Spin dynamics in biological radical reactions

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Valentina Lukinović
School of Chemistry
Outline

List of Figures .................................................................................................................. 5
List of Tables ..................................................................................................................... 10
List of Abbreviations ......................................................................................................... 11
Abstract ............................................................................................................................ 13
Declaration ........................................................................................................................ 14
Copyright Statement .......................................................................................................... 15
Acknowledgments .............................................................................................................. 16

Chapter 1
General introduction
1.1 Continuous-flow experiments ..................................................................................... 18
  1.1.1 Continuous-flow in EPR ..................................................................................... 18
  1.1.2 Continuous-flow in other techniques ................................................................. 19
1.2 Electron paramagnetic resonance (EPR) spectroscopy ................................................. 20
  1.2.1 Basics of continuous-wave EPR ..................................................................... 20
    1.2.1.2 Boltzmann distribution .......................................................................... 23
    1.2.1.3 Hyperfine interaction .......................................................................... 23
    1.2.1.4 Application of EPR ............................................................................ 24
  1.2.2 EPR spectrometer .............................................................................................. 25
    1.2.2.1 Resonators .......................................................................................... 25
  1.2.3 Time resolved EPR methods .............................................................................. 28
    1.2.3.1 Continuous-wave detection, direct detection EPR, DD-EPR ............... 28
    1.2.3.2 Pulsed microwave detection ................................................................. 30
1.3 Radical pair theory ..................................................................................................... 32
  1.3.1 Radicals and radical pairs ............................................................................... 32
  1.3.2 Chemically induced dynamic electron polarisation ............................................. 38
    1.3.2.1 Triplet mechanism .............................................................................. 38
    1.3.2.2 RPM from ST₀ mixing ...................................................................... 40
    1.3.2.3 RPM from ST₁ mixing ...................................................................... 41
    1.3.2.2.1 RPM from ST₀ mixing .................................................................. 41
    1.3.2.3 CIDEP from f-pairs ......................................................................... 44
    1.3.2.4 Net and multiplet polarisation ......................................................... 45
  1.3.2.4 RPs studied by EPR and application of CIDEP ........................................ 45
1.3.3 Magnetic field effects (MFE) ................................................................................. 47
  1.3.3.1 Biological significance of MFE and animal magnetoreception ............ 49
1.3.4 Chemically induced dynamic electron polarisation (CIDNP) ................................ 50
1.4 Project aims ............................................................................................................... 51
1.5 References ............................................................................................................... 53

Chapter 2
Materials and methods
2.1 Sample preparation ..................................................................................................... 56
  2.1.1 Photoreduction of duroquinone .................................................................... 56
  2.1.2 Preparation of cobalamins for photolysis followed by TR-EPR .................... 56
  2.1.3 Preparation of free flavin cofactors for UV-Vis and SF/UV-Vis spectroscopy .... 57
  2.1.4 Drosophila melanogaster cryptochrome (DmCry) growth and purification protocol ............................................................... 57
    2.1.4.1 Cell growth ..................................................................................... 57
    2.1.4.2 DmCry purification ......................................................................... 58
Chapter 3
Development of continuous-flow EPR
3.1 Continuous-flow EPR setup ................................................................. 67
  3.1.1 Pump ................................................................. 68
  3.1.2 Quartz ................................................................. 68
  3.1.3 Sample vessel ............................................................... 70
3.2 Test reaction ................................................................................. 71
  3.2.1 9 GHz EPR ........................................................................ 72
  3.2.2 34 GHz EPR ........................................................................ 73
3.3 Triggering setup ........................................................................... 74
  3.3.1 Pulse positions ........................................................................ 74
  3.3.2 Measurements of delay after flash (DAF) .................................. 76
    3.3.2.1 9 GHz EPR ....................................................................... 77
    3.3.2.2 34 GHz EPR ....................................................................... 78
3.4 Determination of signal phase ....................................................... 79
3.5 Conclusions ................................................................................... 80
3.6 References ..................................................................................... 82

Chapter 4
Time-resolved Fourier transform EPR study of the photo-reduction of
duroquinone by chloroquine in 2-propanol
4.1 Introduction .................................................................................... 83
4.2 Results ........................................................................................... 85
  4.2.1 Control experiments ................................................................. 86
  4.2.2 Reaction with the antimalarials in the system ......................... 88
    4.2.3 Concentration dependance experiments ............................... 92
4.3 Discussion ..................................................................................... 93
4.4 Conclusions ................................................................................... 94
4.4 References ..................................................................................... 95

Chapter 5
Photolysis of cobalamins followed by continuous-flow EPR
5.1 Introduction .................................................................................... 97
5.2 Results ........................................................................................... 104
  5.2.1 Simulations of FT-EPR spectra of methylcobalamin ............. 104
  5.2.2 Photolysis of base-on cobalamins ......................................... 106
    5.2.2.1 Photolysis of methylcobalamin experiments using 9 GHz FT-EPR .... 107
      5.2.2.1.1 Photolysis at 532 nm ................................................. 107
      5.2.2.1.2 Concentration dependence of 532 nm FT-EPR .............. 112
      5.2.2.1.3 Viscosity dependence at 532 nm .............................. 114
      5.2.2.1.4 Photolysis at 355 nm ............................................. 116
      5.2.2.1.5 G and f-pair summary .......................................... 118
    5.2.2.2 FT-EPR of methylcobalamin at 35 GHz ......................... 119
5.2.3 FT-EPR of 5'-deoxyadenosylcobalamin ........................................ 122
5.2.4 Photolysis of base-off cobalamins ............................................. 124
  5.2.4.1 Methylcobalamin ................................................................. 125
  5.2.4.2 5'-deoxyadenosylcobalamin ................................................. 130
  5.2.4.3 Summary for photolysis of base-off cobalamin ....................... 131
5.3 Discussion ................................................................. 132
  5.3.1 Photolysis of methylcobalamin ................................................ 132
    5.3.1.1 Photolysis of the base-on structure of MeCbl ...................... 132
    5.3.1.2 Comparison of base-on and base-off MeCbl results ............. 134
  5.3.1.3 35 GHz FT-EPR ............................................................... 137
    5.3.1.4 Choosing the appropriate combination of polarisation patterns 137
  5.3.2 Photolysis of 5'-deoxyadenosylcobalamin .................................. 138
5.4 Conclusions ........................................................................... 139
5.5 References ............................................................................. 141

Chapter 6
Studies on the reoxidation of reduced cryptochromes and potential magnetic field effects
6.1 Introduction ........................................................................... 144
6.2 Results .................................................................................... 150
  6.2.1 Reduction and oxidation reactions of free cofactors ................. 150
    6.2.1.1 Reduction of free flavins .................................................... 150
    6.2.1.2 Magnetic field effect studies of free FMN ......................... 153
  6.2.2 Reduction of Cry proteins ..................................................... 155
    6.2.2.1 Reduction of Drosophila melanogaster cryptochromes, DmCry 155
    6.2.2.2 Reduction of Arabidopsis thaliana cryptochromes, AtCry .......... 158
  6.2.3 Potentiometry experiments .................................................... 159
  6.2.4 Oxidation of Cry proteins .................................................... 162
  6.2.5 Magnetic field effect studies ................................................ 165
    6.2.5.1 Magnetic field effects on oxidation of AtCry ...................... 165
    6.2.5.2 Magnetic field effects on oxidation of DmCry .................... 167
      6.2.5.2.1 Oxidation of DmCry ..................................................... 167
      6.2.5.2.2 Oxidation of DmCry with intervals of presence and absence of 171
        MF during acquisition ................................................................ 171
    6.2.5.3 The oxidation of DmCry studied under continuous illumination 174
    6.2.5.4 Control experiments .......................................................... 176
  6.3 Discussion and conclusions ..................................................... 178
  6.4 References ............................................................................. 183

Chapter 7
Summary of conclusions and future work
7.1 Photoreduction of duroquinone by chloroquine ............................... 186
7.2 Photolysis of cobalamins ......................................................... 187
7.3 Reoxidation of reduced cryptochromes and MFEs ................................ 190
7.4 References ............................................................................. 192

Appendix
A – Construction of FT-EPR spectra
  A.1 Data processing for FT-EPR ...................................................... 193
  A.2 Code for plotting from data processed with Xepr ........................ 194
A.3 Code for simulation ................................................................. 195

B - Cobalamins

B.1 Simulation of Me radical without contribution of f-pairs .................. 198
B.2 Calculation for diffusion ............................................................. 199
B.3 Linear prediction ..................................................................... 200
B.4 Direct detection EPR of photolysis of MeCbl ................................ 206
B.5 FT-EPR of MeCbl at low concentration ......................................... 206

References ...................................................................................... 207

Word count: 47,175
List of figures

- **Chapter 1**

  **Figure 1.1**  A) Zeeman energy splitting diagram; right panel presents absorption and derivative curves as example of EPR signal; B) The most common frequency bands in EPR spectroscopy with the corresponding magnetic field strengths and microwave frequencies.

  **Figure 1.2.**  Position of the sample in the cavity.

  **Figure 1.3.**  A: ‘Dip’ due to cavity absorption and calculation of Q factor. B: over-coupled and critically coupled resonators dips.

  **Figure 1.4.**  Magnetization around the z axis rotates into the xy plane after application of a π/2 microwave pulse.

  **Figure 1.5.**  Continuous-wave direct detection mode.

  **Figure 1.6.**  Fourier transform EPR.

  **Figure 1.7.**  A) Lineshape of the radical decay with regards to the position of laser and microwave pulse. B) Characteristics of the π/2 microwave pulse, which turns the magnetization into the xy plane and creates transverse magnetization.

  **Figure 1.8.**  Different reaction routes that could results with generation of radicals.

  **Figure 1.9.**  Scheme of possible outcomes of RPs, including the possibility of separation by diffusion, escaping from the radical cage and later recombination.

  **Figure 1.10.**  Formation of different energy states following absorption of a photon.

  **Figure 1.11.**  The lifetime of triplet-born RP after photoexcitation.

  **Figure 1.12.**  Diagram summarizing the different ways of generating spin polarized spectra.

  **Figure 1.13.**  Development of spin polarisation by the triplet mechanism (TM).

  **Figure 1.14.**  Energies of the singlet and triplet energy levels as a function of inter-radical distance.

  **Figure 1.15.**  Vector model of ST₀ mixing in time. B₀ and S₁ and S₂ represent the magnetic field and two spin states, respectively.

  **Figure 1.16.**  RP spin states in MF. A) Vector model of RP spin states in a MF, with arrows indicating spin orientation regards to the magnetic field.

  **Figure 1.17.**  The effect on a reaction yield of singlet (red) and triplet (blue) born RPs depending on the magnetic field strength.

- **Chapter 2**

  **Figure 2.1.**  UV-Vis spectrum of isolated DmCry.

  **Figure 2.2.**  Fractions of purification resolved by SDS-PAGE.

  **Figure 2.3.**  Fractions of protein peak resolved by SDS-PAGE.

  **Figure 2.4.**  Schematic of a standard SF/UV-Vis spectrophotometer.
Chapter 3

Figure 3.1. Quartz tubing used for different experimental setup presented in the Table 3.1.

Figure 3.2. 1) Flow cell inserted in the dielectric ring resonator for low temperature continuous-flow experiments, 2) Quartz tubing connected to the silicone tubing, 3) Flow cell from 2 inserted into the dielectric resonator for RT measurement, in situ, 4) Three neck round bottom flask used for the experiment, purged with Ar, 5) 34 GHz resonator

Figure 3.3. A) Power dependence of duroquinone radical anion in FID detection. B) FT-EPR spectrum of duroquinone radical anion.

Figure 3.4. A) Power dependence of the radical. B) FTEPR spectra of duroquinone radical anion dependent on the position of the microwave pulse.

Figure 3.5 Positions of the pulses in the experimental setup.

Figure 3.6. A) Oscilloscope traces showing the difference between positions of the laser flash and microwave pulse in the Signal Channel. B) The difference spectrum between the transmitter, receiver monitor and Signal Channel.

Figure 3.7 A) Time domain spectrum of the -31 MHz peak with varying DAF. B) FT amplitude of the highest intensity peak (-31 MHz peak) dependent on the time difference between the laser flash and the microwave pulse in the Signal Channel, after the quadrature detection.

Figure 3.8. FT amplitude of the highest intensity peak (-31 MHz peak) on variation of the DAF.

Figure 3.9. Time domain spectra of four different field positions of the propan-2-oyl radical.

Figure 3.10. FT-EPR spectrum of BP-ketyl radical (central line) and propan-2-oyl radical (outer lines) built, after obtaining a FID at each field position.

Figure 3.11. Continuous-flow EPR setup developed for time-resolved EPR studies.

Chapter 4

Figure 4.1. 1) Duroquinone 2) 4,7-dichloroquinoline 3) Novol diamine (2-amino-5-diethylamino pentane) 4) Triethylamine 5) Chloroquine 6) PGEAM#97 7) Bis-dealkylated trifluralin 8) Trifluralin 9) Deaminated chloro trifluralin.

Figure 4.2. Durosemiquinone radical generated after photoexcitation of duroquinone in 2-propanol using 355 nm laser light.

Figure 4.3. Structures of the durosemiquinone radical (DQH•) and the duroquinone radical anion (DQ•−) with their respective hyperfine values.

Figure 4.4. Duroquinone radical anion generated after photoexcitation of DQ with TEA present in the solution using 355 nm laser light.

Figure 4.5. FT-EPR spectra of durosemiquinone radical generated after photoexcitation of DQ with 3 different compounds.

Figure 4.6. Staggered FT-EPR spectra of duroquinone radical anion at delay times ranging from 0.08 to 80.08 μs in 2-propanol.
Figure 4.7. Staggered FT-EPR spectra of duroquinone radical anion at delay times ranging from 0.08 to 80.08 μs in 2-propanol.

Figure 4.8. FT-EPR spectra generated after photoexcitation of DQ with 4,7-dichloroquine or PGEAM#97 in the solution.

Figure 4.9. A) FT-EPR spectra of radicals observed at different concentrations of DQ. B) Concentration dependence of the FID amplitude.

Chapter 5

Figure 5.1. Structures of B_{12} derivatives with various upper and lower axial ligands.

Figure 5.2. Scheme presenting the differences in photophysics of MeCbl and AdoCbl considering the excitation wavelength.

Figure 5.3. Theoretical spectra for different spin polarization mechanisms of the methyl radical.

Figure 5.4. Reaction scheme with suggested reaction mechanism of photolysis of MeCbl.

Figure 5.5. A) Fourier transform EPR spectra of the methyl radical after photoexcitation at 532 nm, at five different DAF. B) Average peak intensity values for two concentrations of methyl radical and three delay times.

Figure 5.6. Simulation of FT-EPR spectra using combinations of TM / ST_{0,T-born} / f-pairs (A) and TM / ST_{0.5-born} / f-pairs (B).

Figure 5.7. Simulation of methyl radical generated after photolysis of MeCbl at 532 nm.

Figure 5.8. (A) Polarization by f-pairs examined by measuring at three different concentrations of MeCbl at five DAF. (B) Fit of free induction decay at one of the field positions.

Figure 5.9. (A) Experimental data of photolysis of MeCbl in 50 % glycerol; (B) Peak intensities of Me radical in 50 % glycerol solution with standard deviation as an error; (C) Simulated data with combination of g-pairs and f-pairs, as in Figure 5.7; (D) Final f-pair contribution from the simulated spectra for four [MeCbl]. Experimental parameters: buffer 20 mM HEPES, pH 7.5, 50% glycerol; 532 nm, 20 mJ; 16 ns microwave pulse, 8 dB microwave attenuation.

Figure 5.10. FT-EPR spectra of methyl radicals generated using 355 nm excitation.

Figure 5.11. Contribution of f-pair polarisation.

Figure 5.12. FT-EPR experimental and simulated spectra of methyl radical followed at 35 GHz, after photolysis of MeCbl in viscous (A) and non-viscous (B) solution.

Figure 5.13. FT-EPR experimental and simulated spectra of methyl radical followed at 35 GHz, after photolysis of MeCbl in non-viscous solution.

Figure 5.14. Comparison of FT-EPR spectra of Ado⁺ radical generated by photoexcitation at 532 (A) and 355 nm (B).

Figure 5.15. Schematic presentation of the mechanism of photolysis of cobalamin in the base-off form.

Figure 5.16. FT-EPR of Me radical generated after photoexcitation of base-off form of MeCbl.

Figure 5.17. Experimental data of methyl radical after photolysis of base-off MeCbl.
Figure 5.18. Experimental data of methyl radical after photolysis of base-off MeCbl.  
Figure 5.19. Comparison of FT-EPR spectra of Ado• radical generated from photolysis of the base-off form AdoCbl.  
Figure 5.20. Final polarization mechanism.  
Figure 5.21. Comparison of FT-EPR spectra of methyl radical after photolysis of base-on and base-off MeCbl.

Chapter 6

Figure 6.1. Three loops characteristic for type I Crys.  
Figure 6.2. Light-induced photocycle in Cry and redox states of the FAD cofactor upon photoactivation.  
Figure 6.3. Photoreduction of Cry.  
Figure 6.4. Structures of the flavin cofactors studied in this chapter.  
Figure 6.5. UV-Vis spectra of chemically reduced and photoreduced FAD.  
Figure 6.6. Reoxidation of FAD.  
Figure 6.7. Stopped-flow transients recorded at 450 nm upon mixing 5 μM FMN with 100 μM O₂ in the presence and absence of a 100 mT MF with the corresponding difference trace after subtracting the MF off data from the MF on data.  
Figure 6.8. Bar chart diagrams with observed rate constant measured from the SF for 5 μM (A) and 10 μM (B) FMN.  
Figure 6.9. UV-Vis spectra of reduction of DmCry.  
Figure 6.10. UV-Vis spectrum of reduction of DmCry using sodium dithionite, DT as reductant.  
Figure 6.11. UV-Vis spectrum of reduction of 12 μM DmCry using 455 nm LED.  
Figure 6.12. UV-Vis spectra of reduction of AtCry with or without addition of DTT at two pH values (A and C). UV-Vis scanning kinetics spectra of reaction of reoxidation of 38 μM AtCry (B and D).  
Figure 6.13. UV-Vis spectrum of DmCry (A) and AtCry (B) obtained during potentiometry experiment, at different potentials.  
Figure 6.14. Change in absorption at 454 nm observed during reductive titration.  
Figure 6.15. Reduction and reoxidation reactions of protein cofactor.  
Figure 6.16. UV-Vis spectrum obtained with PDA experiment in UV-Vis stopped flow experiment.  
Figure 6.17. EAS calculated by global analysis of the experimental data from oxidation of AtCry.  
Figure 6.18. Fit (dash) of the experimental data (solid) for oxidation of AtCry.  
Figure 6.19. Oxidation of AtCry.  
Figure 6.20. Bar chart diagrams with observed rate constant measured from the SF for AtCry.  
Figure 6.21. Averaged kinetic traces followed at 580 nm in oxidation reaction of AtCry.  
Figure 6.22. Oxidation of DmCry semiquinone.
Figure 6.23. Fitting of the on and off kinetic traces with bar chart diagrams. 169
Figure 6.24. Oxidation of DmCry. 169
Figure 6.25. Oxidation and MF studied of fully reduced DmCry. 171
Figure 6.26. Each kinetic trace obtained for acquisition start for MF off and on. 173
Figure 6.27. Each kinetic trace obtained for acquisition start for MF off and on. 173
Figure 6.28. PDA experiment of photoreduction of DmCry. 174
Figure 6.29. Single wavelength experiment followed at 450 nm. 175
Figure 6.30. Single wavelength experiment followed at 450 nm with intervals measurement. 175
Figure 6.31. Control experiments with intervals for DmCry. 176
Figure 6.32. Each kinetic trace obtained for acquisition start for MF off and on. 177
Figure 6.33. Test with of intervals measurements with MF. 178

List of tables

Table 1.1. Pascal triangle. 24
Table 1.2 Summary of ST\(0/\pm1\) RP mechanism effect on EPR spectra. 46
Table 3.1 Dimensions of the quartz tubing used for different EPR experiments with accordingly calculated flow rates. 69
Table 6.1 Summary of the reaction rates obtained after fitting the data for three chemically reduced or photoreduced cofactors. 153
List of abbreviations

EPR  electron paramagnetic resonance
FT-EPR  Fourier transform EPR
DD-EPR  direct detection EPR
M  magnetization
CW mode  continuous-wave mode
TREPR  time-resolved EPR
CIDEP  chemically induced dynamic electron polarisation
DAF  Delay after flash
CIDNP  chemically induced dynamic nuclear polarisation
MFE  magnetic field effect
s  spin quantum number
RP  radical pair
g-pairs  geminate pairs
f-pairs  freely-diffused radical pairs
T  triplet
S  singlet
J  exchange interaction
k_{ISC}  intersystem crossing, with rate
TM  triplet mechanism
T_{1}  triplet spin-lattice relaxation time
RPM  radical pair mechanism
ω  precession frequencies
NMR  nuclear magnetic resonance
A  absorption
E  emission
TEA  triethylamine
MeCbl  methylcobalamin
AdoCbl  5’-deoxyadenosylcobalamin
Ado^*  5’-deoxyadenosyl radical
HEPES  4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
ε  extinction coefficient
FAD  flavin adenine nucleotide
FMN  flavin mononucleotide
DmCry  Drosophila melanogaster cryptochrome
rpm  revolutions per minute
Tris  trisaminomethane
DT  sodium dithionite
LED  light-emitting diode
k_{OBS}  apparent rate constants
HNQ  2-hydroxy-1,4-naphthoquinone
PMS  phenazine methosulfate
FID  free induction decay
TM  transmitter monitor
RM  receiver monitor
TWT  Travelling Wave Tube
DQ  duroquinone
DQH^*  durosemiquinone radical
DQ^*  duroquinone radical anion
S/N  signal-to-noise
CQ
chloroquine
I
nuclear spin
Abstract
Spin dynamics in biological radical reactions

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Science and Engineering by Valentina Lukinović, 2018.

Biological reactions often involve formation of short-lived intermediates, from which some of them are in form of a radical pair (RP). These intermediates are often difficult to detect, but by observing their spin dynamics, the influence of the environment on reaction mechanism can be elucidated. The aim of this study was to develop the system that will enable following formation and dynamics of these short-lived species. The continuous-flow setup was developed for 9 GHz and 35 GHz EPR frequencies, which enabled observation of short-lived radicals at nanosecond resolution, through an optimised triggering sequence which provided measurement at the shortest possible delay after flash (DAF).

The developed system was exploited to study the yet unknown reaction mechanism of antimalarial drugs and, in greater detail, photochemistry of cofactors of B12 enzymes, reactions which proceed through formation of a RP. The first investigated system involved a question about the phototoxicity of antimalarial drugs and potential electron transfer reactions. From our approach, with using Fourier transform EPR (FT-EPR) to test different drug analogues, it is suggested that the amine functionality is the source of electron transfer in these reactions. The second studied biological system involved investigating spin dynamics of B12 derivatives, using the bespoke continuous-flow system. Methylcobalamin (MeCbl) and 5′-deoxyadenosylcobalamin (AdoCbl) serve as cofactors of enzymes involved in metabolism of vitamin B12, through their reactivity in organometallic reactions. The Co-C bond has a crucial role in these enzymatic reactions. Flash photolysis FT-EPR was used to determine differences between electronic structure of two cofactors and origin of the precursor molecules, since both cofactors produce analogous RPs following photoexcitation. From simulation of the experimental data it was observed that whereas the spin polarisation following the photolysis of AdoCbl suggests signal from predominantly S-born, geminate pairs, MeCbl geminate RPs are born in both S and T spin-states and polarisation from T-born f-pairs also contributes to the signal. Further significant differences in reaction mechanism were identified varying the lower and upper axial ligand, the positions important to B12-dependent enzyme reactions.

Finally, the spin chemistry of the reoxidation of insect and plant cryptochromes (Crys) proteins was studied through investigation of magnetic field effects (MFEs). The proteins have been previously suggested as being involved in magnetoreception. Recent behavioural studies and theoretical calculations have indicated involvement of a RP, potentially formed in the reoxidation step. However, using the MFE stopped-flow device in our study showed no evidence for MFEs on this reaction step and the work will guide future studies to fully understand the reoxidation step.
Declaration

Title: Spin dynamics in biological radical reactions

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Chapter 1

General introduction

This chapter will describe three topics relevant to the work involved in the experimental studies completed and described throughout the thesis. First, previously reported experiments on continuous-flow electron paramagnetic resonance (EPR) spectroscopy will be presented, together with a literature review on continuous-flow used in other techniques. The theory of EPR technique will then be described with a focus on the main principles of time-resolved EPR techniques. Finally, radical pair (RP) theory will be described, together with spin polarisation in EPR spectra, magnetic fields effects, and spin polarisation in NMR.

1.1 Continuous-flow experiments

EPR spectroscopy can be applied to the study of samples containing unpaired electron(s). This includes a wide range of samples, from basic organic and inorganic compounds, to complex biomolecules such as proteins. EPR spectra provide information about the environment of the unpaired electron, as well as its electronic and geometric structure, physical properties, and reactivity and spin dynamics. The advantage of EPR is that it is highly sensitive and has very good specificity when looking only at the region which contains the unpaired electron. These characteristics make the EPR technique useful for the study of proteins involved in important enzymatic reactions such as metallic–cofactor containing enzymes through observing unpaired electron species created in these reactions.

1.1.1 Continuous-flow in EPR

To observe radicals under continuous-flow conditions, Hubbell et al. used a stopped-flow device in combination with a syringe pump and a loop-gap resonator. \[1\] Reactions that were studied include ascorbic acid and Ce(IV) sulfate in acid solution, as well as the reaction between TEMPO and ascorbic acid under basic conditions. For continuous-flow, an important aspect was the experimental “dead-
time”, which is the amount of early time where it is not possible to make a measurement. The ageing times included both the mixing time and dead-time. The authors found that the distribution of ages in the observed volume was an important consideration.

A syringe pump was also used for studying free radicals generated in compressed and supercritical fluids by EPR spectroscopy. [2] The quartz tubes in the flow EPR experiment were positioned in the active area of the cylindrical cavity, with electrically insulated end plates. The system was also used to study photoinduced bond homolysis of the B_{12} coenzyme. [3] Another example of a continuous-flow EPR experiment was the characterisation of the transient histidine radicals, formed in aqueous solutions, in combination with a dielectric resonator equipped with a mixing chamber. [4] The experimental setup for continuous-flow was designed with a syringe pump that ensured a low consumption of reactants. A further example of an in-situ reaction was the photolysis reaction of cytidine and K_{2}S_{2}O_{8}, where the radical lifetime of at least several milliseconds was required. [5] Two studies have been provided by Woodward [6] and Lu [7] on photoactivated reactions utilising time-resolved EPR with continuous-flow when looking into the reaction of H_{2}O_{2} with DMSO or electron transfer or hydrogen transfer to duroquinone.

1.1.2 Continuous-flow in other techniques

Syringe pumps have been used in EPR spectroscopy to obtain continuous-flow, but they also have various applications such as in the continuous delivery of mixture into the instrument for polymerase chain reaction, PCR [8], as well as for flow of solvent in fast atom bombardment mass spectrometry. [9] Amol et al. used a syringe pump, which provided precision fluid feed in small-scale experiments, to deliver supercritical CO_{2}, because formation of nanoparticles of a hydrophilic drug can be understood using enhanced molecular mixing. [9] The pump was specialized for chromatography and other analytical applications requiring extremely low flow noise and ml/min range flows. Motoyama et al. used a high pressure syringe pump
for the proteomic separation of the yeast proteome, to obtain the constant flow mode, which improved separation and increased protein coverage. [10]

As mentioned above, there are two more types of pumps besides syringe pump, which can be integrated into an HPLC system - a constant pressure and a piston pump. At constant pressure, a pneumatic pump has been used for HPLC applications, with high and low pressure tests, as a drift-free, stable flow and low noise pump, where the flow rate in real time was measured with a flow meter and feedback system, enabling flow rate adjustment by varying the temperature of a fine capillary. [11] The second type of HPLC piston pump, can have a single or dual piston configuration. The piston was designed to move backwards and sucks the eluent and once the path for the eluent is opened, liquid passes through it whilst the inlet valve is closed. Dual piston pump enables more pulse-free flow, since the one chamber is filled, while the other pushes liquid out from the other chamber.

1.2 Electron paramagnetic resonance (EPR) spectroscopy

1.2.1 Basics of continuous-wave EPR

Spectroscopy is the branch of science which studies structure of atoms and molecules and its interaction with light or electromagnetic radiation. Different structural and kinetic information can be obtained by spectroscopic studies, depending on the region of studied electromagnetic irradiation. Two basic equations in spectroscopy are:

\[ \nu = \frac{c}{\lambda} \]  
\[ \Delta E = h\nu \]

where \( \nu \) is frequency, \( \lambda \) is wavelength and \( c \) is velocity of light \( (3 \times 10^{-10} \text{ cm s}^{-1}) \), \( E \) is energy and \( h \) is Planck’s constant \( (6.6 \times 10^{-34} \text{ J s}) \). [12] A typical approach in spectroscopy is to tune the frequency over a range of the electromagnetic spectrum. However, different spectroscopic techniques have different approaches. Most commercial EPR spectrometers keep the frequency constant but sweep the magnetic field (MF). Figure 1.1 presents the concept of EPR, presenting splitting in
the energy level diagram that at the resonance frequency results with absorptive and/or 1st derivative spectra. On the bottom panel are presented most used EPR frequencies with according MF strengths.

**Figure 1.1.** A) Zeeman energy splitting diagram; right panel presents absorption and derivative curves as example of EPR signal; B) The most common frequency bands in EPR spectroscopy with the corresponding MF strengths and microwave frequencies.

EPR is a spectroscopic technique that takes into account electron orbitals and spin angular momentum. [13] The magnetic moment of an electron spin placed inside a MF precesses around the applied field, with the frequency known as the Larmor frequency; [14]

$$\omega_0 = -\gamma_e B_0$$  \hspace{1cm}  [Equation 1.3]

where $\gamma_e$ is the gyromagnetic ratio.

Spin is a characteristic of an electron described by its magnitude and direction in space. Two electron spins can be oriented in different directions but with the same magnitude (see Figure 1.1, blue arrows). As long as the space isotropy conditions hold, two states have the same energy - they are degenerate (Figure 1.1, red, low field area). Once the MF is applied, electron spins states separate (Figure 1.1, grey, high field area) in energy. [15] The splitting between two electron spins, due to the MF, is called the Zeeman effect and the interaction of the electron magnetic moment with the external applied MF is called electron Zeeman
interaction. [15] The spin states are divided into low and high energy levels, depending on their orientation to the external MF. The lower energy state is aligned with the external MF and high energy state against it. [16] Transition between two energy states follows once the energy of electromagnetic irradiation is equal to the separation between the spin energy levels (Figure 1.1, red arrow). The microwave irradiation is applied perpendicular to the MF. The transition between these states follows the selection rules, $\Delta m_s = \pm 1$ and $\Delta m_I = 0$. [12] The energy difference is measured based on the relation of $\Delta E$ and the absorption of electromagnetic radiation, as described by the Equation 1.4. However, the fundamental equation of EPR spectroscopy is:

$$\Delta E = h\nu = g\beta_e B_0$$  \hspace{1cm} \text{[Equation 1.4]}$$

where "$g$" is a $g$ value, $\beta_e$ is a natural unit of electronic magnetic moment, named Bohr magneton and $B_0$ stands for MF. From this it is clear that the difference in energy level is dependent on the applied external MF.

The $g$ value is proportionality constant and measures the difference of $m_s = \pm 1/2$ levels in a MF and is characteristic of the chemical species. From Equation 1.4, the equation for calculation of $g$ value can be extrapolated.

$$g = \frac{h\nu}{\beta B} = 0.714484 \times \frac{\nu [MHz]}{B [G]}$$  \hspace{1cm} \text{[Equation 1.5]}$$

The calculated $g$ value for free electron is $2.0032$. The $g$ value can be used to distinguish spins present in the sample and is independent on the frequency. [17] It is approximately $2$ for most organic samples. [12]

The EPR spectrometer sweeps the MF at a constant microwave frequency. In the continuous-wave (CW) mode, sinusoidal modulation of the applied MF, ($B_0$) is used in order to reduce the frequency range of the noise, to get a sufficient signal-to-noise ratio. [14, 18] The result of the field modulation is a first derivative signal since it shows the difference in absorbance between the ends of the modulation amplitude (Figure 1.1).
The most common working frequencies in EPR spectroscopy are presented in the bottom part of Figure 1.1. Working on different frequencies is particularly useful for systems where more than two levels can occur.

### 1.2.1.2 Boltzmann distribution

Absorption detectable in EPR spectra can occur only if there is a greater population in the lower energy level than in the higher. The spin population split in two Zeeman levels is presented in the Figure 1.1. Since the parallel state has lower energy, more electron spins can populate this state according to Boltzmann distribution. At thermal equilibrium and under an external MF these energy levels are populated according to the Boltzmann-Maxwell distribution, which gives relative populations of $|\alpha>m_s = +1/2$ or $|\beta> m_s = -1/2$ states. Equation 1.6 presents the ratio between population levels at equilibrium;

$$\frac{N_{upper}}{N_{lower}} = \frac{-\Delta E}{e^{k_B T}} = e^{\frac{g\mu_B}{k_B T}}$$  \hspace{1cm} [Equation 1.6]

where $N_{upper}$ or $N_{lower}$ present the population of $\beta$ or $\alpha$ states, and $k_B$ presents the Boltzmann constant. At room temperature and moderate MFs, this exponent is very small and estimated to be $e^{-x} \sim 1-x$. Therefore, the equation can be expanded to

$$\frac{N_{upper}}{N_{lower}} = 1 - \frac{g\mu_B}{k_B T}$$  \hspace{1cm} [Equation 1.7]

From the Equations 1.6 and 1.7, it is clear that difference in the population level will depend on the external MF strength, which is proportional to the frequency and the temperature.

### 1.2.1.3 Hyperfine interaction

Another magnetic parameter which can be observed in EPR spectroscopy is the interaction between electrons and nuclei, called the electron-nuclear hyperfine interaction. [17, 18] Nuclei often have a magnetic moment and produce their own local MF and, therefore, influence the nearby electron. [17] Depending on the orientation of this local field, it can add or oppose to the laboratory MF originating from the instrument. The hyperfine interaction present on EPR spectra can be
observed from the appearance of an equal separation of resonance lines called, “splitting”, which is frequency independent. One example is the electron spin interacting with the H atom, which has a nuclear spin of $\frac{1}{2}$. The EPR spectrum of this species is separated by $B_I$ (magnetic moment of the nucleus). If this magnetic moment adds to the resonance field, less of the external field is needed and the resonance field is lowered. The opposite is true when $B_I$ subtracts from the field. This causes separation in the EPR spectra, which is called the hyperfine coupling constant. [17]

The hyperfine interaction is on the order of 1 to 40 Gauss for organic radicals. [18] The number of hyperfine lines, however, is found by the $2n I + 1$ rule, where $n$ presents the number of nuclei with a nuclear spin, $I$. [12] This is useful for determining the radical species observed in EPR. [17] Therefore, the number of lines observed in EPR spectra and their relative intensities can depend on the unpaired electron interacting with multiple equivalent nuclei. Table 1.1 shows the predicted relative intensities at thermal equilibrium for $I = 1/2$. This obeys Pascal’s triangle. It is formed by adding $2I + 1$ numbers to get the number in the next row.

**Table 1.1.** Pascal triangle. Number of lines is dependent on $2n I + 1$.

<table>
<thead>
<tr>
<th>Number of equivalent nuclei, $n$</th>
<th>Relative intensities</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:1</td>
</tr>
<tr>
<td>2</td>
<td>1:2:1</td>
</tr>
<tr>
<td>3</td>
<td>1:3:3:1</td>
</tr>
<tr>
<td>4</td>
<td>1:4:6:4:1</td>
</tr>
<tr>
<td>5</td>
<td>1:5:10:10:5:1</td>
</tr>
</tbody>
</table>

Relative integrated intensities of a sample are dependent on the concentration of excited spins. However, another important parameter is the applied microwave power, where it is important to work under non-saturating conditions where signal amplitude has a linear relationship with the square root of applied power. [17]

1.2.1.4 Application of EPR

From what has been described above, it is clear that the EPR is a technique used for measuring samples with paramagnetic centres, possessing a spin angular momentum. [13] These systems are often in the ground state. However, excited
states can be measured as well if the sample is irradiated. Weil and Bolton [13] described systems that can be measured using EPR: free radicals in different phases, transition ions, point defects, species with conducting electrons or with more than one unpaired electron. Species involving more than one unpaired electron can be in a form of a triplet state, as biradicals or multiradicals. [13]

Examples of reactions forming paramagnetic species, besides reactions of reduction and oxidation or radiolysis, are hydrogen abstraction or addition and photolysis. An example of hydrogen abstraction will be presented later in the experiments using duroquinone as an electron acceptor (Chapters 3 and 4). Photolysis studies are used in the system where photoexcitation generates radical intermediates. If the lifetime of intermediates is long enough, a steady-state approach can be used to describe the kinetics. However, for short-lived intermediates flash-photolysis studies and advanced EPR techniques have to be employed. An example of this study is presented in the Chapter 5 with the photolysis of alkyl cobalamins.

1.2.2 EPR spectrometer

When designing an EPR experiment, sample conditions have to be taken into account. Factors that should be considered are the quantity of the sample and its environmental conditions, as well as the phase of the sample and its spin concentration. The components that are common to most EPR spectrometers are a microwave source, a magnet, a resonator and a detection system. [17] In designing an experiment with continuous-flow with time resolved EPR, the resonator has an important role and is described in more detail below.

