



Australian
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Optical spectroscopy of the red forms of Photosystem II and I

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July 2025

A thesis submitted for the degree of Doctor of Philosophy of
The Australian National University

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of the author's knowledge, it contains no material previously published or written by another person, except where due reference is made in the text.

Jennifer Halle Morton

July 2025

Acknowledgements

In order to make a complex project like this possible, we required assistance from people with expertise in everything from the biochemistry to engineering.

First of all, I would like to thank Nick Cox and Elmars Krausz for their supervision and for providing some of the data and diagrams used. Colin Jackson and Rob Stranger for assistance with supervision. I would also like to thank my lab colleagues Julien, Robin, Martyna and Jeremy for their help and advice. I also need to thank Paul Smith for the spinach PSII samples and advice on EPR.

I would also like to thank the following organisations and individuals for their support:

- Professor Jian-Ren Shen and his colleagues in Japan and China for the cyanobacteria and red algae samples that were the basis of this project.
- Dennis Nürnberg, Bill Rutherford and the group at Imperial College for the samples of chlorophyll f containing cyanobacteria and for hosting a visit.
- Rafael Picorel for various samples including spinach.
- Jeff Reimers for his help with theory.
- Eiri Heyno for the Cyt c550 sample.
- Vincent Craig whose help with the hydrophobic coating on the sample cells which was vital to this project
- Arvi Freiberg and the staff and students at the University of Tartu for hosting me for a visit and letting me use their FLN spectrometer.
- The staff and students at Max Planck Institute for Chemical Energy Conversion for the use of their MCD system.
- Keith Jackman whose sample cells and extensive work on our MCD system made this project possible. I would like to also thank all of the technical staff at the RSC past and present who have helped keep the lab operational for the past 30 years.
- The late Ron Pace for his very artist interpretations of our MCD data.

And finally, I would like to thank my and Ian Morton and Judy Morton, my grandparents Halle Morton, Donald Morton and Curtis Deland for helping to support me through university.

This research is supported by an Australian Government Research Training Program (RTP) Scholarship.

Abstract

Research in photosynthesis encompasses a wide array of complex and interconnected processes, requiring the incorporation of concepts from physics, chemistry and biology.

This thesis contributes to the understanding of photosynthesis by using low temperature optical spectroscopy to study Photosystem II (PSII) and Photosystem I (PSI). These two catalytic protein complexes are responsible for splitting water to provide electrons for the photosynthetic process, and transferring these electrons across the thylakoid membrane, respectively

These enzyme complexes provide a particular challenge for optical spectroscopy as they contain large numbers of chromophores with overlapping absorption bands, which require a combination of optical techniques to separate out. In this work, the techniques of absorption, fluorescence, Circular Dichroism (CD), Magnetic Circular Dichroism and electrochromism have been used to study these enzymes.

This thesis has two main themes: the direct observation of the optical spectroscopic properties of the Oxygen Evolving Complex (OEC) in Photosystem II (Publications 1 and 4) and the location of far-red absorbing chlorophylls in PSII and PSI of a red-light adapted cyanobacterium (Publication 2 and 3)

The OEC of PSII is the tetramanganese cluster where electrons are extracted in a stepwise fashion from water, resulting in the release of oxygen. In this process, the cluster passes through four metastable S-states. In Publication 1, variable temperature and variable field (VTVH) MCD is used as a tool to identify the spectrum of each S-state in an optically transparent window in the region of 720-800nm.

Low temperature MCD spectra of this region shows a series of sharp peaks that are S-state dependent. These features were assigned to a series of Mn(IV) $4A \rightarrow 2E$ "spin

flip” transitions. These spectra and their associated assignments provide a new perspective on the electronic and magnetic structure of the OEC.

Publication 4 follows on from 1, focusing on the two sub-forms of the S₂ state of the OEC. A combination of EPR and MCD enables the identification of these two states as being redox isomers and give insight into the mechanisms of water oxidation.

Chroococcidiopsis thermalis is an extremophile cyanobacteria that can grow in hot and dry conditions. When grown under red-light conditions, this cyanobacteria can produce red absorbing chlorophylls d and f, along with chlorophyll a.

In Publication 2, using a range of biophysical techniques, including low temperature optical spectroscopy, we clearly establish that these red absorbing chlorophylls play a key role in both PSI and PSII and are not just as long wavelength light harvesting pigments as previously reported. Specifically, charge separation in PSI uses chlorophyll f at 745nm and PSII uses chlorophyll d or f at ~727nm. The organism is shown to be able to perform photosynthesis with lower energy light than previously thought possible.

Publication 3 is an extension of Publication 2 where the location and function of the red absorbing chlorophyll at ~727nm of PSII in *C.thermalis* is further investigated. Modelling of low temperature spectroscopic data, CD and electrochromism in particular, yields more information about the location of this pigment within the PSII complex. Previously, Chl_{D1} was assigned as the most likely candidate for the primary donor. However, the analysis in Publication 3 favours P_{D2} as the primary donor. This unexpected result requires further experimentation and calculational studies to verify.

This thesis shows the strength of applying multiple spectroscopy techniques to complex biological systems and how such data can be used to extract information about both the structure and function of these proteins.

List of Abbreviations

6803	<i>Synechocystis sp. PCC 6803</i>
Å	Ångstrom (0.1 nanometers)
A	Absorbance
A _L /A _R	Absorbance of left/right circularly polarised light
Allo	Allophycocyanin
<i>A. marina</i>	<i>Acaryochlorous marina</i>
ATP	Adenosine triphosphate
ΔA	Differential absorbance
CD	Circular Dichroism
Chl	Chlorophyll
<i>C. Thermalis</i>	<i>Chroococcidiopsis thermalis</i>
Cyt	Cytochrome
DRS	Deep Red State
Fe _x	Ferredoxin
FRL	Far Red Light (700nm or longer)
Glassing agent	Glycerol or 1:1 glycerol and ethylene glycol
MCD	Magnetic Circular Dichroism
NADP(H)	Nicotinamide adenine dinucleotide phosphate (Reduced)
OD	Optical Density

OEC	Oxygen Evolving Complex
Pheo	Pheophytin
PQ, PQH ₂	Plastoquinone, Plastoquinol
PsbV	PSII extrinsic protein containing Cytochrome C ₅₅₀
PSI	Photosystem I
PSII	Photosystem II
P680	PSII primary donor
P700	PSI primary donor
Q _x	Plastoquinone (PSII)
RC	Reaction Centre
<i>T. Vulcanus</i>	<i>Thermosyneccocus vulcanus</i>
<i>T. Elongatus</i>	<i>Thermosyneccocus Elongatus</i>
VTVH	Variable Temperature, Variable Field
WL	White Light

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Chapter 1

Introduction to Photosynthesis

1.1 Overview of Oxygenic Photosynthesis:

The evolution of oxygenic photosynthesis profoundly changed the planet leading to the development of multicellular life and an oxygen atmosphere. The key enzyme complexes behind the light driven process are Photosystem I and II. These enzyme complexes efficiently convert light into chemical energy using common metallic elements as catalysts. If this process could be artificially replicated then it could provide a reliable supply of clean energy.

Photosystem II is responsible nearly all the oxygen atmosphere and the evolution of complex multicellular life. Oxygenic photosynthesis first evolved in cyanobacteria. In organisms such as plants and seaweed photosynthesis occurs in organelles known as chloroplasts. These organelles were originally cyanobacteria that were absorbed by a larger eukaryotic cell and eventually full incorporated into it. This process is known as endosymbiosis and is covered in more detail in Section 1.5.

While the general processes behind photosynthesis are well understood, delving into the details provides abundant topics for ongoing research. Of particular interest is the precise mechanism involved in splitting water in Photosystem II.

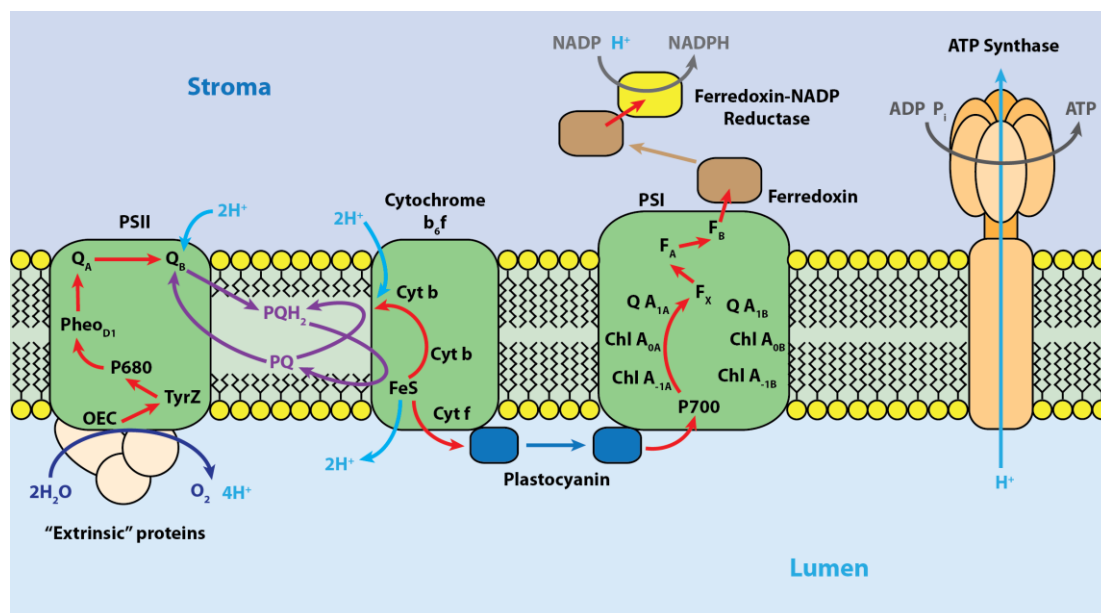
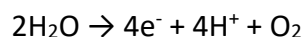


Figure 1.1: overview of the light dependent reactions in oxygenic photosynthesising organisms. Adapted from the diagram in [1]. Light harvesting proteins omitted for clarity

Figure 1.1 shows an overview of the key chemical reactions involved in the light driven process. Photosystem II extracts electrons from water via a four-step process, with one electron extracted for every photon absorbed. This reaction is catalysed by the Oxygen Evolving Complex (OEC), a cluster of four manganese ions.



This process occurs with high quantum efficiency, close to 100% for visible light shorter than 700nm [2]. The electrons extracted from water are used to reduce plastoquinone (PQ) to plastoquinol (PQH₂), which is then re-oxidised by cytochrome b₆f. Those electrons are then transferred to Photosystem I via plastocyanin.

PSI then re-excites these electrons to Ferredoxin, which in turn transferred that electron to Ferredoxin-NADP Reductase. The proton gradient generated during this process powers ATP synthase. The ATP and NADPH generated can then be used to in the carbon fixation (ie the Calvin cycle). PSI together with Cytochrome b₆f can also undergo cyclic electron reactions to produce ATP without PSII. This notably occurs in some nitrogen fixing cyanobacteria.

The fundamental processes involved in oxygenic photosynthesis are highly conserved between different organisms. When significant deviations from the “normal” process are discovered, they are of great interest.

1.2 Structure and function of Photosystem II

The X-ray crystallography structures of PSII and PSI derived from thermophilic cyanobacteria [3, 4] and red algae [5, 6] have been reported.

The smallest unit of Photosystem II that is still capable of evolving oxygen is referred to as the PSII core, weighing in at 350kDa. The crystal structure of the core from cyanobacteria is shown in Figure 1.2 with key cofactors highlighted.

The PSII core consists of the two central reaction centre proteins D1, D2 and cytochrome b_{559} , the antenna complexes CP43 and CP47, and the OEC stabilising proteins, including cytochrome c_{550} in most organisms. In total, the complex contains 35-36 chlorophylls, 2 pheophytin, 11 carotenoids, 1-2 cytochromes and the OEC. The structure and function of the pigments are covered in section 1.4.

The overall structure and function of the PSII core is highly conserved across organisms. One notable difference is the “extrinsic proteins” on the lumen side of the membrane shown in Figure 1.1. Higher plants lack the PsbV subunit that contains cytochrome c_{550} .

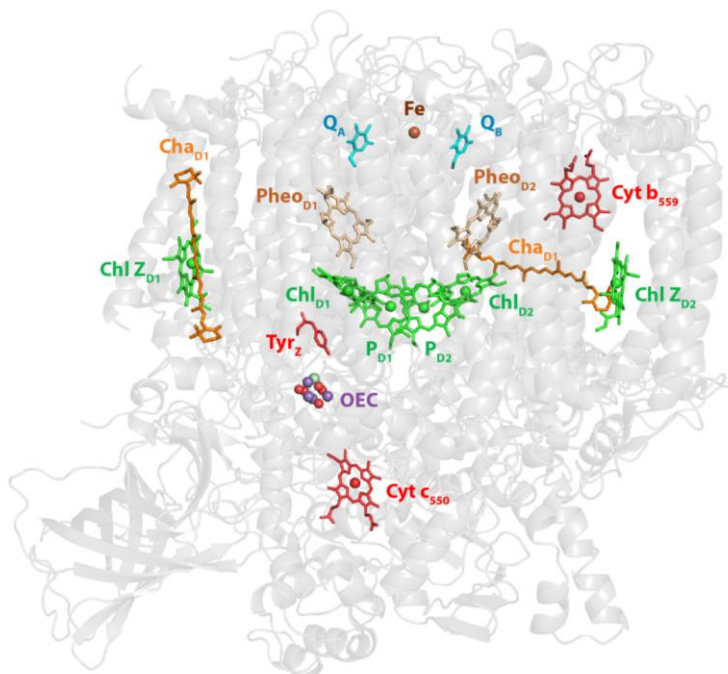


Figure 1.2: Diagram of the PSII core from cyanobacteria, based on the crystal structure from Loll et al (2005)[7]. Chlorophylls and carotenoids in the antenna are excluded for clarity.

1.2.1 Electron transport in Photosystem II

The primary donor is known as P680, which represents four interacting chlorophylls, P_{D1} , P_{D2} , Chl_{D1} and Chl_{D2} [8]. Electron is transferred up to a plastoquinone molecule (Q_A), then across to another plastoquinone (Q_B) that is loosely bound to the PSII complex. Once Q_B is reduced twice, it can detach from PSII and migrate through the thylakoid membrane to cytochrome b_6f .

P680⁺ then extracts an electron from the OEC, which in turn extracts an electron from a molecule of water. To produce one molecule of oxygen the OEC cycles through four stable intermediate states known as S-states. When kept in the dark the OEC rolls back to the S₁ state.

P680 is named for the bleach that appears in the electrochromic spectrum that appear upon the formation of the radical. P680⁺ has an estimated redox potential up to +1.2V which is the highest of any system known in nature. This high potential is required in order to remove electrons from water. This high potential means there is the likelihood that electrons could be extracted from elsewhere, resulting in damage to the complex. Chlorophylls in the antennas are close enough to transfer excitation to P680 but far away enough to minimise the potential for electrons to be extracted from those chlorophylls rather than the OEC.

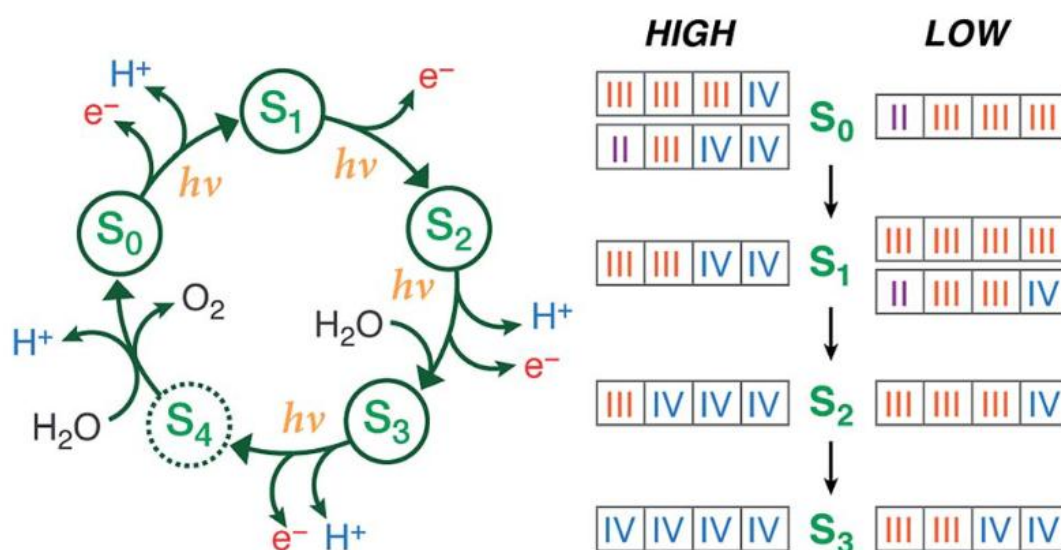


Figure 1.3, the S-state cycle. From Krewald et al. 2015 [9]

In plants, green algae and red algae the membrane the reaction centre is surrounded by light harvesting complexes (LHCs) that funnel and regulate light to the reaction centres (Figure 1.4). LHCII can also move between PSII and PSI reaction centres, balancing the relative amount of light that reaches both centres.

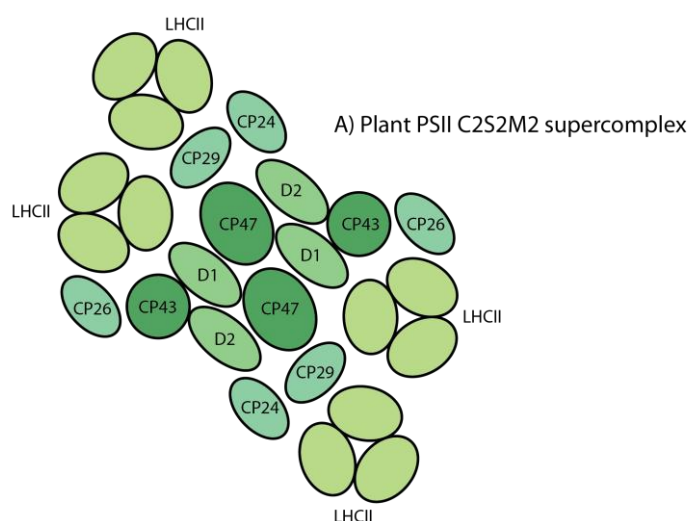


Figure 1.4: One of the possible arrangements of plant Photosystem II subunits and associated light harvesting complexes as seen from the stromal side. Derived from diagram in [10]

In plants there are a diverse number of small LHCs associated with the reaction centres, as well as the larger LHCII trimers. The structure of the LHCII complex changes under different light conditions to favour different pathways [11].

Under high light conditions the role of the LHCs switches from light harvesting to photoprotection. Instead of the excitation being transferred from chlorophyll to chlorophyll into the PSII reaction centre it is instead transferred to a carotenoid and dissipated as heat via a process known as Non Photochemical Quenching (NPQ). This process prevents the formation of long-lived chlorophyll triplet excited state which in turn can lead to the formation of highly reactive singlet oxygen species.

1.2.2 The oxygen evolving complex

The Shen et al (2011) crystal structure at 1.8Å revealed the general structure of the OEC in the S1 state as given in Figure 1.5 below. The cluster self assembles in the cavity from free Mn(II) ions when PSII apo-protein is exposed to light.

Mn_{1,2} and 3 form a cubane like structure along with a calcium atom with Mn₄ referred to as the “dangler” because of its more exposed position. This configuration is flexible, changing its configuration over the course of the cycle.

All the Mn atoms are fully ligated with oxygen, the exception being Mn 1 which is bound to the histidine residue. As indicated in Figure 1.3 there has been a long standing debate as to the oxidation states of the Mn ions of the cluster in all S-states. This is described in terms of two models: the low oxidation state and high oxidation state paradigms. [9]

As well as cycling between different S-states, the OEC can adopt slightly different configurations in a given S-state. This slight change in geometry results in a difference in electronic structure that can be measure using various spectroscopy.

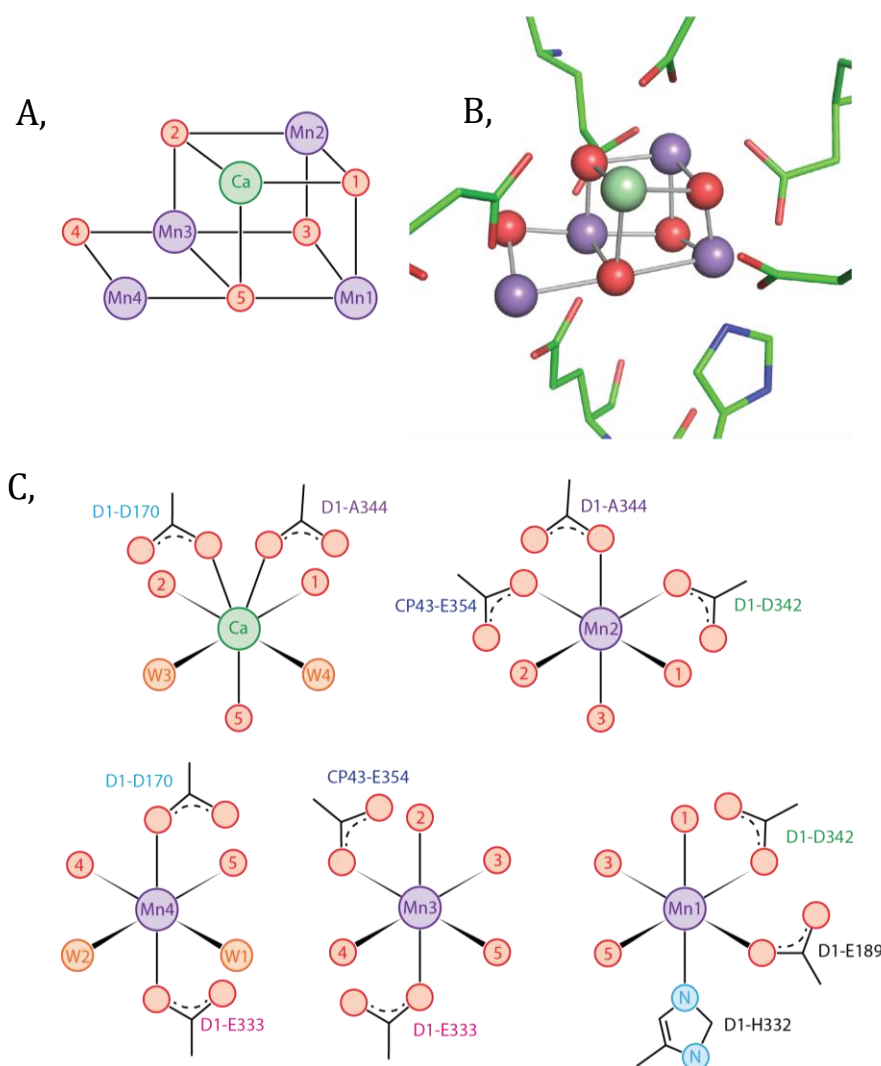


Figure 1.5: Structure of the OEC as a simplified cartoon (A) and based on the crystal structure (B). The ligands around each individual Manganese atom are shown as (C).

Figure 1.5 shows each stage of the cycle. In thermophiles such a *T. vulcanus* the half-life of the other S-states is on the order of minutes, with the exemption of the transitional S₄ state. In theory a series of pulsed bursts from a laser can be used to set the sample into a particular S-state, and then the sample can be quickly frozen to lock the OEC into that configuration.

In practice there are always a number of “misses”, especially in isolated samples. The further along in the cycle the smaller the proportion in that state. The yield of S₂ using

this method was >80%, however the proportion of S3 in the sample that had been flashed twice was usually 20-40%.

When frozen samples are illuminated the electron extracted from P680 transfers to Q_A and remains there. This movement of charge slightly shifts the absorption of nearby pigments. It is important to control the level of light used in measurement so that the spectra does not detectably change during the course of a scan. Changes in the spectrum before and after intense illumination can be used to study the chromophores in the centre of the complex and their role in charge separation. See Section 2.3.

1.3 Structure and function of Photosystem I

While the central focus of this work is on the Photosystem II complex, when studying one photosystem it is often necessary to do parallel experiments on both as there is often a measurable level of one contaminating samples of the other.

The crystal structure trimeric PSI core from *T.elongatus* shows 96 chlorophyll per complex[12] (Figure 1.6). The number per centre varies depending on the species and on the preparation. Also shown are the iron-sulphur clusters (Fe_x , Fe_a and Fe_b)

Light is transferred to central pair of chlorophylls that form P700. From there the excited electron is transferred up to the iron-sulphur clusters, then to ferredoxin and onto NADPH reductase. $P700^+$ can then extract an electron from plastocyanin.

The $P700^+$ radical can be quite stable at room temperature and for some experiments the sample must be chemically reduced to remove it. At low temperatures the electron extracted from P700 remains on one of the iron-sulphur clusters. As with PSII, illumination of the sample at low temperature allows us to measure an electrochromic shift spectrum.

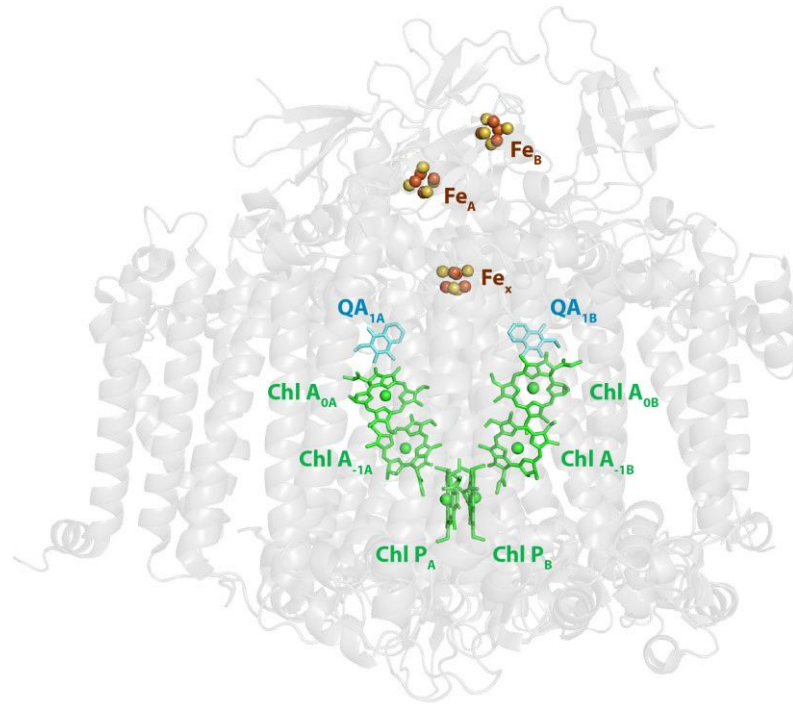


Figure 1.6: Diagram of the PSII core from cyanobacteria, based on the crystal structure from Jordan et al [4]. Carotenoids and antenna chlorophylls excluded for clarity.

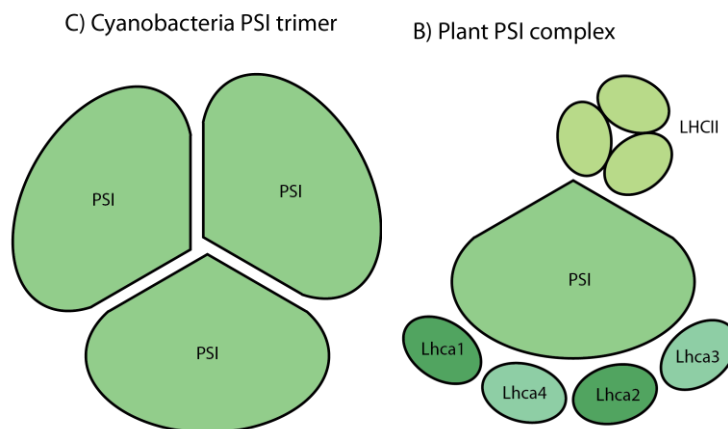


Figure 1.7: Arrangement of the PSI trimer seen in cyanobacteria, and monomeric PSI from plants with associated light harvesting complexes as seen from the stromal side.

1.4 Pigments of the reaction centres of photosynthesis

The twin enzyme complexes Photosystem II and Photosystem I present a unique challenge for optical spectroscopists. Using light to study light-powered enzymes allows us to directly probe the underlying mechanisms.

Both complexes contain a large number of pigment cofactors with overlapping absorption bands. This requires us to use multiple techniques to pull apart the spectra. As these systems are highly sensitive to light the amount of light used has to be carefully managed and the experimental system has to be optimised for these experiments.

1.4.1 Chlorophylls

The key chromophores in photosynthesis are the chlorophylls. These versatile molecules are involved in both light harvesting and charge separation. Chlorophyll a is the most abundant chlorophyll in most organisms, and usually the only one found in the PSI and PSII reaction centres. Isolated chlorophyll a in dry ether has a strong absorption peak at 665nm. This gives chlorophyll its characteristic green colour.

The position of the absorption peaks are modified by the environment surrounding the chlorophyll. When a chlorophyll absorbs a photon it can transfer that excitation to another chlorophyll via a process known as Förster energy transfer. A chlorophyll that absorbs at higher energy transfers the excitation to a lower energy chlorophyll, with the energy difference lost as heat. In the enzyme complexes the chlorophylls are arranged in order to funnel energy into the reaction centres of the photosystems where the chemistry occurs.

It is also possible for lower energy chlorophylls to transfer energy “uphill” with thermal energy making up the difference. Statistically this transfer is mostly one way, but is relevant to discussions about PSI which has antenna chlorophylls absorbing at longer wavelengths.

Figure 1.8 shows the structures of the other three chlorophylls relevant to this thesis. They all differ from chlorophyll a by only one functional group.

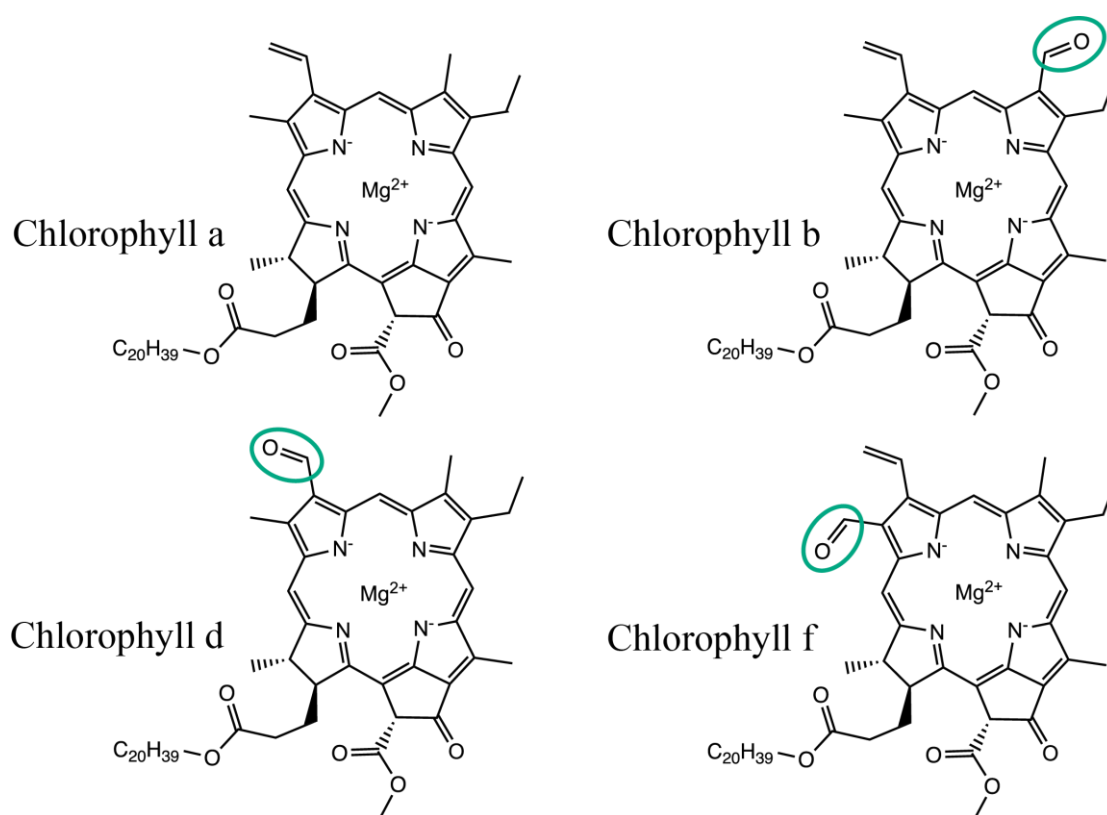


Figure 1.8: Comparison of the different types of chlorophyll relevant to this work.

Chlorophyll a has strong absorption bands in the blue and red regions of the spectrum, resulting in its distinctive green colour. Organisms can use other pigments to bridge this absorption gap under low light conditions, for example chlorophyll b and c which both have Q-bands further to the blue than chlorophyll a.

Chlorophyll b is found in green algae and higher plants, typically within the light harvesting complexes (LHCs) that are small transmembrane proteins associated with PSI and PSII. Plants produce a diverse variety of LHCs which also contain chlorophyll b and carotenoids.

Chlorophyll c (not shown) lacks the phytol chain. It is commonly found in the brown algae such as kelp. Like chlorophyll b it is used primarily as a light harvesting pigment.

In contrast chlorophyll d and chlorophyll f both have absorption maxima further to the red. Chlorophyll f was first reported in 2010, it was first isolated from cyanobacteria growing underneath stromatolites. It has since been found in numerous different strains of cyanobacteria. The existence of chlorophyll e had earlier been reported but the molecule has yet to be properly characterised.

In most organisms chlorophyll a is the only chlorophyll in the PSI and PSII core complexes. Other chlorophylls are found in antenna complexes. The notable exemption is the marine cyanobacteria *Acaryochlorous marina*, where PSI and PSII contain almost entirely chlorophyll d, with at least one chlorophyll a in each enzyme complex.

Another exemption is studied in this work. When grown under low light conditions the cyanobacteria *Chroococcidiopsis thermalis* incorporates chlorophyll f into PSII and PSI, as well as one molecule of chlorophyll d in PSII. While mass spectrometry could show those chlorophylls were in the enzyme complexes, optical spectroscopy was needed to determine the role these chlorophylls.

While not strictly chlorophylls, pheophytin molecules - chlorophylls without the Mg^{2+} ion stabilising the ring – are also important components of the electron transfer pathway in photosynthetic reaction centres. It is more easily reduced than chlorophyll which allows it to act as an electron acceptor in the PSII reaction centre.

1.4.2 Other pigments

Other pigments found in photosynthetic reaction centres are involved in light harvesting and in photoprotection. Two important categories of pigments are the carotenoids, molecules with a long series of conjugated bonds, and open chain tetrapyrroles known as phycobilins.

Carotenoid molecules such as β -carotene, lutein and zeaxanthin are another pigment found in PSI, PSII and its associated light harvesting complexes. They are able to absorb

light and transfer energy to the chlorophylls. They also play an important role in photoprotection.

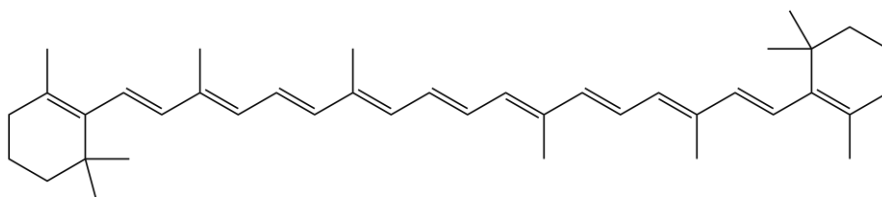


Figure 1.9 Structure of β -carotene

Phycobilins play a vital role in light harvesting in cyanobacteria and are also used by red algae which gives many species their characteristic colour. They are synthesised by opening up the ring of heme molecules by a specialised enzyme. Phycobilins are arranged in large protein complexes known as phycobilisomes that sit above the photosynthetic complexes, as shown in Figure 1.11 with three different forms of phycobilisome.

Phycobilisomes containing higher energy phycobilins are at the edge of the supercomplex funnel energy to lower energy phycobilins and then onto the chlorophylls in the photosystems.

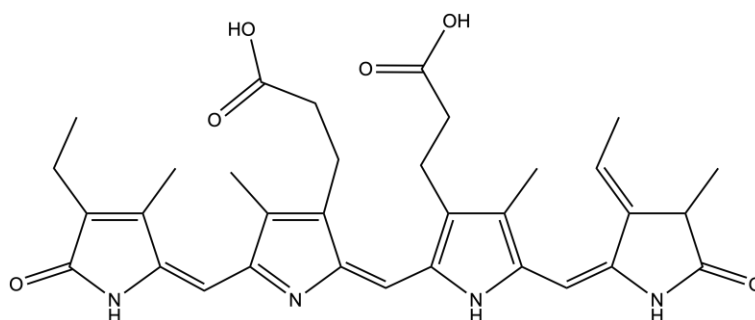


Figure 1.10: Structure of phycocyanobilin, a blue pigment found in phycobilisomes.

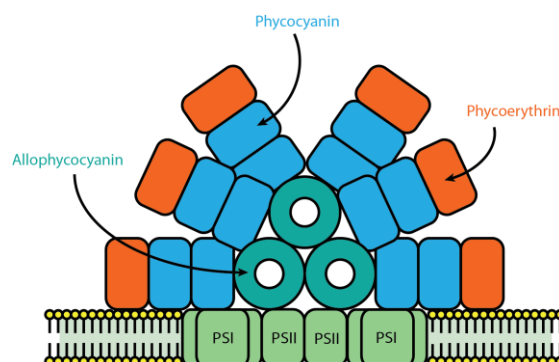
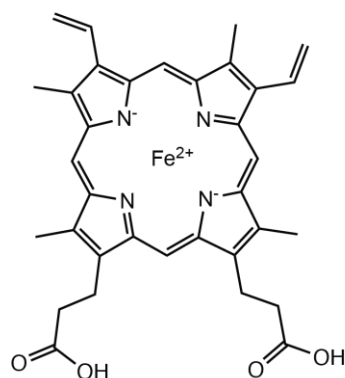


Figure 1.11: One possible arrangement of phycobilisomes above the PSII and PSI core complexes, based on an example from [13]

Cytochromes are heme containing proteins that are typically involved in electron transport and redox reactions. The optical properties of the cytochromes change dramatically depending on the oxidation state of the heme.

There are two different cytochromes in the PSII core. The b_{559} subunit is necessary for the assembly of the complex and is also involved in secondary electron transfer pathways.

Heme b



Heme c

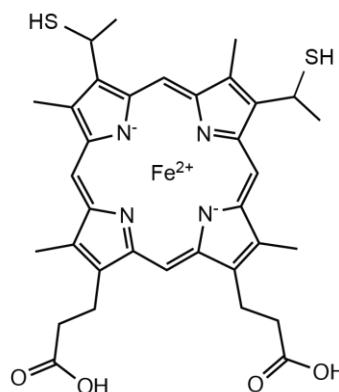


Figure 1.12: Structure of the reduced heme molecules inside the cytochrome.

The precise role of cytochrome c_{550} is unclear, it is not essential for PSII to function as it is not found in the PSII cores of land plants [14] Heme c is bound to the protein via two thioether bonds formed at the location of the sulphurs shown.

1.5 The evolution of photosynthesis

Life on earth is divided up into three domains, bacteria, archaea, and eukaryotes. Bacteria are an ancient and highly diverse domain of organisms that are either single celled or grow in colonies with some specialisation of cells. Eukaryotes include most multicellular lifeforms (plants, animals, fungi and macroalgae), as well as a diverse array of single celled organisms.

Atmospheric oxygen level were low until approximately 2.4 billion years ago [15]. Numerous other bacteria use bacteriochlorophylls which are structurally similar to chlorophyll. These bacteria don't produce oxygen, instead they harvest light to power cyclic electron reactions. These bacteria contain enzyme complexes that resemble PS I and PS II, known as Reaction Centre I (RCI) and Reaction Centre II (RCII). These bacteria can contain either one of both of these complexes.

While RC I and II may appear to be "ancestral" forms of Photosystems I and II, more comprehensive analysis [15] has shown this is likely not the case. RCII notably lacks the ability to split water, instead driving cyclic electron reactions.

Eukaryotes were able to use the oxygen in the environment in respiration. At some point a eukaryotic cell absorbed a cyanobacteria, and incorporated the smaller cell into itself rather than digesting it. The cyanobacteria eventually became an organelle.

This cell was the ancestor of the group Archaeplastida which branched into three main divisions. Viridiplantae, which includes green algae and land plants. Rhodophyta, the red algae. And Glaucophyta, a group of small simple algae.

Secondary endosymbiosis events occurred when the chloroplasts in one species were incorporated into another. This event happened numerous times, resulting in chloroplasts having a different evolutionary relationship to the rest of the organism that contains it. Most photosynthetic eukaryotes have chloroplasts derived from one of two "lineages", those derived from red algae and those from green algae. [16]

Some of the organisms studied in this work are extremophiles, growing in environments considered too harsh for most other lifeforms. For example environments with high temperatures ($>45^{\circ}\text{C}$), low pH, only trace amounts of water and low light levels.

The core complexes extracted from extremophiles are far more robust than those derived from organisms growing in more ambient conditions. This allows the samples to be more easily purified and potentially crystallised. At room temperature the S-states of PSII derived from thermophiles are far longer lived than PSII from other organisms. For instance the S2 state of PSII derived from *T.vulcanus* has a half-life on the order of 10 minutes, compared to that from spinach which decays in a matter of seconds.

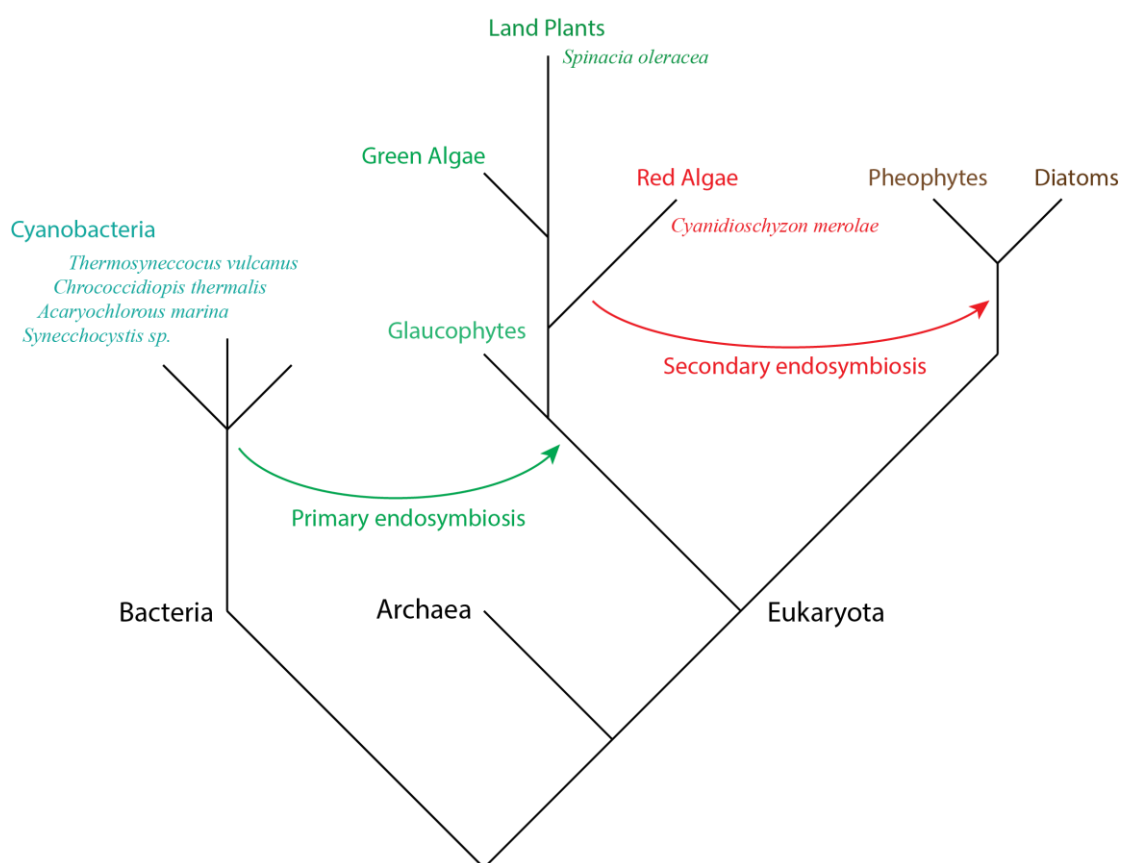


Figure 1.13: Simplified evolutionary relationship between cyanobacteria and eukaryotic photosynthetic organisms. The species mentioned in this work are listed in italics.

1.5.1 Organisms mentioned in this work

Section 3.3.1 details the samples that were used. *Thermosyneccocus vulcanus* and related strain *T. elongatus* are thermophilic cyanobacteria found growing in volcanic hotsprings in Japan. The PSII cores extracted from *T.vulcanus* are significantly more stable than that from land plants, allowing the complex to survive being crystalised. The samples used in this work are from the same research group that reported the structures in [3, 4], though the crystallisation step was considered unnecessary for these experiments.

Cyanidioschyzon merolae is a single celled thermophilic red algae also found growing in volcanic hot springs. It is one of the smallest and simplest known eukaryotes. It and related red algae [5] are of significant interest as they can be used to produce PSII core samples robust enough to be crystalised. Spectroscopy of this organism was carried out by Julien Langley and published in [17].

Chroococcidiopsis thermalis is a cyanobacteria that can be found growing under rocks in deserts. Like other extremophiles, its ability to survive in hot dry conditions results in more durable photosystem samples. Under low light conditions *C.thermalis* and many other cyanobacteria produce chlorophyll d and chlorophyll f alongside chlorophyll a.

Work was also done on organisms that grow in significantly less extreme conditions. Spinach (*Spinacia oleracea*) is one of the land plants commonly used in photosynthetic research. It can produce PSII core samples with no measurable amount of PSI contamination if grown under the right conditions [18]. CP43, CP47 and Reaction Centre samples have all been prepared from spinach.

Synechocystis sp PCC 6803 is another cyanobacteria that is commonly used as a model organism [19]. It can be found growing in warm fresh water.

Acaryochlorous marina is a cyanobacteria first found growing underneath sea squirts [20]. It is highly notable because it contains mostly chlorophyll d with only a comparatively small amount of chlorophyll a.

Chapter 2

Theoretical Background

2.1 Low temperature spectroscopy

In this section I will cover the theoretical background of the work in this thesis. The primary tool used to study Photosystem I and II is low temperature optical spectroscopy. This was complemented by with Electron Paramagnetic Resonance, which is briefly described in Section 2.

While the photosystem complexes from many organisms have been studied by means of x-ray crystallography, this technique has some significant limitations. Optical and EPR spectroscopy enable a wider range of samples to be studied, not just proteins that would survive the crystallisation process.

The spectra reported in this thesis were typically measured at either 1.8K using liquid helium as a coolant, or at 77K using liquid nitrogen, but the spectrometer described in section 3 can also be used at room temperature.

Measuring optical spectra at low temperature has several advantages. These include narrowing of linewidths, which is explained in detail in section 2.1.2. Low temperatures prevent chemical reactions from occurring, particularly important for light sensitive compounds such as the photosystems. Freezing the samples also enables the study of intermediate states. For experiments involving Photosystem II the same sample can be poised in different S-states and then frozen before relaxing back.

Low temperatures also stabilise charge separated species. As discussed in Chapter 1, charge separation occurs in both photosystem I and II. In frozen samples, a mobile acceptor is not able to transfer the electron away from the protein complex. Changes induced by low temperature illumination can be reversed by annealing the sample at room temperature.

Samples studied by optical spectroscopy can be maintained in a wider variety of chemical environments. These techniques can also be non-damaging to the sample.

For low temperature measurements the same sample can be recycled between experiments with only minor degradation with use of the right cryoprotectant.

Low temperature optical spectroscopy also comes with several technical challenges, as described in Chapter 3. These low temperature experiments require either solid state samples or liquids that form a transparent glass upon cooling.

Absorption of light by a molecule occurs when the electric field of the UV-vis light interacts with electrons of the molecule. The electrons are promoted from a resting ground state to an excited state (Figure 2.1). Electronic transitions in organic molecules typically involve s, n and p electrons. Inorganic molecules typically have d-d transitions, ligand-to-metal charge transfer (LMCT) transitions, and metal-to-ligand charge transfer (MLCT) transitions.

The photosynthetic systems investigated in this thesis comprise organic pigment-protein complexes with metals in their active centres. Thus, all these types of transitions are observed. Of particular note are the $p \rightarrow p^*$ transitions seen in chlorophyll and the d-d spin flips observed in the oxygen evolving centre (OEC) of photosystem II. The spectroscopy of chlorophyll is discussed further in section 2.2.1 below. “d-d spin flips” are described in section 2.2.3, paper 1 and its supplementary information.

The absorbance of a sample is given by the simple Beer-Lambert law.

$$A = \epsilon cl.$$

Equation 1.

Where A is the absorbance, ϵ is the molar extinction coefficient ($L \text{ mol}^{-1} \text{ cm}^{-1}$), c is the concentration (mol L^{-1}) and l is the path length in cm.

Once a molecule has absorbed a photon of light there are a number of processes that can follow:

(I) The molecule can relax back to the ground state, releasing a photon as fluorescence.

(II) The absorbed energy can be dissipated as heat. This occurs if there are one or more relaxation pathways. If there are intermediate states can prevent fluorescence by providing alternative pathways for de-excitation, the process known as fluorescence quenching.

(III) The absorbed light provides the energy to drive a chemical reaction.

(IV) The excitation energy can be transferred to another molecule. The process known as Forster resonance energy transfer. This is the mechanism by which light energy can be harvested in photosynthetic systems. Chlorophylls on the periphery of the protein complex absorb at higher energy and funnel the excitation energy to the red shifted chlorophyll in the reaction centre.

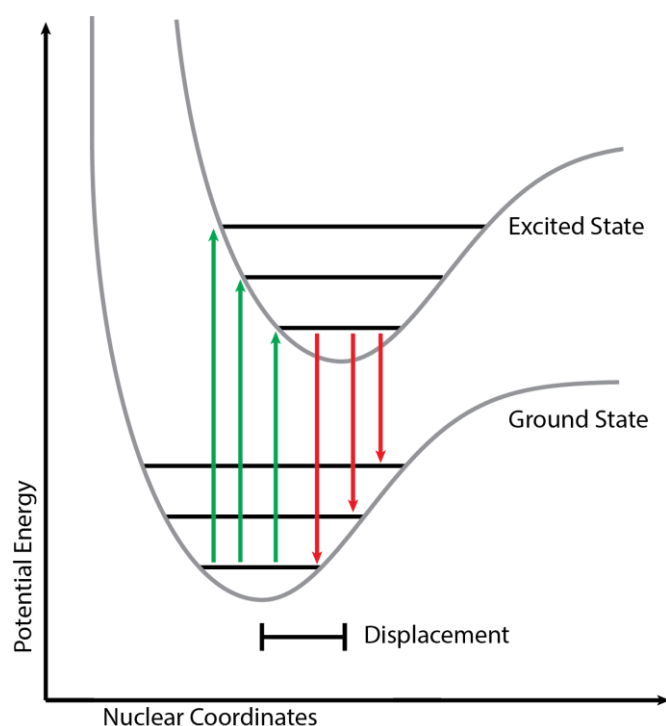


Figure 2.1: Potential energy diagram of a single chromophore. Each electronic transition is coupled to a number of vibrational modes. See figure 2.12 A for corresponding absorption spectra.

Electronic transitions occur through the coupling of the electromagnetic field to the transition dipole moment. Whether or not a transition occurs is given by the transition moment integral:

$$\int \psi_1 \mu \psi_0 d\tau$$

Equation 2.

Where Ψ_0 and Ψ_1 are the wavefunctions of the ground and excited states (Figure 2.1), respectively; and μ is the dipole operator. It is not necessary to evaluate the integral to determine if a transition takes place. We only need to determine if the direct product of the functions forming the integrand is symmetric or antisymmetric (even or odd) with respect to the point group to which the molecule belongs. μ is an odd function. If Ψ_0 and Ψ_1 have the same symmetry, the integrand will be odd and the integral zero. However, if they have different symmetries, the integral will be non-zero.

