

Structural and functional studies on Photosystem II
from *Thermosynechococcus elongatus*

Jeffrey Douglass

Thesis submitted for the Diploma of the Imperial College, PhD degree
of Imperial College London

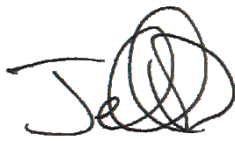
Department of Life Sciences

Division of Molecular Biosciences

2015

Declaration

This thesis, submitted in fulfilment of the requirements for the Diploma of the Imperial College and PhD degree of Imperial College London, unless appropriately referenced represents my own work and has not been previously submitted to this or any other institution for any degree, diploma or qualification.

A handwritten signature in black ink, appearing to be 'Jeffrey Douglass', is centered above a horizontal line.

Jeffrey Douglass

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Abstract

Photosystem II is a membrane-bound complex found in plants, eukaryotic algae and cyanobacteria which converts photo-excitation energy into chemical energy in the form of both oxidising and reducing power, catalysing the oxidation of water and reduction of quinone. The structure of this enzyme is tuned to balancing thermodynamic and quantum efficiency while minimising photodamage. This thesis tests a number of hypotheses regarding structural and functional aspects of this enzyme, addressing (1) the importance of structural differences between normal- and high-light-induced protein isoforms, (2) the binding mode of the inhibitor DCMU, (3) electron transfer from the primary quinone acceptor Q_A to the terminal quinone acceptor Q_B and (4) the structural origins of functional differences between redox-active tyrosines Y_Z and Y_D , using the thermophilic cyanobacterium *Thermosynechococcus elongatus* as a model organism. A crystal structure of PSII containing the PsbA3, the high-light isoform of the D1 protein which binds cofactors involved in the active electron transfer chain, is presented, demonstrating structural similarities with PsbA1. Crystallographic evidence is also presented which supports the binding of DCMU to D2-Ser264 and D2-Phe265 in this isoform, similar to predictions from PsbA1. Kinetic studies show that the half-times of electron transfer from Q_A^- to Q_B and Q_A^- to Q_B^- are around 400 μ s and 1 ms respectively, and the implications of these rates for structural studies are discussed. Finally, electron paramagnetic resonance experiments provide evidence that a hydrogen-bonding network linking Y_D and CP47-Glu364 is not a major determinant of functional differences between Y_Z and Y_D .

Acknowledgements

I would like to sincerely thank my co-supervisors Professors Bill Rutherford and Peter Nixon for their support and guidance throughout my project, and for giving me the opportunity to work with them at Imperial.

I would like to thank all those who supported my work in the lab, particularly Dr Jianfeng Yu, Dr. James Murray, Dr. Woj Bialek and Dr. Karim Maghloui, Fabrice Rappaport and Fraiser Macmillan.

I appreciate the help and support I received from my colleagues, particularly Sven DeCausemaecker, as well as the rest of the members of the 7th floor lab.

I would like to acknowledge the help of collaborators Sidsel Birkelund Schmidt for his ICP-MS analysis of my samples, Alain Boussac for arranging the synthesis of DBM by Christian Herrero and Diana Kirilovsky for providing strains generated by Sabah El Alaoui.

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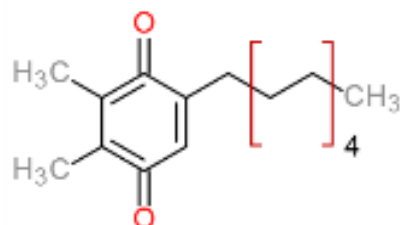
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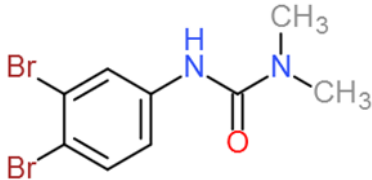
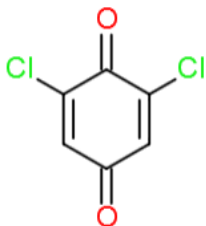
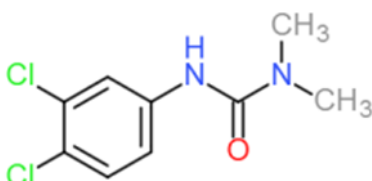
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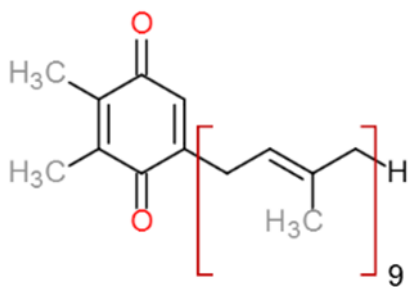
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Abbreviations

Abbreviation	Expansion
10PQ	Decylplastoquinone (Sigma), CAS 112055-76-2, PubChem 24894163
	
43H	<i>T. elongatus</i> strain with C-terminal His ₆ -tagged CP43. (Sugiura and Inoue, 1999)
47H	<i>T. elongatus</i> strain with C-terminal His ₆ -tagged CP47. (Iwai et al., 2010)
APS	Ammonium persulfate, CAS 7727-54-0, PubChem 62648
Arg	Arginine
Asn	Asparagine
BC	Sodium bicarbonate, CAS 144-55-8, PubChem 516892
bRC	Bacterial type II reaction centre, as from <i>R. sphaeroides</i>
BSA	Bovine serum albumin (Sigma), UniProt P02769
β-DDM	n-Dodecyl-β-D-maltoside, Glycon/Biomol
CAS	Chemical Abstracts Service
CAPSO	N-cyclohexyl-3-aminopropanesulfonic acid, CAS 1135-40-6
Chl	Chlorophyll <i>a</i>
Cm	Chloramphenicol (Sigma)
CN-PAGE	Clear native PAGE
CP43	Photosystem II reaction centre antenna subunit encoded by <i>psbC</i>
CP47	Photosystem II reaction centre antenna subunit encoded by <i>psbB</i>
CV	Column volume
D1	Photosystem II reaction centre core subunit encoded by <i>psbA</i>

Abbreviation	Expansion
D2	Photosystem II reaction centre core subunit encoded by <i>psbD</i>
DBMU	3-(3,4-dibromophenyl)-1,1-dimethylurea
	
DCBQ	2,6-dichloro- <i>p</i> -benzoquinone
	
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea, AKA Diuron, CAS 330-54-1, PubChem 3120
	
DFT	Density functional theory
DMSO	Dimethyl sulfoxide, CAS 67-68-5, PubChem 679
DNA	Deoxyribose nucleic acid
DNase 1	Deoxyribonuclease I
DTT	Dithiothreitol, CAS 3483-12-3, PubChem 446094
ECL	Enhanced chemiluminescence
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid, CAS 60-00-4, PubChem 6049
EPR	Electron paramagnetic resonance
FeCN	Potassium ferricyanide, $[\text{Fe}(\text{CN})_6]^{3-}$, PubChem 439210
FPLC	Fast protein liquid chromatography

Abbreviation	Expansion
FWHM	Full width at half maximum; spectral width of an optical peak at half the maximum height
Gln	Glutamine
Glu	Glutamate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, CAS 7365-45-9
His	Histidine
IPTG	Isopropyl β -D-1-thiogalactopyranoside, CAS 367-93-1
Km	Kanamycin sulfate from <i>Streptomyces kanamyceticus</i> (Sigma), CAS 25389-94-0, PubChem 24896240
LC-ICP-MS	Liquid chromatography - inductively coupled plasma mass spectrometry
Leu	Leucine
MES	2-(N-morpholino)ethanesulfonic acid, CAS 4432-31-9
MWCO	Molecular weight cut-off
NT	<i>T. elongatus</i> strain obtained from Dr. Hua-Ling Mi
OD	Optical density
OE-PCR	Overlap-Extension PCR
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
PGA-LM	Low molecular weight (200-400 kDa) poly- γ -glutamic acid
Phe	Phenylalanine
Pheo	Pheophytin

Abbreviation	Expansion
PQ	Native plastoquinone
	
PSI	Photosystem I
PSII	Photosystem II
PVDF	Polyvinylidene difluoride
Q _A	Primary electron acceptor quinone bound to D2
Q _B	Terminal electron acceptor quinone bound to D1
QM/MM	Hybrid quantum mechanics/ molecular mechanics modeling
<i>R. sphaeroides</i>	<i>Rhodobacter sphaeroides</i>
S _{1/2/3/4/n}	The Mn ₄ CaO ₅ cluster in different oxidation states during its reaction cycle
SDS	Sodium dodecyl sulphate, CAS 151–21–3, PubChem 3423265
SEC	Size exclusion chromatography
<i>Synechocystis</i>	<i>Synechocystis</i> sp. PCC 6803
TA cloning	Ligation of adenine-overhang (added by Taq activity) single-strand DNA to linearized thymine-overhang backbone DNA
Taq	Thermostable DNA polymerase from <i>Thermus aquaticus</i> , obtained from NEB
<i>T. elongatus</i>	<i>Thermosynechococcus elongatus</i>
TEMED	Tetramethylethylenediamine, CAS 110-18-9, PubChem 8037
Tris	Tris(hydroxymethyl)aminomethane, CAS 77-86-1, PubChem 6503
Tween 20	Polyoxyethylene (20) sorbitan monolaurate, CAS 9005-64-5
Tyr	Tyrosine

Abbreviation	Expansion
UV	Ultraviolet radiation (10-400 nm)
WT	Wild-type <i>T. elongatus</i> BP1, obtained from Dr. Karim Maghlaoui
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, CAS 7240-90-6
Y _Z	Tyrosine 190 of D1; redox couple with Mn ₄ CaO ₅ cluster and P ⁺
Y _D	Tyrosine 189 of D2; redox couple with P ⁺

Chapter 1: Introduction to Photosystem II

1.1. Importance of Photosystem II

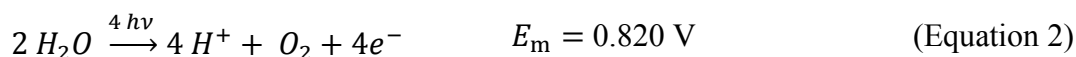
1.1.1. The role of PSII in vivo

Photosystem II (PSII) is a membrane-bound protein complex found in cyanobacteria, algae and higher plants whose water-plastoquinone oxidoreductase activity (Equation 1) constitutes the first of the light-dependent reactions of oxygenic photosynthesis: the conversion of light-induced chlorophyll excitation into electrochemical potential (Cardona et al., 2012; Dau et al., 2012; Rappaport and Diner, 2008; Shen, 2015).



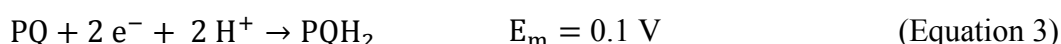
PSII belongs to the Type II reaction centres which are characterised by their sequential two-electron reduction of a quinone acceptor, not all of which are able to oxidise water (Heathcote et al., 2002). This differs from the less complex Type I reaction centres (including Photosystem I (PSI)) which only deal with one-electron chemistry. Together, PSI and PSII generate the reducing power and electrochemical gradients that power the majority of extant life (Nelson and Yocum, 2006).

Photo-excitation of the central chlorophylls (collectively called P_{680} from their overlapping absorbance peak at ~ 680 nm) results in one-electron charge separation between adjacent pigment cofactors in the reaction centre core. This induced electrical potential energy drives the concerted four-electron oxidation of two water molecules at the ‘donor side’, producing molecular oxygen and four protons for every four charge-separation events (Equation 2). These protons are released into the lumen and contribute to a transmembrane proton gradient whose dissipation drives ATP synthase activity (Kramer et al., 2003).



The other half-reaction occurring at the ‘acceptor side’ involves the sequential two-electron reduction of a plastoquinone (PQ) molecule to hydroquinone (PQH₂) (Equation 2) via a

semiquinone radical intermediate, occurring twice for each dioxygen produced. The hydroquinone is an exchangeable electron carrier that equilibrates with a quinone pool in the photosynthetic membrane. Quinone oxidation by the cytochrome *b₆f* complex at the luminal side of the membrane is coupled to the reduction of a soluble electron carrier (plastocyanin or cytochrome *c*₆) which provides electrons to subsequent reactions in the photosynthetic electron transport chain. Cytochrome *b₆f* transfers an additional proton per electron from the stromal to the luminal side during the Q-cycle, further contributing to the transmembrane electrochemical gradient.



Linking the electron donor (H₂O), charge separation (P) and the electron acceptor (PQ) is a series of redox-active cofactors whose redox midpoint potentials are regulated by their protein environment to achieve a high quantum yield of forward electron transfer with reasonable thermodynamic efficiency. The reaction sequence is driven forwards by the increasing stability of each radical pair: the reduction potentials of the cofactors increases towards the acceptor side and decreases towards the donor side. While unavoidable, this reduces the free energy of the photo-excited state available to do useful work. Kinetic controls imposed by cofactor positioning and protein conformational changes contribute further to the reduction in wasteful and potentially harmful back- and side-reactions. The different redox reaction stoichiometries are reconciled in part by redox-state-dependent protonation of cofactors or their ligands that neutralise charges in order to reduce the oxidising or reducing power required for subsequent redox reaction to occur. These molecular mechanisms represent a delicate balance between maintaining sufficiently high-energy states to oxidise water while preventing and protecting against the damage that these states can cause. What remains is an overall ΔE of 0.72 V for the reaction described in (Equation 1. Furthermore, the transmembrane ΔpH generated by this activity of ~ 1.5 units increases this ΔE by ~ 90 mV and can be dissipated to drive ATPase activity.