1.2.2.1 Resonators

Resonators are parts of the EPR spectrometer that increase the sensitivity, so that it is possible to observe weak EPR signals by concentrating the microwave power at the sample and storing the microwave energy. [17-19] They are used to excite the electron spins of the sample with the microwave field \( B_{1} \), which is oriented perpendicular to the static MF (Figure 1.2). The sample is generally placed in a region with the minimum electric field and the maximum microwave field,
therefore enhancing the EPR signal. Those factors make the sample size and its placement in the cavity important.

**Figure 1.2.** Position of the sample in the cavity. The sample should be positioned in the area of max $B_1$ and minimum electric field. *Taken from [20].*

The iris is an adjustable device on a resonator used for matching impedance of the cavity (resonator) to the waveguide. The cavity is critically coupled when the impedance of iris and cavity combination is identical to the impedance of waveguide at the resonant frequency. In order to observe the resonance frequency, *Tune* mode of the spectrometer is followed. In that mode, the microwave frequency is swept and the reflected microwave power is measured as signal. The EPR signal is the voltage generated by magnetization from the sample since the precessing spins in the sample produce a voltage in the resonator, under the applied MF. [14, 19]

One important consideration is the reflection of microwave power to the detector. The Q-factor is the quality factor of the resonator that expresses the efficiency of storage of the microwave energy at the sample, described by Figure 1.3 and Equations 1.8.

$$Q = \frac{2\pi \text{energy stored}}{\text{energy dissipated per cycle}}$$  \[Equation 1.8a\]

$$Q = \frac{v_{res}}{\Delta v}$$  \[Equation 1.8b\]

Dissipated energy is energy lost per cycle because microwaves can generate electric current, which in turn can generate heat. Heat can also be generated in the experimental setup with lasers used for photoexcitation of the sample, and can
therefore cause changes in the Q-factor. If there is change in the Q value, then there is change in the reflected power from the resonator to the detector. [19] In addition to the resonator’s Q, EPR signal intensity is proportional to the filling factor, which is a fraction of the microwave power effective in EPR transitions. [21]

![Figure 1.3](image)

**Figure 1.3.** A) ‘Dip’ due to cavity absorption and calculation of Q-factor. B) Overcoupled and critically coupled resonators dips.

Depending on the EPR method, the Q-factor may vary, as shown in Figure 1.3. In a pulsed EPR experiment, the resonator should be overcoupled, in order to decrease the Q value and minimize the spectrometer dead-time. However, in a CW experiment, the resonator should be critically coupled so that there is a minimum of microwave power reflected back from the resonator. Further, in a CW EPR experiment, the signal is proportional to the resonator Q, whereas in a pulsed EPR experiment, the signal is proportional to the $\sqrt{Q}$.

Based on their Q-factors, resonators are divided into three types: dielectric ring, cavity (rectangular, cylindrical) and loop-gap resonators (split-ring). [22] A loop-gap resonator has a high filling factor and a low Q value. It is much smaller in size comparing to standard cavities and it is less sensitive to dielectric losses. Splitting and dielectric ring resonators are characterized by their high filling factor, resulting in maximum sensitivity. For Bruker 9 GHz FT-EPR resonators (MD5 and MS5/3), the high filling factors are complemented by low dead-time and variable Q-factors (Figure 1.3). A resonator’s Q-factor can be changed continuously from very high to very low (Figure 1.3), which allows matching of the resonator bandwidth to a specific experimental task, i.e. the lowest Q for the shortest dead-time and largest
bandwidth. This versatility is further enhanced by the (optional) optical window, which allows irradiation of the total active resonator volume. The response times of the split-ring and dielectric resonators are as follows [23]:

- A split ring resonator - 20 ns;
- A dielectric ring resonator - 100 – 200 ns.

1.2.3 Time resolved EPR methods

There are several EPR methods but the focus here will be on the time-resolved EPR techniques. So far, the concepts described above involved the theory of CW EPR, where radical species are measured on the ms timescale. However, the short lifetime of paramagnetic intermediates requires the application of advanced EPR techniques. Some intermediates generated in photochemical reactions can be detected by special methods, such as continuous-wave direct detection (DD) and pulsed microwave detection (Fourier transform EPR, FT-EPR or Electron-spin echo, ESE). The main objective of the time-resolved EPR (TREPR) technique is to monitor the magnetization along the field direction (z magnetization) associated with the formation and decay of free radicals (Figure 1.4). Magnetization (M) appears as a result of the interaction of the electron spins with the lattice and with each other. It can be described as the sum of magnetic moments of individual spins. [15]

![Figure 1.4](image)

Figure 1.4. Magnetization around the z axis rotates into the xy plane after application of a π/2 microwave pulse. Magnetization along the xy plane is named transverse magnetization, since it is in the plane perpendicular to the MF and is measured with pulsed TREPR.

1.2.3.1 Continuous-wave detection, direct detection EPR, DD-EPR

In this technique, microwaves are applied in the same way as in the standard CW mode, but the signal is measured with respect to time (nanosecond resolution). However, the field modulation is not used in this experiment, since it limits detection. Spectra are taken directly from a preamplifier, and a series of spectra at
different delay times are collected and reconstructed at different MFs. [24] This results with the signal in absorptive and not derivative mode. The experimental setup is based on time detection after a laser pulse, as shown in Figure 1.5. The earliest detectable signal after the laser pulse is at 20-150 ns.

Magnetization from the initial $M_z$ direction after the laser pulse becomes a $M_y$ component caused by rotation influenced by the resonant microwave field. [25] A series of complete TREPR spectra are obtained by slowly stepping the MF, to build a 2D spectrum, with field on the one axis and time on the other. Typically, the signal initially increases, after light excitation. Spectra at early times show the interaction of the spin system with microwaves. However, at later times the signal decays due to relaxation processes, caused by diffusion or re-encountering of free radicals. [23] Deviation from thermal equilibrium or polarization can be generated at any point during these times. The changes in the signal are related to the chemically induced dynamic electron polarisation (CIDEP) effect. This effect arises from spin systems of free radicals not being at thermal equilibrium and will be described in the next section. The signal acquired at certain time delays ($\tau_D$) after the laser excitation can be affected with CIDEP, which holds important information about the nature of the early time intermediates. [26] The influence of the microwave field also has to be considered in the time between formation of a radical and signal detection. [26] It is known that the off-resonance signal can have some slow decaying signal caused by laser light. The laser light causes heating, which can result in de-tuning of the resonator. [23] Therefore, the off-resonance signal has to be removed from the recorded data.
Figure 1.5. Continuous-wave direct detection mode. Microwaves are constantly applied during the experiment. The signal is measured after applying light (photoactivation of the sample). The signal intensity is monitored as a function of time at different MFs.

1.2.3.2 Pulsed microwave detection

Even though DD-EPR has longer dead times, it is useful in the measurement of wide spectra. [24] On the other hand, FT-EPR technique is best suited for systems with narrow spectral widths. [27] FT-EPR is useful to obtain additional information about the spin system that cannot be obtained from CW EPR, by using a single pulse. This technique is often used for the observation of short-lived free radicals generated in photochemical reactions, due to its high resolution. [27] Compared to DD-EPR, this method is simpler given the absence of microwaves in the moment of measurement (Figure 1.6). [26] Pulsed EPR spectroscopy, combined with laser excitation, is used with electron spin echo detection or Fourier transform detection, with a potential time resolution of about 10 ns. [23]

In the FT-EPR experiment, electron spins are excited and generated by applying a short laser pulse. A $\pi/2$ microwave pulse is applied at selected delay times (DAF) (Figure 1.6). At the time of applying the microwave pulse, the magnetization lies in the direction of the external MF (z-plane). The $\pi/2$ microwave pulse flips magnetization from the z-plane into the transverse (xy-plane) and measures transverse magnetization (Figure 1.4). After application of the microwave pulse, microwaves are absent and what is observed is the time domain spectrum of magnetization decay from the xy-plane back to the z-plane, and is named a free induction decay (FID). In order to obtain information on the spin dynamics of the system, the time domain spectra are transformed to frequency domain by Fourier transform. The result is FT-EPR spectra. FT-EPR spectra are not influenced by microwave radiation like in DD mode. The spectra depend only on the dynamics of the spin system.
Figure 1.6. Fourier-transform EPR. After applying light (orange rectangle) after certain delay times ($t_o$), a microwave pulse (blue rectangle) is applied and the signal is observed. After Fourier transformation, the EPR spectrum in a frequency domain is obtained.

Evolution of species can be observed after collecting the FIDs at different DAF. Figure 1.7 illustrates the characteristics of the applied microwave pulse as well as the lineshape of radical concentration in relation to the position of light and a microwave pulse.

Figure 1.7. a) Lineshape of the radical decay with regards to the position of laser and microwave pulse. b) Characteristics of the $\pi/2$ microwave pulse, which turns the magnetization into the $xy$ plane and creates transverse magnetization. Note: shape of the radical decay will be different for different species; this is an illustrative example. Adjusted from [25].

The FT-EPR technique is often used for studying transient radicals. [6, 25, 26, 29, 30] Free radicals can be formed as a result of photoexcitation, where the microwave pulse is applied at certain delay times after laser pulse. The formed spectra are the outcome of magnetization influenced by Boltzmann equilibrium signals as well as the signals originated from CIDEF effects (non-equilibrium signals) derived from triplet or RP mechanisms (See Section 1.3.2). Therefore, this technique with high time resolution can be useful for studying spin polarized RPs.

One of the examples of studying spin dynamics is an experiment conducted by Bittl et al. where they looked into the electron transfer reactions. [31] The authors
used transient EPR and echo-detected pulsed methods to study protein functions. The studied systems concerned the generation of RPs in the photoinduced reaction of photosynthesis, where the EPR technique was used to study cofactor distances, orientation and functional aspects. Another example of a system described with generated RPs is the photoreduction of flavin, as a cofactor of photolyase, which is involved in enzymatic repair of DNA. [31] Advanced pulsed techniques were used in studying radicals in flavoproteins [32, 33], protein science, catalysis and dynamics of nucleic acids. [34]

Since the RPs play a role in enzymatic reactions, can be light induced, and therefore studied with TREPR, their generation will be discussed in further detail. In the end, it is important to mention the dead-time present in FT-EPR, which disables observation of the signal within 50-100 ns after applying the π/2 pulse. [30] Dead-time of the FT-EPR technique can be removed by linear extrapolation of the FID signal, where it reaches the time resolution of about 10 ns, quite close to the time of applied microwave pulse. [24] Finally, both DD-EPR and FT-EPR have their advantages and together can complement one another measurements of transient radicals within in the range 10 -150 ns.

1.3 Radical pair theory

This section will describe spin chemistry theory involved and used in this thesis. Basic theory on radicals and RPs will be followed by theoretical background on spin dynamic phenomena, which results from the generation of RPs on the reaction pathway. Phenomena described in this section involve chemically induced electron and nuclear polarisation (CIDEP and CIDNP) and magnetic field effects (MFE). Also, the relationship between the EPR experiments and theory will be summarised.

1.3.1 Radicals and radical pairs

Radicals are unstable, reactive intermediates that have one or more unpaired electrons. Methods of generating free radicals are presented below in Figure 1.8.
Many reactions can generate pairs of radicals simultaneously as indicated in Figure 1.8. Some of the examples of RP formation are bond homolysis, resulting in electrons located on different fragments, or electron transfer between molecules. One of the characteristics of RP is that there is no bond between two members making up the pair. However, the spins of this g-pair are initially correlated with a spin-state defined by the multiplicity of the precursor, because of spin conversation. For example, if the precursor molecules A and B are in the singlet (S) state, the RP \([A^+ + B^-]\) is S-born as well. The same rule of spin selectivity is valid for reverse reaction where \(A + B\) state can occur from the S state. [35]

There are two types of RPs, which can be generated as result of processes presented above. [27, 36] The RPs generated initially are called geminate pairs, g-pairs, and have pure spin state due to the conservation of a spin (Figure 1.9).

However, if these radicals diffuse apart from the cage and, then, randomly re-encounter with other free radicals they are called freely-diffused RPs, or f-pairs (Figure 1.9). The possible events that can occur in RP reactions and will be discussed in greater detail later (Figure 1.11).
An important method for generating free radicals and RPs is in photochemical reactions. RPs can be formed from interaction of a molecule with light, reaction which can be matter of photochemistry and photophysics studies. Both photochemistry and photophysics hold great interest in modern technologies and are interesting fields to be studied, ranging from photosynthesis, and converting sunlight into fuels, to photophysics being applied to material sciences, such as using fluorescence for sensors. The branch of photochemistry is following net chemical changes that occur with this absorption of photon, whereas in photophysics net physical changes are studied. [37] The most common pathway after absorption of photon by molecule A is through formation of intermediate, which in most cases has characteristics of a RP, biradicals or zwitter ions. [37] Therefore, in photochemistry, molecule A absorbs a photon and results in formation of an excited state, A*, from which reaction intermediates can form. If an intermediate contains two highly reactive fragments with correlated spins, formed after photoexcitation, it is called a RP. The spin-correlation of these two spins is originating from the conservation of spin from the excited state.

**Figure 1.10.** Formation of different energy states following absorption of a photon. The first step is the absorption of light (yellow arrow) by a molecule, which undergoes a transition from ground to one of the excited states (S). Energy dissipation can occur via fluorescence (green arrow), dissipation through phosphorescence (red line), internal conversion (curvy arrow) or intersystem crossing to excited triplet state (T1). kISC is a constant for the intersystem crossing that can occur between those two states and kIC rate of internal conversion. The spin-state configurations for each energy level are indicated.
As mentioned, RPs can be formed by absorption of light, and possible outcomes of these photophysical processes are displayed in Figure 1.10, presenting an example of a Jablonski diagram for a typical organic chromophore, with a ground state molecule in an S state. In these reactions, molecules absorb light energy, which results in formation of an intermediate state (Figure 1.10, yellow arrow), with different properties from the ground state molecule. Photoexcitation of a molecule results in population of one of the vibrational sublevels of the S excited states (S₁, S₂, S₃). Photogenerated energy of an excited state can be dissipated via different routes, some of which are radiative (e.g. fluorescence, phosphorescence) and some non-radiative (e.g. IC, ISC). One of the processes that often occurs is fluorescence, where energy is dissipated between one of the S levels and ground state molecule in an S state (Figure 1.10, green arrow). One of the pathways for dissociation of energy of the excited state is internal conversion and change of multiplicity from S to triplet (T) via intersystem crossing, with rate $k_{ISC}$. This transition is a forbidden transition in origin. However, if the vibrational transitions are allowed in the electronic structure, this process becomes weakly allowed. It is a slow non-irradiative process occurring in the time range of $10^{-8}$ to $10^{-3}$ s. [38] Energy dissipation is, then, possible through phosphorescence, which occurs on a much slower timescale than fluorescence (Figure 1.10, red arrow), and is a transition between states of different multiplicities, whereas the fluorescence is between the states of the same multiplicity. The multiplicity of the precursor molecule (which reflects the number of possible spin alignments) can be identified through the conservation of a spin as discussed above, since the spin of the ground state molecule is reflected on the excited state molecules.

Therefore, spins of unpaired electrons on each radical are correlated depending on the multiplicity of the precursor; so S precursor results with S-born RP, and T precursor with T-born RP. The energy separation between S and T states of the RP corresponds to $J(r)$, where $J$ is the exchange interaction influenced by the inter-radical distance ($r$). [27] As the geminate radicals separate, $J(r)$ drops rapidly and S and T states become close in energy. At a separation of at least 0.1 nm, $J(r)$ reaches the order of the magnitude of average hyperfine coupling constants for
organic radicals. At this point spin-state mixing starts to become energetically tractable.

Spin-state mixing originates from the fact that S or T are not stationary states and they oscillate by certain frequency, which is dependent on the hyperfine interaction. [35] This interaction of an unpaired electron with magnetic nuclei causes the oscillation between these two spin states and their interconversion is dependent on the Larmor frequency of each unpaired electron spin, defined by:

\[
\omega = \frac{g \mu_B B_{\text{local}}}{\hbar}
\]  

[Equation 1.9]

From this, each unpaired electron spin will oscillate with a different frequency, caused by the difference in hyperfine interaction and/or g-values. Lack of the hyperfine interactions would result in the absence of oscillations, and therefore the RP would remain in the original state until the system ultimately relaxes to equilibrium.

This process of interconversion is a coherent process. However, spin-correlation can be lost through relaxation to an equilibrium distribution of RP spin-states, which is an incoherent process in kinetic competition with coherent spin-state interconversion. The ratio of these processes is important parameter which can be influenced by external MF.

In zero applied MF field coherent spin-state interconversion is not possible if \( J(r) \) is too large. As mentioned above, two unpaired spins have to separate, for example in a diffusive step, and afterwards, energetically, the interconversion can be achieved. This process can occur within the solvent cage. The outcome of the re-encounter depends on the spin states. If the RP reencounter in the S spin-state, they can recombine and react. Next, if the reencounters are in the T spin-state they usually cannot recombine due to the Pauli exclusion principle. However, they can diffuse apart again, undergo spin-state mixing, reencounter and recombine, as illustrated in the Figure 1.11. If these reactions or radical recombination occur within the solvent cage, products of these reactions are called cage products. Cage
products are products of either primary reactions of geminate pairs or reaction dynamics after the formation of f-pairs. [39] Furthermore, T-born RPs (S-born as well, but on a smaller scale) often proceed to the forward direction and escape the radical cage, therefore forming freely diffusing radicals, f-pairs. These radicals can randomly encounter and form escape products.

**Figure 1.11.** The lifetime of T-born RP after photoexcitation. After radical formation, a spin correlated RP is born. RPs in a T state encounter are more likely to escape from the cage (escape products) than S-born pairs. If spin mixing occurs, radicals recombine and the reaction can proceed (cage products). *Adapted from [27].*

Overall, movement of two radicals in the pair is restricted, inside the solvent cage, which enables interaction between spins of radical species. Even though there is no direct bond between them, from their interactions as intermediates, final experimental results can contain information on these species. [27] These interactions and their spin-state mixing can be influenced by external MF, resulting with the change in rate and product yield in RP reactions (further in Section 1.3.3). The RP term was first used to describe anomalies in intensities in NMR spectra. This phenomenon was later described as chemically induced dynamic nuclear polarization (CIDNP). CIDEP was first reported in 1963. where the hydrogen atom spectrum exhibited the low-field line in emission and the high-field line in absorption. [40]
1.3.2 Chemically induced dynamic electron polarisation (CIDEP)

The spin system of photochemically generated radicals will not be at thermal equilibrium at the time of formation. This population difference can affect the EPR signal intensities at any time before a radical is relaxed and it can also change the time evolution of a signal [23]. CIDEP can arise from different phenomena, which are summarised for a T-born RP in Figure 1.12. The main processes studied in this thesis are the triplet mechanism (TM) and radical pair mechanism (RPM), and are described in detail further below.

![Diagram summarizing the different ways of generating spin polarized spectra.](image)

**Figure 1.12.** Diagram summarizing the different ways of generating spin polarized spectra. *Taken from [40].*

### 1.3.2.1 Triplet mechanism

The TM is formed through a series of photochemical events as depicted in Figure 1.13. Firstly, the S state upon light excitation absorbs light and forms an excited S state ($^1{\text{MX^*}}$, Figure 1.13). The S state via ISC forms a T state, which causes net spin polarisation. However, the T state formed is different from a thermally generated T state. T states generated in photochemical reactions can undergo selective populations of the different sublevels in the molecular frame. [26] The selective population of $T_x$, $T_y$, $T_z$ is often transferred to the laboratory frame (high field). As a result, there can be a difference in population between $T_{11}$, $T_0$, $T_{-1}$ levels, which can cause absorptive or emissive spin-polarised EPR spectra in the subsequent RP signals.
This polarisation occurs in the subsequent RP signals only if the rate of the chemical reaction is within the spin-lattice relaxation time of a radical. [40] The spin-lattice relaxation time of the generated T state is short, on the order of a few ns. One of the reactions examples where the unstable, short-lived T state can be quenched is through a mediator in the system which enables an electron transfer reaction. The polarization by TM is, then, transferred to the formed doublet radical after the quenching reaction. The lifetime of this species is usually long enough for TM polarization to be detected in EPR spectra. [41] It results with a spectrum being in one phase (net polarised) without distortion of the line intensities. The phase of the signal, absorption or emission, depends on increase in population of higher or lower energy state. [23, 41]

The time development of the TM signal can be described using Equation 1.10;

$$s_{TM} = \frac{k_f P_T}{k_f + T_1^{-1}} \left[ 1 - \exp \left\{ -\left( k_f + T_1^{-1}\right) t\right\} \right]$$  \[Equation 1.10\]

where $T_1$ represents the T spin-lattice relaxation time, $k_f$ the first-order radical formation constant and $P_T$ the initial population difference. [42, 43] The population difference that causes the polarization can reach up to 600 times from the equilibrium. [40] However, spin polarization occurs only when these states are near degenerate or populate similar energy levels. Since the T sublevels are non-degenerate in this experiment, the electron exchange interaction is crucial for mixing of one of the T sublevels with an S state. The mixing of different energy
levels causing CIDEP was extensively described by McLauchlan, with the origin of CIDEP in TM and RPM. [44, 45]

This process usually occurs in diffusion controlled reactions. The magnitude of the TM is a function of the rotational correlation time of the precursor molecule, the zero field splitting constants D and E, the spin-lattice relaxation time, the chemical lifetime of the T state and the experimental MF. [27] The magnitude of this polarisation, however, mainly depends on the rotational correlation time and is described by Equation 1.11.

\[
P_T = \frac{-4DK\omega_0}{15} \left( \frac{4}{4\omega_0^2 + [k_T + \tau_R^{-1}]^2} + \frac{1}{\omega_0^2 + [k_T + \tau_R^{-1}]^2} \right) \quad \text{[Equation 1.11a]}
\]

\[
K = (k_\perp - k_\parallel) / k \quad \text{[Equation 1.11b]}
\]

In these equations; D is the zero-field constant, \( \omega_0 \) is the resonator frequency. \( K \) is described by Equation 1.9b and presents anisotropy of ISC rates perpendicular and parallel to the main axes, with \( k \) as the total value of both ISC rates \( (k_\perp \text{ and } k_\parallel) \). \( k_T \) is the rate of formation of T state and \( \tau_R \) is the rotational correlation time, both dependent on solvent viscosity. [45]

Once spin-lattice relaxation occurs, polarisation by the TM disappears. [45] Therefore, if polarised radicals are to occur, the spin-polarisation by triplets is in competition with the rates of spin-lattice relaxation. This is described by Equation 1.12, for the spin-polarised radical, \( P_R \). \( k_D \) presents the diffusion-controlled constant for the T molecule and substrate M, \( P_T^{eq} \) is the population of thermally equilibrated triplets and \( T_{T1} \) is the spin-lattice relaxation time.

\[
P_R = \frac{p_T k_D[M] + p_T^{eq} T_{T1}^{-1}}{k_D[M] + T_{T1}^{-1}} \quad \text{[Equation 1.12]}
\]

1.3.2.2 Radical pair mechanism (RPM)

An RPM is more common than the TM and arises in photochemical and thermal processes. It is generated from the interaction of two members in the RP since their electron spins are spin-correlated. Spin polarization in this system arises from mixing of one of the non-degenerate \( T_{+1,0,-1} \) states with the S state, with \( J \) as an
important parameter (Figure 1.14). [23] Mixing between states is caused by the difference in MF experienced by the unpaired electrons and is therefore dependent on the hyperfine interaction of each radical.

![Figure 1.14](image)

**Figure 1.14.** Energies of the S and T energy levels as a function of inter-radical distance. If $J \sim \Delta \omega$, ST₀ mixing can occur, whereas if $J < 0$ ST₁ mixing is possible (red dotted circles). *Taken from* [26].

### 1.3.2.2.1 RPM from ST₀ mixing

The Hamiltonian that describes spin dynamics for ST₀ mixing contains exchange and magnetic terms for describing interactions within the radicals. It is shown in the Equations 1.13. [39, 46]

\[
\mathcal{H}^{(ex)} = -J(r)(2\hat{S}_1\hat{S}_2 + \frac{1}{2}) \quad [\text{Equation 1.13a}]
\]

\[
\mathcal{H}^{(m)} = \mu_B(g_1S_1 + g_2S_2)H_0 + \sum_n A_{1n}l_{1n}S_1 + \sum_m A_{2m}l_{2m}S_2 \quad [\text{Equation 1.13b}]
\]

These interactions within radicals are characterised by the Bohr magneton, $\mu_B$, and the electron spins of each member of the RP, $S_1$ and $S_2$. $A_1$ and $l_1$ are the hyperfine coupling constant and nuclear spin of the nucleus of radical 1, $A_2$ and $l_2$ for radical 2, respectively, and finally $g_1$ and $g_2$ which are the $g$-factors of each radical. The difference between two $g$ factors and in hyperfine coupling constants within members of the RP has an influence on ST₀ mixing. The difference in precession frequencies ($\Delta \omega$) of the two spins also has an effect on ST₀ mixing (when $J = 0$), which involves a three step process – generation of a RP, diffusion of radicals within the solvent cage and reencountering of free radicals. This process occurs within a few ns. Typical organic free radicals can give rise to a frequency of $10^7$-10⁸ rad/sec,
even though this value can be larger, depending on the difference in g-value and hyperfine couplings in a RP. [36]

ST₀ mixing can be simply described by using a vector model as presented in Figure 1.15. The difference in precession frequencies (ω₁-ω₂) between two spins (S₁ and S₂) causes them to oscillate between the T₀ and S state. The T₀ state with time evolves into the S state through an intermediate state at certain frequencies, and vice versa for the S→T₀ oscillation.

![Figure 1.15. Vector model of ST₀ mixing in time. B₀ and S₁ and S₂ represent the MF and two spin states, respectively. Taken from [46].](image)

When describing the polarization caused by ST₀ RPM, several parameters have to be considered. The constant c used in Equation 1.14 describes a radical that is formed in terms of the solution used for experiment and temperature. [23] The polarization generated in the RP depends on the Q (Equation 1.15), which is mixing parameter between two states (mixing depends on the square root of Q).

\[
P \propto \left[ Q^{\frac{1}{2}} - cQ \right]
\]

\[
Q = \frac{1}{2} (g₁ - g₂) \mu_B B + \frac{1}{2} \sum_n a_{1n} m_{1n}^{(a)} - \frac{1}{2} \sum_m a_{1m} m_{1m}^{(b)}
\]

The Q^{1/2} is a term which describes the separation or re-encountering processes; the exchange interaction is negligible and each electron spin precesses around MF in its environment with its own frequency. The observed polarization can also be described as a change in one of the EPR line intensities as a result of radical interaction of one of the members of the RP with the other member in all of its
hyperfine states (Equation 1.16). In this case, nuclear spin and the Zeeman state of
nucleus $n$, $b_n$, respectively, have to be considered.

$$ P_{1,a} = \frac{1}{x_2} \sum_n P_{1,a}b_n \quad \text{[Equation 1.16]} $$

As mentioned earlier, polarization by TM results in net absorption or emission. RPM polarization, on the other hand, usually results in mixed phase signals, with part of the signal in absorption and part of it in emission, with final polarization of spectra depending on the spin-multiplicity of the molecule precursor (see Section 1.3.2.4). Secondly, if the origin of a precursor molecule is in a T state, ST$_0$ mixing will result in a higher number of cage products because more S will be formed, which then can form a reaction product; if the precursor is in a S state, with ST$_0$ mixing more T state is generated and will give less cage related products, these radicals then can diffuse apart and form f-pairs. The sign of the polarization of f-pairs and T pairs is opposite to the S pairs.

The theory above does not take $J$ into account since $J$ decreases as the pair separates (ST$_0$ usually calculated for $J=0$), but in viscous media $J(r)$ does not become irrelevant. Members of the RP stay in close proximity to each other and the spectrum now can be polarized by the RP itself and result in additional line splitting. ST$_0$ mixing is still possible even at higher fields because these two levels are almost degenerate when the radical separation is a few molecular diameters.

1.3.2.2.2 RPM from ST$_{-1}$ mixing

The second RPM polarization effect which can occur in viscous media is mixing of S and T$_{-1}$ states. This effect can also result from the influence of hyperfine interaction and therefore enhanced spin-state mixing. [23] It is described by Equation 1.17;

$$ P_{ST_{-1}} \propto a^2 [I(I + 1) - m(m + 1)] \quad \text{[Equation 1.17]} $$

where $I$ is nuclear spin, $m$ nuclear magnetic quantum number and $a$ hyperfine coupling constant. T$_{-1}$ and S levels differ by 1 in the nuclear magnetic quantum numbers.
ST₁ mixing is influenced by $J(r)$. This interaction is relevant since in viscous media it is not zero immediately, and S and T₁ levels can be close to each other in energy (see Figure 1.14), as long as S lies under T₀ in the energy levels. [23] When $J < 0$ enhanced emission and absorption will depend on the multiplicity of the precursor molecule. If it is a T state, the signal will be in emission on the low frequency side and in absorption on the high frequency side. If the precursor is a A state, the signal is reversed. If $J > 0$ ST₁ mixing occurs at the higher MF (Figure 1.13), [26] this polarization is strongly inhibited in high fields and is observed in combination with ST₀ mixing and TM in some cases. [46]

1.3.2.3 CIDEP from f-pairs

The polarization described above originates from the RPM, which occurs from within a few ns of the generation of RPs. However, polarization can occur from RPs formed after diffusion and random encountering, the effect that is important at high radical concentrations. [40] In this case, RP spin states are still correlated where S-born pairs can recombine and T-born pairs can cause polarization development. Therefore, all f-pairs are T-born. Polarization from these RP generated by free diffusion dominates over later times. This is one of the facts which distinguish the interpretation of data concerning the origin of the polarization between geminate pairs and f-pairs. [23] The other parameter that can be examined is the concentration dependence. The f-pairs’ encounter is a second order reaction and, therefore, concentration dependent, which is not the case for g-pairs. What can also be examined is the phase of the signal; since the phase can be different between the two origins of polarization. f-pairs in general behave similar as T-born geminate pairs (since they are T-born in origin). [46]

A possible explanation for multiple effects (obvious in mixed phase signal) in f-pairs is in the changing $J(r)$ value since the radicals diffuse apart and as a consequence $J$ changes. Therefore, a change in the phase with time does not have to be caused by polarization effects but due to the interaction with the nucleus, observed in electron-nucleus cross relaxation. [23] What also has to be considered in flow experiments is that CIDEP is mostly caused by f-pairs unless the mixing is
completed before entering the cavity. The polarisation by f-pairs is therefore important in the experiments that are to be studied as part of this project, together with potential TM and RPM that can occur with it as well.

1.3.2.4 Net and multiplet polarisation

Some of the rules describing polarization in CIDEP have been presented in this chapter. In terms of polarization causing net (single phase) or multiplet (more phases) effects, rules can be summarised through using terms $\Lambda_{ne}$ for net effects and $\Lambda_{me}$ for multiplet effects.

$$\Lambda_{ne} = \mu J \Delta g \begin{cases} + & : A \\ - & : E \end{cases}$$

$$\Lambda_{me} = \mu J \begin{cases} + & : A/E \\ - & : E/A \end{cases}$$

The multiplicity factor $\mu$ is defined as “+” for T precursor and f-pair and “-“ for S precursor. In summary, all net effects originate from TM mechanism whereas RPM causes a multiplet effect. At low radical concentration it can happen that the polarization by TM is so strong that any contribution of the RPM cannot be detected. Also, in flow experiments, only polarization caused by a re-encountering process can be observed. The polarization at time of formation of RP could be observed if mixing is not completed before the sample enters the cavity. This was discussed in terms of the flow experiments where formation of benzoyl and t-butyl RP was followed. In general, the RPM can occur with polarization by TM and f-pairs, the effect mainly appear with neutral radicals. When simulating EPR spectra of RP formed by photolysis, relative contributions from each polarization (TM or RPM) have to be considered. Therefore, calculation of individual polarisation pattern is the first step in analysis of CIDEP data. Afterwards, they are added or combined together in certain ratio to reproduce the experimental spectrum.

1.3.2.5 RPs studied by EPR and application of CIDEP

Since CIDEP signals are short-lived, they can be followed by TREPR techniques. TREPR spectrum can provide information about the multiplicity of the system,
approximate $g$-values and information about the exchange interaction in relation to the absorption/emission pattern in the MF. Table 1.2 shows a summary of what theory predicts for the EPR spectra affected by the electron polarization. In the Table 1.2 various options are summarised for resulting spectra polarized by RPM. For the TM, it is known that it results in a net polarization and emissive or absorptive spectrum, depending on the population difference between states.

**Table 1.2** Summary of $ST_{0/1}$ RP mechanism effect on EPR spectra.

<table>
<thead>
<tr>
<th>RPM</th>
<th>Precursor molecule</th>
<th>Spectrum</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>$ST_{0}$ RPM</td>
<td>J&lt;0</td>
<td>Singlet</td>
<td>A/E</td>
</tr>
<tr>
<td></td>
<td>J&lt;0</td>
<td>Triplet</td>
<td>E/A</td>
</tr>
<tr>
<td></td>
<td>J&gt;0</td>
<td>Singlet</td>
<td>E/A</td>
</tr>
<tr>
<td></td>
<td>J&gt;0</td>
<td>Triplet</td>
<td>A/E</td>
</tr>
<tr>
<td>$ST_{-1}$ RPM</td>
<td>A&lt;0</td>
<td>Singlet</td>
<td>A (low field*)</td>
</tr>
<tr>
<td></td>
<td>A&lt;0</td>
<td>Triplet</td>
<td>E (high field*)</td>
</tr>
<tr>
<td></td>
<td>A&gt;0</td>
<td>Singlet</td>
<td>A (high field*)</td>
</tr>
<tr>
<td></td>
<td>A&gt;0</td>
<td>Triplet</td>
<td>E (low field*)</td>
</tr>
</tbody>
</table>

* denotes enhancement of one of the MF sides

CIDEP occurring in products of photochemical reactions can serve as an indication of reaction precursors or intermediates. Martino et al. reported that TM CIDEP is generated in single bond photolysis (such as in methylcobalamin) of radicals, created on a subnanosecond timescale. [26] Another example of a CIDEP study is the photoexcitation and scavenging of a T state of quinones in different solutions; where both hydrogen and electron transfer take place resulting in the formation of paramagnetic species detectable by EPR. [7, 48] FT-EPR was useful for detection of aceton-ketyl radicals, peroxides, and photoinduced electron transfer in homogeneous or heterogeneous media. [25, 43] Van Willigen and Levstein summarised the CIDEP effect found in heterogeneous media, such as electron transfer from porphyrines to quinones in micellar systems. [42] Another example of a reaction followed by TREPR through CIDEP is the reaction between $H_{2}O_{2}$ and DMSO, where polarisation by f-pairs was observed. [6]
1.3.3 Magnetic field effects (MFE)

So far it was demonstrated that RP reactions can be influenced by the difference in populations of spin states, differences in hyperfine couplings and $g$-values of each radical and solvent conditions/cage effects. However, spin motion of radicals in the RP is also dependent on an applied MF. A different dependence on spin motion is generated depending on the field strength (non-linear response). One of the important experimental parameters is the viscosity of the solvent since spin rotation changes in the viscous solutions and changes the lifetime of the RP pair. [39]

The RP spin states in a MF are presented using vector model displayed in Figure 1.16A. These vectors are precessing about the applied external MF with its Larmor frequency defined by the Equation 1.9. From the Figure 1.16A it is clear that the $S$ state is non-magnetic and the bulk magnetization vector of the $T_0$ state is orthogonal to the applied field. These two states are separate in energy only by exchange interaction, $J(r)$. However, magnetisation of $T_{+1}$ and $T_{-1}$ is anti-parallel and parallel to the applied field, respectively, which is the origin of their energy dependence on the external field.

**Figure 1.16.** RP spin states in MF. A) Vector model of RP spin states in a MF, with arrows indicating spin orientation regards to the MF. Orientation in relation to the MF is indicated with green arrows. B) Splitting of the two coupled electrons in different energy levels due to the Zeeman interaction. The relative spin orientation are presented with combination of spin orientations $\alpha (|\uparrow\rangle)$ and $\beta (|\downarrow\rangle)$ as $\alpha_1\alpha_2, \beta_1\beta_2$ and their combinations. Spin states are also presented as four energy levels that could occur: singlet ($S$) and three T sublevels ($T_{+1}$, $T_0$, $T_{-1}$), which are non-degenerate in the zero MF.
The coupled spin states of two radicals in RP can be described as the interaction of two independent spins, $\alpha (|\uparrow>)$ and $\beta(|\downarrow>)$, as presented in the Figure 1.16. The spin states of a RP are degenerate at zero MF (as long as the exchange interaction is negligible), where interconversion of all the spin states is still possible (Figure 1.16B, yellow rectangular). When these coupled electrons are influenced by an external MF, splitting in the energy level diagram of a RP, and four possible states can be envisaged (Figure 1.16B). Figure 1.16B shows that on increasing the field, $S$ and $T_0$ remain the same in energy whereas $T_{+1}$ increases in energy and $T_{-1}$ decreases (it is more stable because the magnetization of $T_{-1}$ state is parallel to the field). Therefore, at higher fields these levels are non-degenerate due to the Zeeman splitting. Due to Zeeman effect, $T_{+1/-1}$ become increasingly less involved in spin-state interconversion until they are removed in energy completely.

If a RP is formed from the S state, applying the MF therefore results in an increase of the population of S and $T_0$ energy levels. [43] Since the relative S population has increased, the recombination probability increases as well, with a decreased possibility of escape products. [27] However, if a RP is born from the T state, because $T_{+1/-1}$ are trapped as T in an applied field, the relative population of S decreases and so does the recombination probability. [27, 43]

This region is characterised as hyperfine dominated region and has origin in Zeeman interaction described above. It is the mid-field region and ranges from $\sim 0.001$ T to $\sim 1$ T. As $T_{+1/-1}$ are removed in energy by increasing the MF, they become less involved in spin-state interconversion until they are no longer involved (saturation of the MFE).

