.

Overall, the new highly-sensitive PF MARY experiment has been shown to be suitable for examining a range of MFEs and for monitoring the spin dynamics of geminate RPs confined to micelles, including how these factors are affected by altering the components of the RP and/or the environment of the micelle.

4

Photosignalling in *DmCry*

As discussed in previous chapters, the photochemistry of cryptochromes, including *DmCry*, has been explored using a variety of optical spectroscopies, ranging from TA to cavity-based techniques [42, 54, 63]. However, whilst the photochemistry of animal cryptochromes is now beginning to be well understood, the same cannot be said for the mechanism of signal transduction. The mechanism by which the photochemistry is linked to the photosignalling method is still somewhat unclear.

In this chapter, the connection between the photochemistry and signal transduction pathway in *DmCry* is investigated, and variations in blue-light induced conformational changes studied, initially using partial proteolysis techniques but then by the more quantitative HDX-MS. Both *DmCry* wild type (WT) and its mutants *DmCry* W342F and *DmCry* W394F are investigated: the two mutants, *DmCry* W342F and *DmCry* W394F, are identical to *DmCry* WT except a redox inert phenylalanine (F) replaces the third and fourth tryptophan respectively in the ET chain. The redox inert phenylalanine should interrupt the ET chain and provide an insight into the role of these tryptophans in the signal transduction pathway in *DmCry*. The HDX work presented in this chapter has been done in collaboration with Shane Chandler (Justin Benesch group, University of Oxford).

4.1 Introduction

Cryptochromes are flavoproteins comprising a conserved photolyase homology (PHR) domain which non-covalently binds an FAD molecule. The CTT is a part of the protein that varies in length from species to species. In *DmCry* the CTT is made up of residues 528-539 and forms a α -helix (α -23), shown in red in Figure 4.1. In the dark the CTT occupies a region of space similar to where damaged DNA substrates would bind in photolyases. Figure 4.1 depicts the X-ray crystal structure of *DmCry* WT in the dark state, with structural elements surrounding the CTT highlighted. These regions include the C-terminal lid (residues 420-446, pink), the protrusion motif (residues 288-306, orange), the phosphate binding loop (residues 249-263, blue), and the CTT base loop (residues 154-160, dark green). The FAD and tryptophan tetrad are shown in yellow and green respectively.

In *DmCry* the ground state FAD is unprotonated, and is photoexcited to the first excited singlet state, $^1\text{FAD}^*$, upon exposure to blue-light. From here the molecule is reduced, with the neighbouring tryptophan (W) residues acting as the source of electrons, to form a singlet SCRPs, $^1[\text{FAD}^{\cdot-} + \text{WH}^+]$. Unlike in plant cryptochromes such as *AtCry* where ET stops at the third tryptophan, all four tryptophans in the ET chain are involved in photo-reduction of the FAD in *DmCry* [108]. The 4thFAD-W RP is the first photo-induced state with a lifetime greater than a microsecond. The longer lifetime of this RP makes it too long lived for spin-selective recombination to compete effectively with spin relaxation and so this RP is not magnetically sensitive [51].

The singlet SCRPs is formed initially due to conservation of total spin angular momentum, however, the singlet and triplet states may interconvert via ST mixing (as discussed in Section 1.6). These RPs are known as radical pair 1 (RP1). RP1 has a lifetime of $\sim 1 \mu\text{s}$ which is likely too short-lived to be the signalling state of the molecule, however, both the singlet and triplet SCRPs in RP1 may be converted to a longer-lived radical pair 2 (RP2) state via a change in protonation state to $[\text{FADH}^{\cdot} + \text{W}]$. RP2 has a lifetime of milliseconds *in vitro* and is proposed to lead to the signalling state of the molecule. It is proposed that it is this change in oxidation

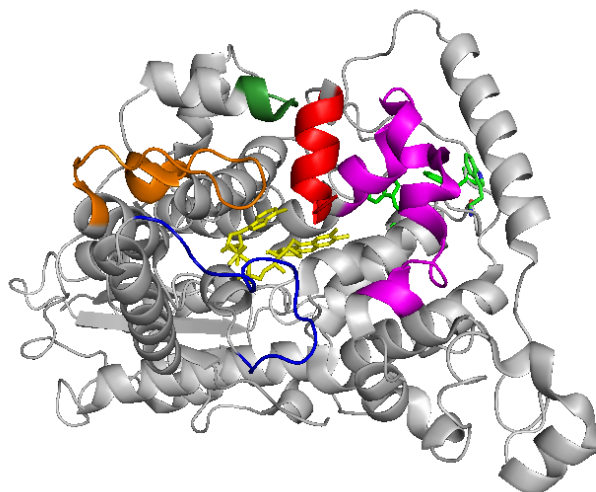


Figure 4.1: *DmCry* X-ray crystal structure (PDB: 4GU5) in the dark state conformation. The bound cofactor, FAD (yellow), and corresponding tryptophan residues (light green) result in the formation of a radical pair upon blue light activation. The C-terminal tail (CTT) α -23 helix, a region of significant structural interest, is shown in red. Motifs localised close to the C-terminal tail are also highlighted: C-terminal lid (pink), the protrusion motif (orange), the phosphate binding loop (blue), and the CTT base loop (dark green).

state of FAD to $\text{FADH}^\cdot$ that triggers the signalling process in cryptochromes [56, 109–111]. After formation of the longer-lived RP2 state from RP1, the RP2 state could be further stabilised to reverse ET (by reduction of a tryptophan from an extrinsic ED) to extend the lifetime of the RP2 state and lead to the signalling state.

Exactly how the photochemistry discussed here is related to signal transduction pathway, however, is still not well understood. One widely held model for cryptochrome signal transduction suggests that blue light induces a conformational change in the protein that enables interactions with downstream partners and thus induces the signalling cascade [112–114]. More specifically, it is believed that a conformational change centred about a region of the protein known as the C-terminal tail (CTT) could leave the protein more susceptible to proteolysis, inducing interactions with downstream signalling partners.

4.2 Partial Proteolysis

Initial evidence for involvement of a conformational change in the signalling pathway has been found using partial proteolysis techniques, with proteolytic protection assays showing that the CTT becomes more exposed when exposed to light [112, 113, 115, 116]. Vaidya *et al.* [112] showed that the CTT and associated regions are more susceptible to proteolysis when *DmCry* WT is exposed to light compared to when it is kept under dark conditions.

Partial proteolysis can be used as a crude method of observing conformational changes in proteins. Proteases, such as trypsin, have specific cleavage sites to digest cryptochrome in precise locations. The number of these active sites that are surface accessible will determine the number and nature of polypeptide fragments that result from the digestion. Consequently, the conformation of the protein should have a direct effect on the polypeptide fragments produced; if the protein is in a different conformation in dark and light then different cleavage sites will be accessible and this should be reflected in the partial proteolysis pattern, which can be resolved using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).

For an initial attempt at observing a blue-light induced conformational change in *DmCry* WT, I spent time in Freiburg learning how to carry out partial proteolysis experiments on cryptochromes and how to handle the proteins themselves. The results from this technique provided qualitative confirmation of a conformational change, and although not very sophisticated, the technique highlights that a conformational change is present and demonstrates the requirement for a more quantitative technique (namely, HDX-MS which used for experiments in the rest of the Chapter).

4.2.1 Experimental

Materials

All *DmCry* WT proteins were produced and purified in Freiburg; samples were expressed in *E. coli* following procedures described in Ref [108]. Solutions of *DmCry* WT were prepared to 20 μ M concentrations in a buffer of 50 mM HEPES, 100 mM

NaCl, 20% v/v glycerol. Trypsin solutions (from bovine pancreas) were made up to concentrations of 200 μ M, and trypsin inhibitor (from soybean) solutions were at concentrations of 400 μ M. All chemicals were purchased from Sigma Aldrich and used without further purification.

Method

A 2 μ L sample of *DmCry* (extracted from *E. Coli* cells) was split between two eppendorfs: one kept under a dim red light ('dark' conditions), and the other exposed to 455 nm blue light ('light' conditions) at a power of <0.5 mW. For the dark sample, 2 μ L trypsin is added to start the digestion and then aliquots of the sample are removed, mixed with 2 μ L of trypsin inhibitor and flash frozen in N_{2(l)} to quench the proteolysis at various time points (1, 2, 4 and 10 minutes) after trypsin addition. For the light experiments, the same protocol is followed, however, the sample is pre-exposed to 455 nm blue light for 2 minutes prior to trypsin addition, and then for the continuation of the experiment. The products in each aliquot were heated to 90°C before being resolved using SDS-PAGE whereby the polypeptide bands are separated according to their molecular weight. Gels were stained with Coomassie brilliant blue in order to observe the peptide bands.

4.2.2 Preliminary results

Early attempts at observing a blue-light induced conformational change were with the simple partial proteolysis technique described above. Figure 4.2A shows a sample SDS-PAGE gel for such an experiment. The lane furthest left corresponds to a Protein Standard marker which indicates which points on the gel correspond to what molecular weight. Lanes 2-6 show digestion of *DmCry* WT under dark conditions for 0, 1, 2, 4 and 10 minutes of trypsin digestion, respectively; and the next 5 lanes (lanes 7-11) are for the same respective trypsin digestion times but under blue-light conditions.

A slight qualitative difference can be seen in the band patterns between the light and dark samples, specifically when looking at the bands at 10 minutes (lanes D-10

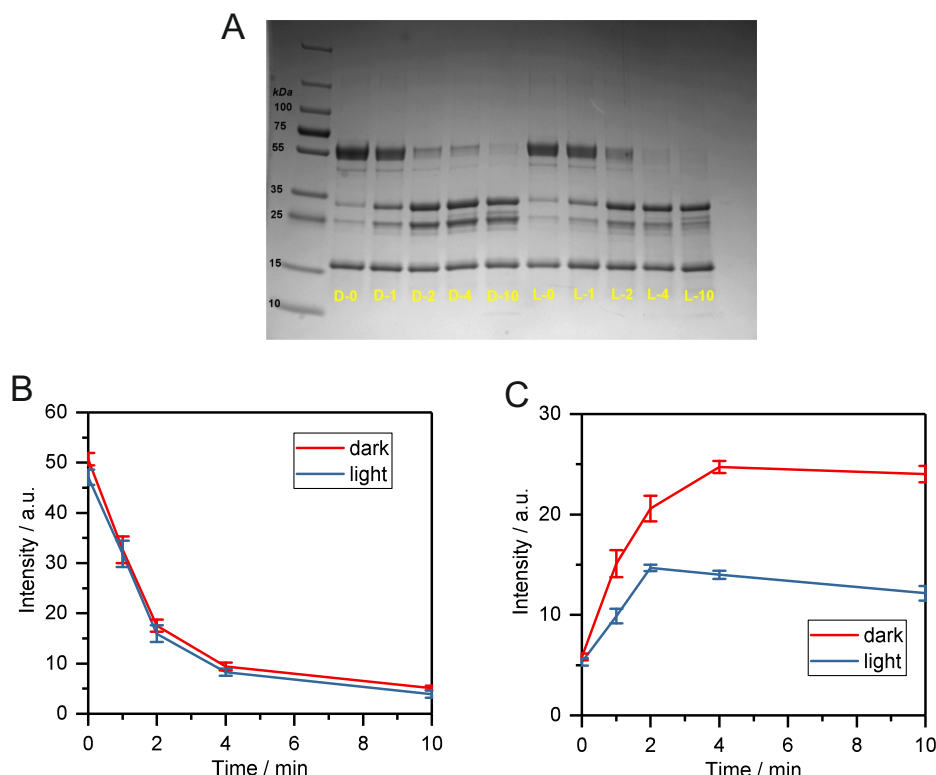


Figure 4.2: **A** SDS-PAGE gel of *DmCry* WT. Each band represents a different polypeptide band resulting from the digestion of the full length protein (~62 kDa), the molecular weights of which are indicated by the marker (lane 1). Lanes from left to right: marker, dark 0, 1, 2, 4, 10 mins; light 0, 1, 2, 4, 10 mins after trypsin digestion was initiated. **B** An example of a band which shows no difference in rate of change in light vs. dark conditions. This band corresponds to the upper most band on the gel at a molecular weight of ~62 kDa (*i.e.* the full length protein) **C** A band which shows a difference in rate of change in light vs. dark conditions (band at a molecular weight ~25 kDa). Uncertainty bars represent the standard error of the mean of seven gels/repeats.

and L-10). The relative intensities of the bands at ~25 kDa are slightly different for light and dark conditions: in light (lane L-10) the heavier band is more concentrated, whereas in dark (lane D-10) the two bands are of similar intensities. Although qualitative differences can be seen, the effects are very small. Qualitatively, it is hard to discern if there is any significant light-induced change in the digestion patterns.