The oxygenation of the atmosphere 2.4 billion years ago gives a lower limit to the age of oxygenic Type II reaction centres (Cardona et al., 2015; Dismukes et al., 2001; Hohmann-Marriott and Blankenship, 2011). This enzyme enabled organisms to access water as an abundant electron source to generate reducing power using sunlight as a source of excitation energy in order to produce organic molecules (Barber, 2008). The competitive advantage that

this provides in the majority of habitats on earth over existing alternative sources of inorganic reducing power (e.g. chemotrophy (Sousa et al., 2013)) has led to its involvement in the vast majority of primary productivity. The resulting availability of oxygen has also driven the increasing complexity and size of heterotrophic organisms through increased respiratory efficiency, the formation of the UV-blocking atmospheric ozone layer and other effects, radically changing earth's biome (Lyons et al., 2014).

1.1.2. The anthropocentric view of PSII

Humans rely on current biological photosynthesis to produce energy, materials and, most importantly, food. In this context it is important to understand the mechanisms by which this process takes place if we are to improve or emulate its productivity. We also rely similarly on ancient biological photosynthesis whose products have been converted into fossil fuels, giving us access to thousands of years of photosynthetic output. While our total power consumption exceeds the rate of renewable (eventually, solar) energy capture, fossil fuel deposits will diminish. From an energetic standpoint, as fossil fuels become more scarce and their extraction less efficient the useable proportion of gross energy production will decrease. The long-term requirement for a sustainable source of useful energy is evident, as is the best solution that has arisen by natural selection. Biological solutions such as biohydrogen production (H. Sakurai et al., 2013) make use of photosynthesis in its entirety while synthetic catalyst design (Zhang et al., 2015) focuses on PSII activity, moving towards the 'artificial leaf' (Olmos and Kargul, 2015). From an environmental standpoint, the costs of burning fossil fuels is huge (Barbir et al., 1990). Photosynthetic activity could play an important role in mitigating these effects as a CO₂ sink (Battle et al., 2000).

Despite the long evolutionary timescale and the wide extant genetic and phenotypic variation observed, the global biological photosynthetic conversion efficiency from sunlight to biomass is estimated to be just 0.2% (Barber, 2007; Ruimy et al., 1994). This is far below the estimated theoretical maximum efficiency of photosynthesis which has been estimated for decades to be around 5%. Efforts to improve this efficiency have mainly involved spending fossil-derived energy on (1) controlling environments and (2) supplying reduced substrate in the form of fertilisers. With a better understanding of the kinetic and energetic constraints on PSII function it is possible that improvements can be engineered at a molecular level which improve yields and render photosynthesis a viable alternative to fossil fuel production.

1.2. The Structure of the PSII complex and its place in the cell

1.2.1. PSII in its photosynthetic membrane

Mature PSII particles are found in specialised photosynthetic membranes with characteristic lipid composition (I. Sakurai et al., 2006) which can contain photosynthetic antenna proteins (Croce and van Amerongen, 2014) and other proteins involved in the light-reactions of photosynthesis. Reaction centres in the early-diverging purple and green bacteria are found on the cytoplasmic membrane (Drews and Golecki, 1995; Oelze and Golecki, 1995). In cyanobacteria, internal thylakoid membranes contain PSI, PSII and the *b₆f* complex as well as quinone electron carriers (Bald et al., 1996), with associated soluble phycobilisome antennae complexes attached to PSI/PSII (Glazer, 1985). In eukaryotic thylakoids the soluble antennae is replaced by membrane-bound LHCII complexes (Dekker and Boekema, 2005). Dynamic re-arrangement of the antenna systems balances excitation between PSI and PSII (Allen and Pfannschmidt, 2000). Hydroquinone generated by PSII activity is oxidised by cytochrome *b₆f* (Allen, 2004) and the electron passed to plastocyanin, the substrate of PSI (Amunts and Nelson, 2008) in the classical Z-scheme of photosynthesis (Hill and Bendall, 1960). PSII can be found as monomer and homodimer in different parts of plant thylakoid membranes (Danielsson et al., 2006). Its most active form *in vivo* is the homodimer, while its oligomeric state *in vivo* is contested (T. Takahashi et al., 2009; Watanabe et al., 2009; Zheleva et al., 1998). Numerous other membrane-bound and soluble accessory proteins are also involved in PSII assembly, turnover and repair (Nixon et al., 2010).

1.2.2. The cyanobacterial PSII

The first successful crystal X-ray diffraction measurements on an oxygenic PSII were made using *Thermosynechococcus elongatus* (herein *T. elongatus*) (Zouni, Witt, et al., 2001). These showed that the cyanobacterial PSII monomer is composed of 17 transmembrane subunits, three luminal subunits, two pheophytins, two plastoquinones, the catalytic Mn₄CaO₅ cluster, one heme *b*, one heme *c*, one non-heme iron, and two bicarbonates (Ferreira et al., 2004). This has been confirmed with subsequent improvement in resolution, with the most recent crystal structure also able to resolve 1300 water molecules, including potential substrates in the Mn₄CaO₅ cluster (Umena et al., 2011).

X-ray diffraction data have been collected from PSII crystals derived from *T. elongatus* (Ferreira et al., 2004; Guskov et al., 2009; Kamiya and Shen, 2003; Kern et al., 2012, 2005; Kuhl et al., 2000; Zouni, Witt, et al., 2001) and its close relative *T. vulcanus* (Kamiya and Shen, 2003; Shen and Kamiya, 2000; Shen et al., 2011; Umena et al., 2011), from which the 3D structure of the proteins and their cofactors have been determined to a resolution of 1.9Å. The PSII from these two species, while showing small structural differences, are virtually identical in their primary sequence. The crystal structure has also recently been determined for PSII bound to the inhibitor terbutryn (Gabdulkhakov et al., 2011), in which the Ca^{2+} ion in the active site is substituted with Sr^{2+} (Koua et al., 2013) and as a monomer (Broser et al., 2010). Chapter 3 describes work using similar methods to address structural questions regarding the high-light-induced D1 isoform PsbA3.

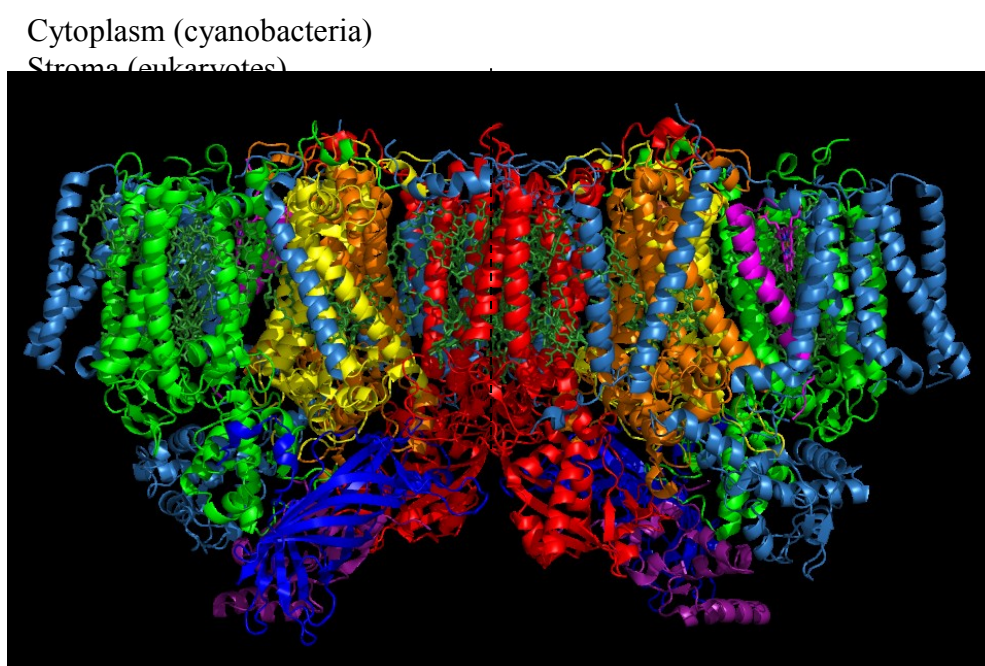


Figure 1-1: Cartoon showing the backbone structure and bound cofactors of PSII, including core subunits CP47 (red), CP43 (light green), D1 (yellow) and D2 (orange). Model generated using data from Umena et al. (2011).

D1 and D2 form a heterodimer at the core of the reaction centre and ligate all of the cofactors involved in electron transfer (see Figure 1-2). CP47 CP43 are intrinsic antennae complexes, containing chlorophylls excitonically linked to the site of charge separation (Bricker and Frankel, 2002) There are two heme-containing cytochromes b_{559} , involved in cyclic electron transfer (Shinopoulos and Brudvig, 2012; D. H. Stewart and Brudvig, 1998) and may play a

role in reducing photoinhibition (Sugiura, Nakamura, et al., 2014), and *c₅₅₀* (Shen et al., 1992) whose role is less well understood (Roncel et al., 2012) and has been related to maintaining Mn₄CaO₅ cluster stability (Shen et al., 1998).

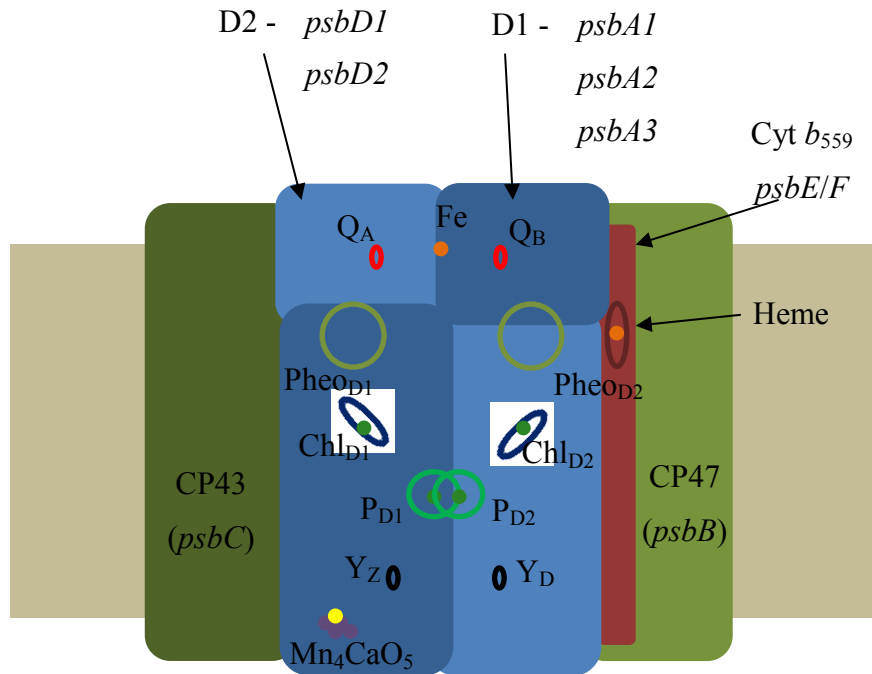


Figure 1-2: Cartoon representation of the cyanobacterial PSII reaction centre showing subunit and cofactor arrangements. Multiple gene names are given for the different isoforms of D1 and D2 in *T. elongatus*.

1.2.3. PSII cofactor arrangement

Pigments involved in electron transfer are arranged in two symmetrical branches associated with the D1 and D2 subunits, forming the active and photoprotective pathways respectively (Diner and Rappaport, 2002; Martínez-Junza et al., 2008). These include two central chlorophylls (P_{D1} and P_{D2}), two secondary chlorophylls (Chl_{D1} and Chl_{D2}), two pheophytins (Pheo_{D1} and Pheo_{D2}) and two plastoquinones (Q_A and Q_B). Two redox-active tyrosines Y_Z and Y_D, D1-Tyr161 and D2-Tyr160 respectively are also involved in electron transfer (Styring et al., 2012).

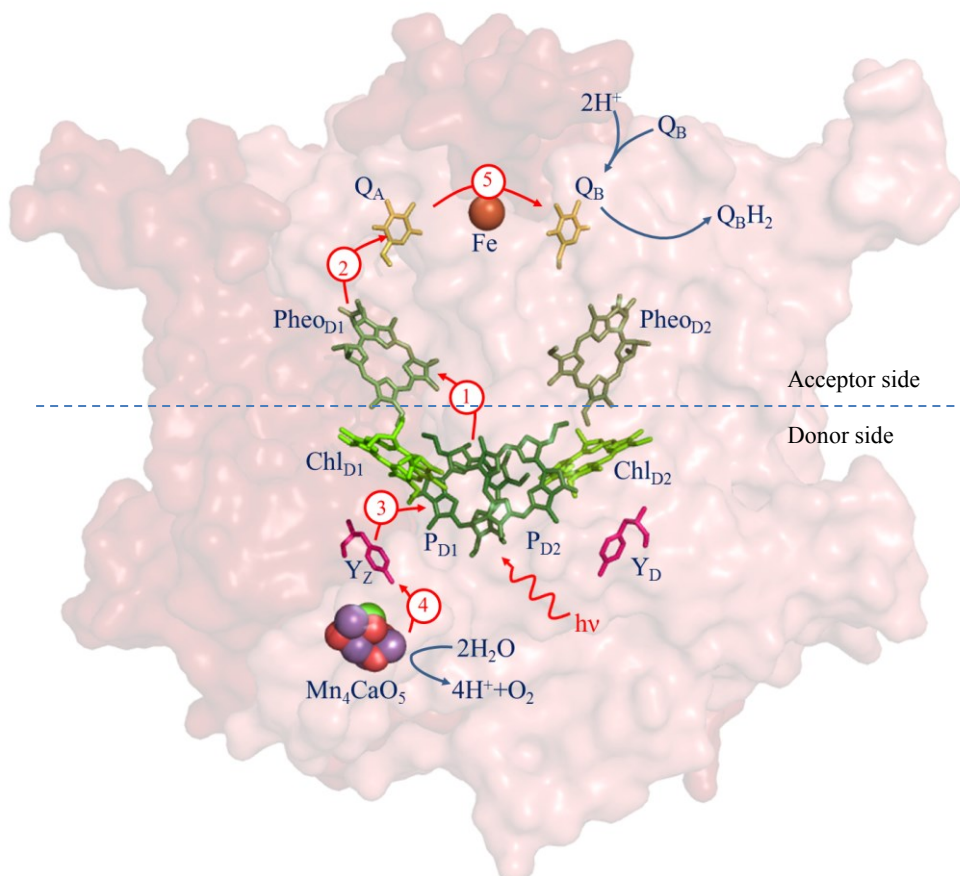


Figure 1-3: Cartoon showing the redox-active cofactors in the reaction centre core consisting of D1 (dark red) and D2 (light red). The sequence of electron transfer reactions between the Mn_4CaO_5 cluster and Q_B is shown in red arrows and the terminal electron donor and acceptors are shown with blue arrows.