Once $T_{+1/-1}$ are removed in energy at higher fields, ST$_0$ mixing becomes more efficient again due to the different $g$-values of the RP. This is characterised in $\Delta g$ dominated region at higher fields (Figure 1.17, dark green). [27] Since the applied MF, $B$, and $\Delta g$ are part of the same term of the spin Hamiltonian (Equation 1.15), at higher MF small $\Delta g$ for organic radicals is not negligible anymore, which results in an increase in the ST$_0$ mixing rate.
Figure 1.17. The effect on a reaction yield of S- (red) and T- (blue) born RPs depending on the MF strength. Three different regions of MF strengths are indicated with different background colours – low field region, hyperfine dominated region and $\Delta g$ dominated region.

The last of three distinct regions where MFE occur is low field region (Figure 1.17, yellow section). In zero applied MF, there are numerous states each with a varying degree of S and T character and some of these states are degenerate. The spin-states interconvert with a frequency proportional to their energy separation. When a weak field is applied, some of the degeneracies are lifted, thus increasing the extent of spin-state interconversion. However, in this region conditions have to be right for the interconversion: RP lifetimes need to be longer and competing processes must therefore be slower. The lifetime can be increased by controlling diffusion of RPs or creating cage effect (glycerol).

1.3.3.1 Biological significance of MFE and animal magnetoreception

There have been studies on proteins where the influence on MF was investigated. From previous investigations, it is known that animals such as salmon, sea turtles, and honey bees can detect the geomagnetic field, which could help them in migration. It is also interesting to study the impact of MF on biology because of the potential influence of MFs from human sources, such as from mobile phones, on human health. [49] One of the mentioned examples of possible health effects of MF exposure is the association between residential proximity to high voltage power lines and increased risk of childhood leukaemia. [49]

Therefore, it was shown that MF studies could be used as a tool for studying the potential influence of RP reactions on the reaction pathways of proteins. [49]
One of the examples of MF sensitivity is the ion RP generated in the photosynthetic centre of *R. sphaeroides*. The sensitivity ranged up to 200 mT and the experimental results agreed with a RP mechanism. Another studied system was with coenzyme B<sub>12</sub>, which has various functions, from photoreceptor chromophores to the cofactor of the enzymes involved in the metabolism. The recombination of free 5'-deoxyadenosylcobalamin (AdoCbl) after photolysis showed itself as the magnetically sensitive process. [49, 50] In experiments with enzyme-bound AdoCbl, an enhanced MF dependence was observed, with formation of S-born RP. This MFE was observed in the absence of substrate, and is probably caused by the cage effect of the protein. This cage effect prolongs the lifetime of the RP and therefore enables larger changes caused by MFEs. However, once there is substrate is bound to an enzyme, the 5'-deoxyadenosyl radical is quenched by the substrate, resulting with limited recombination of radicals. [49]

Another aspect of MF effects has been to study magnetoreception. Magnetoreception has been a general mystery for a long time and the biophysical process of this is still not clear. The simple question is: how does the bird know how to orient towards south and, how, when it’s nested, finds the same nest the next year? What is known so far is that the avian compass is an inclination compass [35, 39] and light dependent, which is known from behavioural studies with good orientation in blue and green, and disorientation in red. There are three basic stages for magnetoreception. Photons have enough energy to activate the light sensitive molecules in birds’ eye. This can cause the formation of RPs via electron transfer. [51-53] Finally, different concentrations of an activated state of a biomolecule can be generated depending on the interaction with present MFs. Yield of these products will influence the orientation of the bird.

### 1.3.4 Chemically induced dynamic nuclear polarisation (CIDNP)

Another consequence of generation of a RP is CIDNP, which can be observed as anomalies in NMR spectra. It was described above how mixing of S and T states can cause a population difference. This can be reflected in a difference in population of any of the nuclear spin levels and causing CIDNP. Depending on which state is more...
populated, the signal can be in absorptive or emissive mode. The absorptive mode is observed when the higher energy state is populated, and emission for the lower energy state. [27]

Mixing between S and T states caused by a difference in precession frequency of two radicals making up the pair is necessary for this effect to occur. For a small difference, recombination reaction won’t be fast, which results in T-born pairs escaping the radical cage and S-born pairs recombining. However, for the higher differences in precision frequencies, mixing is more likely to happen and subsequent recombination to S state and re-encountering. This causes different distribution in nuclear-states of cage and escape products.

Kaptein developed rules for prediction of polarization by CIDNP, even though exemptions are found later. [27, 36] Shortly, rules describe that the effects are dependent on: the sign of hyperfine coupling constant, \( \Delta g \) sign, how the RP is born – in S or T states or reencountering, identity of cage or escape products and sign of the nuclear-nuclear spin contact. Exemptions to the rule include the MF, scalar exchange coupling, nuclear relaxation times of the radical and the molecule, and lifetime of the RP. [36] This effect can arise as well after thermal or photochemical reactions.

### 1.4 Project aims

The general aim of the study was to investigate the biological reactions that involve generation of transient radicals on the reaction pathway. Various reactions involving formation of free radicals were studied using FT-EPR. Also, potential MFEs in cryotochrome as biological system were followed using fast-mixing spectroscopic technique.

The first aim of the study was the development of a continuous-flow EPR setup to enable the study of short-lived radicals involved in various biological reactions involving spin chemistry. The development of the system included development of the sample cell its optimisation depending on the EPR experiment that it is to be
conducted and solvent properties that was to be used. Another optimisation was the triggering sequence to ensure the minimal delay after flash (DAF). The initial test reaction for the developed system was with a previously studied system using photoexcitation of duroquinone in isopropanol. The development of the continuous-flow setup and its testing are described in Chapter 3.

The development of continuous-flow EPR setup enabled studying the potential mechanism of antimalarial drugs. Since the exact mechanism of these drugs is still not clear, with this approach we wanted to investigate whether it involves formation of free radicals. This was investigated in the reaction of electron transfer to duroquinone where the drug or its derivatives were used as an electron donor. Results of this study are presented in the Chapter 4.

The next question which the continuous-flow setup helped to answer was about the origin of the precursor molecule on the reaction pathway of methylcobalamin and 5’-deoxyadenosylcobalamin; the cofactors involved in the metabolism of vitamin B\textsubscript{12}. This was extensively investigated through various solvent conditions, experimental parameters such as excitation wavelengths and concentrations, and ligation. Even though this system has previously been studied extensively by flash photolysis and computational studies, it still presents an experimental and theoretical phenomenon with contradicting results. Chapter 5 presents important progress in clarifying this reaction pathway, together with a contribution to the identities of the different electronic structures. CIDEP reflected on experimental spectra was analysed by simulation.

Finally, another aspect of spin chemistry in biology, in a view of MFEs was studied on cryptochromes; proteins that are potentially involved in migration. It was known previously that the signalling state involving reactions of reduction of these proteins is magnetically sensitive. However, the resting state or reactions of oxidation that serves to complete the photocycle have not been investigated in the sense of magnetosensitivity. Here, we studied the potential MFE on the oxidation reaction of two types of cry, plant and animal, using home-built MFE UV-Vis
spectrophotometer. Chapter 6 presents results of this study, together with experiments of reduction studies with cofactors that are not bound to the protein.

Summary and future plans for each of the studied systems are presented in Chapter 7. Additional experimental details are presented in Appendix.

1.5 References


Chapter 2

Materials and methods

2.1 Sample preparation

All chemical were purchased from Sigma Aldrich unless stated otherwise.

2.1.1 Photoreduction of duroquinone

The photoreduction of duroquinone was followed with or without amine in solution as a potential electron donor. The experiments were completed in propan-2-ol as a solvent and duroquinone as the chromophore (both Sigma-Aldrich). Triethylamine (TEA) was purchased from Fischer Scientific. Chloroquine and other potential antimalarials were synthesised by collaborators from the Natural Product and Medicinal Chemistry Research Group at Liverpool John Moores University. The samples were freshly prepared before each measurement to the desired concentrations and degassed with Ar at least 5 mins prior to the measurement and continued to be purged during the experiment. The concentration of each sample component is indicated in the respective figure legends.

2.1.2 Preparation of cobalamins for photolysis followed by TR-EPR

Methylcobalamin (MeCbl) and 5’-deoxyadenosylcobalamin (AdoCbl) were used without further purification. The powder was dissolved in different buffers, depending on the form of cobalamins to be studied. The buffers that were used are presented below.

<table>
<thead>
<tr>
<th>Base-on cobalamins</th>
<th>Base-off cobalamins</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM HEPES pH 7.5</td>
<td>HCl:glycine buffer pH 2.2</td>
</tr>
<tr>
<td>0 % or 50 % glycerol</td>
<td>0 % or 50 % glycerol</td>
</tr>
</tbody>
</table>

Buffers were filtered and degassed with nitrogen and stored in an anaerobic glovebox (Belle Technology) at least one day before the measurements to ensure full removal of oxygen. Samples were dissolved in the respective anaerobic buffer
under red light and the concentration was checked using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies). The extinction coefficients used for calculating the concentrations for base-on forms of cofactors were $\varepsilon_{520\text{ nm}} = 7.7 \times 10^3$ M$^{-1}$ cm$^{-1}$ for MeCbl and $\varepsilon_{522\text{ nm}} = 8 \times 10^3$ M$^{-1}$ cm$^{-1}$ for AdoCbl. Concentrations of base-off forms of cofactors were calculated using $\varepsilon_{450\text{ nm}} = 5.4 \times 10^3$ M$^{-1}$ cm$^{-1}$ for MeCbl and $\varepsilon_{405\text{ nm}} = 7.4 \times 10^3$ M$^{-1}$ cm$^{-1}$ for AdoCbl. All sample vessels were closed with Suba-seals and parafilm to reduce exposure to oxygen while moving samples from the anaerobic glovebox to the EPR experiment. Prior to EPR measurements samples were purged with Ar and also during the EPR measurement.

2.1.3 Preparation of free flavin cofactors for UV-Vis and SF/UV-Vis spectroscopy

The buffer used for investigation of reduction and oxidation of free flavin cofactors was 50 mM HEPES, 150 mM NaCl, 10 % glycerol, pH 8. The buffer was filtered and degassed with nitrogen for a minimum of 1 h and stored in an anaerobic glovebox (Belle Technology) at least 72 h prior to the measurement to remove any residual oxygen. Stock solutions of FAD, FMN and lumiflavin-3-acetic acid were prepared and diluted to desired concentrations by measuring the absorbance in a Cary 60 UV-Vis spectrophotometer (Agilent Technologies). An extinction coefficient of $11 \times 10^3$ M$^{-1}$ cm$^{-1}$ at 450 nm was used. The concentrations of the flavin cofactors are stated in respective figure captions. Sodium dithionite (DT) used for reduction was freshly prepared for each experiment.

2.1.4 Drosophila melanogaster cryptochrome (DmCry) growth and purification protocol

DmCry was expressed in and purified from Escherichia coli SoluBL21 cells as described by Kutta et al. [1] The experimental details are provided below.

2.1.4.1 Cell growth

Cells were grown in TB Medium containing 432 g of yeast extract and 216 g of tryptone for 18 L media preparation, with 0.17 M KH$_2$PO$_4$ and 0.72 M K$_2$HPO$_4$. 18 L of media was then divided into 30 x 600 ml in 2 litre conical flasks. Media was autoclaved at 2 bar, 121°C for about 20 minutes.
For preparation of starter culture 250 μl of 100 mg/ml stock solution of ampicillin was added to 250 ml of autoclaved TB media. On the tip of pipette (~50 μl) frozen glycerol stock of the culture (soluBL21 containing expression vector pRSET-A with sequence DmCry 1-539 coding for DmCry) was added to the media and incubated overnight at 37°C in a shaker incubator (Infors Ltd) at 220 rpm. The starter culture was used for inoculation of large scale culture (30 x 600 ml culture). 600 μl of 100 mg/ml stock solution of ampicillin was added to the 600 ml of autoclaved media in each flask. Under sterile conditions, 6 ml of the starter culture was added to each flask containing 600 ml TB media. Cultures were incubated at 24°C in a shaker incubator (Infors Ltd) at 180 rpm.

After 5 h of incubation, 1 ml aliquots were aseptically removed from two or three flasks and the O.D. of the culture was measured. When the O.D. reached between 0.5 – 0.7, 600 μl of 1 M IPTG was added under aseptic conditions for induction. Cultures were incubated overnight at 24°C and 180 rpm. Cultures were centrifuged at 6000 rpm for 10 minutes at 4°C and pellets were stored at -20°C.

2.1.4.2 DmCry purification

Buffers used for the purification of DmCry are shown below. All buffers were filtered and degassed using Nalgene reusable water top filters. The proteins were purified using an Äkta purifying system (GE Healthcare).

<table>
<thead>
<tr>
<th>Buffers for affinity chromatography</th>
<th>Buffers for desalting column and anion exchange chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Binding buffer</strong></td>
<td><strong>Start buffer 2</strong></td>
</tr>
<tr>
<td>50 mM Tris/HCl</td>
<td>50 mM HEPES/NaOH</td>
</tr>
<tr>
<td>500 mM NaCl</td>
<td>50 mM NaCl</td>
</tr>
<tr>
<td>10 mM Imidazole</td>
<td>10 % Glycerol</td>
</tr>
<tr>
<td>20 % Glycerol</td>
<td>pH = 8.0</td>
</tr>
<tr>
<td>pH = 7.5</td>
<td></td>
</tr>
<tr>
<td><strong>Start buffer 1</strong></td>
<td><strong>Buffer A</strong></td>
</tr>
<tr>
<td>50 mM Tris/HCl</td>
<td>50 mM HEPES/NaOH</td>
</tr>
<tr>
<td>500 mM NaCl</td>
<td>10 % Glycerol</td>
</tr>
<tr>
<td>20 % Glycerol</td>
<td>pH = 8.0</td>
</tr>
<tr>
<td>pH = 7.5</td>
<td></td>
</tr>
<tr>
<td><strong>Elution buffer</strong></td>
<td><strong>Buffer B</strong></td>
</tr>
<tr>
<td>50 mM Tris/HCl</td>
<td>50 mM HEPES/NaOH</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Cell pellets were resuspended in approximately 80 mL binding buffer, for about 50 g of pellets. The volume of buffer was higher for larger amount of the culture. Usually 50 – 70 g of cell pellets was collected from 6 L of culture. After full resuspension a Complete® EDTA-free protease inhibitor tablet (1 required per 100 ml solution), 10 mg/mL DNAsel, 5-10 mM 1M MgCl$_2$, 10 mg/mL lysozyme and 0.025 g/mL CellLytic™ Express (Sigma Aldrich) were added. The suspension was incubated for 20 min at room temperature before centrifuging at 20 000 rpm for 30 min at 4°C. The supernatant was filtered using a 0.45 μm Sartorious syringe filter attached to a pre-cooled bottle. The filtered supernatant was transferred to a super loop and connected to an AKTA Pure instrument.

The 6xHis-tagged proteins were purified using a Ni-NTA affinity chromatography (HisTrap FF, GE Healthcare) in Tris buffer (pH 7.5). After the column was washed with dH$_2$O it was equilibrated with 3 % elution buffer. Sample was loaded onto the column from the super-loop and washed with 3 % elution buffer. The column was washed further with 10 % elution buffer before the protein was eluted with 100 % elution buffer containing 500 mM NaCl and 300 mM imidazole. The protein was desalted using CentriPure P100 desalting column (Generon; start buffer 2). Next, the protein was purified further by anion exchange chromatography. This was completed by using a linear gradient from 50 to 300 mM NaCl on a ResourceQ column (GE Healthcare). If further purification was required an additional anion exchange chromatography column MonoQ 10/100 GL (GE Healthcare) was also used. In this step the column was washed with dH$_2$O and buffer B and later equilibrated with 5 % buffer B prior to loading the sample. The column was washed with 5 % buffer B and protein eluted by a linear gradient from 5 % - 30 % buffer B. A final wash with 100 % buffer B was used.

After purification, proteins were exchanged into the desired buffer by using a CentriPure P100 desalting column (Generon). Samples were concentrated to the

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 mM NaCl</td>
<td>1 M NaCl</td>
<td></td>
</tr>
<tr>
<td>300 mM Imidazole</td>
<td>10 % Glycerol</td>
<td></td>
</tr>
<tr>
<td>20 % Glycerol</td>
<td>pH = 8.0</td>
<td></td>
</tr>
<tr>
<td>pH = 7.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

59
required concentration using Viva-Spin concentrators with a molecular weight cut-off of 30,000 Da (Sartorius), snap frozen in liquid N₂ and stored at -80°C until required. An example of UV-Vis spectrum after purification is displayed in Figure 2.1.

![UV-Vis spectrum](image)

**Figure 2.1.** UV-Vis spectrum of isolated DmCry.

### 2.1.4.3 Determination of sample purity using SDS-PAGE

The sample purity was determined after each purification step using 10 % polyacrylamide gels (Mini-Protean TGX Stain-Free™ Gel with Precision Plus Protein™ Unstained Standards, BioRad). Samples were mixed with concentrated SDS loading dye and heated for 5 min at 95°C before loading on the gel. The gels were run in SDS buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1 % SDS) at 300 V for ~ 15 min. The gels were stained afterwards using Coomasie Blue stain (Expedeon) and visualised with a Bio-Rad Gel Doc EZ imager. The purification process is indicated in the protein gels in Figures 2.2 and 2.3.
**Figure 2.2.** Fractions of purification resolved by SDS-PAGE. M- standard size marker with sizes indicated in kDa, SL – cell extract in super loop, loaded on NiNTA, N- protein solution after NiNTA and concentration, before resource Q; P1-P6 different peaks from the chromatogram, where nothing after P4 was taken for concentrating the sample for further experiments due to high impurity; red arrow indicates the DmCry band.

**Figure 2.3.** Fractions of protein peak resolved by SDS-PAGE. M – marker, wells 1-8 indicate different wells combined for concentrating the protein, with well 4 indicating max peak intensity and highest protein concentration; red arrow indicates the DmCry band.
2.2 Spectroscopic techniques

2.2.1 UV-vis spectroscopy

UV-Vis spectroscopy has also been used in this study to follow reactions of reduction and oxidation through following changes in different bands of the UV-Vis spectra. UV-Vis absorbance measurements were carried out using a Cary 50 Bio UV-Vis spectrophotometer (Agilent Technologies) placed in an anaerobic box. Changes at 450 nm were followed as an indication of change of oxidation state of the flavin cofactor. The concentrations of oxidised flavin species were calculated using an extinction coefficient of $1.1 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$ at 450 nm.

Oxygen was removed from fresh samples by passing them through an Econo-Pac* desalting prepacked gravity flow column (Bio-Rad) that had been pre-equilibrated with the anaerobic buffer previously stored in the anaerobic box. The experiments were conducted using two buffers – HEPES or phosphate buffer, concentrations presented below.

<table>
<thead>
<tr>
<th>HEPES (1)</th>
<th>Phosphate buffer (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM HEPES</td>
<td>0.1 M phosphate buffer</td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>0.1 M NaCl</td>
</tr>
<tr>
<td>10 % glycerol</td>
<td>(10 % glycerol)</td>
</tr>
<tr>
<td>pH 8</td>
<td>pH 7.5</td>
</tr>
</tbody>
</table>

The flavin chromophore was reduced using sodium dithionite (DT) or photoreduced using 455 nm ThorLabs M455L3 LED. The length of the illumination varied between the samples, as well as the amount of the added DT. After the reduction, the sample was diluted to the defined concentration and oxidation was followed using the UV-Vis stopped flow instrument.

2.2.2 Rapid mixing techniques

The oxidation of free cofactors and proteins was followed using an Applied Photophysics UV-Vis stopped-flow instrument (SX20) placed in an anaerobic glove box (Belle Technology) to ensure the oxygen level was below 2 ppm. The experimental setup is presented in Figure 2.4 and briefly involves individual syringes with two reagents driven to a mixing chamber and into an observation cell. This
pushes sample through to a stop syringe, which triggers the detection/acquisition of the spectral changes that occur in the sample in the observation cell on the millisecond timescale. [2] A photodiode array (PDA) detector was used to collect the whole spectra at different time points and a photomultiplier tube (PMT) detector was used in conjunction with a monochromator in experiments where measurements were conducted at a single wavelength.

**Figure 2.4.** Schematic of a standard SF/UV-Vis spectrophotometer.

The oxidation was initiated by mixing the anaerobic cofactor or protein solution with oxidised buffer at 25°C. In the experiments where the oxygen concentration dependence was studied, one syringe was filled with 10 μM free cofactor and the other one with oxygenated buffer with different concentrations of O₂. Concentration range of 25 – 200 μM O₂ was obtained by mixing different ratios of aerobic and anaerobic buffer. In the oxidation experiments with proteins, all samples were mixed against air saturated buffer (O₂ concentration ~ 200 μM) to yield a final O₂ concentration of ~ 100 μM in the observation cell. After mixing of both solutions (cofactor/protein vs buffer) the increase in absorbance at 450 nm was used to follow the oxidation reaction. Measurements were repeated several times (indicated in the figure legend) and transients were fitted to the sum of exponentials using Origin software, to generate apparent rate constants (k_{OBS}). The data collected with the PDA detector were analysed using Glotaran, a global
analysis software. [3] The simplest sequential model was used for all fits, therefore excluding the possible reverse reaction.

Another modification of the SF/UV-Vis that was used in these experiments enabled the measurement of any possible magnetic field effects (MFE) on the rate of reaction. In this modified instrument the observation cell was located inside a plastic holder, instead of the metal one that is in the conventional SF device. This enables positioning magnets on either side of the observation cell. Permanent magnets were used with MF strength of 100 mT. The instrument used in the experiments described in Chapter 6 was an Applied Photophysics SX20 and was housed in a Belle Technology anaerobic box. In the experiments of photoreduction and oxidation of DmCry followed by UV-Vis (Section 6.2.6.3) an optical bandpass filter (450FS10-25, Thorlabs) was used for removal of fluorescence.

2.2.3 Potentiometry

In order to measure redox potentials a Metler Toledo potentiometer was used in combination with an Agilent Technologies Cary 60 UV-Vis spectrophotometer. Samples contained the following mediators: 0.3 μM methylviologen, 1 μM benzylviologen, 7 μM 2-hydroxy-1,4-naphthoquinone (HNQ) and 2 μM phenazine methosulfate (PMS). After adding the mediators, the solution was left to equilibrate for 1 h. The reduction of 13 μM DmCry and 10 μM AtCry was followed by the addition of either sodium dithionite (DT) or titanium (III) citrate and UV-Vis spectra were recorded at different potentials. The reaction was conducted in an anaerobic glovebox (Belle Technology) under red light at room temperature. A correction factor of + 207 mV was applied to the electrode reading.

Preparation of Ti(III) citrate for reduction of DmCry

Ti(III) citrate was used for potentiometric titration of DmCry because it has a more negative potential than DT. Its potential at pH 7 is \( E_0 = -480 \text{ mV} \). [4] Since titanium precipitates in hydroxide this is avoided by adding citrate and the stable complex is formed. It was prepared by the addition of a 15 % solution of Ti(III) chloride to 50 ml of 0.2 M sodium citrate, in an anaerobic glovebox (Belle
Technology). The solution was later neutralised by the addition of saturated sodium carbonate.

### 2.2.4 EPR

Electron paramagnetic resonance (EPR) spectroscopy is a technique used to follow reactions described in Chapters 3, 4 and 5. There are slightly different setups for each of the experiments. Details of the different setups are described below; together with the laser setup, as a source of photoexcitation.

Time-resolved EPR techniques can be used to get a better insight into photoinduced reactions that occur on a short timescale. Our approach was to design a continuous-flow experiment (see Chapter 3), which would enable constant delivery of fresh sample into the microwave cavity and allow excitation with a laser flash. From the literature research presented in the Chapter 1, it was found that HPLC pumps provide the most accurate, pulseless, flow, in modern applications. A single piston HPLC Gilson 305 pump was used for constant delivery of the sample into the cavity. All experiments were completed at room temperature and the samples were purged with Ar prior and during the measurement.

The reaction was followed on a Bruker E580X EPR instrument with a Bruker ER4118X-MD5 dielectric resonator. A $\pi/2$ pulse of 16 ns was used to generate the free induction decay (FID), with 8 dB microwave attenuation; if different it is stated in the figure caption. The spectra were collected using four-step phase cycling in PulseSpel, at different delay after flash (DAFs). FIDs were transformed using Bruker Xepr software [5] as described in Appendix A.1. For the experiments described in Chapter 5, not all of the resonance lines can be collected at once. Therefore, each resonance lines was collected at separate field values and spectra were assembled later using Matlab (code presented in Appendix A.2). For higher frequency experiments, the reaction was followed on a E580Q EPR spectrophotometer with Bruker EN 5107D2 resonator. A $\pi/2$ pulse of 20 ns was used to generate FIDs with 0 dB microwave attenuation; unless otherwise stated. After FIDs were collected, the same procedure was followed as described above. One of the options for data
treatment is using linear prediction (LP) to extrapolate early time of FID. This process is described in Appendix B.3.

A second and third harmonic of Brilliant blue q-switched Nd:Yag was used for photoexcitation of the system at 532 nm and 355 nm with 20 mJ/pulse and repetition rate of 10 Hz. In order to observe these short-lived radicals, dead time has to be kept to a minimum. A delay generator Stanford DG535 was used to externally trigger both the laser and spectrometer and the pulses were monitored using a Tektronix oscilloscope, and the laser flash was detected using a Thorlabs DETa Si detector. For the higher frequency experiments, a laser Continuum Minilite II was used as the photoexcitation source at 532 nm and 20 mJ, or 355 nm with 3-4 mJ.

2.3 References

Chapter 3  
Development of continuous-flow EPR

Important biological events, such as reactions of vitamin B$_{12}$ derivatives that are the subject of a later chapter, involve the generation of short-lived radicals. These radicals cannot be easily observed at room temperature because of their transient nature. Therefore, the design and application of continuous-flow EPR apparatus was needed to enable the study of these transient radicals. The continuous-flow EPR method allows the replacement of reactants within the resonator, with the new sample being excited with each laser flash. It enables accumulation of the signal derived from paramagnetic species and sample averaging.

During the design of the experiment, several components had to be considered. First, the flow system for the resonators used for the time-resolved EPR studies and loading of the sample had to be developed. We tested the new flow system with a known electron transfer reaction that generates radicals with a lifetime of a few $\mu$s. Afterwards, pulses used to trigger different parts of the setup were defined in relation to each other to ensure minimum dead-time during the Fourier transform (FT-EPR) experiment. When the dead time was defined, the continuous-flow EPR setup was ready to be used to study photochemically generated short-lived radical species in biological systems.

3.1 Continuous-flow EPR setup

Due to the dimensions of typical pulse resonators (split and dielectric), commercially available products for obtaining flow through the active zone of the resonator, such as flow and flat cells, cannot be used. Therefore, the flow cell for this system was developed using customised quartz tubing, with both ends connected to silicone tubing. Constant delivery of fresh sample into the active zone of the EPR resonator needed to be maintained when using continuous-flow and
various factors had to be considered, such as pump design, quartz diameters and flow rate. The following sections give a summary of the main considerations.

3.1.1 Pump

The pumping system has to provide a pulseless flow in order to avoid any influence on the EPR signal. Therefore, literature research on pumps used for different flowing techniques was conducted. It was found that syringe pumps provide pulse-free flow and have been used in different experiments for continuous-flow measurements. [1-3] However, since the systems that we would like to study are air sensitive, using syringe pumps was not a convenient solution. A second option that satisfies the condition of pulseless flow was the use of HPLC pumps. [4] Therefore, the pump used for flow flash EPR studies was a reciprocating single piston HPLC pump.

The HPLC pump was suitable for these types of experiments, not just because of its pulseless flow, but also because it required smaller sample quantities, as thinner tubing can be used. This characteristic makes it convenient for working with biological samples where often only small sample volumes are available. One of the other advantages was the ability to keep the sample under anaerobic conditions during the measurement, as the sample vessels can be purged with Ar gas during delivery of the sample. When using the HPLC pump, it is important that the pump head cannot run dry and, therefore, priming of the pump has to be done. The best solution for this experiment was to pump the solvent completely through the tubing system.

3.1.2 Quartz

Several dimensions of quartz tubing were used, depending on the experiment that was conducted (Figure 3.1). The main constraint that determined the size of tubing required was the solvent used for the studied system. Solvents that have high dielectric constants can lower the resonator Q and cause loss of microwaves. In order to overcome this we reduced the volume of the solvent inside the resonator by using narrower tubing. Generally, resonator coupling is important in both direct-detection EPR (DD-EPR) and FT-EPR; for DD-EPR experiments the
resonator has to be critically coupled and for FT-EPR it must be overcoupled (Chapter 1, section 1.2.2.1). Care must be taken in both experiments to ensure adequate efficiency of the resonator. It was found to be particularly important in DD-experiments to use a smaller inner diameter of the quartz. Testing experiments (see Section 3.2), showed that for aqueous solutions at 9 GHz, quartz tubing of O.D. 3 mm and I.D. 1 mm was optimal for FT-EPR experiments. Flow through the 34 GHz resonator needed longer quartz tubing connected to the PEEK tubing. The O.D. of this tubing was 1.6 mm, which can be used for some alcohol solvents. However, for aqueous solutions, the inner diameter had to be smaller. For experiments with methylcobalamin (MeCbl) in water, a capillary with O.D. of 0.5 mm was used, which displayed minimal influence on the mechanical movements of the resonator. A summary of the quartz dimensions used for different experiments is shown in Table 3.1 and Figure 3.1.

**Table 3.1.** Dimensions of the quartz tubing used for different EPR experiments with calculated flow rates.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>O.D. / mm</th>
<th>I.D. / mm</th>
<th>Flow / ml min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 FT-EPR</td>
<td>1.6</td>
<td>1.2</td>
<td>2</td>
</tr>
<tr>
<td>2 FT-EPR_{aqueous}</td>
<td>3</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>3 DDEPR</td>
<td>1</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>4 34 GHz</td>
<td>1</td>
<td>~0.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**Figure 3.1.** Quartz tubing used for different experimental setups presented in Table 3.1. 1-4 is correlated to the numbers in the table, which present different experiments.
The next step for designing a flow experiment using EPR was to determine the flow rate, which is dependent on the quartz tubing used for the experiments. The calculation was based on the area of the laser beam, thickness of the sample exposed to the laser flash, and repetition rate of the laser. A summary of the flow rates used in the experiment using different dimensions of quartz tubing is also summarised in Table 3.1. An example of the calculation of flow rates for DD-EPR experiment is presented below:

Volume of the sample in 0.74 mm ID tube exposed to the laser flash (beam size 3 mm):

$$V = \frac{r^2 \pi h}{2} = \frac{(0.74)^2 \pi \times 3}{2} = 1.29 \text{ mm}^3$$  \hspace{1cm} [Equation 3.1]

For the repetition rate of the laser 10 Hz:

$$Q = 1.29 \text{ mm}^3 \times 10 \text{ Hz} = 12.89 \text{ mm}^3/s = 0.77 \text{ ml/min}$$

3.1.3 Sample vessel

Samples were prepared in an anaerobic glove box. A three neck bottle was used as the sample vessel, closed with Suba-seals to keep the sample anaerobic during the measurement. One of the bottle necks was used as a port for degassing the sample during the measurement. The sample was purged with argon for five minutes before the experiment and during the measurement. Figure 3.2 presents the continuous-flow EPR setup with the flow cell (2) inserted into the resonator (1 and 3), sample vessel (4) and dimension of the EN5107D2 35 GHz EPR resonator (5).

A sample rod was also modified to allow low temperature continuous-flow experiments since the resonator has to fit into the cryostat. The setup for it is shown in Figure 3.2 (1).
Figure 3.2. 1) Flow cell inserted in the dielectric ring resonator for low temperature continuous-flow experiments, 2) Quartz tubing connected to the silicone tubing, 3) Flow cell from 2 inserted into the dielectric resonator for RT measurement, in situ, 4) Three neck round bottom flask used for the experiment, purged with Ar, 5) 34 GHz resonator

3.2 Test reaction

The system used as a test reaction for flow through the dielectric ring resonator (ER4118X-MD5) was electron transfer between the photoexcited triplet state of duroquinone and amine present in the solution, which has previously been studied by EPR. [5] The reaction was followed at 9 and 35 GHz EPR using the continuous-flow EPR setup with quartz dimensions of O.D. 1.6 mm. Photoexcitation was conducted with a Nd:YAG Minilite laser source, operating at a 355 nm wavelength and a pulse energy of 4 mJ.

The initially generated singlet state undergoes a process of intersystem crossing resulting in formation of a triplet state. Depending on the environment, the photoactivated triplet state can be deactivated by proton or electron transfer, depending on the properties of the solution. With amine present in the solution several reactions of electron transfer, hydrogen transfer and escape from radical cage occur resulting in an equilibrium between the duroquinone radical anion and the ground state of duroquinone. In the system studied here, the formed triplet state is reduced by electron transfer from triethylamine present in the solution. The final result of this reaction is the generation of the duroquinone radical anion observed in EPR. Its formation was followed firstly by CW EPR, then with free induction decay (FID) detection. Even though the CW EPR measures in the
millisecond domain, it is useful to find the resonance field and afterwards to proceed with FID detected FT-EPR. The duroquinone radical anion has twelve equivalent protons but not all thirteen lines in the EPR spectrum can be observed due to the low intensity of the outer lines and probably a bandwidth issue to excite all the lines (Figure 3.3).

3.2.1 9 GHz Fourier transform EPR

The photoexcitation reaction of duroquinone was followed using FT-EPR at a fixed field value (Figure 3.3). Figure 3.3A presents a time domain spectrum used to determine the optimum power level for the experiment. The spectrum shows the duroquinone radical anion has a lifetime of approximately 3 μs. Conversion of the time domain spectrum to the frequency domain results in a spectrum with eight clear peaks (Figure 3.4B). The hyperfine coupling constant of 5.40 MHz obtained in this experiment is comparable to previously published results. [5]

From the previous study it is known that the frequency domain spectrum is in emission at early times. Measuring at longer delay times, the signal of the duroquinone radical changes from emissively polarised to absorptive. [5] A similar result was observed in this study, with the generation of an emissive spectrum (Figure 3.3B), intensity of which decreases at later times, which could indicate a change towards the absorptive spectrum (Figure 3.4B). The frequency domain signal has an asymmetry, with the low frequency side enhanced compared to the high frequency side. The asymmetry can be assigned to the radical pair mechanism (RPM, as described in Chapter 1, Section 1.3). The obtained information confirmed that the multiplicity of the precursor molecule is a triplet state. The observed results are in the agreement with the previously published data. [5]
**Figure 3.3.** A) Power dependence of duroquinone radical anion in FID detection. B) FT-EPR spectrum of duroquinone radical anion at 7 dB. Sample: duroquinone + TEA in propan-2-ol. Experimental conditions: 345.8 mT centre field, wavelength 355 nm, pulse energy 4 mJ, repetition rate 10 Hz.

### 3.2.2 35 GHz FT-EPR

The test reaction was used to measure at higher frequency EPR as well. The power dependence experiment was conducted in order to optimise the power level for the experiment at 35 GHz. The results are presented in Figure 3.4A. The final setup used for FT-EPR measurements at 35 GHz was a microwave attenuation of 0 dB and 20 ns microwave pulse. The signal of the duroquinone radical anion was followed in the time domain, while changing the position of the microwave pulse with respect to the laser. After transforming the spectra to the frequency domain, seven lines were observed. It was also observed that the resonance position of certain peaks changed with the increasing changing delay times (Figure 3.4B). The spectra indicate a change from emission to absorption with the increasing delay after flash (DAF), and were consistent with that shown in the literature.
3.3 Triggering setup

One of the reasons for the development of the continuous-flow apparatus is for each laser pulse to excite new sample, allowing averaging and the detection of short-lived radicals. In order to observe these radicals the dead-time had to be kept to a minimum. Therefore, the initiating laser light and EPR spectrometer had to be synchronised. A delay generator was used to precisely trigger both the laser and spectrometer in order to achieve a short dead-time following the reaction. The pulse positions and the experimental setup are described below, with the experimental measurements at two different EPR frequencies.

3.3.1 Pulse positions

A Stanford DG535 delay generator was used in order to define correct timings between the pulses. An external trigger box was used to trigger the laser flash lamp and Q-switch, as well as the spectrometer. Figure 3.5 presents the positions of the pulses in the experiment. Pulses sent from the delay generator were followed using a Tektronix oscilloscope, and the laser flash from Continuum Minilite II or Brilliant blue, was detected using a Thorlabs DETa Si detector. The trigger for the flash lamp was held at a constant value and can be considered as time zero. The position of the pulse triggering the Q-switch varied slightly (+/- 5ns) depending on the laser power, typically it was around 331 μs after the flash lamp for 20 mJ laser power.
The position of the pulse triggering the spectrometer was changed with respect to the laser flash depending on the desired DAF (Figure 3.5, lower panel). The laser flash was monitored using a photodiode as mentioned above. The spectrometer has to be fired first (ahead of the Q-switch) for a DAF < 320 ns because of the through-put time of the instrument to generate the microwave pulse.

![Diagram of pulse sequence](image)

**Figure 3.5.** Positions of the pulses in the experimental setup. Top panel: delay region (solid grey) is the region that can be varied depending on the desired DAF, with indicated pulse lengths. Lower panel: pulse sequences illustrating the observed pulses on oscilloscope – pulse triggering spectrometer (yellow), Q-switch trigger (blue), laser flash (green), the microwave pulse in Signal Channel after quadrature detection (red); delay between red and green can be varied and determines the DAF.