In an attempt to apply a more quantitative approach to analysing SDS-PAGE gels, the gels were quantified (using a PEQLAB Gel Doc) and normalised to the

signal in lane 1 (containing the standardised Protein Label marker). The intensity of each band was calculated from the density of pixels in the region of the gel occupied by the band, so that the rate of change of each band as a function of trypsin digestion time could be compared between light and dark. The average intensities for seven gels are shown for 2 representative bands in Figure 4.2. Figure 4.2B shows the band at a molecular weight of ~ 62 kDa and plots the intensity of the band as a function of trypsin digestion time, showing no significant difference in behaviour between the light and dark conditions. The full length protein band (~ 62 kDa) decays at a similar rate in both light and dark conditions.

Figure 4.2C corresponds to the band at ~ 25 kDa, and the final intensity (at 10 minutes) in dark conditions is approximately twice as strong as in the light. A peptide of weight ~ 25 kDa could correspond to a region of the protein making up part of the C-terminus (from the C-terminus up to part of the C-terminal lid, 420-446), however it could equally be from the N-terminus of the protein, or from subsequent digestion of a ‘main body’ peptide. Regardless, the quantitative difference observed suggests that the protein is in a different, more exposed conformation in the light state which results in a more rapid digestion by trypsin. If the light-state conformer is more open, then trypsin may be able to more easily digest the peptide (as more tryptic cleavage sites are accessible) and so a more rapid digestion, and thus depletion, of this peptide is observed in light conditions.

Whilst it can be surmised that *DmCry* WT is behaving differently in light and dark conditions and that this is likely to be due to the protein undergoing a light-induced conformational change, it is hard to conclude much else with any real certainty. It is not easily possible to tell what specific peptide the band at ~ 25 kDa (or any other band for that matter) corresponds to without doing further analysis¹. Furthermore, both the experimental technique and the method of quantification lack any real resolution, and are very crude, being subject to several sources of error.

¹It is possible to cut out specific bands from an SDS-PAGE gel for mass spectral analysis, however this process was not available whilst doing these experiments in Freiburg. Furthermore, this process is an inefficient and tedious way to get peptidic information for the whole protein, normally just a few bands are analysed via this method.

Sources of error

The quantification technique of the bands observed on the SDS-PAGE gels simply looks at the density of pixels for the defined area of the gel making up each band. This process is heavily prone to human error as the edges of the bands are not always clear cut and human judgement is relied upon to determine band boundaries. Even slight variations in boundaries can have vast effects on the trends observed.

The partial proteolysis technique and experimental procedure are also very basic. Once again, the process is heavily susceptible to human error as the even small variations in factors such as trypsin digestion time or the amount of sample loaded onto the SDS-PAGE gel can have notable effects on the results obtained. The technique lacks any real reproducibility. Furthermore, despite best efforts, partial proteolysis is not a very quantitative technique and is limited in the regions of the protein that one can investigate by the regions that trypsin will digest and that are resolved on the SDS-PAGE gel. This technique does not have the ability to reliably look at conformational changes on a near-residue level.

Overall, these data highlight a potential a conformational change, but they also highlight the pitfalls and lack of sensitivity of this partial proteolysis method. A more rigorous, reproducible and quantitative technique is required for any in-depth analysis.

4.3 Hydrogen Deuterium Exchange Mass Spectrometry

As discussed above, whilst partial proteolysis techniques have provided initial evidence for light-induced conformational changes in *DmCry*, these techniques are inherently low resolution and the sensitivity to change is limited by the mechanism of cleavage. Furthermore, these techniques suffer with low reproducibility between runs as human error can vastly affect the results.

A more quantitative approach can be adopted, however, in the form of Hydrogen/Deuterium exchange coupled with mass spectrometry (HDX-MS). This

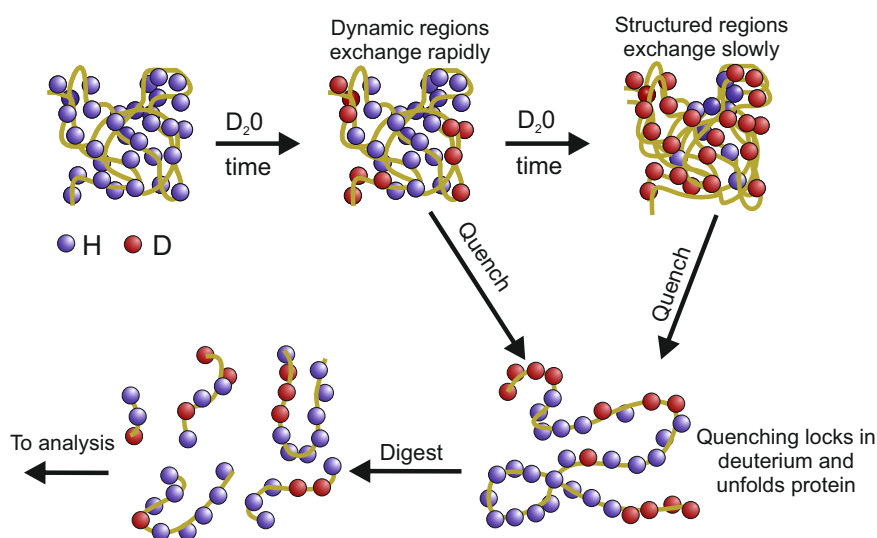


Figure 4.3: Principles of HDX

technique provides a labelling method that can report on the local structural changes for each backbone amide, allowing investigation of blue-light induced conformational changes in *DmCry* at a much more quantitative level that is also more reproducible.

HDX-MS is a well-established technique for probing protein conformation [117–119]. A schematic showing the principles of HDX-MS is shown in Figure 4.3. The technique relies on amide hydrogens in the protein backbone being labile and exchanging with protons in solution. Although other protons do exchange, only the amide protons do so on the timescale of the experiment and thus these are the protons that are probed. The H/D exchange rate of the labile backbone amide hydrogens is extremely sensitive to their accessibility to a deuterated solvent as well as to restrictions arising from the local hydrogen-bonding network. Consequently, upon exposure to a deuterated solvent, dynamic regions will exchange quickly, whereas structured regions that are less solvent accessible will exchange more slowly. These features make monitoring the rate of exchange a powerful means of understanding protein structure and corresponding dynamic motions [117, 118, 120, 121].

After labelling the protein with deuterium, the H/D exchange is quenched and the protein digested into peptides so that the isotopic incorporation can be measured

using mass spectrometry and deuterium uptake localised to near-residue level. By comparing the rate of deuterium uptake in both darkness and under blue-light conditions it is possible to probe blue-light induced conformational changes in *DmCry* and thus investigate how the blue-light photochemistry occurring may affect protein conformation and thus any signal transduction pathway. A more detailed, technical explanation of the HDX-MS experiment can be found below, (Section 4.4.2).

4.4 Experimental

4.4.1 Materials

DmCry expression was once again performed in Freiburg; samples were expressed in *E. coli* following procedures described in Ref [108]. *DmCry* W342F and W394F mutants were expressed in the same way as *DmCry* WT but with a point mutation replacing the 3rd and 4th tryptophan in the ET transfer chain, respectively, with a phenylalanine. Solutions of *DmCry* were prepared to $\sim 20 \mu\text{M}$ concentrations in a buffer of 50 mM HEPES, 100 mM NaCl, 20% v/v glycerol. The native equilibration buffer was 5 mM potassium phosphate (H_2O), pH 7.4; the labelling buffer was 5 mM potassium phosphate (D_2O), pD 7.4; and the quench buffer was 50 mM potassium phosphate (H_2O), pH 1.9. All chemicals and reagents were purchased from Sigma Aldrich and used without further purification.

4.4.2 Hydrogen-Deuterium Exchange Mass Spectrometry

A schematic for the process of an HDX experiment is shown in Figure 4.4. For labelling experiments, 5 μL of protein sample was diluted with 55 μL of D_2O labelling buffer of the same composition and corresponding pD at 20°C. The resulting labelling solution was therefore 92 % D_2O , and an ~ 12 fold dilution of the initial protein sample. Although it is preferable to work at lower temperatures than 20°C when handling cryptochromes, a compromise had to be made between having an optimal handling temperature and a reasonable temperature for labelling so that experiments were not unreasonably long.

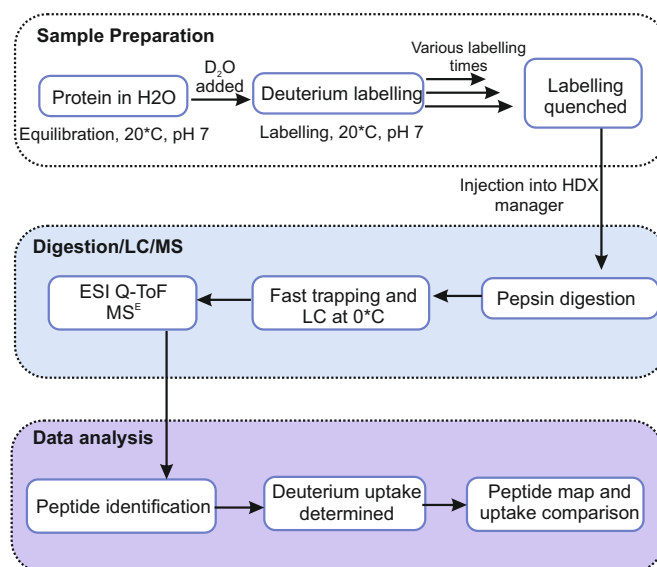


Figure 4.4: Schematic of HDX experiment. In the sample preparation phase, samples are labelling with D₂O for various labelling times before labelling is quenched and the sample is injected into the HDX manager. Pepsin digestion follows, with peptide products collected on a trapping column before being injected into a Q-ToF mass spectrometer. Data analysis involves peptide identification and determination of deuterium uptake, which allows a peptide map and uptake comparison to be constructed.

Labelling was quenched after specific incubation periods (0 sec, 15 sec, 1 min, 5 min, 30 min, 1 hour) by adding 50 μ L of labelled sample to 50 μ L of quench solution (pH 2, 2.5 °C), which had been pre-aliquoted and cooled in a separate vial. The quench acts to lower the temperature and reduce the pH of the sample to 2.3, which both quenches the labelling reaction and unfolds the protein.

The resulting solution was quickly injected into an on-line HDX manager (Waters, Milford, MA, USA). Initially, the sample was injected onto a 50 μ L sample loop at 0 °C, before being passed over an immobilised pepsin column (Enzymate Pepsin (Waters)), at 20 °C using an isocratic H₂O (0.1 % v/v) formic acid solution at a rate of 200 μ L/min.

A trapping column (BEH C18 (Waters)), held at 0 °C, was used to collect the peptide products from the pepsin column. Following a 2 minute collection period, the peptides were de-salted and eluted onto an analytical column. The analytical column acted to separate the peptides using a reverse-phase gradient, set at a flow

rate of 40 $\mu\text{L}/\text{min}$. The resulting sample was electrosprayed directly into a Synapt G2Si (Waters, Wilmslow, UK) Q-ToF instrument for mass analysis.

All sample handling was partially automated through the use of a robotic liquid handling HDX system (LEAP technologies, Ringwood, Australia). This technique allowed for more reproducibility in timings, and is consequently much more reproducible than the previously discussed partial proteolysis method as the susceptibility to human error is removed/reduced. A cleaning injection cycle was performed in between each labelling experiment to ensure no sample build up between runs, with a blank injection between each replicate used to verify the cleaning process.

For all HDX experiments, protein samples were kept in complete darkness, with any essential sample handling done in under dim red lights. All experiments were performed in a dedicated darkroom to control light-intensity throughout. For the dark experiments, conditions of complete darkness were maintained throughout all stages of the experiment. For the blue-light experiments, samples were handled in darkness, but then continuously illuminated with 450 nm LEDs at a power of < 0.1 mW for the duration of the labelling step and a prior equilibration period of 20 minutes. Samples were illuminated using a cage of LEDs built to surround the samples on all sides at a fixed distance of ~ 2 cm. The labelling chamber is temperature controlled but an external temperature probe was introduced to confirm that the addition of LEDs did not induce any temperature fluctuations.