Two quinones, Q_A and Q_B , are bound either side of a non-heme iron at the acceptor side of the complex to D2 and D1 respectively (Mieghem et al., 1989). While Q_A is tightly bound, Q_B is exchangeable in its oxidised (quinone) or reduced (quinol) forms (Hasegawa and Noguchi, 2014). A hydrogen bonding network between them bridges the D1 and D2 subunits via a non-heme iron bound to D1-His215, D1-His272, D2-His214 and D2-His268, and its associated bicarbonate ion (Müh et al., 2012). A third quinone binding site was proposed by Kruk and Strzalka (2001) and a candidate Q_C identified in one of the published crystal structures (Guskov et al., 2009; Krivanek et al., 2007), although it does not fit with the original predictions and is missing from the most recent high-resolution structure (Umena et al., 2011). The Q_B site is also the binding location for a variety of PSII inhibitors including DCMU, whose mode of binding will be investigated further in Chapter 3. The non-heme iron modifies the strength of hydrogen bonds between the two acceptor quinones and their

bridging histidines (Lin and O'Malley, 2011), while the bicarbonate also contributes to inter-quinone electron transfer (Chernev et al., 2011).

While Y_Z plays an essential role in forward electron transfer, reducing photo-generated P_{680}^+ and oxidising the Mn_4CaO_5 cluster (Ahlbrink et al., 1998), the role of Y_D is less well understood (Ananyev et al., 2002). Both tyrosines are deprotonated in their oxidised form but the mechanism by which this occurs is different, resulting from differences in their environment. Electron paramagnetic resonance (EPR) has been used extensively to study these residues which form interacted radicals in their oxidised states (Mino and Kawamori, 2008). The structural origins of the difference between Y_Z and Y_D are addressed further in Chapter 5.

During its catalytic cycle, the Mn_4CaO_5 cluster undergoes sequential oxidation and deprotonation reactions as it transitions through five metastable S-states S_{0-4} (Dau and Haumann, 2007; Kok et al., 1970). The structure of the dark-stable S_1 state has been determined in the high-resolution crystal structure, although questions remain about the extent of X-ray-induced oxidative damage (Kawakami et al., 2011). Structural studies on other S-states are limited by their short lifetime and the treatments required to generate homogenous samples. This problem is addressed in Chapter 4 in the context of recent time-resolved crystallographic studies which claim to have measured the structure of the S_3 state.

1.3. PSII Reaction mechanism

Pigments in the PSII core ($D1$, $D2$, $CP47$ and $CP43$) and surrounding antennae proteins ($LHCII$, phycobilisomes (Croce and van Amerongen, 2014)) are excited by the absorption of a photon. This excitation is rapidly equilibrated across all of the chlorophylls in the complex by radiation-less, dipole–dipole-coupling-mediated Förster-type resonance energy transfer (Förster, 1946, 2012; Şener et al., 2011) and other coherence effects (Fuller et al., 2014). The coupling of the central chlorophylls that constitute P_{680} , named for its absorbance maximum at 680 nm (1.82 eV, $175.9 \text{ kJ}\cdot\text{mol}^{-1}$) (Barber, 2002), creates a lower-energy ‘shallow trap’ for this excitation. Mixing of excited and charge transfer states of these coupled central chlorin pigments (Madjet et al., 2009; Romero et al., 2012) results in a primary charge separation event between one of two pairs of pigments, $Chl_{D1}/Pheo_{D1}$ or P_{D1}/P_{D2} , which converge to a $P_{D1}^+/Pheo_{D1}^-$ state with an apparent time constant of 20 ps (Novoderezhkin et al., 2011; Renger and Schlodder, 2011). Subsequent electron transfer generates a series of radical pairs

involving pheophytin and quinone anion radicals on the electron acceptor side, and a neutral tyrosyl radical (Y_Z ; D1-Tyr161) and higher-valence Mn ions on the donor side of the complex (Figure 1-4).

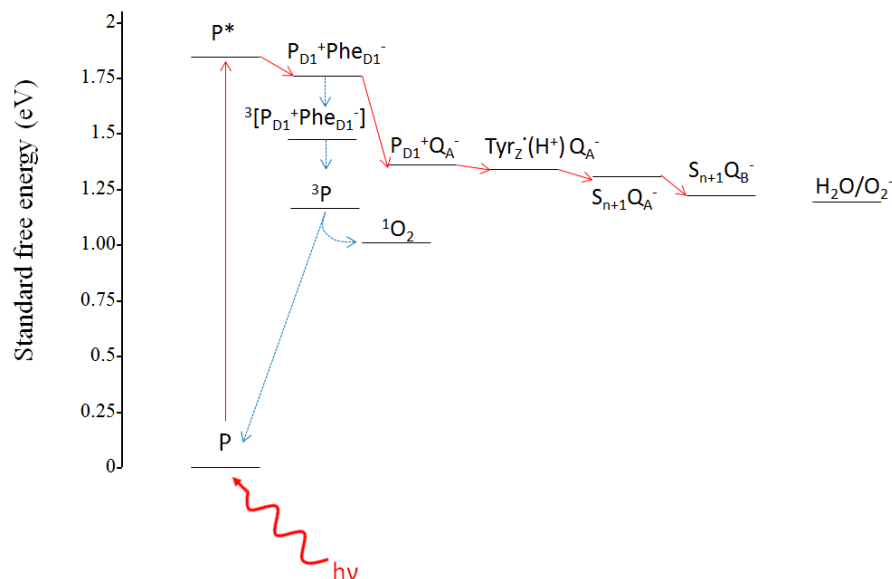


Figure 1-4: An energy scheme of the electron transfer steps in PSII occurring after the absorption of one red photon in the dark adapted stable state, S_1 . Forward electron transfer is favoured by the increasing stability of radical pairs whose redox potentials determine the rates of forward and back reactions. Also shown is the energy required for water oxidation and the triplet formation pathway, major route of photodamage.

1.3.1. Electron transfer between Q_A and Q_B

The reduced primary quinone acceptor Q_A^- is stabilised, possibly by hydrogen bonding with nearby polar D2-Thr217 (Ishikita and Knapp, 2005a), resulting in the loss of a significant portion of the energy of the primary charge separated state. While apparently wasteful, this promotes forward electron transfer and reduces the yield of damaging backwards reactions (Rutherford et al., 2012). The electron is then donated to Q_B which, in contrast to the role of Q_A as a redox mediator, is loosely bound and in equilibrium with the membrane plastoquinone pool. The pH dependence of the equilibrium constant of $Q_AQ_B^-/Q_A^-Q_B$ (~ 20 at pH 7.5, (Diner, 1977), higher at lower pH) of this donation suggests that Q_B^- is stabilised by the protonation of a nearby residue. Recent QM/MM calculations suggests that this residue could be D1-His252 and a resulting re-orientation of the D1-Ser264 hydroxyl group towards a carbonyl group of the quinone could stabilise Q_B^- , promoting forward electron transfer (Ishikita and Knapp, 2005a).

A second photochemical charge separation event within the lifetime of Q_B^- results in Q_B^- protonation, reduction and a second protonation producing Q_BH_2 which is exchanged with quinones in the membrane pool. Calculations suggested that D1-His252(H^+) donates its proton to Q_B^- via D1-Ser264 which re-orientates and forms a hydrogen bond to $Q_BH^\cdot$, and that this event is favoured when Q_A is reduced (Saito et al., 2013a). The second electron is then transferred from Q_A^- to $Q_BH^\cdot$ to form Q_BH^- , in agreement with theoretical analysis of bacterial reaction centres (Graige and Paddock, 1996; Okamura et al., 2000). The identity of the proton donor in the second protonation event is less clear as PSII lacks equivalent residues to those thought to be involved in the better understood bacterial reaction centre, specifically L-Glu212 (Michel and Deisenhofer, 1988). A study of the kinetics of these reactions in *T. elongatus* cells and isolated PSII cores is presented in Chapter 3.

The midpoint potential of Q_A/Q_A^- affects the rates and yields of different oxidation reactions (Fufezan et al., 2007). It has been measured electrochemically in different preparations to be around -70 mV in intact PSII from *T. elongatus*; ~120 mV higher without the Mn_4CaO_5 cluster (60 ± 25 mV) and 52 mV higher in the presence of DCMU (Ido et al., 2011; Johnson et al., 1995; Krieger-Liszkay and Rutherford, 1998). A lower set of potentials have also been reported for the intact and Mn-depleted centres of -140 mV and 20 mV respectively (Shibamoto et al., 2009). Indirect estimates of the Q_B/Q_B^- midpoint potential suggest that it is 83 mV to 120 mV more positive compared to Q_A (Crofts and Wraight, 1983; Minagawa et al., 1999; Robinson and Crofts, 1983) although no direct measurement have been made.

1.3.2. Kinetic assay of Q_A^- oxidation - Chlorophyll fluorescence

The quantum efficiency of charge separation in active ‘open’ reaction centres is around 90% at room temperature (Dau et al., 2012) and close to 100% at 1.7 K (Hughes et al., 2006). This is determined by the competition between forward reactions and decay of the excited singlet states of chlorophylls in the antenna. Fluorescence from PSII results from radiative de-excitation of the central chlorophyll from the singlet excited state to the ground state with the emission of a 680 nm photon (Krause and Weis, 1991). These losses are separated into two categories, depending on their mechanism. Prompt fluorescence (PF), which occurs in the ps-to-ns time scale after illumination, includes fluorescence from Chl^* before any photochemical processes have occurred, as well as luminescence resulting from recombination of the fast-decaying $P^+/Pheo_{D1}^-$ state re-forming Chl^* (Papageorgiou, 2007).

Delayed fluorescence (DF), which occurs on the μs -to-h time scale after illumination, arises from Chl^* whose excitation comes from charge recombination of more stable charge separated states. The large time range of this phenomenon is due to the different stabilities of the many charge-separated states that can be generated and the effect this has on the probability of recombination over time (Goltsev et al., 2009).

The PF yield of a photo-excited sample (from chlorophylls and other pigments e.g. phycobilisomes) gives a comparative measurement of the yields of different quenching reactions (photochemical and non-photochemical) in the sample (Dau, 1994; Krause and Weis, 1991). This technique has many uses in vivo, assessing photosynthetic performance and adaptive quenching responses to illumination (Baker, 2008; Murchie and Lawson, 2013), as well as measuring reactions occurring in PSII itself. Generally, short ($<10 \mu\text{s}$) detection pulses are given by LEDs (because of their short rise and fall times in the ns range) and the number of photons used is adjusted with flash duration and intensity such that sufficiently large fluorescence signal is produced while minimising their photochemical effect on the sample.

During the lifetime of Q_A^- , the fluorescence yield is increased (Schatz and Holzwarth, 1986). This phenomenon has been related to an electrostatic effect on Pheo_{D1} , reducing the yield of charge separation and increasing the decay of $\text{P}^+\text{Pheo}_{\text{D1}}^-$, although the exact mechanism is contested (Ishikita et al., 2006; M. Szczepaniak et al., 2009). These centres in which the photochemical yield is zero are said to be ‘closed’. The increase in the fluorescence yield immediately after illumination can be used as a measurement of the Q_A redox state, or the proportion of PSII which are ‘closed’ by the illumination, and its decay as a measurement of the oxidation of Q_A^- (Krause and Weis, 1991). Illuminating PSII increases the fluorescence yield (F) from a fully-open F_0 to a light-induced F_M' and eventually a fully-closed F_M . The amount of Q_A^- generated by a flash is calculated as $F_V = F_M' - F_0$.

The fluorescence yield is measured by applying a weak flash and measuring PF over 4-10 μs . Typical experiments consist of a series of weak ‘detection’ flashes designed to probe the fluorescence yield and ‘actinic’ flashes or illuminations designed to excite P_{680} . In order to limit each reaction centre to a single excitation, short (flash length $\ll$ shortest decay half time) single-turnover flashes can be used. In order to achieve excitation in all PSII centres (saturation) in a sample, laser pulses are often used. The fluorescence yield immediately after a single-turnover saturating flash does not represent a true F_M as 1) the quantum yield of

charge separation < 1 , and 2) the signal from centres in which Q_A^- is oxidised in the interval between excitation and the first measurement is lost. In order to address 1) a sample can be illuminated with high intensity light for enough time that open centres whose first excitation decayed as PF receive further excitation. In order to address 2) herbicides such as DCMU are used which compete with quinone for binding in the Q_B site and eliminate the fast oxidation of Q_A^- by Q_B/Q_B^- .