Positions of the microwave pulses were recorded on the oscilloscope (Figure 3.6) in order to define the exact timing of events including the first time point where the signal can be detected. The spectrometer allows various points to monitor the microwave pulse; the transmitter monitor (TM) is a spectrometer
channel detecting microwave pulses going to the TWT (Travelling Wave Tube) before the resonator, whereas the receiver monitor (RM) detects the pulse that is reflected from the resonator. [6] The Signal Channel is after quadrature detection and contains the EPR FID signal. The measurements showed that there was a difference of 40 ns between the TM and the Signal Channel (Figure 3.6). This causes some uncertainty about the precise point the micro pulse contacts the sample because of the delays through amplification and detection. Measurements were then made to monitor the signal with delay time.

\[\text{Figure 3.6. A) Oscilloscope traces showing the difference between positions of the laser flash and microwave pulse in the Signal Channel. Position of microwave pulse observed in signal channel (red) regards to positions of laser flash (green). The x-axis is a relative time in units of nanoseconds. Presented separation corresponds to the DAF 80 ns. Positions set on the delay generator: Q-switch trigger = 351 \mu s after flash lamp, spectrometer trigger = 261 ns before the Q-switch trigger. B) The difference spectrum between the transmitter, receiver monitor and Signal Channel.}\]

### 3.3.2 Measurements of delay after flash (DAF)

The pulse positions between the laser flash and microwave pulse were changed in order to experimentally monitor the time evolution of the spectra from the generated free radicals. In the research experiments, the photo-generated species was typically the methyl radical formed by photolysis of methylcobalamin. Several pulse positions were used, ranging from applying the microwave pulse before the laser flash, overlapping of the two pulses, and increasing the time between the laser and following microwave pulse. After obtaining these spectra, information on
the optimum DAF for the experiment was obtained and the measurement can be conducted at the optimal time for radical generation.

3.3.2.1 9 GHz EPR

The intensity of the FT signal of the highest intensity peak line of the methyl radical was followed while changing the DAF in order to determine the first appearance of the signal. The experiment was conducted under different solvent conditions and laser wavelengths and is presented in Figure 3.7. Figure 3.7A presents the different time domain spectra obtained after changing the DAF. It was observed that after the initial appearance of the signal, increasing the DAF decreases the signal intensity. This trend is highlighted in Figure 3.7B and we can conclude that the maximum signal appears at DAF of 80 ns. This DAF originates from the relative distance between the laser flash (Figure 3.6A, green) and the microwave pulse observed in Signal Channel (Figure 3.6A, red) corresponding to 120 ns. The 80 ns is, then, calculated by subtracting 40 ns from 120 ns (Figure 3.8B), and represents the DAF calculated from the difference between the laser flash and TM mode. This accounts for an internal dead-time caused by the detection method reflected in the absence of signal at early time in Figure 3.7B.

![Figure 3.7](image)

**Figure 3.7.** A) Time domain spectrum of the -31 MHz peak with varying DAF. B) FT amplitude of the highest intensity peak (-31 MHz peak) dependent on the time difference between the laser flash and the microwave pulse in the Signal Channel, after the quadrature detection. Zero time is the time when two pulses overlap, or there is no time between them. Spectra are obtained from 0.5 mM MeCbl in HEPES, pH 7.5, 50 % glycerol after photoexcitation at 532 nm, 20 mJ pulse energy, at room temperature.
3.3.2.2 35 GHz FT-EPR

The same experiment was repeated on a 35 GHz EPR spectrometer, since a different laser source was used. The Continuum Minilite II was the laser used for these experiments and was also triggered externally using the same delay generator. The second harmonic of the laser was used for the excitation since it can provide us with sufficient power at 532 nm. The delay between the flash lamp and the Q-switch, which provides the least jitter, was previously established to be 167 μs. The time between the laser flash and the microwave pulse was varied in order to determine the optimal delay time. The experimental results are presented in Figure 3.8.

![Figure 3.8. FT amplitude of the highest intensity peak (~31 MHz peak) on variation of the DAF. Experimental conditions: 1 mM (A) or 0.5 mM (B) MeCbl in HEPES, pH 7.5, 50 % glycerol; photoexcitation at 532 nm, 20 mJ pulse energy, at room temperature.](image)

The intensity of the signal increases at approximately the same rate as in the 9 GHz EPR experiment. The highest intensity peak is at ~ 100 ns. After that, interestingly, intensity remains relatively constant over a longer period of time. This could be caused by the fact that the relaxation properties of the radical changes with an increase in the frequency. Increasing the frequency of the experiment increases the spin-lattice relaxation time, which could result in the longer lifetime of the radical.
3.4 Determination of signal phase

Since the spectrum of the methyl radical, as one of the studied radicals in this thesis, exceeds the bandwidth of the technique, each of the four resonance lines had to be recorded separately. Therefore, to investigate the correct phase of each of the resonance lines, a comparison was made to a published study of the benzophenone (BP)-ketyl and propan-2-olyl radicals, which were recorded using FT-EPR. [7] They were shown to have polarisation characteristics from the RPM in emission/absorption spectra in addition to the triplet mechanism (TM), which is indicated in the emissive spectrum of BP-ketyl radical. Results after photoexcitation of 40 mM benzophenone in propan-2-ol using 355 nm, 20 mJ laser light at DAF 120 ns obtained using the continuous-flow FT-EPR experiment are presented below (Figures 3.9 and 3.10). The spectra were obtained using PulseSPEL with 100 transient averages, 4-step phase cycling (same setup in the software that was used for the experiment with MeCbl). The signal was phased in order to get the microwave pulse in a positive upward direction on the oscilloscope in the Signal Channel as an internal phase reference point.

Data were recorded at five different field positions, which were chosen to show the propan-2-olyl radical (resonance lines observed at 3412.15 G, 3432.15 G, 3471.7 G and 3491.7 G) and BP-ketyl radical (at 3451 G). The raw data for the propan-2-olyl radical at this field position showed opposite phase, where the lower field lines were in A and the higher in E. There was a clear change between phases at different field positions (Figure 3.9). The phase of BP-ketyl radical showed the same trend, where the FID was in absorption. The experiment was repeated using direct detection and resulted in the same E/A pattern. Therefore, by comparison to the expected polarisation pattern (above), in order to build the EPR spectrum, intensities were multiplied by -1 (Figure 3.10) when the microwave pulse was phased in a positive upward direction.
Figure 3.9. Time domain spectra of four different field positions of the propan-2-olyl radical. The FIDs were obtained after photoexcitation of 40 mM benzophenone in propan-2-ol at 355 nm, 20 mJ.

Figure 3.10. FT-EPR spectrum of BP-ketyl radical (central line) and propan-2-olyl radical (outer lines) built, after obtaining a FID at each field position. Experimental conditions: 40 mM benzophenone in propan-2-ol at 355 nm, 20 mJ.

3.5 Conclusions

The main aim of this chapter was the development of a flow system, which will enable the study of B12 dependent coenzymes, using TREPR. Having established which pumps might be best suited for the flow system known chemistry was used to test performance. The experiments resulted in the testing of a full array of quartz
tubing to be used in different microwave resonators; quartz tubing of various dimensions was exploited. Next, measurements were carried out to study the dependence of the intensity of signal on the DAF. These experiments provided an insight into the rise of the signal and the earliest generation of paramagnetic species. Finally, using the new apparatus, the photochemistry of B₁₂ coenzymes can be examined using different transient EPR techniques and at two different EPR frequencies.

In summary, Figure 3.11 presents the simplified version of the experimental setup for the continuous-flow EPR studies. The main components of the continuous-flow apparatus are HPLC pump and the delay generator used to coordinate the pulse timings between triggering the laser and the spectrometer. This pulse sequence was followed using the oscilloscope to get the exact information on the timings in the experiment.

![Figure 3.11](image.png)

**Figure 3.11.** Continuous-flow EPR setup developed for time-resolved EPR studies. The EPR spectrometer is simplified showing only the resonator and the magnets. Pulses that are sent from the delay generator to the laser and the spectrometer are followed on the oscilloscope using BNC cables.
3.6 References


Chapter 4

Time-resolved Fourier transform EPR study of the photo-reduction of duroquinone by chloroquine in 2-propanol

4.1 Introduction

Malaria presents a risk for the world population with 212 million malaria cases in 2015, and an estimated 429 000 malaria deaths. [1] Chloroquine 5, a 4-aminoquinoline, is a medication used to prevent and to treat malaria but its mechanism is still not fully understood although the critical step of drug binding to heme is generally accepted by investigators. [2] 5 is favourable because of its price, and more importantly it is safe for infants, children and pregnant women. [3] It is also used in the treatment of autoimmune diseases such as rheumatoid arthritis. However, it is known to possess undesirable phototoxic properties that produce dangerous side effects both in the skin and in the eyes such as Bull’s eye maculopathy. [4, 5] Another example of potential phototoxicity is in the 5 melanin complex observed and reported by Buszman et al., [6] involving formation of a charge transfer reaction. In this experiment it was demonstrated that binding of 5 to melanin was responsible for free radical formation in melanin.

The negative side of using 5 as a drug in malaria treatment is the growing parasitic resistance to it. [2, 7] Previously, it was reported that the resistance to 5 and quinolines originates in drug accumulation in digestive vacuole of an insect. [8] The exact mechanism was not clear. A different study, however, implied there was accumulation of undigested hemoglobin in the parasite. [2] In 2001 Wellems and Plowe [9] reported that malaria resistance is caused by a mutation in the expression of a protein transporter in the digestive system of the parasite, when comparing two types of parasites. This provided better insight into the resistance to 5 treatments. However, in 2006 Laufer et al. [3] in their clinical trial observed the return of the efficacy of 5 in malarial treatment in Malawi. The study compared
current anti-malarial treatments including sulfadoxine and pyrimethamine against 5 where 5 was more effective. However, it is still not recommended to be used as a stand-alone drug because of the potential return of resistance, which was observed in other African countries. It is considered as a good potential drug, which could be used in combination therapies.

Chloroquine is known to absorb mainly UVA and UVB light. [10] However, the mechanism is unknown, with singlet oxygen, superoxide and peroxyl adducts being invoked as possible causes of the phototoxicity. Here, we examine the structure of 5 to investigate an alternative pathway to phototoxicity through electron transfer to photoexcited triplet (T) states. The photoexcited T state is less stable than the ground state and can be easily oxidised or reduced and, therefore, can be quenched by reactions of reduction or oxidation, depending on the redox potential. Amines can be electron donors and have been used as electron transfer agents in these types of reactions. [11] Our study involves an electron transfer study between the T state of duroquinone (DQ) and 5 and several other potential antimalarials containing the amine group. There have been studies presenting the photo production of amine radicals and later involvement in electron transfer reactions. [12, 13] Chiavarino et al. [14] described oxidation of tertiary amines. This process occurs in catalysis with heme enzymes with the electron transfer and formation of a cation radical followed by deprotonation resulting in the formation of a neutral radical. Hu et al. [11] also reported amine radical cations formed by oxidation of amines. The oxidation was followed through using UV and visible mediated photochemistry.

Steady state methods and time-resolved techniques such as laser photolysis with EPR detection have been used to investigate the photoreduction of quinones by amines. [15-18] Guttenplan and Cohen [17] were the first to propose that the primary reaction of the photoreduction was an electron transfer from amine in its ground state to the quinone T, forming a charge-transfer complex. McLachlan and co-workers carried out the first photo-CIDEP studies of the photoreduction of DQ by triethylamine (TEA) in 2-propanol solution. [19, 20] They observed the chemically induced electron polarization (CIDEP) of duroquinone radical anion by
transient direct detection EPR in the microsecond time regime. The full mechanism of the reaction was later understood by Lu and Beckert using Fourier transform (FT) EPR spectroscopy by detection of all the relevant paramagnetic intermediates. [21] The initially photo-generated singlet state of DQ undergoes the process of intersystem crossing, which allows transition from the short-lived generated singlet state to the longer lived photoexcited T state. Depending on the environment, the T state can be scavenged by protons or electron transfer, depending on its surroundings. Lifetimes of a T state were within the time-scale of the electron transfer reaction with oxidising agent. One such agent can be amine. [11]

In this work, we use FT-EPR to investigate electron transfer of 5 and its derivatives in the presence of DQ in 2-propanol solution on response to laser excitation at 355 nm. Although, the scavenging reactions of DQ have been reported previously [21, 22], here we studied the potential mechanism and generation of radicals using antimalarial drugs as a source of amine in the solution. In these experiments we observed formation of duroquinone radical anion (DQ•−) and durosemiquinone radical (DQH•), depending on the drug used. The cation radical was not observed. Derivatives of 5 were used to study the source of the electron transfer to the DQ in terms of functional groups. Overall motivation stems from the cause of the phototoxicity of 5 and evidence sought for potential to undergo electron transfer.

4.2 Results

The molecules used in this study are shown below in Figure 4.1. Details of the experimental set-up and materials can be found in Chapter 2, Sections 2.1.1 and 2.2.4.
4.2.1 Control experiments

Figures 4.3 and 4.4 represent control experiments in this study. They show time and frequency domain spectra after photoexcitation of DQ in isopropanol in the presence and absence of amine. Without the TEA present in solution, the neutral DQH\(^{•}\) radical was detected and is presented in Figure 4.2. The DQH\(^{•}\) radical is generated by abstraction of a hydrogen atom from the solvent. Three groups of septets could be observed from the expected septet of septets. The other weaker resonance lines were not well-resolved under the experimental conditions at room temperature. The observed hyperfine values were comparable to the previously published values shown in Figure 4.3.
Figure 4.2. DQH$^\cdot$ radical generated after photoexcitation of DQ in 2-propanol using 355 nm laser light. A) The free induction decay (FID) of the radical. B) The Fourier transformed EPR spectrum of the radical. Sample: 20 mM DQ in 2-propanol. Experimental conditions: centre field 344.0 mT, 355 nm Nd:YAG Minilite, power 4 mJ, 10 Hz.

![Figure 4.2]

Figure 4.3. Structures of DQH$^\cdot$ and DQ$^\cdot$ with their respective hyperfine values.

However, with the amine present in the solution, the T state of DQ is reduced by the amine group. Figure 4.4 displays the observed spectrum showing seven lines of DQ$^\cdot$. Not all thirteen lines from the protons can be observed; however, the observed proton hyperfine value of the radical of 5.2 MHz is comparable to the previously published value for this radical. [21] The left panel of Figure 4.4 shows the time dependence of the signal of DQ$^\cdot$ measured using delay after flashes (DAFs) ranging from 0.08 to 104 μs. The right panel presents FT-EPR spectra of the radical for the same range of time. Signal intensity is in emission at early reaction times. The signal changes to absorptive after a 2 μs delay. At early DAFs it was also observed that the signal intensity was enhanced on the high frequency side. This enhancement was lost with the later delay times where the signal shows Boltzmann intensity. The decrease in initial Boltzmann intensity was caused by the back transfer reactions in radical pair (Chapter 3, Section 3.2). [21]
Figure 4.4. DQ$^-$ generated after photoexcitation of DQ with TEA present in the solution using 355 nm laser light. A) The FID of the radical in the range of time from 0.08 to 104.08 μs. B) The FT-EPR spectrum of the radical in the same time range. Sample: 20 mM DQ with 20 mM TEA in 2-propanol. Experimental conditions: centre field 344.4 mT, 355 nm Nd:YAG Minilite, power 4 mJ, 10 Hz.

The control experiments described above served as an indicator of the type of radical that is formed upon photoexcitation of the system. The information gained from these experiments can help to elucidate the reaction pathway that occurs when different potential antimalarial drugs are used in the FT-EPR study.

4.2.2 Reaction with the antimalarials in the system

The same experiment of photoexcitation of solution with DQ and potential electron donor was repeated with several of the antimalarial drugs shown in Figure 4.1. The compounds were chosen depending on the presence or absence of an amine group. The possibility of electron transfer between DQ and one of the drugs can be concluded from the FT-EPR spectra depending on whether DQH$^+$ or DQ$^-$ radical is observed. Compounds 7-9 were initially tested and Figure 4.5 displays representative spectra. The spectra showed the presence of DQH$^+$ by the observation of seven groups of seven lines. Again, due to the experimental conditions at the room temperature not all the lines were completely resolved (Figure 4.5). The spectra show pronounced distortion because of CIDEP effects, which is not the subject of this study.
Figure 4.5. FT-EPR spectra of DQH• radical generated after photoexcitation of DQ with three different compounds (Figure 4.1) present in the solution using 355 nm, 4 mJ laser light. Sample: 20 mM DQ with 20 mM of compound 7, 8 or 9 in 2-propanol.

Formation of emissive DQH• was observed for the three studied compounds presented in Figure 4.5. This was expected for the compounds 7 and 9 but this observation was interesting for the compound 8 where there is an amine in the structure but formation of DQH• was still observed.

Chloroquine 5 was studied under the same conditions. Figure 4.6 displays spectra obtained after photoreduction at 355 nm laser light of DQ with 5 (Figure 4.1) present in the solution. The FT-EPR spectra contain seven visible resonance lines of the possible thirteen with a hyperfine coupling constant of 5.3 MHz, which is comparable with the previously published hyperfine values for DQ•-. [18] The time domain spectrum reveals DQ• with a lifetime around 2 μs, similar to the $T_1$ published, previously. [21] The spectra can be compared to the control experiment (Figure 4.4) and confirms formation of the same radical species. The generation of DQ• radical is most likely caused by electron transfer from the amine group of chloroquine after photoexcitation. From Figure 4.6, it is clear that radical signal is in emission up to about 4 μs delay time. Afterwards, it changes to absorptive signal at later times. This is most likely caused by the triplet mechanism that occurs in the reaction. [22] Also, small asymmetry in the spectra was observed. This could be caused by the RPM.
Figure 4.6. Staggered FT-EPR spectra of DQ•⁻ at delay times ranging from 0.08 to 80.08 μs in 2-propanol. Sample: 20 mM DQ, with 20 mM 5 in 2-propanol. Experimental conditions: centre field 349.8 mT, 355 nm Nd:YAG Minilite, power 4 mJ, 10 Hz.

The FT-EPR experiment at 355 nm was repeated using the novol diamine, 3 (Figure 4.1). After photoexcitation of this system, formation of DQ•⁻ was observed. The FT-EPR spectra start with an emissive signal at early times, with the highest signal intensity at a delay of 1 μs. At early time there is intensity enhancement of the high field side compared to the low field side. The signal is emissive up to about 3-4 μs. After a delay time of 4 μs, the signal changes to absorptive. At later times, there is a smaller difference between the signal intensity of the high and low field side. Overall, the signal intensity is stronger by ~1.5 times than found on use of 5 for all studied delay times. It appears that the signal is long lived since it can still be observed at a delay time of 80.08 μs with good signal-to-noise. The changes of the signal from emissive to absorptive occur at about the same time point for both compounds 5 and 3.
Figure 4.7. Staggered FT-EPR spectra of DQ$^-$ at delay times ranging from 0.08 to 80.08 μs in 2-propanol. Sample: 20 mM DQ with 20 mM 3 in 2-propanol. Experimental conditions: centre field 349.4 mT, 355 nm Nd:YAG Minilite, power 4 mJ, 10 Hz.

The study was expanded to further include compounds with molecular functionality similar to 5. Figure 4.8 presents the results of the experiments using 4,7-dichloroquine 2 and PGEAM#97 6 in the solution with DQ. Figure 4.8A for 2 shows the emissive FT-EPR spectrum after formation of DQH$^*$. There was a slight shift in frequency caused through observing at a frequency that was not perfectly on resonance. However, information on the formed radical still can be obtained. The hyperfine values were comparable to the control study with no amine present in the solution and, therefore, no evidence of electron transfer was found (Figure 4.3). However, if there 6 was present in the solution (Figure 4.8B), the result was different. The FT-EPR spectrum was still emissive but it appears that the spectrum displays a mixture of DQH$^*$ and DQ$^-$ radicals by comparison of the hyperfine values. This spectrum is similar to the spectrum obtained using a lower concentration of TEA as an electron donor compared to DQ as shown in the work of Lu et al. [21]. This could imply that there is an electron transfer occurring in the system but at a slower rate compared to when there is a tertiary amine present in the solution.
4.2.3 Concentration dependence experiments

The photoreduction of DQ was investigated by varying concentrations of chloroquine 5. From the previous concentration studies with DQ and TEA [21] it was observed that at lower concentration of the amine both semi-reduced and fully reduced quinone was observed. In this system, the 5 concentration dependence was followed in the range from 1 mM to 100 mM 5. The results are presented in Figure 4.9. At concentrations lower than 10 mM, DQH• and DQ• were superimposed in the emissive FT-EPR spectra (Figure 4.9A, 1 mM and 5 mM). It was observed that on increasing the amine concentration from 1 mM to 80 mM 5, the DQ• radical dominates and was the sole radical observed (Figure 4.9A). This result is comparable to the previous study with TEA as an electron donor. [21]

Figure 4.9B displays a graph of the FID intensity of the central resonance at 0 MHz at the different 5 concentrations. At 5 concentrations above 40 mM, the FID intensity tended to saturate. This was an indication of spectra starting to have Boltzmann distribution of spins and, thus, fewer enhancements through polarisation. Also, the plateau is comparable to the study with DQ and amine in the solution [21] and explained by the yield of the reaction being at an equilibrium between the electron transfer to the formed radical pair and hydrogen abstraction from the solvent cage.
4.3 Discussion

Mechanism of antimalarial drugs is still not fully understood or their apparent phototoxicity. In this study, FT-EPR was used as a technique to investigate reaction pathways of potential antimalarial drugs. Compounds that were different variations of the structure of quinoline drugs were used in order to understand their electron transfer properties. [7] Previously, it was claimed that mechanism of drug action and phototoxicity can involve formation of free radicals by electron transfer. [24] It was found that if there was a tertiary amine present in the solution, DQ$^-$, was observed (Figures 4.6 and 4.7). However, with a secondary amine, 6, formation of both fully reduced and semireduced radical was detected (Figure 4.8B). Without the amine present in the solution, only DQH$^+$ was detected (Figures 4.5 and 4.8A). This is consistent with the formation of a stable cation in the presence of a tertiary group.

Experiments with compounds 2, 7, 8, 9 did not show formation of DQ$^-$. Considering the structure of these compounds this result was expected, except for compound 8 which is a tertiary amine. It suggests the presence of the aromatic ring does not favour the oxidised form in this case; preferring to keep its aromaticity.

Compounds 5 and 3 both produce DQ$^-$ as does TEA. The same alkyl amine group (end tail), in both structures of 3 and 5 makes it the likely site of the electron
transfer. In the time dependence measurements on the DQ\(^{\cdot}\) generated after photoexcitation of the system with 5 or 3, a similar pattern was observed for both compounds. The spectrum at earliest time appeared in the emission. Increasing the DAF caused the signal to change from emissively polarised to absorptive (indicating change in population of T levels, from T\(_{-1}\) to T\(_{+1}\)). Interestingly, it is known from previous work that the "antimalarial tail" of 3 has the ability to form radicals. [24]

On increasing the concentration of 5, additional electron transfer molecules were added to the reaction route and it appeared that the drug reacted faster at higher concentrations. This caused the electron transfer reaction to dominate at higher concentrations, and resulted in the observation of only DQ\(^{\cdot}\) (Figure 4.9). The amount of the DQ T state deactivated by electron transfer varied with changing concentration of 5. At lower amine concentrations, the electron transfer reaction for T deactivation was in a competition with spin relaxation and hydrogen abstraction from the solvent. Therefore, both DQH\(^{\cdot}\) and DQ\(^{\cdot}\) were observed (Figure 4.9, left). Further increases in the amine concentration caused the DQ\(^{\cdot}\) to dominate, which implies a faster rate of electron transfer. The intensity increase of the DQ\(^{\cdot}\) with increase of the concentration of 5 was in the agreement with previous studies with TEA as an electron donor to DQ in methanol. [21, 22] The intensity increase was greater than for the system with TEA in the current study. The signal of the cation radical was not observed in any of the studied systems, possibly because of a fast spin-spin (T\(_2\)) relaxation time. However, the absence of this signal could be caused by the secondary reaction of cation with DQ.

4.4 Conclusions

The study shows for the first time that antimalarials drugs can undergo electron transfer on photoexcitation of DQ. Several potential antimalarial drugs were used in order to assess their ability to act as an electron transfer agent. It was observed that with a secondary or tertiary amine in the solution, formation of the radical anion was detected. However, in the case where the drug does not contain the amine, the neutral radical was detected and, therefore, the triplet was
deactivated by the hydrogen transfer from the solvent. Thus, the prerequisite for
electron transfer is the presence of the amine tail. The application of photo FT-EPR
made it possible to follow the time profile of the reaction. The results suggest a
new route for phototoxicity through photoexcitation of quinone molecules present
in biology. Further experiments are needed to test this hypothesis and measure
kinetics, as well as with the chemistry of the 5 melanine complex. These
experiments might also provide us with further insight into the mechanism of
antimalarial drugs, which are not fully understood and may operate using electron
transfer.

4.5 References

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Chapter 5

Photolysis of cobalamins followed by continuous-flow EPR

5.1 Introduction

Biologically-active derivatives of vitamin B\textsubscript{12} can be fully synthesised by microorganisms. Food from animal sources is the only source of vitamin B\textsubscript{12} in human diet, since humans are not able to synthesise this vitamin. Therefore, the conversion of B\textsubscript{12} to metabolically active cofactors strongly depends on its uptake. Vitamin B\textsubscript{12} belongs to a group of corrinoids with a cobalt ion in its centre, therefore belonging to a group of cobalamins. [1] Cobalamins as cofactors are involved in several enzymatic reactions usually presenting a source of organic radicals. Cobalamins are present in DNA synthesising cells and cells of central nervous system. [2, 3] Therefore, cobalamin deficiency represents a potential risk for development of neurodegenerative diseases.

The structure of cobalamins contains the corrin ring with a six-coordinated cobalt in the centre (Figure 5.1). The corrin ring is quite flexible and its planarity changes depending on the upper axial ligand. [4] The lower axial ligand is 5,6-dimethylbenzimidazole (DMB) in the base-on form. The function of B\textsubscript{12}-dependent enzymes involved in metabolism depends on the upper axial ligand present in the cobalamin structure. [5] Even though there are only two B\textsubscript{12}-dependent enzymes in humans, there are many of them present in bacteria. They are divided into few major classes such as methyltransferases, mutases / eliminases, aminomutases and dehalogenases. [2] Methylcobalamin (MeCbl) and 5'-deoxyadenosylcobalamin (AdoCbl) are biologically active cobalt corrinoids relevant in metabolism. The upper axial ligand in MeCbl is a methyl group and in AdoCbl it is 5'-deoxyadnosyl (Figure 5.1). Six-coordinated cobalt, Co(III), is diamagnetic and has two axial ligands. Decreasing the number of axial ligands results in the formation of cobalt in different oxidation states, such as paramagnetic penta-coordinated cobalt, Co(II) or even Co(I), occurring in methyl transfer in methyltransferases reaction. The corrin ring
without axial ligands has diamagnetic Co(I) in its centre. [1] This implies that redox reactions of cobalamins include a change in the Co oxidation state, which is accompanied by a change in number of axial ligands. Redox reactions of vitamin B$_{12}$-derivatives are relevant for their biological functions, with Co-C bond breakage as crucial step. This can occur via two mechanisms: homolytic or heterolytic bond cleavage. [1, 5] Homolytic bond breakage occurs in enzymatic reactions with coenzyme B$_{12}$ (AdoCbl) as a cofactor whereas heterolytic mode occurs in reactions with MeCbl.

A crucial and first event in the biological activity of these cofactors is the cleavage of the Co-C bond after substrate binding, for both cobalamin dependent isomerase and transferase. In AdoCbl dependent enzymes, such as glutamate mutase, methymalonyl-CoA mutase and ribonucleotide reductase, which catalyse reactions of isomerization or reductions, the Co-C bond is subject to homolytic bond cleavage generating the 5'-deoxyadenosyl radical, Ado$^*$, and cob(II)alamin radical. This process is usually triggered by substrate binding. The radical generation is followed by hydrogen abstraction, radical rearrangement and regeneration of catalyst. [1, 5] Methionine synthase and DNA-methylase are examples of the MeCbl dependent enzymes, which have a reaction mechanism that differs from AdoCbl-dependent enzymes, even though their cofactors are structurally quite similar. The difference in mechanism starts with the first step of heterolytic cleavage of the Co-C bond, which results in reduction of Co(III) to Co(I) and transfer of the methyl cation. MeCbl is then regenerated by transfer of a methyl cation from donor molecules, such as methyltetrahydrofolate to cob(I)alamin. This transition of cofactor from hexa-coordinated Co(III) to tetra-coordinated Co(I) is accompanied by structural changes in protein environment. [1, 6, 7] The Co-C bond can also be cleaved by light where the same radicals as in the enzymatic reaction of AdoCbl enzymes are formed. In photolysis of Co-C bond in the MeCbl cofactor, methyl/cob(II)alamin RP is generated, not a methyl cation / cob(I)alamin ion pair as in enzymatic reaction.
Figure 5.1. Structures of B$_{12}$ derivatives with various upper and lower axial ligands. 5,6-dimethylbenzimidazole (DMB) as lower axial ligand is presented in green and two upper axial ligands for two studied cofactors in red. Base-off form of the molecule is presented on the right where DMB is replaced by water molecule (blue).

As previously mentioned, the photochemistry of MeCbl and AdoCbl results in cleavage of the Co-C bond and formation of the alkyl/cob(II)alamin radical pair (RP). The photophysical pathway to the reaction product is still the subject of different studies and presents an experimental and computational challenge. Transient absorption (TA) experiments of the photolysis of MeCbl show two dissociation pathways, dependent on the excitation wavelength (Figure 5.2). [8-10] After photoexcitation at 530 nm, a lower energy ion-pair like S$_1$ state is populated, where 85 % relaxes back to the ground state and 15 % forms a RP between the methyl and cobalamin radical. However, if the photoexcitation is conducted at 400 nm, a higher energy state is populated, which leads directly to prompt bond homolysis with a yield of 25 %. The remaining 75 % relaxes to the same S$_1$ state which then relaxes to the ground state or forms a RP, as after photoexcitation at 530 nm. Photolysis of AdoCbl did not show a wavelength dependence in both TA and computational studies. [9]
Figure 5.2. Scheme presenting the differences in photophysics of MeCbl and AdoCbl considering the excitation wavelength. The symbol H₃C⁺•Cob represents the methyl/cob(II)alamin RP, in the solvent cage when written with brackets. {H₃C⁺•Cob} represents the MLCT state. Rate constants k₁ and k₂ represent formation of geminate RP (k₁) or metastable photoproduct (k₂), k.escape describes escape from the radical cage, k.homolysis formation of RP from {H₃C⁺•Cob} and k.recovery its recovery to the ground state (adapted from Shiang et al. [9]).

Computational studies also confirmed different pathways occurring to different extents at different excitation wavelengths by following which bond between axial ligands elongates first for MeCbl. [4] Bond length is one of the parameters when following the structural change in cobalamins and it is dependent on the upper axial ligand. [4] Comparison of the ground and excited states of computationally predicted spectra shows that the Co-N_im bond length decreases whereas the bond between Co and upper axial ligand in AdoCbl and MeCbl extends prior to cleavage [11] but overall structural changes in photolysis of MeCbl are smaller. Theoretical studies of these molecules are challenging because of the sensitivity of input parameters, scalability, large structure of cofactors with many atoms, and mixing of π-orbitals of the corrin ring and d-orbitals of cobalt. [4] However, computational studies have identified the S₁ state as being a metal-to-ligand-charge-transfer complex, MLCT. [12] The lowest energy state, S₁ state is d_Co → π*corrin and π_corrin →
\[ \pi^*_{\text{corrin}} \] is character. [10] However, if the excitation of MeCbl was carried out at 400 nm, the higher energy state is populated and RPs are generated directly, with the remainder producing RPs via the MLCT state with a ratio of 3:1. [12] This was also observed in TA spectroscopy studies as described above. [9] Additionally, these studies looked into the potential energy surfaces (PES) as an indicator of reaction pathways, using time dependent-density functional theory, TD-DFT, because of complexity of the system. [10, 13] The calculations showed that the potential energy surface of \( S_1 \) state was close to the proximity of several energy surfaces of triplet (T) states and, therefore, the T state as an origin of the precursor molecule cannot be excluded. Also, the most recent TD-DFT studies observed a drop in energy of the T state, which implied that the origin of precursor molecule was a T state for both forms of MeCbl. [10] This appears to be not consistent with previous theoretical studies [13, 14] or magnetic field effect studies, which suggest a singlet (S) RP precursor. [15]

Studying Co-C photolysis properties in AdoCbl from a theoretical perspective is quite challenging because of size of the molecule and the interactions within it. By putting a ribose as the upper axial ligand, the \( S_1 \) state was identified as MLCT, as in MeCbl. [4] However, the experimental data show that the photoexcitation pathway is different. [9, 11] Therefore, new ways of identifying the lowest excited states will have to be determined. The possible reason for obtaining results similar to the \( S_1 \) of MeCbl is the higher density of excited states in AdoCbl, which are in close proximity to the \( S_1 \) state. [4] Later studies used the complete AdoCbl molecule in their computational approaches. [16, 17] TD-DFT studies used the \( S_1 \) state as the starting and the key point for generation of S-born RP in photolysis of AdoCbl. To relate computational studies with experimental model, the potential energy surfaces were studied. The \( S_1 \) state was identified as a MLCT state and the origin from which the homolysis starts. Afterwards, energetically favourable stretching of the Co-C bond occurs. Further on this path, bond homolysis occurs from ligand field (LF) state where the axial ligand is partially detached. [16]

So far, the base-on structure of a cofactor has been described, with DMB as lower axial ligand. B_{12} cofactors can also be found in the ‘base-off’ configuration,
where the coordinating nitrogen of the DMB is instead protonated and the lower axial ligand is a water molecule (Figure 5.1, right). This can be achieved in solution at low pH. [18, 19] Alternatively, many B₁₂-dependent enzymes bind ‘base-off/His-on’ structure, with a lower axial histidine. One example is human methionine synthase, which with MeCbl as its cofactor catalyses synthesis of methionine from homocysteine in cytosol. [1] Also, the intermediate state in the photolysis of AdoCbl, presented in the lower panel in Figure 5.2, has been identified as being in the base-off state, where the lower nitrogenous axial ligand was detached. [20] From the ultrafast photolysis studies it was observed that when the electronic structure of the cofactor is modified to the base-off form, a channel for non-radiative decay from excited state opens, which was clear from the great increase in rate for internal conversion. However, the main characteristics of this channel are not clear yet from computational studies. [4] What is identified so far is that in the base-off form the reaction yield depends on competition between internal conversion and bond photolysis. [18] This is different from the base-on form where reaction yield depends on competition between radical diffusion after bond homolysis and geminate recombination. Computational studies of base-off AdoCbl are yet to be investigated.

Thus, studies have mainly indicated the singlet born (S-born) RP in the photolysis of MeCbl, as well as AdoCbl. However, the T state should not be excluded, especially for the base-off form of MeCbl, which was indicated in a few computational studies through looking into the PES and bond elongation. [4, 7, 12, 21] These studies also indicated a difference in photochemistry between MeCbl and AdoCbl through the difference in wavelength dependence, energy minima and barriers related to different reaction paths. [16] The CIDNP study on model alkylcobalamins indicated the S-born RP from studying line intensities of methyl radical in °H NMR. [21] This was in the agreement with S-born pairs being observable and magnetically sensitive in the MFE study. [15] However, it is in disagreement with chemically induced dynamic electron polarisation (CIDEP) Fourier transform (FT-EPR) study [7], where only T-born pairs were observed through the TM pathway. This discrepancy was explained by the reaction path
involving the S state being observable only in CIDNP and MFE, but not through CIDEP. [21] Therefore, our approach is to use time-resolved EPR and the CIDEP effect to help elucidate and clarify the origin of the precursor molecule. Once the geminate RP is generated, diffusional processes and recombination have been shown to be dependent on temperature and viscosity in the base-on form of cobalamins. [1, 9, 22, 23] Since previous studies had conflicting results about the origin of the precursor molecule our approach was to use the time-resolved EPR (TREPR) because of its high spectral and time resolution to get an insight on the dynamics and formation of the alkyl radicals through studying CIDEP effects. FT-EPR was previously used to study radicals generated after bond homolysis in organometallic complexes. [7, 24, 25] FT-EPR has the advantage of measuring the radical signals as soon as possible after their creation and provides transient information on reaction dynamics on a sub-microsecond timescale. Since the signal of the photochemically generated species is not at thermal equilibrium, information about the precursor can be obtained from the polarized spectra. Interpretation of spectra is more complicated since they contain information on spin dynamics as well as the chemical system, but at the same time it is a powerful technique for obtaining this information. Despite earlier FT-EPR work on this system [26], theoretical interpretation of CIDEP was not fully described and concentration and time dependence was not studied. Here, we present a study on photodissociation and spin polarisation followed at two EPR frequencies, two wavelengths, as well as different solvent conditions, concentrations and delay times. In this chapter, description of simulations and theoretical interpretation of polarisation of methyl radical is presented first, followed by FT-EPR results of photolysis of the base-on and base-off form of MeCbl. The same experimental approach was conducted at higher EPR frequency, and, to our knowledge, has generated the first reported pulsed continuous-flow EPR spectra at 35 GHz. Afterwards, a study of photolysis of AdoCbl followed by FT-EPR is reported. Finally, discussions of results together with conclusions are presented for each cofactor.
5.2 Results

5.2.1 Simulations of FT-EPR spectra of MeCbl

The EPR spectrum of the methyl radical is built from four lines since the radical has three equivalent nuclei interacting with the unpaired electron. The expected relative intensities of electron interaction with nuclei in non-polarised EPR spectra are 1:3:3:1. Since the FT-EPR spectra of the photochemically generated methyl radicals are not in thermal equilibrium at time of formation, there is distortion from this pattern and Boltzmann equilibrium. This distortion can be caused by several polarization mechanisms, which are used to describe the experimental spectra aimed to help determine the photochemical pathway. Simulations of different polarization patterns of the methyl radical with a negative hyperfine value are presented in Figure 5.3. Each of the polarisation mechanisms causes spin polarization in the system resulting in different intensity patterns.