Initial light experiments were performed in the same manner as those with blue-light, but with white light rather than 450 nm LEDs. The white light comprised a high proportion of blue-light, and no difference between the white and blue light experiments was observed. The light source was eventually refined to be LEDs centred around 450 nm in order to prove that the blue component of the white light is what is important for activating cryptochrome proteins. For completeness, the spectra of all lights used in these experiments is shown in Figure 4.5.

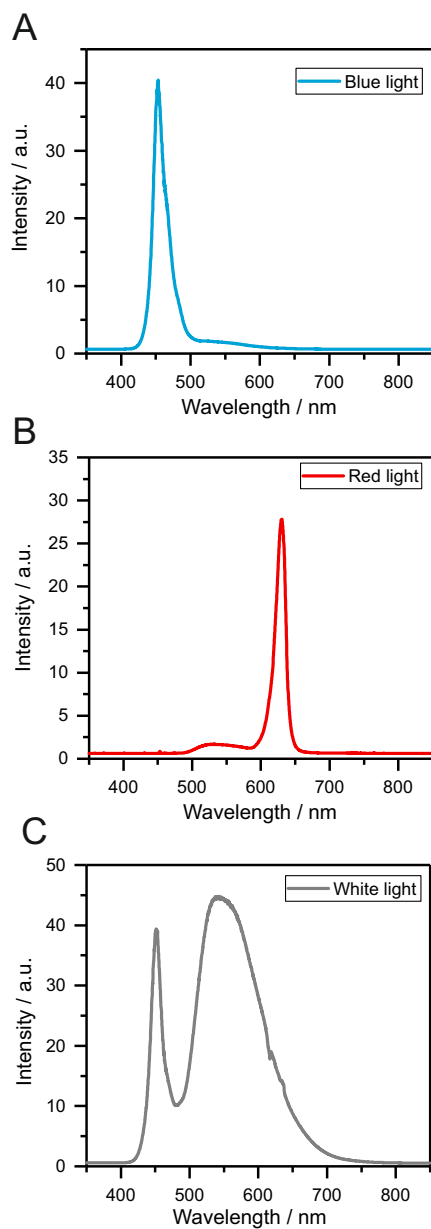


Figure 4.5: Spectra of lights used in HDX-MS experiments. **A** Spectrum of blue lights used in light experiments. The peak is fairly sharp and centred around 453 nm. **B** Spectrum of red lights used in control experiment, importantly showing no blue-light component. **C** Spectrum of the white lights used in initial HDX-MS experiments. The spectrum is broad, as expected, and has a high intensity of blue light.

4.4.3 Data Analysis

Technology known as ‘MS^E’ is used in the MS process, and allows fragmentation spectra to be scored in terms of their similarity to spectra predicted from peptide sequences from a database (Protein Lynx Global server 3.0 software (Waters)). In order to build up a list of peptides for the protein, a similar experiment to that described above was used but with the absence of the labelling step. Instead, 5 μ L of protein sample was diluted into 55 μ L of a un-deuterated buffer. Peptides were then identified by ‘MS^E’ analysis and a database search.

The list of peptides generated from this step was then filtered according to factors such as fragmentation quality, where the minimum number of fragmentation products per amino acid was set at 0.2, and reproducibility, so only peptides which were found in all MS^E repeats were kept. Only after this were they integrated into HDX analysis. The data were processed in DynamX 3.0 software (Waters) which automatically carried out peptide assignments. However, all of these peptide assignments were manually checked and verified, and any noisy spectra found were removed.

4.5 Results

4.5.1 Dark state dynamics of *DmCry* WT

An HDX-MS experiment was performed on *DmCry* WT under dark conditions. The deuterium incorporation for each peptide over a range of deuteration times (15 s, 1 min, 5 min, 30 min and 1 hour) was determined using the methods described above. This experiment was carried out in order establish the basal dynamics of *DmCry* WT; the resulting dark state dynamics are reported in Figure 4.6.

Figure 4.6A depicts a heat-map for the relative deuterium uptake for each time point (15 s, 1 min, 5 min, 30 min and 1 hour) across the entire *DmCry* WT sequence. A sequence coverage of 80.5% was obtained after data processing, with white areas on the heat map indicating areas of no coverage. Large redundancies were observed at the N- and C- termini where many overlapping peptides were generated.

The resulting uptake for each time point is shown across the entire sequence, with uptake values ranging from 0-70% (blue-red). Values of 62-72% can be considered fairly close to saturation if considering the maximum relative uptake to be 92% (due to residual H₂O in solution) and accounting for back-exchange of deuterium at 20-30%. The majority of the protein shows very low uptake, with only three regions of the protein reaching the estimated saturation (peptides 289-293, 439-444, and 502-509). After 15 s of continuous deuterium exposure, only 56% of the protein shows a relative uptake of >5%, and even after an hour this figure is still ~64%, suggesting the protein is fairly rigidly structured. These regions include three of the tryptophans involved in the ET chain: W420, W397, W394, corresponding to positions one, two and four in the ET chain, respectively (see Figure 1.3). The third tryptophan, W342, was not directly observed in the peptidic products but is closely surrounded by regions of low uptake.

Although the majority of the protein shows low uptake, some regions do display a fast exchange, specifically residues 187-192, 210-214, 289-303 and 333-336, showing an uptake of over 50%. Of the three aforementioned regions that reach the estimated saturation value (289-293, 439-444, and 502-509), the exposed 289-303 peptide reaches the limit extremely rapidly, saturating after only 15 s. Consequently, this peptide region is unlikely to show any marked difference in uptake when compared to the light state.

For the shortest (15 s) and longest (1 hour) labelling time, the deuterium uptake heat-maps for *DmCry* WT in the dark were mapped on to the X-ray crystal structure of the protein (PDB: 4GU5) and the resulting structures are shown in Figure 4.6B. Areas of the protein that are solvent accessible or loosely structured can be seen to readily and rapidly uptake deuterium, but more structured, solvent-inaccessible regions show less rapid uptake and only begin to show a change after 1 hour of labelling. Overall, there is a good correlation between deuterium incorporation and secondary structural features; less solvent accessible (more buried) regions see slow exchange whereas solvent exposed regions show much quicker exchange. In particular, the α -22 helix sees a notable increase in deuterium uptake over time,

going from an uptake of $\sim 10\%$ after 15 s to $\sim 63\%$ after an hour. Furthermore, this α -22 helix also shows much greater deuterium uptake than the other α -helices. Conversely, the α -23 helix (the CTT) undergoes minimal deuterium incorporation in the dark, reaching only $\sim 15\%$ after 1 hour of labelling.

4.5.2 Blue-light induced conformation changes in *DmCry*

In order to investigate whether there is a blue-light induced conformational change in *DmCry* WT, the deuterium uptake of the protein in the dark must be compared to the rate of uptake in the light. After having established the basal dynamics of *DmCry* WT in the dark, the rate of deuterium uptake upon exposure to blue light was observed by exposing *DmCry* WT to 453 nm light for the duration of labelling and equilibration stages of the experiment, as discussed in Section 4.4.2. Deuterium uptake levels were compared to data taken within the same 12-hour time period.

A differential heat-map for the blue-light induced conformational change observed in *DmCry* WT is shown in Figure 4.7, with areas of the protein that show no difference in uptake in light and dark conditions depicted in dark blue, and those that show an increased uptake in light conditions in red. It is notable just how much of the protein shows no significant change in deuterium uptake when exposed to blue-light (as indicated by dark-blue regions on the heat-map). 95% of the protein shows a less than 10% increase in uptake after 15 s of deuterium exposure implying a minimal blue-light induced change, and this only slightly decreases to 92% of the protein after 1 hour of exposure.

Despite the fact that a large proportion of protein shows minimal light-induced difference in deuterium incorporation, some regions do display a marked increase in uptake. The α -23 helix (the CTT) and α -22 helix (located proximal to the CTT) both show a large increase in uptake during exposure to blue-light after just 15 seconds of labelling. That the majority of the sequence undergoes little change, therefore acting as an internal control, implies that these specific regions underwent a conformational change to become more loosely structured or solvent accessible upon exposure to blue light, whilst the rest of the protein remains

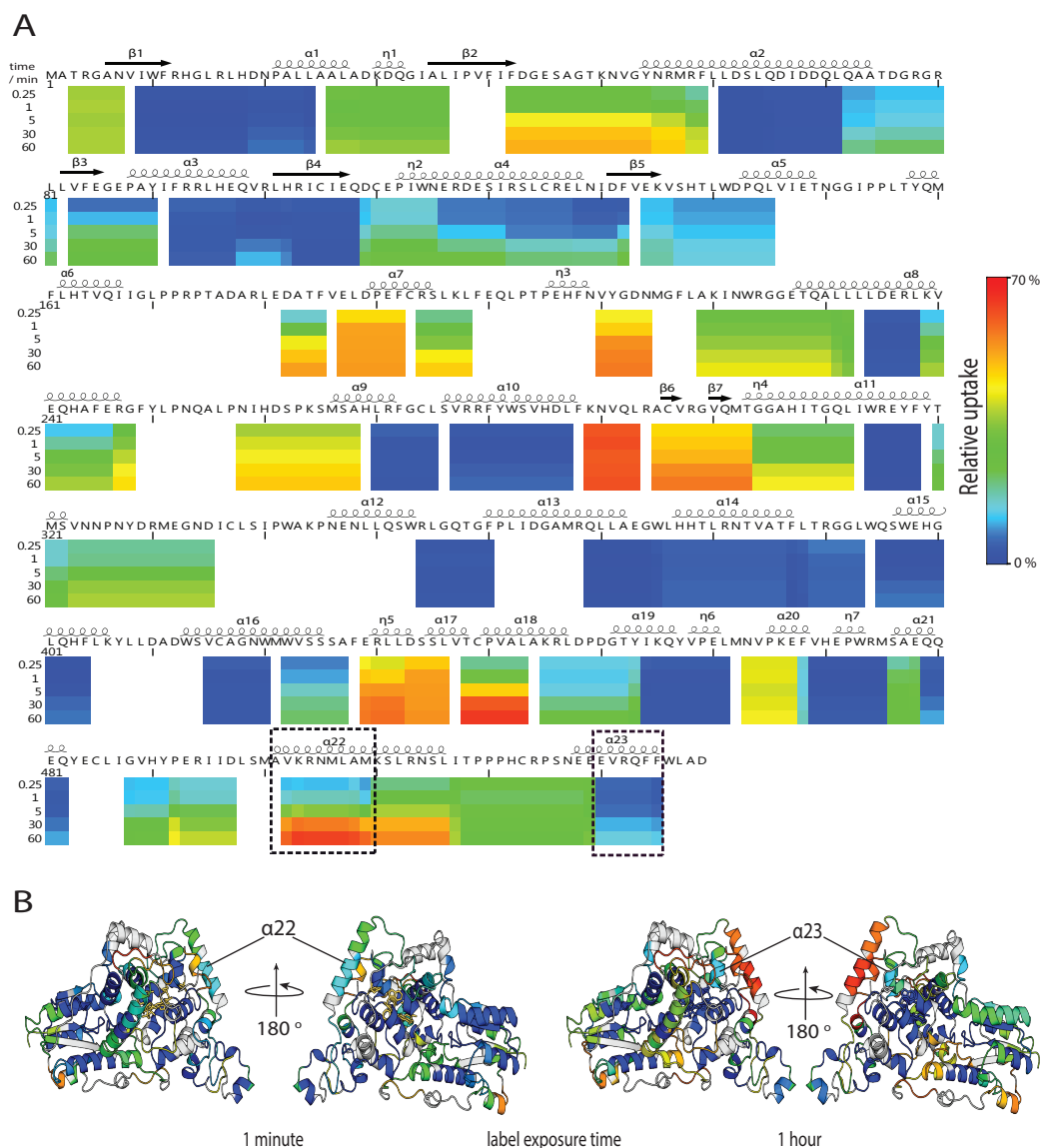


Figure 4.6: Dark state dynamics of *DmCry* WT as observed by HDX-MS. A The deuterium uptake heat-map for *DmCry* under dark conditions. HDX-MS was performed on *DmCry* after exposure to a deuterated buffer solution for 0.25, 1, 5, 30 and 60 minute incubation periods. Estimates of the uptake at each residue are shown having been normalised to the theoretical maximum and correspond to values between 0 % (blue) and 70% (red) as indicated in the colour bar. A sequence coverage of 80.5% was observed with regions of no coverage shown in white. Secondary structure elements, α -helices, β -sheets, 310-helices (η), are depicted above the sequence. B Uptake heat-maps are mapped on to the crystal structure (PDB: 4GU5) for the time points 15 seconds (left) and 1 hour (right). C-terminal helices (α -22 and α -23) are marked.