The overall rate at which F decays (i.e. the rate at which Q_A^- is oxidised) is dependent on the distribution of redox states of the electron acceptors downstream of Q_A^- and the kinetics of electron transfer between them. In an ideal 100% dark-adapted sample (S_1 , Y_Z , Y_D , P_{680} , $Pheo_{D1}$, Q_A , Q_B) containing only fully-active PSII, the fluorescence decay kinetic following a single-turnover flash would follow a single-exponential decay: the first-order electron transfer from Q_A^- to Q_B . The rate constant for this transfer is dependent on ΔG^0 (in turn dependent on the E_m of Q_A/Q_A^- and Q_B/Q_B^-), the distance and the nature of the intervening medium according to Marcus theory (R. A. Marcus, 1964). In experimental conditions, polyphasic decays are seen due to the presence of competing reactions and heterogeneity in the sample in terms of initial state. These processes will be analysed further in chapter 3.

1.4. *T. elongatus*, a model cyanobacterium

The thermophilic cyanobacterium *T. elongatus* BP-1 (formerly *Synechococcus elongatus*, Toray), isolated from volcanic hot-springs in Japan (Komárek, 2010; Yamaoka et al., 1978), is a common model species for studying PSII and is the principal biological material for the present study. It is a non-nitrogen-fixing species which grows as a single cell or as undifferentiated filaments. The high stability of PSII in thermophilic species facilitates the extraction and purification of active, homogenous PSII complex samples making it suitable material for biophysical and biochemical studies aimed at understanding the structure/function aspects of the enzyme.

The complete genome sequence of *T. elongatus* was published in 2002 (Y. Nakamura et al., 2002), enabling the identification of potential genes as well as the application of targeted molecular mutagenesis techniques. It contains three genes coding for isoforms of the D1 subunit (*psbA1* - tlr1843, *psbA2* - tlr1844 and *psbA3* - tlr1477; Figure 1-5 (A) and (B)) and two genes coding for isoforms the D2 subunit (*psbD1* - tlr1630 and *psbD2* - tlr0455; Figure 1-5 (C) and (D)) (see Figure 1-2 for subunit and gene nomenclature). In order to generate

PSII samples consisting of homogenous mutated reaction centre proteins it is necessary to knock out all but one of the alternative genes.

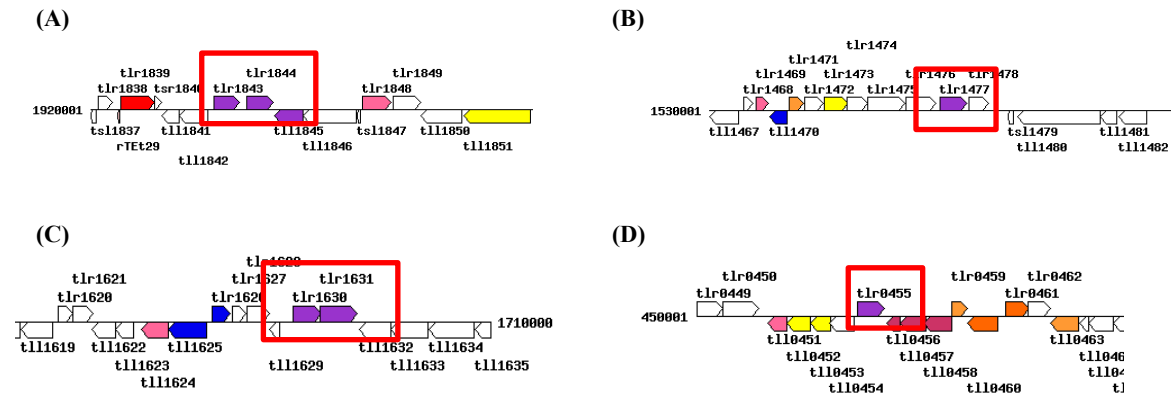


Figure 1-5: Target loci in the *T. elongatus* genome. Region surrounding (A) *tlr1843* (*psbA1*) and *tlr1844* (*psbA2*); (B) *tlr1477* (*psbA3*); (C) *tlr1630* (*psbD1*) and *tlr1631* (*psbC*) and (D) *tlr0455* (*psbD2*) in the *T. elongatus* genome. Obtained from the KEGG genome database at <http://www.genome.jp/>.

While ploidy has not been reported in *T. elongatus* it can be inferred from that of related species. Chromosomal copy number is highly growth-phase dependent in the more commonly used *Synechocystis* sp. PCC 6803 (herein *Synechocystis*), ranging from 218 copies per cell during exponential growth phase to 58 copies per cell during linear and stationary growth phases (Griese et al., 2011). The chromosomal copy number in *T. elongatus* is likely to be much lower and less variable, similar to the more closely related (Schirrmeyer et al., 2011) *Synechococcus* sp. WH8101 (Armbrust et al., 1989) and *Synechococcus* sp. PCC 6301 (Binder and Chisholm, 1990) strains which have 1 and 1-6 copies respectively. This increases the complexity of segregation following transformation.

1.5. Genes and proteins of the D1 subunit

1.5.1. PsbA isoforms, functions and expression profiles

The D1 subunit of the PSII complex coordinates all of the cofactors involved in the active forward electron transfer pathway except Q_A (Figure 1-2). The *psbA* gene encoding the D1 subunit of the PSII core has three different isoforms in *T. elongatus* encoded by three different alleles (Y. Nakamura et al., 2002). These are preferentially expressed under normal (*psbA1*), microaerobic (*psbA2*) and high-light (*psbA3*) conditions. In order to study the properties of the high-light, isoform wild-type cultures have been exposed to high-intensity

light ($>500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) which results in a transient increase in *psbA3* mRNA over ~30 min to $>90\%$ (Kós et al., 2008). This is correlated with an increase in the proportion of PsbA3 protein to ~80% after 1.5 h (Sander et al., 2010). In order to generate homogenous PsbA3-containing PSII, however, it is necessary to knock out the other *psbA* genes 1 and 2 (Sander et al., 2010; Sugiura et al., 2008).

The D1 core subunit of PSII harbours the cofactors which constitute the active electron transport chain from D1-Y161 to Q_A , as well as contributing most of the coordinating residues of the Mn_4CaO_5 cluster. As is it the primary site of photochemistry it is also the primary site of photoinhibition and photodamage when the complex is under photo-oxidative stress (Vass, 2012). Damaged D1 proteins are rapidly re-cycled, as frequently as every 20 min in high light (Ohad et al., 1984; Tyystjärvi et al., 1994).

Whereas higher plants only have a single copy of the *psbA* gene (besides a duplication in some conifer chloroplasts), many cyanobacteria including *T. elongatus* have multiple *psbA* genes coding for isoforms of the D1 protein. The translation of these different genes has been shown to be dependent on culture conditions including the intensity of UV-B and visible light (Clarke et al., 1993), and temperature. While the terms D1:1, D1:2 and D1' are used consistently across species to refer to homologues of the isoforms expressed under normal, high-light and microaerobic conditions respectively, the gene nomenclature varies. Combining the adaptive significance of this behaviour with measurements of difference in electron transfer properties between D1 isoforms to determine the role of each (Golden, 1995; Mulo et al., 2009). The relationship between changes in cofactor potentials that influence electron transfer and structural differences between the isoforms can be further tested using molecular modelling software (Loll et al., 2008).

1.5.2. *psbA* transcription controls isoform switching

There have been many studies demonstrating similar *psbA* isoform transcription regulation in response to culture conditions in a number of different cyanobacteria (Golden, 1995; Mulo et al., 2009; Sugiura and Boussac, 2014b). In *T. elongatus*, *psbA1* codes for D1:1 and constitutes around 95% of *psbA* mRNA under normal ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$) light conditions, with the remaining transcripts originating from the D2:2 isoform *psbA3*. Under high light conditions ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$) this distribution is reversed with *psbA3* constituting 98% of *psbA* mRNA. Transcription of *psbA3* is also up-regulated to a lower degree under UV-B

irradiation ($12 \mu\text{mol m}^{-2} \text{s}^{-1}$) to 20% of the pool. The D1' isoform *psbA2* remained at trace levels under these conditions (Kós et al., 2008).

Similar *psbA* regulation has been shown in *Synechococcus elongatus* PCC 7942 (referred to here as *S. elongatus*) (Bustos et al., 1990; Clarke et al., 1993; Golden et al., 1986; Kulkarni and Golden, 1994), *Anabaena* sp. PCC 7120 (Sicora et al., 2006) and *Synechocystis* (Tichý et al., 2003). In contrast, two of the five *psbA* genes found in *Gloeobacter violaceus* PCC 7421 are constitutively expressed, while the expression of another is up regulated under high light all three of which correspond to the D1:2 isoform (Sicora et al., 2008). Low temperature treatments have also been shown to induce expression of the D2:2 isoform in *S. elongatus* (D. A. Campbell et al., 1995; Sane et al., 2002; Sippola et al., 1998).

The high turnover of D1, measured under high intensity light by the effect of a protein synthesis inhibitor lincomycin, suggested that 65% of D1 proteins would be D1:2 after 1.5 h of exposure, based on 75% being synthesised from an mRNA pool of roughly 80–90% *psbA3* transcripts (Kós et al., 2008). Later studies in which the protein variants could be identified by mass spectrometry showed similar expression patterns and confirmed this prediction measuring 98% of transcripts and 42% of proteins derived from *psbA3* after 1.5 h of high light, reaching a steady 70-80% of both when continuous (Sander et al., 2010).

The effect of D1:1/D1:2 variations on PSII activity, measured as O_2 evolution, have been demonstrated in *T. elongatus* strains expressing either *psbA1* or *psbA3*. In cells expressing only *psbA1*, the addition of lincomycin did not affect the rate constant for the decrease of the O_2 evolution activity under high light, indicating that *psbA1* expression is not able to compensate. Furthermore, on exposure to high intensity light O_2 evolution rates decreased faster in *psbA1*- than *psbA3*-expressing cells both with lincomycin, indicating the resistance to photo-oxidative stress, and without lincomycin, including the effects of expression regulation. In these studies the rate of the decrease in D1 concentration is similar in both *psbA1* and *psbA3* cells and much slower than the rate of decrease in O_2 evolution. Compared to the difference in O_2 evolution this demonstrates the importance of photo-protection and protein turnover rather than the level of expression. The faster decrease in O_2 evolution in cells containing mostly PsbA1 than those containing mostly PsbA3 correlates with a much faster inhibition of the S2-state formation, giving some indication of the relative and increasing proportion of damaged reaction centres (Ogami et al., 2012).

Wild-type growth rates have been measured for mutant *T. elongatus* lacking either *psbA1* and *psbA2* or *psbA3* under normal growth conditions, indicating that D1 function is not limiting growth (Sander et al., 2010). The *psbA3* knockout shows minimal growth under high light condition, probably due to the down regulation of *psbA1* and the resulting decrease in D1 concentration. Similarly, a higher light and UV-B sensitivity, partly due to higher rate of photodamage and partly due to the less efficient PSII repair, has been observed in *Synechocystis* expressing D1:1 protein compared to the D1:2 from *S. elongatus* (Tichý et al., 2003)

In studies of cyanobacterial *psbA* family genes, *S. elongatus* is the only species to lack a D1' isoform. As well as constitutive expression low-levels in many species, an increase in expression of the D1' isoform in wild-type cells has been demonstrated under anaerobic or microanaerobic ($1.3 \pm 1\%$ (v/v) O_2) conditions in *Cyanothece* sp. ATCC 51142, *Synechocystis*, *Anabaena* sp. PCC 7120 and *T. elongatus* (Sicora et al., 2009; Summerfield et al., 2008).

Promoter regions affecting *psbA* expression have been analysed in *S. elongatus*. Based on the length of transcripts, the transcription of all three *psbA* genes in *S. elongatus* begins 50 bp upstream of the start codon (Golden et al., 1986). The sequence from 236 bp upstream of the translational start codon was shown to be involved in expression *psbA1* using luciferase and LacZ reporter genes, with transcription starting 53 bp upstream of translation. Minimal promoters for *psbA1*, *psbA2* and *psbA3* expression were shown to be -54 bp to +1 bp, -39 bp to +12 bp and -39 bp to -1 bp relative to the transcription start site. (Li et al., 1995; Nair et al., 2001). Transcription initiation around -50 bp and promoter regions starting around -86 bp are similar to *psbA* genes found in *Synechocystis* and *Anabena* PCC 7120 (Mohamed et al., 1993; Shibato et al., 1998). The *psbA3* gene was found to have a longer 5' UTR than the other isoforms at 88 bp, more similar to those of plant *psbA* genes.

1.5.3. Structure and function of the high-light D1:2

The D1:1 and D1:2 proteins in *T. elongatus* *psbA1* and *psbA3* differ at 21 amino acid positions, 6 of which are located in the N-terminal tail (Kós et al., 2008). This region has been implicated in the recognition and selective degradation of damaged D1 by FtsH and its replacement in *Synechocystis* (Komenda et al., 2007). It is therefore likely that the photoprotective role of D1:2 is also related to an increase in the rate or efficiency of in D1 turnover which is necessary under conditions where protein damage is likely such as photo-

oxidative stress or UV radiation. This effect could explain the expression of D1:2 under low temperature conditions where lipid fluidity could reduce D1 turnover (Sippola et al., 1998) and when UV-induced protein damage requires rapid repair.