![Figure 5.3](image)

Figure 5.3. Theoretical spectra for different spin polarization mechanisms of the methyl radical. The negative hyperfine value for methyl radical is taken into account. TM stands for triplet mechanism, a-absorptive, e-emissive; RPM for radical pair mechanism, originating from mixing of S and T₀ or T₋₁ states from T-born (T) or S-born (S) pairs. Equations for calculation of these spectra are based on the standard CIDEP model as presented in Chapter 1, Section 1.3.2, with line intensities based on Q half dependence, where Q is a spin matrix using custom home-written software (work by Jonathan Woodward, University of Tokyo).
The triplet mechanism (TM) (Figs 5.3A, 5.3B) causes net polarisation with an absorptive or emissive signal, holding to the 1:3:3:1 pattern. The radical pair mechanism (RPM) can originate in mixing of S state, and one of the T sublevels. At magnetic fields of lower strengths, mixing of ST₀ can occur whereas at higher frequencies, mixing can arise between S and T₁ state as well (Chapter 1, Figure 1.14). Enhancement of the low or high field side is observed due to RPM. Therefore, this polarisation causes distortion from the 1:3:3:1 pattern. Intensities that will be enhanced depend on the origin of precursor molecule. Signal is also varied between emissive and absorptive. Figures 5.3C-F presents different distortions of FT-EPR spectra of the methyl radical caused by the origin of precursor molecule in either the S or T state. Overall, the shape and phase of RPM spectra is caused by the large Δg between the two radicals. However, at RT, only the methyl radical is clear and visible and, therefore, only half of the spectrum is displayed since the methyl radical is so far from cobalamin radical, rather than presenting the mixture of both radicals. Depending on the precursor state, the displayed spectrum from the RPM is in emissive or absorptive phase (Chapter 1, section 1.3.2.5, Table 1.2). Further, FT-EPR spectra can be distorted by spin polarisation occurring from interaction of randomly diffused RPs, f-pairs, which causes a symmetrical but mixed phase signal. A theoretical spectrum for methyl /methyl -pairs (f-pairs) is presented in Figure 5.3G. Equations presented in the Chapter 1, Section 1.3.2 were used for simulating the spectra in Figure 5.3. However, overall observed polarisation, \( P_{OBS} \) can be described by Equation 5.1

\[
P_{OBS} \approx \left[ k_{ISC}/(k_{ISC} + k_{CV})\right]P_{TM} + \left[ k_{CV}/(k_{ISC} + k_{CV})\right]P_{RP} \tag{Equation 5.1}
\]

\( P_{RPM} \) is usually estimated to be 0.01 – 0.03 for non-equilibrium systems. \( P_{TM} \) is larger and it is in the range of 0.07-0.45. \( k_{ISC} \) and \( k_{CV} \) describe rates of intersystem crossing (ISC) and bond cleavage, respectively. [21]

Finally, the calculated spectra described above were used to simulate and theoretically describe the FT-EPR spectra generated after photolysis of MeCbl. Depending on the intensity enhancement of methyl radical resonance lines, first conclusions on the origin of precursor molecules can be formed. Then, through
combination of the theoretical spectra each contribution to the polarisation mechanism for different reaction conditions was determined. At first, a combination of TM and ST\textsubscript{0} from S- and T-born RPM were used. Since the polarisation patterns from ST\textsubscript{0} S-born and ST\textsubscript{0} T-born are mirror images of each other when added together in the same amount they result with zero polarisation by RPM. However, in any combination where one of them is dominant, an increase in emissive (S-born) or absorptive (T-born) phase will be observed. However, since this change in phase is not enough to simulate the experimental data, the importance of including f-pair polarisation pattern will be a key pattern to obtain the final polarisation pattern with its mixed phase spectrum.

5.2.2 Photolysis of base-on cobalamins

Photolysis of MeCbl and AdoCbl was followed after photoexcitation using 355 and 532 nm excitation wavelengths, using a custom-built continuous-flow cell (see Chapter 3) and FT-EPR at 9 GHz. After the photolysis of Co-C bond in MeCbl, only the methyl radical was observed since measurements were conducted at room temperature. Each free induction decay (FID) of the resonance lines of the methyl radical was recorded at a distinct field value, from which and the final FT-EPR spectra were then built. FT-EPR spectra did not show the contribution of cob(II)alamin radical because of its short $T_2$ at room temperature. Therefore, relaxation of this radical was too fast and within the dead time of the instrument. The direct detection EPR (DD-EPR) method was used for analogous experiments. However, even though early time spectra showed similar polarisation patterns, time dependent oscillations present in the background made interpretation ambiguous (Appendix B.4).

The FT-EPR experiments were repeated four times and an estimation of the error in the intensity of the resonance line intensities is presented in Figure 5.5B. Influence of the environment on the reactivity of the Co-C bond was studied by varying solvent conditions. The experiments with higher concentration of glycerol were made to increase $T_2$ and understand the influence of viscosity. Another aspect that was considered was conducting the FT-EPR experiment at higher frequency in
order to investigate the contribution of additional polarisation. Figure 5.4 presents the simplified version of the photolytic pathway in the photolysis of MeCbl, where the conversion of S to T state occurs via ISC. From these states, bond homolysis can occur with rate $k_{CV}$, generating a RP that can be polarized by RPM. After methyl radicals escape the radical cage, they can form f-pairs which contribute to the overall polarization observed in FT-EPR spectrum. This pathway will be further discussed in this section through experimental and simulated evidence.

![Reaction scheme with suggested reaction mechanism of photolysis of MeCbl.](image)

**Figure 5.4.** Reaction scheme with suggested reaction mechanism of photolysis of MeCbl. The reactions occurring inside grey area describe dynamics of g-pairs, including bond homolysis and spin-state mixing by $S_{0}$. $k_{ISC}$ presents rate of intersystem crossing and $k_{CV}$ rate of bond cleavage. Reactions that occur outside the grey area describe f-pair formation, which are T-born.

### 5.2.2.1 Photolysis of MeCbl experiments using 9 GHz FT-EPR

#### 5.2.2.1.1 Photolysis at 532 nm

Figure 5.5 shows the FT-EPR spectrum of the methyl radical generated after photoexcitation of 1 mM MeCbl in 0 % glycerol at 532 nm at five different delay after flash (DAF). The hyperfine value of 2.26 mT is in agreement with previously published data. [24, 27] Since the photochemically generated RP are not in thermal equilibrium at the time of their formation, the reaction results in a non-Boltzmann
population distribution and deviation from the typical 1:3:3:1 pattern. The earliest
time point spectrum taken at a DAF of 80 ns shows that the resonance lines are in
absorption with distortion away from the typical 1:3:3:1 pattern with the high field
lines enhanced compared to the low field side. This pattern remains the same
through all measured DAF with an increase in distortion of the spectra with time
(Figure 5.5A). The biggest change can be observed in the intensity of the lowest
field resonance line since it changes from an absorptive signal at earlier times, to an
emissive signal at later times. Figure 5.5B presents the average values for peak
intensities for each of the resonance lines with the standard deviation as an error
for two concentrations of MeCbl. From this figure, it is suggested that the low field
resonance remains absorptive, even within experimental error.

Figure 5.5. A) Fourier transform EPR spectra of the methyl radical after photoexcitation at
532 nm, at five different DAF. B) Average peak intensity values for two concentrations of
methyl radical and three delay times. Error bars are calculated using standard deviation.
Experimental conditions: 1 mM MeCbl, 20 mM HEPES, pH 7.5, 0 % glycerol; 532 nm, 20 mJ;
16 ns microwave pulse.
Comparison of line intensities on the low and high field side, and absorption/emission pattern indicates the origin of spin polarization of the methyl radical and therefore the multiplicity of the precursor molecule. Simulation of experimental data was conducted using a combination of the previously calculated theoretical spectra (Figure 5.3). An absorptive FT-EPR spectrum could originate from spin polarisation of methyl radical by TM. TM can cause polarization in this system because it arises from spin-selective process of intersystem crossing (ISC) between S and T, which gets transformed to radicals because of conversion of spin. [24] From the Figure 5.5, the pattern of the spin polarized signal of methyl radical is consistent with that originating from mixing of the S, and one of the sublevels of a T state, causing the polarization by the RPM of predominantly T-born pairs (as shown in Fig 5.3C). Important determinates are the exchange interaction, $J$, between the radicals as well as the sign of hyperfine coupling constant, $a$, of the methyl radical and the difference in $g$-values between members of the RP. [28, 29] However, the intensity of resonance lines in experimental and theoretical spectra was not entirely consistent. Also, the contribution of TM is expected in this system since in most cases photoexcitation of a molecule results with relaxation to the excited S state from which then T state is formed via ISC. [7] TM is usually stronger than RPM, as presented in section 5.2. However, when trying to simulate the experimental data using these two polarization mechanisms, distortion of the experimental spectra, unsurprisingly, cannot be completely matched using a combination of polarisation by TM and ST$_0$ T born RPM. The results of this simulation overlaid with the FT-EPR experimental data at 532 are presented in Appendix B.1, Figure B.1. Disagreement in resonance line intensities between experimental and simulated spectra is most obvious at the low field lines. This was especially clear for experimental data collected after excitation at 355 nm, presented in Appendix B.1, Figure B.1, lower panel.

Since the combination of spin-polarised spectra by TM and RPM described above does not describe the experimental data well, potential additional polarisation effects that could cause spectral distortion were investigated. From the Figure 5.5A, it can be observed that the distortion of spectra from the 1:3:3:1
pattern increased in time which implied that there is an additional polarisation mechanism. From this observation, it is clear that this mechanism was time dependant. In the theoretical spectra presented in Figure 5.3, polarisation by f-pairs is expected to be time dependent whereas polarisation caused by TM appears at the early reaction stages and does not accumulate any further. Even when adding a greater contribution of RPM mechanism, the simulation did not fully describe the experimental data. Therefore, it was considered that more f-pairs were generated when looking at later reaction times, since the radicals can interact with each other and form methyl/methyl radical interactions. Due to this phenomenon, at longer times polarisation by methyl/methyl f-pairs could cause a larger distortion of the FT-EPR spectra, which was observed and is presented in Figure 5.5A. From the theoretical spectra of f-pairs, a mixed phase signal was expected, with low field lines in emission and high field lines in absorption (Figure 5.3G). Finally, through adding this pattern to the previous combinations of theoretical spectra, the simulation of the experimental data was improved (Figure 5.6). Simulated spectra in Figure 5.6 involve polarisation caused by the absorptive TM, RPM and polarisation by f-pairs. The ratio between S/T-born RPM and TM was held at a constant value of T = 0.8 x TM for T-born pairs and S = 0.5 x TM for S-born pairs. Using this approach, it was possible to observe the change of f-pair contribution with time.

As mentioned, RPM can be caused by ST₀ T-born and S-born pairs, where ST₀ T-born means that that the T-born pairs are dominant compared to the S-born pairs, and in ST₀ S-born S-born pairs are outnumbering T-born pairs. Therefore, both combinations contain a mixed population of these two states. From Figure 5.6 it is clear that both RPM polarisation patterns originating from T- or S-born g-pairs can theoretically describe experimental spectra. This could explain the disagreement in the literature on the origin of precursor molecule. Simulations using both combinations are shown in Figure 5.6. The simulated spectrum in Figure 5.6A was constructed from polarisation by absorptive TM, predominantly ST₀ T-born pairs, which are both in the same phase, and f-pairs polarisation. However, Figure 5.6B was simulated using the same TM and predominantly ST₀ S-born RP, which are in the opposite phases, which, then, results in greater distortion of the spectrum, and
subsequently less correction of distortion by f-pairs is needed. Therefore, overall, the spectra can be simulated by different combination of both S- and T-born pairs, the polarisation patterns that give the same effect, but in the opposite directions.

**Figure 5.6.** Simulation of FT-EPR spectra using combinations of TM / ST\textsubscript{T-born} / f-pairs (A) and TM / ST\textsubscript{S-born} / f-pairs (B). Contribution of each polarisation pattern is stated in each figure. Experimental parameters: 1 mM MeCbl, 20 mM HEPES, pH 7.5, 0 % glycerol; 532 nm, 20 mJ; 16 ns microwave pulse.

Since both RPM polarisation patterns originating from T- or S-born g-pairs successfully simulated the experimental spectra, in order to clarify the origin of the dominant precursor molecule, MFE studies were taken into consideration (work by Teresa Marafa). The MFE data are consistent with magnetically-sensitive RPs being predominantly S-born, which is in agreement with previously published MFE data. [15] When combining the TR-EPR and MFE data, it appears that the photolysis of MeCbl generates a mixture of S- and T-born RPs, with S-born pairs being predominantly magnetically sensitive in MFE studies. This observation of simultaneous formation of both S- and T-born pairs (mixed population) could originate from the similar kinetics of ISC and bond cleavage. In this situation, T-born pairs are still present, but are less magnetically sensitive in the MFE study. Therefore, combinations of polarisations involved in this system should include RPM from S-born pairs being dominant; RPM caused by S-born pairs was used in further simulations.
5.2.2.1.2 Concentration dependence of 532 nm FT-EPR

Another characteristic that one might expect from polarisation by f-pairs, besides time dependence, is concentration dependence. The concentration dependence of the generation of f-pairs obeys second order kinetics. Therefore, if f-pair polarisation is indeed required, one would expect to observe MeCbl concentration dependence on the FT-EPR signal. Therefore, photolysis experiments in a range of concentrations from 0.3 mM to 3 mM were completed. These experiments were also followed in time, at five DAFs. The experimental data obtained for each concentration and DAF are fitted using the combination of polarisation patterns $T_{a}/S_{T0}$ $s$-born/f-pairs and are presented in Figure 5.7. Ratios of RPM and TM were held at constant value, where $S = 0.5 \times TM$.

![Figure 5.7. Simulation of methyl radical generated after photolysis of MeCbl at 532 nm. Combination of used polarisation is absorptive TM, ST$_0$ $s$-born, RPM and methyl/methyl-pairs. Experimental parameters: 3 mM MeCbl, 20 mM HEPES, pH 7.5, 0 % glycerol; 532 nm, 20 mJ; 16 ns microwave pulse, 8 dB microwave attenuation.](image)

Results presented in Figure 5.7 show the time dependence for 3 mM MeCbl. The concentration dependent experiments were carried out between 0.3 and 3 mM MeCbl but working at lower concentration resulted with poor S/N, which caused uncertainty in simulation of the spectra (Appendix B.5, Figure B.11). Methyl/methyl-pair contribution increases with time for all investigated concentrations, which is in the agreement with assumption of generating more f-pairs with time. However, the expected pattern for the concentration dependence was not observed since there was no clear difference between contributions of f-pairs for different [MeCbl]. A small difference observed in contribution of 0.3 mM
spectra could originate from the poorer simulated fit of experimental data collected at this concentration. Therefore, measurements at 0.3 mM are excluded from this analysis and are presented in Appendix B.5. This absence of a concentration dependence could originate from the difference in O.D. for different sample concentration and subsequent light absorption. For MeCbl at neutral pH, the extinction coefficient at 450 nm is $7.7 \times 10^3$ M$^{-1}$ cm$^{-1}$, therefore the absorbance of 3 mM sample at 1 mm path length is 2.31 which results with 99.51 % photons being absorbed and 0.49 % transmitted. Since almost 100 % of photons is absorbed at 3 mM MeCbl, no measurements at higher concentrations were completed to further investigate the [MeCbl]-dependence. For the 0.3 mM sample and the same path length absorption is 0.231 with 58.75 % transmission and 41.25 % absorption. The absence of clear [MeCbl]-dependence might also be explained by looking at the diffusion kinetics and relaxation rates for methyl radical. Diffusion kinetics for 0.5-3 mM [MeCbl] is about 30-5 ns (calculation presented in Appendix B.2), therefore formation of the methyl/methyl-pairs is quick and the majority of f-pairs are formed within 80 ns, which is the first measured time delay. Afterwards, it reaches something approximating a steady state concentration, which could be achieved if new f-pairs were formed at a rate similar to their removal by reaction. Therefore, during acquisition the concentration dependence of f-pairs is removed and non-detectable by our approach. Why do we then see the time dependence of polarisation caused by f-pairs? This could be explained by examination of diffusion and relaxation rates of the methyl radical. Considering the relaxation rate of methyl with published $T_1$ in water/DMSO is about 200 ns [30], both the diffusion and relaxation rates are within the time scale of our measurements. A monoexponential fit of FID was obtained in our experiments for the experiments in buffer with 0 % glycerol and gave a $T_2$ of $\sim$ 130 ns. The result of the fit to the time decay is presented in Figure 5.8B. It is known that the $T_1$ must be always longer or equal to $T_2$. However, with increasing the temperature, $T_1$ decreases and $T_1 = T_2$. Linewidth can give measurements of $T_2$ and therefore at room temperature single Lorentzian line it will involve relaxation times $T_1$ and $T_2$. [31]
Figure 5.8. (A) Polarization by f-pairs examined by measuring at three different concentrations of MeCbl at five DAF. (B) Fit of free induction decay at one of the field positions. Experimental parameters: buffer 20 mM HEPES, pH 7.5, 0 % glycerol; 532 nm, 20 mJ; 16 ns microwave pulse.

Thus, the observed time dependence of the f-pairs is entirely apparent because of the decay of the methyl radical because of relaxation. Thus, both relaxation data and diffusion rate constant support the observed time dependence of methyl / methyl-pairs and absence of [MeCbl]-dependence. However, there is possibility of forming a different type of f-pair, between diffused methyl radical and cob(II)alamin radical. Spectral distortion, therefore, to a smaller extent, can originate in formation of these methyl/Cbl(II) f-pairs. Even though these f-pairs can impact the overall polarisation, compared to the methyl radicals, which are formed on a faster time scale, this effect is insignificant. Also, the symmetrical spectrum of methyl f-pairs is the one that supports the observed distortion in the experimental data.

5.2.2.1.3 Viscosity dependence at 532 nm

RP and spin dynamics are strongly dependent on solvent properties, such as viscosity, so are diffusion kinetics and hence f-pair formation; therefore, comparison of results in buffer solutions with or without glycerol was conducted. Addition of glycerol to buffer solution in a 1:1 ratio resulted in the increase of the resonance line intensities, probably due to the cage effect of glycerol on the RP. The results of photoexcitation of MeCbl in viscous solution at 532 nm are presented in Figure 5.9. The ratio of resonance line intensity varied due to the CIDEF effect, which can be caused by several mechanisms, as previously described. The same polarisation pattern is observed, here, as in non-viscous solution, with high field
enhancement. This implies that the origin of the precursor molecule was not influenced substantially by changes in its surroundings. Figure 5.9A shows development of the radical signal with time. Spectral distortion increases with time as well, but to a smaller extent than in non-viscous solution. From the error analysis displayed in the Figure 5.9B, a smaller error between the experiments was observed, probably due to the increased intensity of the signal and, therefore, better S/N ratio. Simulated spectra of FT-EPR experimental data in viscous solution are presented in Figure 5.9C. The polarization pattern in the experimental data of both viscous and non-viscous solutions can be described with absorptive TM, rapidly formed f-pairs and decay of $ST_0$ polarization from predominantly S-born g-pairs. The cage effect of glycerol on the methyl radical was observed in a smaller contribution of f-pairs with time. The early time data are closer to the 1:3:3:1 pattern than spectra obtained in solution without glycerol. This effect of viscosity is strong on the contribution of f-pairs and this end result is presented in Figure 5.9D. The same pattern was observed as with the non-viscous data of an increase in methyl/methyl-pair contribution with time, with a concentration dependence pattern (Figure 5.9D). The concentration dependence is consistent with slower radical diffusion and, therefore, slower growth of the f-pair contribution. The concentration dependence observed here, but not in the solution without glycerol, is expected, since the f-pairs in the latter are already generated at the time of acquisition, whereas the generation of f-pairs in glycerol is slower. Analysis of time profiles at different DAF determined a spin-lattice relaxation time of ~ 140 ns in viscous solution.
5.2.2.1.4 Photolysis at 355 nm

As mentioned earlier, previously published TA data and theoretical studies [9, 13] showed that different reaction routes occur after photolysis of MeCbl at different wavelengths. The polarization pattern of the quartet signal of the methyl
radical generated by photolysis at 355 nm continued the same trend of high field side enhancement (Figure 5.10). This implies that the precursor multiplicity after photoexcitation at 532 and 355 nm is likely to be in many ways similar. However, distortion of the resonance line intensities is greater and occurs sooner at 355 nm compared to 532 nm. This is shown in Figure 5.10A and is observed in the low field lines. The magnitude of the resonance line intensities differs due to a changed photophysical route. This change originates from different reaction pathway when using 355 nm excitation wavelength. The experimental data can also be described using $T_{M_a}/S_{T_0}$ s-born/f-pairs theory. Simulations for non-viscous solutions are presented in the Figure 5.10B. The results obtained support the earlier femtosecond to nanosecond flash photolysis studies with the addition of prompt bond homolysis with direct formation of $g$-pairs. [9, 32] If it is assumed that the prompt bond homolysis results with $S$-born $g$-pairs there is no additional polarisation by TM. However, methyl / methyl f-pairs, that are by definition T-born, are rapidly formed due to the additional kinetic energy of the methyl radical. Therefore, there is a greater proportion of f-pairs at early times compared to 532 nm and thus greater distortion of 1:3:3:1 pattern.

This is supported by the viscosity-dependence of distortion. A resonance line increase of intensity was, again, observed when looking at the polarization pattern in viscous solution; experimental results together with simulations are presented in Figure 5.10C. The polarization pattern still remained the same, as photolysis at 532 nm in glycerol (Figure 5.10B), which suggests that the viscosity neither affects the nature of RP precursor molecule nor the early spin dynamics. As mentioned in introduction above (Section 5.1), from the TA it is known that photoexcitation at 355 nm results in prompt bond homolysis and, therefore, formation of methyl radicals with high kinetic energy. These pairs rapidly escape the radical cage in non-viscous solution (causing greater spectral distortion, Figure 5.10A) However, in viscous solution these radicals are trapped for longer time, with their diffusion being limited, which is causing a smaller rate of formation of f-pairs. Therefore, from Figure 5.10B, it is clear that there is a similar amount of escaping radicals, and that both pathways that produce radicals (355 or 532 nm), even though to different
extent, are influenced by the solvent cage. From the great similarity in spectra at 355 and 532 nm, it can be concluded that distortion at 355 nm is reduced, either through the formation of f-pairs, and potentially through change in the recombination within geminate pairs. From the TA studies it was found that the cage escape rate constant was strongly dependent on the solvent. [33] Therefore, due to the smaller number of escaped radicals, lower number of formed f-pairs is observed in higher viscosity solvent.

![Figure 5.10. FT-EPR spectra of methyl radicals generated using 355 nm excitation. FT-EPR spectra of methyl radicals generated after 355 nm (red) and 532 nm (green) in buffer with 0 % glycerol for DAF 120 ns (A) and in buffer with 50 % glycerol for DAF 160 ns (B). (C) Experimental and overlaid simulated spectra of for 355 nm FT-EPR spectra of methyl radical for DAF 80, 160 and 360 ns, in 0 % glycerol solution. (D) Experimental and overlaid simulated spectra of for 355 nm FT-EPR spectra of methyl radical for DAF 80, 160 and 360 ns, in 50 % glycerol solution.](image)

**5.2.2.1.5 G and f-pair summary**

This work presented experimental and simulated data of the photolysis of base-on MeCbl. After photoexcitation at 532 and 355 nm, FT-EPR spectra showed
spin polarised methyl radicals. Using simulations, we determined that the polarisation of the radical was defined through combinations of $\text{TM}_a/\text{ST}_0$-born RPM/methyl/methyl-pairs. The apparent time dependence of polarisation by methyl/methyl-pairs, but absence of concentration dependence, can be explained with diffusion kinetics and involvement of relaxation of other polarisation mechanisms. f-pair polarisation by methyl/methyl-pairs is more readily observed at 355 nm, which supports the two different pathways identified in TA and computational studies. Overall, because of direct g-pair formation at 355 nm, and hence more subsequent f-pair contribution at early times, greater polarization contribution and spectral distortion are observed (Figure 5.11). It can be concluded that the methyl/methyl-pairs polarisation appears quickly and is a substantial component at both wavelengths.

\[ \text{Figure 5.11. Contribution of f-pair polarisation. Comparison of f-pair contribution between two wavelengths, dependent on the origin of ST}_0\text{ RPM polarisation (T-born or S-born), for 1 mM MeCbl.} \]

**5.2.2.2 FT-EPR of MeCbl at 35 GHz**

In order to examine the origin of mixing by RPM causing the polarization, similar experiments were performed at 35 GHz EPR. The experiments at higher frequency provide insight into the influence of the magnetic field on CIDEp. By working at higher frequencies, and, therefore, higher fields, the spin lattice relaxation time, $T_1$, can be increased [34] and influence on the reaction channel can
be discerned. Also, importantly, the difference between the Zeeman energy levels of the two radicals increases, which could result in stronger RPM polarisation, depending on the spin population in the excited state. The polarization in this high frequency experiment could be changed by greater splitting in energy levels of $S$ and $T$ states due to the Zeeman effect with the $J$ as a function of magnetic field. The energy level diagram shows that when the magnetic field is applied, mixing of the spin states can occur not only between $S$ and $T_0$ but the $S$ and $T_{-1}$ state, depending on the value of $J$. If $J$ is small, $T$ sublevels are further apart in energy, which make the $ST_{-1}$ mixing process less likely to occur. This will result in a weaker interaction of $S$ and $T_{-1}$ levels and the repression of a polarization effect potentially caused by this mechanism. However, if $J$ is large, the mixing will be enhanced and the polarization pattern causes greater spectral distortion. [28]

FT-EPR experiments at higher frequency are conducted using only 532 nm excitation wavelength due to the limitation in equipment. A detailed experimental setup is described in Chapter 3, Section 3.3.2. Figure 5.12 (black) shows the 35 GHz FT-EPR spectrum of the methyl radical after photolysis in viscous and non-viscous solution. These spectra deviate from 1:3:3:1 pattern, with high field side enhanced comparing to the low field side, for both solutions. Resonance line intensities, again, increase in viscous solution.

Comparing these spectra with results obtained with 9 GHz FT-EPR, there is no suggestion of a strong change in the polarization pattern. This could imply that the possible polarisation caused by RPM of $ST_{-1}$ mixing is weak or does not exist. In order to identify the polarisation pattern present in this experiment, simulation of experimental data was conducted. Figure 5.12 presents the theoretical description of experimental data. Simulations were completed using the combination of absorptive TM, predominantly $ST_0$ s-born RPM and polarisation by f-pairs. The ratio of contributions for each polarisation is presented in the figure.
Figure 5.12. FT-EPR experimental and simulated spectra of methyl radical followed at 35 GHz, after photolysis of MeCbl in viscous (A) and non-viscous (B) solution. Simulations are completed using the combination of absorptive TM, S-born ST₀ RPM and polarisation by f-pairs. Ratio of each contribution is presented in the figure in the same order. Experimental conditions: 1 mM MeCbl, 20 mM HEPES, pH 7.5, 0 % or 50 % glycerol; 532 nm, 20 mJ; 20 ns microwave pulse, 0 dB microwave attenuation.

In order to examine the additional polarisation mechanism of ST₁ S-born RPM, a small contribution of this RPM was observed in the spectra generated in non-viscous solution (Figure 5.13A), with simulated spectra having big similarity with simulated spectrum obtained without contribution of ST₁ RPM. In non-viscous solution, no contribution of ST₁ RPM was observed and the simulated spectrum is identical to the Figure 5.12A. When attempting to investigate a potential contribution from ST₁ RPM from T-born pairs for the non-viscous solution, the simulation was not successful and the best fit was with combination of TMa and ST₁ T-born, without other contributions and resonance line splitting was observed (Figure 5.13B), with a small change in intensities, but no change was observed for viscous solution. This observation again implies that there is no great contribution of polarisation by ST₁ RPM.
Figure 5.13. FT-EPR experimental and simulated spectra of methyl radical followed at 35 GHz, after photolysis of MeCbl in non-viscous solution. Simulations are completed using the combination of (A) absorptive TM, S-born ST\textsubscript{0} RPM and ST\textsubscript{1} RPM, and polarisation by f-pairs or (B) absorptive TM, T-born ST\textsubscript{0} RPM and ST\textsubscript{1} RPM, and polarisation by f-pairs Ratio of each contribution is presented in the figure in the same order. Experimental conditions: 1 mM MeCbl, 20 mM HEPES, pH 7.5, 0 %; 532 nm, 20 mJ; 20 ns microwave pulse, 0 dB microwave attenuation.

Finally, the motivation to work at higher frequencies was to investigate whether the polarisation is a result of additional RPM mechanism, caused by ST\textsubscript{1} mixing. This mechanism becomes possible at higher frequencies because of the greater splitting in energy levels; it is expected to produce greater distortion in FT-EPR spectra of methyl radical. This, however, was not observed or it was not detectable by our approach. Therefore, it can be concluded that this polarisation mechanism does not have any or any significant contribution to the generation of spin polarised signal after photoexcitation of MeCbl at 532 nm.

5.2.3 Photolysis of 5’-deoxyadenosylcobalamin

Another studied system was photolysis of AdoCbl, or coenzyme B\textsubscript{12}. Upon Co-C bond homolysis, the 5’-deoxyadenosyl radical, Ado\textsuperscript{*}/cobalamin RP is generated. From TA studies, it is known that photoexcitation of AdoCbl is wavelength independent. [9] Our approach was to use FT-EPR to follow Co-C bond homolysis in AdoCbl using two excitation wavelengths. Since the FT-EPR experiment is conducted in aqueous solution at room temperature, only formation of the Ado\textsuperscript{*}, was observed. Figure 5.14 presents the absorptive FT-EPR spectra of Ado\textsuperscript{*} generated after photoexcitation of the base-on form of AdoCbl using 355 and 532 nm excitation wavelengths. The spectra show splitting into a triplet with 2.21 mT
splitting, and two doublets splitting with values of 0.181 mT and 0.078 mT. These results correspond to the predicted values for Ado• radical and are close to the previously published values. [7] When attempting to conduct experiment in solution of higher viscosity, no FID decay was detected. This could be caused by the fact that the rate constant for radical cage escape is viscosity dependent and recombination is enhanced in solution with higher viscosity (i.e. the radical yield is low). [20] Therefore, due to the limitations in the experimental approach, because it is within dead-time of instrument, no signal of Ado• was observed in viscous solution. In the monoexponential decay fit of FID traces, $T_2$ value for Ado radical was about 25-30 ns in non-viscous solution.

Figure 5.14. Comparison of FT-EPR spectra of Ado• radical generated by photoexcitation at 532 (A) and 355 nm (B). The radical is generated by photolysis of Co-C bond from base on form AdoCbl. Experimental: 1 mM AdoCbl in 20 mM HEPES, pH 7.5, 0 % glycerol; 532 nm or 355 nm, both 20 mJ; 16 ns microwave pulse, 8 dB microwave attenuation, video gain 39 dB.

From Figure 5.14 when comparing spectra generated at two wavelengths, there is no clear wavelength dependence when looking at the polarisation pattern or spectra intensity, which is in agreement with the published TA. [9] All spectra show enhancement of the low field side compared to the high field side, which implies an S state precursor. [7] Deviation from this pattern does not change for the three measured DAFs. The spectral pattern remains the same but there is a decrease in the resonance line intensities with increase in DAF. This is different when compared to the methyl radical, where distortion of the spectra increased with time, together with decrease in intensity. The formation of f-pairs in photolysis of Ado• occurs on a smaller scale than with small molecules such as methyl radicals,
which diffuse faster and have higher probability of f-pair formation. Therefore, only relaxation of other polarisation mechanisms is observed to a significant extent in a decay of signal intensity at later times, without further signal distortion, as observed in Figure 5.14.

Finally, after significant effort, FT-EPR spectra of Ado• radical were obtained, revealing the expected S precursor for Co-C bond photolysis in AdoCbl. Differences in spectra were followed when collecting spectra after photoexcitation at two wavelengths at different DAF. It was observed that there was no wavelength-dependence and greater distortion of spectral intensity ratios at later times; again, as expected. The influence of the electronic structure was also followed through the different forms of the cofactor (results presented in section below).

### 5.2.4 Photolysis of base-off cobalamins

Another important question was to investigate differences between base-on and -off structures, which could potentially cause different reaction mechanisms. Through these experiments the role of the ligand in the bond photolysis was investigated. Structures with DMB as the lower axial ligand, base-on, and with a water molecule as lower axial ligand, base-off, are compared. The yield for the base-on reaction depends on the rates of radical escape and geminate recombination, whereas for the base-off form it depends on the rates of radical formation and internal conversion, as schematically presented in Figure 5.15. In this section, the preliminary results with hypothesis on photolysis of the base-off structure will be presented.
5.2.4.1 Methylcobalamin

The role of the lower axial ligands was investigated and, therefore, their influence on the electronic structure of the cofactor on the reaction pathway. In B_{12}-dependent enzymes this ligand can be DMB or histidine. The length of the lower axial bond can also be varied by the protein and / or substrate binding. So far, photolysis of base-on MeCbl was described, where DMB is lower axial ligand in the structure. In the base-off structure DMB is detached and replaced by a water molecule. [10, 18] This structure can be formed under acidic conditions (pH ~ 2), where the coordinating nitrogen of DMB is protonated and is presented in Figure 5.1 (page 3). From previous TA studies, it was observed that this changes the electronic structure of the molecule and opens new reaction channels for non-radiative decay. From these studies it was concluded that the reaction yield after photoexcitation of this form of MeCbl now depends on competition between the newly identified channel and bond photolysis. [10] Our approach was to use the FT-EPR technique to compare results between two MeCbl forms.

Photolysis of base-off MeCbl was followed using the same experimental setup as described in Chapter 3. Photoexcitation was completed using 355 and 532 nm excitation wavelengths. Figure 5.16 shows FT-EPR spectra of the methyl radical generated after photoexcitation of base-off MeCbl using 532 and 355 nm excitation.
wavelengths. It was observed that the FT-EPR spectra of base-off MeCbl results in a spectrum slightly distorted from the 1:3:3:1 pattern. Distortion of the spectra increased with increasing DAF, for both wavelengths. This can be observed in the change of the resonance lines intensities, especially for the low field lines. The same pattern was observed for both 532 and 355 nm spectra. When comparing FT-EPR spectra at the two different wavelengths, the intensity of the resonance lines was increased at 355 nm spectra. This observation could support the theory about the wavelength dependence present in the base-off form (described below). [18]

![Figure 5.16. FT-EPR of methyl radical generated after photoexcitation of base-off form of MeCbl. Used excitation wavelengths are 355 nm and 532 nm. Colour map was used to illustrate the different intensities between wavelengths. Experimental parameters: buffer HCL/glycine, pH 2.2, 0 % glycerol; 532 nm, 355 nm, both 20 mJ; 16 ns microwave pulse, 8 dB microwave attenuation, video gain for 532 nm 39 dB and for 355 nm 30 dB.](image)

From the FT-EPR experiments for the base-on form described in Section 5.2.2.1, it was observed that there is a wavelength dependence of photolysis of MeCbl. However, the wavelength dependence for the base-off data is less clear in the distortion of the line intensities, but is more apparent in the observed increase of resonant intensities. This is probably caused by the higher yield of methyl radical per photon at 355 nm. The wavelength dependence of photoexcitation of the base-off structure of MeCbl was also found in TA studies where there is additional wavelength dependent relaxation component added to the global analysis of the data. [18] From this study of excited states of MeCbl, a stronger transition was observed at 465 nm than at 530 nm, with $S_1 \rightarrow S_n$ as the lowest lying $S$ state. Wavelength dependence is usually observed in computational studies through the reaction proceeding by different paths, depending which bond elongates first (as
mentioned in introduction). However, for the base-off form it was observed that only path B is present, where the bond between Co and the lower axial ligand elongates first, due to the high energy of breakage of methyl ligand. These calculations predicted T-born pairs. [4]

Triplet pathways are also indicated as possible in photodissociation by crossing between PES and through study of the spin-orbit coupling (SOC) of different electronic states of MeCbl. [10] From study of the PES of S1, it crosses several T energy levels (illustrated in Figure 5.15), resulting in a number of candidates potentially involved in spin-forbidden crossing. It was found that the electronic structure of base-off MeCbl has large SOC magnitudes. [10] The final outcome of this study were two possibilities occurring as transitions in excited states. They involve either transition from S to T via ISC and then to 3(σ → σ*) or from S directly to 3(σ → σ*), occurring at ~ 2.5 Å bond length. This would support the opposite observation in FT-EPR spectra of base-off and base-on MeCbl, where RPs are predominantly S-born.

From the experimental data presented in Figure 5.17 it was observed that the spectral distortion, and contribution of f-pairs increases with time, even though to a smaller extent than when compared to results obtained with the base-on structure. This is probably due to the smaller spectral distortion overall, which is likely to be a result of more TM masking the effect of f-pairs, which could be either because of higher initial radical yield for the base-off structure, or more ISC. Hence, making comparison to the base-on structure, photoexcitation of the electronically different base-off structure might result in populating more T levels. Therefore, if the excitation results in a higher excited state and generates triplets directly, there is greater polarisation by TM. If it is considered that the origin of the precursor molecule on the photophysical pathway of base-off is predominantly a T-state, greater contribution of TM is expected, which is a stronger polarisation mechanism (indicated in Section 5.2.1).

When comparing the polarisation pattern between two wavelengths, it appears that smaller spectral distortion was observed for spectra taken at 355 nm
compared to at 532 nm, which is opposite to the results obtained for base-on MeCbl. Despite the computational work suggesting just a single pathway from base-off, regardless of wavelength, our data suggest that there is still a wavelength-dependence. It is therefore possible that at 355 nm more T states are populated, there is more TM polarisation, and therefore the spectral distortion owing to f-pairs is masked to a greater extent in 355 nm spectra than in the 532 nm spectra.