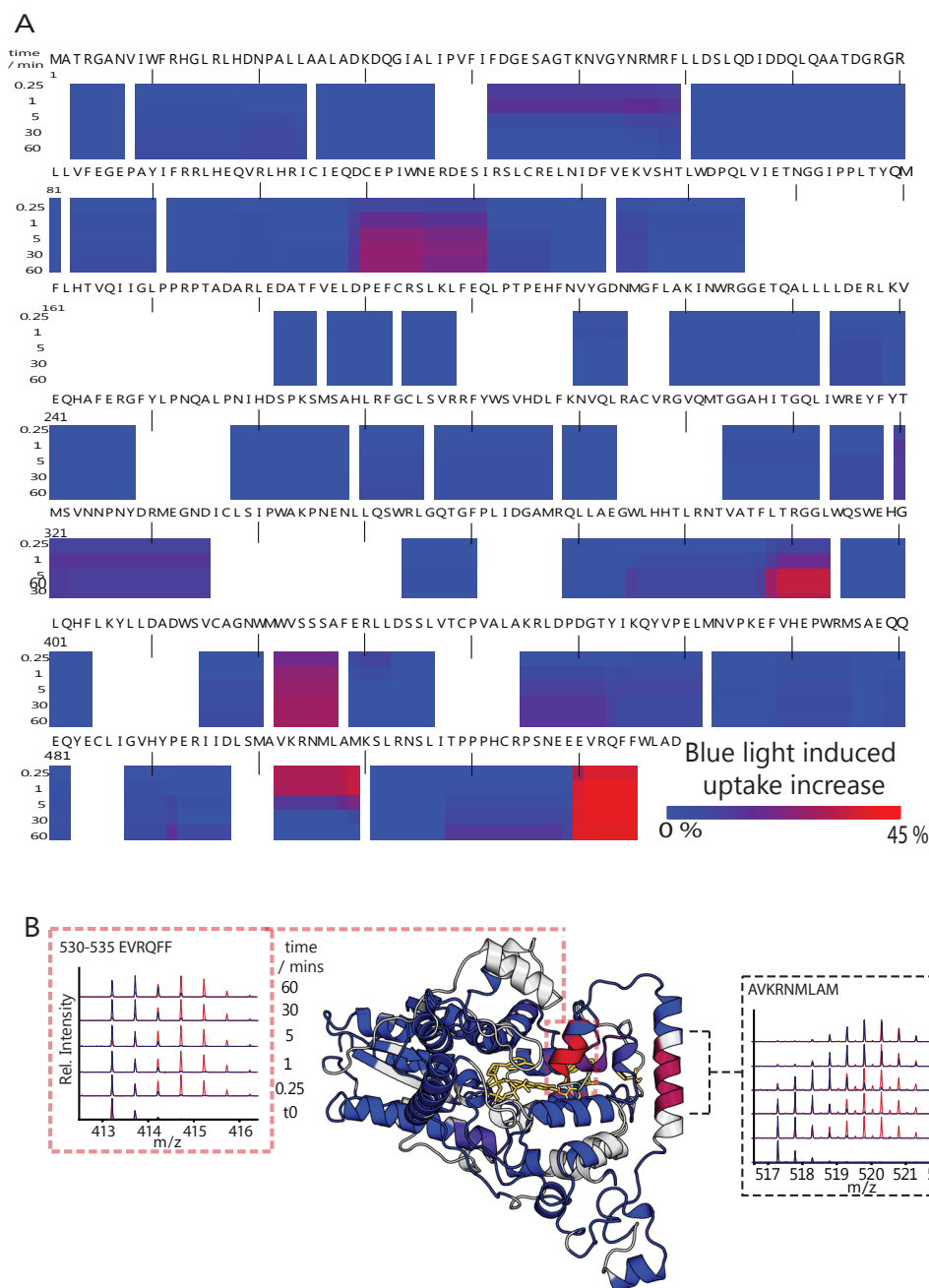


Figure 4.7: Blue-light induced conformational change of *DmCry* WT shown by differences in the rate of deuterium uptake in light and dark conditions. **A** Deuterium uptake difference plot of *DmCry* WT under continuous blue light illumination and in the dark; regions in red indicate an increased uptake in blue light, whereas blue regions imply minimal change in uptake for the two conditions. The majority of the protein undergoing no change acts as an internal control. The CTT shows a large increase in uptake implying it undergoes a conformational change, becoming more loosely structured upon exposure to light. **B** Uptake difference plot after 15 s of labelling mapped onto protein structure (PDB: 4GU5). Representative mass spectra are shown for 2 peptides in α -23 (CTT) and α -22 helices, which both show a large blue-light-induced increase in deuterium uptake. White regions indicate no coverage and the FAD/W residues are shown in yellow.

relatively static. There were a minimum of 3 overlapping peptides in each region (α -23 and α -22), all of which showed the same behaviour across 3 repeats, providing some certainty to these results.

This CTT-centred conformational change is further highlighted in Figure 4.7B which maps the difference in uptake after 15 s of labelling to the 3 dimensional protein structure. Mass spectra are shown for peptides spanning the regions 530-535 (EVRQFF) and 501-509 (AVKRNMLAM), which correspond to peptides in the α -23 and α -22 helices respectively, along with a reference undeuterated time zero, t_0 , spectrum. The mass spectra show a shift to higher $\frac{m}{z}$ in blue-light conditions implying an increase in incorporation of deuterium in light conditions. The peptide in the α -23 region shows limited uptake throughout the duration of the experiment in the dark; however, under blue light a rapid increase in mass is seen after just 15 s of labelling, implying rapid deuterium incorporation and a light-induced structural relaxation. Whilst the α -22 helix also displays an increase in the rate of uptake in light conditions, this region shows different dark-state behaviour to the α -23 helix: the α -22 helix sees dark-state uptake increasing and then converging with light-state uptake. This explains the reduction in the light-dark difference observed over time for the α -22 helix in comparison to the large light-dark difference persisting, even increasing, throughout the entirety of the experiment for the α -23 helix.

A selection of areas outside of the α -22 and α -23 regions also show an increase in uptake after 1 hour, specifically peptides 110-121, 389-393 and 422-428. With the exception of peptide 110-121, which is situated far away from the other peptides, these increases could be explained by the proximity of the regions to the CTT. Region 389-393 is located before the fourth tryptophan in the ET chain (W394) and is buried by the α -22 helix; peptide 422-428 lies close to W420 in the ET chain and corresponds to the N-terminal part of a region of the cryptochrome known as the C-terminal lid, a structural element surrounding the CTT (see Figure 4.1). As the α -22 and α -23 helices become more loosely-structured upon exposure to blue-light, this could result in the proximal 389-393 and 422-428 regions becoming less protected and thus also seeing an increase in uptake.

The signalling state of cryptochrome has to be relatively long-lived in order for a signal to be effectively transduced. Whilst movement of the α -23 helix is proposed to be related to the mechanism of signal transduction (by allowing the protein to better interact with downstream signalling partners [112, 113]), the movement of the α -22 helix and other aforementioned areas of the protein could be a result of a stabilising mechanism. The α -22 helix is positioned in close proximity to α -23 and could act to stabilise the movement of the CTT. Moreover as mentioned above, the 389-393 region is proximal to the most terminal tryptophan in the ET chain, and the conformational change here seems to increase over time. Considering the positions of these regions, it is not inconceivable that the conformational changes observed are from a stabilising motion to prolong the lifetime of the signalling state of the molecule.

These findings complement previously published results by Ozturk [113] and Vaidya [112], with the data largely agreeing with the regions of the protein they found to be more exposed/undergo a conformational change after exposure to blue light. Using limited proteolysis Ozturk and Vaidya reported 3 of the same light sensitive cleavage sites (R430, K503, 520-542), as well as non-light sensitive sites (K289), which agrees well with the results above. However, Vaidya also reported on additional light sensitive sites, as well as regions that become more protected in light. Of these additional sites, R354 agrees with the above data, however the light-induced change at the peptide around residue R494 is very small/negligible and we do not have data for 2 regions (R368 and R179) as no peptides were found here. Overall, however, the data highlight similar areas of the protein to be light sensitive/insensitive, but due to the nature of HDX-MS it is possible to go further and quantitatively report on over 80% of the protein sequence rather than just focussing on a few distinct sites.

Blue-light intensity and red light control

In addition to investigating the light sensitive regions of the protein, the response to blue-light intensity was also investigated. Figure 4.8 illustrates how deuterium uptake varies as a function of light intensity. The centroids of the mass spectra as a

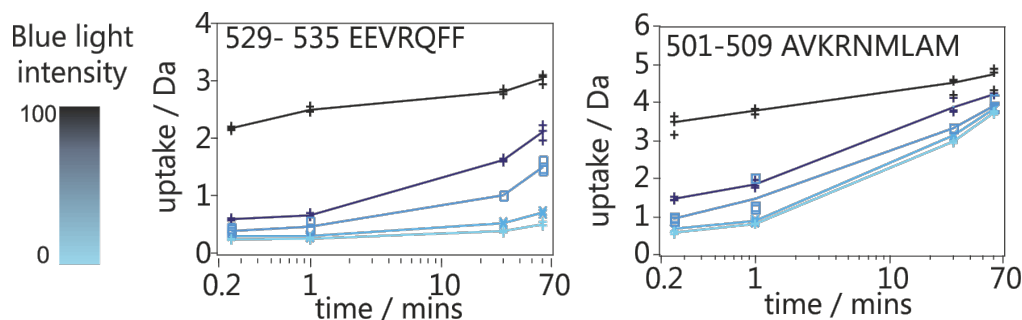


Figure 4.8: Blue-light intensity variation for two representative CTT peptides highlighted in Figure 4.7B: peptides 529-535 and 501-509, from the α -23 and α -22 helices respectively. The data demonstrate an increase in uptake for increased intensity of blue-light in *DmCry* WT.

function of labelling time (0.25-60 min) are shown for both previously discussed peptides, 530-535 (α -23) and 501-509 (α -22), corresponding to the CTT and a region close to the CTT, respectively.

Increasing light intensity (light blue to dark blue) results in an increase in deuterium uptake resulting from a local unfolding of the α -helices and/or the region becoming more loosely structured. These data clearly show that photo-activation does not result in a binary response; instead the rate of conformational change is related to the intensity of the light source. Both α -23 and α -22 peptides show an increased uptake with more intense light, however their behaviour is somewhat different to each other. The terminal tip of the CTT appears to be the most sensitive to the intensity of light. α -22 peptides converge towards an upper limit of 4.8 Da, and do so at a faster rate with increased light intensity; the α -23 peptides, however, do not show any sign of converging to a maximum.

A control experiment was performed on *DmCry* WT under a comparable intensity of red light to confirm the light-induced conformational changes observed are indeed a result of the FAD/W photochemistry in cryptochrome. Figure 4.9 shows the deuterium uptake as a function of labelling time for the same two CTT peptides, 529-535 (α -23) and 501-509 (α -22), under blue light, red light and dark conditions. It is hypothesised that if cryptochrome is responsible for the light-induced conformational change then the wavelength of the light-activation will have a significant effect

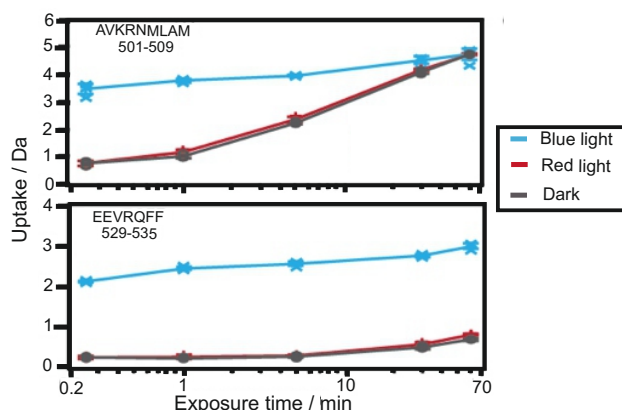


Figure 4.9: Deuterium uptake as a function of time for two CTT peptides, AVKRNMLAM (α -22) and EEVRQFF (α -23), under red light (red), blue light (blue) and dark (grey) conditions. Red light control experiments show no uptake difference to that in the dark state, confirming the involvement of the FAD/W photochemistry in the light-induced conformational change.

on the results. As discussed in Section 1, cryptochromes have a light-dependence and require blue light to function.