A key substitution in the D1:1 isoform is a glutamine at position 130 which is replaced with a glutamate in the D1:2 isoform. This residue interacts with a key pheophytin co-factor, influencing its functional characteristics. Comparisons of the absorption spectra of PSII containing different D1 isoforms has been used to measure differences in the electronic transitions of their bound cofactors (Hughes et al., 2010). A red-shift in the absorption peak assigned to Pheo_{D1} between D1:2- and D1:1-containing PSII from *T. elongatus* D1:1, from 547.3nm to 544.3nm, has been ascribed to its hydrogen bond between Pheo_{D1} and D1-Glu130 (Shibuya et al., 2010). The same study found for the D1:2 isoform that the quantum efficiency of photo-induced oxidation of side-pathway donors was higher than with D1:1.

Thermoluminescence (TL) measurements of the temperatures at which back-reactions occur following a saturating flash and rapid freezing can be used to characterise PSII by its electron transport properties. The rate of recombination from the charge-separated S₂Q_A⁻ and S₂Q_B⁻ states trapped by the low temperature determines the height and the temperature at which the luminescence peak is measured (Rappaport et al., 2005; Rappaport and Lavergne, 2009). *T. elongatus* cells expressing only *psbA3* showed TL peaks of lower amplitude and at lower temperatures compared to the wild type (Kós et al., 2008), indicating a faster S₂Q_A⁻ recombination with an approximately 20 meV smaller ΔG (Cser and Vass, 2007). This upshift in the midpoint potential of Pheo_{D1} has been measured spectro-electrochemically to be 17mV (Sugiura, Kato, et al., 2010) and is supported by a decreased half-time of fluorescence decay resulting from recombination of S₂Q_A⁻ (Kós et al., 2008), although subsequent studies have not seen this effect (Sander et al., 2010). These results are supported by similar inactivation mutations in *S. elongatus* (Sane et al., 2002), site-directed mutagenesis studies in *Synechocystis* (Cser and Vass, 2007; Rappaport et al., 2002) and heterologous expression of *S. elongatus* D1 proteins (Tichý et al., 2003; Vinyard et al., 2013) which confirm the effect of the D1-Glu130Gln substitution.

Conflicting TL measurements on purified protein samples show higher TL peaks for D1:2- compared to D1:1-containing PSII in the presence of DCMU with no difference in temperature. This effect has been attributed to differences in the acceptor side which affect DCMU binding, and hence the Q_A/Q_B⁻ $\leftrightarrow$ Q_A⁻/Q_B $\rightarrow$ Q_A⁻/DMCU equilibrium, favouring the

reduced form of Q_A in D1:1 PSII. No differences in Q_A/Q_B electron transfer were observed as measured by the decay in flash-induced prompt fluorescence (Sugiura, Kato, et al., 2010). Subsequent spectroelectrochemical measurements have also demonstrated a 38mV higher Q_A potential in D1:2 than D1:1 that was previously discounted, possibly related to residue substitutions near Q_B (Kato et al., 2012). Crystallographic work addressing the impact of structural modifications in PsbA3, as well as the binding of urea-type herbicides, is presented in Chapter 3.

1.5.4. Structure and function of the microanaerobic D1'

Analysis of the primary sequence of D1' homologues show only three conserved amino acids that differ in all other D1 forms: Gly80Ala, Phe158Leu and Thr286Ala (Sicora et al., 2009). Two of these residues, F158L and T286A, lie close to Chl_{D1} and P_{D1} and the difference in size of the residues could have a steric effect on the orientation of the cofactors. The effects of these and other residue substitutions have been studied using single site-directed mutants. Studies in *Synechocystis* demonstrate that its D1' protein (*psbA1*) is able to sustain phototrophic growth when its expression is driven by the promoter from a D1:1 protein (*psbA2*) (Salih and Jansson, 1997).

Thermoluminescence peaks at higher temperatures and increased time constants of flash-induced chlorophyll fluorescence decay indicate the stabilization of the S_2/Q_A^- and S_2/Q_B^- charge pairs is increased in D1'-containing PSII in strains expressing the D1' isoform with a D1:1 promoter by about 20meV. Site-directed mutagenesis to introduce D1' substitutions (Phe186Leu, Phe186Ala and Phe186Leu/Pro162Ser) into D1:1 proteins shows similar Q_A^- stabilisation, identifying residues which lead to functional variation. Differences in the slow component of recombination in these mutants which relates to the free energy gap between Q_A and Q_B via the $Q_A/Q_B^- \leftrightarrow Q_A^-/Q_B$ equilibrium shows a decrease in the redox gap between Q_A and Q_B . In the native D1', however, these acceptor side effects are not seen, presumably compensated by other substitutions. This supports the hypothesis that the S_2/Q_A^- and S_2/Q_B^- stabilisation is due to decreased redox potential of the S_2 state (Sicora et al., 2004).

A 50% reduction of variable fluorescence, related to the production of Q_A^- , in a D1'-expressing strain indicates that D1' centres are less efficient than normal D1:1 in terms of quantum yield. The substitution Phe186Leu, located near D1-Tyr161, gives similar results suggesting that this effect is due to a slower re-reduction of P_{680}^+ (Funk et al., 2001). Further

study of the environment of D1-Tyr161 in D1' PSII, using EPR spectroscopy to detect D1-Tyr161 radical formation, have also demonstrated a modified bonding geometry and slower electron transfer processes which are limited by proton transfer to D1-Tyr161 (Sugiura et al., 2012).

1.6. Genes and proteins of the D2 subunit

The D2 core subunit of PSII harbours the cofactors which constitute the photoprotective electron transport chain including the redox active D2-Tyr160 (Rutherford et al., 2004), arranged symmetrically to those in D1. The estimated redox potential of D1-Tyr161 (~0.76 V) is around 0.25 V more negative than that of D2-Tyr160 (~1 V) (Boussac and Etienne, 1984; Tommos and Babcock, 2000; Vass and Styring, 1991), hence the faster rate of electron donation to the P⁺ cation after primary charge separation. D2-Tyr160 can be oxidised and reduced by the Mn₄CaO₅ cluster in different S-states, indicating a possible role in photoactivation (Ananyev et al., 2002; Rutherford et al., 2004). It is also oxidised by P⁺ (Faller et al., 2001) and the positive charge left after deprotonation could promote localisation of the cation resulting from charge on P_{D1}, accelerating D1-Tyr161 oxidation (Styring et al., 2012).

1.6.1. *psbD* expression in cyanobacteria controls turnover

In *T. elongatus*, *Synechocystis* and *S. elongatus*, *psbD1* is cotranscribed as a discistronic mRNA with *psbC* (CP43) whereas *psbD2* is monocistronic. Unlike the *psbA* gene family, *psbD* genes code for identical polypeptides with minor neutral variation in coding sequence. The reason for conserving two genes can therefore not be explained by functional variation.

Quantification of D2 mRNA using Northern blots in *S. elongatus* showed a 5-fold increase in *psbD2* transcription and no change in *psbD1/psbC* transcription, resulting in a 2.5-fold increase in D2 concentration, during 12 h incubation under high intensity light. Similar experiments on a *psbD2*-less strain showed a decrease in D2 concentration by 50% (Bustos and Golden, 1992). This suggests that *psbD2* is up-regulated to compensate for increased D2 degradation under oxidative stress.

Although the absolute concentrations were not reported, experiments in *T. elongatus* showed that a majority (88%) of D2 mRNA from *psbD1* under normal light at 40°C was increased to

90% at 60 °C, decreased to 70% under high intensity light and decreased to 81% under 12 $\mu\text{mol m}^{-2} \text{s}^{-1}$ UV-B irradiation (Kós et al., 2008). As CP43 is less likely to receive photodamage under photo-oxidative stress than D2, this change in relative expression levels could be seen as a way to regulate D2 expression independently of CP43.

1.7. Cyanobacterial transformation

1.7.1. DNA uptake and replication

Transformation, the uptake and replication of DNA from the environment, is part of the normal physiology of many bacterial species and plays a role analogous to sexual reproduction. (G. J. Stewart and Carlson, 1986). For successful transformation, DNA must 1) cross the cells membrane, 2) avoid DNase activity and 3) recombine with the genome or replicate individually. In order to increase the chance of success, 1) chemical, physical and enzymatic treatments have been developed to enable transformation in strains which do not exhibit natural transformation and improve transformation efficiency by inducing competency or disrupting the cells' outer membrane, improving the permeability of macromolecules; 2) DNA can be specifically methylated prior to transformation to prevent degradation and strains have been created in which nuclease gene have been disrupted; and 3) large homologous flanking regions are incorporated into plasmids to promote recombination (Aune and Aachmann, 2010).

The first successful cyanobacterial transformation was reported in 1970, where incubation with genomic DNA extracted from an antibiotic-resistant strain of *S. elongatus* transferred antibiotic resistance to wild-type cells (Shestakov and Khyen, 1970). Natural transformation was first demonstrated in *Synechocystis* in 1982 (Grigorieva and Shestakov, 1982) and has been shown to be related to expression of pili genes (Yoshihara et al., 2002), many of which have homologues in *T. elongatus*. This species which has become a commonly used model organism partly due to the isolation of a strain (Williams, 1988) which can grow heterotrophically, enabling the disruption of photosynthetic genes. While DNA is likely to enter the cell as a single strand, *Synechocystis* can be transformed with linear or circular, single or double stranded DNA (Barten and Lill, 1995). Recombination of linear DNA fragments with the chromosomal DNA requires longer homologous regions (>500 bp) flanking the incorporated region, possibly due to exonuclease activity shortening these

regions inside the cell. Methylation of single-stranded fragments could therefore significantly increase transformation efficiency as well due to the inhibitory effect on exonuclease activity (Vermaas, 1996, 1998).

Electroporation is a technique in which a re-arrangement of a cell membrane's molecular structure resulting in pore formation and increased permeability is triggered by a ns-pulsed electric field (Chen et al., 2006). This technique has enabled the transformation of many species and cell types which do not exhibit natural transformation (Shigekawa and Dower, 1988), although it can introduce random mutations (Bruns et al., 1989). It was first demonstrated in cyanobacteria in *Anabaena* sp. strain M131 (Thiel and Poo, 1989), avoiding DNase activity by transferring a self-replicating plasmid between strains.

Although it lacks an exploitable endogenous plasmid, *T. elongatus* was the first thermophilic cyanobacteria to be transformed by electroporation, using a *E.coli*-derived IncQ plasmid vector which was replicated by the host and recombined with chromosomal DNA disrupting expression of PSI genes (Mühlenhoff and Chauvat, 1996). Although this study concluded that the species was not naturally transformable after protocols designed for *Synechocystis* and chemical treatments which increased transformation efficiency in *E. coli* were unsuccessful, conjugation with *E. coli* was successful in producing transformants. Natural transformation in a *T. elongatus* strain was first mentioned in 2002 (Y. Nakamura et al., 2002) and first reported in 2004 (Iwai et al., 2004; Onai et al., 2004).

Many variables affecting electroporation efficiency have been optimised for *T. elongatus* (Iwai et al., 2004; Mühlenhoff and Chauvat, 1996; Onai et al., 2004). Studies show that the number of colony-forming units after selection is dependent on 1) increasing transformation efficiency and death rate with field strength with an optimum between 10 and 11 kv/cm; 2) growth phase of transformed cell with an optimum at during 'log phase' ($0.5 < OD_{730} < 0.8$, 5×10^8 cells/ml); 3) increasing transformation efficiency with DNA concentration; 4) recovery conditions, with no colonies forming without 24h incubation in liquid culture under optimal growth conditions; 5) selection conditions, with more slower growing colonies forming when embedded in soft agar. Optimised natural transformation protocols consist of a period of incubation of cells and DNA at high concentration before similar recovery and selection.

Further improvements to transformation efficiency have been achieved by modifying the transformation plasmid (using a thermostable codon-optimised resistance cassette,

incorporating native promoter sequences) (Onai et al., 2004; Vinyard et al., 2013) and the strain (disrupting the putative type I restriction endonuclease) (Iwai et al., 2004).

1.7.2. Selection, segregation and the problem of ploidy

Polyploidy is common in cyanobacteria and while the chromosomal copy number in *T. elongatus* has not been directly measured, comparison to related species suggests it is likely to be close to four (Griese et al., 2011). After the uptake of plasmid DNA into a cell there may be only one recombination event between homologous sequences, leaving mutant cells with multiple copies of the wild type sequence on non-recombinant chromosomes. With the incorporation of an antibiotic resistance cassette that is closely linked to a desired mutation, positive selection for the recombinant sequence can be introduced by antibiotic stress. This leads to an increase in the proportion of chromosomes that contain the recombinant sequence in a clonal population. In the absence of this selective pressure, however, reversion in such incompletely segregated lines is common as the mutation itself or the expression of an antibiotic resistance cassette are likely reduce a cell's reproduction rate, favouring wild-type sequences. Over successive generations, and with gradually increasing antibiotic concentrations, cell lines with more completely segregated genomes can be selected until fixation of the mutant sequence is achieved and the wild-type sequence cannot be detected.