**Figure 5.17.** Experimental data of methyl radical after photolysis of base-off MeCbl. Experimental parameters: buffer HCL/glycine, pH 2.2, 0 % glycerol; 532 nm, 355 nm, both 20 mJ; 16 ns microwave pulse, 8 dB microwave attenuation, video gain for 532 nm 39 dB and for 355 nm 30 dB.

Since the RPM is strongly dependent on solvent properties, such as viscosity, comparison of results in buffer solutions with or without glycerol was conducted. In viscous solution, the intensity pattern was quite close to the typical Boltzmann equilibrium 1:3:3:1 pattern for methyl radical at early times. Again, at longer delay times, the distortion of the spectral ratios increases (Figure 5.18). The distortion of the spectra was greater for 532 nm spectra, as was the same for non-viscous solution. Therefore, the wavelength dependence was still preserved even in the different solvent environment, and viscosity does not influence the origin of the precursor molecule. It also appears, here, that the cage effect of glycerol reduced the effect of polarisation by f-pairs. In this TREPR study of Co-complexes, the spin polarized signal of methyl radical varied with viscosity of the solvent, dielectric constants and temperature. [7] The signals varied between absorption, emission and mixed phased signals, depending on the solvent. There is no overall change in
the polarisation pattern with change in viscosity, only in the distortion. The distortion in polarisation is only result of f-pairs, which is viscosity dependent.

![Figure 5.18](image)

**Figure 5.18.** Experimental data of methyl radical after photolysis of base-off MeCbl. Experimental parameters: buffer HCL/glycine, pH 2.2, 50 % glycerol; 532 nm, 355 nm, both 20 mJ; 16 ns microwave pulse, 8 dB microwave attenuation, video gain for 532 nm 39 dB and for 355 nm 30 dB.

It was observed that the contribution of f-pairs increased with time for both wavelengths, and is higher at 532 nm. The same effect was observed in non-viscous solution. For both situations, the spectral distortion is greater at 532 nm, which is the opposite to what was observed after photolysis of base-on MeCbl. From this, our hypothesis is that the increase in cage efficiency results in reducing the amount of formed f-pairs and polarisation is strongly influenced by TM. This could be correlated to TA studies which described a difference in photolysis reaction yield between base-on and base-off form of cofactor. The reaction yield in photolysis of base-off MeCbl is dependent on equilibrium between internal conversion to the ground state and bond homolysis, without geminate recombination, which is opposite to studies with base-on structure. [18]

From the TA studies, which studied conformational relaxation, it was found that reaction yield in photolysis of base-off MeCbl depends on the internal conversion and generation of RP. [18] In the base-off form, energetically favourable processes involve detachment of water followed by dissociation of Co-C bond. [10] Overall, differences in intensities between the two wavelengths in the FT-EPR
spectra show that there is wavelength dependence in the base-off form of MeCbl. There was a solvent dependence on the spectra, the same as in experimental data obtained for base-on structure. In this case, the intensity of resonance lines increased and the signal was closer to the 1:3:3:1 pattern at early reaction times for viscous solutions.

5.2.4.2 5’-deoxyadenosylcobalamin

To investigate the influence of the electronic structure on radical formation, photoexcitation of base-off AdoCbl was followed by FT-EPR. The base-off form of AdoCbl has different properties than the base-on form, and was obtained by creating more acidic environment as for MeCbl. [16, 18] The results of FT-EPR experiments are presented in Figure 5.19. To our knowledge this is the first FT-EPR spectrum of Ado’ generated from the base-off form of AdoCbl. Comparison between two wavelengths and three DAF was carried out. From the Figure 5.19 it can be seen that the polarisation pattern remains the same, with the low field side enhanced compared to the high field side, for both wavelengths. This was also observed on increasing the DAF, the intensity pattern remained the same for both wavelengths. The only change observed with increasing DAF was in signal intensity, as expected due to the relaxation of polarisation mechanisms. However, when comparing signal intensities between wavelengths, a higher intensity was observed in the 355 nm spectra. The same effect was observed for the base-off form of MeCbl. This could potentially be caused because of the different reaction mechanism of photolysis of base-off AdoCbl. In the base-on form, the reaction quantum yield of bond photolysis is dependent on the geminate recombination and diffusion of generated radicals. In the base-off form, however, the reaction yield is also dependent on internal conversion and bond homolysis. [18]

From the TA it is known that in the photolysis of AdoCbl in the base-off form, the quantum yield reduces compared to the photolysis of base-on form. The TA was investigated at one excitation wavelength. However, from 355 and 532 nm FT-EPR data it appears that the reduction in quantum yield is wavelength dependent. Assuming that the difference in quantum yield does not originate from the
difference in laser energy and number of absorbed photons, this apparent difference in radical yield could originate from populating higher energy levels at 355 nm. This hypothesis, set from these FT-EPR data, along with TA study, however, cannot provide any firm conclusions. Without lack of further evidence further experiments to investigate this are needed.

**Figure 5.19.** Comparison of FT-EPR spectra of Ado• radical generated from photolysis of the base-off form AdoCbl. Experimental conditions: 1 mM AdoCbl in HCl/ glyine buffer, 0 % glycerol, pH 2.2; 532 nm or 355 nm, both 20 mJ; 16 ns microwave pulse, 8 dB microwave attenuation, video gain 39 dB. Note: signal appearing about 338.4 mT in all spectra is due to impurity.

### 5.2.4.3 Summary for photolysis of base-off cobalamin

The preliminary works on base-off forms of MeCbl and AdoCbl have given an insight about the mechanism of bond homolysis. So far from TA (pump wavelength 405 - 410 nm) studies it is known that the quantum yield for bond homolysis in base-on cofactors is 0.35 ± 0.03 for MeCbl and ~ 1 for AdoCbl [35], and for the base-off 0.65 (+0.15, -0.25) for MeCbl and 0.12 ± 0.06 for AdoCbl. Therefore, the quantum yield goes down for AdoCbl and up for MeCbl when going from base-on to base-off form.

The photolysis mechanism of base-off MeCbl is described by hypothesis based on populating more of T sublevels, therefore TM mechanism being main polarisation mechanism in the FT-EPR spectra. The wavelength dependence is clear from the smaller distortion of 355 nm spectra, where f-pair polarisation is masked.
by stronger influence of TM. Overall, higher initial yield or RP is observed for photolysis of the base-off form.

In the FT-EPR spectra of Ado$^\ast$ radical, it is expected to observe lower radical yield in the base-off forms due to more rapid internal conversion. The increase in the internal conversion for the base-off AdoCbl is by $\sim$ 10-50 fold compared to its base-on form (increases $\sim$ 10 for MeCbl). [18] In the base-off form of AdoCbl there was observed a potential wavelength dependence, which was not observed for the base-on form. On comparison with MeCbl, the signal intensity increases with photolysis of base-off form at 355 nm, but on a smaller scale for 532 nm. As mentioned, this apparent wavelength dependence could originate from the internal conversion competing to lesser extent with bond homolysis at 355 nm. However, final conclusions on the observed wavelength-dependences for either AdoCbl or MeCbl require further study (e.g., wavelength-dependence of the TA signals).

5.3 Discussion

5.3.1 Photolysis of MeCbl

5.3.1.1 Photolysis of the base-on structure of MeCbl

The FT-EPR technique was used to follow part of the reaction mechanism of MeCbl, involving a generation of spin polarised spectra of the methyl radical after bond homolysis. Previous work using magnetic field effect and time-resolved absorption studies have proposed one of the reaction steps as a predominantly S-born RP. [9, 36] These complementary techniques, together with FT-EPR, can provide details on electronic structure of different cobalamins and thus inform on why there might be different enzymatic pathways for MeCbl- and AdoCbl-dependent enzymes. The work herein through observation of the methyl radical using a continuous-flow EPR setup revealed new information on the reaction pathway. There was little intensity change observed in the ratio of resonance lines, which was different to the changes observed with FT-EPR studies of cobaloximes, compounds structurally similar to cobalamines. [7] This spectral distortion was present in viscous solution but to a smaller extent. Combinations of theoretical
polarisation of the methyl radical were used to explain the origin of spin polarisation in this system by simulating the experimental spectra. In our approach, it was found that the methyl radical is spin polarised by the TM and RPM, which was independent of the reaction conditions. It appears that the multiplicity of the precursor molecule does not change in the measured experimental conditions. Instead, increasing contributions to the polarisation pattern from methyl/methyl f-pairs contribute to the increasing distortion of the spin polarised FT-EPR spectra from the 1:3:3:1 pattern, which was confirmed by a better fit to the experimental data when this polarisation was included in the simulation. Looking at the contribution of f-pairs to the overall polarisation of the FT-EPR spectra, a higher contribution was observed at earlier times at 355 nm. This result is in agreement with flash photolysis studies that showed prompt bond homolysis for excitation at 400 nm, with a rate constant of 1 ± 0.1 ns. [9]

Figure 5.20. Final polarization mechanism. Comparison of f-pair contribution between 532 and 355 nm spectra. Experimental conditions: 20 mM HEPES, pH 7.5, 0 % glycerol; 532 nm or 355 nm 20 mJ; 16 ns microwave pulse, 8 dB microwave attenuation.

Figure 5.20 compares f-pair contribution between two photoexcitation wavelengths at different DAF. From the Figure 5.20 a greater contribution of methyl/methyl-pairs for 355 nm spectra was observed. This observation could be explained by results observed in TA where at 400 nm there is prompt bond homolysis and direct formation of methyl/Cbl(II) RPs from the higher energy levels, rather than via the S₁ state. Therefore, this could be reflected in the FT-EPR experiment since there is greater amount of generated methyl radical after photoexcitation and 355 nm. With bond homolysis and generation of freely
diffusing methyl radicals, methyl/ methyl f-pairs are formed. Fast build-up of methyl radical leads to the greater formation of these f-pairs which then causes bigger distortion in spectra. There is potentially smaller contribution of polarisation by methyl/Cbl(II) f-pairs, but it has insignificant contribution compared to the other polarisation mechanisms present. The theory with polarisation by f-pairs is supported by the concept that the increased distortion of FT-EPR spectra is caused by relaxation of other polarisation mechanisms and does not present an increase in absolute amount of methyl/ methyl-pairs after early DAF times.

5.3.1.2 Comparison of base-on and base-off MeCbl results

Since in some cases B$_{12}$ cofactors bind to their dependent enzyme in the base-off form, information on the multiplicity of the precursor molecule can help inform understanding of the effect of the axial ligands in an enzymatic reaction. However, it is important to mention that in solution, for the base-off form, the lower axial ligand is water whereas when bound to an enzyme it is histidine. The same intensity pattern of the methyl radical was observed at different pH values (base-on and base-off form of cobalamin) with the high field side enhanced compared to the low field side. The intensity of resonance lines changed depending on the pH value, as shown at Figure 5.21. A higher rate of bond homolysis should result in a higher polarisation of the methyl radical and, therefore, FT-EPR signals with greater S/N. This is supported by the higher energy barrier for breaking the bond in base-on form is (about 0.5 eV higher) then for the base-off form, which supports our results and other computational approach. [3] The FT-EPR spectra obtained at different pH values showed a decrease in resonance intensities when increasing the pH value. When photoexcitation was conducted with 355 nm, a higher intensity of resonance lines was observed at low pH compared to at 532 nm. This is consistent with TA studies where the yield for base-off form increased to 0.65 (+ 0.15, - 0.25), from yield of 0.34 (± 0.04) calculated for base-on form. [18, 32]
Figure 5.21. Comparison of FT-EPR spectra of methyl radical after photolysis of base-on and base-off MeCbl. Presented results are for solutions with and without glycerol. Base-on buffer: 20 mM HEPES, pH 7.5, 0 % glycerol; base-off buffer: HCl/glycine, pH 2.2, 50 % glycerol; 532 nm, 355 nm, both 20 mJ.

Distortion of spectra is greater for base-on spectra and intensities are smaller when compared to the base-off form for results in 0 % glycerol solution. From the Figure 5.21, it is clear that there is a different mechanism between excitation of the two forms of MeCbl, present at both wavelengths, which implies different reaction mechanism between forms. Smaller difference in intensities was observed when photolysis was studied in viscous solution (Figure 5.21B). Another difference between forms was the wavelength dependence of the f-pairs. Even though there was wavelength dependence for both forms, for base-on form, a higher contribution of f-pairs was present at 355 nm. This contribution also occurs earlier than at 532 nm spectra. However, for base-off structure, this is opposite and contribution of f-pairs was greater at 532 nm. These results can be supported by previous TA and computational studies about different mechanisms between two MeCbl forms. It is known that the reaction yield for the base-on form of MeCbl depends on the branching ratios between bond homolysis, forming metastable state and radical escape. [4, 9] However, since the electronic structure of MeCbl changes in the base-off form, new channels for non-irradiative decay is opened and no big geminate recombination for MeCbl was observed. [11, 18] Because of this channel, reaction yield for this structure depends on the internal conversion and radical production by Co-C bond photolysis. [18] Unfortunately, the nature of this channel is still not described.
The molecular origin of these differences could originate in the population of different states at depending on the cofactor structure. The photolysis of base-on structure at 355 nm results with populating higher energy level and prompt bond homolysis, continuing with branching mechanism as observed for 532 nm. In the FT-EPR spectra this was observed in greater distortion at 355 nm spectra. Since the opposite effect was observed for photolysis of the base-off structure it can be assumed that the different excited states are populated after photoexcitation of base-off form and there is no prompt bond homolysis at 355 nm. Considering the finding that $S_1/S_2$ states are close to the several T states which have low energy ($T_4,T_5,T_6,T_7$), smaller distortion of 355 nm spectra compared to 532 nm could be explained through having a higher population in T states and stronger TM polarisation, which is “masking” the amount of polarisation and, therefore, distortion caused by f-pairs. From the TA studies it was found that the internal conversion, from the similar values for both base-on AdoCbl and MeCbl, changes when the base-off form was investigated. It increases by ~10 fold for MeCbl.

From the theoretical studies it was observed that the energy for breakage of the Co-C bond is higher for base-on than for the base-off form [3] with a large change in absorption spectrum. Spin forbidden crossing depends on the crossing of the potential energy surfaces (PES), which depend on the distance of PES to minimum-energy crossing point (MECP). Large magnitudes of MECP are observed for the base-off structure, which makes it an interesting subject of investigation with CIDEP. Results obtained with FT-EPR can be correlated with this theoretical study through the signal intensities, which are greater after photoexcitation of the base-off form. The signal intensity could be higher because of greater polarisation after photoexcitation of the base-off form. In this photoexcitation, based on theoretical studies, there is the possibility of populating T states, which originates from few overlapping MECP and SOC magnitudes. This is clear from the Figure 5.21 where the signal intensity was weaker for base-on form of MeCbl. One of the differences that computational studies found between two forms of MeCbl is in their energy minimum. For the base-on form, the energy minimum is described as a MLCT state [9, 10, 14] whereas for the base-off form it is preferably a LF state. [4]
The latter state has antibonding character and, therefore, in photodissociation studies bond homolysis should occur faster. [4] This can be again supported by the FT-EPR data with greater intensity of the spectra obtained after photoexcitation of base-off form of MeCbl in non-viscous solution (Figure 5.26A). The signal intensities after photolysis of base-off AdoCbl show the same trend as observed in FT-EPR of base-off MeCbl, however, there is still no evidence of T-born RPs for base-off AdoCbl. Therefore, this could also originate from large MCEP, but in the absence of further experimental or computational evidence, strong conclusions cannot be offered. The large density of excited states in photolysis of AdoCbl was indicated as a subject of further computational studies [4] and can potentially be supported by our observation.

5.3.1.3 35 GHz FT-EPR

Another approach to study CIDEP in this system was to work at higher frequency where energy levels are further separated. Results obtained at 35 GHz FT-EPR show the same polarisation pattern as at 9 GHz. This observation was not dependent on solvent conditions. The only observed difference was an increase of resonance intensities in higher viscosity solvent, as for 9 GHz FT-EPR. Since the spectrum at higher frequency does not show any greater distortion, we can rule out an additional contribution from mixing by ST\textsubscript{1} RPM, or it is small and not detectable by our approach.

5.3.1.4 Choosing the appropriate combination of polarisation patterns

Combination of three polarisation patterns was used, as described previously, to simulate the experimental data. Since the simulation was similar to the RPM mechanism originating from both S and T born pairs, conclusions from MFE data were used to progress further with simulation and to decide on final combination of polarisation pattern (MFE work by Teresa Marafa, University of Manchester). Therefore, a spectrum of ST\textsubscript{0} S-born RPM methyl radical was used, together with TM and f-pairs. Even though data from MFE are helpful, it is important to state that there is difference between this experiment and our approach. In the MFE studies, accumulation of cobalamin radical is followed to determine origin of the precursor
molecule whereas in FT-EPR approach, changes in the spectrum of methyl radical are followed. However, since TM polarisation is much larger than RPM, even small proportions of T pairs could hide the signal originating from S-born pairs. Therefore, it is possible that the proportion of S-pairs could be only visible by MFE studies.

5.3.2 Photolysis of AdoCbl

After photoexcitation of AdoCbl, excited electronic state, S₁, is populated, and is the initial state from where Co-C bond homolysis occurs. [16] Bond homolysis results in the formation of geminate pairs on a picosecond timescale. The quantum yield of this bond homolysis depends on the geminate recombination and formation formatting freely diffusing radicals, escaped from the radical cage. The origin of the precursor molecule in the photolysis of AdoCbl has been confirmed as an S state. [9, 15] In our study, FT-EPR was used to follow bond homolysis in the base-on and base-off forms of AdoCbl, by observing formation of the Ado⁺ radical.

From the FT-EPR experiments it was confirmed that there is no wavelength dependence apparent in the products of photoexcitation of AdoCbl, at least when using 355 nm and 532 nm as excitation wavelengths. This was also observed in earlier TA and MFE studies. [9, 15] Previously, it was identified that Co-C bond homolysis proceeds through few intermediate states (Figure 5.2). [23] This can also be seen in FT-EPR spectra where the difference in resonance line intensities presented in our study was dependent on the electronic structure of the cofactor.

Previous experimental and computational studies help to elucidate results obtained from the continuous-flow FT-EPR experiments. [9, 11, 16, 17] Studying the FT-EPR results, there is an evidence that there could potentially be different pathways or different intermediates with different forms of AdoCbl. The difference in resonance line intensities between the two forms could indicate that the LF state for the base-off form occurs earlier on the reaction pathway and, therefore, there was a higher yield, which causes greater signal intensity (Figure 5.20). This is in the agreement with computational studies but in disagreement with the TA studies which showed that the quantum yield of bond photolysis changes from 0.2 ± 0.03 for the base-on to 0.12 ± 0.06 to the base-off AdoCbl. One other possible reason
causing the increase in intensity between wavelengths for the base-off form could be a smaller rate of recombination.

When comparing the FT-EPR spectra of methyl and Ado* radicals in non-viscous solution, the opposite polarisation patterns were observed. In the FT-EPR spectra of the Ado* radical, the low field side was enhanced compared to the high field side indicating that the origin of the precursor molecule is an S state. This is in agreement with previous TA and MFE studies. [9, 15] The difference was also observed studying the signal progression with time. For the methyl radical, spectral distortion increased with time, especially at 355 nm spectra. However, regarding the Ado* radical, distortion of the spectra did not change with time, for either wavelength. The origin of this could be in the different mechanisms of the two cobalamins (Figure 5.2). In the photoexcitation of MeCbl, the reaction yield depends on the branching between bond homolysis, formation of the metastable product and recovery to the ground state. Also, because of the size of methyl radical, increased formation of escape products helps formation of f-pairs, which then can contribute to the spectral distortion. Formation of escape products and formation of f-pairs in photolysis of AdoCbl occurs with a slower rate and therefore distortion of FT-EPR spectra does not change with time and the resonance intensity lines decrease due to the other relaxation mechanisms present in the system.

5.4 Conclusions

In summary, FT-EPR was useful in explaining the origin of the precursor molecule in the reaction of MeCbl. The photophysical pathway is still the subject of debate because of the differences in experimental type and the nature of experimental and computational phenomenon. From previous studies various conclusions were brought. CIDEP is reflected on intensities of the resonance lines in FT-EPR spectra, from which two reaction routes depending on the excitation wavelengths were defined. This observation confirmed results from previous transient absorption studies. [9] With our study we can confirm, through experimental and simulated data that the reaction involves both S- and T-born pairs.
on the early reaction pathway. This idea was indicated previously that the photoexcitation of MeCbl could result in a mixture of both S- and T-born pairs [9], especially for the base-off form. [12] This is now confirmed by looking into CIDEP mechanism through FT-EPR. This is the first time that photolysis of MeCbl followed by FT-EPR was studied in terms of time and concentration dependence with supported theoretical interpretation and simulation. From this approach information on cause of spectral distortion is expanded which helped understanding early stage of the enzymatic reaction.

Overall, continuous-flow FT-EPR was successfully exploited to study free radicals formed after photolysis of MeCbl and AdoCbl. The experiments allowed study of radical development with time at two different wavelengths. Comparisons could be made between base-on and -off forms. To our knowledge, these are the first spectra of the methyl and Ado* radicals in FT-EPR generated after photoexcitation of the base-off form of MeCbl and AdoCbl, respectively.

Table with summarised conclusions:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeCbl</td>
<td>Absorptive FT-EPR spectra of methyl radical with hyperfine splitting of 2.26 mT; Wavelength dependence; spectra distortion greater at 355 nm spectra; From the simulations: origin of spin polarisation in absorptive TM, S born ST₀ RPM and f-pairs; F-pair contribution greater for 355 nm; FT-EPR spectra for both wavelengths of base-off form have greater intensity implying energy for breakage of Co-C bond and energy minima for two electronic structures.</td>
</tr>
<tr>
<td>AdoCbl</td>
<td>Absorptive FT-EPR spectra of Ado* radical with hyperfine splitting of 2.21 mT, and two doublets splitting of 0.181 mT and 0.078 mT; No wavelength dependence observed between 532 and 355 nm spectra; Greater intensity of base-off AdoCbl FT-EPR spectra compared to the base-on spectra; Spectra distortion does not increase with time which indicates that there is no or less formation of f-pairs than with MeCbl.</td>
</tr>
</tbody>
</table>
5.5 References


18. Peng, J., Tang, K.C., McLoughlin, K., Yang, Y., Forgach, D., Sension, R.J., Ultrafast Excited-State Dynamics and Photolysis in Base-Off B₁₂ Coenzymes and Analogues:


34. UCL, Spin-lattice and spin-spin relaxation, in NMR spectroscopy.

Chapter 6

Studies on the reoxidation of reduced cryptochromes and potential magnetic field effects

6.1 Introduction

Different organisms have the ability to perceive the Earth’s magnetic field (MF) and orient themselves under its influence. The biophysical mechanism of this phenomenon is still not fully understood even though the influence of the MF on orientation has been studied in many species, such as salmon, sea turtles and honey bees. [1, 2] The main studies in terms of orientation observed in migratory birds with the significant magnetic field effect (MFE) have focussed on the European robin. [2-5] The basic theory of magnetoreception involves magnetically sensitive molecules in the bird’s eye which are also light sensitive. Studies show that the magnetosensitivity is wavelength dependent, where the correct orientation occurs in blue and green light and disorientation occurs in red or orange light. [3, 4, 6] The avian magnetic compass was also identified as an inclination compass. [2, 3, 5, 7] There are two main theories to explain magnetoreception, through either magnetite or radical pair chemistry. The first one involves the presence of small magnetically sensitive, but light insensitive particles located in birds’ beaks. [2] The second theory involves chemical reactions in birds’ eye and the formation of a radical pair as a magnetically sensitive intermediate. [3, 4, 8-10] Based on the observation of migratory birds, Ritz et al. suggest that cryptochrome (Cry) proteins may be potential magnetoreceptors. [4] Crys were identified as the only molecules present in vertebrates capable of forming radical pairs (RPs), which is necessary for magnetic sensitivity to occur. Therefore, these proteins could be primary sensory molecules in radical-based magnetoreception [3, 4, 11], which was supported by studies showing that the Cry present in the retina could have a role in the avian compass. [12]
Cry proteins are structurally similar to photolyases (PL), the DNA-repair enzymes that use light for DNA repair. [3, 11, 13] They are the evolutionary descendants of DNA photolyases and the main difference between these two proteins is Cry have lost the DNA repair ability and have instead gained a new signalling role. [13, 14] Crys have a signalling function and are involved in controlling the circadian rhythm through regulating different responses in animals and plants. For example, in the Drosophila circadian clock, Cry is used to lengthen the period at limiting light intensity by interaction with protein TIM, process regulated by blue-light activation. [13, 14] Cry proteins are classified into three groups - plant Crys, animal Crys, and CRY-DASH proteins, depending on their role in the circadian clock.

The Cry/photolyase family consists of 55-70 kDa proteins with two non-covalently bound prosthetic groups, a photoredox-active FAD and a pterin or flavin type antenna chromophore. Most Crys have a C-terminal extension of 20-200 amino acids, which distinguish them from photolyase. [3, 13] This C-terminal end varies in size depending on the Cry type/origin and its function. [11] Even though Cry and photolyases have different roles, the three-dimensional structures and folding pattern remain similar [15, 16], characterised by an α/β domain and a helical domain. Three characteristic loops present in Cry are the phosphate binding loop, with a protrusion motif close to it as an extended surface loop, and a C-terminal lid located against helix C. [16, 17] These regions provide a cavity for the C-terminal tail (CTT) (Figure 6.1). [16] Two different conformers obtained from the crystal structure indicated that the reduced form of FAD influences nearby residues, which change the stability of the CTT. Comparing structures of Drosophila melanogaster (DmCry) with photolyase, the CTT in DmCry stands in the region where the substrates are bound in photolyase during DNA repair. [17]
Figure 6.1. Three loops characteristic for type I Cry. The phosphate binding loop (purple), protrusion motif (yellow) and C-terminal lid (red) create a cavity to bind the CTT. Taken from [16].

The C-terminal end, which is a part of the α-domain and is needed for light signalling, provides a pocket for the FAD cofactor and a conserved Trp tetrad. [18] Upon light exposure the cofactor is reduced, which results in a conformational change of the CTT. [17] The effect of observing only conformational change without chemical modification was supported by reversibility showed in proteolytic assay caused by light exposure. [17] Photoreduction of plant Crys results with proton transfer following the one electron reduction, which appears to be different from DmCry. The complete reduction of FAD in Arabidopsis thaliana (AtCry) is only possible using high intensity illumination and strong reducing agents. [19] The photoexcitation cycle and the respective redox states of the FAD cofactor are displayed in Figure 6.2, although different states are generated depending on the origin of the Cry. Cysteine residues in the proximity of the FAD binding pocket may influence the redox function of the protein [16] through regulating the redox potential of Trp electron transfer chain. [8] In DmCry, upon light excitation the FAD is reduced to the anionic semiquinone radical (FAD•⁻) whereas in PL this cofactor reacts through the fully reduced flavin (FADH) state. [17, 18, 20]
Figure 6.2. Light-induced photocycle in Cry and redox states of the FAD cofactor upon photoactivation. The signalling state of Cry involves different oxidation states of its cofactor FAD$_{ox}$, FAD$^+$, FADH$^+$ and FADH$. The inactive form accumulates in the dark and is the non-signalling state. The semiquinone states are the signalling states of the protein. The excited states (shown in the orange rectangular boxes) involve short-lived intermediates in the photocycle of Cry. [21]

Looking into change of redox potential upon photoexcitation, it was suggested that redox reactions play a key role in the response of Crys to light. [14] Flavin photoreduction in Crys occurs via the tryptophan tetrad where the photoexcited state of the flavin abstracts an electron from a nearby Trp via an electron transfer cascade (Figure 6.3). [22-24] This pathway is used to accumulate flavin radical, which is generally relatively stable in vivo and in vitro, in anaerobic conditions. [25] The photoreduction results in the formation of two RPs (brown rectangular box in Figure 6.3). [12, 26, 27] RP1 consists of an FAD$^+$ and a Trp radical and is a singlet (S) born. This RP is magnetically sensitive where the S-triplet (T) interconversion is influenced by a MF. RP1 can relax to the ground state because of spin-selective reverse electron transfer within the S state. [3, 12, 26] The MFE on the reaction yield of electron transfer induced by light indicates that this protein can be involved in the magnetic sensing compass. The second possible route of RP1 is the formation of RP2 by spin-independent (de)protonation of one or both of the radicals. The RP2 is magnetically insensitive. This MF insensitivity potentially originates from protein stabilization by reduction with Trp radical which leaves the FAD$^{++}$ radical in a long-lived signalling state. [12, 26] Ritz and Schulten [4] claim that compass based on the radical pair chemistry requires formation of a spin-correlated radical pair with interconversion between the S and T state. The S state can be modulated by Zeeman interaction and a small inter-radical exchange. This spin-correlated RP can be influenced by the external MF as described in Chapter 1. For MFs to have an
influence the RP has to be long-lived but the spin-correlation between the members has to have a lifetime of at least 1 μs. [3] Therefore, product yield in this reaction could be altered at different MF strengths. [12]

Figure 6.3. Photoreduction of Cry. Left panel: Electron transfer chain of photoreduction of FAD via tryptophan triad. Right panel: Green arrows indicate interconversion of S and T states of RP1 which can be influenced by a MF. The photocycle of AtCry contains FADH⁺ instead of FAD•− (Taken from [28] and [26]).

Reduction of these proteins has been extensively studied by different techniques. Time resolved EPR was one of the techniques used to study Cry proteins, [22, 29, 30] which confirmed that reduction of the cofactor occurred via the Trp tetrad in DmCry. It should be noted that this was initially thought to be triad as in other Cry (e.g. AtCry1) and it was shown that modifications to the Trp triad cause different responses, where mutation of local Trp can influence the electron transfer. [30] Light dependence was also showed in behavioural studies with fruit flies where signal transduction in Cry was correlated to behavioural response. [6]

The electron transfer cascade that occurs upon light exposure results in the signalling state and it is known that the yield of this reaction is influenced by external MFs. Predicted changes in product yield of the RP reaction is ~ 25 %, although the value might vary under real biological conditions. [4] However, in darkness and under aerobic conditions Cry is in a resting state and oxidation occurs spontaneously, which serves as regeneration of cofactor for the next cycle. Therefore, the activation of Cry depends on the equilibrium between the photoreduction reaction and oxidation reaction. However, the oxidation chemistry in Crys has been studied in much less detail than the photoreduction chemistry and therefore, this step has now been investigated in this chapter. The current
proposed mechanism of oxidation involves an electron transfer from reduced flavin to O$_2$. This results in the formation of a flavin / superoxide radical pair with a subsequent, spin-selective electron transfer forming H$_2$O$_2$ and oxidised flavin (FAD$_{ox}$). In the resting state it was observed that oxidation of FAD$^*$ in DmCry is the rate-limiting step. [17] Therefore, a RP between the reduced flavin and superoxide radical was suggested to be involved in the oxidation reaction. [10, 31] This is supported by the theory that in order to observe changes under the influence of the Earth’s MF, one of the hyperfine constants in the formed RP has to be extremely small to match the Zeeman interaction. Therefore, there is a proposal that the RP is formed between reduced FAD and molecular oxygen, which has no nuclear spin. [9, 10, 12, 32, 33] Questions about alternative RPs arose from the need for anisotropy in the RP, greater than the one in the [FAD$^*$ + $^*^*$Trp] RP. If one of the members of the RP is FAD$^*$, then the other member cannot have significant hyperfine couplings [10, 12, 23, 34], and from calculations magnetic sensitivity drops by 2x comparing the FAD$^*$ paired with a radical with low (insignificant) hyperfine interaction, for the [FAD$^*$ + $^*^*$Trp] RP. However, it is important to note that the orientation dependence is different between triad and a tetrad, which is expected to have significant influence on real hyperfine interaction. Previously, it was suggested that sensitivity of Cry to a MF could originate from two MF sensitive reactions, from both the signalling and resting state. [34] This prediction has been studied by UV-Vis, SF UV-Vis and EPR spectroscopy, along with computational approaches, where computational prediction qualitatively showed that the potential reaction [FADH$^+$+O$_2$$^*$] $\rightarrow$ [FADH$^+$+O$_2$] could be magnetically sensitive. [9]

In this chapter, results on the reduction and oxidation reactions of two types of Cry proteins will be reported. The chapter starts with reduction and oxidation studies on free flavin cofactors at a range of oxygen concentrations. Further studies are then described on two types of Crys, including various approaches to measure the potential MFE. The outcomes of this research could potentially inform the results from previous behavioural studies with E. robin when magnetoreception is investigated in the dark, with potential role of re-oxidation reactions in it. This study
showed that photomagnetoreception still works in birds when MF and light pulses alternate without any direct overlap between the two. [35]

6.2 Results

6.2.1 Reduction and oxidation reactions of free cofactors

Preliminary studies were carried out using free flavin cofactors in order to establish a method of investigating the reoxidation of reduced free cofactors before it gets implemented to study the reoxidation of proteins. The reduction and oxidation reactions for three different flavin cofactors, FAD, FMN and lumiflavin (Figure 6.4), were studied. All experiments were carried out in an anaerobic glove box so that the oxygen concentration could be controlled, at 25 °C. Reduction of the flavin cofactors was followed using UV-Vis absorbance spectroscopy and the oxidation reactions were followed using both time-resolved UV-Vis stopped-flow studies and static UV-Vis absorbance spectroscopy as described in the Materials and Methods. The flavin cofactors were either photoreduced, using illumination from an 455 nm LED, or chemically reduced using sodium dithionite (DT) (example data is shown in Figure 6.5). For all 3 flavins, formation of the same reduced species
was observed upon photoreduction or chemical reduction, with a disappearance of the 375 nm and 450 nm bands, indicating formation of the fully reduced flavin species.

Figure 6.5. UV-Vis spectra of chemically reduced and photoreduced FAD. A: chemical reduction using sodium dithionite (DT). B: cofactor reduction using blue light illumination. Sample: 95 μM FAD in 50 mM HEPES, 150 mM NaCl, 50 % glycerol, pH 8 buffer.

The oxidation of the three reduced flavin cofactors was followed using stopped flow UV-Vis spectroscopy, under anaerobic conditions and 25 °C, by mixing the reduced cofactor with oxygenated buffer. Initially, entire spectra were recorded over time between 300-700 nm using a photodiode array detector, at a single concentration of oxygen. This experiment was conducted in order to check that there are no additional intermediates formed in oxidation reaction. Results obtained with this approach are presented in Figure 6.6A. From global analysis of these data it was confirmed that there is no intermediate species (Figure 6.6B) and the reoxidation proceeds with the rate constant of $1.613 \pm 1.550 \times 10^{-3}$ s$^{-1}$ following the photoreduction of FAD. In the global analysis two components were used but value of the second one was fixed to an infinite value. This could originate from the component which lifetime exceeds the time of recorded data and therefore software is limited by number of points with which it can calculate it.
Figure 6.6. Reoxidation of FAD. A) Raw data of PDA spectrum of reoxidation of FAD with selected time traces. B) Global analysis of data from panel A with an insert representing the residuals of the fit, for two presented time points. C) Oxidation spectrum followed at 450 nm with exponential fit for two of seven measured concentrations. D) Observed reaction rates dependent on concentration of oxygen, average of 5 kinetic traces. Experimental conditions: 5 μM FAD, buffer: 50 mM HEPES, 150 mM NaCl, 50 % glycerol, pH 8 buffer.

Consequently, for all subsequent measurements the time-dependent absorbance change at 450 nm was used to follow the oxidation kinetics upon mixing oxygenated buffer with reduced cofactor (Figure 6.6C). The experimental data were then fitted to a single exponential equation to calculate the observed rate constants. In order to ensure pseudo first order reaction conditions, the minimum oxygen that was used was at least 10-fold higher than the concentration of the free flavin cofactor. As the O₂ concentration of air saturated buffer solution can be assumed to be ~ 200 μM at 25 °C [19] then the rate of oxidation was measured at a range of oxygen concentrations from 25 to 100 μM by mixing aerobic and anaerobic buffer. Observed rate constants at each oxygen concentration were calculated (each trace was fitted independently and then averaged for all of the calculated rates) and plotted to show the dependence of the observed oxidation rate on the oxygen concentration with standard deviation at each point (Figure 6.6D). For both photoreduced and chemically reduced FAD the rate of oxidation is
linearly dependent on oxygen concentration and can be fitted to provide a second order rate constant. The intercept on the y-axis rate provides an estimated rate constant for the back reaction. These measurements have been repeated for the photoreduced and chemically reduced forms of all three flavins and the results are summarised in Table 6.1. Similar oxidation rates are obtained for FAD and FMN, but the rate of oxidation is approximately 2-fold higher for lumiflavin. In addition, a small reduction in the oxidation rate is observed for the photoreduced species compared to the chemically reduced flavins in all cases. There is consistent difference between these rates even considering the error, but not by much. These differences could potentially be caused in the different amount of fully reduced cofactor which is later used for oxidation step. Considering that cofactor reduction results with formation of mixture of semiquinone and hydroquinone, relative amounts of these forms could be slightly different which could result with difference in rate of bimolecular reaction. Therefore, with chemical reduction slightly more of hydroquinone could be formed than in photoreduction, which result with slightly higher rates.

Table 6.1. Summary of the reaction rates obtained after fitting the data for three chemically reduced or photoreduced cofactors.