As can be seen from Figure 4.9, the deuterium uptake for red light exposure is very similar to that of the dark state. This suggests that it is specifically blue rather than red light which is required to trigger the conformational change, which correlates with cryptochrome being a blue-light photoreceptor and therefore not being active in red light. These results confirm that the FAD/W photochemistry is responsible for the conformational change.

4.5.3 *DmCry* W342F and *DmCry* W394F

The effect of disrupting the ET chain in *DmCry* by mutating tryptophans in the tryptophan tetrad to redox inactive phenylalanines (F) has been shown to have significant effects on the photochemistry. The impact of the disrupting the photochemistry on the observed conformational change in *DmCry* was investigated by probing the conformational dynamics of point mutants of *DmCry*, W342F and W394F, corresponding to the 3rd and 4th tryptophans in the tryptophan tetrad, respectively. These experiments provide a platform for deepening our understanding

of how the photochemistry occurring in *DmCry* impacts the signal transduction pathway via a blue-light induced conformational change.

HDX experiments were performed on both *DmCry* W342F and *DmCry* W394F under identical experimental conditions as for *DmCry* WT. Differences in uptake in light and dark conditions were compared for each *DmCry* mutant just as before.

As for the WT system, both mutants showed no marked light-dark uptake differences across the bulk of the sequence length and as so the following discussion is focussed on the regions α -23 and α -22.

A comparison of the rate of uptake over time (0.25-60 min) for *DmCry* WT, W394F and W342F is shown in Figure 4.10A. Initially considering region 529-534, the α -23 peptide (Figure 4.10A, top), it is worth noting just how different the rate of uptake is for each variant even when solely comparing the dark-state behaviour. Compared to *DmCry* WT, *DmCry* W394F sees both a slower increase in uptake with labelling time, as well a slower uptake after 1 hour of labelling; *DmCry* W342, however, displays not only a faster uptake compared to that of the *DmCry* WT, but also a larger final uptake value after 1 hour of labelling. The dark state dynamics for each protein are markedly different in this region implying different levels of protection in the dark.

Comparing the difference between light and dark uptake for peptide 529-534, the general trend for *DmCry* WT shows that after 1 hour of labelling the increase in deuterium uptake in blue-light is over double that of the dark, complementing results shown in Figure 4.7. Conversely, both the mutants show a much smaller difference under identical blue-light conditions when compared to their dark-uptake. Even after 1 hour of labelling *DmCry* W342F only shows $\sim 15\%$ increase in deuterium uptake in the light, and in *DmCry* W394F there is no significant change in uptake when exposed to blue-light at all.

The dark-state dynamics at region 501-509, the α -22 peptide, (4.10A, bottom) display comparable behaviour for *DmCry* W394F and *DmCry* WT, both showing a steady increase in uptake with increased labelling time and reaching similar values of ~ 4 Da after 1 hour of labelling. *DmCry* W342F, however, shows a much more

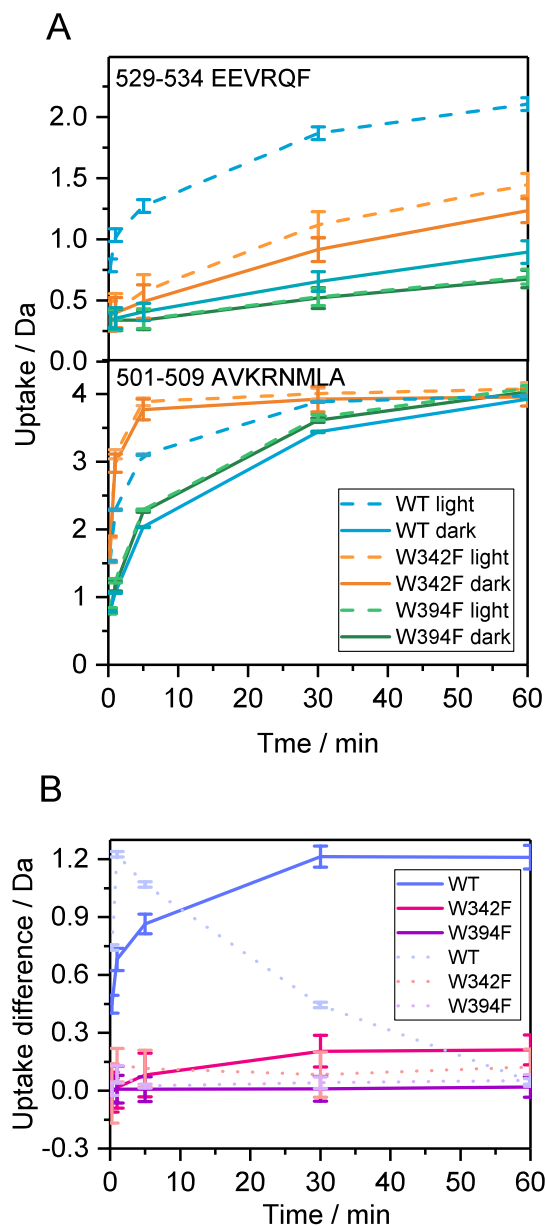


Figure 4.10: Comparison of blue-light induced differences in deuterium uptake for *DmCry* WT, *DmCry* W394F and *DmCry* W342F for peptides located near the CTT: 529-534 (EEVRQF) and 501-509 (AVKRNMLA). **A** Rate of deuterium uptake in both light (dashed lines) and dark (solid lines) conditions for all *DmCry* for a representative peptide in the α -23 (top), and α -22 (bottom), helices. Differing dark state kinetics are observed both between different peptides and between different *DmCry* variants. **B** Difference in deuterium uptake for *DmCry* WT, *DmCry* W342F and *DmCry* W394F, plotted as a function of time. The 529-534 region is represented by solid lines and the 501-509 region is shown as dotted lines. A large increase in uptake in blue-light conditions is observed for *DmCry* WT in both peptide regions; however this difference is drastically decreased when the ET chain is interrupted in *DmCry* W342F and *DmCry* W394F.

rapid initial increase in uptake, reaching 3.77 Da after just 5 minutes, and then increasing much more steadily to a maximum of ~ 4 Da after 1 hour labelling.

The difference in protection of this α -22 peptide for each cryptochrome, however, does not appear to affect the light-induced uptake. Analogous to α -23, *DmCry* WT shows the only distinct change when exposed to blue-light, seeing an almost two-fold increase in uptake after just 15s of labelling. Although this difference decreases in magnitude over time, a clear light-induced increase remains present for all labelling times. Conversely, *DmCry* W342F shows a much smaller, almost negligible change across all labelling times, never exceeding 5% (with a maximum difference reached after 1 minute of labelling), and *DmCry* W394F shows no observable change at all. Despite α -22 and α -23 being less protected in *DmCry* W342F than *DmCry* WT or W394F, both mutants still see no large change in uptake in light, whereas a clear increase is observed for *DmCry* WT. The *DmCry* mutants do not respond to blue light in the same pronounced way as *DmCry* WT does.

The difference in deuterium uptake for each *DmCry* protein (WT, W342F and W394F) over time (0.25-60min) is illustrated in Figure 4.10B. Solid lines represent peptide 529-534 (α -23) and dotted lines represent peptide 501-509 (α -22). As previously discussed, the change in uptake for *DmCry* WT increases steadily over time at the α -23 helix, but at α -22 the difference peaks after 1 minute and then diminishes. The reduced difference over time can be accounted for by the dark-uptake increasing at a faster rate than the light uptake at long exposure times. This behaviour is not seen for 529-534 region, as the dark uptake is relatively more flat over labelling time and does not converge with the rapidly increasing light uptake. The most significant point to note, however, is that neither *DmCry* W342F or W394F show a significant difference in uptake, as seen by the relatively flat lines for these mutants in both peptide regions.

Overall, these data imply the blue-light induced conformational change observed in *DmCry* WT is significantly reduced when you interrupt the tryptophan ET chain; without the third tryptophan the conformational change is extremely reduced in magnitude and with no fourth tryptophan the conformational change effectively

disappears. These data highlight the importance of the fourth, and to a lesser extent the third, tryptophan in the ET chain for the signalling mechanism in cryptochromes and are in line with previous initial findings obtained through partial proteolysis techniques. Complementary to data above, Lin *et al.* found that *DmCry* W342F has considerably lessened conformational sensitivity compared to *DmCry* WT and *DmCry* W394F undergoes little/no conformational activation in light at all.

Ultimately, the data presented in this chapter may help explain recent results that show the *DmCry* W394F mutant to have a very large magnetic field effect (MFE), even larger than that of the WT [80]. Independent experiments carried out on *Drosophila* also showed that MFEs are present when the fourth tryptophan in the ET chain (W394) is replaced with a redox inert residue, but they are abolished when the C-Terminal Tail (CTT) is removed [48, 49, 122, 123]. It appears as though an intact CTT is required for an MFE to be observed, but the fourth tryptophan is not essential. Indeed, the MFE is larger when the fourth tryptophan is not there [80]. This begs the question of why nature has evolved to have a four-tryptophan chain if the MFE is considerably larger in the W394F mutant where the fourth tryptophan is missing. Here we show that although these other experiments may have found the MFE to be larger in *DmCry* W394F, there is little to no conformational change occurring in this mutant. If it is not possible to induce a conformational change without the fourth tryptophan, and thus transduce a signal, then the size of the MFE is irrelevant: there is a balance struck between a large MFE and ability to transmit a signal.

The CTT is essential for an MFE to be observed and for signal transduction to occur. The fourth tryptophan (W394), however, whilst not being necessary for an MFE to be observed, is required for the cryptochrome to undergo a conformational change. Given the location of the fourth tryptophan in the protein, and its proximity to the 389-393 region and the α -22 helix (which both show a conformational change under blue-light), it is possible that the fourth tryptophan leads to the signalling state of the molecule which is stabilised by regions of the protein such as 389-393 and the α -22 helix.

4.6 Conclusions

In this Chapter it has been conclusively shown that a blue-light induced conformational change occurs in *DmCry* WT, and more specifically, due to the quantitative nature of HDX-MS, this conformational change has been located to CTT peptides. Blue light exposure results in the CTT becoming more solvent accessible, consistent with theories that propose a CTT conformational change to be key in the signal transduction pathway, with the CTT becoming more exposed and thus enabling interactions with signalling partners.

HDX-MS experiments on *DmCry* WT under red-light conditions have been shown to be comparable to those in the dark, consistent with cryptochromes being a blue-light photoreceptor. This result supports the theory that the conformational change occurring is a result of the photochemistry of FAD/W in the cryptochrome.

Furthermore, by comparing conformational changes in *DmCry* WT with those in *DmCry* W342F and *DmCry* W394F, the effect that photochemistry has on the conformational change, and thus signal transduction pathway, has been explored. Upon replacing the third tryptophan in the ET chain with a phenylalanine, as in *DmCry* W342F, the conformational change drastically decreases and is effectively abolished. Even more significantly, however, these results have shown the importance of the fourth tryptophan in the signal transduction pathway: when this tryptophan is replaced with a phenylalanine, as in *DmCry* W394F, the conformational change becomes non-existent. The 4th (and 3rd) tryptophan have been shown to be essential for the signal transduction pathway in cryptochromes.

4.6.1 Future work: searching for MFEs

As the light-induced conformational change observed in *DmCry* has been shown to be affected by the FAD/W photochemistry occurring, which in turn is affected by magnetic fields, it would not be unreasonable to expect the conformational change to have a magnetic field dependence. The signalling pathway is expected to be sensitive to MFEs arising from the FAD/W photochemical system.

As previously discussed, magnetic fields alter the ratio of singlet to triplet SCRP in the geminate cage. These states, which we denote RP1, are directly affected by the presence of a magnetic field and are magnetically sensitive, however, as the states are fairly short lived ($\sim 1 \mu\text{s}$) RP1 is unlikely to be the signalling state of the molecule. These states may, however, form a longer-lived RP2 state via a change in protonation state over time. Formation of this RP2 state, with a lifetime of milliseconds, is hypothesized to lead to the signalling state of the molecule. Although RP2 is in itself magnetically insensitive, magnetic fields may still have an indirect effect on the signalling state by the MFE on RP1 being transferred to the longer-lived RP2 state. Consequently it would not be unwarranted to presume that the blue-light induced conformational change discussed above would be somehow affected by a magnetic field.