The likely phenotypic effect of a mutation should also be considered as it will affect the balance of selective pressures during segregation. The selective advantage of antibiotic resistance could be reduced as mutants may not be viable, and are at least likely to have slower reproduction, under growth conditions optimised for wild-type cells. This consideration is particularly important in the present study as mutations are being made that affect the cell's primary phototropic machinery. In order to remove the selective disadvantage of PSII subunit gene knockouts in the photoheterotrophic species *Synechocystis* sp. PCC 6803, for example, herbicides which inhibit PSII activity such as DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) are often used (Golden and Sherman, 1984; Jansson et al., 1987; Metz et al., 1989; Nixon et al., 1992). This approach, however, is not viable in *T. elongatus* as it is obligatorily phototrophic and no culture conditions have been found which sustain heterotrophic or photoheterotrophic growth.

Mutant strains have been produced with affinity-tagged subunits CP43 (Sugiura and Inoue, 1999) and CP47 (Iwai et al., 2010; Sedoud, Kastner, et al., 2011) to improve purification,

while functional modifications include both the alteration of the primary structure of PSII subunits (Fufezan et al., 2007; Kirilovsky et al., 2004; Sugiura et al., 2008, 2009; Un et al., 2007) and blocking of PSII subunit expression (i.e. knockout) (Iwai et al., 2010; Katoh et al., 2001; Sugiura et al., 2008, 2012, 2004; Sugiura, Iwai, et al., 2010).

The aim of this work is to develop and apply structural and functional analytical techniques to wild-type and modified PSII purified using His-tag affinity chromatography. This approach was chosen in order to try to directly link structural features to their functional effects, providing a better understanding of this relationship. Such information could improve our understanding of the diversity of PSII subunits within and between species, the design of modified photosystems and electron transfer reactions in other proteins. In Chapter 3, data obtained from X-ray crystallography is presented which addresses the differences between normal and high-light isoforms of PSII as well as the mode of action of the herbicide DCMU. This technique is ideally suited to provide detailed structural information and has been demonstrated successfully on different PSII samples. The high-light PSII isoform is interesting for its similarity to PSII in higher plants and its role in reducing photoinhibition, while understanding herbicide binding could allow the design of herbicide-resistant PSII. In Chapter 4, time-resolved measurements of fluorescence yield are presented which show the rates of electron transfer processes at the acceptor side of the protein. These data give important information about the energetics and efficiency of charge stabilisation and are relevant to the design of studies which rely on photochemistry to advance the enzyme through its catalytic cycle. In Chapter 5, EPR data is presented which addresses hypotheses regarding the structural origin of functional differences between the symmetrical tyrosines Y_Z and Y_D (D1-Tyr161 and D2-Tyr160).

Chapter 2: Materials and Methods

2.1. *T. elongatus* strains and culture methods

Wild type *T. elongatus* BP1 (taxonomy ID: 197221) laboratory strains were obtained from Karim Maghlaoui (designated WT) and Dr. Hua-Ling Mi (designated NT). A *T. elongatus* strain expressing a C-terminal His₆-tagged CP47 (designated 47H) described by Sedoud et al. (2011), as well as variants of this strain containing CP47-Glu364Gln or CP47-Glu364Leu point mutations were generously provided by Diana Kirilovsky (Saclay). All cyanobacteria were grown in DTN medium (Castenholz, 1988; Mühlenhoff and Chauvat, 1996) supplemented with 10 mM NaHCO₃ (BC) at 45 °C. Liquid cultures of 5-30 ml and 50-150 ml were grown in sterile 60 ml and 225 ml vented flasks (Corning) respectively, shaking at 150 rpm under 20 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (18 W white fluorescent tubes) light in an incubated shaker (Agitorb 200C, Aralab). Liquid cultures of 200 ml, 1 l and 3 l were grown in 500 ml, 3 l and 5 l wide-necked Erlenmeyer flasks respectively, with cotton plugs shaking at 120 rpm under 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in an incubated shaker (Innova 44, New Brunswick Scientific). CO₂-supplemented cultures were sparged with filter-sterilised 5% (v/v) CO₂ in air at 150 ml·min⁻¹. Cultures were also maintained on solid DNT with 1.4% (w/v) Bacto™ Agar (BD), 20 mM NaHCO₃ in petri dishes. Where required, mutant culture medium was supplemented with 50 $\mu\text{g}\cdot\text{ml}^{-1}$ (100 μM) kanamycin sulphate (Sigma) or 5 $\mu\text{g}\cdot\text{ml}^{-1}$ (15 μM) chloramphenicol (Sigma).

Table 1: Table of the *T. elongatus* strains used in this study.

Strain	Phenotype	Resistance	Origin
WT	wild-type	N/A	Karim Maghlaoui
NT	wild-type	N/A	Dr. Hua-Ling Mi
47H	C-terminal His ₆ -tagged CP47	50 $\mu\text{g}\cdot\text{ml}^{-1}$ kanamycin	(Sedoud, Kastner, et al., 2011)
$\Delta psbA1/\Delta psbA2$	C-terminal His ₆ -tagged CP47 <i>psbA1</i> and <i>psbA2</i> knockout	50 $\mu\text{g}\cdot\text{ml}^{-1}$ kanamycin 5 $\mu\text{g}\cdot\text{ml}^{-1}$ chloramphenicol	this study, generated from 47H strain
$\Delta psbD2$	<i>psbD2</i> knockout	50 $\mu\text{g}\cdot\text{ml}^{-1}$ kanamycin	this study, generated from NT strain
CP47-Glu364Gln	C-terminal His ₆ -tagged CP47 CP47-Glu364 replaced with Gln	50 $\mu\text{g}\cdot\text{ml}^{-1}$ kanamycin	Diana Kirilovsky
CP47-Glu364Leu	C-terminal His ₆ -tagged CP47 CP47-Glu364 replaced with Leu	50 $\mu\text{g}\cdot\text{ml}^{-1}$ kanamycin	Diana Kirilovsky

2.1.1. Optical density and absorption spectra measurements

Absorbance spectra and wavelength-specific optical density (OD) measurements were made using a UV/visible spectrophotometer (Shimadzu UV-1601) with 1 cm path-length disposable (VWR) or quartz (Helma) cuvettes. The cell densities of liquid cultures were used to track growth phases, measured by optical density at 730 nm. The minimal absorption at this wavelength means that the majority of OD is due to light scattering by cells, roughly proportional to cell density and independent of cell pigment concentration. Cell density was found to be proportional to OD₇₃₀ over the range measured at $6.5 \times 10^7 \text{ cells} \cdot \text{ml}^{-1} \cdot \text{OD}_{730}^{-1}$, measured using a haemocytometer. Growth assays were carried out in glass tubes continuously bubbled with air using a MC 1000 OD Multi-Cultivator (PSI), measuring OD at 680 nm and 720 nm. Cultures were incubated in 100 ml vented flasks until they reached an OD₇₃₀ between 0.5 and 0.7 before being diluted and transferred into the tubes.

2.1.2. Purification of *T. elongatus* genomic DNA

Pure cyanobacterial genomic DNA was prepared using ZR Fungal/Bacterial DNA MicroPrep™ (Zymogen). Cell lysate produced by vortexing with ZR BashingBead™ was filtered and passed through a Zymo-Spin™ IC Column to bind DNA before washing and elution with TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). Cell boilates used to screen for transformant colonies were prepared by suspending a sample taken with a sterile pipette tip of colonies from plates in 10 µl of purified H₂O and heating to 98 °C for 7 min.

2.2. Molecular Biological techniques

2.2.1. *E. coli* strains and growth conditions

The high-efficiency chemically competent *E. coli* strain 10β (NEB) was used during cloning to propagate plasmid DNA (Genotype: araD139 Δ(ara-leu)7697 galU fhuA lacX74 galK (φ80 Δ(lacZ)M15) recA1 endA1 nupG rpsL (StrR) mcrA Δ(mrr-hsdRMS-mcrBC)). *E. coli* cells were grown in Lysogeny Broth (LB) (1% (w/v) NaCl, 1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract) and incubated at 37 °C in a thermostatic incubator (Raven). Cultures grown in LB were placed on an orbital shaker (SSCI, Stuart) set to 200 rpm. LB was supplemented with 1.5% (w/v) Agar for cultures grown on solid Lysogeny Agar (LA) in plates. For long-

term storage cells were suspended in LB medium containing 10% (^w/_v) glycerol, flash frozen in liquid nitrogen and stored at -80 °C. Antibiotics for selective growth in both liquid and solid LB medium were used at the following concentrations: ampicillin 100 mg·ml⁻¹; kanamycin 30 mg·ml⁻¹; chloramphenicol 30 mg·ml⁻¹; spectinomycin 50 mg·ml⁻¹.

2.2.2. PCR amplification

DNA cloning by PCR was carried out in an automatic thermocycler (peqSTAR, Peqlab). Diagnostic amplicons were generated in 10 µl reactions using Taq polymerase (NEB), FailSafe™ buffer G (epicentre) and 1 mM primers using the cycle conditions outlined below. Construction amplicons were generated in 50 µl reactions using Phusion® polymerase (NEB) in Phusion HF buffer, 3% (^v/_v) dimethyl sulfoxide (DMSO) and 1 mM primers using the cycle conditions outlined below. Template DNA was 50-250 ng purified genomic DNA, 1 pg-10 ng plasmid DNA or 2 µl cyanobacterial boilate (for colony PCR). A-tail reactions were carried out in 50 µl reactions containing 30 µl of gel-purified DNA (40-60 ng/µl), 5 U Taq polymerase, 0.2 mM dATP (NEB), and standard Taq buffer (NEB), incubated for 20 min at 70 °C.

Table 2: Thermocycle conditions used for Taq and Phusion® polymerase reactions. Optimisation of annealing temperature was required for some primer-template combination

Taq polymerase	Phusion® HF polymerase
Heat lid to 98 °C	Heat lid to 98 °C
95 °C for 2min	98 °C for 30s
30 ×	30 ×
95 °C for 15 s	98 °C 8 s
60-63 °C for 30 s	60-63 °C for 20 s
68 °C for 1 min·kb ⁻¹	72 °C for 25 s·kb ⁻¹
68 °C for 5 min	72 °C for 5 min
Store at 4 °C	Store at 4 °C

2.2.3. DNA gel electrophoresis

DNA fragments of different lengths were separated by gel electrophoresis in TAE buffer (40 mM Tris-HCL (pH 8.0), 20 mM acetic acid, 1 mM EDTA,) through 0.7-1% (^w/_v) agarose containing SYBR Safe® stain (Invitrogen) diluted 20000-fold at 120 V. DNA bands were visualised using a GelDoc-It™ imaging system (UVP) under UV transillumination or using a Visi-Blue™ converter plate (UVP) to avoid DNA damage. A DNA standard ladder (2-log, NEB) was used to determine the length of DNA in bands after gel electrophoresis.

2.2.4. DNA purification from reactions, agarose gels

DNA was purified from agarose gels and enzymatic reactions using commercially available PCR purification kits (QIAprep, Qiagen or GeneJET™, Fisher Scientific).

2.2.5. pGEM-T Easy vector

The pGEM-T Easy vector (Promega, Figure 2-1) enabling blue-white screening and conferring ampicillin resistance as a selectable marker was used for TA cloning of cyanobacterial gene constructs (L. Marcus et al., 1996).

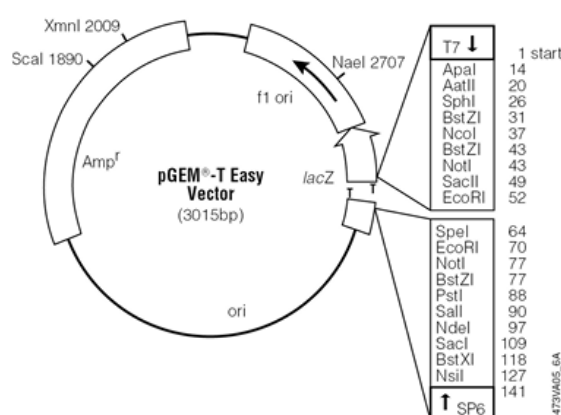


Figure 2-1: pGEM®-T Easy Vector map.

2.2.6. Restriction digests

Digests were carried out using restriction enzymes and buffers obtained from New England Biolabs (NEB). Analytical digests were carried out in 10 µl reaction containing 200 ng of DNA. Blunt digests were carried out in 30 µl reactions containing 2 µg of midprepped plasmid and 5 U nuclease in the appropriate buffer and incubated overnight.

2.2.7. Resistance cassettes

Kanamycin and chloramphenicol resistance cassettes of 1252 bp and 1486 bp respectively were gel purified after release from a pBluescript backbone by *HincII* restriction digestion.



Figure 2-2: Antibiotic resistance cassettes used. Maps of (A) Kan-pBS and (B) Cam-pBS with annotated *HincII* sites used to release the resistance cassettes.

2.2.8. TA and blunt cloning

T. elongatus genes were cloned in 10 μ l reactions containing Rapid Ligation Buffer, 3 U T4 DNA Ligase, 50 ng pGEM-T easy vector (Promega) and a 3:1 molar insert:vector ratio, incubated overnight at room temperature according to manufacturer instructions. Linearized plasmids incubated with 1 U Calf Intestinal Alkaline Phosphatase (NEB) in NEB buffer 3 for 60 minutes at 37 °C and re-purified. Blunt cloning of resistance cassettes was carried out in 5 μ l reactions containing T4 DNA Ligase Reaction Buffer, 3 U T4 DNA Ligase (NEB), 50 ng blunt-digested dephosphorylated plasmid and a 3:1 molar cassette:plasmid ratio, incubated overnight at room temperature.