2.1.1 Selection rules

The role of the symmetry (or parity, as it is often known) of a wavefunction leads to an important selection rule called the Laporte selection rule: transitions in centrosymmetric molecules can only occur between states of different parity. States with even parity are labelled “g” *gerade*, German for “even”; whilst states with odd parity are labelled “u” *ungerade*, German for odd. Thus, only transitions that involve a g→u or u→g transitions are allowed. This automatically forbids transitions within one type of orbital i.e. s→s, p→p, d→d, as such transitions will not involve a change in parity. Similarly, there is the spin selection rule, which states that for a transition to take place, the total spin must not change, i.e. $\Delta s=0$.

These selection rules can all be broken, enabling nominally forbidden transitions to become at least partially allowed. d-d transitions are frequently observed, for example. The Laporte selection rule can be relaxed through a distortion of some kind so that the molecule no longer has its centre of symmetry. This distortion may be

induced by the environment the molecule is in, or the electronic transition may couple with an asymmetric vibration that causes a temporary distortion of the molecule. This latter mechanism is called vibronic coupling. Spin forbidden transitions may become partially allowed through spin-orbit coupling. Here, the magnetic fields created by the spin and orbital motions of the electrons couple with each other, enabling spin-forbidden transitions to take place.

2.1.2 Spectral line widths

The absorption spectrum of a single chromophore consists of an electronic origin, a series of vibrational sidelines, and phonon wings, shown below in fig 2.2.

Phonons are low frequency vibrations within the lattice of ordered systems.

While proteins frozen within a water/cryoprotectant glass mixture are not crystals, some short-range ordering still occurs.

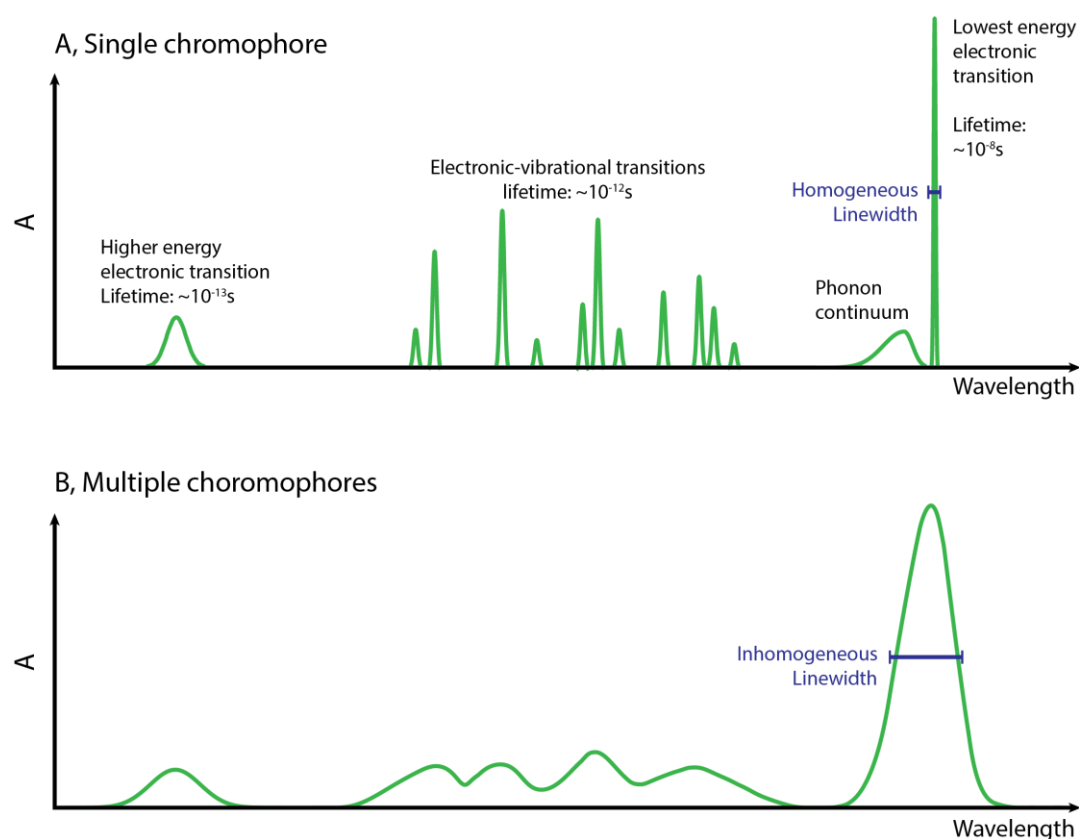


Figure 2.2: Hypothetical low temperature spectrum of a single chromophore (A) and a large population of chromophores (B). Adapted from diagram by Hughes (2007)[21]

The optical linewidth of the electronic origin is given by the so-called homogeneous linewidth, which is determined by the lifetime of the excited state and the influence of the local environment

The equation for the minimum homogeneous linewidth, Γ_{hom} , is shown below, where Γ_{hom} is the homogeneous linewidth given in Hz and T_2 is the total relaxation time given in seconds. [22]

$$\Gamma_{hom} = \frac{1}{\pi T_2}$$

Equation 3.1.

T_2 has temperature dependent (T_1) and independent (T_2^*) components.

$$\frac{1}{T_2} = \frac{1}{2T_1} + \frac{1}{T_2^*}$$

Equation 3.2.

T_2^* is the phase relaxation time, which decreases with increasing temperature. Dephasing is due to fluctuations in the local environment leading to small changes in the excitation energy.

T_1 is the temperature independent component, based on the radiative (fluorescence) and non-radiative (heat dissipation and other mechanisms) lifetime.

$$\frac{1}{T_1} = \frac{1}{T_{1rad}} + \frac{1}{T_{1nrad}}$$

Equation 3.3

Thus, the lowest excited state in a system often has the longest lifetime and subsequently the narrowest band as it has the fewest relaxation pathways. Vibrational sidelines have a shorter lifetime, as they have both radiative and non-radiative relaxation pathway. Generally, the more decay pathways a system has, the shorter the lifetime and subsequently the broader the band.

Inhomogeneous broadening is the broadening of spectral lines that occurs as a result of a population of chromophores being in slightly different local environments. For

instance, proteins will have slightly different conformations when frozen which also contributes to inhomogeneous broadening. Inhomogeneous broadening is more significant in an amorphous glass than in a ordered crystal, as the orientation of molecules is more variable. [23]

Pigments bound to different sites within the protein form distinct subpopulations whose spectra can be buried under the inhomogeneous broadening. This effect is sometimes known as heterogeneous broadening [24].

These different local environments shift the transition energies of the pigments by small amounts. In an amorphous host material, pigment transition energies have a Gaussian distribution that renders spectral features broad and featureless.

Selective spectroscopy techniques such as fluorescence line-narrowing and spectral hole burning can be used to study subpopulations within homogeneously broadened bands. These techniques can be used to distinguish between homogeneous and inhomogeneous broadening and determine homogeneous linewidths.

2.2.1 Spectroscopy of chlorophyll

Chlorophyll absorption is modelled as a four-level system, based upon the Gouterman four orbital model [25]. There are two sets of $\pi \rightarrow \pi^*$ transitions that result absorption in the visible region: the B-bands, also known as Soret bands, that absorb in the blue-near UV region; and the Q transitions that absorb in the red. The asymmetry of the molecule splits each transition in two components polarised in the x and y directions as shown on the structure in Figure 2.3. [26]

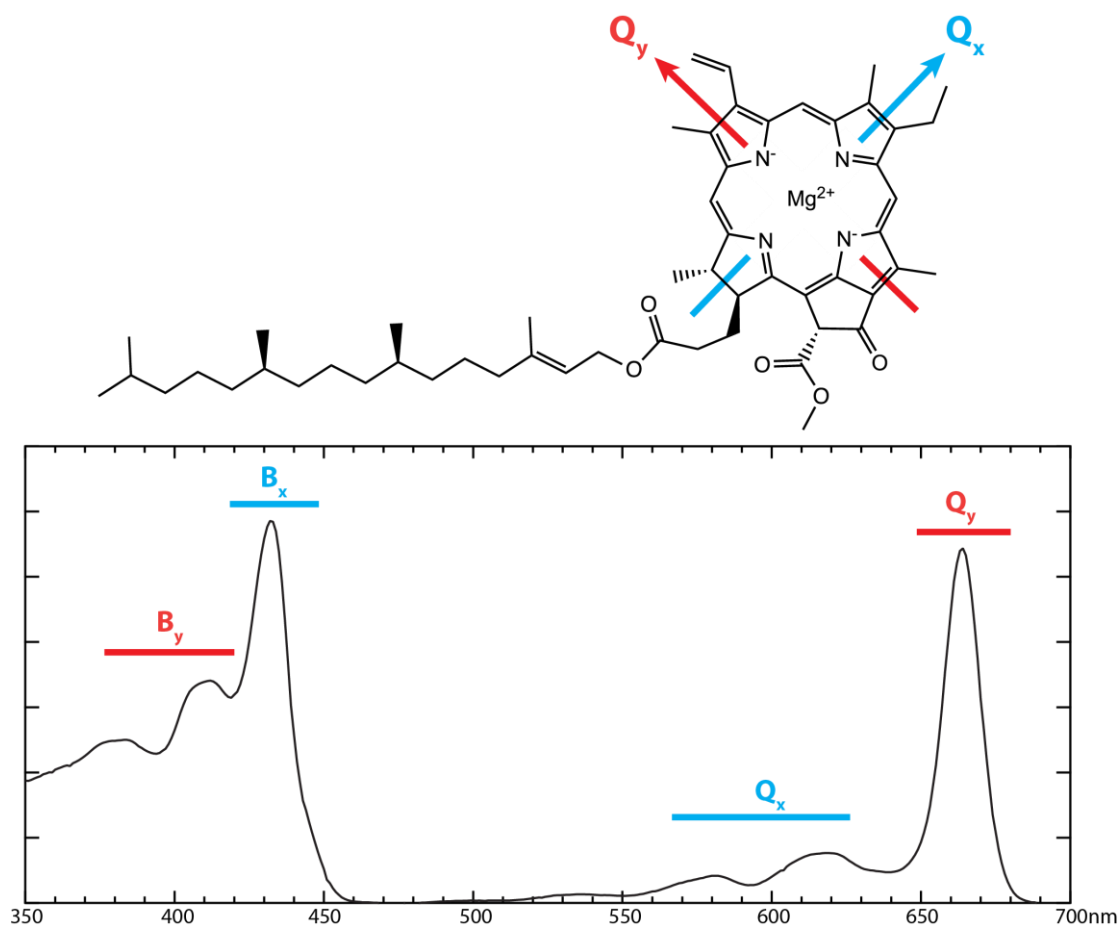


Figure 2.3. Structure of chlorophyll a and its absorption spectrum at 150K in dry diethyl ether. Data provided by Elmars Krausz.

The spectrum shown in figure 2.3 is for chlorophyll a in dry ether. However, in proteins, the transition energy of the absorption of chlorophyll is modified by the protein environment, leading to a distribution of absorption bands and relatively broad and featureless spectra for pigment-protein complexes, as can be seen in section 2.2.2 below.

Furthermore, in some pigment-protein complexes, there are other asymmetric porphyrins such as pheophytins and hemes. The spectroscopy of these pigments can also be understood with the Gouterman four orbital model, but the transitions are at different energies from those of chlorophyll.

2.2.2 Optical Spectroscopy of Photosystems I and II

The absorption spectrum of PSII derived from *T.vulcanus* is shown in Figure 2.4. It shows the numerous overlapping absorption bands from the different chromophores in PSII as well as measurable (~1%) contamination from PSI.

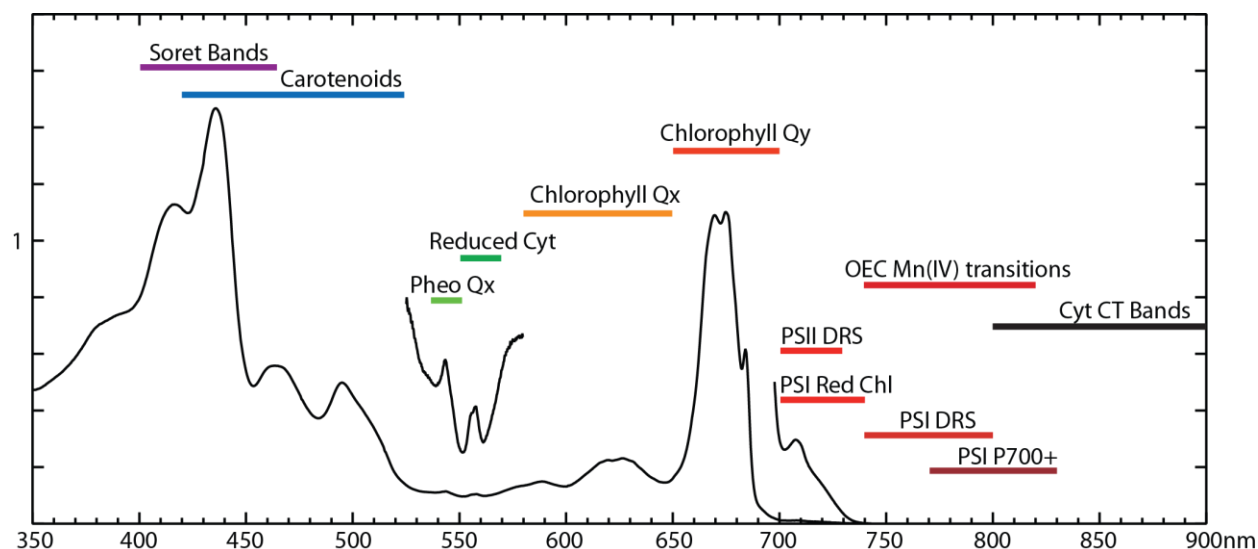


Figure 2.4: Overview of the optical absorption spectrum of isolated PSII cores derived from *T.vulcanus* at 1.8K. Spectrum originally published in [27]

Plant PSII lacks cytochrome c550, and samples often show absorption bands at around 680nm from light harvesting complexes.

When reduced the cytochromes have an absorption peak at around 560nm. Adjacent to these bands is a Q_x transition from pheophytin. Upon illumination the cytochrome bands weaken as some of them are oxidised, while the pheophytin transition shifts.

The low Optical Density (OD) of this region means it can be measured even in highly concentrated samples. It is typically measured in experiments also looking at the far-red region in order to check the quality of the sample.

The PSII deep red state (DRS) is a weak charge transfer band that extends out to 730nm at low temperatures. An equivalent band in PSI extends further out to 800nm.

It was observed in EPR experiments that illumination between 720 - 820nm lead to changes in the structure of the OEC. As this goes beyond the absorption of the chlorophylls, it was concluded that this was due to direct excitation of the OEC.

Photosystem I shows considerable variation in its absorption spectrum depending on the species and sample preparation, as shown in Figure 2.5. Even species that contain only chlorophyll a show highly variable absorption bands into the near IR.

In both *T.vulcanus* [28] and *Arthrospira platensis* [29] the furthest red absorption in trimers is from a pair of chlorophylls located between the PSI units. This absorption band is lost when the complex is broken up into monomers. In land plants the furthest red chlorophylls are located in the small LHCs arranged around the complex, located between the LHCs and the PSI unit. See figure 1.7.

In highly concentrated samples it is possible to observe a weak absorption band at 810nm from the P700⁺ radical.[27]

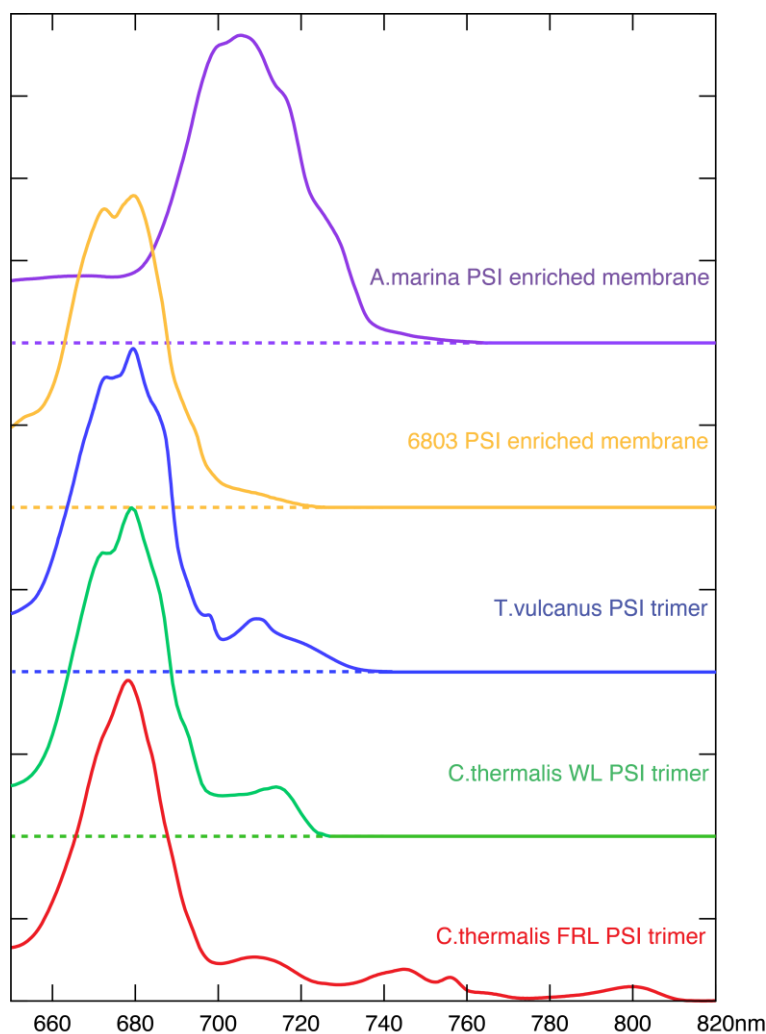


Figure 2.5: Comparison of the absorption spectra of PSI from different species. Data for *A.marina* measured by Elmars Krausz and Sindra Paterson. Based on the design of the diagram from Gobets and van Grondelle (2001).[30]

2.2.3 “d-d spin flip” transitions

As the name suggests, a spin flip transition is one where a spin ‘flips’ direction from spin up to spin down, or vice versa within a single orbital configuration. Since such a transition involves a change in total spin, they are spin forbidden, but have been known to become allowed through spin-orbit coupling.[31] The intensity of such transitions can be further enhanced in dimers (or larger clusters) if a spin on another metal centre within the cluster simultaneously changes spin such that the total spin remains $\Delta s=0$ [32]. See Paper 1 and its supplementary information for further discussion of this subject.

2.3 Electrochromism

As described in sections 1.2 and 1.3, PSII and PSI can form stable, separated charges when illuminated at low temperature. The absorption bands of pigments near the photoinduced charges shift slightly because of the interaction between the transition dipole and the electric field from nearby charge. Furthermore, the radical species have very different absorption spectra from those of the neutral species, leading to spectral bleaches where they used to absorb. Although these spectral changes are small, they are readily measurable through taking absorption spectra before and after illumination and subtracting the spectrum before illumination from that after illumination. This results in an absorption difference or electrochromism spectrum. The process is depicted in Figure 2.6 below.

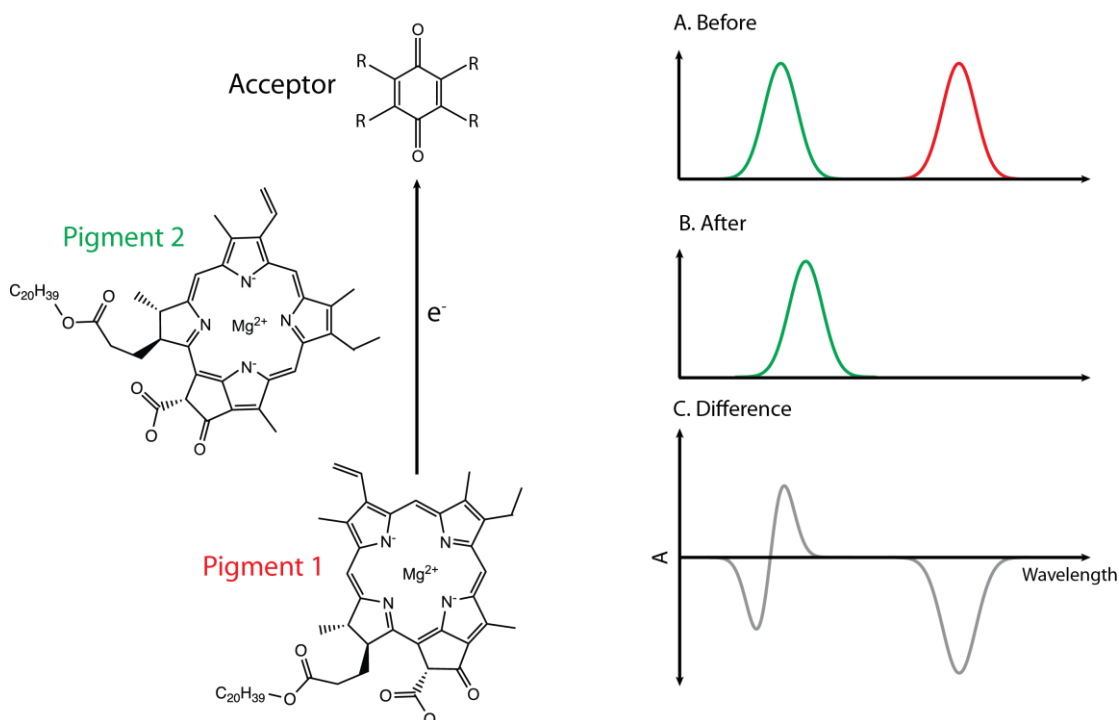


Figure 2.6 Change observed in the absorption spectrum due to the movement of an electron.

Electrochromism can be used to study the pigments in the centre of PSI and PSII complexes and their role in charge separation. The magnitude and direction of the electrochromic shift of a given pigment is determined by its distance from the photogenerated charge(s) and the relative orientation of its change in static dipole moment between ground and excited state. With the help of simulations,

electrochromic spectra can be used to assign absorption wavelengths to particular pigments based on their location and orientation relative to the charged species.

Of particular interest in this thesis is the cyanobacterium *C. thermalis*, which, when grown under red light, replaces some of the chlorophyll a molecules in PSI and PSII with the more red-absorbing chlorophylls d and f. These can be seen clearly in their absorption spectra. Electrochromism is a technique that we have used successfully to identify which chlorophylls are substituted.

2.4.1 Circular Dichroism and Magnetic Circular Dichroism.

As stated earlier, light is an electromagnetic wave. The electric and magnetic fields oscillate perpendicular to one another and to the direction in which the light is propagating. When the electric field oscillates in a fixed plane then the light is said to be linearly polarised. If instead the electric field rotates around the propagation direction the light is circularly polarised, in spectroscopy, circularly polarised light is right-handed if the electric field rotates clockwise when viewed by the receiver, and left-handed when it rotates anticlockwise. Circularly polarised light occurs when two perpendicular plane waves of equal magnitude oscillate 90° , or a quarter of a wavelength, out of phase with each other. It is generated by first linearly polarising the light with a polariser and then passing it through a quarter-waveplate at an angle of 45° with respect to the direction of polarisation to produce the 90° phase shift. For the experiments described in this thesis, we used alternating left and right circularly polarised light. This is generated with a polariser followed by a photoelastic modulator. The modulator acts as a variable waveplate, producing an alternating $\pm 90^\circ$ phase shift at a frequency of ~ 60 kHz, thus producing alternating left and right circularly polarised light at ~ 60 kHz. For further experimental details see section 3.1.

2.4.2 Circular dichroism

Circular dichroism (CD) is the differential absorption of left and right circularly polarised light. It is associated with the absorption bands of chiral systems (i.e.

systems without a centre of symmetry). These so-called optically active materials absorb left and right circularly polarised light differently. This difference is given by:

$$\frac{A_L - A_R}{A_L + A_R}$$

Equation 4

By this notation, greater absorption of left circularly polarised light gives a positive signal and greater absorption of right circularly polarised light gives a negative signal.

Since chlorophyll possesses a centre of symmetry, the CD of isolated chlorophyll is very weak. In contrast, chlorophylls within a protein show comparatively strong CD. This arises because chlorophylls within a protein are coupled to each other and have a chiral arrangement imposed upon them by the protein structure.

The intensity of the observed CD transitions depends on the distance between the molecules and their relative orientation. This enables it to be used to probe the relative positions and orientations of the chlorophylls within the protein. For instance, extensive modelling of the CD of the PSII RC preparations from spinach has been reported. [33]

2.4.3 Magnetic circular dichroism

Magnetic Circular Dichroism (MCD) is the difference in absorption between left and right circularly polarised light induced by a magnetic field applied in the direction of light propagation. Like CD, MCD is measured in wavelength regions of optical absorption. It is a manifestation of the Zeeman effect.[34]

Experimentally speaking, MCD is measured in the same way as CD, but with the application of a magnetic field. Samples with inherent CD will also have MCD. The circular dichroism is measured with and without the magnetic field, the purely magnetically induced component is then isolated by subtracting off the zero-field spectrum.

The mechanisms behind CD and MCD are different. This means that the two techniques yield different information. Whilst CD provides structural information, MCD probes the electronic structure of states. In particular, it can provide information about the symmetry of transitions, (magnetically-induced) coupling between electronic levels, vibronic coupling [35], and identify bands in congested spectra. MCD is a multidimensional technique in that it can be measured as a function of temperature, field and wavelength. It probes both ground and excited states.

This technique is complementary to EPR (covered in the next section) as the Zeeman splitting that gives rise to EPR signals also produce an MCD signal. This can be used to link an observed EPR transition to an optical transition. It has the advantage over EPR in that it reports the electronic structure of both the ground and excited states.

At low temperature (<10K) the MCD signal of paramagnetic species is enhanced relative to that from other chromophores. This is particularly useful when studying weakly absorbing paramagnetic species on a background of strongly absorbing diamagnetic species.

MCD can be described using the following equation [36]:

$$\begin{aligned} \frac{\Delta\epsilon}{E} &= \frac{\epsilon_{LCP} - \epsilon_{RCP}}{E} = \frac{1}{dc} \frac{A_{LCP} - A_{RCP}}{E} \\ &= \gamma\beta_B B \left[\left(-\frac{\partial f(E)}{\partial E} \right) A_1 + \left(B_0 + \frac{C_0}{kT} \right) f(E) \right] \end{aligned}$$

Equation 5.

E is the energy, $f(E)$ is the line shape function, T is the temperature, B is the applied magnetic field, β_B is the Bohr magneton, k is Boltzmann constant, γ is a constant, d is the path length, c is the concentration.[37]

The A_1 , B_0 and C_0 terms are coefficients. They relate to the influence the magnetic field has on the electronic states under investigation. A-terms arise from the magnetic-field-induced lifting of ground or excited state degeneracies. This leads to a

characteristic derivative-shaped spectral feature. B-terms result from mixing of either the ground or the excited state with other electronic states. B-terms have the same shape as the absorption feature that they correspond to. The intensity of a B-term arising from the mixing of excited states increases linearly with increasing field and is mostly temperature independent. However, B-terms arising from ground state mixing can show temperature dependence. An example of a B-term found in this work is the mixing of Q_x and Q_y transitions in chlorophyll (See Section 2.2.1). C-terms are caused by magnetic-field-induced splitting of ground state degeneracies. When ground-state degeneracies are lifted in a magnetic field, the population levels in the different states are changed by means of a new Boltzmann distribution. C-terms have the same shape as the absorption feature they correspond to, but are temperature dependent. MCD signals may be either positive or negative. The sign of the MCD signal is determined by the change in orbital angular momentum between ground and excited states. The three MCD terms are summarised in Figure 2.7 below.

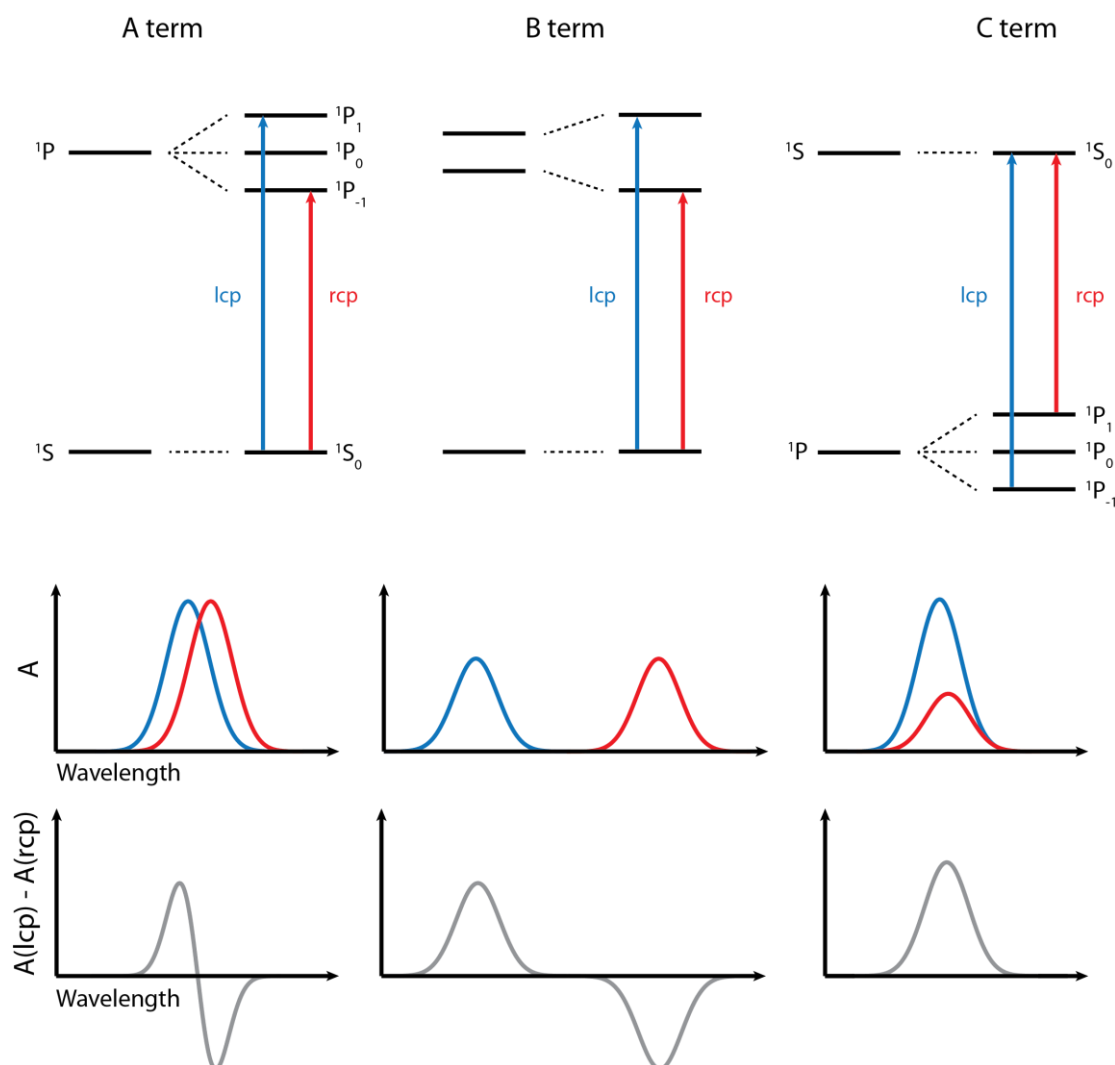


Figure 2.7, The origins of the A, B and C term MCD signals. Adapted from Piepho and Schatz [36] Note that the splitting on the energy scale is exaggerated.

At higher fields ($>1\text{T}$) and lower temperatures ($<10\text{K}$), C-terms no longer scale linearly with field and temperature. The intensity of a simple purely C-term transition is given by:

$$\frac{\Delta\epsilon}{E} = A_{limit} \tanh\left(\frac{g\beta_B B}{2kT}\right) f(E)$$

Equation 6

T is the temperature, B is the applied magnetic field, β_B is the Bohr magneton, k is the Boltzmann constant and g is the g-value.

This saturation behaviour is useful in separating overlapping MCD signals. As well as this non-linear behaviour being useful for excluding contributions from A and B terms, it can be used to separate overlapping bands with different g-values.

The phrase “Pseudo C-term” can be used to describe any absorption band that behaves similarly to a simple C-term in Variable Temperature Variable Field (VTVH) measurements but has a more complicated electronic structure.

In the case of complex ground state splitting a more rigorous treatment is necessary. For instance when describing the MCD behaviour of magnetically coupled atoms. A paper by Neese et al [38] examines weakly coupled metal dimers. The examples shown demonstrate complex field and temperature dependant behaviour. In one case the MCD absorption band reversed sign with increasing temperature.

The ground state splittings that lead to MCD C-terms are also the origin of electron paramagnetic resonance (EPR) signals. EPR is a spectroscopic technique used for studying systems with unpaired electrons. In EPR, transitions between the electronic ground states that are split in the magnetic field are probed directly with microwaves. The transition energy in an EPR experiment is given by:

$$\Delta E = h\nu = g\mu_B B_0$$

Equation 7

Where g is the g-value, h is Planck’s constant, μ_B is the Bohr magneton and B_0 is the applied magnetic field.

In contrast to an MCD experiment where the magnetic field is kept fixed and the wavelength is scanned, in EPR the microwave source is kept at a fixed energy and the magnetic field is scanned. A comparison of EPR and MCD is shown in Figure 2.8 below. The energy of the optical transition is on the order of 10^4 times greater than an EPR transition.

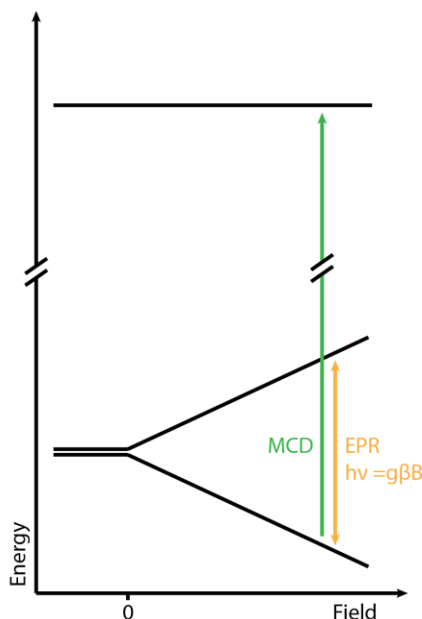


Figure 2.8: Comparison of an EPR transition and MCD C-term.

EPR does not require samples of high optical quality as MCD does. EPR studies on Photosystem II for instance are regularly made using PSII-enriched thylakoid membranes. Such preparations scatter light too much to produce MCD spectra of useful quality.

It is important to note that spin forbidden transitions that can be seen in MCD are not equivalent to the change in spin measured in EPR. The MCD signal arises from a change in spin of one electron relative to the other electrons on that atom, while EPR reports the change in net spin of all the electrons on that atom.

The shape and position of an EPR transition is influenced by the environment in which the electron(s) under investigation are in. For example, an unpaired electron can gain or lose angular momentum, which can change its g -value; when multiple unpaired electrons are present, they can interact with each other leading to zero-field splittings and exchange coupling; if nearby nuclei have a non-zero nuclear spin, they will affect any unpaired electrons associated with that atom leading to hyperfine splitting, which splits the EPR resonance signal into multiplets. All these effects are often orientation dependent and so can provide information about the atomic or molecular orbital containing the unpaired electron. Detailed information is usually obtained from EPR spectra by modelling the band position and shape.

EPR and MCD are complementary techniques. For example, modelling a measured saturation curve of an MCD signal can give a g -value. This enables a signal observed in EPR to be linked to an optical transition. The two methods yield interrelated information about a system and their combined use can be very insightful.

The example below is the spectrum of the isolated PsbV subunit, which contains cytochrome c550. MCD and EPR have been used extensively in the study of cytochromes. A review by Cheesman et al [39] covered the applications of MCD to hemoproteins, with the technique often applied to various types of cytochrome in larger complexes.

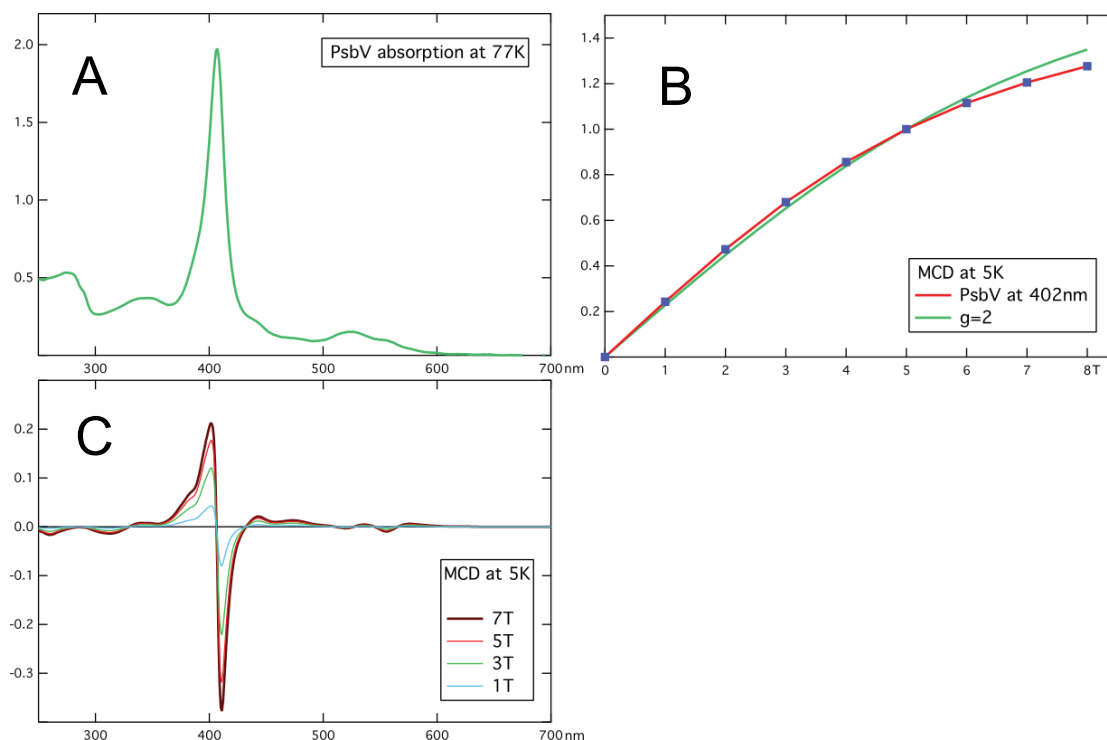


Figure 2.9: The absorption spectrum (A), field dependent intensity curve (B) and MCD field dependent spectra (C), of oxidised isolated cytochrome c550.

In EPR, the isolated PsbV subunit from *T.elongatus* shows up as multiple peaks at $g_z=2.94$, $g_y=2.24$ and $g_x=1.49$ [14]. Measuring the VTVH curve of cyt c550 gives a curve close to the hypothetical $g=2$ line from Equation 6. The deviation from the simple curve is consistent with the g -value having different directional components.

Chapter 3

Technical and Experimental

3.1 The spectrometer system

The key experiments done for this thesis were performed on our optical spectroscopy system. The current configuration of the system dates back to 1986, with a number of incremental changes made since. Several improvements have been made to the system during this doctoral work. These are described in section 3.1.1 below, and also documented in detail in the appendix of Publication 1.

Measurements can be taken on any sufficiently transparent sample that fits on the sample rod. Initial work using this system primarily involved solid state samples. However the work described here was carried out on frozen aqueous solutions. Details of the cells used for such samples are described in section 3.3.

One key advantage of this system is that it can be switched between different modes without perturbing the sample. It can simultaneously record absorption, CD and MCD spectra. It can also be rapidly reconfigured to measure emission and excitation spectra, without disturbing the cryogenic sample. The flexibility of the system allows us to easily test modifications to the system that were needed during various experiments. The different configurations are described in section 3.1.1

3.1.1 Absorption configuration

The configuration of the system for absorption is shown in Figure 3.1 below, which was adapted from a paper by E. Krausz [40]. with modifications to show the specific configurations used for the experiments in this thesis.

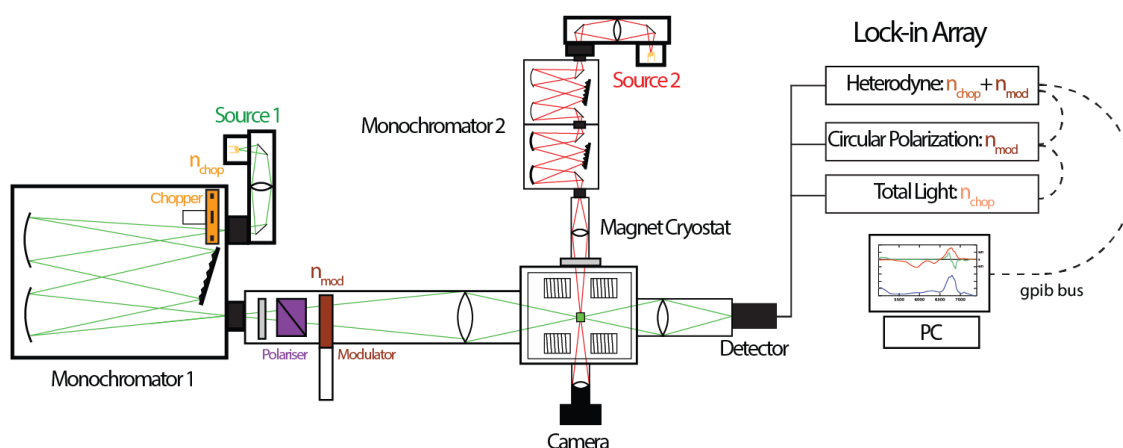


Figure 3.1, overview of the spectrometer as set up for absorption and CD/MCD measurements. Modified from the original version provided by E. Krausz [40]

Measurement light in this configuration is provided by Source 1. A mechanical chopper chops the light at 483 Hz. This light is then passed through a 0.75 m Czerny-Turner Spex 1402 dual single/double monochromator. For the work here, it was always operated as a single monochromator. The output of the monochromator is passed through an order sorting filter, a linear polariser and a photo-elastic modulator (PEM), the last of which is modulated at ~ 60 kHz. The polariser PEM combination produces alternating left and right circularly polarised light for the (M)CD measurements. The resulting light is then imaged on the sample. Light that is transmitted through the sample is collected and imaged on the detector. The system has three lock-in amplifiers in a heterodyning arrangement. The circular polarisation lock-in measures the signal at 60 kHz. Its output is sent to the heterodyne lock-in amplifier, which is referenced to the 483 Hz chopper frequency. The heterodyne lock-in thus provides a signal that is modulated at both 483 Hz and 60 kHz, and consequently strongly rejects the interference from either the modulator or the chopper. The detector output is also fed to the total light lock-in referenced to the chopper frequency that measures the total light level.

The spectrometer depicted above can reliably measure absorbance up to OD ~ 2 . Above this absorption level, spectra can become distorted, with peaks appearing to be clipped. This is due to stray light at other wavelength being of greater intensity than the transmitted light at the measurement wavelength.

In this configuration, monochromator 2 (Spex 0.25 m) can be used to illuminate the sample by rotating the sample rod. This enables the measurement of wavelength-dependent electrochromic shift spectra. Monochromator 2 can also be used to back illuminate the sample so it can be photographed (see section 3.1.3). The sample can also be illuminated with other light sources (such as a laser) by removing the detector or camera.

3.1.3: Fluorescence configuration

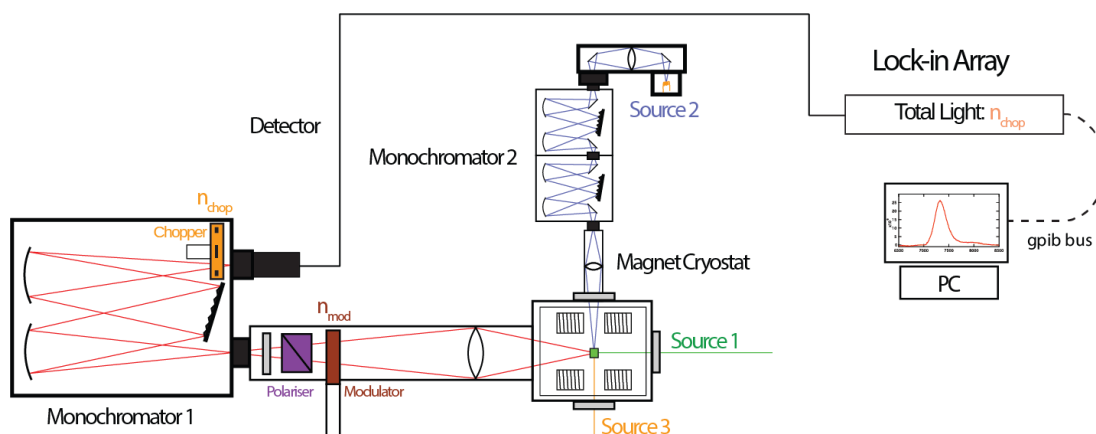


Figure 3.2, system as configured for fluorescence experiments. Modified from the original version provided by E. Krausz [40]

Figure 3.2 above shows the spectrometer in its fluorescence configuration. Fluorescence can be excited using a laser at either at the source 1 or source 3 positions or with a lamp (source 2) via Monochromator 2. This last option is especially attractive as it is a highly tuneable, stable light source with a well characterised output. Because of its tunability, this source can also be used for excitation spectra. With the modulator and polariser in place, circular and magnetically circular polarised luminescence (CPL and MCPL) can be measured using a laser at source 1.

3.1.4 Light sources:

There are two light sources used for absorption measurements: a quartz halogen lamp, and a laser driven light source (LDLS). For exciting emission, either the quartz halogen lamp is used in conjunction with a small double monochromator (monochromator 2), or a laser. The most commonly used laser in this thesis was a titanium sapphire laser.

The quartz halogen lamp is the most commonly used light source for absorption experiments, including all the absorption spectra in this thesis. Being based on a tungsten filament, this lamp good output from 420 nm to a few micrometres into the near infrared. It consists of a low cost 24 V, 250 W quartz halogen lamp in a cooled housing operated with a stabilised power supply. The result is a highly

stable light source with an apparent change in absorption arising from instability in the lamp on the order of 10^{-5} over the course of 5 hours.

The laser driven light source (Energetiq model EQ-1500 LDLS) uses a focused laser beam to generate and maintain plasma between the discharge electrodes of a xenon gas filled bulb. Being based on a xenon discharge, it has a wavelength range from ~ 200 nm to ~ 2 mm. Since it has good output in the UV region, it is an excellent complement to the halogen lamp. The spectrum of PSII in Figure 2.13 for example is a composite of two spectra measured with the two different light sources.

The Titanium sapphire (Ti:S) laser is an infrared laser with a tuneable output from 690 – 900 nm. The laser used here is a Schwartz EO Ti:S laser pumped with a 532 nm Continuum Verdi G. The output of the Ti:S can be up to 400mW, filters were used to reduce the intensity of the beam at the sample.

3.1.5 The detectors:

Different detectors were used for different wavelength ranges. It was often necessary to use two or more detectors in the course of a single experiment. Changing detectors is a relatively straight forward procedure. The detectors used in this doctoral work are a photomultiplier, a silicon avalanche photodiode, and an InSb detector. An example where multiple detectors are used is in concentrated PSII samples where the pheophytin and cytochrome peaks in the “green window” (see Figure 2.4) are measured using the photomultiplier, and the avalanche diode for the near-IR MCD experiments.

For the visible region (400 – 800 nm) a Hamamatusu R669 photomultiplier is used. This tube was chosen for optimal stability and baseline performance. Photomultiplier tubes do suffer baseline shifts induced by the magnetic field. These can be mitigated to some extent through the use of shielding, and can be corrected.

Measurements further to the red (500 – 1100 nm) were made with a temperature stabilised 5 mm Advanced Photonix silicon avalanche photodiode. A important advantage of this detector is that it is significantly less sensitive to magnetic fields than a photomultiplier tube. This makes it a good choice of detector for magnetic-field dependent measurements. For measurements further into the infrared, a 1 mm, liquid nitrogen cooled InSb detector system with condensing optics was used. This detector has a wavelength range from 700 nm to about 5 mm.

3.1.6 The camera

The camera is a Canon DSLR with the IR filter removed. As PSII and PSI samples are mostly transparent beyond 740nm, light at longer wavelengths can be used to inspect the sample without altering it. Bubbles, ice crystals and excessive cracking of the sample can result in artefacts in the spectra. Other problems with transmission such as condensation on the outer windows or obstruction from items that have fallen into the cryostat can be seen with the camera before time and helium are wasted taking measurements. Examples of images taken with the camera are shown in Figure 3.7.

3.2. Cryostat

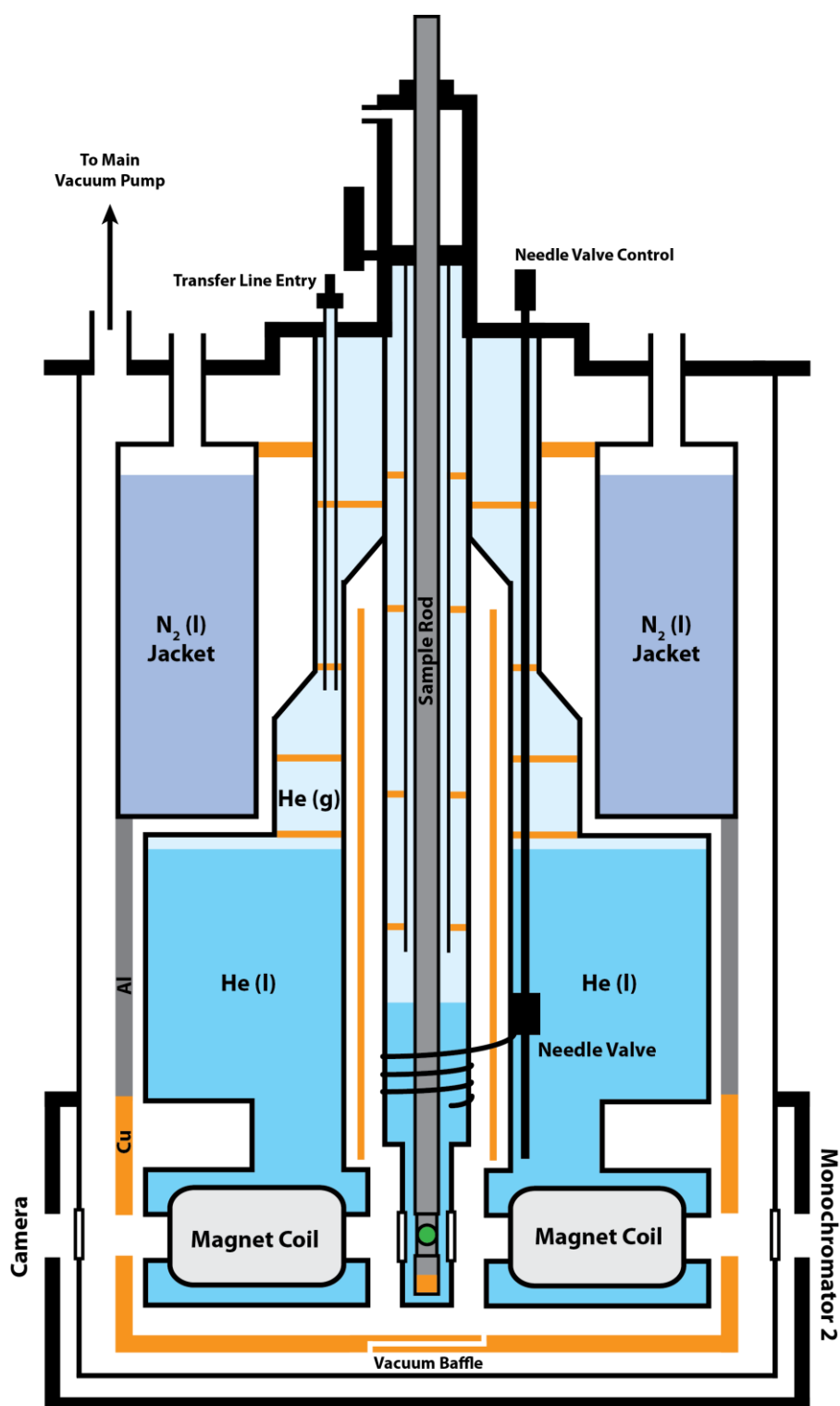


Figure 3.3: The cryostat housing the sample as viewed from the detector side in Figure 3.1 and 2. Based on original diagram by S. Peterson.