Preliminary attempts to investigate MFEs on the conformational change in *DmCry* WT were carried out, employing a similar methodology to that discussed in Section 4.4, however, the setup was modified in order to accommodate a series of permanent neodymium bar magnets underneath the sample holder. Consequently, protein samples were subjected to a magnetic field for the duration of the labelling process, and experienced a field strength of $\sim 100 \text{ mT}$ (however the field produced was by no means homogeneous). All other experimental parameters were the same as those laid out in Section 4.4.

Figure 4.11 shows the initial attempts to observe an MFE on the conformational change in *DmCry* WT by this method. The deuterium uptake for the CTT peptide EEVRQF (α -23) is shown for conditions of blue light (blue), blue light and a magnetic field (red), dark (green), and dark with a magnetic field (purple). Whilst both dark conditions show no marked difference regardless of the presence of a magnetic field or not, a clear difference is observed in the uptake values for the blue light conditions with a magnetic field. The presence of a magnetic field (in light) appears to act to increase the extent of deuterium uptake, implying that the CTT undergoes an even greater conformational change/becomes even more solvent accessible when subjected to a magnetic field. These data appear to suggest

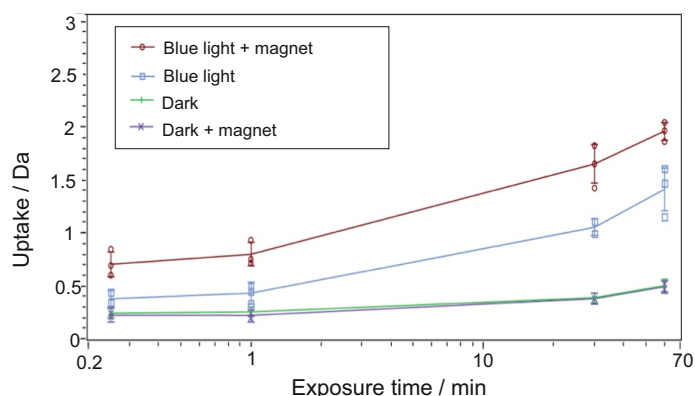


Figure 4.11: Deuterium uptake plots as a function of labelling time for the CTT peptide EEVRQF under: i) blue light and in the presence of a ~ 100 mT magnetic field (red), ii) blue light and no magnetic field (blue), iii) dark conditions (green), and iv) dark conditions and a ~ 100 mT magnetic field (purple). A clear difference in deuterium uptake is observed when a magnetic field is applied.

that an MFE may somehow be translated to the conformational change observed in *DmCry* WT. The results suggest that the RP is triplet-born, which is hard to explain if considering the magnetic sensor to be the $[\text{FADH} \cdot \text{W}]$ RP, but could be explained if an alternative RP, $[\text{FADH} \cdot \text{Z}]$, was responsible (see Section 1.2). Moreover, as a 100 mT magnetic field appears to increase the extent of CTT movement, if an LFE was present this may mean that a $50 \mu\text{T}$ Earth strength field would act to reduce the extent of CTT movement and thus suppress the active concentration of the signalling state of cryptochrome.

However, whilst these initial attempts at observing an MFE appear promising, for unknown reasons any attempt at repeating these results have thus far been unsuccessful. Therefore, care should be taken not to read too much into these results, however they do provide hope that an MFE may be observed on the conformational change in cryptochromes in the future.

In the future, it would be useful to re-attempt these MFE experiments with a more sophisticated magnetic field source to ensure a more homogeneous field. Whilst the reasoning behind the difficulties in reproducing the above data are unclear, the lack of homogeneity experienced by the samples is not ideal regardless.

Furthermore, repeating the HDX experiments detailed above (including ones with a magnetic field) on even more biologically relevant protein samples for magnetoreception such as cryptochrome from the European robin, *Erithacus rubecula* cryptochrome (*ErCry*) may be beneficial. MFEs observed on the *ErCry* using optical spectroscopy such as TA and CRDS have been shown to be orders of magnitude larger than those observed for *DmCry* [80], which has a maximum MFE of only $\sim 1\%$ [54]. Although there is no guarantee that the size of the MFE observed by these methods would be reflected in the size of MFE observed in the conformational change, it may be the case that the MFE on the conformational change in *ErCry* would be larger than that of *DmCry* and so would be observed more easily in these HDX MS experiments.

Regardless of whether an MFE is observed or not, HDX experiments on *ErCry* would still provide a good comparison to those from *DmCry*. These experiments would help conclude if a similar light-induced conformational change is present in avian cryptochrome, and they would act as a means to compare the conformational change, and thus signal transduction pathway, between cryptochrome from two distinct species.

5

Conclusions

The work presented in this thesis demonstrates significant progress towards more fully understanding the photochemistry and photosignalling mechanisms occurring in cryptochromes. In terms of photochemistry, a highly sensitive fluorescence-based detection method, PF MARY, has been redesigned to increase sensitivity and to make it more suitable for exploring the photochemistry of biological samples. Using this technique, first steps were made towards observing MFEs on cryptochromes *in vivo*. The re-designed PF MARY was further used to investigate the photochemistry of thionine RPs in micelles, exploring LFEs and the effects of altering the micelle environment on the RP photochemistry. The photosignalling pathway in *DmCry* was also investigated, and through the use of HDX-MS the existence of a blue-light induced conformational change centred at the CTT was confirmed. Furthermore, the third and fourth tryptophans in the ET chain were shown to have a vital importance this photosignalling pathway in *DmCry*.

The context of this thesis was introduced in Chapter 1, where the concepts of magnetoreception and the RPM were introduced, highlighting the likely involvement of cryptochrome as the magnetoreceptor molecule. The RPM is one of the leading hypothesis to explain how some animals are able use the Earth's $\sim 50 \mu\text{T}$ magnetic field as a navigation cue during long-distance migrations. Consequently, it is of huge importance to understand the photochemistry and photosignalling mechanisms

occurring in cryptochromes. The structure and function of cryptochromes was presented and the theories of photosignalling discussed, explaining how, to date, minimal is known about the signal transduction pathway in these molecules (specifically in animal cryptochromes). The chapter went on to introduce the relevant concepts of spin chemistry needed to understand the photochemistry discussed in this thesis.

Chapter 2 describes how the already highly sensitive PF MARY technique was modified in order to further increase sensitivity and make the experiment more suitable for studying biological samples. Amongst other modifications, replacing the sample cell and introducing a peristaltic pump meant that it was possible to reduce the volume of the setup from tens of millilitres to just 1.5 ml, and allow small volumes of sample to be flowed/recirculated. This is particularly important when measuring precious biological samples which are only available at low concentrations and volumes and so cannot afford to be flown to waste or used on high volume experiments. The re-designed PF MARY was shown to be superior to the previous setup, with large sensitivity gains and a reduction in SNR observed when comparing the MARY plot of a model system of RF/W between the two setups. Increased sensitivity allows for the detection of even smaller MFEs; this ability was highlighted in experiments throughout this thesis but specifically during attempts to observe MFEs on *DmCry* in *Sf9* cells.

The fact that the PF MARY technique is now well-suited to monitor MFEs on biological samples, in particular cryptochromes *in vivo*, has been a huge advancement towards observing MFEs on systems that are even closer to biology. Although attempts at observing an MFE in *DmCry* WT and *DmCry* W4 were not successful, this was largely due to the presence of RF in the medium which was likely undergoing undesirable photochemistry with an ED to give rise to an unwanted MFE that obscured the MFE of interest (from the *DmCry Sf9* samples). If the medium that the *Sf9* cells are grown in is exchanged for another buffer without any undesirable chemicals in, then the PF MARY system is in a very good position to be able to observe an MFE on this system.

In Chapter 3, the redesigned fluorescence experiment was used to investigate the effects of confining a RP system, specifically one containing thionine and an electron donor, to a micelle. Micelles act as nanocontainers for chemical reactions, restricting the diffusion of RP partners which results in a longer lived RP. A comparison of the MFE in a system of thionine and DABCO in the presence and absence of SDS demonstrated the effect a micelle can have on the RP photochemistry. Micelle confinement was shown to increase the magnitude of the MFE by more than a factor of 5. An LFE was also observed on the thionine/DABCO/SDS system, which was accounted to a large RP lifetime (brought about by RP being in SDS micelles) in comparison to the average hyperfine coupling of the thionine/DABCO RP.

The effect of changing micelle size was also investigated by exchanging SDS for SDeS or SOS in the thionine/DABCO system. Even though the variation in micelle size for the systems discussed in this Chapter was minimal (the monomers SDS, SDeS and SOS vary by only two and four carbons in the alkyl chain respectively), the sensitive PF MARY technique was able to distinguish between the systems. An increased micelle size shown to cause a small decrease in the MFE, which is accounted to an increased number of molecules being able to occupy the hydrophobic core in larger micelles, leading to increased quenching and a smaller MFE. The importance of micelle charge was demonstrated: changing the micelle from anionic (SDS) to cationic (DTAC) for a system of thionine/DABCO acted to drastically reduce the MFE (the MFE was over 20 times smaller in the DTAC system). This was accounted for by the relative positions of the RP partners in the micelle. In DTAC micelles the unfavourable charges repel the RP partners away from each other, so they are not held in close proximity and the RP lifetime is not increased and so the MFE is not enhanced.

The photochemistry of thionine/aniline/SDS was also explored. This system involves a slightly altered photochemical reaction scheme to others studied thus far as thionine undergoes π -stacking with aniline, resulting in MFEs arising from the triplet mechanism. Consequently, the photochemistry can be affected by altering the heavy atom substituent on the aniline as the triplet mechanism is dominated

by the rate of SOC. A trend was observed whereby the MFE was more rapidly quenched with increasing SOC.

In Chapter 4, the photosignalling mechanism of *DmCry* was explored. Initially a partial proteolysis technique was used, but the results were limited by the crude nature of this technique and the many potential sources of error. The Chapter went on to introduce a much more highly quantitative technique that is capable of monitoring conformational change at a near-residue level: HDX-MS. By comparing the rate of deuterium uptake across the protein sequence in the presence and absence of light, a blue-light induced conformational change was observed on *DmCry* WT, and the change was able to be localised to the CTT. A control experiment under red light confirmed the requirement for blue-light activation to bring about the conformational change, in-line with cryptochrome being a blue-light photoreceptor, and proving that the FAD/W photochemistry is responsible for initiating the conformational change.

In addition, the effect of the 3rd and 4th tryptophans in the ET chain has on the photosignalling process was highlighted. HDX-MS experiments were performed on the *DmCry* mutants *DmCry* W342F and *DmCry* W394F, whereby the 3rd and 4th tryptophans respectively were replaced by the redox inert phenylalanine. These mutations result in the ET chain being interrupted, and resulted in an effective abolishment of the conformational change in both cases. Although a small conformational change was still observed in *DmCry* W342F, this was lost entirely in *DmCry* W394F. These results are hugely significant as they may help explain why cryptochromes have evolved to have four tryptophans involved in the ET chain despite the fact that MFEs observed on the *DmCry* W394F mutant (i.e. with no 4th tryptophan) are orders of magnitude larger than in *DmCry* WT. If all four tryptophans are not present then although the MFE may be larger, no conformational change occurs and thus the photosignalling pathway is prohibited. It does not matter if a large MFE is present if signal transduction is not possible. Both the 3rd and 4th tryptophans in the ET chain in cryptochromes are important for effective signal transduction.