2.2.9. *E. coli* transformation

50 μ l culture aliquots stored at -80 °C were thawed on ice, mixed with 5 μ l of completed ligation reaction and incubated on ice for 30 min. Cells were then subjected to a heat shock at 42 °C for 30 s (Inoue et al., 1990) and immediately chilled on ice for 10 min. A 450 μ l aliquot of room temperature Super Optimal Broth with Catabolite repression (SOC: 2% (w/v) tryptone, 0.5% (w/v) Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was then added and the cells were incubated at 37°C for 1 h to recover before plating on to LA supplemented with 40 μ g·ml⁻¹ X-gal and 0.1 mM IPTG for blue/white screening.

2.2.10. Plasmid purification from *E. coli*

Plasmid DNA was purified from 2 ml and 100 ml *E. coli* cultures in using QIAprep Spin Miniprep Kit and QIAfilter Plasmid Midi Kit respectively (Qiagen). Cell pellets are lysed in an alkali NaOH/SDS solution containing RNase A (Birnboim & Doly 1979; Birnboim 1983) which is then neutralized and adjusted to high-salt binding conditions causing the co-precipitation of denatured proteins, chromosomal DNA, cellular debris and SDS. Smaller plasmid DNA remains in solution which is separated by centrifugation or filtration. DNA is then bound to a QIAGEN-tip anion exchange column or silica QIAprep Spin Column membrane, washed to remove endonucleases and remaining salt before being eluted in TE buffer (see 2.1.2) or purified H₂O.

2.3. Vector construction

Considering their wild-type expression, *psbA1* and *psbD1* are the ideal targets for mutagenesis. In this study, knockout and mutagenesis vectors were designed for each of the genes, with the exception of *psbA2* for which only knockout vectors were designed. The cloning of intact photosynthetic genes for mutagenesis was unsuccessful, probably relating to leaky expression and toxicity in the *E. coli* strain used, and not described further. *T. elongatus* genome sequence for target genes and flanking regions were obtained from the Kegg genome database (Kanehisa and Goto, 2000). Prokaryote promoter prediction webserver PePPER (<http://pepper.molgenrug.nl/>) and SoftBerry BPROM (<http://linux1.softberry.com/berry.phtml>) were used to identify putative functional sequences in non-coding regions so that their modification could be avoided.

Primers described in Table 3 were designed to amplify *T. elongatus* fragments with overlapping sequences which could be used as templates in overlap-extension PCR (Bryksin and Matsumura, 2010) to create fusion fragments¹. All primers were designed to have a

¹ Primers are named with the gene they are targeting, the letter 'P' for primer, a number (1-4) indicating their annealing order along the region in each set, the letter 'F' or 'R' indicating the synthesis direction and, where necessary, the letter 'D' or 'C' indicating a member of a deletion or cloning set respectively. Fragments produced are named with the gene(s) they are targeting, the letter 'F' for fragment, the letter 'D' or 'C'

melting temperature close to 60 °C for both chromosome-annealing and overlapping sequences using Primer3Plus (Untergasser et al., 2007) and PrimerX (Lapid and Gao, 2006). The overlapping regions contain unique restriction sites in order to insert an antibiotic resistance cassette. A 5' adenine overhang could then be added using a non-proofreading polymerase, complementary to 3' thymine overhang of pGEM-T Easy to facilitate cloning (Zhou and Gomez-sanchez, 2000). In order for a sufficiently high chance of recombination, fragments were designed with ~1 kb upstream and downstream flanking regions. Two fragments were designed for each target loci, one lacking the coding region of the target gene (D) to create knockout mutants and one containing the full sequence (C) into which point mutations will be introduced using a quick-change method (Zheng et al., 2004).

indicating a member of a deletion or cloning set respectively and a number (1 or 2) indicating their order in the wild type sequence. Improved versions are designated using decimal format i.e. 1 → 1.1, 1.2 etc.

2.3.1. Primers

Table 3: Labels used for annotating primers to identify added restriction sites, point mutations, sequence annealing to *T. elongatus* genomic DNA and overlapping fragment regions.

CA*TATG – NdeI	GAT*ATC – EcoRV	A*CTAGT – SpeI
CCC*GGG – SmaI	AAT*ATT – SspI	T*CTAGA – XbaI
XXXXX – point mutation	XXXXX – annealing sequence	XXXX – fragment overlap

Table 4: Primers used to amplify fragments from *T. elongatus*, annotated as outlined in Table 1.

Name	Sequence
A1,2-P1F	GCCACCACTAAATCCGTAGC
A1,2-P4R	ACTATTGCCATTGCCCTGTT
A1,2-P2RD	ACCTTACTAGTGATATCCATATG AGTCGTGATAAGTCCAAATATATTGATT
A1,2-P3FD	CGACTCATATGGATATCACTAGT AAGGTCGCTCCTAAACCTCTCT
D1-P1F	CAGGTGCAAATTGTGGCTG
D1-P4R	TGAACCAGACAAAGCAGGTG
D1-P2RD	TTCATTCTAGAGATATCCATATG
D1-P3FD	ATTCCCATATGGATATCTCTAGA
D2-P1F	CATCAAACTACCGCCAACA
D2-P4R	TAGAGGAAATTGCCCTGCAC
D2-P2RD	GCCCTCTAGAGATATCCATATG AAAATTCAACTCCGAAGGGC
D2-P3FD	TTTTCATATGGATATCTCTAGA GGGCGTAAGAAACCTTATCCTT

2.3.2. Map of A1,2FD

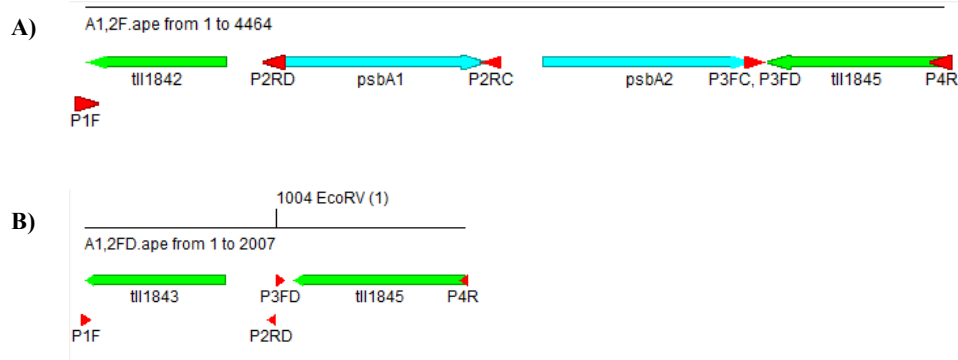


Figure 2-3: Maps showing the deletion of *psbA1/psbA2* (tlr1843/tlr1844). Translated regions and primer binding are shown for (A) wild-type sequence and (B) A1,2FD. A1,2FD lacks the region from the translation start codon of *psbA1* to the stop codon of *psbA2*.

2.3.3. Map of D1FD

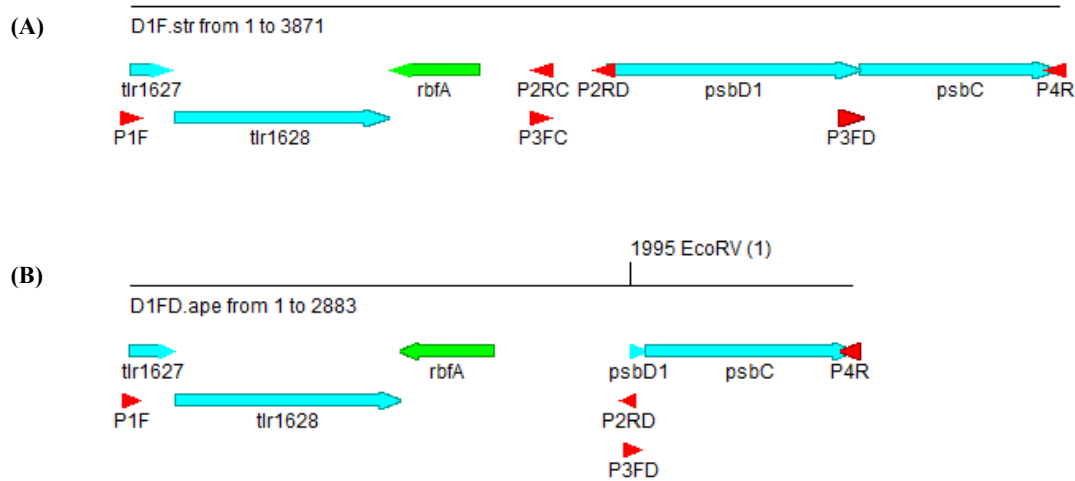


Figure 2-4: Maps showing the deletion of *psbD1* (tlr1630). Translated regions and primer binding are shown for (A) wild-type sequence (tlr1629 annotated as *rbfA*), and (B) D1FD lacking the region from the start codon of *psbD1* to the start codon of *psbC* (tlr1631).

2.3.4. Map of D2FD

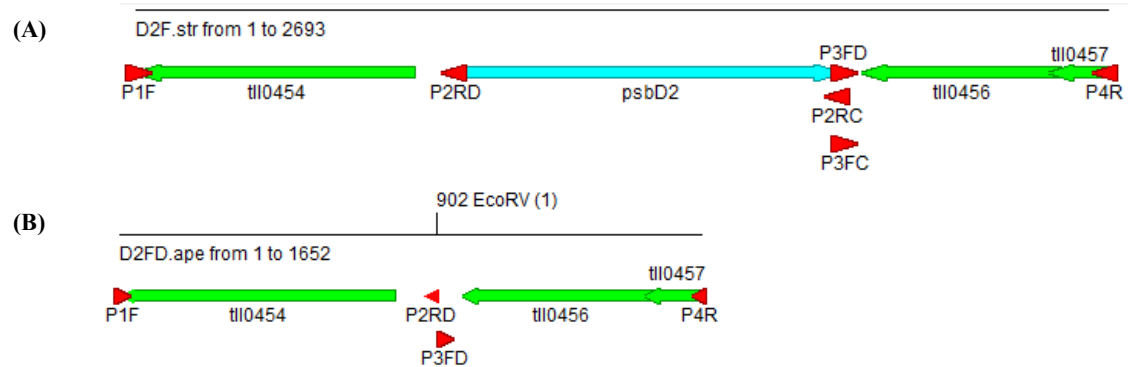


Figure 2-5: Maps showing the deletion of *psbD2* (tlr0455). Translated regions and primer binding in (A) wild-type sequence, (B) D2FD lacking the coding region of *psbD2*.

2.4. *T. elongatus* electrotransformation

Cells from a 50 ml sample of *T. elongatus* culture at $0.5 < OD_{730} < 1$ were harvested by centrifugation at 3500 g at room temperature for 5 min. The cell pellet was washed with 50 ml of 2 mM Tricine-NaOH (pH 8.0), 1 mM EDTA solution and then three times in

18.2 MΩ H₂O before being suspended in 18.2 MΩ H₂O at 20<OD₇₃₀<100 (measured by diluting 5 µl in 995 µl H₂O) and kept at room temperature. A 40 µl concentrated cell sample was mixed with 1-10 µl of salt-free DNA dissolved in 18.2 MΩ H₂O in ice-cold 2 mm-gap electroporation cuvettes (BioRad) and incubated on ice for 2 min. A 5 ms exponentially-decaying electric field pulse was then applied at 5-15 kV·cm⁻¹. The samples were immediately diluted with 1-3 ml of 45 °C DTN + BC and transferred to a sealed tube for a 1-5 day recovery period at 45 °C under low light (5-10 µmol·m⁻²·s⁻¹) shaking at 150 rpm. The samples were then spread, with or without mixing with 3 volumes of DTN containing 0.35% (^{w/v}) Bacto™ Agar (BD), onto DTN plates without antibiotics, with 12.5 µg·ml⁻¹ kanamycin or with 6.25 µg·ml⁻¹ spectinomycin. Colonies were re-streaked onto DTN plated containing 25 and then 50 µg·ml⁻¹ kanamycin or with 12.5 and then 25 µg·ml⁻¹ spectinomycin.