The cryostat is an Oxford Instruments SM-4 magneto-optical cryostat. A cross-section of this cryostat is shown in Figure 3.3 above. It enables the temperature of the sample to be controlled between 1.8 K and room temperature, and magnetic fields of 0 – 5 T to be applied. The sample is attached to the end of a rod inside the cryostat, and can easily be rotated and moved up and down within the cryostat. The top of the rod is clamped into position while experiments are being run. To do the measurements, the sample is lowered directly into a bath of nitrogen or helium, cooling it rapidly. This is vital for experiments involving PSII poised in higher S-states, as these states have a relatively short lifetime. The magnet could be energised up to 5T in approximately 10 minutes. Being able to rapidly change the field was important for making multiple measurements at different fields in one day.

During the course of the work described in this thesis, a number of improvements have been made to the cryostat. These are: a lock for isolating the sample was added to the top of the cryostat, a guide was added to the bottom of the cryostat to keep the sample still, the temperature stability of the sample was improved.

A lock was built at the top of the cryostat. This enabled the sample to be raised out the liquid helium bath and brought to a given temperature without exposing the sample to light or air. This addition was vital for annealing samples and allowing changes to take place that can only take place at temperatures above liquid helium or liquid nitrogen temperatures. Furthermore, the lock has a coverable window in it, enabling the sample to be “flashed” to initiate photochemistry that can only take place at temperatures above liquid helium or liquid nitrogen temperatures.

The sample was found to undergo very small movements in the magnetic field of the cryostat. These movements cause errors in the baseline of MCD measurements. Accordingly, a small sprung guide was placed at the bottom of the cryostat to hold the bottom of the sample rod in place.

As the temperature of the sample compartment in the cryostat changes, the length of the sample rod changes slightly. This leads to small, but measurable, drifts in

the MCD baseline. To solve this problem, a system with a solenoid-operated pump valve switched according to a set point on a carbon resistor at the sample was developed. This maintains the temperature of the sample at 1.8 ± 0.01 K. In this way, the sample space can be kept at 1.8 K for 1-2 hours: enough time to do a full series of measurements from 0T to 6T.

3.3. Samples and sample handling

Samples used in this work were generously provided by others.

Thermosynechococcus vulcanus samples were provided to us by Jian-Ren Shen's from Okayama University. The PSII samples are the same preparations that are used to produce crystals for X-ray crystallography [41]. This group also provided samples of the red algae *Cyanozadum merole*.

Chroococcidiopsis thermalis provided by Dennis Nurnberg and Bill Rutherford from Imperial College London.

Spinach (*Spinacia oleracea*) is typically used because it has a high concentration of chlorophyll, low levels of starch and grows quickly. Spinach samples were produced by Paul Smith who previously worked at the ANU.

PSI, PSII and other samples are stored in buffer either in the -40C freezer or under liquid nitrogen, with glassing agent added before the experiment. It was important to minimise freeze/thawing cycles, as this can potentially damage the samples, even with the addition of some cryoprotectant. Changes that can be observed optically include the oxidation of c550 in *T.vulcanus* PSII with subsequent freeze/thawing, and the detachment of LHC subunits from the PSII of spinach[18].

Experimental set up designed to allow us to manage the light exposure to the sample. Green LEDs were used to provide enough light to safely work with. These LEDs emit light at the absorption minimum of chlorophyll containing samples. Once the sample cell has been filled and fitted to the sample rod, it can be kept in the dark and subsequent light exposure can be carefully controlled.

The volume of sample typically used in an experiment is between 5-50 μ L. Small volume glass syringes (10-200 μ L) are used to measure out the sample and to add the mixture to the sample cells. The buffer and antifreeze are blended together in an Eppendorf tube, along with any other additives, before the photosynthetic sample is added. Image of the sample being loaded into the cell is shown in the supplemental of Publication 1.

Concentrations of samples provided for these experiments were typically between 0.5-10mg/ml of chlorophyll. Highly concentrated PSII samples contain approximately 65mg/ml of protein, this combined with buffer and 45% glassing agent results in a highly viscous sample. ~5% variability in the concentration of the sample. For EPR controls, *T.vulcanus* sample had to be diluted by a factor of about 10, but contained the same ratio of buffer and glassing agent.

There are a number of chemical treatments that can be applied to the samples. Potassium ferricyanide is typically used to chemically oxidise P700 in PSI. Sodium dithionite solutions could be used to reduce the cytochromes in PSII and is another method to remove the OEC.

Oxidants and reductants are diluted in the buffer before the protein sample is added to the mixture. A super-saturated solution in distilled water is mixed up right before use. This reduces the dilution of the sample, and in the case of sodium dithionite solutions to reduce the amount of time spent preparing the solution as sodium dithionite decays in water.

Photosynthetic samples contain a significant amount of detergent that acts as a substitute for the lipid membrane that originally surrounded the enzyme. High detergent levels lead to the sample wicking out of the sample cell. This was less likely to occur in cells with hydrophobic coatings. Bubbles formed in the mixture if not handled carefully. The presence of bubbles resulted in “clipping” of the spectra, where the tops of high OD peaks were flattened. Bubbles were especially problematic when measuring difference spectra, as a slight movement of sample containing bubbles resulted significant apparent changes to spectra.

3.3.2 Sample cell designs

Photosynthetic samples are injected into quartz cells with a small syringe. The sample is loaded under low light conditions, with green LEDs providing illumination. The loaded sample cell is then affixed to the end of the sample rod and crash cooled by rapidly lowering it into the cryostat directly into either liquid nitrogen or liquid helium. Cells must be able to survive repeated rapid cooling. Cells made entirely out of quartz have been used; however, they are at a greater risk of shattering from thermal shock. Polymer cuvettes are highly birefringent, and so are of little use for CD/MCD experiments. This means that MCD cells tend to have a more sophisticated design than standard spectroscopic cuvettes.

A standard MCD cell, often referred to as a Solomon cell, consists of two quartz windows separated by an O-ring and held in place by a frame. Cells with copper and Teflon frames were used in Figure 3.6. Such standard cells have the benefit of being simple to construct and easy to clean because they can be disassembled.

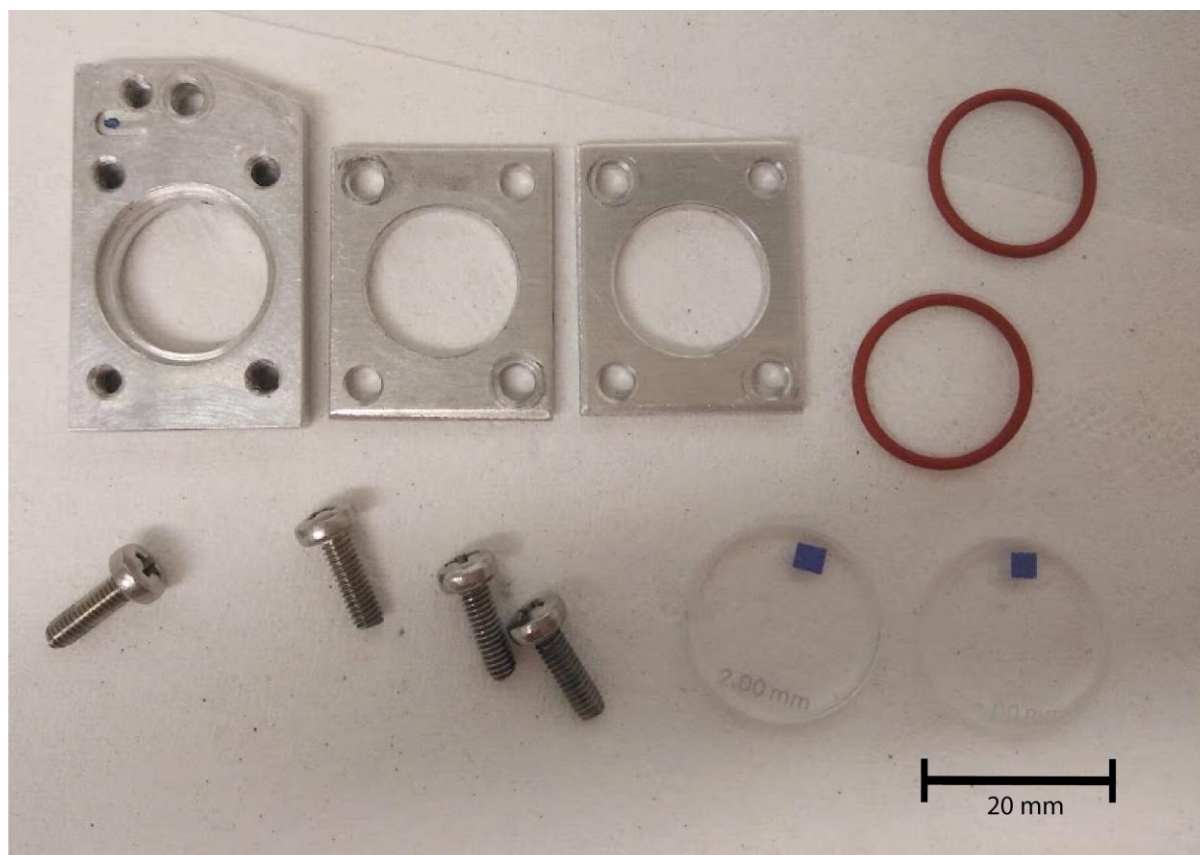


Figure 3.4.1. "Standard" MCD cell in a disassembled state.

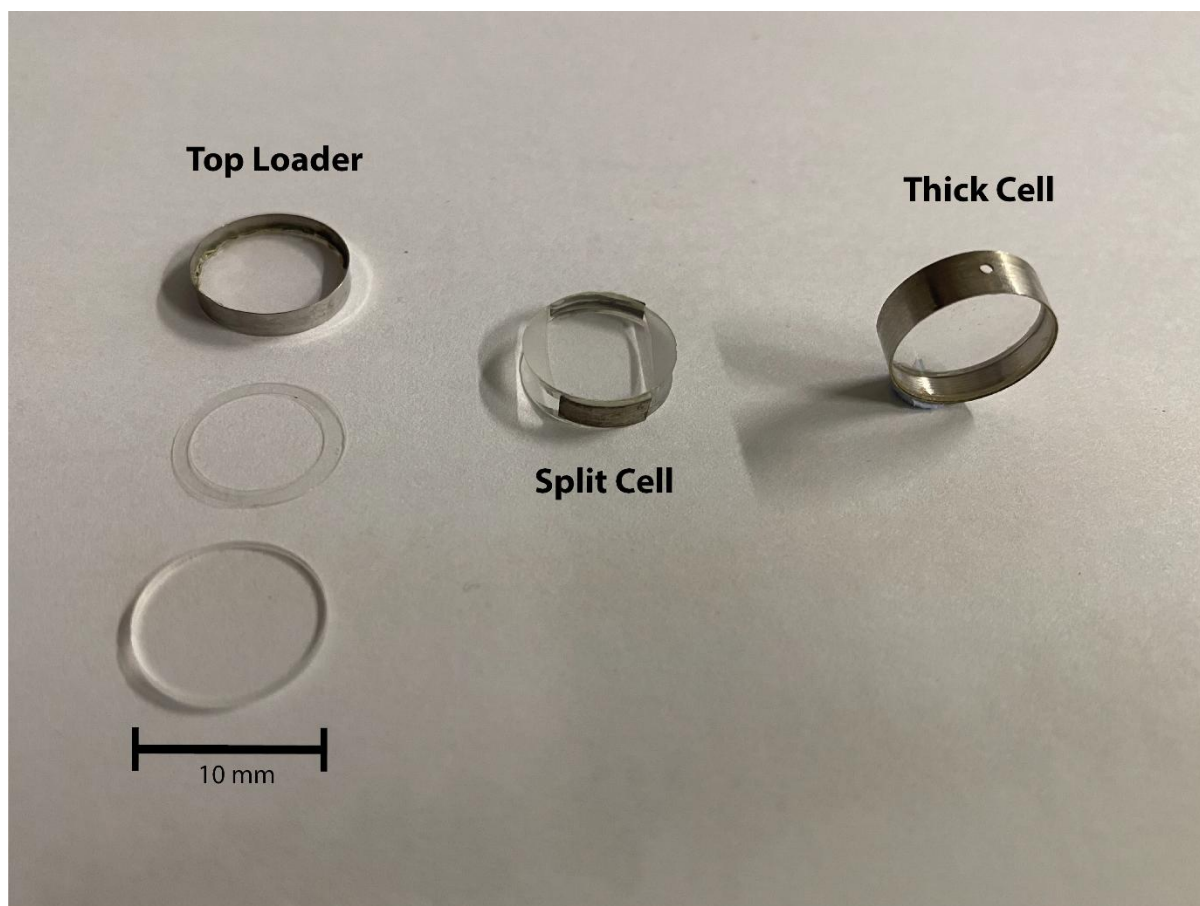


Figure 3.4.2. Three different “ANU” sample cells

At the Australian National University, we have designed a number of novel MCD sample cells. These sample cells have evolved over the years. Different cells were designed for different applications.

The “ANU thick cells” were used for most of the OEC work in Paper 1. These cells consist of two 1 mm thick, 12 mm diameter quartz windows held together with a titanium ring and Ecobond™ glue. They have a pathlength of 0.5 to 2 mm. The cell is filled and emptied through a small hole in the titanium ring. Titanium and quartz both have low thermal expansion coefficients, reducing the risk the cell will crack, and because of the small size of the cell the strain across the quartz windows is negligible. The downside to these cells is they are quite difficult to construct. Eventually, freezing and thawing the sample can open gaps in the Ecobond, allowing the sample to wick out, and ultimately the sample cell falls apart.

For the work in this thesis, the thick cells had a hydrophobic coating applied to the inside of the cell. This is detailed further in section 3.3.3.

“ANU split cells” are described in [40]. Two 1 mm thick, 12 mm diameter quartz windows are held 200 μm apart with strips of titanium as spacers. The sample is held in place through surface tension. The cell is filled and emptied through a gap between the titanium spacers.

With “ANU top-loader cells”, the sample is sandwiched between two 1 mm thick, 12 mm diameter quartz windows with a mylar spacer between them. A titanium ring around one window prevents the sample from wicking out. The spacers are 10 μm and 50 μm thick. After the mylar spacer and sample have been put in the ring-window assembly, the second window is simply dropped on top to create a very short path length sample. These cells have been used to measure low OD spectra of concentrated samples.

3.3.3 Sample glassing and hydrophobic coating:

With the exemption of a few model compounds mentioned in paper 1, all the MCD work in this thesis was done using aqueous solutions.

When water freezes at atmospheric pressure it forms crystalline ice, resulting in an opaque sample. The concomitant expansion of the aqueous solution can also damage the sample cell and the protein itself. To prevent this, a glassing agent is added to the sample. Glycerol, ethylene glycol and sucrose are typical glassing agents for protein samples. [21]

The use of glassing agents does have an impact on the photosystem cores, particularly the OEC in PSII. Sucrose solutions have the potential to “dehydrate” samples. By weight, sucrose solutions require more sucrose than water in the sample to form a glass. Glycerol and ethylene glycol are small enough to travel up the water channel, displacing water molecules. This is a problem for S-state dependent PSII experiments where water must be at the active site. This can suppress the formation of the high-g form of S₂ and reduce the yield of the S₃ state.

The physical processes determining glass formation are less simple than they first appear. A paper by Murata and Tanaka [42] examines phase transitions in water/glycerol mixtures.

The cooled water/glycerol liquid is metastable above ~150 K, below this temperature, it forms a glass. This phase change can be seen in our work with the sample becoming foggy at or slightly above 150 K until it melts entirely at about 200 K.

An ideal glass has high transmission, no cracks and low strain. In practice though this is not achievable, as cracks in the sample reduced strain. For our MCD experiments, the best results were obtained from samples with a few large cracks. The glassing quality was often unpredictable. In a 1mm cell the concentrated *T.vulcanus* samples would have a transmission of less than 15% at 800nm.

The inspiration for applying a hydrophobic treatment to the cells came after it was noted that the transmission of the sample was noticeably worse after the sample cell had been thoroughly cleaned. Protein samples can leave a white residue inside the sample cell that builds up over time. While this layer is undesirable because it is opaque, it also makes the inside of the cell slightly more hydrophobic than clean quartz. Ironically, this results in improved spectra.

The aqueous mixture in the cell has a higher thermal expansion coefficient than the quartz and titanium. As the sample cools and contracts, it becomes strained unless it can detach from one or both windows of the cell.

The hydrophobic coating was added to replicate the effect of the protein residue, but in a more controlled manner and with a transparent substance. Applying a hydrophobic coating to the inside of the sample cell leads to an increase in total transmission from 2 - 15 % to 45 – 55 %. The total strain of the sample decreases, as seen in the CD spectra in Figure 3.6.

A solution of 1H,1H,2H,2H-perfluorooctyltrichlorosilane was used to form the monolayer. The method used was based on Liu et al (2011) [43] This method was chosen because the coating would not reduce the transmission of the sample cells, would be able to survive repeated freeze/thawing cycles down to 1.8K and could be exposed to a variety of solvents. Any light scattering from the polymerised siloxane is negligible compared to the increased transmission of the sample.

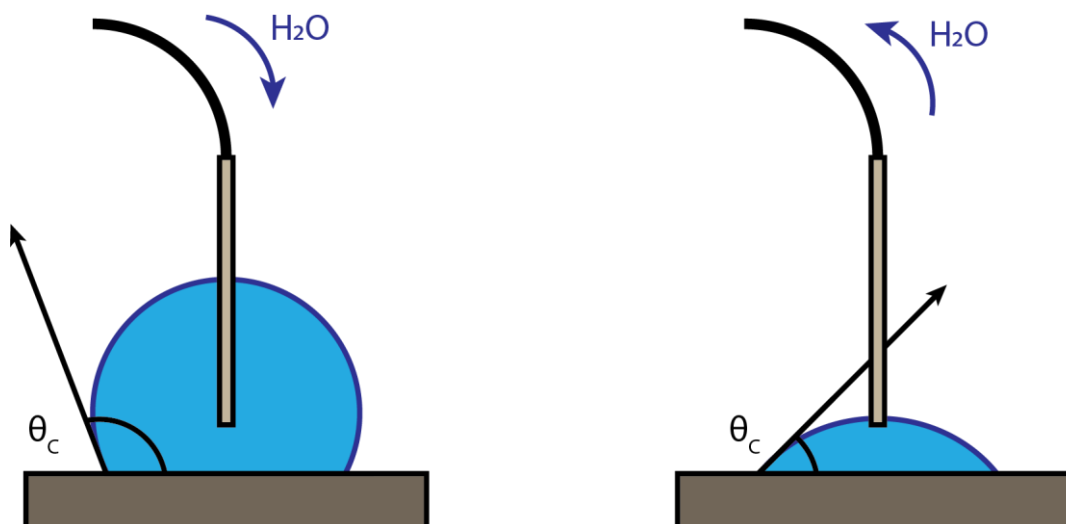


Figure 3.5, Measurement of the advancing and receding contact angles on a surface treated with a hydrophobic coating. The lower the contact angle (θ_c), the more effective the treatment.

The contact angle of the quartz window was measured to test the effectiveness of the treatment. Advancing and receding contact angles were measured using a KSV CAM200 Goniometer instrument.[43] The instrument consists of a syringe that deposits a droplet of the test solution onto the treated surface, and a camera. The contact angle of distilled water on the treated quartz surface matched that given in literature on a flat surface [44]. The effective contact angle for a clean quartz surface is zero, a droplet deposited on the surface forms a thin layer. For a surface that hasn't been thoroughly clean, the contact angle is typically less than 10° .

The test sample and the buffer solution both had significantly lower contact angles compared to distilled water. This indicates that the hydrophobic treatment might be less effective in samples with significant amounts of detergent, but this does not appear to increase the strain seen in the frozen samples.

The effect of strain is demonstrated in Figure 3.6 below. The zero tesla (green) line shows the apparent CD in the sample arising from stain. The samples are transparent water/glycerol/ethylene glycol mixtures.

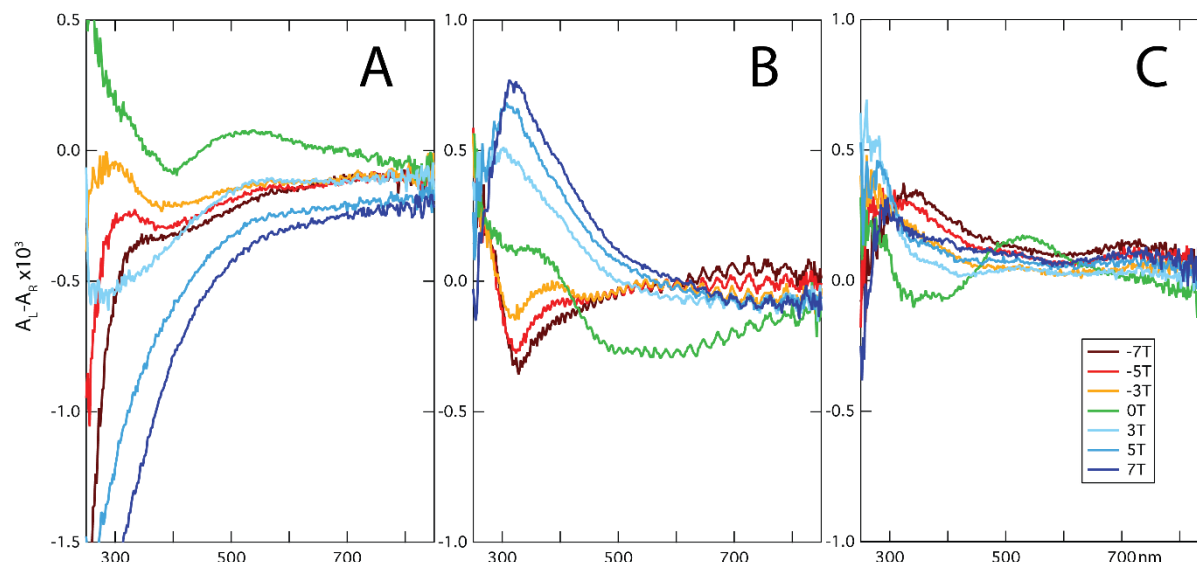


Figure 3.6 -7T to +7T 5K apparent MCD spectrum of 2:1:2 water/glycerol/ethylene glycol mixture in: **A**, Cu/quartz 3mm standard cell. **B**, Teflon/quartz 2mm cell and **C**, Ti/quartz coated 2mm ANU cell.

Zero Tesla (green) line shows the apparent CD in the sample arising from stain. As the magnetic field is applied, the sample moves slightly, resulting in an apparent MCD signal if the strain is not homogeneous. The ripples seen in spectrum B are approximately the same intensity and the MCD signal from the OEC measured in Paper 1. The reduction in strain achieved by using the hydrophobic ANU thick cell made it possible to measure such low intensity signals.

The addition of 40% of a 1:1 mixture of glycerol and ethylene glycol had previously been found to be the minimum amount of cryoprotectant that would reliably produce a glass in the ANU cells.[21]

After applying the hydrophobic coating to the inside of the cells, different mixtures of cryoprotectant were tested, with the results recorded with both the spectrometer and the new IR camera. Photographs of some of the samples are shown in Figure 3.7.

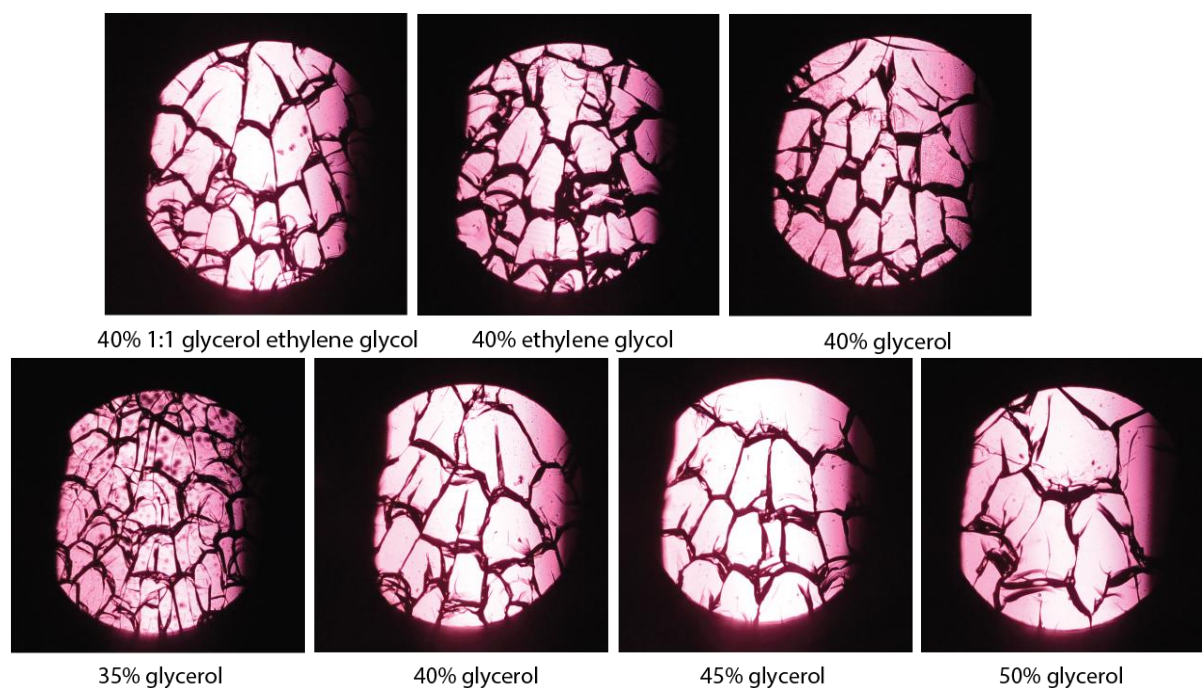


Figure 3.7, Photographs of sample cells containing buffer/antifreeze mixtures at 77K.

The least amount of glycerol useable is 35 %. At this concentration ice appears as dark spots on transmission photos as shown in Figure 3.7 There is no observed difference in transmission between samples with 40 % glycerol and samples with 40 % 1:1 glycerol/ethylene glycol. However, samples with 40 % glassing agent become opaque overnight as the inner of the cryostat warms to ~ 90 K. 45 % v/v is therefore a better minimum amount of glassing agent to add to a sample. Adding additional glycerol above 45 % does not improve the transmission of the sample. Addition of protein has little impact on the appearance of the sample and its transmission at 800 nm.

With 40 – 45 % glycerol, water will form crystals if given nucleation points and cooled slowly. The hydrophobic coating on the inside of the cell may prevent the former as water cannot stick to the surface. Rapid freezing of the sample in the cryostat does not allow time for water crystals to form. The samples used in the work here have thickness between 50 μm and 2 mm, allowing the whole sample to cool rapidly and homogeneously.

Chapter 4

Mn(IV) d-d “spin flip” transitions in the near infrared Magnetic Circular Dichroism spectra of PSII

4.1.1 Introduction to Publication 1

The initial aim of this work was to measure the optical absorption of the oxygen evolving complex of Photosystem II. EPR studies by Boussac et al [45] showed that illuminating a PSII sample in the S2 or S3 state with 720-860 nm light could lead to changes in the EPR spectrum of the OEC. As this is too far to the red for chlorophyll absorption, it can be assumed that the OEC must be directly absorbing the light. As shown in Figure 2.4, the spectrum of PSII is dominated by chlorophyll absorption up to 700 nm. The region between 710-1000nm appears empty. Weak absorption from the OEC could hypothetically be measured in this region with a sufficiently concentrated sample at low temperature. However, the variable scattering of light in a real sample makes measuring these bands with optical absorption alone impractical.

Low temperature magnetic circular dichroism was used to optically characterise the absorption of the OEC. This technique has a number of advantages, as described in Section 2.4.3. In particular, it excludes light from scattering and enhances the absorption of paramagnetic species relative to other bands. The field dependent behaviour of different chromophores can be used to uncouple overlapping signals, and can be compared to EPR data of the same samples.

In this work, PSII extracted from *T.vulcanus* was used. There were a number of reasons for this choice. The half-life of the S-states formed at room temperature is long enough to enable the sample to be loaded and frozen before significant roll back occurs. Previous spectroscopic studies have shown consistency between batches [18]. Samples are robust enough that they can be highly concentrated and retain activity through multiple freeze-thaw cycles. The PSI content of the PSII samples is on the order of 1% (see section 3.3).

A number of improvements were made to the spectrometer and sample handling for the work in this publication, as detailed in Section 3.3.3. In particular, baseline issues encountered in earlier experiments [27] were resolved by applying a hydrophobic coating to the sample cells, producing a more homogeneous strain in the samples and therefore less baseline shift. Figure 3.6

shows an example of the apparent MCD spectra that can be generated from movement of a sample. Furthermore, the sample in the earlier experiment was an uncontrolled mixture of S-states. By dark adapting the sample and then applying controlled pulses of light with a laser, we obtained spectra of each S-state. The S1 → S2 conversion was reliably ~ 80%, and the S2 → S3 conversion was at most 50%, declining with repeated freeze-thaw samples.

4.1.2. Key findings from Publication 1

Variable temperature variable field MCD was reported for the OEC in each S state. The dark-adapted sample was entirely in the S1 state, and displayed a broad negative feature that was weakly diamagnetic. After a single flash from the pulsed laser, a highly-structured MCD signal developed that could be attributed to the S2 state. Second and third pulses advance the OEC to the S3 and S0 states, respectively, which also have structured, but distinctly different MCD spectra. Differences in field and temperature dependencies enable the MCD spectra of the various S states of the OEC and cytochromes and chlorophylls to be distinguished from each other. By subtracting the various appropriately scaled components from each other, spectra of the S1, S2, S3 and S0 OEC states could be obtained.

The spectral features in the MCD of the S1, S2 and S3 states are only 10 – 20 nm FWHM. This, in combination with transition energies and relative intensities of these features enabled us to definitively assign the sharp features seen to $4A \rightarrow 2E$ “spin-flips” from Mn(IV). Furthermore, it was noted that the shapes of the S state MCD signals, that of S2 in particular, bore a strong resemblance to that of the OEC mimic manganese cubane, further strengthening our assignment of spin flips in the PSII OEC. The possibility that the far-red absorption was due to these spin-forbidden transitions had not been previously considered. Section 2.2.3 details the mechanism by which spin flips within a cluster can gain intensity.

4.1.3. Additional notes

We were not able to generate the “high-g” form of the S2 state following the protocol in Bossa et al [45]. This is followed up in Publication 4.

While MCD spectra in the region 710 – 1000 nm of other samples were measured, only those from *T.vulcanus* were worth publishing. MCD spectra of spinach in this region show features similar to those seen in *T.vulcanus*, along with a significant amount of PSI. No serious attempt was made to measure the OEC in *C. thermalis*. The absorption bands of the far-red chlorophylls overlap with the OEC region. There was no sign of an OEC signal in the 710-900 nm region of *Synechocystis* 6803 PSII. *C. merole* shows a negative MCD band with $g=2$ saturation behaviour, which is too intense to be from the Mn cluster.

4.2 Statement of Contribution

**Statement of Contribution**

This thesis is submitted as a Thesis by Compilation in accordance with https://policies.anu.edu.au/ppl/document/ANUP_003405

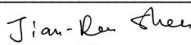
I declare that the research presented in this Thesis represents original work that I carried out during my candidature at the Australian National University, except for contributions to multi-author papers incorporated in the Thesis where my contributions are specified in this Statement of Contribution.

Title and authors: Structured near-infrared Magnetic Circular Dichroism spectra of the Mn₄CaO₅ cluster of PSII in *T. vulcanus* are dominated by Mn(IV) d-d 'spin flip' transitions
Jennifer Morton, Maria Chrysina, Vincent S.J. Craig, Eusamichi Akita, Yoshiki Nakajima, Wolfgang Lubitz, Nicholas Cox, Jian-Ren Shen, Elmars Krausz

Current status of paper: Published

Contribution to paper: 90% of the data in the main paper and supplementary, 10% of the writing. Key parts of the analysis.

Senior author or collaborating authors endorsement:

Nicholas Cox		10/6/25
Jian-Ren Shen		24/6/25
Elmars Krausz		10/6/25

Jennifer Morton		1/5/25
_____ Candidate	_____ Signature	_____ Date

Endorsed

Nicholas Cox		25/6/2025
Chair of Supervisory Panel	_____ Signature	_____ Date
Mark Humphrey		27 June 2025
Delegated Authority	_____ Signature	_____ Date

4.3 Publication 1

BBA - Bioenergetics 1859 (2018) 88–98



Contents lists available at ScienceDirect

BBA - Bioenergetics

journal homepage: www.elsevier.com/locate/bbabio

Structured near-infrared Magnetic Circular Dichroism spectra of the Mn_4CaO_5 cluster of PSII in *T. vulcanus* are dominated by Mn(IV) d-d 'spin-flip' transitions

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ARTICLE INFO

Keywords:
Photosystem II
S-states
Oxygen Evolving Center
Charge-transfer
Magnetic Circular Dichroism

ABSTRACT

Photosystem II passes through four metastable S-states in catalysing light-driven water oxidation. Variable temperature variable field (VT-VH) Magnetic Circular Dichroism (MCD) spectra in PSII of *Thermosynochococcus* (*T. vulcanus*) for each S-state are reported. These spectra, along with assignments, provide a new window into the electronic and magnetic structure of Mn_4CaO_5 . VT-VH MCD spectra taken in the S_2 state provide a clear $g = 2$, $S = 1/2$ paramagnetic characteristic, which is entirely consistent with that known by EPR. The three features, seen as positive (+) at 749 nm, negative (−) at 773 nm and (+) at 808 nm are assigned as ${}^4\text{A} \rightarrow {}^2\text{E}$ spin-flips within the d^3 configuration of the Mn(IV) centres present. This assignment is supported by comparison(s) to spin-flips seen in a range of Mn(IV) materials. S_3 exhibits a more intense (−) MCD peak at 764 nm and has a stronger MCD saturation characteristic. This S_3 MCD saturation behaviour can be accurately modelled using parameters taken directly from analyses of EPR spectra. We see no evidence for Mn(III) d-d absorption in the near-IR of any S-state. We suggest that Mn(IV)-based absorption may be responsible for the well-known near-IR induced changes induced in S_2 EPR spectra of *T. vulcanus* and not Mn(III)-based, as has been commonly assumed. Through an analysis of the nephelauxetic effect, the excitation energy of S-state dependent spin-flips seen may help identify coordination characteristics and changes at each Mn(IV). A prospectus as to what more detailed S-state dependent MCD studies promise to achieve is outlined.

1. Introduction

The light driven chemical conversion of water to molecular oxygen and protons is performed in nature by a single enzyme, the Photosystem II (PSII) pigment-protein supercomplex [1–5]. Light absorption is followed by transfer of excitation to the reaction centre of PSII, leading to subsequent charge separation and the creation of a powerful Chl- α based oxidant, $\text{P680}^{+\bullet}$. This radical cation has the potential needed to oxidise water. Water oxidation is performed in a step-wise fashion through the coupling of $\text{P680}^{+\bullet}$ to the Mn_4CaO_5 cluster of the oxygen-evolving complex (OEC) via a redox active tyrosine residue (Tyr_Z). Each light absorption event, followed by charge separation, then proceeds to

oxidise the cluster by extracting a single electron. After four oxidation events, the cluster then takes four electrons from two molecules of water, releasing molecular oxygen whilst returning the OEC to its least oxidised configuration [6]. The catalytic cycle of PSII is described in terms of a series of intermediate (S_i) states, where the subscript i denotes the number of stored oxidising equivalents in the manganese cluster.

Significant advances have recently been made in a broad range of experimental studies [7] focusing on the OEC and its Mn_4CaO_5 cluster in particular. The imperative has been to determine a detailed mechanism of water oxidation. Of greatest significance in this quest have been improvements in structural data [2], including the spectacular

Abbreviations: PSII, Photosystem II; near-IR, near infrared; UV/Vis, ultra violet/visible; CD, circular dichroism; MCD, Magnetic Circular Dichroism; VT-VH, variable temperature variable field; EPR, electron paramagnetic resonance; OEC, oxygen evolving complex; LMCT, Ligand-to-Metal Charge Transfer; MnCat, manganese catalase; Chl- α , chlorophyll- α ; Tris, tris (hydroxymethyl)aminomethane; CW, continuous wave; SI, supplementary information

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<https://doi.org/10.1016/j.bbabio.2017.10.004>

Received 21 April 2017; Received in revised form 17 October 2017; Accepted 19 October 2017

Available online 21 October 2017

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development of free electron laser-based femtosecond X-ray techniques [8–10]. Structures now exist for the resting state S_1 as well as S_3 , which is the ultimate metastable state prior to O–O bond formation.

Crystallographic data alone cannot provide the level of detail needed to determine the electronic structure of the OEC and subsequently the mechanism of water oxidation. In this paper we describe the utility of Magnetic Circular Dichroism (MCD) as an incisive probe of the electronic structure of the Mn_4CaO_5 cluster, as poised in each metastable S-state. The MCD technique [11] is a natural partner to EPR spectroscopies [12,13]. It has particular sensitivity towards open-shell metal systems. This characteristic allows the weakly absorbing features associated with d-d transitions of manganese ions within the Mn_4CaO_5 cluster to be distinguished from (the MCD of) far stronger chromophores present in PSII. The MCD technique also has the advantage of not being compromised by rapid magnetic relaxation processes. MCD displays equivalent sensitivity in both even and odd electron systems. Strong magnetic fields (> 1 T) and low temperatures (< 4 K) can create a situation where Zeeman splittings are comparable to both kT and ground state electronic energy spacings. MCD signals then become non-linear in magnetic field strength [14,15]. Analysis of these curvatures is very sensitive to magnetic coupling between metal ions [16]. MCD thus promises to be a powerful and multi-faceted window on and into the Mn_4CaO_5 cluster.

It has long been known that near-IR illumination of PSII in the 700 nm–900 nm region gives rise to dramatic changes in the EPR spectra of the Mn_4CaO_5 cluster. In particular, near-IR illumination of the S_2 state at 140 K has been shown to change the spin configuration of the S_2 ground state [17–19]. There are two electronic configurations of S_2 , a low spin ($S = 1/2$, multiline) state and a high spin ($S = 5/2$, $g \geq 4.1$) state. These have been assigned to two proposed structural forms of the Mn_4CaO_5 cluster [20,21].

Near-IR illumination at lower temperatures (< 20 K) can facilitate the formation of intermediates in which an electron is taken from Tyr_Z and returned to the Mn_4CaO_5 cluster [22]. For the S_3 state, near-IR illumination at ≈ 10 K generates a distinctive EPR ‘split signal’, characteristic of an organic radical species coupled to a high-spin metal-based magnetic moment. This split signal for S_3 is ascribed to an $S_2Y_Z^*$ -like intermediate [23] [24,25] [26,27]. The EPR action spectra of the S_2 and S_3 near-IR induced photo-conversion processes, as well overall yields, are similar. Both peak at ≈ 760 nm [28] and both processes are reversible upon warming.

The Mn_4CaO_5 based absorption responsible for the S_2 photo-activity has variously been attributed [17] [29] [30] to a d-d transition within the Mn(III) d^4 configuration(s) or a charge transfer (mixed valence) absorption(s) of neighbouring Mn(III)-Mn(IV) pair(s). By contrast, the photophysical activity in S_3 has also been addressed by proposing the presence of a spin-allowed, low energy d-d transition of a trigonal-bipyramidal Mn(IV) structure proposed [31] for the ‘open’ form of the S_3 state.

We recently reported [32] OEC based MCD features in PSII core complexes of *T. vulcanus* evident at 770 nm and 800 nm. These MCD features were strongly temperature dependent and saturated at high magnetic fields, indicating a predominantly paramagnetic species. The 770 nm-centered feature seen was provisionally assigned to a $d_{x^2-y^2} \rightarrow d_{xz}$ transition of a Mn(III) within the Mn_4CaO_5 cluster of the OEC, based on an analogy to a feature of similar amplitude seen in a manganese catalase (MnCat) [33–35], an enzyme that is structurally related to the OEC [36].

This study was able to clearly establish the chromophores responsible for stronger MCD features that overlap with weaker MCD signals associated with the OEC. The interfering signals at shorter wavelengths are primarily due to temperature-independent, B-term MCD arising from Chl-*a* based excitations. These originate from both a small (1%) component [37] of PS I present in the PSII preparation, as well as long-wavelength charge transfer absorption [38] of PSII. Strongly temperature dependent (C-term) MCD at longer wavelengths is due to

Ligand-to-Metal Charge Transfer (LMCT) transitions of oxidised cyt b_{559} and cyt c_{550} . The MCD C-term characteristics of these LMCT transitions [39] make them difficult to separate from the (weaker) OEC based bands, as the latter have similar field and temperature dependencies.

In the initial report, our PSII core complexes were not poised in a specific S-state of the enzyme. After handling the sample in minimal light and a brief (5 min) dark adaption period, the sample would have been predominantly in the (stable) S_1 state, with perhaps some S_2 and S_0 contributions. In response to the limitations of the exploratory study, we have made significant improvements in sample illumination protocols, sample cell design and overall spectrometer performance. These enhancements have led to a ≈ 10 fold increase in sensitivity as well as a ≈ 100 fold improvement in baseline stability.

In this paper, we report variable-temperature, variable-field (VT/VH) MCD spectra for PSII which has been pre-flashed to create a well-defined S_1 state and advanced further to the S_2 , S_3 and S_0 states in the usual way, i.e. via one or more closely spaced (≈ 3 Hz) and intense nanosecond YAG laser-flashes. We also report the four-flash spectrum, which restarts the Kok cycle. Apart from a pre-flashed and fully dark-adapted S_1 state, laser-flashed samples are invariably mixtures of S-states, owing to S-state advancement ‘misses’ and ‘double hits’. We use the labels $f0(S_1)$, $f1(S_2)$, $f2(S_3)$, $f3(S_0)$ for spectra of the 0, 1, 2, 3 flash-advanced samples, having S_1 , S_2 , S_3 and S_0 as targeted components. Appropriate calibration and manipulation of these spectra allow individual S-state spectra to be determined.

The phenomenology seen, that of a stepwise increase in the number of Mn_4CaO_5 features in MCD spectra in moving from S_1 to S_2 , corresponding to an oxidation of a Mn(III) to a Mn(IV) at each step combined with the narrowness of spectral features observed, has led us to consider the possibility of an assignment of these features as arising from ‘spin-flips’ of the d^3 configuration of the Mn(IV) ions and *not* assignments based on transitions between Jahn-Teller split levels of tetragonally-distorted (six coordinate) d^4 Mn(III) configuration which, in all the examples in the literature, exhibit broader spectral features in absorption and MCD.

2. Materials and methods

2.1. Spectrometer system improvements

The basic spectrometer system utilised, capable of measuring absorption, CD and MCD spectra, has been previously described [40–41]. A number of significant enhancements to the spectrometer system have been introduced. These improvements have led to a greatly increased sensitivity and stability of the system. In particular, the issue of zero levels and field dependent baseline in MCD has been fully resolved. The enhancements implemented and performance characteristics achieved are provided in the SI_2.1.

2.2. Hydrophobic/lipophobic sample cells

The increased stability and sensitivity of our spectrometer has placed a far greater emphasis on limitations arising from the poor and variable (2–10%) transmission of samples at low temperatures. This low transmission was due to the characteristic opalescence of thick, low temperature glasses formed when using sample cell designs developed previously [42]. We were able to create highly reproducible samples with ten times greater transmission, by coating the sample cell with a thin but robust hydrophobic/lipophobic film. Samples frozen in these coated cells also exhibited far less strain birefringence, which enabled reproducible MCD baselines. A description of the development, production, and performance characteristics of these cells is provided in SI_2.2.

2.3. Sample illumination systems and protocols

Samples were advanced into the higher S-states using the well-established ‘saturating flash’ technique of illumination by intense 5 ns flashes from a high power Q-switched YAG laser operating at 532 nm, in our case an injection-seeded Continuum Powerlite 8000. In our optical experiments, samples needed to be flashed with the sample cell already loaded with PSII sample and the cell installed in the sample rod. As the optical density of the sample at 532 nm was ≈ 4 OD units, the ≈ 300 mJ pulses from the laser were split into two equal intensity components, illuminating both the back and front of the sample cell simultaneously.

To poll a pre-loaded PSII sample accurately into a uniform S_1 state, the sample was pre-flashed once and left in the dark for 60 min before transfer to the cryostat. S_1 is the resting state of the OEC. However, a fraction of the S_1 population will have been formed by the slow decay of an S_0 component of the population, S_0 having been created when the sample was exposed to light. This S_0 decay process leaves a corresponding fraction of the Tyr_D population reduced. A uniform S_1 population is created by the pre-flash protocol. Upon flashing, the S_1 population with Tyr_D reduced is activated to oxidise the Tyr_D and not the OEC. Any S_0 population present is advanced to S_1 and higher S_2 and S_3 populations created by the flash decay back to S_1 during the 60 min dark adaptation period.

To poise the sample in higher S-states, so as to enable the measurement of $f1(S_2)$, $f2(S_3)$ and $f3(S_3)$ spectra, the pre-flash procedure was repeated and the sample then flashed 1, 2 or 3 times, with pulses spaced by 0.3 s. The sample rod was then transferred to the superconducting magnet cryostat via a lock and frozen within ≈ 1 –2 min. This process is rapid enough for thermophilic cyanobacterial samples, as they display [43] slow S-state deactivation rates. The rapid-action docking system was constructed so that the transfer to the cryostat could be performed with the sample kept in complete darkness, thus avoiding unintentional scrambling of S-states. The apparatus is described in SI.2.3.

For near-IR illuminations at 775 nm, a ≈ 300 mW continuous wave (CW) Ti:S laser (Schwartz EO) pumped with a Continuum Verdi G CW laser operating at 532 nm was used. Significant care was taken to remove all extraneous visible light from the illumination beam (see SI.2.3).

2.4. Multifrequency EPR measurements

CW X-Band EPR measurements were performed at 5–15 K using a Bruker E500 spectrometer, equipped with an Oxford Instruments ESR 935 cryostat and ITC4 temperature controller. W-band EPR experiments were performed at 4.8 and 7 K using a W-band EPR spectrometer (Bruker ELEXSYS E680) operating at ≈ 94 GHz, as previously described [44,45]. A Hahn echo sequence was used to collect the EPR spectra i.e. t_p - τ - $2t_p$ - τ -echo. The length of the $\pi/2$ microwave pulse was generally set to $t_p = 8$ ns (S_3 signal) and 40 ns (S_2 signal). The interpulse distance was ≈ 260 ns.

2.5. MCD and EPR S-state controls for *T. vulcanus* PSII

The PSII samples were very similar to those previously used [32–46]. The oxygen evolving capacity of the non-recrystallised preparations is typically $3000 \mu\text{mol of O}_2 (\text{mg Chl-a})^{-1} \text{h}^{-1}$. Tris(hydroxymethyl)aminomethane-washed (Tris-washed) PSII samples were prepared by treating the PSII samples with 1 M Tris at pH 8.5 twice at 0.5 mg Chl-a/ml, each with 30 min on ice and precipitated by centrifugation following addition of 12.5% polyethylene glycol. This results in the complete removal of three extrinsic proteins PsbU, PsbV and PsbO, as well as the Mn_4CaO_5 cluster.

The PSII samples used were diluted $\approx 45\%$ (v/v) with a 1:1 (v/v) mixture of ethylene glycol and glycerol as a glassing agent immediately

before use and transferred to a 1 mm path length hydrophobic/lipophobic sample cell. The final PSII concentration (after dilution with glassing agent) of both active and Tris-washed PSII was ≈ 5 mg Chl-a/ml.

The precision of PSII MCD spectral baselines, when utilising the upgraded spectrometer and hydrophobic/hydrophilic sample cells, was established by a comparison with spectra taken at 5 T and -5 T (Fig SI.2.5a). Previously published PSII MCD spectra of *T. vulcanus* in the S_1 state, once corrected for baseline offsets, were seen to be essentially identical to those [32] presented previously (Fig SI.2.5b). Tris-washed samples show (Fig. SI.2.5c) a reduction in C-term MCD. This is attributable to a loss of $\text{cyt } c_{550}$. In the Tris-washed samples, the structured OEC MCD characteristic in S_1 , S_2 and S_3 is absent.

X- and W-band EPR experiments were performed to monitor the behaviour of the same batch of active PSII preparation, in the same glassing mixture and buffer as was used in the optical experiments, albeit at lower concentrations for X-band measurements. S_2 multiline signals were readily obtained from the sample with yields of $\approx 80\%$. Yields of S_3 , as monitored by the reduction of S_2 multiline intensity upon two saturating YAG laser-flashes were $\approx 40\%$. This is comparable to what other workers have obtained [47].

Near-IR illuminations of the S_2 sample at 140 K led to minimal conversion to the S_2 -g4 form of S_2 , as monitored by the depletion of the S_2 multiline EPR signal at X-band. This is currently attributed to the use of the ethylene glycol/glycerol glassing agent needed for optical experiments. Similarly near-IR illumination of the S_3 state led to minimal yield of any Tyr_Z split signal at W-band.

3. Results

3.1. S-state dependent PSII MCD overview

Fig. 1 shows the distinctive changes in MCD spectra occurring upon YAG laser-flash advancing the PSII sample from S_1 to S_2 , S_3 and S_0 states. In this series of measurements, the sample was first pre-flashed and dark adapted for 60 min at 20°C before being cooled to 1.80 K in the spectrometer, to create a well-poised S_1 state. Spectra were recorded at 0 T and at a range of applied magnetic fields. The $f0(S_1)$ spectra in Fig. 1 shows the variation of MCD spectra with magnetic field intensity. The sample was then returned to room temperature in the dark, pre-flashed again and dark-adapted for a further 60 min so as to recalibrate the S_1 state, i.e. free of higher S-states and without Tyr_Z oxidation.

The sample was then YAG laser-flashed so as to advance it to S_2 of the Kok cycle. The field dependent measurements for $f1(S_2)$ are shown in Fig. 1. This procedure was then repeated to explore the $f2(S_3)$, $f3(S_0)$ and $f4(S_1)$ configurations of the sample (SI.3.2). The same series of measurements, all made on the same sample, also included measurements of $f1^*(S_2)$ and $f2^*(S_3)$ for which the sample was laser-illuminated at 780 nm (SI.3.2). Finally, the (scrambled) $f4(S_1)$ sample was dark-adapted for 60 min and shown to quantitatively reproduce the $f0(S_1)$ spectrum.

Except for the $f0(S_1)$ spectrum, for which the entire PSII sample rests in the S_1 state, subsequent measurements shown in Fig. 1 and elsewhere interrogate heterogeneous samples in which a number of S-states are present. The single-flash spectral set of Fig. 1, labelled as $f1(S_2)$, exhibits three narrow features, at 749 nm, 773 nm and 808 nm compared to the two features previously reported [32] in S_1 at 770 nm and 800 nm. The three features in $f1(S_2)$, display the same field dependence and the features can be attributed to the multiline ($g = 2$) form of the S_2 state. As the YAG laser-flash induced yield of S_2 obtained in EPR control experiments has been determined to be $\approx 80\%$, any residual S_1 MCD component in $f1(S_2)$ spectra is small, especially as the OEC MCD of S_1 is weaker than that of S_2 .