<table>
<thead>
<tr>
<th></th>
<th>Rate of back reaction (s(^{-1}))</th>
<th>Rate of bimolecular reaction (s(^{-1}) M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD, DT reduced</td>
<td>0.462 ± 0.050</td>
<td>(8.480 ± 0.749) x 10(^3)</td>
</tr>
<tr>
<td>FAD, photoreduced</td>
<td>0.380 ± 0.016</td>
<td>(5.850 ± 0.186) x 10(^3)</td>
</tr>
<tr>
<td>FMN, DT reduced</td>
<td>0.385 ± 0.009</td>
<td>(7.760 ± 0.225) x 10(^3)</td>
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<tr>
<td>FMN, photoreduced</td>
<td>0.453 ± 0.044</td>
<td>(5.070 ± 0.656) x 10(^3)</td>
</tr>
<tr>
<td>Lumiflavin, DT reduced</td>
<td>0.898 ± 0.104</td>
<td>(16.350 ± 1.540) x 10(^3)</td>
</tr>
<tr>
<td>Lumiflavin, photoreduced</td>
<td>1.031 ± 0.069</td>
<td>(11.930 ± 1.020) x 10(^3)</td>
</tr>
</tbody>
</table>

6.2.1.2 Magnetic field effect studies of free FMN

The effect of an external MF on the oxidation reaction of free FMN was investigated using the MFE stopped flow instrument as described in Chapter 2, Section 2.2.2. The experiments were carried out in an anaerobic glovebox at 25 °C with all samples in 0.1 M phosphate buffer pH 7.5 containing 0.1 M NaCl and 50 % glycerol. High concentrations of viscogen were used to provide the best mimic of the protein environment and to slow down the oxidation process, which should
make the identification of any MFEs easier. The FMN (at final concentrations of both 5 and 10 µM) was photoreduced immediately prior to the experiment by illumination of the samples in the syringes using a 455 nm LED. Transients were recorded at 450 nm in the presence and absence of a 100 mT MF (Figure 6.7).

Figure 6.7. Stopped-flow transients recorded at 450 nm upon mixing 5 µM FMN with 100 µM O₂ in the presence and absence of a 100 mT MF with the corresponding difference trace after subtracting the MF off data from the MF on data. All transients were recorded in 0.1 M phosphate buffer-HCl, pH 7.5, 50 % glycerol and the data shown are the average of 15 independent experiments.

All kinetic traces were fitted to a single exponential equation to obtain observed rate constants for the oxidation of FMN in the presence and absence of the MF (Figure 6.8). However, small reduction in observed rate constant was observed on the oxidation reaction for either concentration of FMN, but within the error, without any significant MFE. There could be a small MFE but the error of the method is too high to prove it. The data were also fitted to a double exponential, which again showed no significant change between the observed rate of oxidation of FMN with the MF on or off. Also, the difference trace (Figure 6.7, blue) is insignificant on the scale of followed reaction.
Figure 6.8. Bar chart diagrams with observed rate constant measured from the SF for 5 μM (A) and 10 μM (B) FMN. Errors were calculated based on 15 repeats for reoxidation of FMN, with comparison of rates in the presence and absence of a 100 mT MF. All transients were recorded in 0.1 M phosphate buffer-HCl, pH 7.5, 50 % glycerol with 5 μM or 10 μM FMN.

6.2.2 Reduction of Cry proteins

Upon completion of the experiments on the free flavin cofactors in solution, studies were then focussed on the Cry proteins themselves. Two Cry proteins were studied, one from *Drosophila melanogaster* (DmCry) and the other from *Arabidopsis thaliana* (AtCry). The reduction of Crys has been extensively studied previously (see Section 6.1 above) but in the present work the chemical reduction and photoreduction of DmCry and AtCry have been studied and compared. The redox states of the FAD were followed using UV-Vis spectroscopy. The spectrum of FAD$_{ox}$ bound to the protein has absorbance peaks at 375 nm and 450 nm (extinction coefficient of 11 × 10$^3$ M$^{-1}$ cm$^{-1}$) and two smaller shoulders at 420 nm and 480 nm, whereas the fully reduced form has absorbance peak at approximately 400 nm. There are two potential radical forms that can occur in these redox reactions. The FADH$^+$ absorbs between 580 and 620 nm, whereas the FAD$^-$ absorbs at 380 nm, with a sharp peak at 400 nm. [33] The overall aim of the present work is to investigate whether the oxidation chemistry proceeds via a RP that is magnetically sensitive. However, prior to that it was important to understand the reduction chemistry in more detail to provide a basis for studies of the oxidation chemistry.

6.2.2.1 Reduction of *Drosophila melanogaster* cryptochrome, DmCry

DmCry was purified as described in Chapter 2, section 2.1.4 and reduced either chemically with DT or by photoreduction with blue light ($\lambda_{455\text{nm}}$). Both reactions
resulted in the formation of the same FAD$^-$ species (Figure 6.9). This is shown by the increase in the absorbance bands at 360 nm and 400 nm, together with the disappearance of peaks at 450 nm and 480 nm. Increasing the illumination time or illumination with green light did not result in any further reduction. This observation is in agreement with the literature with only formation of FAD$^-$ in reduction of DmCry. [17, 18, 31]

Since these approaches did not result in formation of fully reduced flavin, it appears that the second electron transfer is gated in some way. Additional experiments at lower pH values and in the presence of dithiotreitol (DTT) also only resulted in formation of the FAD$^-$ species. However, prolonged incubation of the DmCry protein with 4 mM DT for 16 h eventually resulted in the formation of the fully reduced species, although the additional presence of 3 mM DTT and 4 mM TCEP in the buffer reduced the time required to fully reduce the protein to 320 min (Figure 6.10). This fully reduced species returned to the fully oxidized form of FAD upon exposure to oxygen (Figure 6.10B). Also, comparing the spectra before reduction and after oxidation (Figure 6.10D) it was observed that there is no great difference. These results can be correlated with results shown by Vaidya et al. [17] of studying interaction of DmCry with proteins involved in circadian clock. It was presented that the reduction of DmCry with DT, and later oxidation followed in proteolytic assay does not cause protein degradation. The slow reduction of DmCry from FAD$^-$ to

![Figure 6.9. UV-Vis spectra of reduction of DmCry. A: Photoreduction of 7.3 μM DmCry in combination with chemical reduction; B: photoreduction of 36.5 μM DmCry with 455 nm LED. All spectra were recorded in 50 mM HEPES, 150 mM NaCl, 10 % glycerol, pH 8 buffer.](image-url)
FADH\(^-\), together with conformational change of the protein was explained by protonation of the flavin as a rate-limiting step.

**Figure 6.10.** UV-Vis spectrum of reduction of DmCry using sodium dithionite, DT as reductant. Prior to the measurement buffer was exchanged in the glove box to the same anaerobic buffer. Sodium dithionite was added (4 mM) and the spectral changes were followed each 15 min (A and C). The sample was left outside the glove box to follow the reoxidation and the spectrum was recorded the day after (B). The sample was left outside the glove box to follow the reoxidation and the spectrum was recorded the day after and compared to the original spectrum (D). Experimental conditions: A/B 14 μM protein solution was in 150 mM NaCl, 50 mM HEPES, pH 8 buffer; C/D 14 μM DmCry was in 150 mM NaCl, 50 mM HEPES, pH 8, 3 mM DTT, 4 mM TCEP buffer.

The same approach with reductants present in the buffer was used to investigate the photoreduction of the protein. The protein was again incubated with DTT and TCEP but in contrast to the chemical reduction, illumination of the sample with blue light only resulted in formation of the FAD\(^-\) (Figure 6.11). This is again in the agreement with literature where prolonged light illumination did not result with further reduction of flavin cofactor. [17] The photoreduction of DmCry causes the conformational changes, probably affecting the CTT region. The negative charge develops near the N(1) position in FAD\(^-\) which causes further electron
transfer as unfavourable process [36] and could explain only formation of FAD$^-$ in the cofactor.

Figure 6.11. UV-Vis spectrum of reduction of 12 μM DmCry using 455 nm LED. Prior to the measurement buffer was exchanged in the glove box to the same anaerobic buffer. Protein solution was stored overnight in the 150 mM NaCl, 50 mM HEPES, pH 8 buffer.

6.2.2.2 Reduction of Arabidopsis thaliana cryptochrome, AtCry

In contrast to DmCry it was much easier to fully reduce the AtCry protein upon illumination with blue light at different pH values and in the presence or absence of DTT (Figure 6.12). This is caused by the aspartic acid residue opposite to N(5) in flavin which favours proton transfer. DmCry has cysteine in this position, with pKa about 4 units higher than in AtCry, which reduces the proton transfer in insect protein. [18, 36] Also, the difference in reduction could originate from residues stabilizing the negative charge developed near N(1), with arginine for DmCry and histidine for AtCry. [31, 36] In all cases there is initially a decrease in the 445 nm and 468 nm bands and an increase in the 580 nm – 620 nm range, with an isosbestic point at approximately 485 nm, indicating the formation of a FADH$^+$ radical state. Upon further illumination there is an additional decrease at 450 nm and decrease in the 580 nm - 620 nm features, which indicates formation of the fully reduced species. Moreover, the illumination time needed for reduction decreases at lower pH values from 140 min at pH 7.5 to 90 min at pH 6.5 and this decreases further in the presence of DTT. These results are in the agreement with
literature where it was found that strong illumination and oxygen-free conditions are needed for protein reduction. [19]

**Figure 6.12.** UV-Vis spectra of reduction of AtCry with or without addition of DTT at two pH values (A and C). UV-Vis scanning kinetics spectra of reaction of reoxidation of 38 μM AtCry (B and D). Buffer used for these experiments is 0.1 M phosphate buffer, 0.1 M NaCl, pH 7.5. Stock solution of 100 mM DTT was used for titration (about 15 μl was added – 3 mM final concentration of DTT)

### 6.2.3 Potentiometry experiments

Both AtCry and DmCry non-covalently bind FAD as a cofactor, where its photoexcitation or reduction triggers conformational changes in the protein. [20] However, as the reduction of DmCry results in the formation of FAD•− whereas the fully reduced species is formed in AtCry, via the FADH•+, spectroelectrochemical titrations have now been used to measure the redox potentials of the two proteins. Redox titrations were carried out using a potentiometer as described in Chapter 2, Section 2.2.3 under anaerobic conditions and in the presence of red light to avoid photochemical reduction. UV-Vis spectra were recorded at a range of potentials between -393 and 412 mV (Figure 6.13). It should be noted that it was necessary to include 10 % glycerol in the buffers to improve the stability of the protein during the course of these measurements. As none of the mediators used in these
experiments absorb at 454 nm the reduction of the FAD cofactor was followed at this wavelength for both proteins.

**Figure 6.13.** UV-Vis spectrum of DmCry (A) and AtCry (B) obtained during potentiometry experiment, at different potentials. Spectra of reduction of 13 μM DmCry were recorded in 150 mM NaCl, 50 mM HEPES, 10 % glycerol, pH 8 buffer using λ<sub>435 nm</sub> and Ti(III) citrate for reduction. Spectra of reduction of 10 μM AtCry were recorded in 0.1 M phosphate buffer, 0.1 M NaCl, pH 7.5 buffer using DT as reductant. Both experiments were completed in anaerobic conditions under red light. *Note: species with λ<sub>max</sub> ~ 600 nm is reduced mediator(s).*

Afterwards, the absorbance at 454 nm was then plotted against a reference potential (Figure 6.14). Data were then fitted to the Nernst equation (Equation 6.1) used to calculate the mid-point redox potentials for the electron transitions in both proteins.

\[
y=A_0^+ + \frac{A_1}{1+\exp\left(\frac{E_1-X}{n_1}\right)} + \frac{A_2}{1+\exp\left(\frac{E_2-X}{n_2}\right)}
\]

**[Equation 6.1]**

From the titration curve of two proteins, different potentials are observed. Reduction of DmCry resulted with formation of FAD<sup>+</sup> in the single electron reduction step with E = -200 mV. The potential for second electron transfer in DmCry is below -400 mV. The reduction of AtCry resulted with the formation of fully reduced species in one two electron reduction step with the reduction potential of -150 mV. The less negative redox potential of AtCry indicates that this protein gains electrons more easily and therefore reduces more readily to the fully reduced flavin. Therefore, the more negative redox potential of DmCry indicates
that it loses electrons easier, and the process of full reduction is more difficult. This experiment supports the results from UV-Vis spectroscopy presented in the section above where reduction of AtCry occurs via different route and more readily than reduction of DmCry.

Figure 6.14. Change in absorption at 454 nm observed during reductive titration. A: reduction of 13 μM DmCry using $\lambda_{455}$ nm and Ti-citrate, in 50 mM HEPES, 150 mM NaCl, 10 % glycerol, pH 8; B: reduction of 10 μM AtCry using DT, in 0.1 M phosphate buffer, 0.1 M NaCl, pH 7.5. Notes: 1) Y-axis presents an absorbance at 454 nm subtracted with absorbance at 800 nm; 2) The dip at low potential in B caused by the mediators.

Therefore, from the results collected using UV-Vis spectroscopy and potentiometry the differences of reduction of DmCry and AtCry were investigated. It was observed that these two proteins, even though they belong to the same family, have different routes of light-induced photoreduction or chemical reduction. In AtCry, reduction proceeds via a FAD$^+$ radical whereas the formation of the FAD$^-$ is observed in reduction of DmCry. The potentiometry experiments indicated low redox potential of one electron reduct ion followed by depletion of FAD$^{\text{ox}}$ at 450 nm. The absence of further reduction in DmCry could originate from whether or not nearby residues to the N(5) of FAD can act as a source of protons. In AtCry that residue is aspartic acid whereas there is no ready source of protons in DmCry. Therefore, the initial reduction step in AtCry to give the FAD$^+$ is followed by protonation, which neutralises the negative charge, resulting with formation of FADH$^+$. This protonation step results in an increase in the redox potential of the FAD in AtCry, which is more likely accept electrons and further reduce. On the other hand, formation of FAD$^-$ after reduction step, and lack of proton donor in DmCry, results with the absence of protonation step. Therefore, the FAD$^-$ state remains as
6.2.4 Oxidation of Cry proteins

The re-oxidation of the reduced FAD cofactors in Cry proteins has not been fully explored and it is unknown whether it results in the formation of magnetically sensitive RPs. Therefore, the oxidation reaction has now been investigated in both DmCry and AtCry upon mixing reduced protein with oxygenated buffer in the stopped-flow. In the experiments both DmCry and AtCry were first chemically reduced using DT as reductant and then excess DT was removed by passing the protein solution through a desalting column. Samples were then continually illuminated with blue light through the sample syringes to ensure reduction prior to the measurement (summarised in Figure 6.15).

![Figure 6.15 Reduction and reoxidation reactions of protein cofactor. The experimental details are briefly described. A) Reduction to FAD\(^\text{**}\) or B) complete reduction.](image)

Initially, entire spectra were recorded over time between 300-700 nm using a photodiode array detector, at a single concentration of oxygen (Figure 6.16). The oxygen concentration was not measured as for free cofactor because the rate of oxidation is too slow to measure at low O\(_2\) concentrations. Measurements were repeated for two reduced forms of DmCry. The FAD\(^\text{**}\) which is mainly formed if there are no reductants in the buffer and the fully reduced form that is formed when there are additional reductants in the buffer (see Section 6.2.5.2). For AtCry the oxidation reaction has only been studied from the fully reduced species that is formed upon addition of DT. It should be noted that the intensity of the white light
source used in these experiments was kept to a minimum to mitigate photoreduction of the flavin during measurement and in general transients could be recorded for ~ 400 seconds without any observed background photoreduction (except in the spectra of fully reduced DmCry where it appears to reach a steady state at the FAD•, see Figure 6.16B). It has been proposed that electron transfer from reduced flavin to molecular oxygen results in the formation of the FADH• radical and superoxide radical. The next step in this reaction is the formation of FAD$_{ox}$ and H$_2$O$_2$. Formation of FAD$_{ox}$ can easily be followed by observing the change in absorbance at 450 nm and in theory, it could be possible to follow the formation of superoxide at 270 nm, but this is not straightforward as the fully oxidised flavin also has a strong absorbance at 270 nm.

**Figure 6.16.** UV-Vis spectrum obtained with PDA experiment in UV-Vis stopped flow experiment. Full spectral data sets presented are A) semireduced DmCry, B) fully reduced DmCry, C) fully reduced AtCry. Buffer A and C: 0.1 M phosphate buffer, 0.1 M NaCl, 10% glycerol, pH 7.5; Buffer B: 50 mM HEPES, 150 mM NaCl, 10% glycerol, pH 8, 3 mM DTT, 4 mM TCEP.

The global analysis was used as a tool to identify possible intermediates on the reaction pathway, which then later can be followed in single wavelength experiment. From the global analysis of PDA data of AtCry two steps of oxidation were observed, going from fully reduced to oxidised species. EAS spectra from global analysis are presented in Figure 6.17, with insert presenting decay of species formation in time. Although the outcome of this analysis did not yield distinct species spectra (Figure 6.17, red), there is an indication of formation of semiquinone. This spectrum (Figure 6.14, red) most likely presents a mixture of FAD$_{ox}$ and FADH•, and it is confirmed that there is at least one species intermediate that can be followed in single wavelength experiment. A simple, sequential model
was used with only irreversible steps; physical reality is likely to be far more complex and a full and accurate kinetic model will require further study. With this result no meaningful kinetic information can therefore be extracted from this simple analysis.

Results of the fit for oxidation of AtCry presented in Figure 6.18A displays fit for the wavelengths at different time points and Figure 6.18B displaying fit at two single wavelengths at 450 and 580 nm. The oxidation step will therefore be followed at these two wavelengths in single wavelength experiments. Global analysis of the oxidation of anionic form of DmCry did not result with formation of any intermediates. The oxidation of fully reduced DmCry proceeds only to anionic form on the timescale of the experiment (Figure 6.16B). From this, it appears that the kinetics of oxidation are very slow. This is consistent with the reoxidation observed for fully reduced DmCry followed by UV-VIS presented in Figure 6.10; the spectrum of fully oxidised FAD bound to DmCRY was acquired the day after the fully reduced form was exposed to oxygen. Therefore, unless in vivo conditions are meaningfully different, slow kinetics of this reaction could indicate the presence of a very long lived RP, which is highly unlikely to behave as a magnetic sensor.

**Figure 6.17.** EAS calculated by global analysis of the experimental data from oxidation of AtCry.
6.2.5 Magnetic field effects studies

The potential influence of external MFs on the oxidation reaction was then investigated in the two Cry proteins. The aim was to test whether the reaction yield of the formed RP can be varied by an external MF. Measurements to follow the oxidation reaction were carried out on fully reduced AtCry, and on semi and fully reduced DmCry using the MFE stopped flow in an anaerobic glovebox as described in Chapter 2, Section 2.2.2. Additional experiments, where the MF was switched on and off and different intervals were carried out for DmCry. In all cases, the magnitude of the MF used in these experiments was 100 mT.

6.2.5.1 Magnetic field effects on oxidation of AtCry

In order to identify any potential MFES on the oxidation reaction of AtCry the kinetic traces at 450 nm and 580 nm were followed. The increase at 450 nm indicates the formation of the fully oxidised FAD and the changes at 580 nm reflect the formation and disappearance of the FADH\(^*\). Firstly, transients recorded in the presence and absence of the MF at 450 nm show comparable rates for the formation of the fully oxidised species (Figure 6.19). The calculated averaged rates based on 4 kinetic transients for both field on and off are \(k_1 = 2.393 \pm 0.102 \text{ s}^{-1}\) and \(k_2 = (1.134 \pm 0.029) \times 10^{-2} \text{ s}^{-1}\) for the field off, and for the field on were \(k_1 = 2.522 \pm 0.173 \text{ s}^{-1}\) and \(k_2 = (1.158 \pm 0.035) \times 10^{-2} \text{ s}^{-1}\) (Figure 6.20). In order to confirm the
apparent lack of a MFE at this wavelength the difference transient (MF on minus MF off) also shows that there are only minor differences between the kinetic traces. In contrast it was possible to observe differences in the kinetic transients at 580 nm in the presence and absence of the MF (Figure 6.21). The initial increase at 580 nm, which represents the formation of the FADH$_2^*$, is similar in both cases and does not appear to be magnetically-sensitive. However, on longer timescales there are clear differences in the disappearance of the semiquinone species. Since the absorbance of MF on transients at later times is higher compared to the transients in the absence of MF, this could indicate that the final step of oxidation is magnetically-sensitive. However, there was no observed effect at 450 nm transients (Figure 6.19), where there is bigger signal-to-noise ratio than at 580 nm. Therefore, in the absence of further evidence it can be concluded that the apparent change at 580 nm is likely to be artefactual and no MFE can be concluded just based on these experiments.

**Figure 6.19.** Oxidation of AtCry. Averaged kinetic traces (4) followed at 450 nm in oxidation reaction of AtCry, with MF on and off together with subtracted spectrum of MF on and off (blue, zoom in bottom panel). Buffer: 0.1 M phosphate buffer, 0.1 M NaCl, 10% glycerol, pH 7.5.
Figure 6.20. Bar chart diagrams with observed rate constant measured from the SF for AtCry. Errors were calculated based on 4 repeats for reoxidation of AtCry, with comparison of rates in the presence and absence of a 100 mT MF, with standard deviation as an error. All transients were recorded in 0.1 M phosphate buffer-HCl, pH 7.5.

Figure 6.21. Averaged kinetic traces followed at 580 nm in oxidation reaction of AtCry. Because of truncation of the early time data ΔAbs = 0 is where AtCRY has ends up after 500 s at FAD\text{ox} for the MF off measurement. Buffer: 0.1 M phosphate buffer, 0.1 M NaCl, 10% glycerol, pH 7.5.

6.2.5.2 Magnetic field effects on oxidation of DmCry

6.2.5.2.1 Oxidation of DmCry

From the anionic semiquinone

The oxidation of DmCry was monitored by measuring kinetic traces at 450 nm in an anaerobic glovebox in the presence and absence of a MF (Figure 6.22). Initially, there were problems with maintaining consistent oxygen concentrations in the stopped flow in the anaerobic glovebox so gas tight syringes were subsequently chosen for the measurement (Figure 6.22B). However, although this only slightly
improved the shot to shot variability it is still difficult to obtain any useful information by overlaying MF off or on data. Kinetic traces, in the presence and absence of a MF, were fitted to a double exponential fit, which showed that it appears that there is no field dependence on the observed rates of oxidation (Figure 6.23), which is expected as the reaction starts from the radical. Averaged rate constants for the field off were \((1.297 \pm 0.157) \times 10^{-2}\) and \((0.265 \pm 0.014) \times 10^{-2}\) s\(^{-1}\), and for the MF on \((1.269 \pm 0.235) \times 10^{-2}\) s\(^{-1}\) and \((0.255 \pm 0.026) \times 10^{-2}\) s\(^{-1}\), respectively. Similarly, overlaid averaged transients, together with the difference data, for MF on and off also shows no MF dependence on the oxidation of semireduced DmCry (Figure 6.24).

![Figure 6.22.](image)

**Figure 6.22.** Oxidation of DmCry semiquinone. Change in absorbance followed at 450 nm. Kinetic traces for acquisition without MF A) plastic syringe; B) gas tight syringes. Buffer: 50 mM HEPES, 150 mM NaCl, 10 % glycerol, pH 8.
Figure 6.23. Fitting of the on and off kinetic traces with bar chart diagrams. Exponential fit of kinetic trace at 450 nm for the A) field off, B) field on; bar chart diagram (C) presenting averaged observed rate constants obtained with using gas tight syringes from 6 kinetic transients with standard deviation as an error. Buffer: 50 mM HEPES, 150 mM NaCl, 10 % glycerol, pH 8.

Figure 6.24. Oxidation of DmCry. Upper panel: averaged kinetic traces (9) followed at 450 nm in oxidation reaction of DmCry, with MF on and off and subtracted spectrum of MF on and off. Buffer: 50 mM HEPES, 150 mM NaCl, 10 % glycerol, pH 8.

The outcomes of studying oxidation of FAD$^+$ to FAD$_{ox}$ resulted without any significant MFE. Both slower and faster observed rate constants were similar and any differences are within the experimental error. Taking into account the spin chemistry of this reaction this result was not surprising since the reaction starts
from the radical species and results with formation of paramagnetic molecule, without having radical intermediates. Even though there is no apparent MFE in this reaction, it is important to consider the possibility of forming the f-pairs between T state of O$_2$ and FAD$^*$. This was investigated and described in Section 6.2.5.2.2 below.

**Fully reduced DmCRY**

The influence of an MF on the oxidation of fully reduced DmCry was also investigated. Since in the reaction between fully reduced FAD and O$_2$ formation of geminate RPs is possible (unlike from oxidation of semiquinone where formation of T / radical f-pairs is possible, detailed in section below, pg. 175), and the yield of this reaction could potentially be modified by external MF. The RP generated in this reaction is between flavin and the superoxide radical. As described above, the protein was reduced overnight with excess DT and the oxidation was followed by measuring the absorbance change at 450 nm in the presence and absence of the MF (Figure 6.25A/B) fitted to a sum of three exponentials. Obtained rate constants for field off are 5.031 ± 2.172, 0.028 ± 0.002 and (2.945 ± 0.274) x 10$^{-3}$ s$^{-1}$ and for the field on 4.346 ± 0.986, 0.026 ± 0.002 and (1.673 ± 0.909) x 10$^{-3}$ s$^{-1}$. Figure 6.25C displays the average of 4 transients, showing similar amplitudes in the absence or presence of the MF. The observed rate constants obtained from fitting the data for MF on and off are shown in Figure 6.25D. From the bar chart diagram there is a difference in observed rate constants for MF on and off. Any observed change upon applying the MF is expected on the kinetics of oxidation, since the MF should not affect the final yield of the fully oxidised form, only the transient yield of the radical form. It appears that the rate of reaction decreases with applying the MF, but this result is within the error so it should be taken with caution. If the error could be reduced, better conclusion on MFE could be drawn from these investigations. One of the potential reasons causing this error could be in the O$_2$ concentration in buffer. The results presented here are based on the assumption that concentration of O$_2$ in air-saturated buffer is ~ 200 μM. However, since the reaction of oxidation follows second order kinetics, even a relatively minor uncertainty in the concentration could cause quite significant kinetic effects, which could hide any
potential MFE in the system. Therefore, no firm conclusions can be made from observed differences in rate constants at this point. Error of these differences could potentially be reduced by using the oxygen monitor. When comparing the amplitudes (Figure 6.25C) there is no great difference between the presence and absence of MF. The difference spectrum showed the potential MFE of 0.03 % (insert, Figure 6.25B).

![Figure 6.25](image)

**Figure 6.25.** Oxidation and MF studied of fully reduced DmCry. A) Fitting of kinetic trace for acquisition without MF. B) Fitting of kinetic trace for acquisition without MF. Both A and B are averages of 4 traces. C) Overlaid averages of MF on and off traces with insert of presenting data on the log scale. D) Bar chart diagram comparing observed rate constants after fitting data with field on and field off for 4 kinetic transients with standard deviation as an error. Buffer: 50 mM HEPES, 150 mM NaCl, 10 % glycerol, pH 8, 3 mM DTT, 4 mM TCEP.

### 6.2.5.2.2 Oxidation of DmCry with intervals of presence and absence of MF during acquisition

As there was too much variation in kinetic transients to definitively observe a reliable MFE on the oxidation of DmCry an alternative approach was used where
kinetic transients were recorded at 450 nm and the MF introduced periodically at different intervals. The experiments were repeated with both semi-reduced and fully reduced DmCry.

**Oxidation and MFE from semireduced DmCry**

Firstly, the oxidation of the semiquinone form of DmCry was measured at 450 nm and the MF introduced and then removed in 2 min intervals. The magnetosensitivity in this oxidation step could originate from potential formation of FAD$^*/$O$_2$ T f-pairs. Although FAD in this experiment with DmCry is not fully reduced and no RPs are formed in reaction of oxidation, interaction of FAD$^*$ with O$_2$ in a T state and formation of f-pairs is possible, potentially exhibiting MFEs. Some encounters will react immediately and proceed to formation of FAD$_{ox}$, but for other encounters reoxidation will be spin forbidden and they will produce spin-correlated/ T f-pairs which have to interconvert to undergo the electron transfer and generate FAD$_{ox}$. This interconversion could potentially be magnetically sensitive.

Combinations of data were acquired starting with either the MF on or off (Figure 6.26). By using this approach it is clear that there is a deviation in the signal when the MF is applied or removed. The absorbance at 450 nm appears to decrease upon applying the MF and then increases when the MF is removed, and this observation is independent of whether the MF is on or off at the start of the experiment (Figure 6.26). The measured amplitude difference for the start-MF off transient, the average difference is $0.326 \pm 0.153$ mO.D., and for start-MF on spectra $0.452 \pm 0.204$ mO.D.
Figure 6.26. Each kinetic trace obtained for acquisition start for MF off and on. A) Data collected where the acquisition started with a field off; B) Data collected where the acquisition started with field on; C) Average values for A and B, for 2 min intervals. Sample: 11 μM, 0.1 M phosphate buffer, 0.1 M NaCl, 10 % glycerol, pH 7.5.

Oxidation and MFE experiment of fully reduced DmCry

The same experiment was conducted for the fully reduced DmCry. Again, the experiment with the MF applied at intervals showed a difference in the amplitude upon applying the MF (Figure 6.27). The absorbance at 450 nm increases in the absence of the MF and decreases when 100 mT MF is present. The measured amplitude difference for the start-MF off transient, the average difference is 0.285 ± 0.053 mO.D., and for start-MF on spectra 0.141 ± 0.424 mO.D. Comparing outcome of the oxidation of fully reduced form, MFEs are smaller than in the oxidation of semireduced form.

Figure 6.27. Each kinetic trace obtained for acquisition start for MF off and on. A) Data collected where the acquisition started with a field off; B) Data collected where the acquisition started with field on; C) Average values for A and B, for 1 min intervals. Buffer: 50 mM HEPES, 150 mM NaCl, 10 % glycerol, pH 8, 3 mM DTT, 4 mM TCEP.
6.2.5.3 The oxidation of DmCry studied under continuous illumination

The oxidation of DmCry has also been investigated whilst continuously illuminating the sample with blue light through the measurement cell of the MFE SF in order to try to replicate the situation inside the cell. This experiment has physiological relevance since the white light illumination from PDA can mimic the sunlight and the method can follow spin chemistry reactions that occur under these conditions, with or without MF. In this approach oxidised DmCry protein in anaerobic solution was mixed against anaerobic and aerobic buffer and kinetic traces were recorded using both the photodiode array detector and in single wavelength mode at 450 nm (Figure 6.28). When protein was mixed with anaerobic buffer only the photoreduction step was observed, leading to formation of the FAD•- (Figure 6.28A). However, when protein was mixed with aerobic buffer the subsequent oxidation of the FAD was observed following photoreduction (Figure 6.28B). In the single wavelength mode a 450 nm bandpass filter was used between the measurement cell and the detector in order to remove additional signals that result from fluorescence of the sample (Figure 6.29, insert). The kinetic transients were followed at 450 nm in the presence and absence of a MF (Figure 6.29), yielding rate constants for the oxidation step of 0.091 ± 0.004 and (5.950 ± 0.265) x 10^{-3} s^{-1}. Comparing the average transients for MF on and off, there was again no significant difference between the traces.

![Figure 6.28. PDA experiment of photoreduction of DmCry. Protein solution was mixed with anaerobic buffer (A) and aerobic buffer (B) for comparison. Inserts: extracted time traces at 450 nm. Buffer: 0.1 M phosphate buffer, 0.1 M NaCl, 10% glycerol, pH 7.5.](image-url)
Figure 6.29. Single wavelength experiment followed at 450 nm. First two panels are presenting kinetic traces obtained in the absence or presence of MF. The last panel compares averaged kinetic transients for field on and off with inset presenting the UV-Vis spectrum of filter that was used to remove fluorescence from the sample. Buffer: 0.1 M phosphate buffer, 0.1 M NaCl, 10 % glycerol, pH 7.5.

Again, oscillations were observed when the field was introduced or removed (Figure 6.30). The measured amplitude difference for the start-MF off transient, the average difference is 0.485 ± 0.556 mO.D., and for start-MF on spectra 0.598 ± 0.023 mO.D. The signal amplitude decreases at later times, after ~ 800 s, probably due to the used oxygen. Comparing the difference in amplitude in oxidation step followed by this approach, the differences are slightly higher regards to the oxidation followed from semireduced DmCry (Section 6.2.5.2.2).

Figure 6.30. Single wavelength experiment followed at 450 nm with intervals measurement. First two panels are presenting kinetic traces obtained with starting with field on or field off. The last panel compares averaged kinetic transients for field on and off. Measured intervals are 60 s. Buffer: 0.1 M phosphate buffer, 0.1 M NaCl, 10 % glycerol, pH 7.5.
6.2.5.4 Control experiments

In order to examine if the change in signal amplitude upon applying the MF were caused by any mechanical movements around the cell, a number of control experiments were carried out. Firstly, instead of introducing magnets to the side of the cell (as is the case when changing the MF) non-magnetic plastic caps were placed on the side of the measurement cell of the instrument. Transients were recorded at 450 nm upon oxidation of fully reduced DmCry (Figure 6.31). It is clear that no oscillations in signal amplitude can be observed when using non-magnetic material instead of magnets for the experiments with either the black caps on or off at the start of the measurement. MFE measurements, using the same approach but with magnets at two minutes intervals, were carried out on the same samples to ensure that the MFE was still observable (Figure 6.31B).

![Figure 6.31. Control experiments with intervals for DmCry. A) Each kinetic trace obtained for acquisition start for black plastic caps off and on. Left panel presents the data where the acquisition started with caps on, right panel where the acquisition started with caps off. B) Each kinetic trace obtained for acquisition start for MF off and on. Left panel presents the data where the acquisition started with a field off, right panel where the acquisition started with field on. The experiment was conducted in two minutes intervals for both black caps and magnets. Buffer: 0.1 M phosphate buffer, 0.1 M NaCl, 10 % glycerol, pH 7.5.](image-url)
An additional control involved carrying out similar MFE experiments on samples that were not expected to be magnetically sensitive in order to see if movement of the magnets causes difference in the signal amplitude. The sample used for this experiment was FMN in 10% and 50% glycerol buffer, higher glycerol concentration to mimic the protein environment. However, it is important to mention that the FMN reoxidation is not the most appropriate control since it proceeds via RP intermediates, and therefore the results should be taken with caution. Small oscillations in the absorbance change at 450 nm could still be observed upon applying and removing the MF (Figure 6.32). This change has a smaller intensity than the difference observed with the protein but it is still present, where the amplitude still decreases with applied MF. Hence, although the observed difference may be caused by the change in reaction it also appears that there may be an additional contribution from interaction of the magnets with surroundings.

**Figure 6.32.** Each kinetic trace obtained for acquisition start for MF off and on. Left panel presents the data where the acquisition started with a field off, right panel where the acquisition started with field on. Sample: FMN in 0.1 M phosphate buffer, 0.1 M NaCl, 50% glycerol, pH 7.5.

Since the same effect was observed for the reoxidation of FMN and because oxidation of FMN also proceeds via RP a further control had to be done. Furthermore, when the storage place for magnets during the acquisition without MF changed, a change in amplitude of the opposite phase was observed (Figure 6.27A/B, ~200 ns). This change could either indicate a potential anisotropic effect of MF, or indicate an experimental artefact. Therefore, further control was needed and a simple experiment with water was conducted.
In this simple experiment with water and removing the magnets on and off during acquisition, a reproducible effect of absorbance amplitude dropping was observed when magnets were on (Figure 6.33). This drop was ~ 0.5 mAbs, which is similar to the values observed in experiments with proteins and FMN (change somewhat lower for fully reduced DmCry). Therefore, it appears that the observed effect is caused by magnets affecting one of the instrumentation parts. Even if there is any chemical origin in the oxidation step, it is probably hidden by this experimental artefact.

![Graph](image)

**Figure 6.33.** Test with of intervals measurements with MF. The acquisition starting with MF off (A) and MF on (B) is followed with water in the measurement cell.

### 6.3 Discussion and conclusions

This chapter presented work on the reduction and oxidation reactions of Cry, a protein that is potentially involved in MF sensing in bird orientation. Previously it was found that the reduction reaction or reactions of the signalling state of Cry are magnetically sensitive. [26, 37] However, the oxidation reaction or resting state of the protein was not examined in terms of magnetic sensitivity. Here we investigated the oxidation of a plant and animal type Cry (AtCry and DmCry) and potential variability in the reaction when a MF was applied. Since AtCry sample was limited, extensive measurements were not possible. The oxidation was carried out by mixing reduced forms of both proteins with oxygenated buffer since it is known that the oxygen can diffuse through protein channels caused by its thermal motion.
Initial measurements, following the oxidation of the free flavin cofactors, FAD, FMN and lumiflavin, in solution showed a linear dependence on the concentration of oxygen over the range from 25 to 100 μM, as expected for bimolecular reaction. The bimolecular rate constants obtained from this analysis were about $8 \times 10^3 \text{ s}^{-1} \text{ M}^{-1}$ for chemically reduced FAD and FMN and about $5 \times 10^3 \text{ s}^{-1} \text{ M}^{-1}$ for the same photoreduced cofactors. Rate constants were higher for lumiflavin, with $16 \times 10^3 \text{ s}^{-1} \text{ M}^{-1}$ for chemically reduced and $12 \times 10^3 \text{ s}^{-1} \text{ M}^{-1}$ for photoreduced flavin. In addition, there was no detectable MFE on the oxidation of free FMN in 50 % glycerol solution and hence, it can be concluded that no MFE is measurable using this method (Figures 6.7 and 6.8).