References

- [1] A. T. von Middendorff. *Karten-Atlas zu Dr. AV Middendorff's Reise in den Aussersten Norden und Osten Sibiriens*. Buchdr. der K. Akademie der Wissenschaften, 1859.
- [2] W. Wiltschko and F. W. Merkel. "Orientierung zugunruhiger Rotkehlchen im statischen Magnetfeld". In: *Verh Dtsch Zool Ges* 59.362.367 (1966).
- [3] W. Wiltschko and R. Wiltschko. "Magnetic orientation in birds". In: *J. Exp. Biol.* 199.1 (1996), pp. 29–38.
- [4] S. Johnsen and K. J. Lohmann. "The physics and neurobiology of magnetoreception." In: *Nat. Rev. Neurosci.* 6.9 (2005), pp. 703–712.
- [5] G. C. Nordmann, T. Hochstoeger, and D. A. Keays. "Unsolved mysteries: Magnetoreception—A sense without a receptor". In: *PLoS Biol.* 15.10 (2017).
- [6] K. Maeda et al. "Chemical compass model of avian magnetoreception." In: *Nature* 453.7193 (2008), pp. 387–390.
- [7] T Ritz, S Adem, and K Schulten. "A model for photoreceptor-based magnetoreception in birds." In: *Biophys. J.* 78.2 (2000), pp. 707–718.
- [8] K. Schulten, C. E. Swenberg, and A. Weiler. "A Biomagnetic Sensory Mechanism Based on Magnetic Field Modulated Coherent Electron Spin Motion". In: *Zeitschrift fur Phys. Chemie* 111.1 (1978), pp. 1–5.
- [9] T. P. Quinn. "An Experimental Approach to Fish Compass and Map Orientation". In: *Mech. Migr. Fishes*. Springer US, 1984, pp. 113–123.
- [10] D. Shcherbakov et al. *Magnetosensation in zebrafish*. 2005.
- [11] P. Light, M. Salmon, and K. J. Lohmann. "Geomagnetic orientation of loggerhead sea turtles: evidence for an inclination compass". In: *J. Exp. Biol.* 182.1 (1993).
- [12] J. B. Phillips. "Two magnetoreception pathways in a migratory salamander." In: *Science* 233.4765 (1986), pp. 765–7.
- [13] R. Wehner and Th Labhart. "Perception of the geomagnetic field in the fly *Drosophila melanogaster*". In: *Experientia* 26.9 (1970), pp. 967–968.
- [14] P. Nemec et al. "Neuroanatomy of magnetoreception: the superior colliculus involved in magnetic orientation in a mammal." In: *Science* 294.5541 (2001), pp. 366–8.
- [15] S. Marhold, W. Wiltschko, and H. Burda. "A magnetic polarity compass for direction finding in a subterranean mammal". In: *Naturwissenschaften* 84.9 (1997), pp. 421–423.
- [16] J. L. Kirschvink, M. M. Walker, and C. E. Diebel. "Magnetite-based magnetoreception". In: *Curr. Opin. Neurobiol.* 11.4 (2001), pp. 462–467.

- [17] R. Wiltschko and W. Wiltschko. *The magnetite-based receptors in the beak of birds and their role in avian navigation*. 2013.
- [18] C. T. Rodgers and P. J. Hore. “Chemical magnetoreception in birds: the radical pair mechanism.” In: *Proc. Natl. Acad. Sci. U. S. A.* 106.2 (2009), pp. 353–360.
- [19] C. E. Diebel et al. “Magnetite defines a vertebrate magnetoreceptor.” In: *Nature* 406.6793 (2000), pp. 299–302.
- [20] A. F. Davila et al. “A new model for a magnetoreceptor in homing pigeons based on interacting clusters of superparamagnetic magnetite”. In: *Phys. Chem. Earth* 28.16-19 (2003), pp. 647–652.
- [21] M. N. Williams and J. M. M. Wild. “Trigeminally innervated iron-containing structures in the beak of homing pigeons, and other birds”. In: *Brain Res.* 889.1-2 (2001), pp. 243–246.
- [22] R. Beason, N. Dussourd, and M. Deutschlander. “Behavioural evidence for the use of magnetic material in magnetoreception by a migratory bird”. In: *J. Exp. Biol.* 198.1 (1995).
- [23] C. D. Treiber et al. “Clusters of iron-rich cells in the upper beak of pigeons are macrophages not magnetosensitive neurons”. In: *Nature* 484.7394 (2012), 367–U102.
- [24] G. G. Fleissner et al. “Ultrastructural analysis of a putative magnetoreceptor in the beak of homing pigeons.” In: *J. Comp. Neurol.* 458.4 (2003), pp. 350–360.
- [25] G. Falkenberg et al. “Avian magnetoreception: elaborate iron mineral containing dendrites in the upper beak seem to be a common feature of birds.” In: *PLoS One* 5.2 (2010), e9231.
- [26] M. Zapka et al. “Visual but not trigeminal mediation of magnetic compass information in a migratory bird.” In: *Nature* 461.7268 (2009), pp. 1274–1277.
- [27] K. Schulten, C. E. Swenberg, and A. Weller. “Biomagnetic sensory mechanism based on magnetic-field modulated coherent electron-spin motion”. In: *Zeitschrift Fur Pjysikalische Chemie-Frankfurt* 111.1 (1978), pp. 1–5.
- [28] S. T. Emlen and J. T. Emlen. *A technique for recording migratory orientation of captive birds*. Tech. rep.
- [29] C. M. Hein et al. *Robins have a magnetic compass in both eyes*. 2011.
- [30] W. Wiltschko and R. Wiltschko. “Magnetic compass of European robins.” In: *Science* 176.4030 (1972), pp. 62–4.
- [31] W. Wiltschko and R. Wiltschko. “Magnetoreception in birds: Two receptors for two different tasks”. In: *J. Ornithol.* Vol. 148. SUPPL. 1. 2007.
- [32] W. Wiltschko et al. “Red light disrupts magnetic orientation of migratory birds”. In: *Nature* 364.6437 (1993), pp. 525–527.
- [33] W. Wiltschko and R. Wiltschko. “Migratory orientation of European Robins is affected by the wavelength of light as well as by a magnetic pulse”. In: *J. Comp. Physiol. A* 177.3 (1995), pp. 363–369.
- [34] W. Wiltschko and R. Wiltschko. “The effect of yellow and blue light on magnetic compass orientation in European robins, *Erithacus rubecula*”. In: *J. Comp. Physiol. - A Sensory, Neural, Behav. Physiol.* 184.3 (1999), pp. 295–299.

- [35] P. J. Hore and H. Mouritsen. “The Radical-Pair Mechanism of Magnetoreception”. In: *Annu. Rev. Biophys.* 45.1 (2016), pp. 299–344.
- [36] A. M. Edwards. “Structure and general properties of flavins.” In: *Methods Mol. Biol.* 1146 (2014), pp. 3–13.
- [37] S. Weber and E. Schleicher. *Flavins and Flavoproteins Methods and Protocols Methods in Molecular Biology* 1146. Tech. rep.
- [38] K. S. Conrad, C. C. Manahan, and B. R. Crane. *Photochemistry of flavoprotein light sensors*. 2014.
- [39] A. Sancar. “Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors.” In: *Chem. Rev.* 103.6 (2003), pp. 2203–2237.
- [40] M. Liedvogel and H. Mouritsen. “Cryptochromes—a potential magnetoreceptor: what do we know and what do we want to know?” In: *J. R. Soc. Interface* 7 Suppl 2.Suppl_2 (2010), S147–62.
- [41] A. R. Cashmore. “Cryptochromes: Blue Light Receptors for Plants and Animals”. In: *Sci. (80-.)*. 284.5415 (1999), pp. 760–765.
- [42] K. Maeda et al. “Magnetically sensitive light-induced reactions in cryptochrome are consistent with its proposed role as a magnetoreceptor.” In: *Proc. Natl. Acad. Sci. U. S. A.* 109.13 (2012), pp. 4774–4779.
- [43] A. Günther et al. “Double-Cone Localization and Seasonal Expression Pattern Suggest a Role in Magnetoreception for European Robin Cryptochrome 4”. In: *Curr. Biol.* 28.2 (2018), 211–223.e4.
- [44] H. Mouritsen et al. “Cryptochromes and neuronal-activity markers colocalize in the retina of migratory birds during magnetic orientation.” In: *Proc. Natl. Acad. Sci. U. S. A.* 101.39 (2004), pp. 14294–14299.
- [45] R. Wiltschko et al. “Light-dependent magnetoreception in birds: the crucial step occurs in the dark”. In: *Journal of The Royal Society Interface* 13.118 (2016), p. 20151010.
- [46] P. Muller and M. Ahmad. “Light activated cryptochrome reacts with molecular oxygen to form a flavin-superoxide radical pair consistent with magnetoreception”. In: *Journal of Biological Chemistry* (2011).
- [47] A. Lee et al. “n”. In: *Journal of the Royal Society Interface* 11 (Mar. 2014), p. 20131063.
- [48] R. Gegear et al. “Cryptochrome mediates light-dependent magnetosensitivity in *Drosophila*”. In: *Nature* 454 (2008).
- [49] C. N.G. Giachello et al. “Magnetic Fields Modulate Blue-Light-Dependent Regulation of Neuronal Firing by Cryptochrome”. In: *Journal of Neuroscience* 36.42 (2016), pp. 10742–10749.
- [50] K. J. Fogle et al. “Cryptochrome is a blue-light sensor that regulates neuronal firing rate”. In: *Science (New York)* 331 (2011).
- [51] H. Mouritsen and P. J. Hore. “The magnetic retina: light-dependent and trigeminal magnetoreception in migratory birds.” In: *Curr. Opin. Neurobiol.* 22.2 (2012), pp. 343–352.

- [52] T. Ritz et al. "Photoreceptor-based magnetoreception: optimal design of receptor molecules, cells, and neuronal processing." In: *J. R. Soc. Interface* 7 Suppl 2.Suppl_2 (2010), S135–46.
- [53] I. A. Solov'yov et al. "Decrypting cryptochrome: revealing the molecular identity of the photoactivation reaction." In: *J. Am. Chem. Soc.* 134.43 (2012), pp. 18046–18052.
- [54] D. M. W. Shepperd. "Cavity-Enhanced Detection of Biologically Relevant Magnetic Field Effects". DPhil Thesis. University of Oxford, 2016.
- [55] Jiang Li et al. "Dynamics and Mechanism of Repair of UV-induced (6-4) Photoproduct by Photolyase". In: *Nature* 466 (2010), pp. 887–890.
- [56] C. L. Partch et al. "Role of Structural Plasticity in Signal Transduction by the Cryptochrome Blue-Light Photoreceptor". In: *Biochemistry* 44.10 (2005), pp. 3795–3805.
- [57] I. A. Solov'yov, D. E. Chandler, and K. Schulten. "Magnetic Field Effects in Arabidopsis thaliana Cryptochrome-1". In: *Biophysical Journal* 92.8 (2007), pp. 2711–2726.
- [58] A. Agarwal. "Spin Dynamics". In: *Simulation Studies of Recombination Kinetics and Spin Dynamics in Radiation Chemistry*. Cham: Springer International Publishing, 2014, pp. 61–89.
- [59] C. R. Timmel et al. "Effects of weak magnetic fields on free radical recombination reactions". In: *Mol. Phys.* 95.1 (1998), pp. 71–89.
- [60] K.A. McLauchlan and U.E. Steiner. "Invited article: The spin-correlated radical pair as a reaction intermediate". In: *Molecular Physics* 73.2 (1991), pp. 241–263.
- [61] U. Till et al. "The influence of very small magnetic fields on radical recombination reactions in the limit of slow recombination". In: *Chemical Physics Letters* 298 (1998), pp. 7–14.
- [62] M. Murakami, K. Maeda, and T. Arai. "Dynamics of intramolecular electron transfer reaction of FAD studied by magnetic field effects on transient absorption spectra". In: *J. Phys. Chem. A* 109.26 (2005), pp. 5793–5800.
- [63] T. M. Zollitsch et al. "Magnetically Sensitive Radical Photochemistry of Non-natural Flavoproteins". In: *Journal of the American Chemical Society* 140.28 (2018). PMID: 29940116, pp. 8705–8713.
- [64] S. R. T. Neil et al. "Broadband cavity-enhanced detection of magnetic field effects in chemical models of a cryptochrome magnetoreceptor". In: *J. Phys. Chem. B* 118.15 (2014), pp. 4177–4184.
- [65] K. Maeda et al. "Following radical pair reactions in solution: a step change in sensitivity using cavity ring-down detection." In: *J. Am. Chem. Soc.* 133.44 (2011), pp. 17807–17815.
- [66] E. W. Evans et al. "Magnetic field effects in flavoproteins and related systems." In: *Interface Focus* 3.5 (2013), p. 20130037.
- [67] J R. Lakowicz. *Principles of fluorescence spectroscopy*. Springer, 2006, pp. 1–954.