Clear, but less dramatic changes in MCD occur when advancing to the $f2(S_3)$ spectrum. The S_2 to S_3 flash-induced transition was also

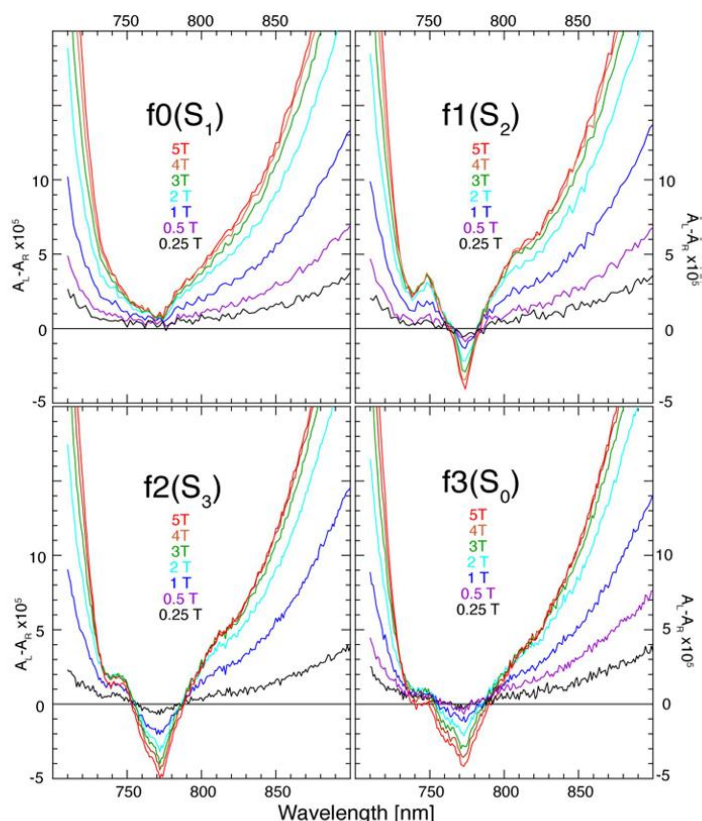


Fig. 1. The four panels show the field dependence of the MCD of *T. vulcanus* at 1.80 K, utilising a pre-flash followed by 60 min dark adaption before 0, 1, 2 or 3 YAG laser-flashes (see text). $\Delta A = 10^{-5}$ corresponds to $\Delta \epsilon$ of $\approx 0.6 \text{ M}^{-1} \text{ cm}^{-1}$ for PSII.

monitored in our samples in EPR. In EPR experiments (Fig. 2), the S_2 to S_3 process was seen to have a yield of $\approx 40\%$, as estimated by the loss of the S_2 state; a lower yield for this step (compared to the S_1 to S_2 step) is mostly due to the accumulation of centres which experience a ‘miss’, i.e. the percentage of centres advanced per flash is maximally 80%, the maximum two flash yield is $0.8 \times 0.8 = 64\%$. With short (5–10 ns) YAG laser-flashes, ‘double hits’ are unlikely. We also note that for the sample to advance past S_2 , the mobile plastoquinone acceptor Q_B needs to be in its binding pocket. In the optical experiments, the yield of S_3 is seen to be very similar to that measured in EPR. The S_3 yield in the optical experiment can be estimated from the amplitude of the narrow S_2 MCD features remaining and scaling to the corresponding narrow S_2 features seen in the $f1(S_2)$ spectra. By this method, the S_3 yield (from S_2) is determined to be $\approx 40\%$. This value is comparable to that seen (i.e. 46%), for PSII microcrystals prepared from the same material *T. vulcanus*, reported in the current XFEL S_3 structure [8], as estimated from parallel FTIR measurements.

As the contribution of S_1 to flash-advanced MCD spectra is small, subtracting 60% of $f1(S_2)$ from $f2(S_3)$ provides a pure S_3 MCD spectrum. This spectrum can then be scaled to the magnitude corresponding to a 100% yield of S_3 from S_1 . The scaled S_3 spectrum is shown in Fig. 3 and exhibits a slightly broader, blue shifted (–) MCD peak at 764 nm, with a resemblance to the weaker 770 nm peak in S_1 and also to the narrower and stronger 773 nm peak in S_2 . The S_2 spectrum in Fig. 3 is also scaled to correspond to a 100% yield from S_1 .

The field dependent behaviour of the S_3 feature in Fig. 1 is distinctly different from that of the S_1 and S_2 features. At 1.80 K the 762 nm S_3 feature approaches saturation between 1 T and 2 T, whereas those of S_2 saturate between 4 T and 5 T.

A first sight, the $f3(S_0)$ MCD spectra in Fig. 2 do not appear to be greatly different to those for $f2(S_3)$. In both, there is a similar shoulder on the remnant of a (narrow) S_2 feature at 773 nm. However, the saturation behaviour of the shoulder near 764 nm in $f3(S_0)$ is different from that of either $f1(S_2)$ or $f2(S_3)$ as seen by the nesting behaviour near 744 nm. The yield from S_2 to S_0 is $\approx 40\%$. This relatively high yield, given the S_2 to S_3 yield was similar, can be attributed to the relatively long lifetime of the S_0 state compared to S_3 and the short period (~ 0.2 s) between the second and third YAG laser-flashes for the $f3(S_0)$ experiment, resulting in negligible S_3 to S_2 decay in this period.

Once the yield of S_0 has been determined, a scaled S_0 spectrum can be obtained from the data in Fig. 1. S_0 exhibits a single, relatively broad negative feature peaking with an apparent minimum near 764 nm. Fig. 3 provides the MCD spectra of PSII in the metastable Kok states, S_1 , S_2 , S_3 and S_0 . These spectra were all generated from the same PSII sample and were measured under an identical set of experimental conditions.

3.2. VTVH MCD spectra

The temperature dependence of MCD spectra in the near-IR region is

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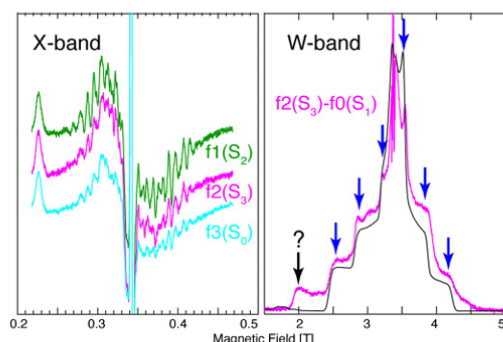


Fig. 2. EPR control measurements of the OEC in PSII of *T. vulcanus* were performed at both W-band, right hand panel, (94 GHz) and X-band, left hand panel, (9 GHz). The right hand panel shows the $f2(S_3)-f1(S_1)$ difference spectrum at W-band (magenta) at 4.8 K as well as the simulated spectrum (black curve). The parameters used in the simulation were those of reference [44], $g = 1.99$, $S = 3$, $D = 0.175 \text{ cm}^{-1}$, $E/D = 0.27$. Turning points of the S_3 state signal ($S = 3$) are marked in blue. An additional $\text{Mn}_2\text{O}_3\text{Ca}$ signal is also observed near 2 T marked as ? which may be due to the influence of the cryoprotectant. Assignment of this feature forms a part of ongoing work. The left hand panel shows the 8 K (S_2 multiline) spectra, for singly doubly and triply flashed samples as indicated. The doubly flashed sample $f2(S_2)$ (magenta) has an S_2 multiline amplitude of 52% of the singly flashed sample $f1(S_2)$ (green) and that of the triply flashed sample $f3(S_0)$ (cyan) falls to 35%.

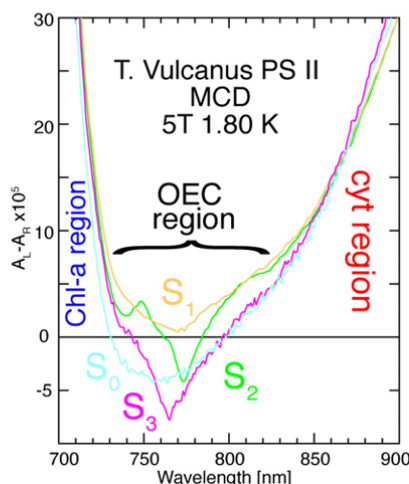


Fig. 3. Scaled Near-IR MCD spectra of PSII for each S-state. The regions dominated by Chl-a (< 730 nm), the OEC (730 nm–820 nm) and cyt $b_{550}/\text{cyt } c_{550}$ (> 820 nm) are annotated.

shown in Fig. 4. These spectra demonstrate the paramagnetic nature of the unstructured MCD at wavelengths longer than 800 nm, present to a very similar extent in each S-state. Such temperature dependent behaviour has been studied previously for S_1 [32] and is due to the high-energy tail of the intense, broad LMCT transitions of oxidised cytochromes which peak near 1600 nm. At wavelengths shorter than 750 nm, Fig. 4 also shows a (smaller) paramagnetic MCD signal present, as well as a B-term MCD component arising from Chl-a based excitations in PSII and the (minor) PS I content of the sample. The purely temperature dependent component of the MCD is provided by

subtracting spectra taken at 1.8 K and 40 K.

The dominantly paramagnetic characteristic of the OEC features in S_1 in *T. vulcanus* at temperatures as low as 1.8 K is unexpected. Previous EPR evidence indicated that in spinach, the S_1 state was shown to be weakly diamagnetic [48–49], having a splitting to a higher lying paramagnetic level of $\approx 2.5 \text{ K}$ (1.7 cm^{-1}).

Fig. SI.3.2a presents higher-sensitivity field dependent MCD spectra of PSII poised in $f0(S_1)$ configuration, whilst also extending the range of measurement to applied magnetic fields of 6 T. These low-noise data enable the field dependence for the OEC-based transitions to be distinguished from that due to the tail of the cytochromes. The field dependence of the 770 nm and 800 nm S_1 OEC MCD features was determined graphically, by resolving the fraction of the 5 T spectrum that accurately nulls S_1 OEC-based MCD at other fields. The resulting field dependence is shown in Fig. 5, along with theoretical saturation curves generated using an effective spin Hamiltonian model for the S_1 state (see extended calculations in SI.3.2b), which treats the cluster as a dimer with two antiferromagnetically coupled $S = 2$ components exchange coupled by a parameter J and excludes the fine structure splitting of the Mn ions. The resulting saturation curves are shown in the left hand panel of Fig. 5, and are normalised at 5 T so as to provide a direct comparison to experimental data.

3.2.1. S_1 OEC MCD characteristics

The modelling in Fig. 5 shows that the experimental data for S_1 can be accounted for by using a J value close to -0.2 cm^{-1} . This corresponds to a splitting between the $S = 0$ lowest state and the $S = 1$ first excited state of the cluster of 0.4 cm^{-1} . This is significantly smaller than the value of 1.7 cm^{-1} inferred from the temperature dependence of the EPR of the S_1 state in spinach. Detailed modelling (Fig. SI.3.2b) shows that at temperatures lower than 1.6 K, the difference between weakly diamagnetic $J = -0.2 \text{ cm}^{-1}$ behaviour and isotropic $\tanh(g\beta H/2kT)$ behaviour becomes easily distinguishable at low fields. Such experiments are feasible and will make quantification of weak diamagnetism more definitive. However as the saturation behaviour seen is indeed similar to that of a $g = 2$ $S = 1/2$ system and diverges significantly from the diamagnetic splitting determined from EPR collected in spinach, we cannot not completely exclude the possibility that the 770 nm feature signal arises from a sub-fraction of centres. This may reflect inhomogeneity of the S_1 state or arise from a small population ($\approx 10\%$) of centres existing in a completely dark stable S_2 state.

3.2.2. S_2 OEC MCD characteristics

The S_2 state of PSII is very well studied. S_2 typically can have a high yield of formation and it exhibits a very well structured and distinctive (multiline) EPR spectrum. With its simple $S = 1/2$, $g = 2$ characteristic, S_2 VTVH behaviour will arise from the Kramers degeneracy in the ground state and have a $\tanh(g\beta H/2kT)$ form. Both the field dependence and temperature dependence of the MCD closely follow this behaviour. At fields as high as 6 T, there is no evidence for a contribution from higher-lying multiplets of the exchange-coupled cluster, which have been implicated in modifying effective spin Hamiltonian parameters, leading to enhanced anisotropy. The absence of any contribution from these states is consistent with EPR results in *T. elongatus*, which have indicated [50] that the energy spacing between the ground and excited spin states of the exchange coupled systems is larger ($> 10 \text{ cm}^{-1}$) than that seen in higher plants.

The well-structured S_2 MCD features associated with the OEC are stronger than those of S_1 . As the yield of S_2 is high ($\approx 80\%$), a subtraction of $f1(S_2)$ and $f0(S_1)$ spectra provides a good approximation for OEC-based MCD of S_2 . Such spectra are provided in Fig SI.3.2c and were used to determine the field dependence in Fig. 5 for S_2 . In Fig SI.3.3a, B-term contributions to the MCD, i.e. from Chl-a absorptions at short wavelengths, are eliminated by the subtraction of corresponding spectra of $f1(S_2)$ and $f0(S_1)$. These B-terms change very little upon flash advancement and can be shown to be almost identical in each step (Fig.

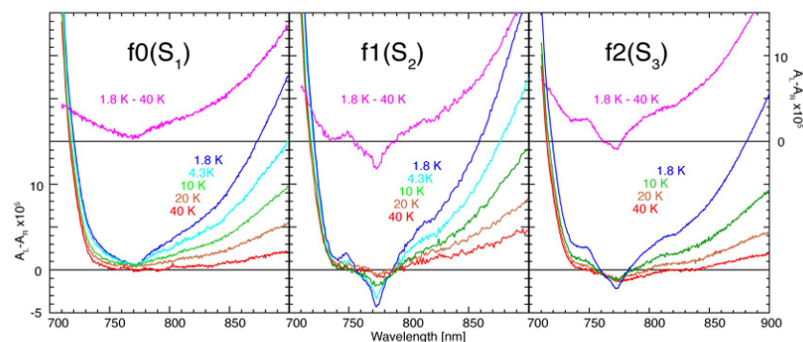


Fig. 4. MCD at 5 T of PSII of *T. vulcanus* for the samples YAG laser-flashed advanced to S_1 , S_2 and S_3 S-states of PSII (see text) the temperatures indicated. The offset (magenta) curves are the difference between spectra taken at 1.8 K and 40 K. Temperature dependent spectra were taken of different samples to the one used in Fig. 1. These latter samples had been thermally cycled more often and exhibited slightly lower yields of S_2 and S_3 as well as exhibiting an increased fraction of oxidised cyt b_{559} . Apart from amplitude, the spectral characteristics of S_1 , S_2 and S_3 were not measurably different and differences do not affect the interpretation of data.

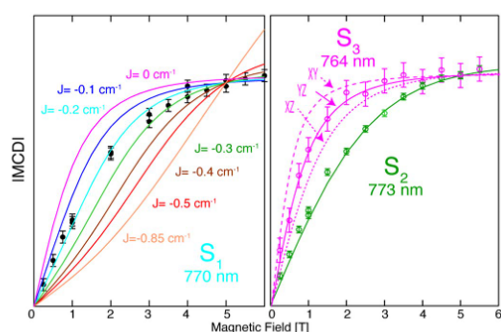


Fig. 5. The magnitude of the field dependence of the 770 nm MCD feature of *T. vulcanus* in the S_1 state at 1.8 K (left hand panel), compared to theoretical curves (see text) for $J = 0, -0.1, -0.2, -0.3, -0.4, -0.5$ and -0.85 cm^{-1} , normalised at 5 T. Analogous saturation curves for the S_2 and S_3 states are shown in the right hand panel as well as theoretical saturation curves for an isotropic $g = 2$, $S = 1/2$ doublet (green) appropriate to S_2 and using the same parameters as used for the simulation of the W-band EPR of S_3 (magenta) used in Fig. 2.

SI.3.2c). LMCT MCD C-term features dominate at longer wavelengths, these arise from both oxidised cytochromes. The cyt b_{559} signal undergoes small changes upon subsequent YAG laser-flashes and/or freeze/thaw cycles and thus are not completely nulled in difference spectra. As mentioned above, MCD amplitudes in cytochrome-based spectral regions increase in older samples, i.e. as cyt b_{559} becomes more oxidised, but cyt b_{559} can also experience reduction. It is known that illumination of PSII samples can lead to cyt b_{559} reduction, presumably due to the production of reduced quinones, in parallel to OEC oxidation.

There are three distinct features in the S_2 OEC-based components in Fig's. 3 and 4; at 749 nm, 773 nm and 808 nm. The two latter features are similar to features seen in S_1 at 770 nm and 800 nm. The purely temperature dependent component of MCD spectra in $f_0(S_1)$ and $f_1(S_2)$ are shown as the (offset) spectra in Fig. 4. These reveal a positive and, paramagnetic signal in both S_1 and S_2 extending under the Chl- a based B-terms. A comparison of the purely temperature dependent component of $f_0(S_1)$, $f_0(S_2)$ and the Tris-washed samples (Fig. 4 and SI.2.4d) establish that such MCD at higher energies is associated with oxidised high-spin Fe(III) cytochromes. The amplitude of the higher energy temperature-dependent component scales well with that of the LMCT band intensity near 900 nm in both $f_0(S_1)$, $f_1(S_2)$ $f_2(S_3)$ and the Tris-

washed sample (Fig. SI.2.5c,d).

When the $f_1(S_2)$ sample was illuminated with a 780 near-IR laser at 140 K, to create $f_1^*(S_2)$, measurable changes were seen in the MCD (Fig. SI.3.2d). Comparing the MCD spectra before and after illumination only small (5–10%) bleach of the S_2 multiline features occurred and a broad negative high- g MCD feature attributable to S_2 -g4 was generated at short wavelengths (Fig. SI.3.2e). EPR measurements confirmed a low yield of S_2 -g4 in our samples.

3.2.3. S_3 OEC MCD characteristics

The $f_2(S_3)$ spectra in Fig. 1 exhibit a feature near 760 nm associated with the S_3 state. A systematic subtraction of narrower (associated with S_2) features provides spectra from which a field dependence of the 760 nm S_3 feature can be obtained. The field dependence is shown in Fig. 5, which demonstrates that S_3 state saturates at low magnetic fields, with its initial slope approximately twice that seen for S_2 .

The high field (W-band) EPR spectrum can be simulated using a model [44] in which the Mn atoms couple to form a well-isolated $S = 3$ ground state. In a high spin system, the unpaired spins of all Mn atoms interact by means of both spin-spin and spin-orbit mechanisms. This breaks the degeneracy of the spin multiplet at zero-field and a fine structure tensor (D) needs to be introduced. The S_3 state EPR data is consistent with the cluster having a small, negative, rhombic zero-field splitting with $D \approx 0.2 \text{ cm}^{-1}$. This relatively small value is consistent with the cluster having four octahedrally coordinated Mn(IV) ions.

The magnetic saturation properties of MCD signal(s) provide additional information, not accessible by EPR. MCD saturation curves depend on the (ground state) spin Hamiltonian terms mentioned above and additional terms associated with the product of transition moment vector components $M_{x,y,z}$ between the ground state and the electronic excited state. Fig. 5 shows that MCD associated with YZ i.e. M_y, M_z polarization product, reproduces the saturation of S_3 seen in the 1.8 K experiment. A determination of the (small) D values in octahedral Mn (IV)s is in principle accessible by means of MCD saturation measurements taken at increasingly low fields and temperatures. Saturation curve nesting, as seen in Fig. SI.3.2b, which shows modelling of the weak diamagnetism of S_1 , is an example of the type of behaviour that could lead to an independent determination of the D parameter. The yield-corrected S -state spectra in Fig. 3 point to a broader negative component(s) associated with S_3 , in the 780 nm to 850 nm region. These broader component(s) have the same (high-spin) saturation behaviour as the main feature.

When the $f_2(S_3)$ sample was illuminated using 775 nm near-IR laser light at 1.8 K, significant spectral changes occurred (Fig. SI.3.2f). A

comparison of MCD spectra taken before and after illumination (Fig. SI.3.2 g) reveal the 20–25% bleach of $g = 2$, S_2 multiline features. A broad positive high- g MCD feature is created at short wavelengths. No measurable change is seen in the S_3 component. In EPR control measurements, there was only a small Tyr₂-like split signal seen. There were some small changes in the (broad) EPR signals associated with the S_3 state. Being able to have samples that have substantial near-IR activity in both S_2 and S_3 is a priority for future work.

3.2.4. S_0 OEC MCD characteristics

The S_0 state of PSII is not as well studied as other S-states, even though S_0 is an odd electron configuration and nominally well-suited to the EPR technique [51–54]. Well-structured S_0 EPR spectra can be obtained from cyanobacterial PSII samples and in spinach samples when methanol is added. There is little doubt that our $f_3(S_0)$ spectrum has advances with good yield to S_0 as the $[f_3(S_0) - f_2(S_3)]$ spectrum closely replicates an inverted S_3 spectrum.

Fig. SI.3.2h shows the field dependent spectra of $f_3(S_0)$, corrected for the S_2 and S_3 components. At first sight, it appears that the OEC of S_0 may exhibit a relatively broad negative MCD signal, evident as a peak near 755 nm. However, this is deceiving. The field dependence of MCD in this region in S_0 is not paramagnetic. It manifests as a linear signal (B-term) to within experimental error (Fig. SI.3.2j).

The top panel of Fig. SI.3.2i displays step-wise changes of S_0 MCD upon each 1 T increase in applied field. For the final (5 T–4 T) step, the cytochrome MCD contribution becomes small as its C-term based intensity is close to saturation. The B-term component associated with each step, owing to Chl- a bands is present, as it is for the other S-states. However, there is a broad negative component extending from 710 nm to ≈ 900 nm that increases linearly with field. This B-term activity is seen clearly in the field dependence of S_0 - S_1 spectra (SI.3.2j, lower panel) in which B-terms associated with Chl- a are eliminated and signals due to cytochrome are strongly reduced. The temperature dependence of the MCD of $f_3(S_0)$ also displays a very broad negative signal near 755 nm up to 40 K, this being consistent with the B Term nature of this band.

When the $f_4(S_1)$ spectrum is compared to that of $f_3(S_0)$ (SD), it is evident that the negative MCD B-term associated with S_0 is reduced (Fig. SI.3.2 k), indicating that a significant fraction of S_0 advances to S_1 . The $f_4(S_1)$ spectrum can be attributed to $\approx 50\%$ S_2 , 10% S_3 and $\approx 10\%$ S_0 , with the remainder ($\approx 30\%$) being S_1 .

There are no identifiable OEC based MCD features in the S_0 with VTVH behaviour similar to those seen for S_1 , S_2 , and S_3 . The very broad and featureless negative B-term MCD appears to be unique to S_0 .

3.3. Determining OEC-based spectra of each S-state

A comparison of S-state MCD spectra in Fig. 3 along with the temperature dependent data in Fig. 4 help discriminate between features due to S-state advancement within the OEC and those due to other cofactors of PSII. There is minimal change in B-terms associated with Chl- a components of PSII in the advancement process (Fig. SI.3.2c). However, strong C-term signals associated with high-spin Fe(III) cytochromes underlie both OEC-based signals as well the Chl- a -based B-term components. Cytochrome-based signals are variable and change with both thermal cycling and YAG-flashing of the sample.

Cytochrome-based MCD changes are less problematic for the S_2 state as the yield is high ($\approx 80\%$). S_2 OEC-based MCD features are narrow and can be identified against a cytochrome background (Fig. SI.3.3a). For S_3 , where yields are lower and where OEC-based features in S_2 and S_3 overlap, alternative procedures are needed to reliably extract an OEC-based S_3 MCD spectrum.

One approach is to use the distinctly different saturation behaviour seen for S_2 and S_3 (Fig. 5). Comparing double difference spectra and taken between the $f_2(S_3)$ spectra and $f_1(S_2)$ spectra at 6 T, where both S_2 and S_3 are saturated and those taken at 2 T where only S_3 is saturated

allows extraction of an OEC-based S_3 spectrum (see Fig. SI.3.3b), independent of any estimate of the yield of S_3 .

A second procedure that leads to less noisy spectra, is to use the yield of S_3 , as determined via a graphics-based determination of the magnitude of the narrow and distinctive S_2 features remaining in $f_2(S_3)$ spectra to determine the OEC-based profile of S_3 . A comparison of S_3 profiles determined by these procedures is provided in Fig. SI.3.3b. Both procedures lead to similar very lineshapes and amplitudes. The yield-based spectrum, determined by difference spectra taken at 2 T as well as the saturation-based spectrum are less prone to errors but are noisier.

The S_0 OEC-based spectrum, having B-term behaviour, is easily distinguishable from other S-states. Once the Chl- a B-term component is accounted for, the S_0 OEC-based profile can be distinguished from both cytochrome signals and other S-state OEC-based components, which become largely saturated at 5 T and 1.8 K.

Having determined OEC-based S-state MCD profiles of S_2 and S_3 using the procedures above, it becomes possible to reliably model both the Chl- a and cytochrome MCD components by fitting these (with 3rd order polynomials) to the yield-corrected experimental data, and subsequently subtract these interfering components. Fig. 6 demonstrates this analysis for S_2 .

The fitted curves for cytochrome and Chl- a components determined in Fig. 6 can, with only minor amplitude changes of the components associated with changes in individual sample concentrations and fraction of oxidised cytochromes, can be used to generate OEC-based MCD spectra of the other S-states. The complete set of OEC-based spectra is shown in Fig. 7. These data have better signal to noise than those generated by double differences etc., and as the spectra contains no components due to PS I, can be put on a PSII molar extinction scale. The narrow S-state dependent features have a $\Delta\epsilon$ between 0.5 and $4 \text{ M}^{-1} \text{ cm}^{-1}$ at 5 T and 1.8 K.

3.4. Narrow Mn(IV) features in the near-IR MCD of model compounds

Weak, narrow MCD features, assigned to $^4A \rightarrow ^2E$ spin forbidden transitions of Mn(IV) have been reported [55] in the near-IR region for a number of Mn(III)-Mn(IV) μ -oxo bridged dimers. These dimers served

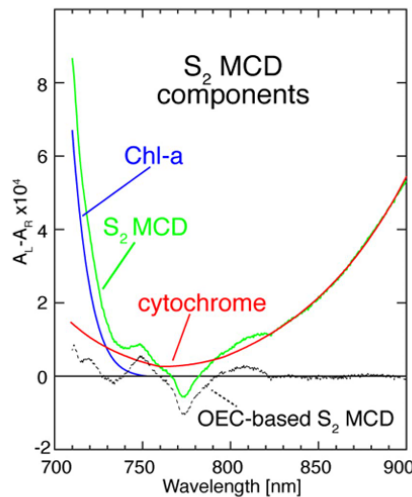


Fig. 6. Near-IR MCD of the S_2 state (green) together with fits to the cytochrome (red) and Chl- a (blue) components (see text). The residual OEC-based S_2 MCD, after these fitted components have been subtracted, is also shown (dotted black).

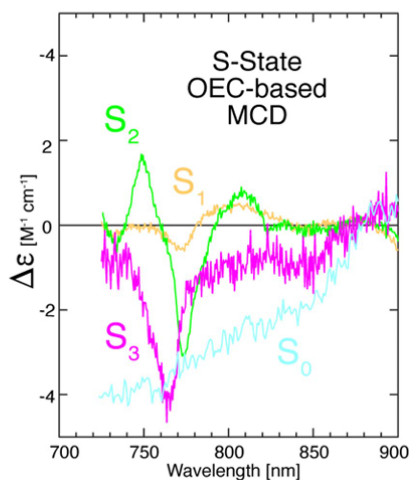


Fig. 7. Calibrated OEC-based near-IR MCD spectra at 5 T and 1.8 K for the S-states of PSII.

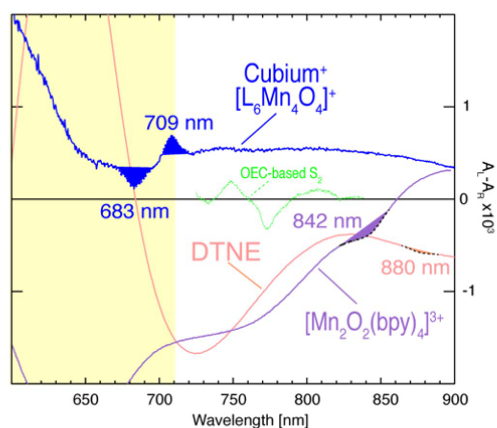


Fig. 8. Near-IR MCD spectra of model compounds exhibiting narrow MCD features (see SI 3.4 for experimental details). Spectra are on the same ordinate scale as Figs. 6 & 7 whilst being extended (yellow shaded region) to where MCD measurements are precluded in PSII by intense Chl-*a* based pigment absorptions. For comparison purposes, the green dotted line is the OEC-based MCD of the S₂ state of PSII taken from Fig. 7 (MCD not to scale).

to model MnCat, which is an enzyme that is structurally related to the OEC. The data in Fig. 8 (see SI 3.4 for details) reproduced measurements on [Mn₂O₂(bpy)₄]³⁺ at higher sensitivity, as well as providing data on a more recent synthesised MnCat model, DNTE [56]. The latter dimer exhibits a weak narrow band at 880 nm. Also shown in Fig. 8 are MCD measurements of the Mn cubane oligomer cubium⁺, which serves as a model compound for the OEC [57]. This system exhibits two narrow MCD features, at 683 nm and 709 nm. This observation is consistent with there being two distinct Mn(VI)s in this system.

Table 1
S-State spin-flips.

S state	λ/nm	Wavenumber/cm ⁻¹	FWHM/nm	Δε/M ⁻¹ cm ⁻¹ 5 T 1.8 K
S ₁	770	13,000	12	(-) 0.6
	800	12,500	20	(+) 0.5
S ₂	749	13,400	13	(+) 1.7
	773	12,900	14	(-) 3.0
S ₃	808	12,400	20	(+) 0.7
	764	13,100	20	(-) 4
	(770–850) ^a	13,000–12,000	> 25	(-) 1.5

^a No clear peak; likely composite.

4. Discussion

4.1. Spectral assignments and the nephelauxetic effect

A key characteristic of the OEC S-state dependent MCD spectra in the S₁, S₂ and S₃ states is that they exhibit features with FWHM widths of only 10–20 nm FWHM (Table 1).

These bands are far narrower than spin-allowed d-d bands of Mn(III) or Mn(IV) which typically have widths 10–20 times larger. Many spin-forbidden bands are also comparably broad. However, the exception is spin-flip d-d bands, which represent inversions of a single spin *within* a single orbital configuration and are, by contrast, narrow.

The lowest-energy orbital configuration of the octahedral d³ ion is t_{2g}³. The lowest energy term is then ⁴A_g, in which each of the three t_{2g} electrons are parallel. The first excited state is ²E_g and it has the *same* orbital configuration as the ground state, t_{2g}³, with one electron spin 'flipped'. This excitation derives its energy from the change in electron repulsion amongst the t_{2g} electrons upon inverting a spin and is affected little by energy changes in low symmetry splittings of t_{2g} and e_g orbitals (which are determined by ligand fields). As the orbital configuration in a spin-flip excitation is not changed, there is little nuclear displacement between the ground and excited state. Consequently, the dominant intensity resides in the *purely electronic* excitation with little intensity occurring in vibrational sidelines. This purely electronic nature ensures spin-flip excitations narrow.

Although spin-flips are ligand-field independent, it was recognised long ago that their excitation energy varied significantly with the nature of the ligands co-ordinating the metal [58]. F⁻ coordination led to excitation energies closest to that of the corresponding atomic, gas phase excitation of the free ion whilst Cl⁻, NH₃ or I⁻ coordination lead to a gradual reduction of the spin-flip excitation energy. This nephelauxetic effect was attributed to the 'hard' or 'soft' nature of ligands and specifically to their 'cloud-expanding' effect on d-orbitals. The simplistic rationale is that electronic wavefunction delocalisation leads to a reduction of the d-electron repulsion terms that define the spin-flip energy. The nephelauxetic effect is larger for metals of higher valence. This is clearly shown in a series of d² systems with increasing metal charge [59]. Nephelauxetic effects in Mn(IV) complexes are thus larger than those in equivalent Mn(III) systems.

For Mn(IV) in a crystalline F⁻ environment, sharp ²E → ⁴A emission occurs near 623 nm [60]. Mn(IV) doped oxides exhibit ²E → ⁴A emission over the 700 nm – 720 nm range [61]. For Mn(IV) an octahedral O-(SeO₄) environment in Mn(SeO₄)₂, the same transition occurs at 755 nm [62]. MCD spectra of the monomeric Mn(IV) complex molecular [MnIV(OMe)₃(Me₃TACN)]⁺ reports [63] a spin-flip feature at 715 nm. Very recently, ²E → ⁴A emission of a *bis*-(tris(carbine) borate) complex of Mn(IV) which has C-(carbine) coordination has been reported [64] at 828 nm.

Spin-flip transitions for both Mn(III) and Mn(IV) are well documented in a series of molecular oxo-bridged dimer model compounds of MnCat [55]. The Mn(III) spin-flips occur in the 500 nm–550 nm spectral region, whereas Mn(IV) spin-flips occur near 806 nm, the latter

having widths of ≈ 10 –20 nm. We have repeated measurements on the $[\text{Mn}_2\text{O}_2(\text{bpy})_4]^{3+}$ dimer and made measurements of an additional dimer, DTNE. These data are shown in Fig. 8 and our measurements for the former system confirm the previous experimental results.

The examples above establish that the $^4\text{A} \rightarrow ^2\text{E}$ spin-flip in Mn(IV) systems occur in the range 620 nm to 880 nm. Although the overall trend is clear, with F ligands leading to the highest energy case and other ligands lower, it is not immediately clear why cubium⁺ with both P-(phosphinate) and O-(cubane-oxo bridge) ligating atoms has spin-flips at quite high energy nor why the $[\text{MnIV}(\text{OMe})_3(\text{Me}_3\text{TACN})]^{+}$ complex with three N-(TACN) and three O-(OMe) ligating atoms has a higher energy transition than $\text{Mn}(\text{SeO}_4)_2$ which has six O-(SeO₄) ligating atoms.

A recent review on the nephelauxetic effect [65] has reiterated the point that there are clear anomalies in attributing the effect simply to delocalisation of d electrons. An alternative approach is to attribute the effect to the polarisability of ligands rather than delocalisation. A differential nephelauxetic effect has been reported between t₂ and e orbitals [66]. This work also indicates a quasi additivity of the nephelauxetic shift in a series of hexadentate complexes in which the ratio of ligating N to S atoms is varied. A beautiful example of a quasi-additivity of shifts is provided by the study of series of mixed Ni(II) doped bromides/chlorides [67]. An analysis of the relativistic nephelauxetic effect establishes that spin orbit coupling on a metal can be significantly reduced [68] via this process.

Spin-flips in the Mn_4CaO_5 cluster (Table 1) occur over a relatively small part of the overall range seen for the Mn(IV) systems discussed above. The data in Table 1 show two similar energy peaks in both S₁ and S₂ i.e. at ≈ 770 nm and ≈ 800 nm. These are provisionally assigned to the Mn2 and Mn3 atoms of the cluster, as they do not change their valence in passing from S₁ to S₂ within the dominant OEC model. The additional sharp positive peak created at 749 nm in S₂, is then assigned to Mn4. More definitive assignments would be greatly assisted by MCD measurements on the near-IR illuminated forms of S₂ and S₃. Our samples showed low yields of these conversions (SI.3.2). The S₂-g4 state shows the emergence a broad putatively Mn(III) d–d band, which could then be associated with Mn4, but more detailed spectra are required.

4.2. Magnitude and sign of spin-flip MCD

The rationale in 4.1 points to an assignment, based on energetics, bandwidths and model compounds, which confirm the narrow bands seen in OEC MCD of the PSII in the 710 nm–800 nm region as spin-flips of Mn(IV)s. The spin-flip absorption intensity for an isolated Mn(IV) species (not magnetically coupled to other ions) is weak, gaining intensity through spin-orbit coupling to spin-allowed transitions. The $^4\text{A} \rightarrow ^2\text{E}$ spin-flip, which can be extremely sharp in some systems, is often only seen in emission. It is far more difficult to see in absorption.

In PSII and in the model compounds of MnCat, spin-flips are clearly visible in MCD. At low temperature and high field conditions, the molar extinction is in the range $\Delta\epsilon = 0.1$ – $3 \text{ M}^{-1} \text{ cm}^{-1}$ (Table 1). Absorption cannot be greater than $2\epsilon\Delta A$ as by definition, $\Delta A = A_L - A_R$ and $A = (A_L + A_R)/2$. Thus PSII spin-flips must have $\epsilon > 0.2 \text{ M}^{-1} \text{ cm}^{-1}$. The ratio $\Delta A/A > 0.1$ in MCD is rarely exceeded, and ϵ is likely to be $\approx 2 \text{ M}^{-1} \text{ cm}^{-1}$ or higher. Even at this intensity, the absorbance due to the OEC in our sample would not exceed 5×10^{-5} and thus be significantly weaker than vibrational overtone features exhibited by the sample.

The relatively strong absorption intensity implied from the intensity of MCD in PSII (and MnCat model compounds) for Mn(IV) spin-flips, arises from the magnetic exchange-coupled electric dipole mechanism [69]. This process is known to dramatically enhance spin-forbidden absorption by compensating the spin change upon electronic excitation of one metal centre by simultaneously changing the spin level of the exchange coupled dimer (or cluster), so that the total $\Delta S = 0$ spin

selection rule for optical excitation is preserved [70].

The absorption mechanism and thus MCD associated with such an exchange-coupled enhanced absorption process is quite distinct from that arising from the normal, single-centre (spin-orbit mixing with spin-allowed states) process. A summary of some of the concepts involved is given in SI.4.2. The consequences of the exchange-coupled mechanism are that not only the intensity of a spin-flip absorption dramatically increases, but also that the relative magnitude of MCD to absorption ($\Delta A/A$) can vary. Even the sign of the MCD can reverse.

The VTVH MCD behaviour exhibited in magnetically-coupled systems is inherently complex but also is both distinctive and informative. In PSII, each Mn in the Mn_4CaO_5 cluster is coupled to the other Mn atoms via exchange parameters $|J|$ of the order of 0.1 – 50 cm^{-1} and the coupling can be either ferromagnetic ($J > 0$) or antiferromagnetic ($J < 0$). These couplings lead to a (magnetic) ground state of the cluster having a particular net total spin value in its lowest magnetic state. This spin value is often determinable through EPR measurements. There will also be a number of (magnetically) excited states of the cluster in which the total spin changes, and these will become populated at higher temperatures. In our MCD measurements, the applied magnetic field is varied between 0 and 6 T. This can lead to Zeeman shifts that are larger than the energy separations in the exchange coupled ladder of the cluster. Zeeman shifts can lead to spin sub-levels of higher excited states being populated at 1.8 K or even becoming the lowest state. An example of this type of behaviour is given in our analysis of the S₁ state, where $J = -0.2 \text{ cm}^{-1}$. The system is nominally diamagnetic but becomes effectively paramagnetic in fields $> 0.2 \text{ T}$.

An instructive example of the influence of magnetic exchange coupling on MCD is provided by the study [16] reported for three isomorphous molecular dimers containing two metal ions. The metal dimers are I Cr(III)-Zn(II), II Gd(III)-Ni(II), III Cr(III)-Ni(II). Zn(II) and Gd(III) are diamagnetic, absorbing minimally through the visible spectral region. Spectra of I and II report the d-d MCD of magnetically isolated Cr(III) and Ni(II). The MCD of III is not however a simple sum of MCD spectra of I and II. Using a spin Hamiltonian model, the authors are able to account fully for the (complex) VTVH MCD of III, from magnetic properties of I and II along with the magnetic susceptibility of III.

Two observations from this study are immediately pertinent to our work. In I, which has a magnetically isolated Cr(III), the $^4\text{A} \rightarrow ^2\text{E}$ transition (which is isoelectronic to the spin-flip in Mn(IV)) is not visible in absorption or MCD. In the exchange coupled dimer III the Cr(III) spin-flip appears as a narrow ($\approx 15 \text{ nm}$) FWHM feature, with $\Delta\epsilon \approx 0.5 \text{ M}^{-1} \text{ cm}^{-1}$ under saturating conditions. Both the width and MCD intensity of the Cr(III) spin-flip parallel the behaviour seen for Mn(IV) spin-flips seen in PSII.

A second relevant characteristic of this work is that the MCD of the lowest energy spin-allowed Ni(II) transition (the $^3\text{T}_2$ manifold) changes in both sign and magnitude in going from the isolated case II to the exchange-coupled case III. The VTVH behaviour of III is described as 'stunningly complex'. A synopsis of the phenomenon is that a) the amplitude and non-linear VTVH behaviour of MCD seen for d-d transitions in open shell metal systems depends critically on spin-orbit coupling at the metal centre undergoing the excitation and b) magnetic coupling of a particular metal centre to other magnetic ion(s) can serve to alter both the magnitude and direction of spin experienced at the centre, i.e. its spin projection. This change in spin projection can then manifest in changes in the MCD magnitude, as well as sign. The spin-Hamiltonian analysis also requires the exchange coupling in the excited state to be incorporated, as well as the direction and magnitude of the electronic transition moments.

In PSII, each Mn(IV) will experience a particular projection of the cluster total spin, relative to its (local) transition moment directions. This will vary with S-state. Each Mn(IV) spin-flip will have an absorption strength (and direction of its optical transition dipoles) according

to the effectiveness of the exchange coupling processes that overcome the spin-forbidden nature of the transition. Measurements of the intensity and sign of each individual spin-flip can then provide, through an appropriate analysis, details of the exchange coupling components within the cluster.

5. Conclusions

MCD measurements in the near-IR region of PSII cores of *T. vulcanus*, between 710 nm and 900 nm, are shown to provide a distinctive optical signature in each metastable S-state. Each S-state has its own MCD spectral profile as well as VTVH behaviour. This new window provides valuable information on both the electronic structure of and magnetic coupling within the Mn_4CaO_5 cluster as it transits through the catalytic Kok cycle of water oxidation.

There remains little doubt that the narrow features seen for OEC based MCD are due to $^4\text{A} \rightarrow ^2\text{E}$ spin-flips of Mn(IV) ions. The sign, magnitude and field dependence of these features depend on rather complex aspects of exchange coupling within the Mn_4CaO_5 cluster. Modelling of the relative positions of the $^4\text{A} \rightarrow ^2\text{E}$ bands by utilising computational chemistry techniques [71] needs to be developed. This will help identify the detailed local nature of each Mn(IV). However, modelling the sign, magnitude and general VTVH behaviour may require an extension of the (dimer) spin-Hamiltonian model to a cluster model. A significant number of parameters may need to be derived computationally.

The weak diamagnetism seen for the S_1 state, analysed to have a paramagnetic level $\approx 0.4 \text{ cm}^{-1}$ above the diamagnetic ground state, compares to a value of 1.7 cm^{-1} reported from EPR measurements using PSII sourced from spinach. The lack of any 'spin-flip' or other paramagnetic OEC based feature in S_0 MCD does not exclude the presence of a Mn(IV) in S_0 as it may be either significantly weaker and/or lie outside the optical window available.

We see no evidence for any broad, temperature-dependent transitions in any of our (non-IR illuminated) OEC based MCD spectra, at a sensitivity level of $\approx \Delta\epsilon$ of $0.06 \text{ M}^{-1} \text{ cm}^{-1}$ under saturation conditions. If present in the 710 nm – 850 nm region, one would expect to be able to see spin-allowed MCD of d-d transitions of Mn(III). This implies that low symmetry d-orbital splittings of Mn(III), which are responsible for the bands seen in the 700 nm – 800 nm region of the MnCat systems and model compounds, are at higher energies in *T. vulcanus* PSII.

Our assignment of two and three spin-flips, and thus at least two or three Mn(IV) sites in S_1 and S_2 states respectively, is consistent with the dominant 'high-paradigm' model [72,73,44,7,74] of the OEC. EPR changes induced by near-IR illumination of S_2 or S_3 states may now considered to be influenced by excitation spin-forbidden excitations of Mn(IV)s.

Having established the feasibility and utility of S-state dependent MCD spectroscopy, now heralded is the opportunity to make parallel S-state dependent MCD measurements on any of the wide range of modified PSII core preparations, especially those targeting the critical and malleable S_3 state. MCD measurements of PSII derived from other organisms will also be invaluable. An immediate example would be to study S_1 in *Synechocystis* sp. PCC 6803, where highly structured EPR has been reported [75]. Another would be to measure S_0 MCD in a sample in which methanol has been added. Methanol has been shown to significantly affect the S_0 EPR of spinach [51].

Transparency document

The <http://dx.doi.org/10.1016/j.bbabi.2017.10.004> associated with this article can be found, in online version.

Acknowledgements

The technical support of Keith Jackman is again gratefully

acknowledged. E.K. gratefully acknowledges correspondence with Bill Rutherford, Daniel Gamellin, Thomas Brunold, Hans Güdel, Andreas Hauser, Christian Reber, Ron Pace, Rob Stranger and Mark Riley. We recognise the support of the Australian Research Council through grants DP110104565 and DP150103137 (E.K.), FT140100834 (N.C.) and MEXT/JSPS of Japan through a Grant-in-Aid for Specially Promoted Research No. 24000018 (J.R.S.).

Appendix A. Supplementary information

Supplementary information to this article can be found online at <https://doi.org/10.1016/j.bbabi.2017.10.004>.

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Chapter 5

Photochemistry beyond the red limit in chlorophyll f–containing photosystems)

5.1.1 Introduction to Publication 2

Chlorophyll a is the most ubiquitous chlorophyll found in photosynthesis. It uses red light (~680 – 700 nm) to convert sunlight into chemical energy. Light with wavelengths longer than this is considered to not have enough energy to drive photosynthesis. As such ~ 700 nm is considered to be the “red limit” of photosynthesis.

Chroococcidiopsis thermalis is a cyanobacteria that produces the far-red absorbing chlorophylls f and d when grown under red light (see section 1.4.1). PSI and PSII in this organism both contain these chlorophylls. A range of biophysical techniques, including low temperature optical spectroscopy, were used to determine the location and function of these red chlorophylls within the photosynthetic complexes and thus determine if photosynthesis was being performed beyond the red limit.

5.1.2 Key findings from Publication 2

The key finding of this publication is that photosynthesis is being performed beyond the red limit. Long-wavelength chlorophylls are found in the reaction centres of both PSI and PSII in *C. thermalis*. Specifically, the primary donor, Chl_{D1}, in PSII is a far-red chlorophyll absorbing at ~727 nm and one or both primary donors, A_{1A} and A_{1B} are chlorophyll f in PSI absorbing at ~745 nm. As such, the red chlorophylls are actively involved in photochemistry using light that is generally considered to have insufficient energy for this process.

The low temperature optical spectroscopy measurements were critical in coming to this conclusion. In particular, the CD and MCD spectra enabled the characterisation of the absorption bands of the individual far-red chlorophylls, yielding information about the number of far-red chlorophylls present and their optical properties. Furthermore, electrochromic shift spectra contributed evidence of the presence of at least one far-red chlorophyll in the reaction centres of both PSII and PSI and assisted in identifying its location.

5.2 Declaration of Contribution

**Statement of Contribution**

This thesis is submitted as a Thesis by Compilation in accordance with https://policies.anu.edu.au/ppi/document/ANUP_003405

I declare that the research presented in this Thesis represents original work that I carried out during my candidature at the Australian National University, except for contributions to multi-author papers incorporated in the Thesis where my contributions are specified in this Statement of Contribution.

Title and authors: Photochemistry beyond the red limit in chlorophyll f-containing photosystems
Dennis J. Nürnberg, Jennifer Morton, Stefano Santabarbara, Alison Telfer, Pierre Joliot, Laura A. Antonaru, Alexander V. Ruban, Tanai Cardona, Elmars Krausz, Alain Boussac, Andrea Fantuzzi, A. William Rutherford

Current status of paper: Published

Contribution to paper: Experiment planning. Low temperature absorption and MCD measurements. Difference spectra used in main paper.

Senior author or collaborating authors endorsement:

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Delegated Authority

5.3 Publication 2

RESEARCH

REPORT

PHOTOSYNTHESIS

Photochemistry beyond the red limit in chlorophyll f-containing photosystems

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Photosystems I and II convert solar energy into the chemical energy that powers life. Chlorophyll a photochemistry, using red light (680 to 700 nm), is near universal and is considered to define the energy “red limit” of oxygenic photosynthesis. We present biophysical studies on the photosystems from a cyanobacterium grown in far-red light (750 nm). The few long-wavelength chlorophylls present are well resolved from each other and from the majority pigment, chlorophyll a. Charge separation in photosystem I and II uses chlorophyll f at 745 nm and chlorophyll f (or d) at 727 nm, respectively. Each photosystem has a few even longer-wavelength chlorophylls f that collect light and pass excitation energy uphill to the photochemically active pigments. These photosystems function beyond the red limit using far-red pigments in only a few key positions.

Oxygenic photosynthesis uses chlorophyll a (chl a) to convert visible light into chemical energy. The photochemically active pigments of the two photosystems, photosystem I (PSI) and photosystem II (PSII), at 700 nm and 680 nm, respectively, represent the energy available for photochemistry (1–3) (see supplementary text S1). This was considered the “red limit” (4), the minimum energy required for oxygenic photosynthesis, until it was found that a cyanobacterium, *Acaryochloris*, extends this limit by using chl d, a pigment absorbing at a wavelength 40 nm longer than chl a (5, 6). *Acaryochloris* photosystems are dominated (~97%) by chl d with one or two chl a located in key positions (6, 7). More recently, chl f, the longest-wavelength chlorophyll known, was discovered (6, 8, 9). Chl f exists as a minority pigment in photosystems that contain ~90% chl a. Chl f is generally assumed to play a purely light-harvesting role, needing heat in the environment, the product of the Boltzmann constant and the temperature ($k_B T$), for uphill excitation transfer to chl a for photochemistry to occur (5, 6, 10–13)

[see, however, (14)]. Thus, the presence of chl f in these photosystems was not considered an extension of the photochemical red limit. Here, we report data that change that view, showing that long-wavelength chlorophylls perform the photochemistry in both photosystems.

Chroococcidiopsis thermalis, a widely spread extremophile cyanobacterium, when grown under 750-nm far-red light (FRL), contains ~90% chl a, ~10% chl f and <1% chl d. The absorption and fluorescence spectra of the cells were shifted to longer wavelengths with a new absorption peak at 709 nm (Fig. 1A) and 80-K fluorescence at 740, 753, and 820 nm, suggesting changes in both photosystems (Fig. 1B) [see (10, 15)]. These data and decay-associated fluorescence (fig. S1) indicate that the FRL chlorophylls are the dominant terminal emitters and are present in essentially all the photosystems [see also (9, 10)]. The wavelength dependence of PSI and PSII activity (action spectra) in FRL cells were red-shifted (Fig. 1C), with new peaks at 745 nm for PSI and 715 nm for PSII. The action spectra correspond well to the absorption spectra of the isolated photosystems (Fig. 1C). P_{700}^{++} formation with far-red light was more efficient in FRL cells (Fig. 1D, right). Primary quinone (Q_A) reduction under far-red excitation in white-light (WL) cells was slow and incomplete, but in FRL cells it was very rapid, faster than in WL cells with visible excitation (Fig. 1D, lower middle). O_2 evolution under far-red light reached the same level as seen under visible light in WL cells (see supplementary text S2).

Thermoluminescence (TL) from $S_2Q_B^-$ (Fig. 2A) and $S_2Q_A^-$ (fig. S2A) (16) in FRL thylakoids is enhanced by a factor of >25 compared with WL

thylakoids. TL arises from repopulation of the excited state of the primary donor chlorophyll, $^*Chl_{D1}$, by charge recombination, which then emits at the same wavelength as the prompt fluorescence (17). The enhancement in TL intensity in FRL PSII indicates a decrease in the energy gap between the precursor charge pair and the luminescent excited-state chlorophyll (17), consistent with a lower-energy, longer-wavelength primary donor/emitter (supplementary text S3). Recombination takes place at temperatures close to those seen in the WL PSII, and the flash-number dependence is nearly identical to that seen in WL PSII, indicating no obvious change in the yields of charge separation and stabilization (fig. S2B).

PSII activity, measured as the rates of Q_A reduction at 293 K (Fig. 2B, inset) and 77 K (Fig. 2B) and as the rate of β -carotene cation radical formation at 15 K (18, 19), were comparable with red and far-red excitation (Fig. 2C and fig. S3). A purely antenna role for chl f (5, 6, 10–13) would require that heat ($k_B T$) drives the excitation from the shortest-wavelength chl f (~720 nm) uphill to P_{680} , the chl a primary donor. The $k_B T$ at room temperature (293 K) is 26 meV and too small to cover the ~100 meV energy gap between 720 and 680 nm. Although some overlap of the absorption bands could allow this process to occur inefficiently at 293 K, thermally induced excitation transfer will become vanishingly weak at 77 K ($k_B T = 6.6$ meV) and 15 K ($k_B T = 1.3$ meV) (supplementary text S4). The matching rates for PSII photochemistry with red and far-red excitation at cryogenic temperatures indicate that a FRL chlorophyll is involved in primary charge separation.