Consequently, the same approach could then be made to investigate the oxidation reaction in the two types of Cry protein. It is known that the reduction of Cry proceeds through the electron transfer chain reaction from nearby Trp residues. [12, 22, 32, 33] From the previous studies on the reduction of AtCry it is known that it occurs in two stages, involving formation of a $\text{FADH}^+$ radical prior to the fully reduced cofactor. [20, 33, 38] In our study it was observed that addition of DTT shortens the illumination time needed for complete reduction. This could originate from some of the residues being chemically reduced. Also, lower pH values results in shorter illumination time for complete reduction which could indicate the possible involvement of a protonation step during the reduction process (Figure 6.9). However, reduction of DmCry was more challenging and it has previously been shown that photoreduction tends to result in the formation of the $\text{FAD}^+$. [18] Formation of the same radical was observed in our study in both photoreduced (blue and green light illumination) and chemically reduced samples, where even excess amount of DT would not completely reduce the cofactor in DmCry. However, from a study by Vaidya et al. [17] it was found that protein stored with two reductants in the buffer could be completely reduced upon addition of DT. The same approach was used in our study and after 16 h incubation the protein was completely reduced, without any additional reductants in the solution. If there were DTT and TCEP in the solution, reduction was completed in over 5 h. This is an indication that the rate of reduction from semiquinone to hydroquinone is slow,
possibly caused by the slow protonation of isoalloxazine N(1), or conformational change required in the protein, since changing the pH value did not allow the reduction to proceed more quickly. However, even though it was eventually possible to fully reduce the cofactor in DmCry this is unlikely to be physiologically or functionally relevant as it takes an unprecedented long time for fully reduced cofactor to form. Therefore, if the fully reduced form of FAD occurs in DmCry [19, 33] then there are probably other factors in the cell that influence its formation.

Finally, when comparing two forms of Cry, it was found that the FAD cofactor in plant Cry cofactor is fully protonated whereas in DmCry it remains in the FAD\(^\cdot\), under physiologically relevant conditions. This is comparable to previous results obtained by transient absorption spectroscopy and steady state UV-Vis kinetics, and can be explained by the difference in amino acid residues in close proximity to the FAD. [16, 18] AtCry and PL have aspartic acid and asparagine residues in this region which can protonate the FAD. In previous studies, potentials of Cry and PL were compared [20] looking at the redox titration of AtCry and PL, using dithionite and home-built potentiometer, in the dark, at 10°C. However, in the DmCry there is cysteine residue in the same position and due to the high pKa of the thiol group the protonation on N(5) and further reduction is likely to be prevented. Mutation of the cysteine residue to asparagine appears to make the protein easier to reduce. [18, 36] Also from previous studies, in order to investigate the active state and equilibrium between signalling and resting state in AtCry, van Wilderen et al. followed the reoxidation of cofactor in AtCry with and without reducing agent in the system. [33] It was observed that FADH\(^\cdot\) is predominately formed if there is no reducing agent in the system. The rate limiting reaction in the proposed mechanism for reaction of resting state is electron transfer reaction from reduced FAD to molecular oxygen. [33] On the other hand, Vaidyaa et al. looked into the oxidation of FAD\(^\cdot\) of DmCry where it was showed that the reoxidation of the FAD\(^\cdot\) was rate limiting step in the dark state recovery and that oxidation state of a cofactor is responsible for structural changes for the signalling state. [17]

Until now it has only been shown that the reduction reaction of Cry is magnetically sensitive. [26, 37] In a recent CIDNP study progress on quantifying the
MFE on the quantum yield of a model compound [39] MFEs ranging from ~ +3 % at low MFs up to ~ -17 % at higher field were obtained. However, there are currently no studies looking at the MFE on the oxidation reaction of Cry proteins. As mentioned previously, from theoretical studies by Lee et al. [10] it was indicated that the RP formed in reaction of oxidation could have a role in magnetoreception, which was supported by behavioural studies as well. [35] It was suggested that a second member of this RP, along with FAD, has insignificant hyperfine interaction, suggesting a superoxide radical. Hence, the aim of this investigation was to investigate the potential formation of flavin/superoxide RP and examine the magnetosensitivity of the oxidation reaction. The oxidation of AtCry, which starts from the fully reduced FADH^- cofactor, proceeds via two phases. Although no clear MFE could be observed at 450 nm kinetic transients at 580 nm, reporting on the formation and disappearance of the neutral FADH^+ radical, did appear to demonstrate a MFE on longer timescales. Hence, as the effect was only observed on the slower decay of the 580 nm signal it is possible that the oxidation from FADH^+ to FAD_{ox} could be magnetically sensitive. Assuming a T-born FADH / superoxide RP, this would have to interconvert to the S RP in order for the second electron transfer from FADH^+ to superoxide to give FAD_{ox} and H_2O_2. Taking this into account, it is expected to observe slower rate constant of formation of FAD_{ox} followed at 450 nm. However, the opposite was observed after fitting the transients, with rates for MF on k_1 = 2.522 ± 0.173 s^{-1} and k_2 = (1.158 ± 0.035) x 10^{-2} s^{-1}, and for MF off k_1 = 2.393 ± 0.102 s^{-1} and k_2 = (1.134 ± 0.029) x 10^{-2} s^{-1}. From Figure 6.21 where the potential MFE was observed in phase at later times, but with lack of MFE at 450 nm and discrepancy of rates observed here, in the absence of further evidence it can be concluded that there is no magnetosensitivity in reoxidation of MFE studied in this system.

However, in studying the oxidation of fully reduced DmCry, it was observed that observed rate constants are smaller in the presence of MF, for example for the field off k_1 = 5.031 ± 2.172 s^{-1} and for the field on k_1 = 4.346 ± 0.986 s^{-1} (Figure 6.25D). This outcome could indicate that that there is potentially a greater MFE in oxidation of DmCry than AtCry. MFE studies on the oxidation reaction of DmCry
were also investigated from the semi-reduced form of the protein. Although it is not expected to observe a MFE on the oxidation of FAD\textsuperscript{•} semiquinone due to the lack of any possible RPs, measurements were still carried out as there is the possibility that FAD\textsuperscript{•}/O\textsubscript{2} T f-pairs could be formed and interconversion of these f-pairs could be magnetically sensitive. However, it was unclear if any MFE could be observed when transients were recorded in the presence or absence of an MF as there was a large amount of variability in the traces. Subsequent experiments, where the MF was periodically switched on and off at different intervals showed oscillations in the oxidation transients for both semi-reduced and fully reduced forms of DmCry, which suggested that the oxidation step is magnetically sensitive. The signal amplitude changes in the presence and absence of the MF, although a similar change in amplitude was also observable in experiments on FMN, which is not expected not to be influenced by an external MF. This could be explained by the reoxidation of FMN proceeding through RP intermediate, which indicates that this experiment cannot be a definite control to exclude the MFE in cry. The amplitude change is smaller than in the protein experiments but is still present suggesting that the oscillations in the data might, at least partially, be an artefact. Final experiment with following changes in absorbance when only water was in the measurement cell, showed the signal changes on similar scale as in measurements with proteins. This indicates that the oscillations could originate from the mechanical effect of moving the magnets in and out of the SF instrument. However, it should also be noted that no changes were observed in similar experiments using non-magnetic caps, which would suggest that physical movement of the magnets near to the cell does not change the amplitude of the absorbance signal owing to changes in light levels. Alternatively, the magnets may influence the light guides used in the SF experiments, although interaction of the magnets with the arc lamp or PMT are less likely as both were placed outside the anaerobic box. Finally, after exploring many different ways there is not convincing evidence that there is measurable MFE in this system, at least with using this approach and further experiments are needed.
6.4 References


Chapter 7

Summary of conclusions and future work

The main motivation behind this PhD project was to develop a flow-flash EPR system, which would allow time-resolved EPR experiments to investigate spin dynamics of complex biological reactions. The development of this setup enabled the study of electron transfer reactions in light activated systems. This allowed the observation of free radicals formed in these reactions on the nanosecond time scale. To test the apparatus, the reaction of known and previously studied spin chemistry of the photoexcitation of duroquinone was used, together with characterisation of time sequences using methylcobalamin. Once developed, the setup was exploited to investigate radical formation in the potential mechanism of antimalarial drugs. A major part of the project used continuous-flow and transient EPR to study the photolysis of B12-derivatives; methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl). The main findings for both of these biological systems will be summarised below, together with potential further analysis. Another aspect of spin dynamics in biological reactions studied in this thesis was to follow the potential reactions involved in magnetoreception by following the reoxidation of cryptochrome (Cry) protein isolated from Drosophila melanogaster and Arabidopsis thaliana. Progress on this aspect of the project and suggested further investigation are also presented.

7.1 Photoreduction of duroquinone by chloroquine

Continuous-flow with Fourier transform EPR (FT-EPR) was used to investigate the reaction mechanism of a series of potential antimalarial drugs. It was found that if there is tertiary amine present in the structure, scavenging of photoexcited duroquinone results in the formation of the radical anion of duroquinone, whereas in the absence of tertiary amine, formation of neutral radical species, or a mixture of neutral and anionic species, were observed.
Although, we have shown that the chloroquine is involved in the formation of free radicals and can serve as an electron donor, these finding can serve as the basis for further study. To completely investigate this reaction and understand the reaction mechanism, further studies on time and concentration dependence would be required. From these experiments, kinetic information on the rate of formation or decay of the radicals could be obtained. When working at higher concentration of the amine donor, there is also a higher possibility of observing the cation radical, which has not been possible so far due to the limitation in the amount of available compounds. The location and observation of cation species would be aided by using direct detection EPR (DD-EPR). From this, further kinetic analysis could be conducted, as mentioned above. Also, different techniques, such as transient absorption spectroscopy, could contribute to the reaction scheme, together with mass spectroscopy that could help in assignment of the generated radical species. After completions of these experiments, it should be possible to test similar drugs as well as different quinone redox partners.

7.2 Photolysis of cobalamins

The second set of biological reactions involving spin chemistry that were studied by using continuous-flow FT-EPR was to follow the reaction mechanism of vitamin B₁₂-derivatives. The origin of the precursor molecule is the subject of various experimental and computational studies and has still not been distinctly identified. In this thesis, chemically induced dynamic electron polarisation (CIDEP) was followed in experimental measurements and simulation of the methyl radical FT-EPR spectra, and information on the precursor species was gained. Although this research left some open questions, our approach was useful in identifying the origin of the precursor molecule during the photolysis of MeCbl, which is an experimental and computational enigma. We found that there is a mixture of singlet (S) and triplet (T) born pairs, with S pairs dominant. This explains the contradictory results that previous studies have shown.
The FT-EPR technique was found to be a useful technique to follow the formation of alkyl radicals after Co-C bond homolysis in base-on and base-off forms of MeCbl and AdoCbl. The results indicated differences between the reaction mechanisms in the two forms of the cofactor. In regards to the MeCbl, one of the differences was indicated through the wavelength dependence where for the base-on MeCbl, ratio of S- and T-born pairs was varied depending on the excitation wavelength. Even though at both wavelengths mixed population of S- and T-born pairs is formed, with S-born dominant at both wavelengths, ratio of these pairs is different for each wavelength. The influence of lower axial ligand was also investigated, by comparing base-on with base-off structure. The importance in studying this is because B$_{12}$ cofactors can bind in either base-on or base-off structure to their dependent enzymes. It was found that by changing the lower axial ligand resulted with differences in FT-EPR spectra of methyl radical after photoexcitation of base-on and base-off MeCbl. From our results and hypothesis for the base-off structure, the potential involvement of spin-orbit coupling between S and T states was indicated. Further simulations of FT-EPR spectra obtained after photolysis of base-off MeCbl complemented with MFE study could additionally improve this hypothesis.

Further experiments are needed to study influence of lower axial ligand, but overall it was found that differences in upper and lower axial ligands play an important role in the reaction pathway, where different spin-states are populated, and therefore different reaction pathways are followed. These differences were expected based on previous TA studies where a new channel for non-irradiative decay has been observed in the photolysis of base-off forms. Future work needs to identify the nature of this channel and obtain more detailed information on it. Further TA experiments could also involve the wavelength dependence of base-off cofactors, since so far the experiment was conducted solely with 400 nm pump light.

The second studied cofactor using FT-EPR was AdoCbl, where the result with base-on structure confirmed previous result of S-born pairs and absence of wavelengths dependence observed previously with MFE and TA studies. However,
preliminary results with base-off form indicated the potential wavelength
dependence. This would have to be subject of further investigation, with initial
investigation of potential laser power dependence. Computational studies on the
base-off form of AdoCbl would help in theoretically explaining the pathway behind
these signals. So far, there have been no theoretical studies on this state. Also, by
simulating the Ado⁰ radical observed by FT-EPR with appropriate hyperfine values,
theoretical spectra could be used to determine the exact polarisation mechanism
on the reaction pathway and contribution of different polarisation effects. In
addition to these experiments on the photolysis of AdoCbl it would be useful to
study the effect of environmental change on signal formation in various solvent
conditions. This was previously studied in ethylene glycol and water where there
was a solvent dependence. [1] In our study we attempted to carry out experiments
using high viscosity solvent (50 % glycerol) but no signal was observed. This could
be caused by an increased recombination rate due to the cage effect. In future it
would be beneficial to use smaller increasing steps in viscosity during the
experimental studies and this could be used to mimic the protein environment.
Furthermore, experiments with cofactor bound to an enzyme could be conducted
as well to see how the protein environment changes the reaction pathway. Some of
the examples are ethanolamine ammonia lyase, which binds cofactor in the base-on
form or methylmalonyl-CoA mutase which binds it in the base-off form. It would be
therefore interesting to investigate these enzymes using TR-EPR. Also, comparison
could be made between the enzymes with base-off bound cofactor, which is His-on
structure, whereas base-off cofactor in solution has a water molecule in a place of
a lower axial ligand.

Further experiments which could compliment the current study are DD-EPR of
MeCbl and AdoCbl. These experiments would have the advantage of measuring
over a longer time-scale. Overall, in this work it was demonstrated that FT-EPR is a
useful technique to follow CIDEP of the alkyl radical formed after Co-C bond
homolysis. The main limitation in this study was caused by the instrumental
parameters of the technique. As described in the experimental section, due to
bandwidth limitation, each resonance line had to be recorded at a certain field
position and FT-EPR spectra are built later on from each separate resonance line. Now, with the instrument development of arbitrary waveform technology, significantly wider bandwidth is possible, providing greater excitation and the possibility to measure the entire spectrum of the methyl radical in one FID. This would surely reduce the experimental error and sample consumption. Another aspect that could be studied is study of the photolysis using a wider range of pH values, which may help to elucidate differences in the electronic structure and reaction pathway. Also, due to the limitation in equipment, Q band EPR experiments at 355 nm excitation were not conducted. This experiment could provide a better insight if there is additional mixing of S and T states contributing to the observed spin polarisation of the methyl radical, and is of particular interest since there is instant bond homolysis at 355 nm.

7.3 Reoxidation of reduced cryptochromes and MFEs

Investigation of cryptochrome (Cry) protein as a potential magnetoreceptor has so far in the literature involved genetic, behavioural, and computational and biophysics studies involving spin dynamics. These studies mainly investigated the reduction of the flavin cofactor in the protein and the dependence of the reaction yield on external magnetic fields. The photoreduction results in electron transfer to photoexcited protein cofactor, FAD, from nearby Trp residues, and this reaction results in the formation of RPs. This reaction has been shown to be magnetically sensitive. From transgenic approaches, however, it has been shown that the Trp triad is not necessary for magnetosensitivity by mutating the Trp residues. [2] Further computational studies and behavioural analysis indicated that there is the potential involvement of different RPs in magnetoreception. [3, 4] Therefore, we investigated the reoxidation reaction of plant (AtCry) and insect (DmCry) Cry and looked for potential evidence of MFEs on this step.

The revealing differences between the plant and insect Cry were observed in potentiometry experiments. Previously, differences were followed between AtCry and photolyase where more negative potential was observed for Cry, which was
assigned to the differences in the residues close to the cofactor. [5] In our study, we compared differences between plant and insect Cry. The results showed more negative redox potential in DmCry when compared to the AtCry. This outcome can be explained by the residues close to the N(5), with aspartic acid in AtCry as ready source of proton. The lower redox potential of DmCry reflected the more negatively charged molecule that cannot be stabilised and the cofactor remains in the anionic form. However, in the AtCry, there is aspartic acid close to N(5) and formed anionic species in reaction of reduction gets protonated and results with formation of neutral radical. This can now be correlated with the observed differences in redox potentials between two Crys.

Another investigation in this study involved MFE in reaction of oxidation of two Crys. In the present work no obvious MFEs were observed on the oxidation of Cry, although better controls will be required for an unequivocal confirmation. Significant variability between traces hampered the interpretation of the results and will require improvements in the quality of the data and signal-to-noise ratio. One of the possible reasons for these fluctuations could be caused by the variability in O$_2$ concentration. As mentioned in Chapter 6, the concentration of O$_2$ in air saturated buffer could be measured with oxygen monitor. Therefore, the second order kinetics of reaction of oxidation could be closely monitored and minimise the potential kinetic effect caused by uncertainty in concentrations which can mask the magnetic field effect in followed reaction.

In terms of the AtCry protein, measurements were limited due to the small amount of sample available so there is certainly scope for further experiments with additional sample. All of the experiments described in this thesis were conducted using the stopped-flow technique, which is useful to follow the kinetics of rapid reactions. However, in the case of Cry the acquisition time of reoxidation is over several hundred seconds and hence, there is no real need to use such rapid mixing techniques to study the reaction. Instead, it would probably be an advantage to use a standard UV-Vis spectrometer, equipped with magnets, to study the reoxidation as this instrument (e.g. lamp stability, detectors, etc) would be more suited to
longer time scale measurements. MFE measurements could also be repeated at various temperatures, which could also influence the potentially observable MFEs.

7.4 References


Appendix

A – Construction of FT-EPR spectra

A.1 Data processing for FT-EPR

As mentioned previously, time domain spectra have to be converted to the frequency domain to get information on the spin dynamics of the system. The main process used is Fast Fourier transform (FFT) with several steps needed before transformation. These steps are described below. In the Fourier transform EPR (FT-EPR) experiments of the photolysis of cobalamins due to limitations in bandwidth, each resonance field position had to be measured separately. The determination of the exact resonance field position was determined by the fact that slower oscillations of free induction decay (FID) occur when the field is closer to the resonance field and faster oscillations when it is further away; the FID has the form of a single exponential decay when it is exactly on resonance. [1]

Each spectrum was collected using a PulseSPEL program, which enable phase-cycling of the signal in order to cancel out offset artefacts. After defining the desired program and variables, 100 transient averages were used with 1 ns time resolution. After subtracting the off-resonance signal, collected data were baseline corrected using a 0th order polynomial (a constant). Traces were phased to obtain pure absorption (real) and dispersion (imaginary) signals. Afterwards, a left-shift was carried out to the first data point to remove any ring-down from the trace. FFT of the data was carried out after zero-filling, which further improves the S/N ratio.

In order to build the field/frequency field spectrum it is worth noting that field and frequency are in opposite direction. [1] Higher field has lower (negative) Larmor frequency as indicated with Equation A.1 and Figure A.1. This fact has to be taken into consideration when building the FT-EPR spectrum. Spectra in the thesis are presented using the magnetic field axis. Detailed Matlab code for building the FT-EPR is presented below (Section A.2). Therefore, FTs of each field position
presented in this thesis are processed using Xepr (as described above), and were constructed in Matlab to the final FT-EPR spectra (code in the section A.2).

\[ \omega_L = -\gamma B_0 \]  \hspace{1cm} [Equation A.1]

![Figure A.1: Position of Larmor frequencies when resonance field is set on the centre line. Taken from [1].](image)

**A.2 Code for plotting from data processed with Xepr**

The Matlab code presented below involves loading processed data with Xepr (FT of each resonance line) as described in the section above, with each field position indicated, and in first step, converting to the field domain from the frequency domain (1). Since the spectrum has to be assembled, the X axis was built in the field domain (as opposed to frequency) with defined step positions (2). In these steps, junctions between each of the resonance peaks were defined. Finally, the Y axis was built by assembling each of the four collected resonance peaks between calculated junctions (3). This was how the spectrum of the methyl radical studied in Chapter 5 was constructed; four resonant lines separated by the hyperfine splitting.

(1)

```matlab
[exp_freq1, exp_intensity1] = eprload('FT2_0p3mM_d400_3414p6.DSC');
exp_field = 1000 * exp_freq1 / 2.8025;
field_position1 = 3414.6;
[exp_freq2, exp_intensity2] = eprload('FT_0p3mM_d400_3437p2.DSC');
field_position2 = 3437.2;
[exp_freq3, exp_intensity3] = eprload('FT_0p3mM_d400_3459p8.DSC');
field_position3 = 3459.8;
[exp_freq4, exp_intensity4] = eprload('FT_0p3mM_d400_3482p55.DSC');
field_position4 = 3482.55;
```
\( f_{\text{step}} = \exp_{\text{field}}(2) - \exp_{\text{field}}(1); \)
\( L = \text{length(}\exp_{\text{field}}); \)
\( X_{\text{axis}} = 3300:f_{\text{step}}:3600; \)
\( \text{step\_position1} = \text{round}((\text{field\_position1}-3300)/f_{\text{step}}); \)
\( \text{step\_position2} = \text{round}((\text{field\_position2}-3300)/f_{\text{step}}); \)
\( \text{step\_position3} = \text{round}((\text{field\_position3}-3300)/f_{\text{step}}); \)
\( \text{step\_position4} = \text{round}((\text{field\_position4}-3300)/f_{\text{step}}); \)
\( \text{junction1} = \text{round}((\text{field\_position1}-3300)/f_{\text{step}}+(\text{field\_position2}-\text{field\_position1})/(f_{\text{step}}*2)); \)
\( \text{junction2} = \text{round}((\text{field\_position3}-\text{field\_position2})/f_{\text{step}}+\text{junction1}); \)
\( \text{junction3} = \text{round}((\text{field\_position4}-\text{field\_position3})/f_{\text{step}}+\text{junction2}); \)
\( \text{Y}_{\text{axis}} = \text{zeros}(1, \text{length(}X_{\text{axis}})); \)
\( \text{Y}_{\text{axis}}(1:\text{junction1}) = \exp_{\text{intensity}1}(\text{floor}(L/2+(\text{step\_position2}-\text{step\_position1})/2)-\text{junction1}:	ext{floor}(L/2+(\text{step\_position2}-\text{step\_position1})/2)-1); \)
\( \text{Y}_{\text{axis}}(\text{junction1}:\text{junction2}) = \exp_{\text{intensity}2}(L/2-(\text{junction2}-\text{junction1})/2:\text{L}/2+(\text{junction2}-\text{junction1})/2); \)
\( \text{Y}_{\text{axis}}(\text{junction2}:\text{junction3}) = \exp_{\text{intensity}3}(L/2-(\text{junction3}-\text{junction2})/2:\text{L}/2+(\text{junction3}-\text{junction2})/2); \)
\( \text{Y}_{\text{axis}}(\text{junction3}:\text{length(}Y_{\text{axis}})) = \exp_{\text{intensity}4}(\text{floor}(L/2-(\text{step\_position4}-\text{step\_position3})/2:\text{L}/2+\text{length(}Y_{\text{axis}})-\text{junction3}-\text{floor((}\text{step\_position4}-\text{step\_position3})/2)-1)); \)
\( \text{plot}(X_{\text{axis}}, Y_{\text{axis}}) \)

A.3 Code for simulation

Theoretical spectra of polarisation patterns that can cause distortion of methyl radical spectra were calculated by Jonny Woodward (University of Tokyo) and presented in Figure 5.3. Here, simulation codes for the experimental data built above are presented. The approach uses a linear combination of basis spectra (those calculated by Jonny Woodward) to construct a simulated spectrum. The relative contribution of each spectrum/polarisation pattern is determined by minimising the RMS difference between peak maxima and minima between the experimental and simulated spectra. Therefore, the final simulation was completed by combination of different potential polarisation patterns. After loading different potential polarisation mechanisms, the X axes for simulated spectra were coded. Afterwards, peaks were identified for each of the polarisation mechanisms (1). The next step involved loading the experimental data and identifying the maximum, for peaks in absorption, or minimum intensity, for peaks in emission, for the four peaks in a spectrum (2). Finally, fitting of the experimental data was conducted by choosing different polarisation mechanisms, with contributions ranging from 0 to 1, with 0.01 steps (can be modified additionally). After obtaining the most similar peak
intensities between experimental and simulated spectra, simulation spectra were plotted against experimental. Peak intensities can also be compared using a simple bar chart diagram as well.

(1)
[I_Sborn]=importdata('singborn.txt');
[I_STminusS]=importdata('singstminus.txt');
[I_TMe]=importdata('tme.txt');
[I_Tborn]=importdata('tripborn.txt');
[I_STminusT]=importdata('tripstminus.txt');
[I_TMa]=importdata('tma.txt');
[I_Fpairs]=importdata('fpair2.txt');
I_Sborn=I_Sborn.data;
I_STminusS=I_STminusS.data;
I_TMa=I_TMa.data;
I_TMe=I_TMe.data;
I_Tborn=I_Tborn.data;
I_STminusT=I_STminusT.data;
I_Fpairs=I_Fpairs.data;
X_theory=3357.6+(0.1785*(1:1024));
all=[I_Sborn I_STminusS I_TMa I_TMe I_Tborn I_STminusT I_Fpairs];
all_peaks=[max(all(1:380,:))+min(all(1:380,:));max(all(380:520,:))+min(all(380:520,:));max(all(520:640,:))+min(all(520:640,:));max(all(640:1000,:))+min(all(640:1000,:))];

(2)
[experimental]=Y_experiment;
[X_exp]=X_experiment;
if max(experimental(1:1450,:))>abs(min(experimental(1:1450,:)))
    exp_peaks(1,1)=max(experimental(1:1450,:));
else
    exp_peaks(1,1)=min(experimental(1:1450,:));
end
if max(experimental(1450:1750,:))>abs(min(experimental(1450:1750,:)))
    exp_peaks(2,1)=max(experimental(1450:1750,:));
else
    exp_peaks(2,1)=min(experimental(1450:1750,:));
end
if max(experimental(1750:2000,:))>abs(min(experimental(1750:2000,:)))
    exp_peaks(3,1)=max(experimental(1750:2000,:));
else
    exp_peaks(3,1)=min(experimental(1750:2000,:));
end
if max(experimental(2000:3000,:)) > abs(min(experimental(2000:3000,:)))
    exp_peaks(4,1) = max(experimental(2000:3000,:));
else
    exp_peaks(4,1) = min(experimental(2000:3000,:));
end

exp_peaks = exp_peaks / max(abs(exp_peaks));

(3)
err_best = 1e99;

for f1 = 0:0.01:1
    for f2 = 0:0.1:1
        for f3 = 0:0.01:1
            for f4 = 0:0.01:1
                for f5 = 0:0.1:1
                    for f6 = 0:0.01:1
                        for f7 = 0:0.01:1
                            f1 = 0;
                            f2 = 0;
                            f3 = 0;
                            f4 = 0;
                            f5 = 0;
                            f6 = 0;
                            f7 = 0;

                            peaks = f1 * all_peaks(:,1) + f2 * all_peaks(:,2) + f3 * all_peaks(:,3) + f4 * all_peaks(:,4) + f5 * all_peaks(:,5) + f6 * all_peaks(:,6) + f7 * all_peaks(:,7);
                            peaks = peaks / max(abs(peaks));
                            err = sum((exp_peaks - peaks).^2);
                            if (err < err_best)
                                err_best = err;
                                f1_best = f1;
                                f2_best = f2;
                                f3_best = f3;
                                f4_best = f4;
                                f5_best = f5;
                                f6_best = f6;
                                f7_best = f7;
                            end
                        end
                    end
                end
            end
        end
    end
end
best = f1_best * all(:,1) + f2_best * all(:,2) + f3_best * all(:,3) + f4_best * all(:,4) + f5_best * all(:,5) + f6_best * all(:,6) + f7_best * all(:,7);
best_peaks = f1_best * all_peaks(:,1) + f2_best * all_peaks(:,2) + f3_best * all_peaks(:,3) + f4_best * all_peaks(:,4) + f5_best * all_peaks(:,5) + f6_best * all_peaks(:,6) + f7_best * all_peaks(:,7);
best_peaks = best_peaks / max(abs(best_peaks));
experimental = experimental / max(abs(experimental));
plot(X_theory, best, X_exp, experimental)

B – Cobalamins

B.1 Simulation of methyl radical without contribution of f-pairs

Different combinations of emissive TM, ST₀ RPM from S- or T-born pairs can be combined as mentioned in Chapter 5, Section 5.2.2. The first step in simulation of the methyl radical was to use a combination of TM and ST₀ RPM. The result of this simulation for 532 and 355 nm spectra in non-viscous solution is presented in Figure B.1. Since the resonance lines intensities for spectrum of simulated and experimental spectra do not match, it is clear that there is contribution of an additional polarisation mechanism. This is particularly obvious for the 355 nm spectra (Figure 5.1, lower panel) where the resonance line of the lowest magnetic field is emissive and the used combination of polarisation patterns is still absorptive. Therefore, polarisation by methyl/methyl f-pairs was added to the simulation code, as discussed in Chapter 5, Section 5.2.2.1.
Figure B.1. Simulation of methyl radical by combination of polarization with absorptive TM and RPM by ST0 T-born. Presented data are for the methyl radical generated after photolysis of 1 mM MeCbl at 532 nm (top) and 355 nm (bottom). Experimental conditions: 1 mM MeCbl, 20 mM HEPES, pH 7.5, 0 % glycerol; 532 nm/355 nm 20 mJ; 16 ns microwave pulse.

B.2 Calculation for diffusion

The diffusion rate constant for the reaction in water at 20 °C is shown in Equation B.1. [2] The formation of the f-pairs for different concentrations of methylcobalamin (MeCbl) at 20 °C is summarised in Table B.1.

\[
k_d = \frac{8RT}{3\eta}
\]

[Equation B.1]

\[
k_d = \frac{8 * 8.31 \text{ J mol}^{-1} \text{K}^{-1} * 293 \text{ K}}{3 * 0.89 \text{ cP}} = 7.29 * 10^{10} \text{ L mol}^{-1} \text{ s}
\]
Table B.1. Formation of the f-pairs for four different concentrations of MeCbl.

<table>
<thead>
<tr>
<th>Concentration MeCbl</th>
<th>Rate of formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 mM</td>
<td>$2.19 \times 10^7$ s$^{-1}$</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>$3.65 \times 10^7$ s$^{-1}$</td>
</tr>
<tr>
<td>1 mM</td>
<td>$7.29 \times 10^7$ s$^{-1}$</td>
</tr>
<tr>
<td>3 mM</td>
<td>$21.87 \times 10^7$ s$^{-1}$</td>
</tr>
</tbody>
</table>

From the previous data on the diffusion of the methyl radical in 2,2,4-trimethylpentane, the diffusion coefficient was estimated to be $1.80 \times 10^{10}$ L/ mol s at 25 °C. [3] For 1 mM cobalamin, the first order rate coefficient for f-pair formation was up to $10^7$ s$^{-1}$. Therefore, the RPM contribution is expected to appear within the first 200 ns.

B.3 Linear prediction

In the photolysis of MeCbl, the FID was generated after applying a 16 ns microwave pulse at different DAF between the laser and the microwave pulse. FIDs were transformed using Bruker’s Xepr software as described in Appendix A.1. [1] Linear prediction (LP) of the data was conducted in order to extrapolate the signal to zero time because of the dead-time created through not being able to measure the FID directly after the pulse. This is not possible because of the defense pulse protection of the amplifier. Equation B.2 states the LP equation, where every data point $x_n$ can be described as a linear combination of $M$ previous ones (sinusoids). [4]

$$x_n = a_1x_{n-1} + a_2x_{n-2} + \cdots + a_Mx_{n-M}$$  \hspace{1cm} [Equation B.2]

An example and description of LP of the real channel for each field value is displayed in Figures B.2, with indicated areas of the microwave pulse and ring down, followed by the FID from the methyl radical. Since the microwave pulse and ring down signals are removed by subtraction of off-resonance signals, for clarity, an example of LP in the real channel is presented in Figure B.3. After assembling FT-EPR spectra of the methyl radical, a comparison of the peak intensities with and without LP was conducted and a bar chart is presented in Figure B.4. Unsurprisingly, it is clear that the intensity of LP spectrum is greater than data without using the LP. Comparison of the relative ratios with and without LP shows broad agreement.
However, the signal-to-noise of the lower field line at 355 nm is particularly weak causing greater uncertainty in the LP fit, which was not removed even with using the zero-filling function.

![Figure B.2](image)

**Figure B.2.** Correction of FID traces using linear prediction. The black line shows recorded FID trace at each field position. The blue line presents the LP of that trace at the time of microwave pulse. The rectangular areas of yellow present microwave pulse are removed by subtracting the off-resonance signal from the raw data. The grey rectangle presents data points used for linear extrapolation. Sample: 1 mM MeCbl at 355 nm for DAF 120 ns.
Figure B.3. Correction of FID traces using linear prediction at each field position of the methyl radical generated after photolysis of 1 mM MeCbl at 355 nm for DAF 120 ns. The traces show the real channel for raw (black) and linearly predicted data (blue, dashed). Sample: 1 mM MeCbl at 355 nm for DAF 120 ns.

Figure B.4. Bar chart diagram comparing peak intensities obtained without (black) and with linear prediction (blue). Relative ratios are stated. Sample: 1 mM MeCbl at 355 nm for DAF 120 ns.

An example comparing LP between two concentrations is presented in Figures B.5 and B.6. Figure B.7 presents the difference in FT-EPR intensity between raw experimental and LP processed data, where, again, poor S/N ratios can cause artefacts in the LP.
Figure B.5. Correction of FID traces using linear prediction at each field position of the methyl radical generated after photolysis of 0.5 mM MeCbl at 532 nm for DAF 120 ns. The traces show the comparison of real channel for raw (black) and linearly predicted data (red, dashed). Sample: 0.5 mM MeCbl at 532 nm for DAF 120 ns.

Figure B.6. Correction of FID traces using linear prediction at each field position of the methyl radical generated after photolysis of 3 mM MeCbl at 532 nm for DAF 120 ns. The traces show the comparison of real channel for raw (black) and linearly predicted data (green, dashed). Sample: 3 mM MeCbl at 532 nm for DAF 120 ns.
Error analysis in relation to LP

Three independent experimental data sets were normalised by division by the maximum intensity line of the FT-EPR spectra, and then, the standard deviation was calculated and presented as the error bar on the chart diagrams. The same process was repeated for solutions with and without glycerol, and the results are plotted against the resonance peak intensities when processed using LP FT-EPR for two delay times. Results are presented in Figure B.8 for non-viscous solution and B.9 for solution with 50 % glycerol. For both solutions the relationship between experimental and LP data are different for each field position. In non-viscous solution, the LP of first and second field position is within the experimental error of reproducibility, for DAF 80 ns. However, LPs on the fourth line at 80 ns and for the 160 ns data are outside the error. In viscous media, experimental error is smaller (compared to the non-viscous) and LP is in general outside this error, with it being quite close on the fourth peak position.
Figure B.8. Average peak intensity values of methyl radical at two delay times, for experimental and LP data. Error bars are calculated using standard deviation. Experimental conditions: 0.5 mM MeCbl, 20 mM HEPES, pH 7.5, 0% glycerol; 532 nm.

Figure B.9. Average peak intensity values of methyl radical at two delay times, for experimental and LP data. Error bars are calculated using standard deviation. Experimental conditions: 0.5 mM MeCbl, 20 mM HEPES, pH 7.5, 50% glycerol; 532 nm.

The advantage of using LP is the correction for dead-time and the increase in signal intensity. However, it was found that poor signal-to-noise can cause uncertainty in the LP as with any fitting method as is very evident in the low field line shown in Figure B3. This can cause greater uncertainty at 355 nm as well as when using longer DAF, where signal-to-noise is weaker. Since many comparisons are being made between data sets, it was decided not to use LP due to this uncertainty (especially at the lowest field position).
B.4 Direct detection EPR of photolysis of MeCbl

Involvement of polarisation by f-pairs was investigated by using a direct-detection EPR (DD-EPR) experiment. This experiment differs from FT-EPR because of the increased dead-time of the technique due to the high Q of the resonator used with Bruker instrumentation. Due to this limitation in instrumentation, early reaction stages are not so readily observed as in the FT-EPR approach. However, it was helpful to follow the reaction using this approach because the signal decays with $T_1$ rather than $T_2$, allowing a longer observation window. The results of this approach are presented in Figure B.10. It can be observed that after the initial signal rise and decay, the signal re-appears after about 2000 ns, during the measuring time of 5000 ns. The signal-to-noise is worse than the FT-EPR traces but the interesting time dependence can be observed, which, however, cannot be easily explained. The early time polarisation is in agreement with results observed in FT-EPR experiments but the quality of the spectra in DD-EPR are poor because of oscillations appearing in the background. Since the analysis is complicated and difficult to explain this is a subject of future studies.

![Figure B.10](image)

**Figure B.10.** DD-EPR spectrum of methyl radical obtained after photoexcitation at 532 nm and 355 nm. Experimental conditions: 0.5 mM MeCbl, buffer 20 mM HEPES, pH 7.5, 50 % glycerol; 532 nm or 355 nm, 20 mJ.

B.5 FT-EPR of MeCbl at low concentration

The concentration dependence experiments of the photolysis of MeCbl was completed in order to establish the influence of concentration and to confirm the presence of f-pairs. The experiment was conducted in a concentration range of 0.3
– 3 mM. However, the poor S/N of the FT-EPR spectra recorded with the lower-concentration samples made reliable fitting of simulated spectra difficult. Since the data quality is low, these spectra were excluded from the analysis.

**Figure B.11.** Simulation of methyl radical generated after photolysis of different [MeCbl] at 532 nm. Combination of used polarisation is absorptive TM, S-born ST_0 RPM and methyl/methyl-pairs. Experimental parameters: 0.3 and 3 mM MeCbl, 20 mM HEPES, pH 7.5, 0 % glycerol; 532 nm, 20 mJ; 16 ns microwave pulse, 8 dB microwave attenuation.

**References**