- [68] K. Schulten et al. "Magnetic field dependence of the geminate recombination of radical ion pairs in polar solvents". In: *Zeitschrift für Phys. Chemie* 101.1-6 (1976), pp. 371–390.
- [69] R. S. Dixon et al. "Effect of a magnetic field on the fluorescence produced in irradiated anthracene solutions". In: *Can. J. Chem.* 55.11 (1977), pp. 2093–2101.
- [70] R. S. Dixon et al. "Fluorescence from γ -irradiated solutions of naphthalene and naphthalene-d8. Effect of an applied magnetic field". In: *Chem. Phys. Lett.* 47.1 (1977), pp. 108–112.
- [71] F. P. Sargent et al. "Pulse radiolysis in an applied magnetic field. The time dependence of the magnetic field enhancement of the fluorescence from solutions of fluorene in squalane". In: *The Journal of Physical Chemistry* 81.9 (1977), pp. 815–819.
- [72] Y. Tanimoto, K. Shimizu, and M. Itoh. "Two-step laser excitation fluorescence study of the magnetic field effect on the hydrogen abstraction reaction of anthraquinone in SDS micellar solution". In: *Chem. Phys. Lett.* 112.3 (1984), pp. 217–219.
- [73] D. Kattnig, E. W. Evans, and C. R. Timmel. "Slow Termination Reactions Amplify Magnetic Field Effects on Radical Pair Processes". 2015.
- [74] S. Salzmann, J. Tatchen, and C. M. Marian. "The photophysics of flavins: What makes the difference between gas phase and aqueous solution?" In: *J. Photochem. Photobiol. A Chem.* 198.2-3 (2008), pp. 221–231.
- [75] P. Cerletti. "Properties of riboflavin phosphates". In: *Anal. Chim. Acta* 20.C (1959), pp. 243–250.
- [76] "Chromatin Assembly In Vitro with Purified Recombinant ACF and NAP-1". In: *RNA Polymerases and Associated Factors, Part D*. Vol. 371. Methods in Enzymology. Academic Press, 2003, pp. 499–515.
- [77] D. L. Jarvis. "Chapter 14 Baculovirus - Insect Cell Expression Systems". In: *Guide to Protein Purification, 2nd Edition*. Ed. by R. R. Burgess and M. P. Deutscher. Vol. 463. Methods in Enzymology. Academic Press, 2009, pp. 191–222.
- [78] J. Shirdel et al. "Absorption and fluorescence spectroscopic characterisation of the circadian blue-light photoreceptor cryptochrome from *Drosophila melanogaster* (dCry)". In: *Chemical Physics* 352.1 (2008), pp. 35–47.
- [79] S. S. Rathi, M. K. Gopalakrishnan K. and Machwe, and J. Kishore. "Fluoremetric studies of solutions of riboflavin and rhodamine-B". In: *Acta Physica Academiae Scientiarum Hungaricae* 34.1 (1973), pp. 41–45.
- [80] T. Zollisch. "Investigation of Magnetic Field Effects on Protein Photochemistry using Cavity Enhanced Spectroscopy". DPhil Thesis. University of Oxford, 2018.
- [81] D. Kattnig et al. "Chemical amplification of magnetic field effects relevant to avian magnetoreception". In: *Nature Chemistry* 8 (2016).
- [82] E. W. Evans et al. "Sub-millitesla magnetic field effects on the recombination reaction of flavin and ascorbic acid radicals". In: *The Journal of Chemical Physics* 145.8 (2016), p. 085101.
- [83] R. Prado-Gotor et al. "Electron transfer reactions in micellar systems". In: *Progress in Reaction Kinetics and Mechanism* 25 (2000), pp. 371–407.

- [84] E. G. Bagraynskaya et al. "Investigation of Radical Pairs in Micelles Using Spin Polarization Techniques". In: *Magnetic Resonance in Colloid and Interface Science*. Ed. by Jacques Fraissard and Olga Lapina. Dordrecht: Springer Netherlands, 2002, pp. 173–184.
- [85] R. Eveson et al. "The effects of weak magnetic fields on radical recombination reactions in micelles". In: *International journal of radiation biology* 76 (2000), pp. 1509–22.
- [86] Malcolm D. E. Forbes, Gregory R. Schulz, and Nikolai I. Avdievich. "Unusual Dynamics of Micellized Radical Pairs Generated from Photochemically Active Amphiphiles". In: *Journal of the American Chemical Society* 118.43 (1996), pp. 10652–10653.
- [87] U. E. Steiner and T. Ulrich. "Magnetic field effects in chemical kinetics and related phenomena". In: *Chemical Reviews* 89.1 (1989), pp. 51–147.
- [88] B. Alberts et al. *Molecular Biology of the Cell. 4th edition*. Garland Science, 2002.
- [89] I.A. Chen and P. Walde. "From self-assembled vesicles to protocells". In: *Cold Spring Harb Perspect Biol.* 2.7 (2010).
- [90] S. Zhao et al. "Hydrocarbon chain packing in the micellar core of surfactants studied by ^1H NMR relaxation". In: *Colloid and Polymer Science* 276.12 (1998), pp. 1125–1130.
- [91] D. Fennell Evans and Håkan Wennerström. "The Colloidal Domain: Where Physics, Chemistry, Biology and Technology Meet". In: 1994.
- [92] B. Brocklehurst. "Magnetic fields and radical reactions: recent developments and their role in nature". In: *Chem. Soc. Rev.* 31 (5 2002), pp. 301–311.
- [93] L. Schaeffer. "Chapter 14 - The Role of Functional Groups in Drugs Receptor Interactions". In: *The Practice of Medicinal Chemistry (Fourth Edition)*. Ed. by Camille Georges Wermuth et al. Fourth Edition. San Diego: Academic Press, 2008, pp. 359–378.
- [94] T. P. Silverstein. "The Real Reason Why Oil and Water Don't Mix". In: *Journal of Chemical Education* 75.1 (1998), p. 116.
- [95] C. B. Grissom. "Magnetic Field Effects in Biology: A Survey of Possible Mechanisms with Emphasis on Radical-Pair Recombination". In: *Chemical Reviews* 95.1 (1995), pp. 3–24.
- [96] L. Maibaum, A. R. Dinner, and D. Chandler. "Micelle Formation and the Hydrophobic Effect". In: *J. Phys. Chem. B* 108.21 (2004), pp. 6778–6781.
- [97] M. Goetz et al. "Quenching Mechanisms and Diffusional Pathways in Micellar Systems Unravelling by Time-Resolved Magnetic-Field Effects". In: *Chemistry (Weinheim an der Bergstrasse, Germany)* 15 (May 2009), pp. 6058–64.
- [98] S. R. T. Neil et al. "Cavity enhanced detection methods for probing the dynamics of spin correlated radical pairs in solution". In: *Mol. Phys.* 108.7-9 (2010), pp. 993–1003.
- [99] J. C. Scaiano, M. Tanner, and D. Weir. "Exploratory study of the intermolecular reactivity of excited diphenylmethyl radicals". In: *Journal of the American Chemical Society* 107.15 (1985), pp. 4396–4403.

- [100] H. Kawasaki et al. "Vesicle-Micelle Transition and the Stability of the Vesicle Dispersion in Mixtures of Tetradecyldimethylamine Oxide Hemihydrochloride and Sodium Naphthalenesulfonate". In: *The Journal of Physical Chemistry B* 107.33 (2003), pp. 8661–8668.
- [101] A. Weller, F. Nolting, and H. Staerk. "A quantitative interpretation of the magnetic field effect on hyperfine-coupling-induced triplet formation from radical ion pairs". In: *Chem. Phys. Lett.* 96.1 (1983), pp. 24–27.
- [102] L. D. Tuck and D. W. Schieser. "Electron spin resonance of some nitrogen-containing aromatic free radicals". In: *The Journal of Physical Chemistry* 66.5 (1962), pp. 937–939.
- [103] M. Kaise and K. Someno. "Simultaneous Observation of Spin Polarized Anion and Cation Radicals in Solution by Flash Photolysis ESR". In: *Chemistry Letters* 16.7 (1987), pp. 1295–1298.
- [104] A. Matsuyama and H. Murai. "Effect of the spin dephasing on the photoconductivity-detected magnetic resonance spectra observed in the photolysis of xanthone and N, N-diethylaniline in 2-propanol". In: *The Journal of Physical Chemistry A* 106.10 (2002), pp. 2227–2231.
- [105] R. Oviedo-Roa et al. "Critical micelle concentration of an ammonium salt through DPD simulations using COSMO-RS-based interaction parameters". In: *AIChE Journal* 59.11 (2013), pp. 4413–4423.
- [106] R. Ranganathan, L. Tran, and B. L. Bales. "Surfactant- and Salt-Induced Growth of Normal Sodium Alkyl Sulfate Micelles Well above Their Critical Micelle Concentrations". In: *The Journal of Physical Chemistry B* 104.10 (2000), pp. 2260–2264.
- [107] A. Rogowska et al. "Theoretical investigation of the EPR hyperfine coupling constants in amino derivatives". In: *Phys. Chem. Chem. Phys.* 9 (7 2007), pp. 828–836.
- [108] D. Nohr et al. "Extended Electron-Transfer in Animal Cryptochromes Mediated by a Tetrad of Aromatic Amino Acids." In: *Biophysical journal* 111 2 (2016), pp. 301–311.
- [109] A. Berndt et al. "A Novel Photoreaction Mechanism for the Circadian Blue Light Photoreceptor Drosophila Cryptochrome". In: *Journal of Biological Chemistry* 282.17 (2007), pp. 13011–13021.
- [110] R. Banerjee et al. "The signaling state of Arabidopsis cryptochrome 2 contains flavin semiquinone". In: *The Journal of biological chemistry* 282 (June 2007), pp. 14916–22.
- [111] V. Herbel et al. "Lifetimes of Arabidopsis cryptochrome signaling states in vivo". In: *The Plant journal : for cell and molecular biology* 74 (Feb. 2013).
- [112] A. T. Vaidya et al. "Flavin reduction activates Drosophila cryptochrome". In: *Proceedings of the National Academy of Sciences* 110.51 (2013), pp. 20455–20460.
- [113] N. Ozturk et al. "Reaction mechanism of Drosophila cryptochrome". In: *Proceedings of the National Academy of Sciences* 108.2 (2011), pp. 516–521.

- [114] M. Kondoh et al. “Light-Induced Conformational Changes in Full-Length Arabidopsis thaliana Cryptochrome”. In: *Journal of molecular biology* 413 (Aug. 2011), pp. 128–37.
- [115] C. Lin et al. “Circadian clock activity of cryptochrome relies on tryptophan-mediated photoreduction”. In: *Proceedings of the National Academy of Sciences* 115.15 (2018), pp. 3822–3827.
- [116] Nuri Ozturk et al. “Mechanism of Photosignaling by Drosophila Cryptochrome: Role of the redox status of the flavin chromophore”. In: *Journal of Biological Chemistry* 289.8 (2014), pp. 4634–4642.
- [117] J. R. Engen. “Analysis of Protein Conformation and Dynamics by Hydrogen/Deuterium Exchange MS”. In: *Analytical Chemistry* 81 (2009), pp. 7870–7875.
- [118] Y. Liu et al. “Phosphorylation-Induced Activation of the Response Regulator VraR from Staphylococcus aureus: Insights from Hydrogen Exchange Mass Spectrometry”. In: *Journal of molecular biology* 391 (July 2009), pp. 149–63.
- [119] G. M. West et al. “Protein conformation ensembles monitored by HDX reveal a structural rationale for abscisic acid signaling protein affinities and activities.” In: *Structure* 21 2 (2013), pp. 229–35.
- [120] J. G. Mandell, A. M. Falick, and E. A. Komives. “Identification of protein-protein interfaces by decreased amide proton solvent accessibility”. In: *Proceedings of the National Academy of Sciences of the United States of America* 95.25 (1998), pp. 14705–14710.
- [121] H. Ehring. “Hydrogen exchange/electrospray ionization mass spectrometry studies of structural features of proteins and protein/protein interactions”. In: *Analytical biochemistry* 267.2 (1999), pp. 252–259.
- [122] G. Fedele et al. “Genetic Analysis of Circadian Responses to Low Frequency Electromagnetic Fields in Drosophila melanogaster”. In: *PLOS Genetics* 10.12 (Dec. 2014), pp. 1–12.
- [123] G. Fedele et al. “An electromagnetic field disrupts negative geotaxis in Drosophila via a CRY-dependent pathway”. In: *Nat Commun* 5 (2014), p. 4391.