When the $Pheo_{D1}^+$ is trapped by illumination in the presence of a reductant (20, 21) the light-minus-dark difference spectrum exhibits the expected bleaches at ~546 and ~680 nm (Fig. 2D) due to the disappearance of the $Pheo_{D1} Q_y$ and Q_x absorptions. In WL PSII, the charge on $Pheo_{D1}^+$ induces a blue shift of the $Chl_{D1} Q_y$ absorption at ~680 nm. This electrochromic shift dominates the difference spectrum (20, 21) but is notably absent in the FRL PSII (Fig. 2D). Instead, a marked blue shift at ~720 nm is seen. This indicates that Chl_{D1} is a long-wavelength chlorophyll in FRL PSII. The smaller blue-shift feature at ~669 nm is attributed to P_{D1}/P_{D2} .

When Q_A^- is trapped by illumination at 77 K (Fig. 2E), the charge created on Q_A^- is known to induce a blue shift of the Q_y band of Chl_{D1} (22). In FRL PSII, the Chl_{D1} blue shift typically seen near ~680 nm (21) is replaced by one at ~727 nm. This indicates that Chl_{D1} is replaced by a FRL chlorophyll (Fig. 2F). The absence of the Chl_{D1} band shift at 680 nm allows a 685-nm red shift to become evident. This corresponds to the expected (but not previously visible) Q_A^- -induced shift of the $Pheo_{D1} Q_y$ excitation (supplementary text S2).

High-performance liquid chromatography (HPLC) indicated that the isolated PSII contained 2 pheo a, 4 chl f, 1 chl d, and 30 chl a per center, based on the 35 chl per PSII (22) (fig. S5A). The 77-K absorption spectrum (Fig. 2E) shows four peaks above 700 nm. The 710-nm

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band is attributed to red-shifted allophycocyanins (9, 23), based on its wavelength, variable amplitude in different preparations, and absence of magnetic circular dichroism (MCD) (fig. S6). The 1.8-K absorption and MCD spectra were fitted with ~5 FRL chlorophylls at 721, 727, 734, 737, and 749 nm (fig. S7).

Intrinsically, chl d absorbs at shorter wavelengths than chl f; thus, it is the most obvious choice for the 721-nm pigment. This pigment could be a linker between the primary donor at 727 nm and the shorter-wavelength antenna (supplementary text S5). Given the small wavelength difference, we need to consider the possibility that chl d is the primary donor; Chl_{D1}. The wavelength (727 nm) of the primary donor is similar to the chl d (725 nm) in this location in *Acaryochloris* (7, 24).

The conserved amino acid changes near Chl_{D1} (figs. S8 and S9) suggest specific H bonding to a formyl group on either the C2 (chl f) or C3 (chl d)

position of Chl_{D1}. This provides further evidence that Chl_{D1} in FRL PSII is a long-wavelength chlorophyll but does not discriminate between chl d and chl f (fig. S8). The three remaining long-wavelength chlorophylls appear to be tuned to span the energy gap from 749 nm up to the photochemically active chlorophyll at 727 nm, with gaps of 10 to 12 nm (23 to 26 meV), appropriate to the value of $k_B T$ at ambient temperatures (~26 meV) (see supplementary text S5 and table S1 for other pigment assignments).

It seems likely that P_{D1}, which bears the key long-lived chlorophyll cation radical, remains a chl a. This suggestion is based on (i) the presence of the blue shifts at 669 nm attributed to P_{D1} (Fig. 2, D and E), (ii) structural considerations (figs. S8 and S12), (iii) the precedence of chl a being P_{D1} in *Acaryochloris* (6, 7, 24), and (iv) conservation of specific chemical properties (e.g. oxidizing power, stability, and reactivity)

without the need for major redox and kinetic tuning. Nevertheless, sequence comparisons indicate changes in the environments of cofactors in the redox core, including P_{D1}, likely reflecting the tuning needed to adjust to the presence of the long-wavelength pigment. (see table S2 and figs. S9 and S12 to S14).

When isolated FRL PSI was excited at 15 K using 610-, 730-, and 750-nm light, comparable photochemistry was seen (Fig. 3A and fig. S15), indicating that the chlorophyll absorbing at ~750 nm is involved in charge separation. In WL PSI, 750-nm excitation resulted in the expected low-quantum-yield photochemistry (Fig. 3A) due to weak optical charge-transfer absorption at 750 nm (supplementary text S4).

Light-minus-dark difference spectra at 293 K in FRL and WL thylakoids are similar to those obtained at 77 and 1.8 K from isolated FRL and WL PSI, except for thermal broadening at higher temperatures (Fig. 3B and fig. S16). The

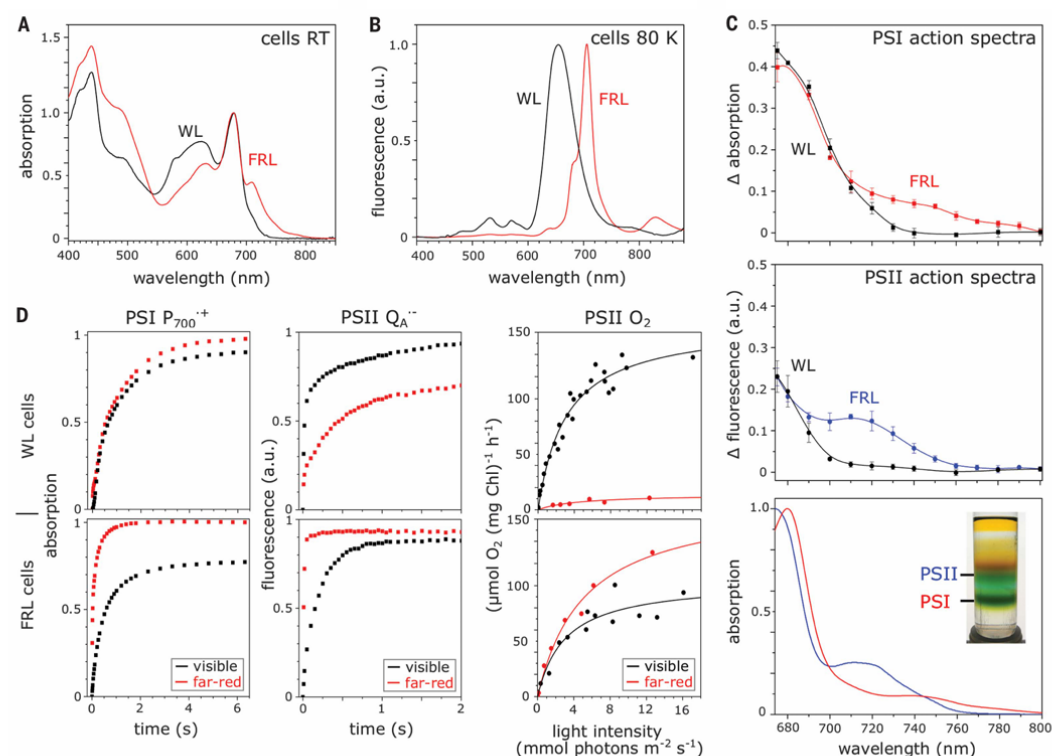


Fig. 1. Changes in the pigments and activities of the photosystems from *C. thermalis* cells grown in 750-nm light. (A) Absorption spectra and (B) 80 K fluorescence spectra of WL and FRL cells. (C) Action spectra for PSI (P_{700}^{+} at 430 nm, 50 μ s after the flash) (top) and PSII (Q_A^{-} + DCMU, measured as fluorescence) (middle) in FRL cells (red and blue, respectively) compared to WL cells (black). Bottom panel: absorp-

tion spectra from isolated PSI (red) and PSII (blue); inset sucrose gradient separating the photosystems. (D) Photosystem activities measured in WL (top) and FRL cells using visible (black) and far-red (red points) excitation. Left: PSI activity (P_{700}^{+} at 705 nm 50 μ s after the flash). Middle: PSII activity (Q_A^{-} as in C). Right: O_2 evolution as a function of light intensity. See materials and methods for details.

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main bleach is at ~ 704 nm with an absorption increase at 692 nm, indicating that the cation is located on the $P_A P_B$ chlorophyll a/a' pair, P_{700}^{++} , as seen in WL PSI. The rest of the spectrum shows marked differences: the sharp trough at 684 nm, the peak at 674 nm (680 nm at 77 K), and the trough at 656 nm (~ 660 nm at 77 K), all of which are present in WL PSI (Fig. 3B and fig. S16) but are absent in FRL PSI. Instead, several changes appear at >700 nm (Fig. 3B). These changes can be attributed to the replacement of chl a band

shifts in WL PSI with long-wavelength chlorophyll band shifts in FRL PSI. The band shifts at >700 nm are better resolved at 77 and 1.8 K and show a broad blue-shift feature (or two overlapping blue shifts) at ~ 745 nm, a sharp red shift at 756 nm, and a weak blue shift at 800 nm. In addition, there is a red shift at ~ 684 nm, which is likely present in WL PSI but now resolved in the FRL PSI.

HPLC shows that FRL PSI contains 0 pheo, 7 to 8 chl f, 0 chl d, and ~ 88 to 89 chl a (fig. S5B), assuming 96 chl per PSI (25). The 1.8 K absorp-

tion and MCD spectra at >700 nm (fig. S17) can be fitted as follows: 1 chl f at 736 nm, 3 chl f at ~ 745 nm, 1 chl f at 756 nm, 1 chl f at ~ 763 nm, and ~ 2 chl f at ~ 800 nm (fig. S18). In addition, a chlorophyll feature at 709 nm is suggested to be a long-wavelength PSI chl a.

Based on the crystal structure (25), the difference spectra (Fig. 3B and fig. S16), the low-temperature photochemistry (Fig. 3A and fig. S15), and structural arguments (figs. S8 and S19), the following model emerges for the FRL PSI redox cofactors: (i) P_A and P_B remain chl a/chl a' and bear the P_{700} cation. (ii) A_{0A} and A_{0B} , the primary acceptors (1, 26), remain chl a, because the redox properties of chl f (13) make it unsuitable for a low potential role. This fits with the assignment of the ~ 684 -nm red shift to A_0 (Fig. 3B) [see (26)] and with A_0 being chl a in *Acaryochloris* (24). (iii) One or both of the primary donors, A_{1A} and A_{1B} (27), are chl f in FRL PSI, absorbing at ~ 745 nm. The difference spectra seem to arise from overlapping blue shifts at this wavelength, replacing the dominant ~ 684 -nm blue shift in the WL PSI. A_{1A} and A_{1B} are the only redox-active chlorophylls oriented to allow a blue shift. Possible locations of the six chl f antenna are discussed in the supplementary materials (supplementary text S6).

The far-red photosystems studied here represent a third paradigm for oxygenic photosynthesis (supplementary text S1). The first is the canonical type that use chl a for both charge separation and antenna absorption. The second is that of *Acaryochloris*, where chl d replaces all chl a for both photochemistry and light harvesting, with the exception of chl a being retained in two key redox roles: P_{D1} , where P_{D1}^{++} is the principle oxidant of PSII, and A_0 , where A_0^{+} is the principle reductant in PSI. The new paradigm maintains chl a in nearly all positions except for a small number of chl f molecules (plus one chl d in PSII). These long-wavelength chlorophylls act as (i) the primary electron donors in both photosystems, (ii) linkers to shorter-wavelength antenna pigments, and (iii) a small, longer-wavelength antenna system. The far-red photosystems provide well-resolved absorption spectra at long wavelengths due to the decreased spectral overlap, which allows more definitive assignments and analyses.

Chl f can absorb at >760 nm, yet the wavelength used to drive photochemistry in the FRL PSII is ~ 727 nm (fig. S20). This wavelength is similar to that of the chl d primary donor (Chl_{D1}) in *Acaryochloris* PSII (6, 7, 24). Thus, both of the known cases of PSII functioning "beyond the red limit" use primary donors with similar energies. This may represent a "second red limit" for PSII, one that applies in the stable, deep-shade environments where far-red photosynthesis occurs in nature. The ~ 110 meV of energy sacrificed by the shift from ~ 680 to 727 nm may correspond to the energy "headroom" needed by PSII to mitigate the photodamage caused by variable light intensities (3, 28, 29). This potential disadvantage may explain why oxygenic photosynthesis using far-red light is restricted to rare, deep-shade

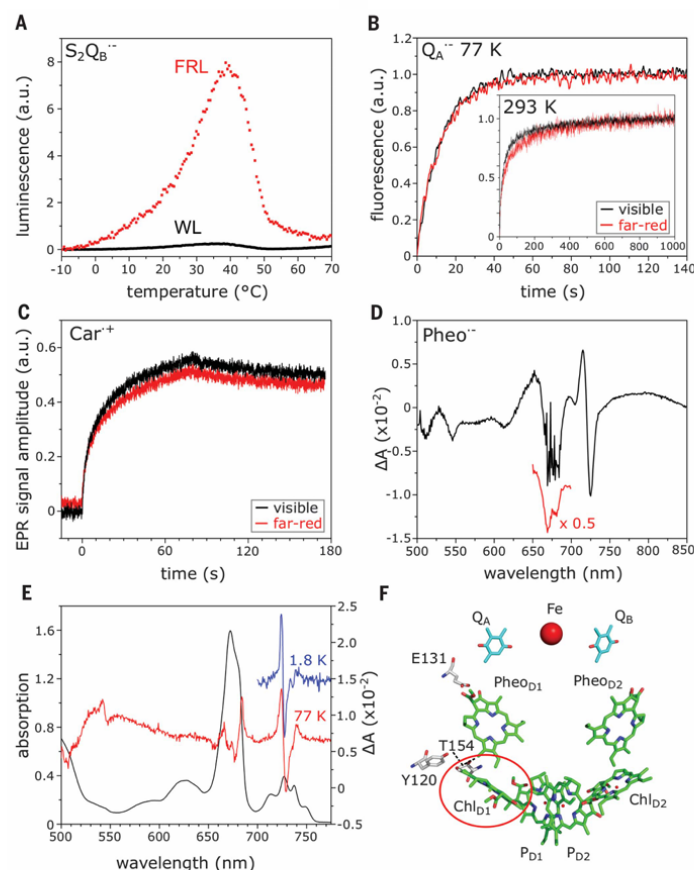


Fig. 2. FRL PSII photochemistry. (A) $S_2Q_B^{--}$ thermoluminescence in WL and FRL thylakoids from *C. thermalis*. (B) Kinetics of Q_A reduction in FRL thylakoids (measured as fluorescence) at 77 K (inset at 293 K). Samples were excited using visible (600-nm) and far-red (710-nm) light. (C) Kinetics of β -carotene cation radical formation in isolated FRL PSII at 15 K illuminated with visible (610-nm) and far-red (730-nm) light. (D) Light-minus-dark visible/near-IR spectrum at 293 K upon $Pheo_{D1}^{+}$ formation in isolated FRL PSII. The 650- to 700-nm region is saturated in the main spectrum (black); the red inset is a 2x-diluted sample. (E) 77-K absorption spectrum of isolated FRL PSII (black) with the 77-K (red) and 1.8-K (blue) 725-nm illuminated-minus-dark difference spectra. (F) Cofactors in PSII showing Chl_{D1} as a FRL chlorophyll (circled) and potential H-bonding amino acids, based on the crystal structure PDB 3WU2 (22).

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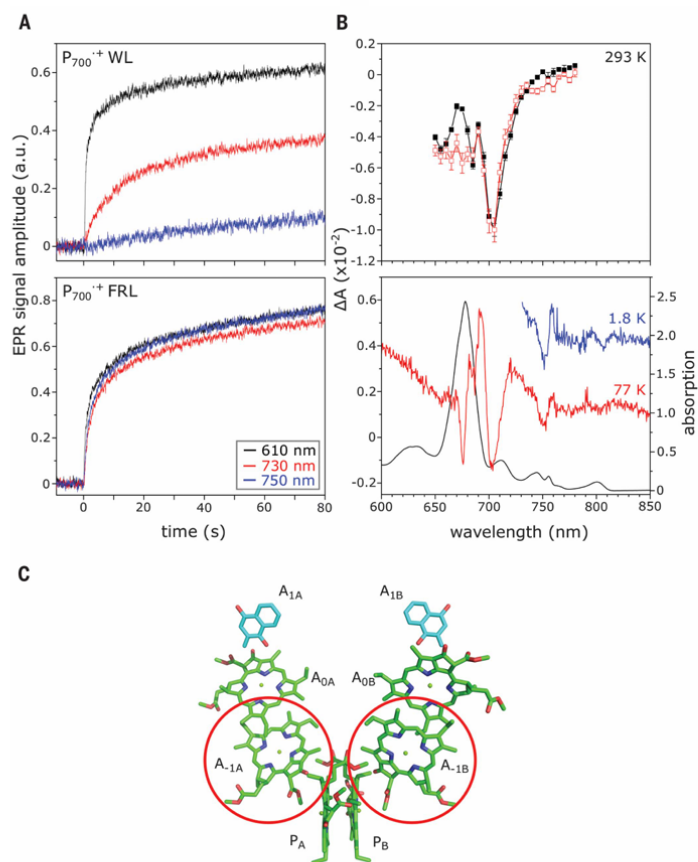


Fig. 3. FRL PSI photochemistry. (A) 15-K photo-accumulation of P_{700}^{+} monitored by EPR using visible and far-red light in (top) isolated FRL PSI and (bottom) isolated WL PSI. (B) (Top) Flash-induced P_{700}^{+} 293-K absorption difference spectrum using FRL and WL thylakoids. (Bottom) P_{700}^{+} 77-K (red) and 1.8-K (blue) difference spectra and 1.8-K absorption spectrum (black) of isolated FRL PSI. (C) FRL PSI model (PDB 1JB0) (25). Red circles show the proposed locations of chl f, with other positions as chl a (see the text).

environments, rich in far-red light (supplementary text S7).

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ACKNOWLEDGMENTS

We gratefully acknowledge our friend and colleague Fabrice Rappaport, who died in January 2016, with whom we worked in the early stages of this project. We thank J. Murray for discussions, B. Nwaobi for technical support, C. Mullineux for access to a 77-K fluorometer, B. Bailleul and W. Remell for technical help in obtaining the absorption spectra, J.-M. Ducruet for lending us the thermoluminescence setup, and L. Haigh for her assistance with the HPLC analysis. **Funding:** This work was supported by BBSRC grants BB/L011506/1 and BB/R001383/1, Leverhulme Trust grant RPG-2017-223 and a Wolfson Merit Award from the Royal Society to A.W.R., Australian Research Council grant DP150103137 to E.K., an Imperial College Junior Research Fellowship to T.C. and an ANR-10-INBS-05 (FRISB) grant to A.B. **Author contributions.** D.J.N. initiated the study, built growth chambers, surveyed, selected and grew the strains. D.J.N., A.F., and A.W.R. conceived of the main experiments, collated results and interpretations, and wrote the article with input, edits, and approval from all authors. D.J.N. with A.T. and A.B. developed and did the isolation of the photosystems. J.M. with E.K. did the low-temperature optical spectroscopy (visible/near IR, CD, MCD, fluorescence). S.S. and D.J.N. did 293 K and 77 K visible/near IR spectroscopy on PSI. D.J.N. and A.T. did the HPLC. A.B. and A.W.R. did the EPR. L.A.A. with D.J.N. did the O_2 evolution. A.F. and D.J.N. did the TL. A.F. did the Pheo⁺ trapping and wavelength dependence of Q_A^+ at 293 and 80 K. P.J. and D.J.N. did the wavelength dependence of PSI and PSI activities in cells. D.J.N. and A.W.R. worked with F. Rappaport to obtain the action spectra with advice from P.J. A.V.R. and D.J.N. did the time-resolved fluorescence studies, and S.S. helped with the interpretation. A.F. and T.C. did the modeling studies. E.K. and D.J.N. did the curve fitting, with input from J.M., S.S., A.F., and A.W.R. **Competing interests:** The authors declare no competing interests. **Data and materials availability:** All data are available in the manuscript or the supplementary material.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/360/6394/1210/suppl/DC1
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Supplementary Text S1 to S7
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Data S1

22 December 2017; accepted 18 April 2018
10.1126/science.aar8313

Chapter 6

**The primary donor of far-red
photosystem II: Chl_{D1} or P_{D2}?**

6.1.1 Introduction to Publication 3

Publication 3 is a further study on the location of far-red-light absorbing chlorophylls in the PSII reaction centre of *Chroococcidiopsis thermalis*. Publication 3 follows on from publication 2. One of the key conclusions of publication 2 is that the primary donor in the reaction centre of PSII in *C. thermalis* is a far-red chlorophyll absorbing at 727 nm. Publication 3 uses a combination of CD, MCD and electrochromic shift spectra as well as spectroscopic modelling to further refine the location and function of the far-red chlorophyll (either d or f) in the centre of PSII.

Particularly critical for the conclusions of this paper were the electrochromic shift spectra and the CD. Modelling of electrochromic shift spectra can yield information about the location of pigments relative to photo-generated charges within a pigment-protein complex and thus be used to link absorption features to specific pigments within a complex. Modelling for this publication was performed by Martyna Judd using a model described in Cox et al. [46]. Figure 2.6 shows a simplified model of an electrochromic shift spectrum containing two chromophores. CD spectroscopy can yield structural information such as the strength of interaction between pigments and how they interact with their nearest neighbours.

6.1.2 Key findings from Publication 3

This publication confirms the conclusion that the chlorophyll absorbing at 726 nm is the primary electron donor. The absorption peak at this wavelength has a $21 \pm 4 \text{ cm}^{-1}$ electrochromic shift arising from the photoproduction of the semiquinone ion $\text{Q}_\text{A}^{\bullet-}$. Detailed modelling of this electrochromic shift shows that both Chl_{D1} and P_{D2} are located at a distance and orientation relative to $\text{Q}_\text{A}^{\bullet-}$ such that either could account for the observed electrochromic shift.

The CD signal at 726 nm is of a magnitude that suggests that the pigment in question is relatively strongly coupled to a neighbouring pigment. P_{D2} is a good candidate for this. In contrast, according to our modelling, Chl_{D1} has negligible CD. Such an

observation favours P_{D2} as the primary donor. This is the first experimental evidence that P_{D2} could be the primary donor in *C. thermalis*. This surprising result needs to be further explored with more experimental and calculational work. This publication demonstrates the efficacy of using multiple optical spectroscopic techniques and modelling on the same sample to obtain detailed structural and functional information.

6.2 Declaration of Contribution

**Statement of Contribution**

This thesis is submitted as a Thesis by Compilation in accordance with https://policies.anu.edu.au/ppl/document/ANUP_003405

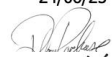
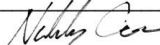
I declare that the research presented in this Thesis represents original work that I carried out during my candidature at the Australian National University, except for contributions to multi-author papers incorporated in the Thesis where my contributions are specified in this Statement of Contribution.

Title and authors: The primary donor of far-red photosystem II: ChlD1 or PD2?
Martyna Judd, Jennifer Morton, Dennis Nürnberg, Andrea Fantuzzi, A. William Rutherford, Robin Purchase, Nicholas Cox, Elmars Krausz

Current status of paper: Published

Contribution to paper: ~95% of the experimental data.

Senior author or collaborating authors endorsement:

Martyna Judd		10/6/25
Andrea Fantuzzi	 24/06/25	
Robin Purchase		10/6/25
Nicholas Cox		10/6/25
Elmars Krausz		10/6/25

Jennifer Morton



1/5/25

Candidate

Signature

Date

EndorsedNicholas Cox
Chair of Supervisory Panel

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6.3 Publication 3

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journal homepage: www.elsevier.com/locate/bbambioThe primary donor of far-red photosystem II: Chl_{D1} or P_{D2}?Martyna Judd^a, Jennifer Morton^a, Dennis Nürnberg^b, Andrea Fantuzzi^b, A. William Rutherford^b, Robin Purchase^a, Nicholas Cox^a, Elmars Krausz^{a,*}^a Research School of Chemistry, Australian National University, Canberra 2600, Australia^b Department of Life Sciences, Imperial College, London SW7 2AZ, United Kingdom

ARTICLE INFO

Keywords:

Photochemistry
Photochemical charge separation
Exciton coupling
Circular dichroism
Magnetic circular dichroism
Fluorescence
Electrochromic shift

ABSTRACT

Far-red light (FRL) Photosystem II (PSII) isolated from *Chroococcidiopsis thermalis* is studied using parallel analyses of low-temperature absorption, circular dichroism (CD) and magnetic circular dichroism (MCD) spectroscopies in conjunction with fluorescence measurements. This extends earlier studies (Nürnberg et al 2018 Science 360 (2018) 1210–1213). We confirm that the chlorophyll absorbing at 726 nm is the primary electron donor. At 1.8 K efficient photochemistry occurs when exciting at 726 nm and shorter wavelengths; but not at wavelengths longer than 726 nm. The 726 nm absorption peak exhibits a $21 \pm 4 \text{ cm}^{-1}$ electrochromic shift due to formation of the semiquinone anion, $\text{Q}_\text{A}^{\bullet-}$. Modelling indicates that no other FRL pigment is located among the 6 central reaction center chlorins: P_{D1}, P_{D2} Chl_{D1}, Chl_{D2}, Pheo_{D1} and Pheo_{D2}. Two of these chlorins, Chl_{D1} and P_{D2}, are located at a distance and orientation relative to $\text{Q}_\text{A}^{\bullet-}$ so as to account for the observed electrochromic shift. Previously, Chl_{D1} was taken as the most likely candidate for the primary donor based on spectroscopy, sequence analysis and mechanistic arguments. Here, a more detailed comparison of the spectroscopic data with exciton modelling of the electrochromic pattern indicates that P_{D2} is at least as likely as Chl_{D1} to be responsible for the 726 nm absorption. The correspondence in sign and magnitude of the CD observed at 726 nm with that predicted from modelling favors P_{D2} as the primary donor. The pros and cons of P_{D2} vs Chl_{D1} as the location of the FRL-primary donor are discussed.

1. Introduction

Oxygenic photosynthesis in the cyanobacteria *Chroococcidiopsis thermalis*, when grown in far-red light (FRL), is based on photochemistry occurring on *chl-f* or *chl-d* [1]. This constitutes an unexpected new paradigm in photosynthesis. Photosystem II core complexes (FRL-PS II) of *C. thermalis* contain 4 *chl-f* molecules and 1 *chl-d*, whilst the majority (~30) of the chlorin pigments remain as *chl-a*.

The original spectroscopic studies of the isolated FRL-PS II provided a model in which one of the long wavelength pigments (FRL-chl) played the role of the primary donor, whilst the 4 remaining FRL-chls served as long wavelength antennas [1]. The primary donor was identified [1] as Chl_{D1} based on the following evidence and arguments: a) trapping of Pheo_{D1}^{•-} at room temperature resulted in electrochromic changes at 720 nm and the apparent loss of the usual dominant blue-shift at ~685 nm attributed to *chl-a* Chl_{D1}; b) $\text{Q}_\text{A}^{\bullet-}$ formation at low temperature resulted in electrochromic changes at 725 nm and again the

apparent loss of the usual dominant blue-shift at ~685 nm attributed to the *chl-a* Chl_{D1}; c) The presence of amino acid sequence changes in the FRL-D1 protein conserved in all known FRL-D1 sequences, which were modelled as potential H-bond donors to the ring I formyl group of either *chl-d* or *chl-f* in the Chl_{D1} location [1]; d) in *Acaryochloris marina*, a similar bandshift was seen (as well as similar H-bonding amino acids) already attributed to *chl-d* at the Chl_{D1} location [2]; e) functional arguments based on Chl_{D1} taking the primary donor role in canonical *chl-a*-PSII (see e.g. [3–7]); f) The chemical properties of *chl-f* [8] and the redox properties [9] of both *chl-d* and *chl-f* favor its location on Chl_{D1} (see below and [1]).

The reaction center of *chl-a*-PS II contains 2 *pheo-a* and 6 *chl-a* molecules. P_{D1} and P_{D2} have partial ring overlap, forming a chlorophyll pair that is the counterpart of the “special pair” in purple bacterial reaction centers. The P_{D1}/P_{D2} exciton coupling term in PS II [10] at 158 cm^{-1} is far smaller than the ‘special pair’ coupling of $\sim 900 \text{ cm}^{-1}$ determined for *Rhodospseudomonas viridis* [11]. Chl_{D1} and Chl_{D2} are

Abbreviations: chl-a-PS II, canonical chlorophyll-a based photosystem II; FRL-PS II, far-red light grown PS II; CD, circular dichroism; MCD, magnetic circular dichroism; chl-a, chlorophyll-a; chl-d, chlorophyll-d; chl-f, chlorophyll-f; FRL-chl, far-red light chlorophyll (chlorophyll-d and/or chlorophyll-f); SI, supplementary information

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Received 31 March 2020; Received in revised form 13 June 2020; Accepted 15 June 2020

Available online 19 June 2020

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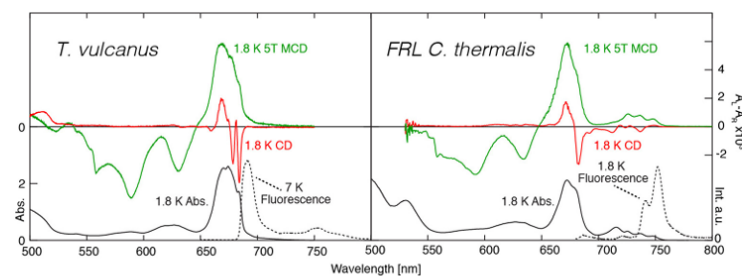


Fig. 1. Low temperature absorption, CD, MCD and fluorescence spectra (as indicated) of chl-a-PS II from *T. vulcanus* (left, adapted from reference [20]) and FRL-PS II of *C. thermalis* (right). Corresponding spectra are set to the same integrated Q_y absorption strengths.

counterparts of the monomeric bacteriochlorophylls, B_L and B_M , of the bacterial reaction center; they are sometimes anomalously referred to as “accessory pigments” despite the demonstrated role Chl_{D1} (and B_L) has in primary charge separation [3]. The two *pheo-a* pigments, $Pheo_{D1}$ and $Pheo_{D2}$, are also core pigments, with $Pheo_{D1}$ having a key electron acceptor role. $ChlZ_{D1}$ and $ChlZ_{D2}$ are not part of the central group of 6 chlorins as they are peripheral and mainly play light-harvesting roles, although $ChlZ_{D2}$ is part of a side-path electron donation system that works at low quantum yield under some circumstances [12,13].

P_{D2} was not considered as a candidate for the primary donor role in FRL-PS II although it is oriented and located in a way that can give rise to an electrochromic shift very similar to that calculated for Chl_{D1} . Here we examine the possibility that P_{D2} is the primary electron donor in FRL-PS II.

2. Materials and methods

FRL-PS II samples were prepared as described in [1].

All spectra were taken on a single, purpose-built multifunctional apparatus [14] built around a 0.75 M Czerny-Turner spectrometer (Spex) and a superconducting magnet cryostat (Oxford Instruments SM-4). The system is able to simultaneously record absorption, CD and MCD spectra at temperatures between 1.8 K and 300 K. This system also can be rapidly reconfigured to measure emission and excitation spectra, without disturbing the cryogenic sample.

The apparatus has a sample handling system [15,16] that minimizes exposure of the sample to oxygen and ambient light, whilst allowing rapid cooling of a room temperature sample to cryogenic temperatures under controlled conditions. Samples were visualized with a near-IR light (> 780 nm) sensitive camera at either room temperature or at cryogenic temperatures. The presence of bubbles, cracks or defects in the prepared sample could be immediately determined without inducing any photoconversion of a dark-adapted sample.

The PSII samples contained cryoprotectant glycerol/ethylene glycol (1:1) at a final concentration of 45% (v/v). Prior to dark adaption, samples were loaded under low light into specially designed [16] strain-free quartz optical cells of 500 μ m, 200 μ m or 50 μ m pathlength. The PSII samples were then dark-adapted for 5 min before being rapidly cooled.

The light source for the absorption measurements was a highly stabilized 250 W quartz-halogen lamp. The fluence of the measurement light at the sample was reduced to a point that absorption measurements did not induce a significant degree of Q_A reduction in the PS II sample. This was established by determining that there was no significant difference between two successive absorption measurements. The necessary reduction in measurement light seen by each PS II assembly involved defocusing the measurement light over a large area (~ 8 mm diameter) sample and narrowing the slit widths of the monochromator, whilst using a large area high quantum efficiency

Hamamatsu R 669 photomultiplier to detect the transmitted light [17]. Electrochromism was induced with a highly stabilized 250 W quartz-halogen lamp passed through either a green bandpass filter combined with a 10 cm pathlength water filter to provide 1–2 mW of broad spectrum green light, or through an optical system [14] based on a Spex Minimate 0.25 M double monochromator, providing 6–8 μ W of light in the 600 nm – 760 nm range and having a bandwidth of 2.4 nm FWHM (see Fig. S2), or a Schwartz EO Ti:S laser pumped with a 532 nm Continuum Verdi G.

CD and MCD measurements required much higher measurement light levels. It is not possible to measure useful CD or MCD spectra and maintain the sample in the Q_A state [18]. Thus, samples were illuminated with green light (300 s at 1–2 mW/cm²) forming the $Q_A^{\cdot-}$ state prior to the measurement [19] to minimize actinic effects during the course of CD and MCD measurements.

Emission was excited with the highly stabilized lamp and Spex Minimate system described above and then detected through the same 0.75 M Czerny-Turner spectrometer as the absorption with a Hamamatsu R 943-02 photomultiplier tube.

Data were analyzed using IGOR pro software and calculations were made using MATLAB.

3. Results and discussion

3.1. Canonical chl-a-PS II and FRL-PS II comparisons

Fig. 1 provides a comparison of low temperature spectra of FRL-PS II core complexes of *C. thermalis* with those of *T. vulcanus*. The latter is a well-determined *chl-a* containing PSII, i.e. *chl-a*-PS II. FRL-PS II exhibits a number of well-resolved absorption features in the 700–760 nm range that are not present in *chl-a*-PS II, as previously reported [1]. Fluorescence spectra of the two systems are also significantly different (Fig. 1 and SI, Fig. S3 of [1]), with *chl-a*-PS II fluorescence peaking near 690 nm and the FR-PS II peaking near 752 nm.

Absorption features in the region beyond ~ 720 nm involve Q_y excitations of 4 *chl-f* molecules and one *chl-d*. Absorption integrals (see SI-1, Fig. S1) are reasonably consistent with there being ~ 30 *chl-a* molecules, 2 *pheo-a* pigments and 5 far-red chls in FRL-PSII, assuming the oscillator strength of each pigment is similar. This result is in agreement with the direct determination of the pigment content of FRL-PS II from the HPLC analyses as previously reported [1].

3.2. Identification of the primary donor

Fig. 2 shows that 735 nm illumination at 1.8 K induces a minimal electrochromic shift amplitude near 726 nm. At 77 K, illumination at 750 nm leads to a minimal shift amplitude whereas illumination at 735 nm leads to a small but significant shift amplitude. The small shift at higher temperatures is attributable to the optically excited lower

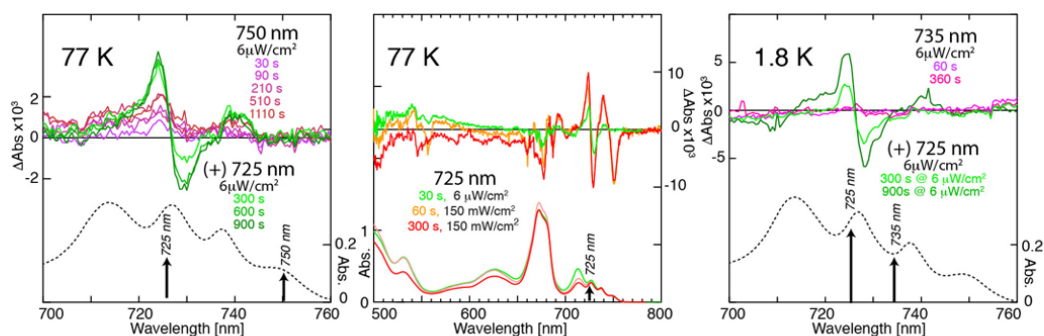


Fig. 2. The left panel shows shift and absorption spectra measured at 77 K, for low power illuminations at 750 nm followed by additional low power illuminations at 725 nm. The central panel compares the 77 K spectrum for a sample illuminated for 30 s with low power 6 $\mu\text{W}/\text{cm}^2$ 725 nm illumination (green), followed by illumination by a 1500 times more powerful, 150 mW/cm^2 Ti:S laser, also at 725 nm for 60 s and 300 s (orange and red traces respectively). The right panel shows difference features induced by illuminating at 1.8 K with low power 735 nm radiation, followed by low power 725 nm illumination.

energy pigment (peaking at 738 nm) leading to charge separation by way of thermally activated access to the primary donor at 726 nm. The energy gap between peaks at 726 nm and 738 nm is 12 nm (220 cm^{-1}). This corresponds to $\sim 4\text{ kT}$ at 77 K pointing to a possible thermally activated primary donor occupation of $\sim 2\%$. We are thus able to confirm that the 726 nm peak is that of the primary donor [1].

At high excitation fluences, achieved by using a Ti:S laser at 725 nm, additional features are generated in difference spectra (central panel in Fig. 2). Most apparent is a bleach at 749 nm, along with an absorption increase in the 738 nm region. The bleach at 749 nm is associated with partial oxidation of the lowest energy FRL-chl. Such photo-oxidation processes are known [17,21]. The peak position of the increased intensity component is at 739.5 nm and does not match the peak position of the band seen in absorption. A blue-shift of this type is characteristic of non-resonant photophysical hole-burning [22–24] although such processes are more pronounced at lower temperatures.

3.3. Variation in allophycocyanin content of FRL-PS II and global analysis

Absorption at wavelengths longer than 700 nm is attributed primarily to FRL-chl. However, there is significant absorption at the shorter wavelength end of the 700 nm to 740 nm region that can be attributed to allophycocyanins [1]. More highly purified FRL-PS II preparations show that the absorption feature near 715 nm becomes depleted, whereas the three other distinct features are retained. Fig. 3 shows FRL-PS II spectra of samples with different allophycocyanin content.

The 700 nm to 715 nm region of FRL-PS II shows little MCD, but

significant CD and absorption. The absorption and CD intensity in this region vary strongly with allophycocyanin content (Fig. 3), showing it to be dominated by allophycocyanins. The fluorescence spectra of high-allophycocyanin FRL-PS II (Fig. 1 and SI-2, Fig. S2) exhibit a weak peak near 730 nm, associated with emission from allophycocyanins, indicating that they are, at least partially, disconnected from PSII.

There are three well-resolved peaks in FRL-PSII spectra at wavelengths longer than 720 nm that are not due to allophycocyanins. These are due to the FRL-chls. An analysis of high and low allophycocyanin FRL-PSII samples (Fig. 3, Table S1 and SI-3) establish small changes in the position and width of these peaks, indicating only a minor disturbance of the FRL-PS II proteins associated with the purification process.

The spectral position and width of all 5 FRL-chl Q_y excitations were determined by a parallel, information-driven, global analysis of simultaneously measured absorption, CD and MCD spectra, such as those seen in Fig. 3. The MCD spectra are key to this analysis as they have no significant contribution from overlapping signals from allophycocyanins. Another important characteristic of the FRL-chl Q_y excitations is the MCD amplitude relative to absorption ratio B/D which in turn is proportional to $\Delta A/A$ at a specific applied magnetic field. This ratio is known to vary and also to be indicative of the role that the pigment takes [18,25,26]. The primary donor, in particular, exhibits a narrow Q_y exciton for which its MCD B/D value is significantly reduced (by $\sim 50\%$) compared to pigments involved in light harvesting, which are less strongly coupled to other chlorophylls [18,27]. All purely electronic excitations (which include exciton coupled Q_y bands) that exhibit B term-type MCD have line-shapes that follow the absorption profile of

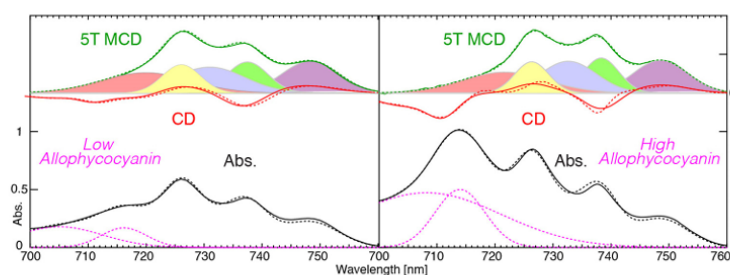


Fig. 3. Absorption (solid black traces), MCD (solid green traces) and CD (solid red traces) of high and low allophycocyanin containing FRL-PS II preparations, scaled to the same area in MCD. A systematic analysis of the MCD (see text and SI-3) leads to the quantification of the 5 individual FRL-chl Q_y MCD components shown in Table S1. The MCD contribution of these 5 Q_y excitons are shown under the MCD spectrum in the figure as coloured overlapping Gaussians. The primary donor (at 726 nm) is yellow. The subsequent fit of absorption spectra (dashed black traces) and CD spectra (dashed red traces), using the positions and widths of the 5 components as determined from the MCD analysis (see text and Table S1) closely follows the experimental data. Allophycocyanin absorption components are estimated (see SI-1) as the dashed magenta curves.

each exciton component [28–30]. Each FRL-chl is taken to have the same integrated Q_y absorption intensity. Exciton coupling-induced intensity re-distribution (Section 3.3) is thus considered to be minor. The differences in molar extinction values between *chl-a*, *chl-d* and *chl-f* have been shown [31] to be relatively small in a range of solvents. Computations at the CAM-B3LYP and SAC-CI levels (Table 2 of [31]) provide similar (to within 10%) oscillator strengths for the three chlorins. As an approximation, we take the oscillator strength of *chl-a*, *chl-d* and *chl-f* Q_y excitations in the protein environment to be the same. Whilst individual pigment absorption strengths are similar, the CD amplitude of each Q_y exciton component band can vary in both magnitude and sign, and is determined by exciton coupling to neighboring pigments [32]. For the purpose of this analysis, the CD bandshapes were assumed to be the same as that of the absorption and MCD. However, CD line shapes may follow those of their absorption features less closely when vibrational sideline features become involved [26,33]. The overall fit to the CD shown in Fig. 3 is less precise than that of the absorption and MCD. This may arise partly as a result of the influence of vibrational sideline CD. Attempts to fit the CD spectrum in the 726 nm region with the CD component of the primary donor constrained to a small relative amplitude were unsuccessful. The positive CD seen in this region is associated with the primary donor (see SI-3 and Fig. S4).

A description of the global analysis is provided in SI-3. Table S1 provides the relevant output. Very similar positions and widths for each FRL-chl Q_y MCD band were obtained from both the low and high allophycocyanin samples shown in Fig. 3. The differences in linewidths between the FRL-chl Q_y features is attributed to a combination of disparate levels of inhomogeneity at each pigment site as well as changes in electron-phonon coupling. There is a variation of relatively strong CD features associated with allophycocyanins present in the high and low allophycocyanin FRL-PS II samples which contributes to uncertainties in determining the CD of the broader FRL-chl Q_y excitons. However, both the sign and magnitude of CD for each of the three narrow FRL-chl Q_y peaks remains well determined. This is considered in detail in SI-3. A graphical presentation of the individual CD components, as seen for the MCD components in Fig. 3 is given in Fig. S4.

3.4. Analysis of electrochromic shifts

Illumination of PS II core complexes at cryogenic temperatures induces metastable spectral shifts. Prominent shifts are attributed to the formation of $Q_A^{\bullet-}$. This radical anion is adjacent to Pheo_{D1} and close to Chl_{D1}. It induces relatively large electrochromic shifts of the Q_x band of Pheo_{D1} (historically named the C550 shift) [34] and of the Q_y band of Chl_{D1} [23,35,36]. Fig. 4 shows both absorption and illumination difference spectra for a FRL-PS II sample.

These spectra are interpretable in terms of previous analyses of PS II core complex electrochromism [23,36]. A comparison of representative electrochromism spectra in PS II core complexes of 4 organisms is shown in Fig. S5. In FRL-PS II the C550 shift of the Pheo_{D1} Q_x feature at

544.5 nm is close to the position in other cyanobacterial PS II systems [23]. In some FRL-PSII preparations (SI, Fig. S5) a bleach upon photoreduction of Q_A identifies (the initially reduced) cytb₅₅₉ absorption in FRL-PSII to be at 556.5 nm. The dominant blue-shift of Q_y , seen at 1.8 K near 683 nm for *chl-a*-PS II [17,37] now appears near 726 nm under similar conditions, as reported earlier [1]. This is consistent with the 726 nm band being associated with the primary donor. In *chl-a*-PS II, the Q_y electrochromic shift pattern is dominated by the Pheo_{D1} and Chl_{D1} excitons. The site-energies of the Chl_{D1} and Pheo_{D1} pigments are similar and there is a significant exciton matrix element between them (Table S3). When pigment excitations are close in energy, this matrix element leads them to interact strongly, with intensity being transferred between the resulting excitations [23]. Calculation of the electrochromism requires independent exciton computations of both Q_A and $Q_A^{\bullet-}$ configurations of PS II. Electrochromic effects displace site energies significantly, causing the mixing of exciton states to change. The situation with FRL-PS II is different, as the primary donor is displaced to lower energy by $\sim 800\text{ cm}^{-1}$ or more from the *chl-a* excitations. Thus, the effects of the coupling matrix elements between the primary donor and spatially adjacent pigments such as Pheo_{D1} and P_{D1} are reduced by the energy differences between them, although the matrix elements themselves do not change.

The spectral isolation of the primary donor Q_y absorption in FRL-PS II assists in the estimation of both its electrochromic shift and the yield of $Q_A^{\bullet-}$ formation. The observed shift appears as a significant fraction of the linewidth and the absorption difference spectrum does not cross precisely at the maximum of absorption (Fig. 4) as would be the case for electrochromic shifts much smaller than the linewidth. The analysis is presented in SI-4 and leads to a yield of $5.5 \pm 0.5\%$ and an initial estimate of the shift at $42 \pm 4\text{ cm}^{-1}$.

However, a quantitative comparison of electrochromic shift data accumulated at 77 K and 1.8 K data taken at higher fluences with low fluence 1.8 K data (Fig. 2), point to a shift magnitude of 42 cm^{-1} to be an overestimate. Instead, it seems that the pigments that photoconvert with high efficiency are only a subset of the total pigment population absorbing at 726 nm. The subset is part of the inhomogeneous distribution of the primary donor excitation energies and this subset is narrower than the total distribution and also shifted (by $\sim 1\text{ nm}$) from the peak maximum measured in absorption. The complete analysis is presented in SI-4 and provides a value for the electrochromic shift of $21 \pm 4\text{ cm}^{-1}$.

There is no observable electrochromic shift of the lowest energy feature at 749 nm. The narrow negative feature peaking at 687.5 nm seen in Fig. 4 is not induced by illumination at 725 nm but only with higher energy (640 nm) illumination. This feature is attributed to electrochromism of *chl-a*-PS II present in the FRL-PS II sample as a contaminant. The data in Figs. 2 and 4, along with the discussion in SI-4 establish that low power 725 nm illumination does not induce significant *chl-a*-PS II photochemistry, i.e. does not induce $Q_A^{\bullet-}$ formation as measured by the characteristic Chl_{D1} electrochromic shift in the 685 nm region.

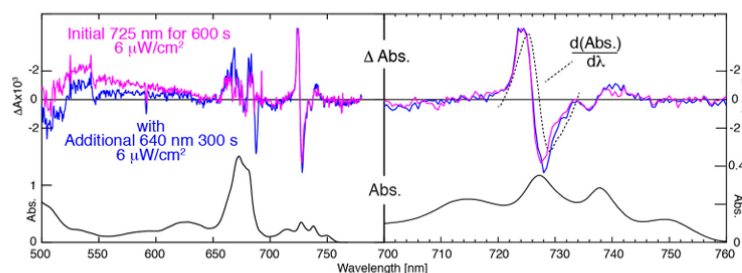
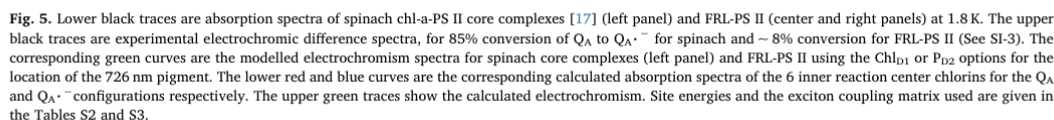


Fig. 4. Lower left panel: 1.8 K absorption spectrum (black line). Upper left panel: Difference spectra after 1. illumination at 725 nm for 600 s with an incident fluence of $6\text{ }\mu\text{W}/\text{cm}^2$ (magenta line), and 2. the total change in absorption induced by the first illumination plus additional illumination at 640 nm (blue line). Right panel: Long-wavelength detail of the absorption and difference spectra shown in the left-hand panels compared to the (scaled) differential of the absorption spectrum taken before illumination (dashed line). This data is analyzed in more detail in SI-4 and Fig. S6.

The yield of Q_A^- compared to that seen for most other organisms remains low even at high fluences (see SI-4, Fig. S5). However, yields are also very low in *Acaryochloris marina* [38], a system in which PS II is dominated by the FRL-*chl chl-d*. Strong illumination of FRL-PS II (SI-4, Fig. S6) leads to increased yields, but also induces additional photochemical and photophysical changes [17].

As a first step, the previously reported analysis [23] of the $Q_A^{\bullet -}$ -induced O_2 electrochromic shift pattern, as calculated for plant PS II

When comparing the calculated electrochromic shift spectra with ChlD_1 or alternatively PD_2 as the primary donor against experimental data (Fig. 5), both options reproduce the sign and amplitude of the shift



seen at 726 nm. The P_{D2} option provides a somewhat better overall fit to the electrochromic pattern in the *chl-a* Q_y region from 650 nm to 690 nm. FRL PS II data in this region suffers from low signal-to-noise due to i) the higher absorbance present in the *chl-a* Q_y spectral region, ii) the requirement of minimal actinic light and iii) the relatively low yield of $Q_A^{\cdot-}$ formation in FRL-PS II (see Fig. S6) compared to spinach *chl-a*-PS II.

The calculated amplitude of the 726 nm $Q_A^{\cdot-}$ shift is slightly smaller for the Chl_{D1} primary donor case than is for the P_{D2} case, despite P_{D2} being further away (2.6 nm vs 2.2 nm) from Q_A . This is a consequence of the orientation of the electric field with respect to $\Delta\mu$ leading to a larger site-energy shift for P_{D2} (Table S4). The chlorin Q_y site energy shifts for $Q_A^{\cdot-}$ formation (Table S4) shows that the P_{D1} and P_{D2} shifts (5.8 cm^{-1} and 18 cm^{-1}) are comparable to the Chl_{D1} shift (14.4 cm^{-1}).

Exciton modelling can be used to simultaneously calculate the lineshape and amplitude of both CD and absorption spectra [26,32] without the introduction of any additional parameters. The amplitude of the CD calculated in this way provides a useful internal check of exciton calculations. For the isolated reaction center preparation RC-6 both absorption and CD line-shapes have been successfully modelled [39]. A quantitative comparison of CD and absorption amplitudes was not made at that time. A detailed comparison of more recent, simultaneously taken, lower-temperature absorption and CD spectra, of both RC-6 and RC-5 [26] show that the calculated CD line-shape as well as amplitude are in agreement with the exciton calculations. The calculations done for RC-6 and RC-5 use the same exciton matrix elements (Table S3) as those used here. The only difference in the former calculations were that the linewidths and site-energies introduced were adapted to take into account the increased line-broadening and site-energy blue shifts present in isolated reaction centers such as RC-6, compared to PS II core complexes.

Isolated FRL-PSII reaction centers have not yet been prepared. Consequently, it is only possible to compare theoretical and experimental CD data for the single FRL-*chl* present in the reaction center component of the FRL-PS II core complex assembly, i.e. the pigment absorbing at 726 nm. The CD characteristics of the other *chl-a* and *pheo-a* FRL-PS II reaction center pigments, i.e. those absorbing in the 650 nm to 690 nm region, are obscured by the dominating CD of *chl-a* present in CP43 and CP47 peripheral antennas (Fig. 1). A more complete analysis would look to calculate the exciton CD of the entire FRL-PS II core assembly but this is not at present feasible. Exciton CD calculations that are restricted to the reaction center pigments of FRL-PS II are a reasonable starting point as couplings between reaction center pigments and pigments in the CP43 and CP47 peripheral antennae are inherently weaker than the (relatively strong) couplings between reaction center pigments (Table S3). This is due to the greater inter-pigment distances involved.

Fig. 6 demonstrates that the computed absorption and CD spectra of the 6 reaction center chlorins in FRL-PS II, shown as coloured green and red profiles respectively, vary significantly depending on whether the primary donor is located at Chl_{D1} or P_{D2} . Of the 6 reaction center chlorins, only the FRL-*chl* primary donor is directly accessible experimentally, because it has CD and absorption features that are not buried under the absorption peak arising from ~30 *chl-a* and 2 *pheo-a* pigments in the 650 nm–690 nm region.

The sign and amplitude of the calculated primary donor CD agrees well with the (positive) experimental CD value when P_{D2} is the primary donor. When Chl_{D1} is the primary donor, the calculated CD is barely visible. It is $30\times$ smaller and is calculated to have a negative sign. The CD spectrum of a multi-chromophore array such as a PS II reaction center arises from a complex interplay between exciton coupling matrix elements (Table S3), site energies (Table S2) and relative orientations of transition dipoles of the interacting chromophores. Thus, it may not be immediately interpretable. However, there is only one FRL-*chl* in the reaction center of FRL-PS II (i.e. the pigment at 726 nm) and the Q_y of

this pigment is displaced by $\sim 1000\text{ cm}^{-1}$ from the other pigments (*chl-a* and *pheo-a*) in the reaction center. This displacement is larger than the values of the exciton coupling matrix elements shown in Table S3. With coupling matrix elements and orientations being held constant, a large red-shift of the chromophore will tend to have the effect of reducing its CD, whether it be located at Chl_{D1} or P_{D2} .

A quantitative measure of the strength of CD is the parameter ρ_A , the ratio of CD amplitude to absorption, $\Delta A/A$. The CD of the *Chl-a* exciton region at 680 nm in RC-6 is experimental and theoretically evaluated as $\rho_A = 1.2 \times 10^{-3}$. This ρ_A value is significantly less than the calculated CD of the Chl_{D1} exciton itself which has $\rho_A = 2.5 \times 10^{-3}$ [26]. The reduction in ρ_A is due to the significant overlap of the Chl_{D1} exciton with nearby excitons having the opposite CD sign. In FRL-PS II the primary donor exciton is well separated from other reaction center excitons and such cancelling does not occur. Thus, we can directly compare the experimental ρ_A of the primary donor in FRL-PS II with the calculated value of $\rho_A = 2.5 \times 10^{-3}$ for the Chl_{D1} exciton in *chl-a*-PS II.

From our global analysis of FRL-PS II data, the CD of the primary donor in FRL-PS II at 726 nm has $\rho_A = 0.5 \times 10^{-3}$. The calculated values for the primary donor CD $\Delta A/A$ at 726 nm is $\rho_A = 0.6 \times 10^{-3}$ when P_{D2} is the primary donor and -0.02×10^{-3} when Chl_{D1} is the primary donor. In both cases, the calculated ρ_A for the primary donor is far less than the value of $\rho_A = 2.5 \times 10^{-3}$ for the Chl_{D1} exciton in *chl-a*-PS II. A significant reduction in CD for the single FRL-*chl* in FRL-PS II can be expected regardless of where it is located. This is due to the relatively large energetic difference between the 5 *chl-a* pigments and the single FRL-*chl* in the reaction center (compared to the coupling matrix elements) reducing the ability of (unchanged) exciton coupling matrix elements to induce CD. The calculated value of $\rho_A = -0.02 \times 10^{-3}$ for the primary donor as Chl_{D1} may appear to be anomalously small given the significant exciton matrix elements between Chl_{D1} and P_{D1} , P_{D2} and *Pheo* $_{D1}$ (Table S4). This result highlights the fact that the CD of an exciton coupled multi-pigment array is not simply reducible to sums of individual couplings.

Preliminary calculations were performed to explore the influence of proximal antenna-based FRL-*chls* on the CD in the 726 nm region. The closest pigment to Chl_{D1} present in CP43, measured edge to edge, is the 'linker' *Chl508* [43]. Exciton coupling matrix elements between the reaction center pigments and *Chl508* were calculated using a point-dipole approximation [41]. The largest matrix elements between *Chl508* and the reaction center pigments were in the range of 1 cm^{-1} to 7 cm^{-1} and significantly smaller than the largest matrix elements within the reaction center (Table S3). Exciton CD calculations were then performed for both the Chl_{D1} and P_{D2} options for the 726 nm pigment, whilst taking *Chl508* to be the FRL-*chl* at 737 nm (Table S1). The CD calculated at 726 nm when the primary donor was Chl_{D1} became significant in magnitude but remained negative having $\rho_A = -0.32 \times 10^{-3}$. With P_{D2} as the primary donor, the calculated CD including *Chl508* remained positive with $\rho_A = 0.36 \times 10^{-3}$ and close to the experimental value of 0.47×10^{-3} . In both cases, the sign of the CD calculated for the 737 nm band was positive and thus not in agreement with experiment (see Fig. 6, Table S1 and Fig. S4). An analogous calculation was made placing an FRL-*chl* at the *Chl505* location. This is the closest CP43 pigment to Chl_{D1} , measured center to center. Although the calculated exciton interaction between Chl_{D1} and *Chl505* is larger than for *Chl508*, the CD with Chl_{D1} is the primary donor is calculated to be significantly smaller than for the *Chl508* case described above and again not in agreement with experiment. A more complete calculation, involving all the pigments FRL-PS II may help identify the location of the proximal antenna FRL-*chls*.

In addition to exciton CD, there is the monomeric CD component of *chl* Q_y transitions. This is independent of, and additive to, exciton CD. Monomeric Q_y CD of *chl-a* is $\rho_A = 0.16 \times 10^{-3}$ [44], -0.07×10^{-3} for *chl-d* [38] and -0.03×10^{-3} for *chl-f* [45]. These values are all small in magnitude and of the opposite sign to the observed value of $\rho_A = 0.47 \times 10^{-3}$ for the 726 nm band in FRL-PS II.

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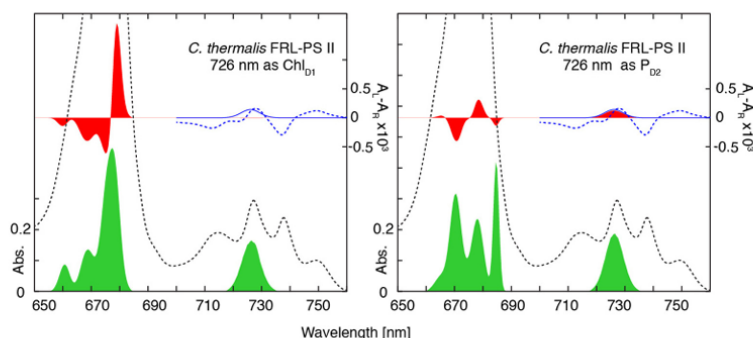


Fig. 6. For both panels, the dashed black trace (left-hand scale) is the 1.8 K absorption spectrum of a FRL-PS II preparation. The dashed blue trace is the simultaneously-recorded CD spectrum in the red region (right-hand scale). The solid green profiles are the exciton-modelled absorption spectra (see text) of the inner reaction center chlorins of FRL-PS II and the red profiles are the corresponding calculated CD spectra, with either Chl_{D1} or P_{D2} as the primary donor as indicated. The solid blue trace shows the CD component of the primary donor at 726 nm. The position, width and amplitude of the primary donor CD at 726 nm (Table S1) was determined by the global fitting procedure described in the text. Experimental FRL-PS II absorption and

CD spectra are scaled to the calculated (reaction center only) spectra by assuming that the integrated absorption area over the entire Q_y region corresponds to a total of ~ 35 chl and 2 pheo-a pigments with the reaction center having 6 chl and 2 pheo-a pigments. Integrals are shown in Fig. S1.

3.6. Arguments in favor of the primary donor being Chl_{D1}

Chl_{D1} is considered to be the primary donor in *chl-a*-PS II [3,5–7,46]. Chl_{D1} is then the obvious pigment to exchange for a long wavelength pigment [1] to create a low energy primary donor. This apparently straightforward option is reinforced by the fact that all known FRL-PS II systems for which sequences are available show a pair of conserved amino acid changes D1Tyr120 and D1Thr155. These can be modelled as providing H-bonds to the extra formyl group that is the substituent on ring I of the chlorophyll that is responsible for the long-wavelength shift in a FRL-chl [1]. This seems to be a genetic/structural “smoking gun”. In contrast, amino acid residues facing the same position in the ring I of P_{D2} appear to be unable to form H-bonds having aliphatic side chains (Val and Leu). These residues were also found to be conserved in both the FRL and WL version of PSII. Nevertheless, we cannot exclude H-bond formation via the amide group of the peptide bond.

The mechanistic and redox arguments cited in the introduction are also strong. Given the similar rates and efficiency of O_2 evolution, it is assumed that the donor-side components of FRL PS II are tuned to have the same redox potentials as in *chl-a*-PS II [1]. Thus, the primary electron donor FRL- chl^+/chl is taken as having a similar potential as its counterpart in *chl-a*-PSII. However, FRL-chl absorbing at 725 nm will have ~ 110 meV less energy available in the excited state than does *chl-a* absorbing at ~ 680 nm. This will result in the FRL- $\text{chl}^+/\text{chl}^*$ couple having higher potentials and therefore FRL- chl^* will have less reducing power than its *chl-a* counterpart [1].

A less reducing FRL- chl^* strongly favors pheo-a as the primary electron acceptor because the pheo-a/pheo-a $^{\cdot-}$ couple has a much higher reduction potential than that of *chl-a*/*chl-a* $^{\cdot-}$. The structure dictates that Pheo-a can only be the primary acceptor when Chl_{D1}^+ is the primary donor and this provides a good argument for the primary donor being Chl_{D1} in FRL-PS II [1]. In models in which P_{D2} is the primary donor, the primary acceptor would be *chl-a* (either P_{D1} or Chl_{D1}). This is expected to be unfavorable in redox terms. A similar argument was made for assigning the *chl-d* primary donor on Chl_{D1} in *Acaryochloris marina* PS II [4]. Along the same lines, recent calculations of the redox potentials of the chlorins in *chl-a*-PSII indicated that Chl_{D1}^+ electron donation to Pheo $_{D1}$ is favorable, but that P_{D1}^+ donation to Chl_{D1} is energetically unfavorable [46]. If this trend is extrapolated to FRL-PSII, FRL- chl^+ is expected to be even less capable of reducing a *chl-a* primary acceptor.

The chemical argument mentioned in the introduction comes from the demonstration that chlorophyll has acid base properties that will favor ligation to water over histidine [47]. In the *chl-a*-PS II reaction center, only the Chl_{D1} and Chl_{D2} pigments have water coordination and

are thus more likely as candidates for replacement by *chl-f* [1]. It is not possible to rule out histidine ligation to *chl-f*, in either the PS II reaction center or in peripheral antenna, but the chemical preference exists.

4. Conclusions

Illumination of FRL-PS II at 1.8 K at a series of wavelengths confirmed that the pigment at 726 nm is the primary electron donor. Charge separation occurred relatively efficiently at wavelengths ≤ 726 nm but became much less efficient at longer wavelengths. This verifies the conclusion made earlier [1].

A global analysis of absorption, CD and MCD data enabled the determination of the exciton spectral amplitudes, positions and widths of all 5 FRL-chl Q_y state spectral components in FRL-PS II. The $Q_A^{\cdot-}$ induced electrochromism in FRL-PS II generated by low temperature and low fluence illumination was modelled by exciton coupling calculations (Figs. 5, 6 and S9). The starting point for these calculations was a new quantitative analysis of existing electrochromism data of *chl-a*-PS II core complexes prepared from spinach, allowing a model-free determination of the value of $\Delta\mu/f$. The FRL-PS II electrochromic shift at 726 nm could be quantitatively accounted for by transferring the Chl_{D1} or P_{D2} *chl-a* site energy appropriate to spinach to the FRL 726 nm position, whilst leaving all exciton coupling terms, other site energies and geometric parameters as well as $\Delta\mu/f$ unchanged. The P_{D2} calculation provided a slightly better fit to the overall electrochromic pattern in the 650 nm to 690 nm region. The exciton modelling was also able to exclude the possibility that there was more than one FRL-chl in the reaction center, again in line with earlier ideas [1].

The CD calculated by the exciton modelling was able to account for the observed value of $p_A = \Delta A/A$ at 726 nm, without the use of additional parameters, when the primary donor was located at P_{D2} . When located at Chl_{D1} the CD calculated was 30 times smaller and of the wrong sign in comparison to experiment. Preliminary exciton calculations of coupling between the reaction center chlorins and the closest chlorins in the proximal antennae (Chl_{508} and Chl_{505}) indicate that coupling an FRL-chl at either of these locations are unlikely to account for the experimentally observed CD amplitude at 726 nm. A more definitive calculation would be to include all the pigments in FRL-PS II or perhaps a subset of the CP43 and CP47 pigments closest to the reaction center pigments. Such calculations may help identify the location of the FRL-chls in the proximal antennae.

There are a number of arguments that make a good case for the Chl_{D1} being the location of the single FRL-chl in the reaction center. However, these arguments are intuitive, mechanistic and, when structural (i.e. the conserved amino acid changes) are yet to be verified by experiment. The current work presents initial experimental evidence,

particularly from the concurrence of calculated and experimental CD at 726 nm, that the primary donor could be located at P_{D2}.

Rather than speculating further on this surprising result, we look to perform additional spectroscopic experiments and calculations that may provide further evidence relevant to the character and location of the primary donor in FRL-PS II.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We recognize the support of the Australian Research Council through grants DP110104565 and DP150103137 (EK), FT140100834 (NC). This work was supported by BBSRC grants BB/L011506/1 and BB/R001383/1 (AWR, AF and DN).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbabi.2020.148248>.

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Chapter 7

Redox isomerism explains the two forms of the S2 state of Nature's water splitting catalyst

7.1.1 Introduction to Publication 4

Publication 4 follows on from Publication 1. In Paper 1, we measured variable temperature variable field MCD spectra of each of the S-states of the OEC in the 710-900 nm region. The MCD of the S-states consists of narrow bands over a relatively small spectral range. We have assigned these narrow bands to Mn(IV) $^4A \rightarrow ^2E$ spin-flip transitions. Each transition corresponds to a different Mn(IV) ion in the OEC structure. Absorption in this region was predicted to occur based on an EPR action spectrum reported in a paper by Boussac et al. [45]

One key intermediate S-state, the S2 state, exists in two distinct forms that differ in their electronic ground state configurations: a low spin state ($S=1/2$) and a high spin state ($S=5/2$). These two S2 sub-states have been studied extensively by EPR and correspond to the low spin “multiline” signal and the high spin $g \geq 4.1$ signal. Boussac et al also showed that illumination in the 710 – 900 nm region caused an interconversion between the two forms of the S2 state. In publication 1, we were not able to observe this interconversion. The experiments in publication 1 used a 1:1 mixture of glycerol and ethylene glycol as a glassing agent. Hydrophobic cells made the addition of ethylene glycol unnecessary, so we attempted the experiment again using only glycerol.

7.1.2: Key findings from Publication 4

Using only glycerol as a glassing agent in the new cells had no measurable impact on the optical quality of the sample. Photographs of clear test samples are shown in figure 3.7

S2 state interconversion was induced with 775 nm laser illumination, and had an efficiency of ~40%, even at ~ 2 K. As with the identification of the MCD of the different S states in publication 1, the distinct field and temperature dependencies of the MCD spectra of the two S2 forms could be used to distinguish them from each other. The field dependencies of the MCD signals of the two S2 sub-states are consistent with $S=1/2$ and $S=5/2$ and a crystal field splitting of $\sim 1 \text{ cm}^{-1}$, respectively. This agrees with

previous EPR reports. Critically, the low spin S2 state has three Mn(IV) spin flip transitions, whilst the high spin S2 state has only two. This observation, in combination with the fact that the interconversion can take place at temperatures as low as 2 K, suggests that one of the Mn(IV) ions is photoreduced to Mn(III) and we conclude that the two S2 state forms are redox isomers.

7.2 Statement of Contribution

**Statement of Contribution**

This thesis is submitted as a Thesis by Compilation in accordance with https://policies.anu.edu.au/ppi/document/ANUP_003405

I declare that the research presented in this Thesis represents original work that I carried out during my candidature at the Australian National University, except for contributions to multi-author papers incorporated in the Thesis where my contributions are specified in this Statement of Contribution.

Title and authors: Redox isomerism explains the two forms of the S_2 state of Nature's water splitting catalyst

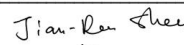
Jennifer Morton, Julien Langley, Jian-Ren Shen, Elmars Krausz, Nicholas Cox

Current status of paper: In preparation

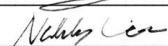
Contribution to paper: Experiment design. All the optical spectra, photographs and the writing.

Senior author or collaborating authors endorsement:

Julien Langley  10/06/25

Jian-Ren Shen  24/06/25

Elmars Krausz  10/6/25

Nicholas Cox  10/6/25

Jennifer Morton



1/5/25

Candidate

Signature

Date

Endorsed

Nicholas Cox

Chair of Supervisory Panel

Signature

Date

25/6/2025

Mark Humphrey

Delegated Authority

Signature

Date

27 June 2025

7.3 Publication 4

Redox isomerism explains the two forms of the S₂ state of Nature's water splitting catalyst

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Supporting Information Placeholder

ABSTRACT: Nature's water-splitting cofactor — a tetra-manganese calcium cluster embedded in the Photosystem II (PSII) supercomplex — undergoes a multistep catalytic cycle. A key metastable intermediate in this cycle is the S₂ state, which exists in two distinct forms that differ in their electronic ground spin states. In this study, we investigate competing models of these two S₂ forms. Using low-temperature magnetic circular dichroism (MCD), we demonstrate that interconversion between the S₂ forms can occur at temperatures as low as 2 K. This interconversion is accompanied by the loss of a Mn(IV) ⁴A → ²E spin-flip transition, attributed to Mn₄, suggesting reduction of this manganese center during the process. Furthermore, using PSII purified from the acid-tolerant *C. merolae*, we show that lowered pH does not change the EPR signals associated with the S₂ to S₃ transition, nor does it stabilize intermediate states such as S₂'. These results suggest the two S₂ state forms are redox isomers and proton egress intermediates do not explain S-state heterogeneity.

Nature's water-splitting catalyst is a tetra-manganese calcium cluster embedded within the Photosystem II (PSII) supercomplex^{1,2}. Its catalytic cycle involves five distinct redox intermediates, known as the S_n states (n = 0–4), where each state represents the accumulation of one oxidizing equivalent³. Progression through the S-states is driven by the PSII reaction center, a multi-chlorophyll pigment assembly. Light absorption initiates charge separation, generating a strong photo-oxidant (P680⁺), which is relayed to the catalyst via a redox-active tyrosine residue (Y_Z). After four such charge separations, the transient S₄ state decays rapidly to S₀, releasing triplet O₂ and binding a new substrate water molecule^{4,5}.

Structural studies using X-ray diffraction (XRD)¹ and X-ray free electron lasers (XFEL)⁶ have revealed that in the early S-states (S₀, S₁, S₂), the cofactor represents a Mn₄O₃Ca complex. The metal ions form a distorted chair-like geometry: Mn₁, Mn₂, Mn₃, and Ca²⁺ constitute the base (an open-cubane Mn₃O₃Ca unit), while Mn₄ (the "dangler") is connected via two μ-oxo bridges (O₄ and O₅). In the S₃ state, the cofactor incorporates an additional oxygen (O₆), likely a hydroxide, forming Mn₄O₆Ca complex^{7–10}. O₅ and O₆ are considered the most probable substrate water molecules^{5,11}.

The S₂ state is unique in that it exists in two distinct ground state electronic configurations: a low-spin state (S_G = 1/2)¹² and a high-spin state (S_G = 5/2).^{13,14} Early S-states (S₁, S₂) typically adopt low-spin configurations, while later states (e.g., S₃) are high-spin (S = 3). These two S₂ forms are distinguished by their EPR signals: the low-spin "multiline" signal¹² and the high-spin g ≥ 4.1 signal.^{13–17} Importantly, these forms are structurally distinct yet interconvertible at cryogenic temperatures (<150 K) following near-infrared (NIR) irradiation.¹⁸ The high-spin form (g ≥ 4.1) is thought to be an intermediate en route to the S₃ state.^{19,20}

Three models have been proposed to explain this S₂ interconversion.²¹ All models assume the same structure for the S₂ multiline state (S₂^{ML}), an open cubane motif (S₂^A) similar to the current XRD^{1,9} and previous DFT²² structures. The Mn^{III} ion located within the cuboidal unit (Mn_i) and is five-coordinate, square pyramidal.²³

i) Redox Isomerism Model²⁴: This model posits that the position of the Mn^{III} ion shifts to the dangler Mn₄ in the S₂ g ≥ 4.1 (S₂^{H4}) state. Oxidation state rearrangement is coupled with movement of the O₅ bridge, leading to a closed cubane structure (S₂^B). While this model accounts for two nearly isoenergetic ground states with a low interconversion barrier, a closed-cubane intermediate has not yet been observed in XFEL measurements.

ii) Proton Isomerism Model^{25,26}: This model posits that the S₂^{H4} state is a proton isomer of the multiline state. A proton migrates from a water in the vicinity of O₄ water channel to the oxo bridge (o₄), disrupting magnetic coupling between Mn₄ and the cubane unit, leading to spin-state conversion. While model complex data does demonstrate spin state tuning via bridge protonation,²⁷ this model predicts the energies of two S₂ forms to differ by >10 kcal/mol, questioning whether both structures could be experimentally observed.²¹

iii) Early Water Binding Model: This model posits that the S₂^{H4} state arises from water binding at Mn₁,^{28,29} a step generally thought to occur during the S₂ to S₃ transition.^{7–9} However, it is unclear what is the thermodynamic driving force for water binding to Mn^{III}, and whether this process can occur at cryogenic temperatures.

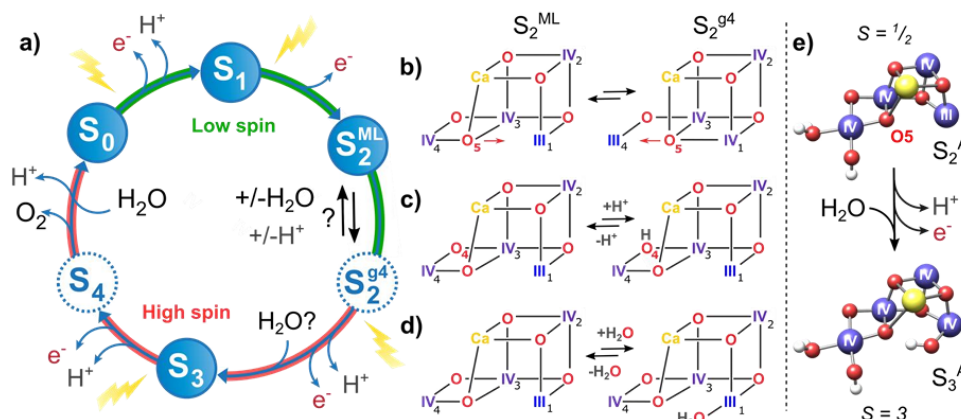


Figure 1: a) The catalytic (Kok/ S-state) cycle of the Mn_4O_5Ca cofactor.^{3, 30} **b, c, d)** S_2 state models of the S_2^{ML} ($S_G = 1/2$) and S_2^{g4} state ($S_G = 5/2$). In all models the S_2^{ML} state corresponds to an open cubane (S_2^A) structure. In the redox isomerism model (**b**) the S_2^{g4} state corresponds to a closed cubane (S_2^B) structure with the two forms interconverting via reorganization of O_5 .²⁴ In the proton isomerism model^{25, 26} (**c**) the S_2^{g4} state corresponds to an open cubane (S_2^A) structure where the O_4 bridge has been protonated. In the early substrate binding model^{28, 29} (**d**) the S_2^{g4} state corresponds to an open cubane (S_2^A) structure where a water molecule has bound to Mn. **e)** The S_2 to S_3 transition leading to open cubane (S_3^A , $S_G = 3$) structure.

Understanding the nature of S_2 interconversion is critical for elucidating the $S_2 \rightarrow S_3$ transition. The redox isomerism model implies that water binds to Mn_4 late in the transition,^{31, 32} while the early water binding model suggests binding at Mn_1 and that this represents the first step in the transition.²⁸ The proton isomerism model also favours water binding to Mn_1 and suggests that S_3 state heterogeneity could be explained in terms of proton isomers, with consequences for proton release/egress.^{26, 33}

To distinguish between these models, we employed two spectroscopic techniques:

Magnetic Circular Dichroism (MCD): In a previous publication we have shown each S-state exhibits a unique MCD fingerprint, characterized by a set of Mn^{IV} $4A \rightarrow {}^2E$ spin-flip transitions.³⁴ We identified transitions for Mn_2 , Mn_3 , and Mn_4 —those coordinated exclusively by oxygen ligands—allowing us to track their oxidation states through the cycle. Here we show that S_2 interconversion occurs efficiently ($\sim 40\%$ yield) even at liquid helium temperatures, where proton tunnelling and substrate binding are switched off. Formation of the $g \geq 4.1$ state results in the complete loss of the Mn_4 spin-flip transition, consistent with Mn_4 photo-reduction—supporting the redox isomerism model.²⁴

High-Field (W-band) EPR Spectroscopy: This technique allows S-state (sub)populations, including those from the integer spin S_3 state to be characterized and quantified.^{7, 32} Here the S_3 -state signals of PSII isolated from the acidophile *Cyanidi-oschyzon merolae* are reported, revealing no pH-dependent changes, other than a uniform reduction in yield, arguing against the proton isomerism model.

RESULTS AND DISCUSSION

S_2 State Photochemistry. As described in Morton et al.,³⁴ each S-state exhibits a distinct magnetic circular dichroism

(MCD) signature, corresponding to sharp, spin-forbidden optical transitions associated with Mn^{IV} ions of the cofactor. The methodology for isolating these overlapping spectral features from background signals is detailed in that work and in the Supporting Information. In brief, the differing magnetic field and temperature dependencies of each S-state—due to their unique ground spin states—enable clean spectral deconvolution via scaled subtraction.

The observed bands are assigned to $4A \rightarrow {}^2E$ spin-flip transitions.³⁴ While these transitions are typically unobservable in monomeric Mn species,³⁵ they have been previously detected in Mn dimers and tetramers.^{34, 36} Their positions can also be inferred from fluorescence data.³⁷ No other transitions in this spectral window can be attributed to the Mn cluster—such as spin-allowed d-d transitions from Mn^{III} , or metal-metal/metal-ligand charge transfer states.

These spin-flip transitions appear over a narrow spectral range and are classically, independent of any crystal field splitting.³⁸ Their precise positions are considered to be modulated by the covalent effects.³⁹ Mn_4 , coordinated by water-derived ligands, is assigned to the bluest (highest energy) positive band, while Mn_2 and Mn_3 correspond to the remaining positive and negative bands.

In magnetically coupled systems, MCD intensities scale with the spin projection factor of each metal ion.⁴⁰ Earlier ^{55}Mn -ENDOR studies of the S_2^{ML} state, have constrained the spin projection factors of Mn_2 , Mn_3 and Mn_4 to be close to 1,^{23, 41} meaning all three Mn^{IV} ions should be observable. This is not the case for other S-states. For example, ^{55}Mn -EDNMR data for the S_3 state reveal an uneven spin density distribution, with Mn_3 and especially Mn_4 exhibiting low spin projection factors.⁷ Consistent with this, the S_3 MCD spectrum is dominated by a single $4A \rightarrow {}^2E$ transition, with the bands associated with Mn_3 and Mn_4 strongly attenuated.³⁴

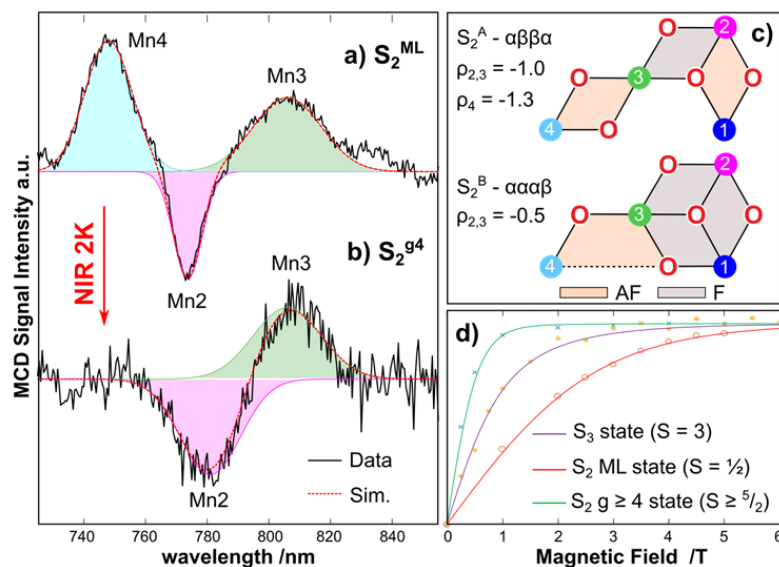


Figure 2. Magnetic Circular Dichroism (MCD) spectra of the **a)** the S_2^{ML} state and **b)** S_2^{g4} state collected at 2 K. The S_2^{g4} state was generated from the S_2^{ML} state in situ using 780nm illumination. The fitted gaussian bands correspond to the observed Mn^{IV} $^4A \rightarrow ^2E$ transitions observed. **c)** Magnetic coupling topologies for the open (S_2^A) and closed (S_2^B) structures, and corresponding spin projection factors.²⁴ In both the S_2^A and S_2^B structure the magnitudes of the spin projection factors of Mn2 and Mn3 are the same. In S_2^B structure Mn4 is poised in the +III oxidation state and thus should not have an observable $^4A \rightarrow ^2E$ transition. Note that the Mn ions are colour coded the same way in panels **a**, **b** and **c**. **d)** Magnetic field dependence of the S_2^{ML} , S_2^{g4} and S_3 states.³⁴ Data processing and fitting are described in the supporting information.

S_2 state interconversion was induced using ~200 mW CW 780 nm laser excitation. As with other S-states, the S_2^{ML} and S_2^{g4} MCD signals can be distinguished by their differing magnetic field dependencies and thus a clean S_2^{g4} can be generated³⁴. It should be noted that the magnetic field dependence of the S_2^{g4} state is consistent with a $S_G = 5/2$, with a zero-field splitting of $\sim 1 \text{ cm}^{-1}$, consistent with previous EPR reports.¹⁴ Importantly, upon conversion to the S_2^{g4} state, the Mn4 associated spin-flip transition disappears entirely, while the Mn2 and Mn3 transitions remain approximately unchanged in both position and intensity.

To validate changes in the MCD data reflect true Mn_4O_5Ca photochemistry, parallel EPR measurements were conducted using identical cryoprotectant conditions (40% glycerol) needed to ensure optical clarity in the MCD experiment. EPR measurements confirmed that the S_2 (ML) state is formed in a majority of centers following advancement from S_1 at 200 K, with a smaller population of the S_2 ($g \geq 4.1$) state. Interconversion between these states was achieved via CW 780 nm laser excitation at temperatures ranging from 140 K to 5 K. Notably, the S_2 ($g \geq 4.1$) EPR spectrum was similar across all temperatures⁴², unlike in higher plants, where the signal lineshape is temperature-dependent.¹⁷ Photoconversion yields at 2 K and 140 K were similar (>30%), based on the loss of the ML signal. We note that photoconversion is highly sensitive to the cryoprotectant used and never exceeds 50%.¹⁸

Redox Isomerism Explains the S_2 ($g \geq 4.1$) MCD Spectrum.

The high photoconversion yield at 2 K argues against models involving stoichiometric changes, such as water binding, or protonation isomerism (proton tunnelling), as both processes are suppressed at cryogenic temperatures. Instead, the complete loss of the Mn4 spin-flip transition is best explained by photoreduction of Mn4 to Mn(III), accompanied by oxidation of Mn1—whose transition lies outside the detectable spectral range.³⁴

The redox isomerism model for the S_2 ($g \geq 4.1$) state also predicts that Mn2 and Mn3 retain similar spin projection factors and relative signs as in the S_2 (ML) state.²⁴

Accordingly, this model predicts that the MCD signals of Mn2 and Mn3 should remain unchanged, which is consistent with experimental observations. In contrast, the protonation isomer model struggles to account for the data. In this model, Mn2, Mn3, and Mn4 all remain in the +IV oxidation state in the S_2 ($g \geq 4.1$) state, and all three ions are expected to have similar spin projection factors.²⁵ Therefore, this model would predict that the S_2 ($g \geq 4.1$) MCD spectrum should exhibit three spin-flip transitions, as observed in the S_2 (ML) state.

Recent X-ray absorption measurements also support the interpretation that the two S_2 state forms represent redox isomers.⁴³ The slight difference in edge positions between the two forms could arise from the Mn^{III} ion occupying distinct environments in each S_2 state.

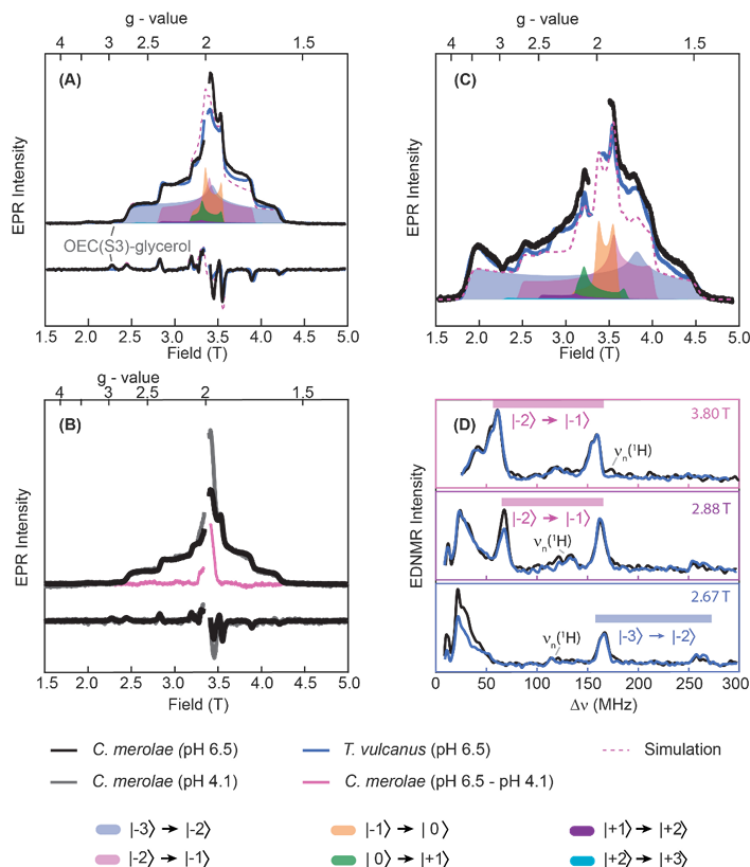


Figure 3. a) W band EPR spectra of the S_3 state of purified PSII prepared from *C. merolae* and *T. vulcanus*. b) Comparison of the S_3 signal in PSII (*C. merolae*) poised at pH 6.5 and pH 4.1. c) Modified S_3 signal seen in PSII (*C. merolae*) upon addition of 3% methanol (v/v). d) Comparison of S_3 state ^{55}Mn -EDNMR spectra collected at the low and high field edge of PSII purified from *C. merolae* and *T. vulcanus*. All data (black lines) represent light-minus-dark difference spectra between samples poised in the S_3 state and poised in the S_1 state. Data in panels a and b represent echo detected absorption spectra and their corresponding processed (pseudo-modulated) spectra. Simulations are shown with red dashed lines. A decomposition of the absorption spectra simulations according to spin transitions are also shown (see color code). Simulation parameters are listed in the SI Appendix (table S1). The asterisk indicates the magnetic field position where ^{55}Mn -EDNMR measurements were performed.

Finally, while recent studies on $\text{Mn}_4\text{O}_5\text{Ca}$ model complexes (cubane-type structures)²⁷ have shown that the ground spin state can be modulated by oxo bridge protonation, this mechanism is unlikely to be accessible for the cofactor—especially in the higher S -states. Except for O_4 and O_5 , the oxo bridges of the cofactor are not solvent-accessible.¹ Moreover, in the S_2 and S_3 states, these bridge Mn ions that are both +IV oxidation state, resulting in these oxo bridges having very low pK_a values. In Mn^{IV} -oxo-bridged dimers, this leads to the bridging oxygens being essentially non-exchangeable.⁴⁴

Table 1: Spin Projection factors for the Mn^{IV} ions of S_2 and S_3 state models

		ρ_2	ρ_3	ρ_4	
$S_2(\text{ML})$		-0.96	-0.98	1.29	Ref. ²⁴
$S_2\text{g}_4$	$S_2\text{B}$	-0.53	-0.50	-	Ref. ²⁴
	O_4H	-0.27	-0.42	-0.59	Ref. ²⁵
S_3		-0.44	-0.18	0.11	Ref. ⁷

Mechanism of S_2 State Photo-Interconversion. A plausible mechanism for spin-state conversion can be proposed based

on the Light-Induced Excited Spin-State Trapping (LIESST) effect,¹⁸ with the key distinction being that two metal ions are involved. Photoexcitation of a Mn^{IV} (a high-spin d^4 ion) generates a strong in situ oxidant that extracts an electron from Mn, the only Mn^{III} ion and thus the Mn with the lowest oxidation potential. This electron transfer traps the system in the S_2 ($g \geq 4.1$) state.

Interestingly, while the S_2 ML and $g \geq 4.1$ states generated by flash advancement at room temperature exhibit similar stabilities,¹⁶ the photoinduced $g \geq 4.1$ state decays back to the ML state on a timescale of seconds (at 140 K) to minutes (at 77 K).^{18,42} This suggests that the photoinduced form may not represent a fully relaxed structure and that electron transfer could occur independently of O₅ bridge movement. This may explain why EXAFS studies^{24,45} has yet been unable to detect changes between the two S_2 forms.

pH dependence of S_2 and S_3 state EPR signal. To further investigate whether protonation state isomerism could explain S-state heterogeneity observed beyond the S_2 , we examined the $\text{S}_2 \rightarrow \text{S}_3$ transition at lowered pH. A major challenge in conducting such measurements is that low pH conditions can lead to disintegration of the $\text{Mn}_4\text{O}_3\text{Ca}$ cofactor, resulting in the appearance of a free $\text{Mn}(\text{II})$ signal that overlaps with the S_2 (ML) spectrum. To mitigate this, we performed our experiments using PSII isolated from the red alga *Cyanidioschyzon merolae*, a close relative of *Cyanidium caldarium*. This acidophilic, thermophilic, unicellular, eukaryotic phototroph thrives in environments with pH 0.2–4 and temperatures of 40–56 °C.⁴⁶ Similar to thermophilic cyanobacteria, members of the Cyanidiales express robust photosystems that resist degradation, enabling experimentation under acidic conditions. PSII from *C. merolae* has been shown to remain stable down to pH ~3.5—lower than canonical photosystems.⁴⁷

As shown in Fig. 3, the EPR signals of the S_3 state in PSII from *C. merolae* are identical to those observed in PSII from *T. vulcanus*. Upon lowering the pH to 4.1, the S_3 signal remained spectrally unchanged compared to that at pH 6.1, although its intensity was reduced to approximately 30–40% of the neutral pH value. In Figure 3, the pH 4.1 spectrum is scaled to match the intensity of the pH 6.1 spectrum. This scaling causes the background Mn^{2+} signal (present in <5% of centers) to appear more prominent in the pH 4.1 spectrum, although its absolute intensity was consistent across all samples. Intermediate pH values (4.5, 5.0, 5.5) are presented in the Supporting Information. As expected, S_3 signal yields decreased with decreasing pH,^{48,49} while S_2 (ML) state yields remained relatively constant.^{50,51}

Both datasets exhibit significant scatter, reflecting the inherent difficulty in reproducibly measuring signal intensities using pulsed W-band EPR. This variability arises in part from challenges in consistent sample loading and maintaining uniform microwave power at the sample.

Crucially, the EPR signature of the S_3' state was not enhanced at lower pH. Instead, formation of this state required the addition of the substrate analogue methanol (MeOH), as seen earlier for the canonical system, *T. elongatus*. This finding is inconsistent with proton isomerism explaining the two S_3 state forms; namely recent models of the S_2 to S_3 state progression,^{33,52} which propose transiently sampled intermediated such as structures in which O₄ is protonated.

Electronic structure of the S_3 and S_3' states. Recent studies have challenged the current assignment of the final S_3 state's ground spin to $S_0 = 3$.^{7,53} We stress this assignment is the only one consistent with multifrequency EPR simulations (X-, Q-, and W-band).^{7,54} Assigning the ground state to $S = 6$, as some have proposed, is incompatible with both the temperature dependence and the overall structure of the EPR signal.^{7,32,55}

It has also been suggested that the spin density distributions of the S_3 and S_3' states are essentially identical.⁵² This claim is based on the re-interpretation of published ⁵⁵Mn-EDNMR data,^{7,32} which did not resolve NMR transitions for two Mn ions—alleging that these transitions were not observed because they fall within the EDNMR experiment's blind spot. This assertion is incorrect. The sensitivity of EDNMR is significantly higher at lower frequencies, where such transitions—if present—are readily observed. As shown in Fig. 3, these low-frequency transitions are much more intense than those at higher frequencies.

Finally, while the S_3 state signals observed in the mesophilic cyanobacterium *Synechocystis* sp. PCC 6803 resemble those seen in thermophilic cyanobacteria, the mesophile is clearly more heterogeneous.⁵⁵ In this respect, they resemble higher plant systems, which historically exhibit more complex S-state signals, which are sensitive to sample preparation and illumination conditions.^{17,56} In the case of the mesophile, at least four overlapping subpopulations contribute to the S_3 signal.⁵⁵ This complexity is not observed in thermophilic cyanobacteria, where the electronic structures of the S_3 and S_3' states can be characterized more cleanly and in isolation.^{7,32}

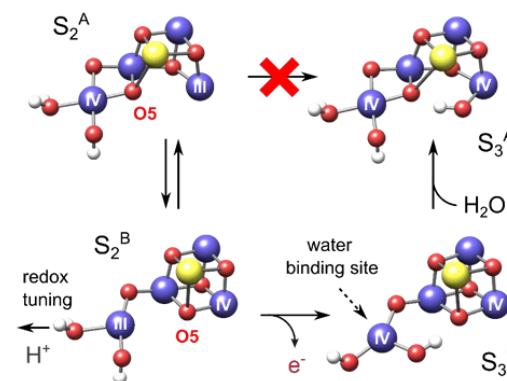


Figure 4. Multistep S_2 to S_3 progression. Redox isomerism in the S_2 state shifts the Mn^{III} ion to the dangle Mn site. Deprotonation of W1/ redox tuning of Mn_4 allows S-state advancement. Facile ligand rearrangement of W1 and W2 opens a coordination site for water binding at the terminus of the O₄ water channel to the five-coordinate Mn_4 . Intramolecular proton transfer results in final S_3^{A} state.

CONCLUSIONS

Mechanistic Insights into the S_2 to S_3 Transition: Role of Proton Release and Redox Shuttling. It is well established that the transition from the S_2 to the S_3 state is triggered by proton release,^{48,49} most likely involving the deprotonation of W1 to Asp61.^{57,58} In contrast, the S_1 to S_2 transition is decoupled from proton release,^{49,50} and consequently is the only S-state transition to occur in the frozen state. The results

presented here are consistent with these earlier finding namely: that S_2 state progression is pH independent,⁵¹ whereas lower pH leads to a decrease in the yield of both S_3 state forms. Data shown here also shows that water binding does not occur in the S_2 state. As a consequence, a plausible interpretation for the presence of two S_3 state forms is that one form (S_3') represents the cofactor prior to water binding. The observation that the S_3' state can be stabilized upon addition of water analogues that associate with the O₄ water channel, supports this notion.

It can therefore be surmised that proton release facilitates both Mn oxidation and water binding.⁵⁹ As described in the introduction, this likely proceeds via the S_2 ($g \geq 4.1$) state,^{19, 20} which is stabilized at elevated pH.⁵⁹ In contrast, the S_2 (ML) state likely represents a protective form of the cofactor, shielding it from oxidative stress. The clearest evidence for this comes from studies on Ca²⁺-depleted PSII, where only the S_2 (ML) form is observed; the S_2 ($g \geq 4.1$) state is completely absent, and water-splitting activity is abolished. If water insertion during the $S_2 \rightarrow S_3$ transition simply involves water binding at Mn1, Ca²⁺ removal should enhance water access to Mn1 and the lower the redox potential of the Ca²⁺ depleted cofactor, make oxidation to the S_3 state more favorable.

Instead, forming the S_2 ($g \geq 4.1$) state - redox shuttling of the Mn^{III} site to the so-called "dangler" Mn - is essential for progression to the S_3 state and for subsequent water splitting activity.^{31, 57} This relocation allows the cofactor to be redox tuned via deprotonation of W1. In this way, proton egress and redox leveling during the $S_2 \rightarrow S_3$ transition are tightly coupled processes.⁵⁹ The formation of a five-coordinate Mn^{IV} ion—a stronger Lewis acid than Mn^{III}—then drives water binding.³¹ Thus, the cofactor is unable to bind both substrate waters until it reaches the S_3 state—the final metastable S-state before O–O bond formation.¹⁹ This mechanism effectively suppresses premature two-electron oxidation chemistry (e.g., peroxide formation), a remarkable feat given the cofactor's aqueous environment.

MATERIALS AND METHODS

PSII core complexes were prepared from *T. vulcanus* as described in ref.³⁴ 45% glycerol v/v added to the sample as glassing agent. PSII was poised into the S_1 and S_2 states by pulsed laser following the illumination protocol used in Morton et al.³⁴ The $g \geq 4.1$ form of S_2 state was generated by illumination with a Ti:S laser (Schwartz EO) pumped with a Continuum Verdi G CW laser at 532nm. Multiple filters and a dispersing prism were used to exclude light from the pump laser and any fluorescent light from the Ti:S laser. The sample was illuminated at 140K with 775 nm ~200mW for 10 minutes. Annealing the sample at ~200K recovered the $g=2$ state. The sample was then illuminated using the same procedure at 2 K.

Samples from the same batch were used in EPR control measurements. The S_2 (multiline) state was generated 200K. The $g \geq 4.1$ form of S_2 state was generated at both 140 K and 6 K by direct illumination using a purpose-built laser diode array that was mounted on the EPR cavity. Varying the glycerol concentration between 10-40% did not substantially change either S_2 state signal or the yield of S_2 state interconversion.

PSII core complexes were prepared from *C. merolae* as described in ref.⁶⁰ Samples were suspended in a buffer containing 0.4 M, 40 mM MES, 10 mM CaCl₂, 0.04% DDM. The pH was controlled by dropwise addition of HCl, KOH under

constant monitoring via a micro pH meter, constant stirring. DDM was added last to prevent excessive foaming during mixing. The concentration of W-band EPR samples (0.7 mm ID) was approximately 4 mg Chl ml⁻¹ Sample were exposed to a short ~520nm RT illumination, in place of a pre-flash to avoid observed sample degradation. Samples were then dark adapted on ice for 20 minutes. Half of the 6 replicates were subsequently frozen to liquid nitrogen (3x S_1 replicates). The other half were doubly flashed by a Nd-YAG laser (~100 mJ, 532 nm) and immediately frozen to liquid nitrogen (3x S_3 replicates). MeOH treated samples were prepared in the same way except MeOH was added to the dilution buffer prior to mixing with PSII. Final MeOH content was 5% (v/v).

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website. Supporting Information includes: additional MCD and EPR measurements and data analysis.

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Funding Sources

No competing financial interests have been declared.

ACKNOWLEDGMENT

Financial support was provided by the Australian Research Council (Grant DP200100338 to N.C.).

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Chapter 8

Appendix

8.1 References for introductory chapters

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9.3 Supplementary to Publication 1

S-state MCD

Revised Supplementary Information

Page 1

Supplementary Information

Structured Near-Infrared Magnetic Circular Dichroism spectra of the Mn_4CaO_5 cluster of PS II in *T. Vulcanus* are dominated by Mn(IV) d-d ‘spin-flip’ transitions

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SI_2.1 Spectrometer System Improvements

The aim of this project was to determine and interpret the OEC-based MCD spectrum of each S state, along with its field and temperature dependence (VTVH). To achieve this goal, the spectrometer system described in previous publications [1] [2, 3] needed to be significantly modified and enhanced.

A: Detector/Sensitivity

Both the gain and spectral response of avalanche diodes vary strongly with temperature. To stabilise the sensitivity of the detector system, the 5 mm Advanced Photonix Si avalanche diode detector was temperature controlled to ± 0.1 °C using a liquid-cooled brass block attached to the detector assembly. Operation of the diode at below room temperature (10⁰ °C -14⁰ °C) increased the avalanche gain significantly. This, along with the greatly improved light throughput of the new sample cell design, enabled the electronic gain in the trans-impedance amplifier connected to the diode to be decreased to 10⁵ V/A. This reduction in amplifier gain enables higher light levels to be accommodated by the detector assembly. As the system sensitivity is shot-noise limited under normal operation, these modifications led to a significant improvement in the signal/noise available in (M)CD spectroscopy. We were able to achieve a sensitivity level of better than peak-to-peak $\Delta A \sim 5 \times 10^{-7}$ with a spectral bandwidth of 2 nm, for an accumulation time of 0.5 s, throughout the 700 nm-1000 nm region.

B: Shielding Removal

The magnetic shielding encasing the spectrometer system split-pair superconducting magnet, initially constructed to shield photomultiplier detectors, was responsible for small movements of the sample (relative to the optical beam) occurring when energising the magnet. These movements led to *large and unpredictable*

changes (relative to sensitivities available) in the CD zero offset and thus spurious and apparent MCD signals, i.e. CD 'signals' that varied with magnetic field. An avalanche diode, unlike a photomultiplier, does not require shielding from stray magnetic fields, thus the magnet housing was replaced with a fully *non-magnetic* assembly.

C: Sample Stability in Field Gradients

More subtle movements of the sample relative to the spectrometer light beam were found to occur upon energising the magnet. These were induced by 'Gouy Balance' forces on the sample rod and/or on the sample itself. Any material placed in a magnetic field gradient experiences a force [4] which scales as the product of the magnetic field and the field gradient $\alpha \mathbf{B} \cdot d\mathbf{B}/dx$. The materials from which the rod/cell/sample are made experience such forces. Significantly, the force does not reverse with the direction of the field as reversing the field also reverses the direction of the gradient. The phenomenology that exposes this effect is that the CD offset induced by energising the magnet does *not* reverse upon reversing the magnetic field magnetic field and is also prone to sudden 'jumps' Such movements of the sample were minimised by installing a beryllium-copper spring guide to actively locate the sample rod position. The guide was located near the bottom of the variable temperature inner cryostat.

D: Temperature Control

A temperature control system, based on a carbon resistance sensor [5] [6] and a solenoid-operated pump valve, was introduced to stabilise the temperature of the superfluid helium bath to $1.80 \text{ K} \pm 0.01 \text{ K}$. Although less precise temperature control may suffice MCD measurements, the control system improved temperature stability and more significantly, reduced offset drift/changes by keeping the column of gas in which the sample rod resides at constant pressure and thus to a constant temperature profile. This minimises movements of the sample associated with lengthening of the rod upon (overall) warming within the inner region of the cryostat.

Obtaining reliable and reproducible spectra at temperatures above 2 K required a careful approach. Between 2 and 4 K bubbling of liquid helium introduced noise. Above 4 K extremely good (better than $\pm 0.1 \text{ K}$) temperature stability was required. The entire sample column of gas above the sample needed to stabilise for a period of 10-30 min before reproducible spectra could be obtained. It was also necessary to record a baseline (0 T) spectrum at each temperature, as residual small shifts in the sample associated with changes in the length of the sample rod with temperature led to significant baseline offsets.

E: Sample near-IR inspection/photography

Being able to inspect a frozen sample to check for cracks, bubbles, leakage and defects (clumps of coagulated protein) is an important feature of our spectrometer system. Thick, concentrated PS II samples have extremely high optical density in the visible region, which precludes inspection with visible light. Additionally, illumination of the sample with visible light leads to reaction centre driven photochemistry. This in turn creates unwanted changes in the state of the redox centres in the sample and can induce changes in near-IR spectra due to carotene radical creation and changes in the deep red charge transfer state of PS II etc.

To visualise the sample without inducing photochemistry, a near-IR enabled Nikon D5000 DSLR, fitted

with a Tamron macro lens, was placed at the transverse window of the split pair of the superconducting cryostat, and was focussed on the sample. The Spex $\frac{1}{4}$ M double monochromator and lamp system, also used to induce fluorescence in samples or drive reaction centre photochemistry, as described in previous publications [2, 7, 8], was used to illuminate the sample with monochromatic radiation in the 800 nm -1000 nm region from the matching transverse window. The optimal wavelength required to visualise the sample is controlled by adjusting the wavelength setting of the double monochromator. In this way, any defects of the sample could be identified before making any spectral measurements. A permanent record of the cracking pattern in each sample was stored by means of the image obtained on the near-IR camera.



Figure SI_2.1a

Shown is a false colour image taken of a 1 mm thick PS II sample, as used in our PS II OEC measurements, taken by back illumination at 800 nm (see text). A mask 6 mm in diameter selects the region of the 12 mm diameter cell having optimal optical characteristics. The sample was at 1.8 K and exhibits a cracking pattern typically seen for our samples, as well as etalon fringing associated with detachment of the PS II glass from the surface treated quartz cell.

SI_2.2 Hydrophobic/lipophobic sample cells

We embarked on an investigative program so as to determine why samples cracked and became opalescent in unpredictable ways and to determine why different sections of a glassy sample exhibited significantly different levels of strain. We established that the critical driver of both strain and fogging characteristics of the sample was the degree of attachment of the frozen sample mixture to the cell, rather than being an intrinsic property of the glass itself. We subsequently developed a procedure to treat the cell with a transparent, robust coating that was *both* highly lipophobic as well as highly hydrophobic [10]. These new generation cells gave rise to frozen samples with ≈ 10 greater transmission than for an untreated cell and the same sample mixture. The treated cells also exhibit far less *strain and less variable* strain over the sample.

Strain-induced birefringence in a sample can, in severe cases, lead to distortions of CD spectra. Lower levels of strain can also lead to an apparent CD signal i.e. offset or baseline problems, even in regions of no absorption. This is because there is *invariably* a level of linear dichroism present in the detector and optical system of the spectrometer. This combines with strain birefringence to produce the CD offset. If the strain is not sufficiently homogeneous, microscopic movements of the sample with respect to the spectrometer beam, induced by energising the magnet, can result in artefacts in MCD spectra, as discussed in various parts of the manuscript and Supplementary Information. These artefacts can be of greater magnitude than the (relatively

small) MCD signal arising from the OEC (see Fig. SI_3.1a). As mentioned above, the transmission of samples was also affected by strain. Strain causes the sample to devitrify, resulting in a translucent (foggy) sample instead of a transparent glass.

To reduce the strain, we looked to apply a strongly hydrophobic coating on the inside of the sample cells used previously. The reduced surface energy resulting from this treatment enabled the sample to detach from either one or both sides of the quartz cell as it cooled and contracted.

The sample cells were prepared by treatment with a low-pressure RF plasma reactor containing water vapour. The power level used was 30 W for 60s. This generated an extremely clean quartz surface having exposed and highly reactive hydroxyl groups. A solution was prepared with 50uL of 1H,1H,2H,2H-perfluorooctyltrichlorosilane added to a dry mixture of 5 mL of hexadecane and 1 mL chloroform. Cells were filled with this solution and left for ~10 minutes. They were then rinsed with ethanol and baked at 100 °C for one hour. The chlorosilane undergoes a reduction reaction with hydroxyl groups on the surface of the quartz to form a monolayer that is both hydrophobic and lipophobic. This procedure formed a coating that did not significantly reduce the transmission of the quartz cell and the coatings were able to survive repeated freeze/thawing cycles down to 1.8 K, as well as being able to survive exposure to a variety of (cleaning) solvents.

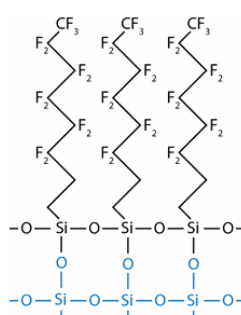


Figure SI_2.2a

Idealised structure of a hydrophobic monolayer (black) on the treated quartz surface (blue).

In principle, there is only a single self-assembled monolayer at the quartz surface [9]. After treatment and cleaning, areas of a white residue remained on the inside of the sample cells, due to polymerisation of the coating. However any scattering from these small residues was negligible. After applying the treatment, the effective transmission of the 1mm sample cell with the same sample, frozen to 1.8 K, increased from nominally 2-15% transmission at 800 nm for untreated cells to 45 - 55 % for the coated cells.

Table SI_2.2a

sample	Contact Angle		
	Advancing	Static	Receding
H ₂ O	111°	111°	45° ± 5°

Buffer	101°	85°	<10°
PS II Test sample	70°	68°	<10°

The contact angle of the coating on an isolated quartz window was measured (Table SI_2.2a) to quantify the effectiveness of the surface treatment. Advancing and receding contact angles were measured using a KSV CAM200 Goniometer instrument [10]. The buffer and cryoprotectant mix used was the same one used to dilute our PSII core samples, it contained ~50% 1:1 glycerol/ethylene glycol, 10mM Mes-NaOH (pH 6.0), 10 mM NaCl, 1.5 mM CaCl₂. The test sample was a mix of PSI and PSII recycled from earlier MCD experiments.

The contact angle of water on the treated quartz surface reproduced the value previously given in the literature for this coating [10]. For a clean (untreated) quartz surface, the contact angle for the test liquids was close to zero, and less than 10° for a surface having some residual protein residues (this replicates the situation when the cells are not effectively cleaned). The test sample had a significantly lower advancing contact angle than the buffer, which is likely due to the high concentration of protein present.



Figure SI_2.2b

The hydrophobic characteristic of the surface treated cell is immediately evident from the nature of the meniscus generated by the solution being transferred. The cell is being filled with a syringe with the PS II preparation at the concentration (~5 mgs/ml Chl-a) used in this paper. The sample rod and 6 ϕ mm mask is seen (left) in the background.

SI_2.3 Sample Illumination Systems and Protocols

The internal components of the YAG flash illuminator are shown in Fig. SI_S2.3a with the top casing of the unit removed for clarity. Laser pulses enter through the shutter aperture and are split 50:50 at the 45° splitter element. The transmitted fraction of the light then passes to the front of the sample (dark disc) whilst the reflected component is further reflected via three fused quartz prisms (acting as 45° reflectors) with 50% of the laser pulse illuminating the rear of the sample. Fig. SI_S2.3a also shows the two halves of the split sample shielding/transport unit with the sample rod placed in the shielded position.

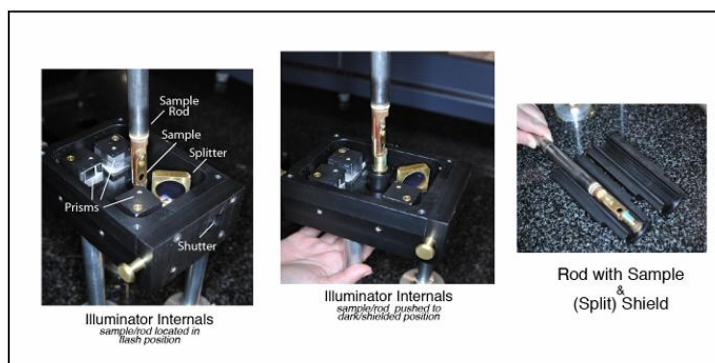


Figure SI_2.3a

Views of the YAG laser-flash illumination unit and sample rod with the light-tight lid removed, as well as the split shield sample transport unit (see text).

Fig. SI_2.3b illustrates the sequence where the sample is YAG laser-flashed, rapidly translated into the assembled split shield and then removed from the illuminator unit in a light-tight transporter unit. The temperature of the illuminator unit/sample can be controlled by passing gas at the appropriate temperature through the unit.

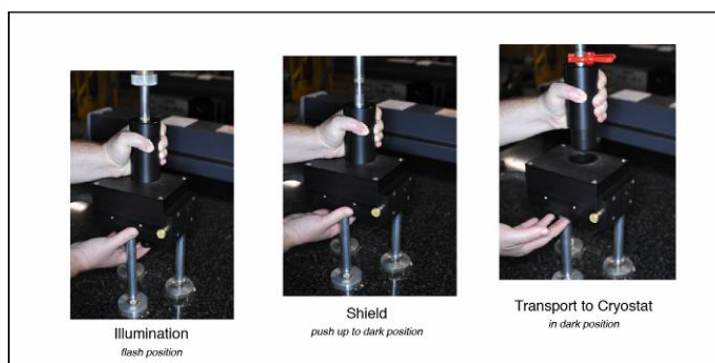


Figure SI_2.3b

The process of sample flash illumination, transfer of the sample to the removable and light-shielded sample/rod holder and the removal and transport of this assembly

The sample is transported to the cryostat in the light-tight shield and fitted on the cryostat lock as shown in Fig. SI_2.3c. The sample can then be lowered into the cryostat and cooled to 4 K within 1-2 minutes of the YAG flash without being influenced by any ambient light in the laboratory. The split lock can then be removed from the sample rod and the sample rod attached to the cryostat with a gas tight seal in the usual way.

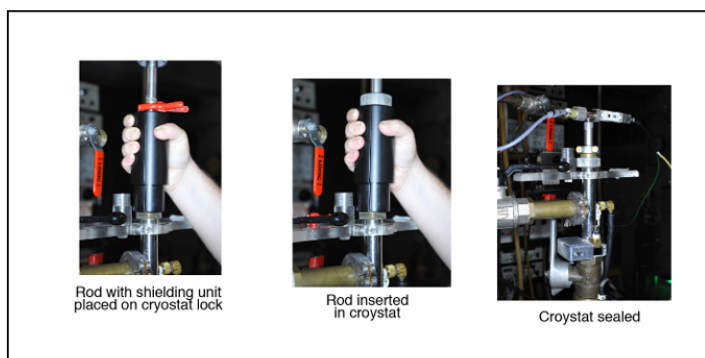


Figure SI_2.3c

Placement of the transportable sample/rod shield on the cryostat lock, cooling of the sample to cryogenic temperatures and sealing of the superconducting magnet cryostat (see text).

CW near-IR illumination was performed with a Schwartz EO Ti:S laser pumped by a Verdi-G laser. Any visible light contamination of the laser source was removed by directing the beam to a Littrow prism and then propagating the beam ~ 20 m to the spectrometer. This eliminated any visible light as well as a large fraction of the Ti:S laser fluorescence pedestal collinear with the laser beam. At the 20 m propagation distance the laser beam was ~ 1 cm in diameter and directed to the sample with plane mirrors. Any spurious room light was eliminated by a Schott RG-720 red pass filter, which was placed in front of the cryostat window.

SI_2.5 MCD and EPR S-state controls for *T. vulcanus* PS II

As mentioned in the introduction section of the manuscript, our initial near-IR MCD measurements exhibited two limitations [ref]. The two key difficulties were that

- The MCD 'zero level' could not be experimentally determined and consequently could only be modelled.
- It was uncertain as to whether S-states other than S_1 contributed significantly to near-IR MCD spectra.

Both of these difficulties have now been resolved by means of the improved spectrometer, effective illumination systems and our hydrophobic/lipophobic quartz cell design.

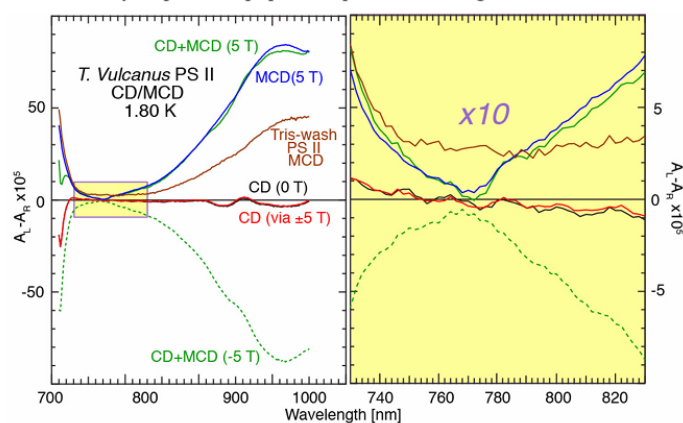


Figure SI_2.5a

The CD (black) is shown along with MCD+CD with alternate 5 T and -5T applied fields (solid and dashed green traces, respectively). Spectra were taken at 1.80 K of a ≈ 5 mg/ml PS II preparation of *T. vulcanus* poised in the S_1 state (see text). The red trace is the CD spectrum calculated by averaging the CD+MCD measured at 5 T and -5 T fields. The blue trace is the corresponding MCD spectrum generated by averaging the subtraction of 5 T and -5 T spectra. The brown trace is the 1.80 K, 5 T MCD spectrum of a Tris-washed PS II preparation of equivalent Chl-a concentration, in which the Mn_4CaO_5 cluster and cyt c_{550} are removed. The right hand panel provides expanded detail in the 730 nm- 830 nm region.

The sensitivity, precision and reproducibility with which MCD spectra can now be measured in the 710 nm – 1050 nm near-IR range is demonstrated in Fig. SI_2.5a. MCD measurements at wavelengths shorter than 710 nm are not practical, because of the rapidly-increasing optical density of the sample. If the sample were made more dilute, MCD signal strengths would become impractically low. There is also the issue of actinic effects of measurement light driving PS II reaction centre photochemistry. For PS II at low temperatures, measuring an absorption spectrum can lead to changes in the absorption spectrum itself. This process is known [7] to change the intensity of the charge transfer state of PS II and its subsequent B-term MCD [3]. Displayed in Fig. SI_2.5a is an *unadjusted* CD spectrum of PS II in the S_1 state, along with ‘raw’ MCD+CD spectra, taken in +5 T and -5 T applied fields. The CD spectrum shows a negative signal at 712 nm associated with the PS I ‘red trap state’. The amplitude of this CD signal serves to quantify the $\approx 1\%$ PS I contamination of our PS II preparation.

The CD spectrum generated by adding the spectra taken at +5T and -5T (thus nulling the MCD component) is *superimposable* on the CD spectrum taken at 0 T. The MCD spectrum thus inverts *quantitatively* with the direction of the applied field. There remains no doubt that the MCD spectrum zero level has now been correctly determined.

We can also demonstrate that illumination protocols described (Section 2.3) to prepare the samples in the S_1 (and higher) states are indeed robust. The spectral profile of the MCD in Fig. SI_2.5a, apart from an offset, is almost identical to that previously reported (Fig. SI_2.5b). A close comparison of spectra in the 770 nm region with those previously published, points to the presence of a very small secondary component in previous spectra. This can be attributed (see Section 3.4) to the presence of a small amount ($<5\%$) of the S_2 state. To ensure that the relatively narrow feature at 772 nm was *not* associated with minor population of S_2 , a PS II sample was pre-flashed and then dark-adapted for 24 hours at 20⁰ C. The subsequent S_1 MCD spectrum was *identical* to that obtained when the sample was dark-adapted for 1 hour. Thus the weak, narrow feature at 770 nm can be confidently attributed to the S_1 state of the OEC in *T. vulcanus*. These new data (Fig. 1) help establish that the LMCT bands of PS II in *T. vulcanus* extend throughout the 730 nm - 800 nm region, extending to the region of Chl-a based signals at shorter wavelengths.

A further control experiment was performed on S_1 to exclude the possibility of near-IR photoactivity in the sample in this S-state. MCD spectra taken before and after 140 K illumination with 200 mW of 775 nm laser light for 10 minutes were compared. There was no detectable change.

The MCD spectrum of a Tris-washed PS II sample, having a Chl-a concentration similar to the active PS II sample, but stripped of its Mn_4CaO_5 cluster and cyt c_{550} , is also shown in Fig. SI_2.5a. This spectrum has a

positive B-term MCD at short wavelengths (arising from Chl-a), which is similar to that seen in the oxygen-evolving sample. The Tris-washed sample has $\approx 1/2$ the LMCT signal, as monitored near 1000 nm. This reflects the fact that one of the two cytochromes of *T. vulcanus* PS II has been removed by means of the Tris-wash. A detailed study of cyt c_{550} in PsbV isolated from *T. elongatus* will be published in a forthcoming article.

The Tris-washed PS II MCD spectrum shown in SI_2.5a establishes that there is no equivalent to the 770 nm feature in the Mn-depleted PS II preparation. The 740 nm to 820 nm spectral region is featureless and confirms that the structure seen is due to the OEC in the S_1 state. The Mn-depleted sample does however exhibit an extremely broad, flat positive MCD signal throughout the important 710-800 nm spectral region. This signal is field dependent, saturating towards 5T at 1.80 K. Detailed field and temperature dependencies for the Tris-washed sample are provided in Figs. SI_2.5c,d.

An indication as to the source of the broad underlying feature is provided by changes in spectra of Mn-depleted samples when pre-flashed, using the same protocols used for the oxygen-evolving PS II samples. Little change is seen in the MCD of Chl-a and LMCT features but there is a ≈ 10 -20% increase in the background signal in the 740 -830 nm region. Also, in oxygen-evolving PS II samples which had been YAG laser-flashed many times, there is a (smaller) build-up of a similar broad, positive and paramagnetic signal. We thus attribute this broad background signal in both active and Mn-depleted PS II preparations to a build-up of a (high-spin) ferrous heme form of cyt b_{559} . The MCD of a high-spin ferrous form of cyt b_0 from *E. coli*, which has similar properties [11] to cyt b_{559} displays just such a broad spectral feature in this spectral region.

The remaining inorganic cofactor of PS II that could potentially contribute to MCD in the near-IR spectral region is the non-heme Fe(II) at the RC acceptor side. This chromophore is expected to exhibit a spin-allowed d-d crystal field transition of the d^6 configuration of octahedral Fe(II). Related non-heme Fe(II) complexes have been studied [12] and exhibit a broad positive MCD peak around ≈ 1000 nm with full width at half maximum (FWHM) of ≈ 150 nm and a magnitude at 5T 1.8K MCD of $\Delta\epsilon \approx 2 \text{ M}^{-1} \text{ cm}^{-1}$. In PS II, this non-heme Fe(II) MCD signal will be subsumed by the prominent cytochrome LMCT 'tail' feature in Fig. 1. This LMCT signal has comparable width to a non-heme Fe(II) band but has an order of magnitude stronger MCD at 5T and 1.8 K, having a $\Delta\epsilon \approx 20 \text{ M}^{-1} \text{ cm}^{-1}$ at 1000 nm.

To confirm that the non-heme Fe(II) does not contribute to S-state MCD spectra significantly, Tris-washed PS II, in which the non-heme iron remains in the native (Fe(II)) form, was treated with ferricyanide. This process is known to oxidise the non-heme Fe(II) to the Fe(III) form. The oxidised form would not be expected to give rise to a near-IR MCD signature. However, ferricyanide may also lead to the oxidation of the high-spin ferrous form of cyt b_{559} and thus reduce any (broad) MCD associated with this species. Ferricyanide treatment of Tris-washed PS II does lead to some reduction in the broad background MCD signal, as well as leading to the generation of distinct MCD features [3] arising from $P700^{++}$. As the signal background in Tris-washed PS II is not fully eliminated, and as it is almost absent in oxygen-evolving PS II, an assignment of the background signal to a high-spin Fe(II) form of cyt b_{559} is justified. EPR control measurements also show that

the high-spin Fe(III) form of cyt b_{559} is significantly more intense in Tris-washed samples, consistent with these observations

The S_1 MCD data previously published [3] can be made consistent with the current data by systematically offsetting spectral curves so as to be compliant with the zero levels established in our current series of measurements.

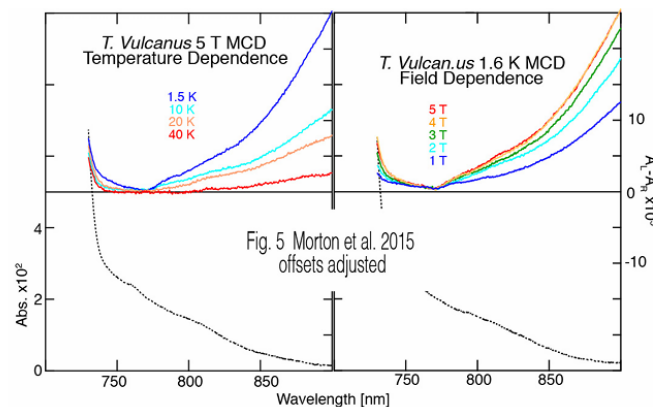


Figure SI_2.5b

This figure shows MCD spectra of *T. vulcanus* by applying appropriate offsets to the data from Fig. 5 in [3] for each field and temperature so as to be compatible with the accurately determined (M)CD zero levels in the current work.

The field dependence of the MCD of Tris-washed PS II is presented in Fig. SI_2.5c. Spectra are shown for a full set of magnetic fields from -6 T to 6 T. The sign of the magnetic field refers to its direction relative to the propagation direction of measuring light. The field dependence seen and mirror symmetry between positive and negative fields confirms that the broad, flat MCD between 750 nm and 800 nm is both real and paramagnetic in nature, as it saturates strongly between 5 T and 6 T.

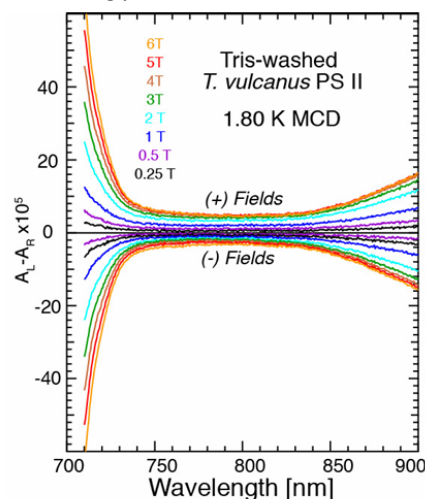


Figure SI_2.5c

Field dependent MCD of Tris-washed *T. vulcanus* PS II core complexes in forward and reversed applied magnetic fields. Spectra are all taken at 1.80 K.

The temperature dependent MCD of the Tris-washed PS II sample (Fig. SI_2.5d) uncovers a positive B-term component in the 750 nm - 850 nm region, which is far less noticeable in the oxygen-evolving PS II core samples. The low energy charge transfer state in PS II has a long-wavelength positive B-term of comparable magnitude but extends only to ≈ 730 nm. One possibility is that the equivalent deep red state [3, 7] moves to longer wavelengths in this treated sample, giving rise to the B-term extending to significantly longer wavelengths.

Spectra of the Tris-washed sample serve to highlight that cyt b_{559} LMCT MCD extends to ~ 830 nm in *T. vulcanus*. At shorter wavelengths the cyt c_{550} LMCT MCD component as seen in intact *T. vulcanus* PS II dominates. Note that the 710 nm - 730 nm temperature dependent component is similar to that seen in intact PS II (Fig. 4 of the main article) and is likely due to cyt b_{559} and not cyt c_{550} .

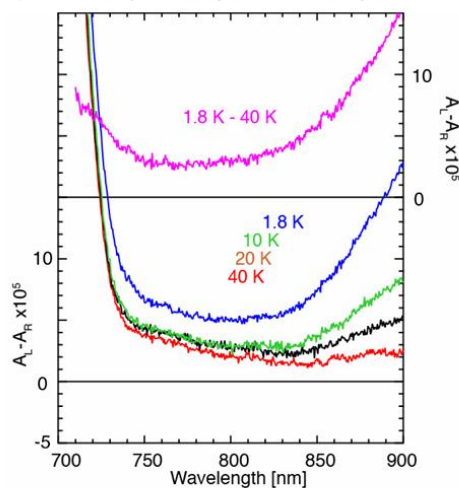


Figure SI_2.5d

Temperature dependence of the MCD of Tris-washed PS II from *T. vulcanus* at 5 T at the temperatures indicated. The purely temperature-dependent component is given in the upper (magenta) trace.

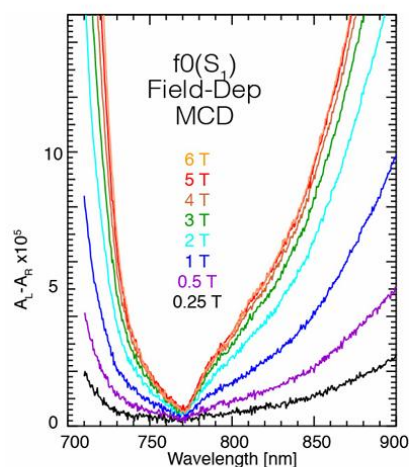
SI_3.2 VTVH MCD spectra

Fig. SI_3.2a

High sensitivity field dependence of the MCD of PS II of *T. vulcanus* in the S_1 state at 1.80 K at fields up to 6 T.

The utility of measurements at low applied magnetic fields, when the temperature of the sample is less than 1.8 K, is demonstrated by the divergence of simple isotropic models for saturation and a weakly diamagnetic exchange coupled case in Fig SI_3.2b. Lower temperatures can be achieved by installing a more powerful pump and having larger diameter pump lines and control valves. Control of the temperature to ± 0.1 C is likely to be equally important to maintain stability of the thermal profile of the sample column in the cryostat and avoid drifting offsets.

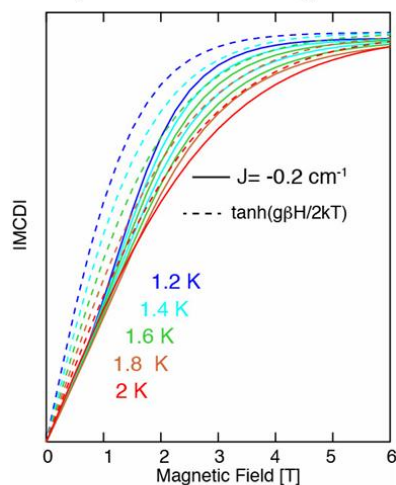


Figure SI_3.2b

Comparison between simple isotropic $g=2$, $S=1/2$ MCD saturation behaviour (dashed lines) between 1.2 K and 2 K compared to the behaviour of the $J = -0.2 \text{ cm}^{-1}$ exchange coupled model (see main article). The curves are normalised at 1.8 K and 5 T for the two models.

The B-term component of $f_0(S_1)$, $f_1(S_2)$ and $f_2(S_3)$ spectra is highlighted in Fig. SI_3.2c. Shown are MCD spectra at 6 T as well as the difference spectra taken at high fields. For (6 T - 5 T) spectra, the paramagnetic MCD component is minimal, as spectra at 5T are close to being saturated. These difference spectra are virtually identical. This establishes not only that the B-terms associated with Chl-a contributions between 710 nm and 730 nm are not changing, but also that no new B-term MCD components are generated in the S_2 or S_3 state. This is in contrast to the S_0 state in which an unidentified and strong negative B-term feature is generated by the third flash (Fig. SI_3.2h).

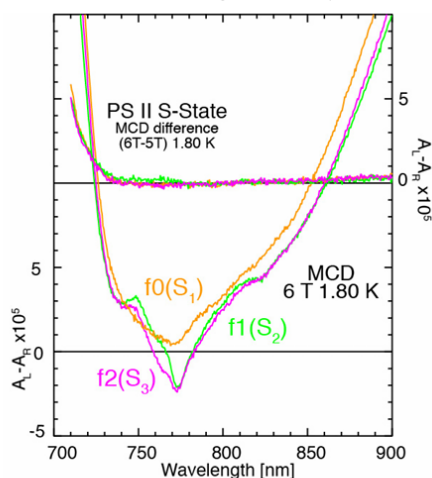


Figure SI 3.2c

The 6 T MCD spectra for $f_0(S_1)$, $f_1(S_2)$ and $f_2(S_3)$ are shown (lower traces) along with (6 T - 5 T) difference spectra for these configurations (upper traces).

Near-IR illumination of the $f_1(S_2)$ configuration at 140 K leads to noticeable changes in the resultant $f_1^*(S_2)$ configuration. These are clear in the field dependent spectra in Fig. SI_3.2d. A significant lowering in MCD intensity in the 750 nm region is obvious.

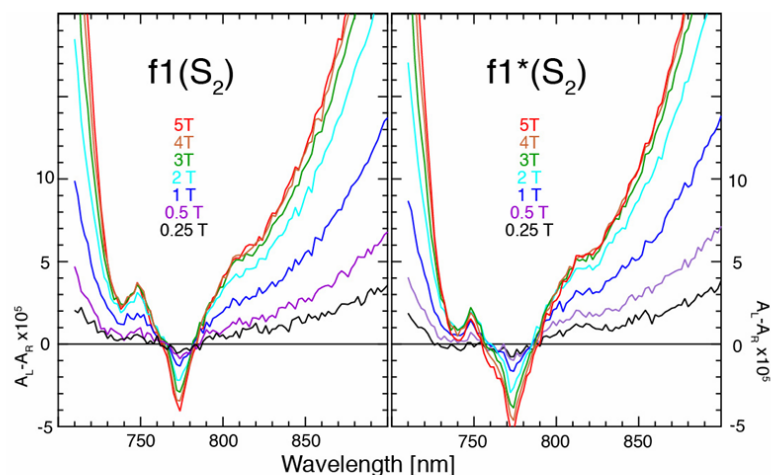


Figure SI_3.2d

Displayed are the field dependent MCD spectra, taken at 1.80 K of the PS II sample in the $f1(S_2)$ configuration and then after near-IR illumination (then labelled as the $f1^*(S_2)$ configuration). Ti:S CW laser illumination was at 775 nm, using 100 mW of laser power for 10 minutes. The sample was illuminated at 140 K in the system cryostat and lowered to 2 K over a 20 min period.

The changes in moving to the $f1^*(S_2)$ configuration are made clearer in Fig. SI_3.2e where MCD difference spectra [$f1^*(S_2) - f1(S_2)$] generated at both 2 T and 5 T are compared. The major change is revealed as a broad negative MCD feature extending to our short wavelength limit of 710 nm. This broad MCD feature has the same amplitude (to within experimental error) at both 2 T and 5 T. Thus this feature is consistent with being associated with the S_2g4 state of PS II.

EPR controls have shown that the yield of S_2g4 is low in our samples, which is in turn consistent with there being a barely visible bleach of the S_2 spin flip bands. This suggests that the broad, high-g S_2g4 feature seen at shorter wavelengths is far stronger than the multiline $g=2$ feature it replaced. A bleach of multiline S_2 is barely visible (Fig. SI_3.2e upper panel) and is <10% of the total S_2 amplitude.

The generation of a broad high-g feature at higher energies by means of 140 K near-IR illumination suggests that this band is a feature of the S_2g4 S-state and associated with a spin-allowed band of Mn(III), as is seen in many MnCat model compounds [13] [14].

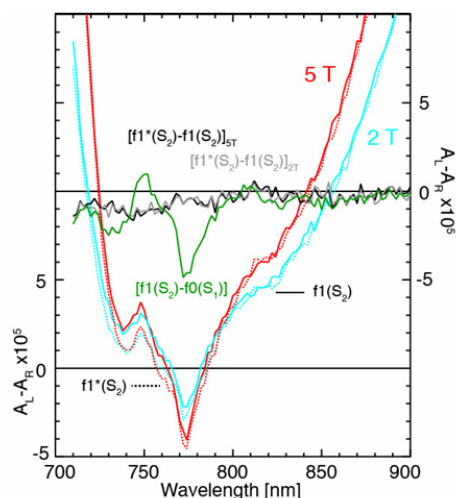


Figure SI_3.2e

Displayed (lower) are the MCD spectra from Fig SI_3.4d of PS II sample in the $f1(S_2)$ and $f1^*(S_2)$ configurations at 2T and 5T. The upper traces show the corresponding MCD differences $[f1^*(S_2) - f1(S_2)]$ at 5T (black trace) and 2T (grey trace). The green trace is $[f1(S_2) - f0(S_1)]$, which approximates an S_2 MCD spectrum, is shown for comparison.

Upon near-IR illumination of the $f2(S_3)$ configuration at 1.8 K (Fig. SI_3.2f), measureable changes in the MCD spectra are seen.

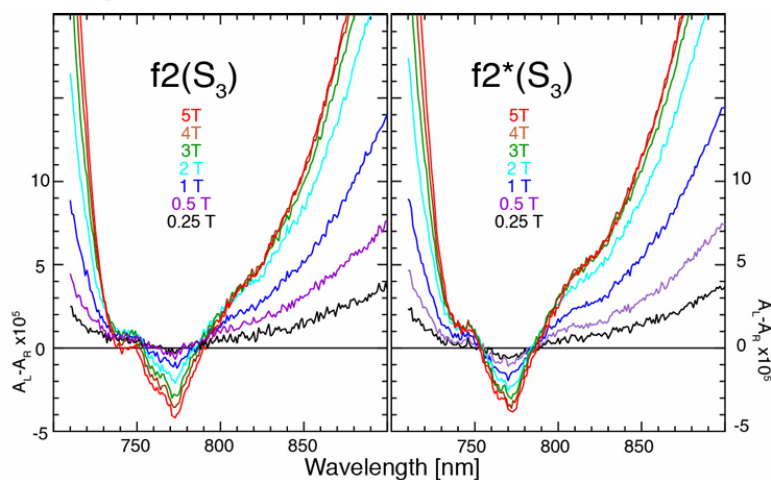


Figure SI_3.2f

Displayed are the field dependent MCD spectra, taken at 1.80 K of the PS II sample in the $f2(S_3)$ configuration and then after near-IR illumination (then labelled as the $f2^*(S_3)$ configuration). Ti:S CW laser illumination was used at 775 nm, with 100 mW of laser power for 10 minutes. The sample was held in superfluid helium near 1.8 K during illumination.

These changes are highlighted in the difference spectra in Fig. SI_3.2g. When compared to the OEC-based S_2 spectrum provided for comparison, it appears that there is a pattern that can be

interpreted as a reduction in OEC-based S_2 MCD upon illumination. The field dependence of this reduction would further point to it being a $g=2$ $s=1/2$ component. There is a positive feature at shorter wavelengths that does not seem to be paramagnetic. However, there is no evidence for a change in the S_3 component upon illumination. EPR control experiments also indicate that the S_3 near-IR photoactivity in our samples is minimal.

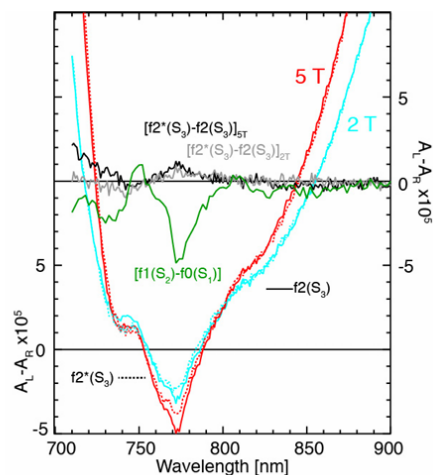


Figure SI_3.2g

Displayed (lower) are the MCD spectra from Fig SI_3.5b of PS II sample in the $f2(S_3)$ and $f2^*(S_3)$ configurations at 2T and 5T. The upper panel show the corresponding MCD differences $[f2^*(S_3) - f2(S_3)]$ at 5T (black trace) and 2T (grey trace). The green trace is $[f1(S_2) - f0(S_1)]$ which approximates an S_2 MCD spectrum is shown for comparison.

One possibility is that illumination of the S_2 component of the $f2(S_3)$ sample leads to a substantial bleach of S_2 due to the formation of the split S_1 -TyrZ⁺ system [15] [16], which would then need to be metastable at 1.8 K.

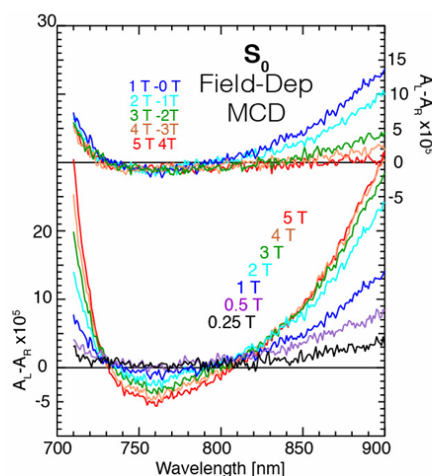


Figure SI_3.2h:

MCD field dependence of the S_0 component (lower traces) of the $f3(S_0)$ spectrum of PSII, corrected for S_3 and S_2 content and rescaled (see text) along with step-wise MCD increments of the MCD (upper traces) for (1T-0T), (2T-1T), (3T-2T), (4T-3T) and (5T-4T).

The shape of the negative B-term induced by YAG laser-flashing S_3 to S_0 becomes quite apparent when the positive B-terms arising from Chl-a is subtracted. This subtraction, and elimination of most of the cytochrome signals is achieved by taking $f3(S_0)$ spectra at each magnetic field, correcting them for S_2 and S_3 content and then scaling for 100% S_0 yield, as described in the manuscript. These corrected S_0 spectra can then be used to create $[S_0-S_1]$ spectra at each applied magnetic field H . These field dependent spectra are displayed in Fig. SI_3.2i, along with an S_1 spectrum for comparison.

This set of MCD difference spectra clearly reveal the build-up of a broad feature, linear in magnetic field, extending from an extreme value near the limit of measurement at 710 nm, to being close to zero near 900 nm. There is a small non-linear (+) feature evident near 770 nm in this set which is the (inverted) S_1 OEC MCD introduced by the subtraction of S_1 MCD in the procedure.

A broad B-term MCD in the near-IR region is characteristic of Chl-a radicals and β -carotene, but these radicals have the opposite MCD sign [3, 17]. The charge transfer state in PS II, also has a positive B-term MCD signal [8]. At this stage, it is not possible to assign the B-term MCD to the Mn_4CaO_5 cluster as the field and temperature dependence of the feature is diamagnetic and the S_0 state is known to be paramagnetic.

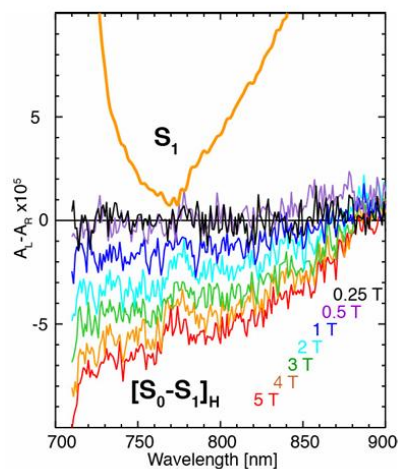


Figure SI_3.2j

Field dependent MCD differences $[S_0 - S_1]$ (lower traces) along with an S_1 reference spectrum recorded at 5T. All spectra are taken at 1.80 K.

So as to further establish the bona fides and characteristics of the S_0 state, MCD spectra of the sample were taken after four flashes. These are compared to the three-flash MCD spectra in Fig SI_3.2j. They show a significant reduction in the B-Term (pseudo)-peak near 760 nm upon flashing the sample four times. Four flashes have clearly advanced a significant proportion of S_0 to S_1 , as discussed in the manuscript.

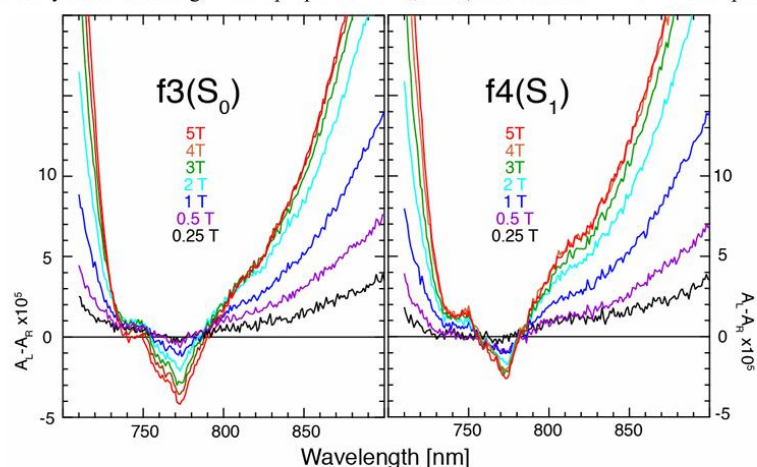


Figure SI_3.2k Field dependent MCD of *T. vulcanus* PS II core complexes, measured after three and four YAG laser-flashes. Spectra are all taken at 1.80 K.

SI 3.3 Determining OEC-based spectra of each S-state

Fig. SI_3.3a displays a set of unadjusted subtractions of low noise data for $[f1(S_2) - f0(S_1)]$ at fields up to 6 T at 1.80 K. The resolution of the spectrometer is ≈ 2 nm and thus the ≈ 10 nm FWHM features seen are fully resolved. The sub-structure seen around the three peaks at is somewhat variable. This may be due to S_2 arising

from centres in which the structure of the Mn_4CaO_5 cluster varies slightly.

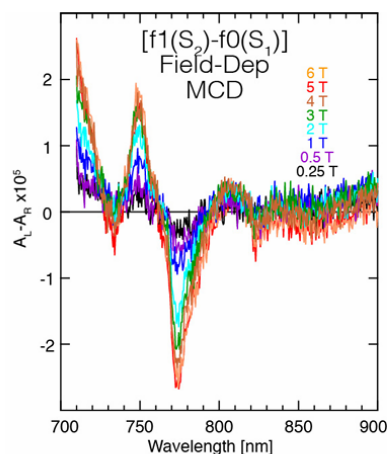


Fig. SI_3.3a

MCD spectral changes upon advancing from S_1 to the S_2 state of PS II, as described in the text as $[f1(S_2)-f0(S_1)]$, as a function of magnetic field.

Systematic procedures were developed to determine the OEC-based S_3 MCD spectrum S_3^{OEC} over the full spectral range. One approach is to use the distinctly different saturation behaviour seen for S_2^{OEC} and S_3^{OEC} . A complicating factor is that the saturation behaviour of the cytochrome tail is similar, but not identical to that of S_2^{OEC} .

As the Chl-a B-terms do not change significantly upon flash advancement and the cytochrome C-terms only vary fractionally, all the B-terms and a dominant fraction of the cytochrome MCD components can be eliminated by subtracting $f1(S_2)$ from $f2(S_3)$ spectra to create MCD difference spectra $\Delta(3-2)_H$ at each applied magnetic field H .

Defining

$$\Delta(3-2)_H = [f2(S_3) - f1(S_2)]_H = [y_3 \cdot S_3^{\text{OEC}} + (1-y_3) \cdot S_2^{\text{OEC}}]_H \quad (1)$$

Where y_3 is the yield of S_3 .

Similarly we can generate $\Delta(2-1)_H$

$$\Delta(2-1)_H = [f1(S_2) - f0(S_1)]_H = [y_2 \cdot S_2^{\text{OEC}} + (1-y_2) \cdot S_1^{\text{OEC}}]_H \approx [y_2 \cdot S_2^{\text{OEC}}]_H \quad (2)$$

At 2 T, S_3 is close to saturation, whereas in S_2 it is not.

$$\delta_{6-2} = [\Delta(3-2)_{6T} - \Delta(3-2)_{2T}] \quad (3)$$

The double difference has minimal contribution from S_3 but a significant amplitude arising from S_2 . The contribution of S_2 to $\Delta(3-2)_{2T}$ can be corrected for, independently of any assumption for the yield y_3 .

The saturation curve for the isotropic $g=2$ $S=1/2$ case appropriate to S_2 shows that the amplitude at 2 T is 65% of that at 6 T. Subtracting $(0.65/0.35)$ times δ_{6-2} provides the S_3 line shape, independent of yield. This lineshape is as the pink curve in Fig. SI_3.3b.

A second procedure that leads to less noisy spectra is to use the yield of S_3 , as determined via a graphics-based determination of the magnitude of the narrow and distinctive S_2 features still remaining in $f2(S_3)$.

As the $y_3=0$ yield amplitude of S_2 is provided by $\Delta(2-1)_H$, multiplied by the known yield of S_2 , the S_2 component in $\Delta(3-2)_H$ can then be subtracted to provide an estimate of S_3^{OEC} . A comparison of S_3^{OEC} determined by these procedures is shown in Fig. SI_3.3b. They lead to similar lineshapes and amplitudes. The yield-based spectrum determined by difference spectra taken at 2 T and the saturation-based spectrum are less prone to errors.

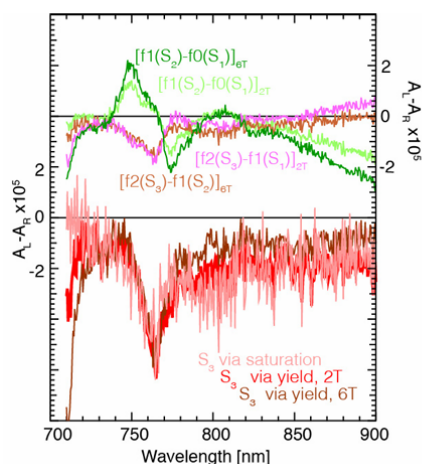


Fig. SI_3.3b

The top panel shows difference spectra (see text) determined at the magnetic fields of 6T and 2T. The lower panel has the resultant OEC-based S_3 MCD spectrum, scaled for 100 % yield from S_2 and saturation conditions, as determined by either subtracting distinctive S_2 features and determining the S_3 yield, or by utilising the very different saturation behaviour of S_2 and S_3 as indicated.

SI_3.4 Narrow Mn(IV) features in the Near-IR MCD of Model Compounds

A series of Mn(III)-Mn(IV) dimer compounds[13] modelling MnCat, displayed Mn(IV) spin flips between 806 nm and 820 nm. We reproduced the previously reported MCD data for $[Mn_2O_2(bpy)_4]^{3+}$ with somewhat better signal to noise ratio. The spectrum shows a clear negative spin flip at 842 nm, built upon a broad background of Mn(III) spin-allowed transitions. We also measured a related dimer[18], DTNE ($[dtneMnIIIMnIV-(i-O)2i-OAc](BPh_4)_2$ (dtne) 1,2-bis(1,4,7-triazacyclonon-1-yl)ethane), which shows a weaker negative spin flip at 880 nm. The nephelauxetic effect for the DTNE system appears to be significantly larger than for most other model compounds.

We also measured the oxidized cubium⁺ [19], a cubane-like $[L_6Mn_4O_4]^+$ system with (L = bis(4-methoxyphenyl)phosphinate). This displays clear Mn(IV) spin flips at 683 nm and 709 nm. These bear a marked resemblance to the OEC features in S_2 but occur at higher energies and have their relative signs inverted.

Measurements of the model compounds and oxidized cubium were made at 1.6 K and an applied magnetic field of 5 T. The glassing medium used was 4:5 propionitrile/butyronitrile (v:v), which was purged with argon shortly before use.

SI_4.2 Magnitude and sign of spin-flip MCD

The enhancement of spin-forbidden d-d transitions by magnetic exchange interactions was first reported in 1959 by means of measurements on the fluorescent ${}^2E \rightarrow {}^4A$ spin flip transition in statistical Cr^{3+} clusters as seen in heavily doped Al_2O_3 (i.e. ruby) [20]. This intensity enhancement phenomenon has since been seen in many systems [21] [22] [23] and is well documented [24] [25] [26]. The magnetic exchange-induced increase in spin-forbidden transition intensities is also seen for molecular dimers and molecular clusters (e.g. basic and acid-rhodo Cr(III) dimers [26]) and also for magnetically ordered, transparent materials such as RbFeF_3 [27]. Intensity enhancements are seen for both similar and dis-similar exchange coupled ions and for both spin-flips as well as d-d excitations involving orbital changes. The enhancement factors observed experimentally vary significantly. The enhancement has been related to the energy and intensity of electron transfer processes coupling the two ions.

A theoretical basis for the effect was developed [28], which modelled the electric field of incident radiation to modulate magnetic exchange interactions between the two metal ions, in both ground and excited states. This mechanism then serves to relieve both the spin selection rule *and* the parity forbidden nature of single ion electric-dipole d-d transitions.

The first report [29] of MCD in an exchange coupled system established that for the system studied, statistical Mn^{2+} clusters in KZnF_3 , exchange coupling led to significantly enhanced absorption intensity for (linear) $\text{Mn}^{2+}\text{-F}^-\text{-Mn}^{2+}$ exchange coupled ions in the cubic lattice, but that their MCD was weak, with single ion (monomer) MCD dominating the spectrum. A weak dimer MCD observed is consistent with the Tanabe exchange-coupled electric-dipole mechanism, as intensity is essentially stolen from a metal-to-metal charge transfer excitation. The charge transfer process is constrained by the structure of cubic perovskites to be dominantly linearly polarized and along the metal-metal axis. A purely linearly polarized transition cannot give rise to MCD [30].

MCD measurements taken at higher sensitivities and with dissimilar ions doped in perovskite fluorides established that weak MCD (relative to absorption) could be seen for exchange-coupled ions. It was later shown that this MCD was due to an ‘interference’ term between single ion and pair transitions [31]. A similar process can be used to explain the (relatively weak) MCD of intervalence charge transfer bands [32] [33], which are also nominally linearly polarized and would thus be expected to have zero MCD in the simplest case.

The MCD seen for spin-flips in the OEC, in MnCat and model compounds as well as in cubium⁺, although being variable in sign and excitation energy, appear to be quite strong relative to absorption. The ‘interference’ MCD mechanism may not suffice to provide sufficient MCD intensity (relative to absorption) for the current systems, as it scales as the *product* of the single ion and pair transition strengths. The single ion

intensity for spin-flips in Mn(IV) is very low. However, interaction pathway(s) between the two metal ions in these systems is qualitatively different to the (linear) metal-ligand-metal super-exchange pathway in perovskites. The bridging μ -oxo/hydroxo metal-metal links in our systems offer both the possibility of direct exchange between the metal ions as well as several (angled) super-exchange pathways through the bridging ligands. This distinctly different characteristic is a likely source of both the variable sign and strong magnitude of the MCD observed. Further theoretical development is underway in our laboratories.

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9.4 Supplementary to Publication 2

This section contains only data from our group along with supporting text.



www.sciencemag.org/content/360/6394/1210/suppl/DC1

Supplementary Materials for

Photochemistry beyond the red limit in chlorophyll f-containing photosystems

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Published 15 June 2018, *Science* **360**, 1210 (2018)
DOI: 10.1126/aar8313

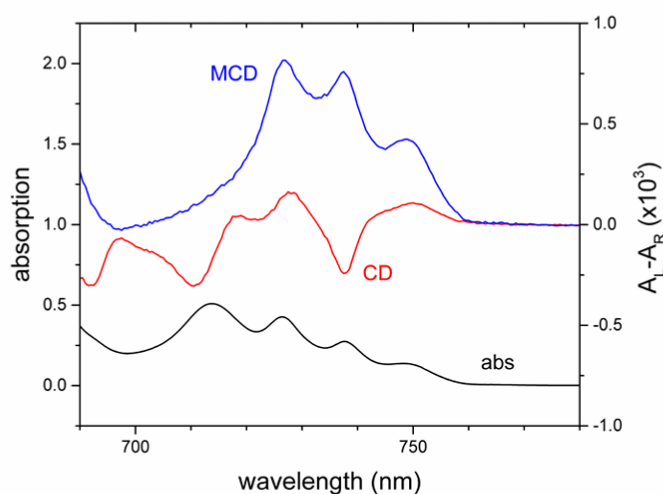
This PDF file includes:

Materials and Methods
Supplementary Text S1 to S7
Figs. S1 to S20
Tables S1 to S3
References

Other Supplementary Material for this manuscript includes the following:
(available at www.sciencemag.org/cgi/content/full/360/6394/1210/DC1)

Data S1 as Excel file

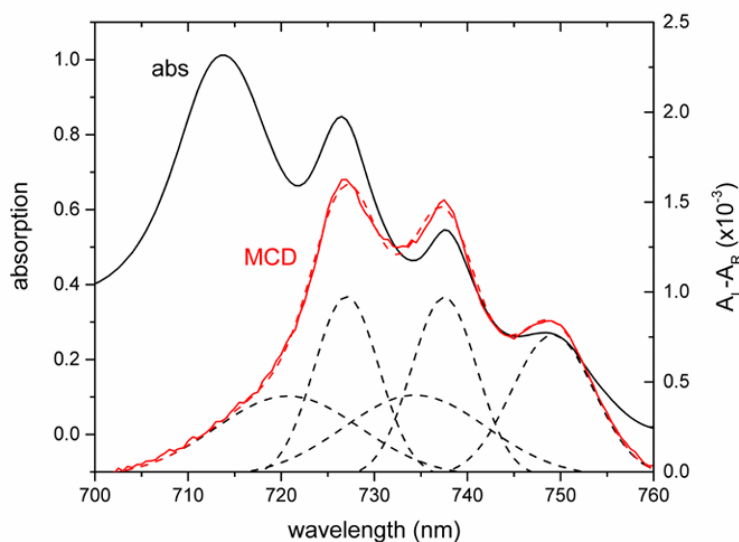
Fig. S6.



Low-temperature (1.8 K) absorption (black), MCD (blue) and CD (red) spectra of isolated FRL-PSII.

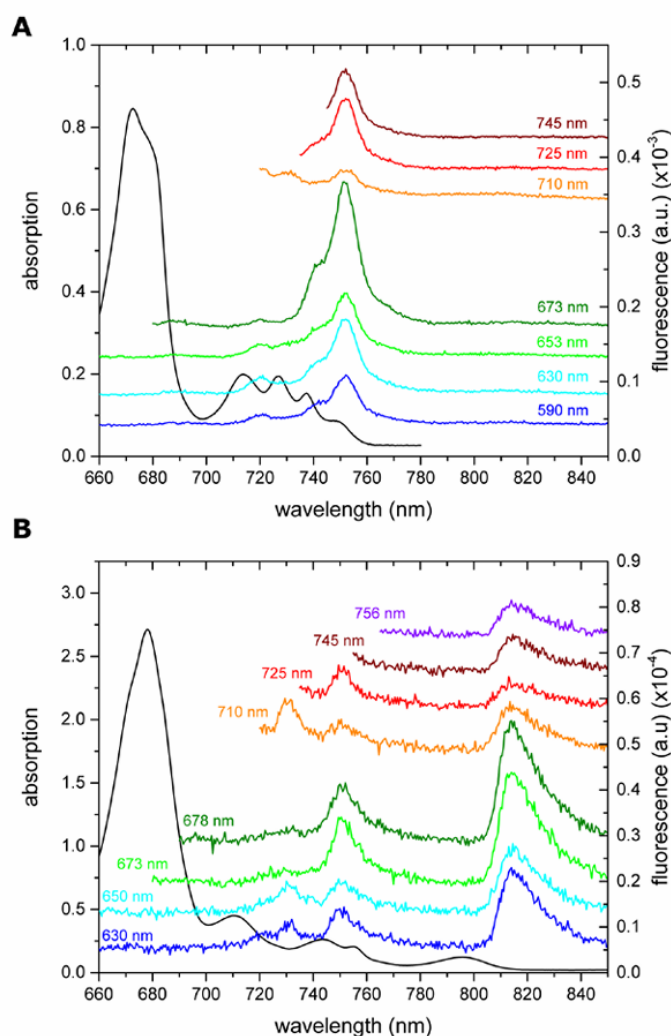
The low temperature absorption spectrum shows four peaks in the long-wavelength region at 712 nm, 727 nm, 737 nm and 748 nm. The 714 nm peak is strongly reduced in the MCD spectrum confirming the assignment of the peak to long-wavelength allophycocyanin. In the CD spectrum the excitonic at (-) 712 nm and (+) 719 nm and also at 794 nm are attributed allophycocyanin. Note that unlike the sample used in Figure 2, this sample had not undergone the ion exchange chromatography step and so showed more contamination from allophycocyanin.

Fig. S7.



Low temperature (1.8 K) MCD (red) and absorption spectrum (black) of isolated PSII and spectral analysis.

The MCD and absorption spectra were obtained at 1.8 K using isolated PSII as described in the materials and methods. Note that the contaminant allophycocyanin with an absorption peak at 712 nm does not show a MCD peak. Thus the MCD spectrum was used first to determine the contributions from five long wavelength chlorophylls. The analysis took into account the different intensities of some peaks in the MCD vs their absorption and this allowed the contributions from the two broader unresolved peaks to be determined. The following results were obtained: FRL-chlorophylls were present at 721, 727, 734, 737 and 749 nm with approximately equal contributions. The individual MCD line-shapes are shown as dashed black lines and the cumulative MCD spectrum is shown as a dashed red line which is compared with the experimental spectrum (solid red line).

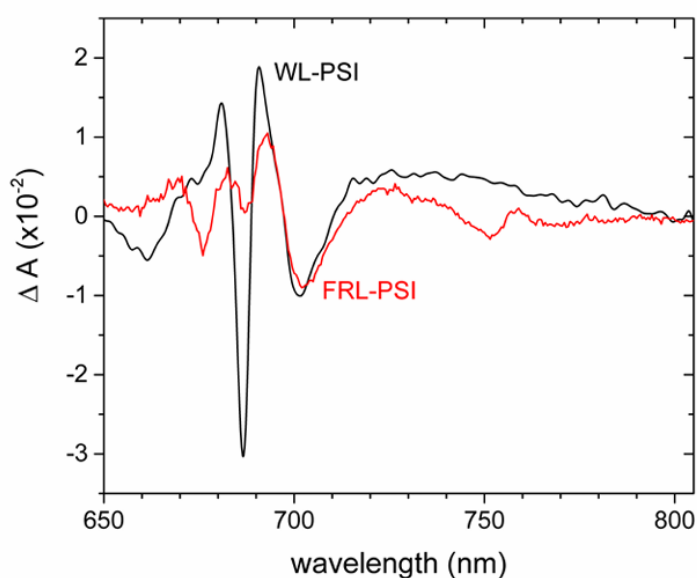
Fig. S10.

Excitation dependence of fluorescence emission at 77 K in isolated FRL-PSII (A) and FRL-PSI (B).

Fluorescence spectra at 77 K were collected at a series of wavelengths from 590 nm to 756 nm using matched excitation intensities ($\sim 6 \mu\text{M photons cm}^{-2}$). The black spectra are the absorption spectra at 77 K of the test sample. Note this preparation had a marked contamination from long-wavelength allophycocyanin (absorption peak at 712 nm) as it had not undergone the anion exchange chromatography step used in most of the other experiments. For PSII the maximum emission is at 755 nm at all excitation wavelengths. The 755 nm emission is lowest when exciting at 710 nm, suggesting that the allophycocyanin (712 nm) is poorly connected to PSII in terms of exciton transfer. The emission peak at 720 nm could be from allophycocyanin but there is also a shoulder at 740 nm which has previously been attributed to the emission from this pigment (63). The 755 nm emission is bigger when excited at 673 nm, the peak of chl *a* absorption, and there is very little chl *a* fluorescence (685-700 nm), indicating that essentially

all the chl*a* is connected directly or indirectly to chl*f*. The fluorescence below 650 nm is emitted from a contaminant of disconnected shorter wavelength phycobilisomes. PSI shows a maximum emission at 812 nm at all excitation wavelengths. Two additional peaks at 730 nm and 755 nm are visible. The 730 nm emission is biggest when excited at 710 nm suggesting that the allophycocyanin is poorly connected to PSI. The emission wavelength fits well with previous observations made for isolated allophycocyanin in other species (23, 88). The emission at 755 nm indicates a minor contamination of the sample with PSII while no PSI contamination in PSII was seen.

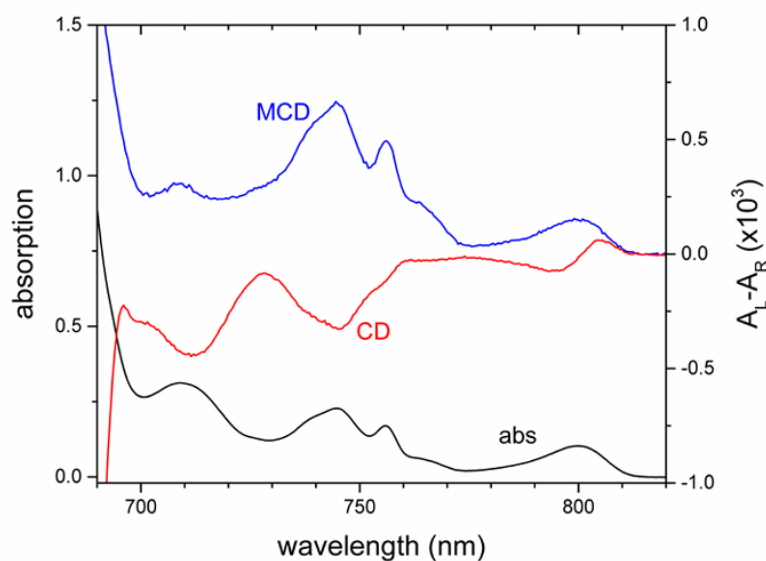
Fig. S16.



Low-temperature (77 K) P_{700}^{++} difference spectrum using isolated FRL-PSI (red) and WL-PSI (black).

The 77 K difference spectra were obtained as described in the materials and methods. Bleaches at 704 nm from P_{700}^{++} are present in both types of PSI but the main band-shifts in the region 675-690 nm in WL-PSI are absent in the FRL-PSI and are replaced by band-shifts at ~745 and 754 nm. The band-shifts in the FRL are better resolved in Figure 3B at higher gain and at 1.8 K.

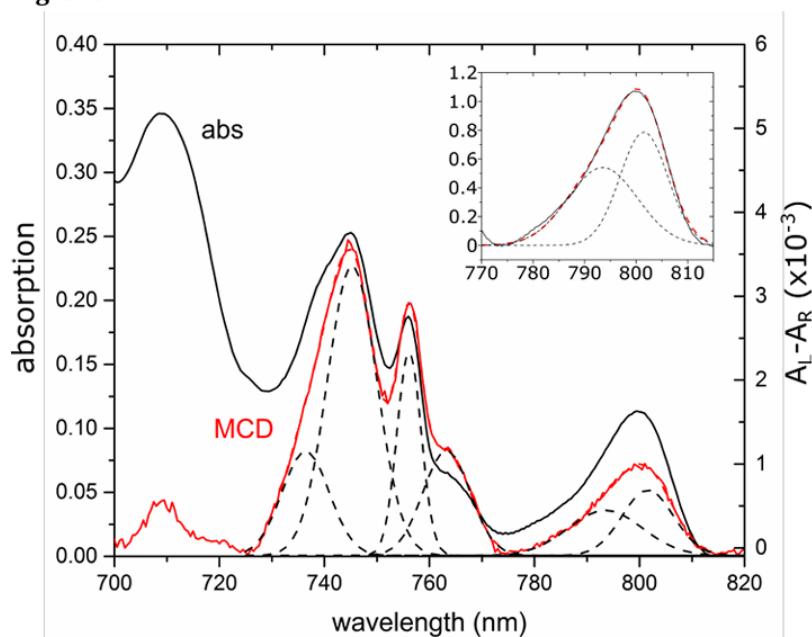
Fig. S17.



Low-temperature (1.8 K) absorption (black), MCD (blue) and CD (red) spectra of isolated FRL-PSI.

The spectra were obtained at 1.8 K using isolated PSII as described in the materials and methods. The low temperature absorption spectrum shows peaks in the long-wavelength region at 712, 745, 756, 764 and 800 nm. The 712 nm peak is essentially absent in the MCD spectrum confirming the assignment of the peak to long-wavelength allophycocyanin. The absence of the allophycocyanin in the MCD reveals a weak narrow peak at ~709 nm which is attributed to a long-wavelength chl a . The strong peak at 745 nm with a shoulder at 736 nm corresponds to several chl f molecules in a range of excitonically-coupled environments and includes some contamination from PSII in this particular preparation. The sharp peak at 756 and weak/broad peak 764 nm are each attributed to a chl f molecule with weak excitonic coupling as indicated by their strong CD and weaker MCD indicating. The broad peak at ~800 nm is attributed to two excitonically coupled chl f molecules, giving typical CD bands of opposite sign and weaker MCD.

Fig. S18.



Low temperature (1.8 K) MCD and absorption spectra of isolated PSI plus curve fitting.

The MCD (red) and absorption spectra (black) were obtained at 1.8 K using isolated PSI as described in the materials and methods. As the allophycocyanin (~712 nm) shows little or no MCD, this spectrum was thus used for the initial fitting. To account for the small contamination of PSII in this particular sample, a weighted fraction of the pure PSII MCD spectrum (Fig. S7) was subtracted to obtain the final the spectrum for the fit, which was done with OriginPro 2015 software. A systematic comparison of the absorption, CD and MCD spectra provided indicated that FRL-chlorophylls at 736, 745 (3x), 756, 763, 794 and 802 nm were present in near equal amounts. The MCD spectrum in the figure is scaled so that it closely matches the absorption profile in the 720 nm to 770 nm region. The weaker MCD signal at around 800 nm (relative to the shorter wavelength peaks) is due to the greater excitonic coupling and leads to an underestimation of the chlorophylls in the fit of the MCD at ~800 nm. When the absorption spectrum above 770 nm itself was used for the fitting (inset), the area of each component peak then corresponds to ~1 chlorophyll. The fitted curves are shown in dashed black lines; the cumulative fits are shown in a dashed red line.

9.5 Supplementary to Publication 3

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Supplementary Data

The primary donor of far-red Photosystem II: Chl_{D1} or P_{D2}?

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SI-1 Allophycocyanin and FRL chlorophyll content of FRL-PS II

The quantification of the five far red chlorophylls in FRL-PS II presents a challenge. Fig. 2 of the manuscript identifies the presence of allophycocyanins which overlap the spectral region where the FRL chlorins, *chl-f* and *chl-d*, absorb.

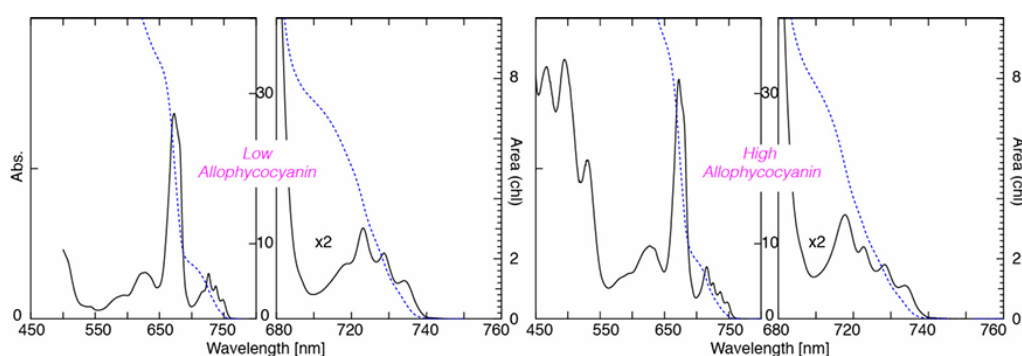


Fig. S1 Absorption spectra of low allophycocyanin and high allophycocyanin FRL-PS II at 1.8 K along with their integrals (dashed curves), scaled to ~35 chl total Q_y absorbance.

Fig. S1 provides the integrals of absorption in the Q_y regions. Both high and low allophycocyanin samples have total absorption in the 700 nm - 800 nm region that exceeds a value corresponding to 5 chls by ~2-3 chl. This assumes that all chls have the same oscillator strength for Q_y . The molar extinction of allophycocyanins isolated from *Synechocystis* is

$\sim 2 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ for both types of allophycocyanin found in this organism. Thus, there are likely to be ~ 1 -2 allophycocyanin per FRL-PS II in our preparations.

Allophycocyanins are known to form both monomers and trimers [1] with trimers being red shifted. This variability precludes a quantitative assessment of the allophycocyanin components. Different monomer and trimer allophycocyanin content is likely to be responsible for the changing spectral profiles in absorption and CD of allophycocyanin components (see Fig. 3 of manuscript).

SI-2 Fluorescence Excitation Spectra and Temperature Dependent Fluorescence

Fig. 1 of the manuscript highlights the difference between the emission spectra of the *chl-a*-PS II and that of FRL-PS II. In the latter the dominant peak is at 752 nm, with a weaker emission in the 720 nm region, attributable to allophycocyanin.

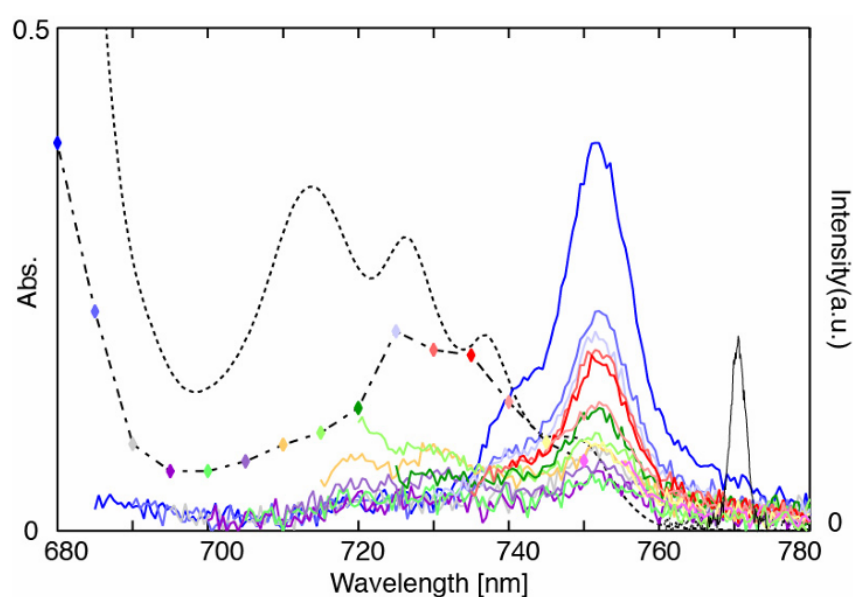


Fig. S2 The absorption spectrum (dotted line) and the emission spectra (coloured lines) of FRL-PSII are shown. Spectra were taken at 77 K of a high-allophycocyanin FRL-PSII sample. The emission spectra were taken with excitation at different wavelengths in 5 nm steps from 680 nm to 755 nm. The corresponding excitation spectrum (composed of coloured diamonds which are joined by a double-dashed line) is generated by plotting the amplitude of the emission peak at 755 nm. The colour of each diamond in the excitation spectrum matches that of the corresponding emission spectrum. The narrow peak seen at 770 nm (black line) is

obtained by measuring 770 nm exciting light. This spectrum serves to calibrate the excitation light bandwidth from the monochromator as ~2.4 nm FWHM.

The spectra in Fig. S2 show that allophycocyanin absorption leads to a low level of emission from FRL-PS II. The excitation spectrum presented in Fig. S2 is similar to the MCD spectra of FRL-PS II in Fig. 1 of the manuscript in that the characteristic 714 nm allophycocyanin peak is missing. Absorption in the 680 nm to 710 nm region leads to the weaker emission peak at ~720 nm which does not correspond to the 714 nm absorption. This emission likely arises from a form of allophycocyanin (monomer, trimer etc.) present in the sample that is not functionally connected to FRL-PS II (see below).

Fig. S3 shows the excitation dependence of the well-resolved fluorescence in a high-allophycocyanin sample at 1.8 K. The weak emission from the contamination by *chl-a*-PS II in the 685nm – 693 nm region, and an allophycocyanin peak at 720 nm discussed above, are apparent, as well as the double peak feature at 741 nm and 752 nm associated with FRL-PS II. The relative intensities of the double peaks in FRL-PS II (Fig. S3) do not vary with excitation wavelength. This indicates that both peaks originate from the FRL PS II component of the FRL-PS II core complex. Additionally, the intensity of the 720 nm peak, when excited at different wavelengths, does not scale with that of the double peaks, indicating it arises from a allophycocyanin in the FRL-PS II preparation and a further indication that the allophycocyanin does not transfer all its excitation energy to a PS II core assembly.

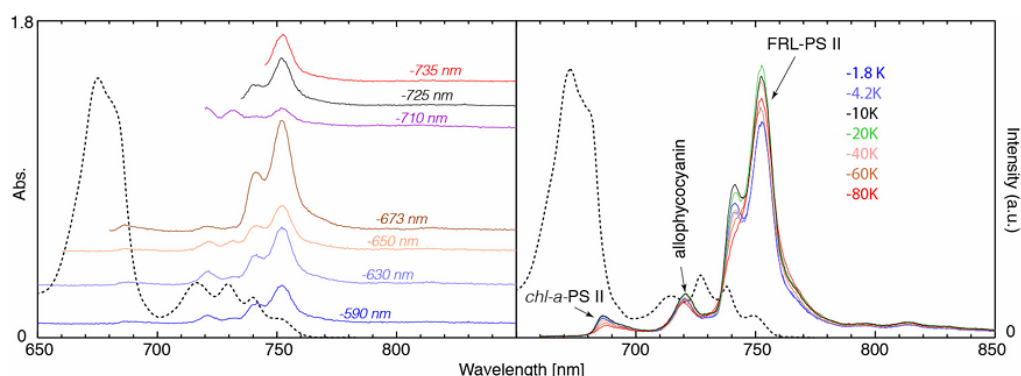


Fig. S3 Excitation dependence of fluorescence from a high-allophycocyanin FRL-PS II preparation at 1.8 K (left panel). Temperature dependent emission of a medium-allophycocyanin sample excited at 532 nm (right panel). Absorption spectra (black dotted lines) are provided for comparison in both panels.

The temperature dependence of the high energy fluorescence component in the 680 nm to 700 nm region (Fig. S3) shows the temperature dependence of *chl-a*-PS II [2], which is clearly present as a contaminant in this sample. Assuming PS II core complexes of FRL-PS II and *chl-a*-PS II have comparable quantum efficiencies in emission and have equivalent absorption strengths at the excitation wavelengths used, the contamination level is estimated to be ~7 %. Fig. S3 identifies a modest decrease in the allophycocyanin fluorescence at 720 nm in the 1.8 K to 80 K range. It exhibits a simple broadening in spectral profile. This behavior is typical for an isolated antenna assembly such as CP43 [3] or CP47 [4].

The PS II core complex emission component of FRL-PSII, having emission peaks at 741 nm and 753 nm, shows a stronger and more complex temperature dependence. The two peaks have distinctly different temperature dependence, with the 742 nm peak reducing in amplitude with temperature more quickly than the 753 nm peak. This temperature dependent behavior is reminiscent of that shown by *chl-a*-PS II where the higher energy component can be attributed [2, 5, 6] to CP43 at the lowest temperatures. This peak becomes less prominent at higher temperatures. The barrier to excitation transfer to the reaction center is overcome by thermal

activation at higher temperatures and consequently emission from the lowest energy excitations of CP43 becomes depleted.

SI-3 Determination of the five FRL chlorophyll Q_y Bands

An initial estimation the peak positions and linewidths of the three distinct FRL chlorophyll features is straightforward. There is some indication of a high-energy shoulder near 713 nm in the MCD spectra (see Fig. 3 of the manuscript) and is also visible in the excitation spectrum shown in Fig. S2.

Attempts to fit MCD spectra with five Gaussians, one for each FRL chlorophyll, did not provide a uniquely defined fit. These attempts do indicate that two FRL Q_y absorptions have greater linewidths than the three obvious features. Absorption and CD spectra do not assist greatly in locating the positions and widths of the two remaining FRL chlorophyll molecules, owing to interference by allophycocyanins.

The amplitude of the MCD relative to the absorption (B/D) of chlorins is known to vary significantly [2, 7-10] with the primary donor having a greatly reduced value of B/D. The approach developed was to subtract a scaled absorption spectrum from the corresponding MCD spectrum and vary the scaling factor until each well-resolved FRL chlorophyll feature in the MCD spectrum was nulled. This procedure worked well and provided relative B/D ratios for each of the three distinct FRL chlorophyll Q_y absorptions.

These pre-determined relative B/D ratios were then held constant in a five band Gaussian fitting of the MCD spectrum. This process determined the position, width and relative B/D of the two remaining FRL chlorophylls, i.e. Chl-3 and Chl-5, as shown in Table S1. The same positions, widths and relative B/D values were obtained in independent analyses of both high and low allophycocyanin MCD spectra.

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The positions and widths of Chl-3 and Chl-5 were not dependent on the precise value of their relative B/D values (these are proportional to the 'Relative Area MCD' column in Table S1). The best overall fit was obtained when Chl-1, Chl-3 and Chl-5 had equal B/D values. The primary donor (Chl-4) had a smaller B/D value. This is consistent with previous observations of B/D for the primary donor in *chl-a*-PS II [2, 7-10] although the full chemical significance of this notable electronic characteristic of primary donors is not understood.

Having determined the position and width of each FRL chlorophyll Q_y excitation (Table S1), the FRL chlorophyll Q_y absorption spectrum was then fitted to five bands of equal area. This process involved a single linear fitting parameter. Two Gaussians were introduced to account for the allophycocyanins absorptions (allophycocyanin-1 and allophycocyanin-2 in Table S1). The narrow allophycocyanin component (allophycocyanin-1) in high-allophycocyanin and low-allophycocyanin samples are similar but allophycocyanin-2 is quite different. The fits for allophycocyanin-1 and allophycocyanin-2 -shown in Fig 3 of the manuscript and Table S1 remain indicative only, as there is significant correlation between fitting parameters. The presence of different forms of allophycocyanins was discussed in SI-1.

In Table S1 the relative area of the Chl-4 absorption was revised to have a value of 1.1 chl. Exciton modelling (SI-4) establishes that the interactions between the FRL chlorophyll primary donor and its *chl-a* neighbors within the reaction center leads to this (relatively low) level of transfer of intensity.

The CD spectra were then fitted, with the amplitude of each Q_y CD feature allowed to vary. The allophycocyanin CD components were allowed unconstrained amplitudes, widths and positions. The widths and positions of allophycocyanin CD features are not those as determined by the corresponding fit of the absorption spectrum. For the five FRL chlorophyll Q_y excitations, each is an individual exciton Q_y component. The CD profile of a single purely electronic Q_y excitation follows that seen in absorption [11]. The absorption profiles of

allophycocyanins are composite of a number of exciton-coupled pigment excitations. Thus allophycocyanin CD profiles do not follow those in absorption [1, 12].

Table S1 FRL-PS II Global Analysis

High allophycocyanin sample

Band	Absorption nm & (FWHM) nm	Energy cm ⁻¹ (Shift [#]) cm ⁻¹	Area Abs.	Area MCD	Area CD
Chl-1	749 (9)	13350 (0)	1	1	1
Chl-2	738 (6)	13550 (200)	1	0.7	-1.4
Chl-3	733 (9)	13640 (310)	1	1	0.3
Chl-4*	726 (6)	13760 (410)	1.1	0.6	0.6
Chl-5	722 (14)	13850 (500)	1	1	0.0
a-1	714 (8)	14010 (660)	1.9	0	-1.5
a-2	708 (25)	14120 (780)	5.3	0	-1.6

Low allophycocyanin sample

Band	Absorption nm (FWHM) nm	Energy cm ⁻¹ (Shift [#]) cm ⁻¹	Area Abs.	Area MCD	Area CD
Chl-1	748 (10)	13370 (0)	1	1	1
Chl-2	737 (7)	13570 (200)	1	0.7	-1.6
Chl-3	731 (12)	13680 (310)	1	1	0.9
Chl-4*	726 (6)	13770 (410)	1.1	0.6	0.6
Chl-5	720 (15)	13890 (520)	1	1	-1.2
a-1	716 (8)	13970 (630)	0.6	0	-0.7
a-2	705 (14)	14180 (820)	1.2	0	-0.4

Areas are relative to the absorption, MCD or CD areas of Chl-1

[#] Shifts are relative to the peak of Chl-1

** Primary donor.*

a-1 - allophycocyanin-1

a-2 - allophycocyanin-2

The right-hand columns in Table S1 show that the area of Q_y CD features associated with Chl-1, Chl-2 and Chl-4 reproduce well between the high and low allophycocyanin samples, whereas discrepancies appear for Chl-3 and Chl-5. Fig S4 provides a comparison of the experimental and fitted CD for high and low allophycocyanin samples, along with the individual CD components determined by the fitting procedure described above.

A significant discrepancy is that Chl-5 is attributed to have minimal CD in the high allophycocyanin sample but a strong negative CD in the low allophycocyanin sample. By

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contrast, the three sharp FRL-chl features, Chl-1, Chl-2 and Chl-4 are well determined in both samples and their CD amplitudes are not significantly correlated. However, the fit of Chl-3, Chl-5, a-1 and a-2 CD features are significantly correlated, particularly for the low allophycocyanin CD data. The region of major discrepancy (720 nm) shows a sharp inflection in both samples that is not reproduced in either absorption or MCD data. A more complete representation of allophycocyanin CD thus requires extra components in the fitting procedure. Additionally, rather than a simple reduction of allophycocyanin content between the two samples, it seems that extra (allophycocyanin) assemblies are introduced via the procedure used to reduce the total allophycocyanin content. These assemblies appear to have significant negative CD in the 720 nm to 730 nm region, making it impossible to disassociate it from CD associated with Chl-5. Without further experimental information, we cannot determine the CD of Chl-5. It seems likely that the CD of Chl-3 is significant and positive.

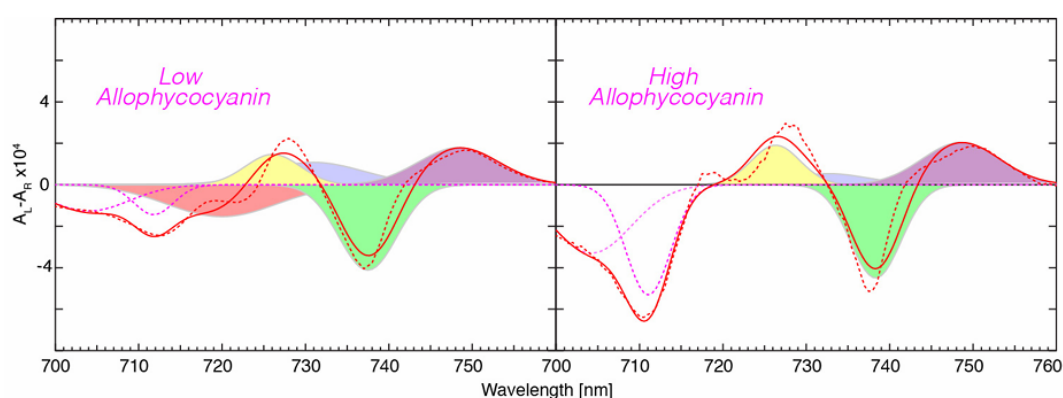


Fig. S4. The 1.8 K experimental CD and corresponding fitted CD spectra of low and high allophycocyanin FRL-PS II samples are shown as dotted and solid red lines respectively. These spectra are also presented in Fig. 3 of the manuscript in less detail and are displayed in conjunction with corresponding absorption and MCD data and analysis. The fitted CD components (see text) are displayed as coloured overlapping Gaussians using the same colour scheme as used in Fig. 3.

SI-4 Electrochromic Shifts

A comparison of Q_A^- formation-induced electrochromic shift patterns for a range of organisms compared to those seen in FRL-PS II, is provided in Fig S5.

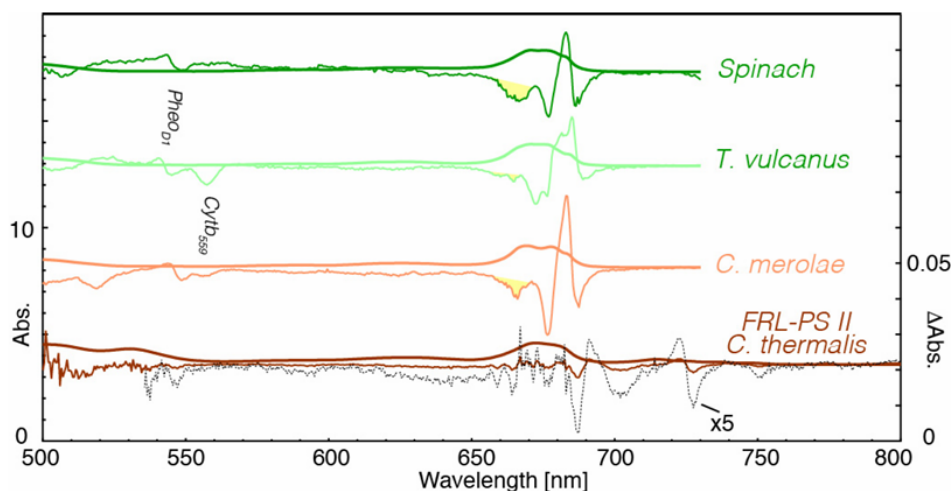


Fig. S5 Absorption (left Scale) and illumination-induced difference spectra (right scale) for PS II core complexes derived from Spinach, *T. vulcanus* [13], *C. merolae* [14] and FRL-PS II *C. thermalis* [15] at 77 K. The shaded yellow regions are assigned to ChlZ_{D2} bleaching (see text). Green light illumination fluences were standardized at 300 s of 5 mW/cm². Spectra have been scaled to comparable Q_y absorption intensities and offset for viewing. Q_A^+ yields in the first three systems are similar (60-80%), whereas the corresponding yield in FRL-PS II is ~10 to 20 times less.

Low temperature electrochromic shifts associated with Q_A^+ formation have been extensively studied [16-21]. In Fig. S5, similar Q_x 'C550' electrochromic blue-shifts [16] of Pheo_{D1} are evident in all organisms. The nearby bleach feature associated with oxidation of reduced *cytb*₅₅₉ is evident in *T. vulcanus* and *C. merolae* preparations. The FRL-PS II preparation of *C. thermalis* used for Fig. S5 did not contain a significant fraction of reduced *cytb*₅₅₉ and thus no bleach is seen, although this spectral feature was evident at 556.5 nm in other sample preparations (see Fig. S6 and [15]). Fig. S5 also shows the bleach near 666 nm, highlighted as a shaded yellow region, associated with the oxidation of a *chl-a* (often considered to be ChlZ_{D2}) for the first three organisms. This oxidation process appears less pronounced in FRL-PS II and other secondary donors such as β -carotenes may be involved [22-24].

The dominant electrochromic features in the Q_y region for *chl-a*-PS II have been identified as a red shift of Pheo_{D1} near 680 nm and blue shift of Chl_{D1} Q_y near 685 nm. The precise position

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and shape of shift features vary with organism. This variation is due to both small changes in pigment site energies with organism, along with changes in the effects of excitonic interactions between pigments associated with relatively small changes in their site energies. This is because the relative energy of sites can be changed quite significantly with respect to the exciton coupling matrix element between pigments. Shift features are doubled in some preparations of *T. vulcanus* and *T. elongatus* i.e. when there are two forms of the D1 protein expressed in the sample [25].

In the FRL-PS II Q_y region, the largest shifted feature is well separated with an intense blue shift feature occurring at 726 nm [15]. Compared to the data in Fig. S5, which are taken at 77 K, those taken at 1.8 K (Fig. 2 and Fig. 4 of manuscript) show better-resolved shift features.

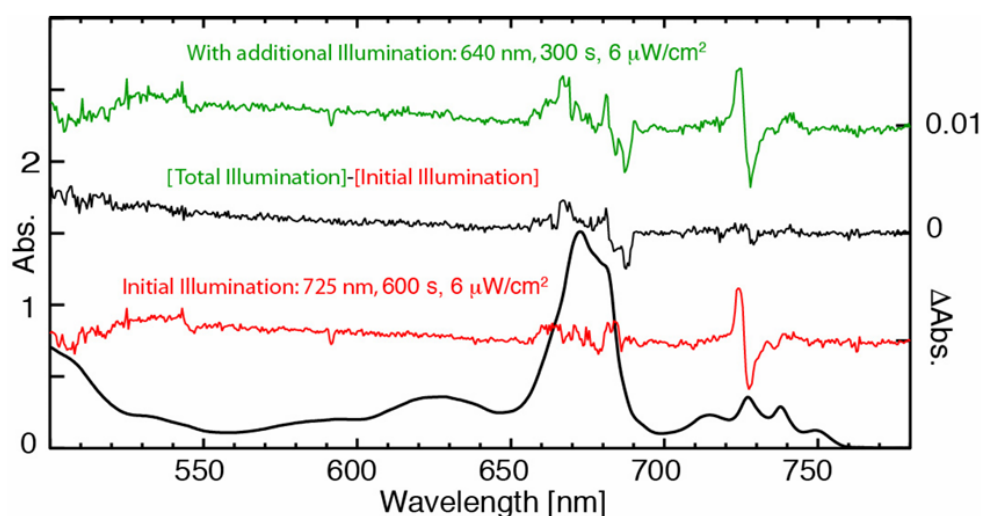


Fig. S6. The bottom black trace is the absorption spectrum (left. scale) of the low-allophycocyanin FRL PS II core complex at 1.8 K. The red trace is the electrochromism spectrum (right scale) induced by illumination at 725 nm with the fluence indicated. The green trace is the accumulated electrochromism after an additional illumination at 640 nm. The thin black trace is the change in electrochromism induced by the 640 nm illumination component.

FRL-PS II core samples contain some *chl-a*-PS II, as evidenced by its characteristic emission spectrum appearing in Fig. 1 of the main manuscript. FRL-PS II samples illuminated with light

at wavelengths significantly shorter than 700 nm, such as the green light used to generate difference spectra in Fig. S5, can be expected to display electrochromism associated with the *chl-a*-PS II component, as well as that of the (majority) FRL PS II component. Illumination of *chl-a*-PS II at 725 nm and 1.8 K is known to induce Q_A^+ formation (and as evidenced by electrochromism) but with low quantum efficiency [20, 26]. The *chl-a*-PSII electrochromism induced by illumination in the 700 nm to 730 nm range is due to absorption by the weak and broad, deep-red charge transfer state of *chl-a*-PS II [14, 20, 26].

Fig. S6 demonstrates that illumination of a low allophycocyanin FRL-PS II core complex sample with 640 nm light, following initial illumination at 725 nm, induces minimal additional electrochromism at 726 nm. The light-induced Q_A^+ formation of the FRL PS II component was essentially completed by the first illumination. However, additional 640 nm illumination leads to an electrochromism spectrum with a negative feature near 686 nm and a positive component near 682 nm. The spectrum exhibits low signal to noise as it was generated as a double-difference spectrum and also recorded in a region of high and rapidly varying absorbance. However, the pattern is compatible with it being due to the Chl_{D1} blue-shift of *chl-a*-PS II of *C. thermalis* [15]. For comparison, the Q_A^+ induced electrochromic shift of *T. vulcanus* (Fig. S5), a *chl-a* PS II thermophilic cyanobacterium has negative and positive peaks at 688 nm and 684 nm respectively.

The red trace in Fig. S6 shows no evidence of the negative feature seen near 685 nm in the black trace. We can conclude that no significant component of the *chl-a*-PS II contaminant of the FRL-PS II sample is photoconverted by 725 nm light. The electrochromism pattern in the red trace is then due only to the FRL PS II majority component in the sample.

The linewidth of the 726 nm feature (7 nm or $\sim 130\text{ cm}^{-1}$ FWHM) allows an initial estimate of the electrochromic shift to be determined, independent of knowledge of the Q_A^+ yield. By measuring the offset, δ , of the derivative of the (initial) absorption spectrum compared to the

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electrochromic shift feature, the electrochromic shift would then be 2δ , i.e. 2.2 nm or equivalently 42 cm^{-1} . This assumes that the relatively small fraction of FRL PS II that undergoes photoconversion has the same spectral distribution as the majority. Those FRL-PS II centers that are not photo-converted to the trapped $Q_A^{\bullet-}$ form do not contribute to the shift feature. The shift feature has a crossing point independent of yield as seen in Fig. S7, and is displaced 1.1 nm from the peak of the absorption, taken before illumination.

A $Q_A^{\bullet-}$ yield can then be estimated by simulating a 100% yield shift feature at 726 nm. This artificial, full-yield spectrum is generated by shifting the absorption spectrum taken before illumination to the blue by 2.2 nm. Subtracting the shifted spectrum from the initial spectrum yields a spectrum corresponding to the entire 726 nm feature being affected. Scaling this shift feature against the experimental 726 nm shift amplitude then provides a $Q_A^{\bullet-}$ yield. Fig. S7 shows fluence-dependent shift spectra with the apparent yield saturating at 10% when using $6\text{ }\mu\text{W}/\text{cm}^2$ excitation at 725 nm.

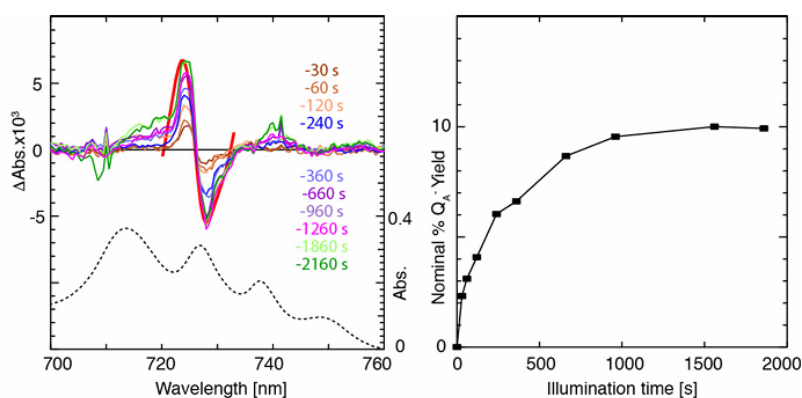


Fig. S7. The left panel shows the fluence dependence of the electrochromic shift of high-allophycocyanin FRL-PS II at 1.8 K illuminated with $6\text{ }\mu\text{W}/\text{cm}^2$ power density light at 725 nm. The red trace in the 726 nm region is the synthetic shift feature created by displacing the initial absorption spectrum by 2.2 nm (42 cm^{-1}) to the blue and subtracting this from the original spectrum. The right panel is the nominal yield of photoconversion to the $Q_A^{\bullet-}$ form of the enzyme versus illumination time, assuming a 2.2 nm electrochromic shift (see revision in text).

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At the lowest yields, a simple 726 nm shift feature dominates. It is narrower than the synthetic shift feature. At higher fluences, shoulders develop which may be associated with photoconversion of a population of FRL-PS II that exhibits the full primary donor spectral distribution. The initial slope of the conversion curve in Fig S7 can be analyzed by gauging the number of absorbed photons for a given excitation fluence. This leads to a direct estimate of the initial quantum efficiency of the Q_A^- conversion as being close to unity. Such high initial quantum efficiencies were also seen in previous studies on spinach PS II core complexes [20] by monitoring the amplitude of the Q_x shift of Pheo_{D1}.

However, at 77 K, shift features at both high and low fluence are displaced from the peak maximum by 0.5 nm, whilst a corresponding synthetic shift feature generated by a 0.5 nm displacement of the 77 K 726 nm absorption accurately reproduces the shift feature shape. This revised estimate of the shift of 0.5 nm leads to a corresponding 2 x increase in our estimate of the Q_A^- yield (i.e. to 20%), compared to that proposed in Fig. S7.

As mentioned above, the low fluence shift feature at 1.8 K arises from a narrower component of the overall primary donor inhomogeneous absorption profile. This narrower component is displaced by 1.1 nm from the peak maximum. This displacement, combined with an actual electrochromic shift of 1 nm, leads to the observed 1.8 K value of 2δ of 2.2 nm. The narrower component has a linewidth close to 4 nm, rather than the overall linewidth of 7-8 nm as given in Table S1. This narrower component of Chl-4 is more in line with the width observed for the Chl_{D1} site energy linewidth in the *chl-a*-PS II core complex (from spinach), which is 1.6 nm (Table S2).

SI-5 Exciton Modelling

Here, we used the same methodology previously used to model the PS II reaction center as present in core complexes of a number of organisms [27] and also for *isolated* PS II (RC-6 and RC-5) [10, 28] reaction centers. We calculated absorption and CD spectra of a range of

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possible configurations of FRL-PS II. Apart from the single FRL chl site, the reaction center parameters used were those for *chl-a*-PS II core complexes isolated from spinach as shown in Table S2. The coupling matrix used is provided in Table S3.

Table S2

Spinach Core Complex PS II reaction center site parameters

	Site Energy nm/cm ⁻¹	FWHM Linewidth nm/cm ⁻¹
Chl _{D1}	684/14620	1.6/40
Pheo _{D2}	678/14750	4.0/100
Pheo _{D1}	676.5/14780	5.6/140
P _{D1}	668.5/14960	6.3/140
P _{D2}	668.5/14960	6.3/140
Chl _{D2}	669/14950	6.1/140
ChlZ _{D1}	672/14880	6.1/140
ChlZ _{D2}	665/15040	6.1/140

Table S3

PS II reaction center exciton coupling matrix elements [29]

Coupling (cm ⁻¹)	Chl _{D1}	Pheo _{D2}	Pheo _{D1}	P _{D1}	P _{D2}	Chl _{D2}	ChlZ _{D1}	ChlZ _{D2}
Chl _{D1}	0	-2.18	43.51	-27.32	-46.77	3.54	1.67	-0.09
Pheo _{D2}	-2.18	0	1.55	12.61	-2.99	41.65	-0.19	-2.57
Pheo _{D1}	43.51	1.55	0	-3.96	15.06	-2.37	-2.52	-0.18
P _{D1}	-27.32	12.61	-3.96	0	158	-41.83	0.45	0.58
P _{D2}	-46.77	-2.99	15.06	158	0	-22.04	0.6	0.61
Chl _{D2}	3.54	41.65	-2.37	-41.83	-22.04	0	-0.05	1.8
ChlZ _{D1}	1.67	-0.19	-2.52	0.45	0.6	-0.05	0	0.15
ChlZ _{D2}	-0.09	-2.57	-0.18	0.58	0.61	1.8	0.15	0

Transition dipole strengths were taken from reference [30] as 5.47 D for *chl-a* and 4.25 D for *pheo-a* and structural data determined from crystal structure data [31].

Electrochromic shifts, Δ , induced by reducing Q_A and $Pheo_{D1}$ forming their anion radical states, can be modelled by considering the interaction of $\vec{\Delta\mu}$ of each reaction center pigment with the electric field associated with a point charge at the position of the reduced species.

$$\Delta = \frac{\vec{\Delta\mu} \cdot \vec{e}}{|\vec{r}|^2}$$

A starting point [30, 32, 33] is to assign $\Delta\mu$ to be 1.8 D for all *chl-a* Q_y excitations and 1.35 D for *pheo-a* Q_y excitations, with e being the electronic charge. This shift, Δ , is then reduced to Δ/f because of the influence of the local dielectric in the protein. When f is taken as 3, the site energy shifts are as given below.

Table S4

PS II Reaction Center Site Energy Shifts for $f = 3$

Shift/cm ⁻¹	$Q_A^{\cdot-}$	$Pheo_{D1}^{\cdot-}$
Chl _{D1}	14.4	77.2
Pheo _{D2}	-9.6	-0.1
Pheo _{D1}	-53.8	
P _{D1}	5.8	0.4
P _{D2}	18.0	45.1
Chl _{D2}	-5.4	-18.8
ChlZ _{D1}	10.0	11.2
ChlZ _{D2}	3.9	2.8

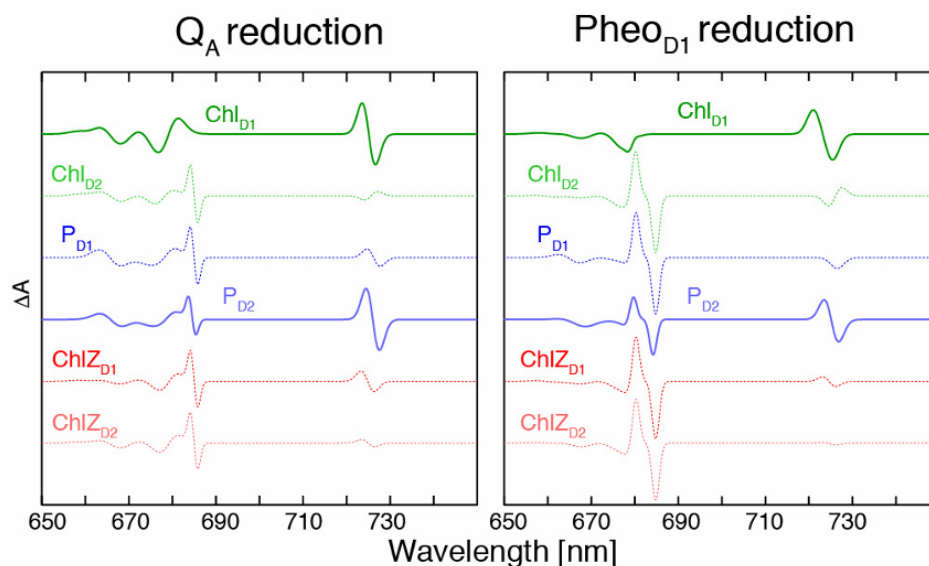


Fig. S8 Left and right panels show calculated electrochromic shift patterns induced by an electron located at the Q_A and $Pheo_{D1}$ positions, respectively. Shift patterns were calculated using the parameters in Tables S2 and S3. Calculations are presented for all locations of a single FRL chlorophyll in the reaction center. The two cases which induce an electrochromic shift near 726 nm compatible with experimental data (Chl_{D1} and P_{D2}) are accentuated (thicker solid lines).

The calculated electrochromic patterns shown in Fig. S8 indicate that $Pheo_{D1}$ reduction experiments could assist in discriminating between the Chl_{D1} and P_{D2} locations for the primary donor. A relatively large primary donor shift for the Chl_{D1} assignment is calculated for the $Pheo_{D1}$ reduction compared to that for the $Q_A^{\cdot-}$ reduction. More useful may be that the associated electrochromic pattern seen in the *chl-a* Q_y spectral region is different for the Chl_{D1} and P_{D2} primary donor assignments. The room temperature $Pheo_{D1}$ reduction spectra previously published in Fig 2D of [15] display a broad negative feature at 658 nm as well a stronger broad positive feature near 650 nm. Neither of these features correspond to the calculated electrochromism (see Fig. S8). In experiments [27, 35] on canonical *chl-a*-PS II preparations, there is no evidence of broad features similar to those seen in Fig. 2D of ref

[15]. It thus seems important to generate the trapped Pheo^- state at low temperature in future experiments.

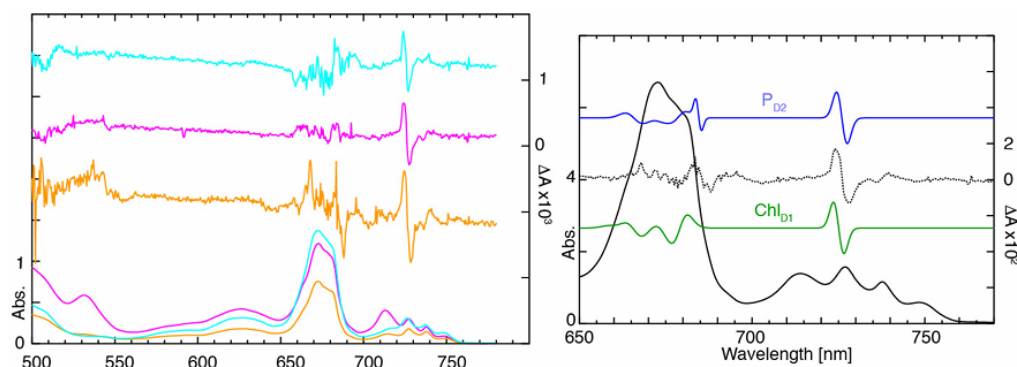


Fig. S9 The left panel shows the absorption (lower traces) and electrochromic shift patterns (each offset for clarity) induced by 725 nm illumination at 1.8 K of three different FRL PS II core complex samples having various allophycocyanin content. The right panel shows the sum of the three absorption spectra (thick black trace) and the sum of the corresponding three electrochromism spectra (thin black trace). Also shown are the calculated electrochromic patterns (from Fig. S8) when the primary donor is located at Chl_{D1} (green trace) and P_{D2} (blue trace). For both cases the calculated patterns have been scaled to match the experimental electrochromism amplitude.

The signal-to-noise ratio and other limitations in the in the 725 nm-induced electrochromism spectra presented in Figs. 2 and 4 of the manuscript and in Fig. S9 makes it difficult to determine which calculated Q_A reduction pattern calculated (Fig. S8) better fits the experimental data in the *chl-a* region. Fig. S9 addresses this difficulty by adding results from identical experiments performed on three different FRL-PS II samples exhibiting differing allophycocyanin concentrations. Illuminations were performed with an identical fluence of 6 $\mu\text{W}/\text{cm}^2$ at a wavelength of 725 nm for 300s. This illumination protocol is shown to correspond to conditions where no significant *chl-a* based photochemistry is induced (see Fig. S6), i.e. that associated with *chl-a*-PS II present in the samples. A comparison of the averaged shift pattern with those calculated for the 680 nm region appears to be more compatible with P_{D2}

being the primary donor. The main feature at ~ 680 nm appears experimentally as a blue shift, as calculated for the P_{D2} case, rather than a red shift as calculated for the Chl_{D1} case.

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