2.5. Preparation and analysis of pure PSII

2.5.1. Chlorophyll concentration measurements

PSII concentrations in pure samples were calculated from their chlorophyll concentrations, assuming that contamination was negligible such that only the 35 chlorophylls associated with the monomeric reaction core were present and that all of the chlorophylls were bound. Chlorophyll concentrations of cell samples were measured spectroscopically after suspending dilute samples in 100% methanol (Lichtenthaler, 1987) and removing insoluble material by pelleting at 8900 g (Fisher AccuSpin Micro, 10000 rpm) for 2 min using:

$$\text{chlorophyll concentration (g} \cdot \text{l}^{-1}\text{)} = \frac{(OD_{665} - OD_{750}) \times \text{dilution}}{79.24} \times 0.894$$

Chlorophyll concentrations of thylakoid or purified PSII samples were measured as above but suspended in 80% acetone (Müh and Zouni, 2005) using:

$$\text{chlorophyll concentration (g} \cdot \text{l}^{-1}\text{)} = \frac{(OD_{663} - OD_{750}) \times \text{dilution}}{76.8} \times 0.849$$

Chlorophyll concentrations of pure PSII samples were also measured in Buffer 1 (Table 4) using:

$$\text{chlorophyll concentration (g} \cdot \text{l}^{-1}\text{)} = \frac{(OD_{674} - OD_{750}) \times \text{dilution}}{70} \times 0.849$$

2.5.2. His₆-tagged PSII Purification

12-18 l of His-tagged *T. elongatus* culture inoculated at $D_{730}=0.1$ were harvested when they reached $0.75 < OD_{730} < 1$ by crossflow filtration (Sartocon® 2 plus holder, Hydrosart® cassette) followed by centrifugation at 4000 g (Beckman J2-21, JA-14 rotor, 6500 rpm), 25 °C for 6 min. The cells were re-suspended in 400 ml Buffer 1, pelleted at 8700 g (as above, 9500 rpm), 25 °C for 12 min, re-suspended in 200 ml Buffer 1 and pelleted again. The cells were finally re-suspended in 50 ml Buffer 1 supplemented with protease inhibitors (1 mM ϵ -aminocaproic acid (Sigma), benzamidine (Sigma) and Pefabloc® (Sigma)), as well as 2 mg·ml⁻¹ (30 μ M) BSA and 50 μ g·ml⁻¹ (1.7 μ M) DNase I, and chilled on ice. Cells were then passed twice through a cell disruptor (T5, Constant Systems) cooled to 4 °C at 25 kpsi to lyse the cells. Unbroken cells were pelleted from the lysate by centrifugation at 1200 g (as above, 3500 rpm), 4 °C for 5 min and discarded.

Membranes were pelleted from the lysate by ultracentrifugation at 150000 g (Beckman Optima™ L-100 XP, TI-70 rotor, 45000 rpm), 4 °C for 25 min and the supernatant discarded before two further washes in an equal volume of Buffer 1 and a final re-suspension in 40 ml Buffer 1. Buffer 1 containing appropriate concentrations of n-Dodecyl- β -D-maltoside (β -DDM; Glycon/Biomol) (M. Nowaczyk et al., 2004) and NaCl was then used to adjust the concentration of chlorophyll to 1 mg·ml⁻¹ (1.1 mM chl; 32 μ M PSII), β -DDM to 1% (w/v) and NaCl to 0.1 M. After stirring for 10 min at 4 °C the sample was spun at 150000 g (as above), 4 °C for 15 min to remove un-solubilised and insoluble membranes. The supernatant was mixed with an equal volume ($>1 \text{ ml} \cdot \text{mg(chl)}^{-1}$) of nickel-chelating resin (ProBond™, Invitrogen) and incubated for 30 min at 4 °C before loading onto a low-pressure 26 mm diameter glass column.

The mobile phase was removed by gravity flow and the column washed in Buffer 2 overnight at $0.5\text{-}1 \text{ ml} \cdot \text{min}^{-1}$ until the flow-through had an $OD_{673} < 0.05$, corresponding to the absorbance peak for chlorophyll bound to PSI and PSII. The absorbance peak wavelength was also monitored to exclude samples with a peak closer to that of PSI (678 nm). Bound PSII was eluted with Buffer 3 and the eluate with $OD_{673} > 0.05$ collected. The sample was concentrating using 100 kDa MWCO filters (Millipore) to $>2 \text{ mg(chl)} \cdot \text{ml}^{-1}$ and used immediately or

washed into Buffer 1, flash-frozen in liquid nitrogen and stored in liquid nitrogen or at -80 °C. The samples produced by this affinity chromatography will herein be referred to as crude PSII in order to differentiate between these and samples separated further using anion-exchange chromatography (see section 2.5.3).

This method was developed throughout the project in order to improve the activity of the purified sample, with help from Wojciech Bialek. NaCl was omitted from the solubilisation step and buffers, and the concentrations of MgCl₂ and CaCl₂ were reduced to 2.5 mM without reducing oxygen evolution activity. With the later availability of an ÄKTA FPLC system, the removal of water-soluble proteins by ultracentrifugation before solubilisation was omitted as the subsequent washing steps could be performed quicker, enabling purification in a single day. Buffers 2 and 3 were modified for compatibility with the ÄKTA system by replacing glycine betaine and glycerol with 0.5 M D-mannitol. Where samples were to be used for immediate anion exchange chromatography, the 15 mM imidazole and 0.3 M imidazole used in buffers 2 and 3 were replaced with 5 mM and 0.1 mM L-histidine respectively in order to avoid the washing step that would be necessary to remove the imidazole before the protein could be bound to the anion exchange column.

Table 5: Buffers for Ni chromatography

Buffer 1	40 mM MES-NaOH (pH 6.5), 10% (v/v) glycerol, 1 M glycine betaine (Sigma), 15 mM MgCl ₂ , 15 mM CaCl ₂ ,
Buffer 2	Buffer 1 plus 0.1 M NaCl, 15 mM imidazole or 5 mM L-histidine, 0.03% (w/v) β -DDM
Buffer 3	Buffer 1 plus 0.2 mM NaCl, 0.3 M imidazole or 0.1 M L-histidine, 0.06% (w/v) β -DDM

2.5.3. Anion-exchange PSII purification

The crude PSII solution was loaded directly onto a Bio-Rad UNO Q-12 column, pre-equilibrated with 2 CV of buffer UnoQ 1 using the ÄKTA Purifier 10 system. The column was then washed with 2 CV of UnoQ 1 or until OD₂₈₀<0.025. PSII complexes were eluted with a linear gradient from 0-60% buffer UnoQ 2 over 180 ml at a flow rate of 4.5 ml·min⁻¹. The resulting peaks were pooled separately and treated as the Ni-column eluate (see 2.5.2). This method consistently yielded four peaks containing inactive monomers (pool 1), active monomers (pool 2), active dimers (pool 3) and inactive dimers (pool 4).

Table 6: Buffers for Anion exchange chromatography

UnoQ 1	20 mM MES-NaOH (pH 6.5), 5 mM MgSO ₄ , 2.5 mM MgCl ₂ , 2.5 mM CaCl ₂ , 0.5 M mannitol, 0.03% β -DDM
UnoQ 2	20 mM MES-NaOH (pH 6.5), 200 mM MgSO ₄ , 2.5 mM MgCl ₂ , 2.5 mM CaCl ₂ , 0.5 M mannitol, 0.03% β -DDM

2.5.4. Size exclusion Chromatography

PSII sample was separated by size into monomer and dimer fractions using a 5 μ m particle size silica gel filtration column (SEC-s3000, Biosep) at a flow rate of 1 ml·min⁻¹.

2.5.5. SDS-PAGE

SDS-PAGE was carried out as following methods described previously (Schägger, 2006). Samples were prepared for SDS-PAGE by mixing 50 μ l of PSII directly from chromatographic fractionation or diluted to 80 μ g(chl)·ml⁻¹ (0.89 mg(PSII)·ml⁻¹, 2.6 μ M PSII) with 12.5 μ l Buffer A (12% (w/v) SDS, 30% (w/v) glycerol, 0.005% (w/v) Coomassie Blue G-250 (National Diagnostics), 150 mM Tris-HCL (pH 7.0)) and 7.5 μ l 10% (w/v) DTT, and incubating at 4 °C for 1 h. Insoluble material was pelleted in a microcentrifuge (maximum speed, room temperature, 2 min). A 15 μ l sample of this mixture was loaded on to a 0.75 mm thick continuous separating gel (9.6% (w/v) acrylamide, 0.4% (w/v) bisacrylamide, 4% (w/v) glycerol, 1 M Tris-HCl (pH 8.45), 0.1% (w/v) SDS, 6 M urea, 0.0005% (v/v) TEMED, 0.05% (w/v) APS) with a stacking gel (3.875% (w/v) acrylamide, 0.125% (w/v) bisacrylamide, 1 M Tris-HCl (pH 8.45), 0.1% (w/v) SDS, 0.00075% (v/v) TEMED, 0.075% (w/v) APS) overlay. Electrophoresis was run at room temperature in a vertical gel tank (Mini-PROTEAN III, PowerPac Basic, Bio-Rad) at 30 V until the protein had completely entered the separating gel and at 150 V until the dye had migrated to the bottom of the gel, generally 2-3 h, using anode (0.1 M Tris-HCl (pH 8.9)) and cathode (0.1 M Tris, 0.1 M Tricine, 0.1% (w/v) SDS) buffers. Gels were stained using either Quick Coomassie Stain (Generon) or SYPRO Orange (Life Technologies) according to manufacturers' instructions and de-stained in 10% (v/v) acetic acid. Pictures were taken using a LAS-3000 CCD digital imaging system (FujiFilm).

2.5.6. CN-PAGE

PSII samples containing 0.7 μ g(chl) (7.8 μ g PSII) per lane were run in 3-12% Bis-Tris gels (NativePAGE™ Novex®, Invitrogen) at 250 V (<10 mA) after 30 min at 100 V using anode

buffer (15 mM BisTris-HCl (pH 7.0)) and cathode buffer (50 mM tricine, 15 mM BisTris-HCl (pH 7.0), 0.05% (w/v) sodium deoxycholate, 0.03% (w/v) β -DDM). Gel imaging was carried out using a LAS-3000 CCD digital imaging system (FujiFilm). Fluorescence emission images were taken using white or blue (460 nm) excitation light and a 670 nm bandpass filter.

2.5.7. Western Blotting

Gels were removed from the cassette and washed in transfer buffer (3 mM Na_2CO_3 , 10 mM NaHCO_3 , 20% methanol). Proteins were transferred to a PVDF membrane (Invitrogen) using a dry blotting system (iBlot[®], Invitrogen) for 1 h at 400 mA. The membrane was removed and incubated in blocking buffer (PBS (137 mM NaCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 2.7 mM KCl, 2.7 mM HCl (pH 7.4)) with additional 0.1% (v/v) Tween 20, 3% (w/v) BSA, 5% (w/v) milk powder (Marvel)) for 1 h to prevent unspecific antibody binding. The membrane was then rinsed with PBS-T (PBS with additional 0.1% (v/v) Tween 20 (Sigma)) and incubated with the primary antibody (1:3000 dilution, anti-His₆, monoclonal, from mouse, Sigma and Invitrogen) at 4 °C overnight. The membrane was then washed three times with PBS-T to remove excess primary antibody before incubating with secondary antibody (anti-mouse IgG, horseradish peroxidase conjugate, 1:10000 dilution in PBS-T, Amersham Pharmacia) at room temperature for 1 h. the membrane was then washed three times in PBS-T to remove unbound secondary antibody and twice in PBS to remove the detergent, for 10 min each time. Binding was detected using enhanced chemiluminescence (ECL; Durrant et al., 1990). The membrane was incubated with a 1:1 mixture of ECL reagent A (100 mM Tris-HCl (pH 8.3) 0.4 mM p-coumaric acid (from 90 mM stock in DMSO), 2.5 mM luminol (from 250 mM stock in DMSO)) and ECL reagent B (100 mM Tris-HCl (pH 8.3), 100 mM H_2O_2) for 1 min before exposure to X-ray film (SuperRX, 100 NIF, 18×24 cm, Fuji Medical) for 5 min. The film was developed according to manufacturer's instructions.

2.5.8. Oxygen Evolution measurements

Oxygen evolution activity of PSII samples was measured in Buffer 1 (see 2.5.2) supplemented with 0.5 mM 2,6-dichloro-*p*-benzoquinone (DCBQ) and 1 mM potassium ferricyanide (FeCN) at 2.5-10 $\mu\text{g}(\text{chl})\cdot\text{ml}^{-1}$ (2.8 μM chl, 80 nM PSII) using a Clark-type

electrode (Oxygraph, Hansatech Instruments Limited) at 25 °C under saturating red light ($>12000 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$).

2.5.9. Crystallisation of dimeric PSII

Purified PSII active monomers and dimers (pools 2 and 3, see 2.5.3) were thawed, washed twice with Crystallisation Buffer (30 mM MES-NaOH (pH 6.0), 3 mM MgCl_2 , 3 mM CaCl_2 , 0.02% β -DDM) using a 100 kDa MWCO centrifugal concentrator (Vivaspin500, Sartorius) and diluted the required chlorophyll concentration in Crystallisation Buffer.

In order to determine the location(s) of zinc binding, zinc chloride was added to the Crystallisation Buffer before adjusting the chlorophyll concentration to make a final concentration of 5 mM. Inhibitor-bound crystals were prepared using DBMU synthesised by Christian Herrero. An aliquot of 100 mM DBMU in DMSO was evaporated in a 2 ml eppendorf tube at 100 °C until dry before adding a PSII sample to a final DBMU concentration of 5 mM on ice and mixing with a pipette for 30 s under $6\text{--}8 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ white light.

Crystallisation conditions were screened using vapour diffusion between 0.2 μl 1:1 sample:buffer sitting drops in 96-well plates and 200 μl well buffer prepared by a liquid handling robot (Mosquito®, TTP LabTech). Initial screening was carried out at 6 and 3 $\text{mg(chl)} \cdot \text{ml}^{-1}$ (0.2 and 0.1 mM PSII) and using MemStart™, MemSys™ and MemGold™ and PGA-LM screens (MD1-41, MD1-33 and MD1-51, Molecular Dimensions). Further buffer testing was carried out using various precipitant concentrations and buffers taken from the literature (Shen et al., 2011). Optimisation was carried out using 2 μl 1:1 and 3 μl 1:2 sample:buffer sitting drops in hand-prepared 48-well plates (MD11-00-100, Molecular Dimensions) with 200 μl well buffer and in 24-well plates (Crychem, Hampton Research) with 400 μl well buffer; using 2 μl hanging drops suspended from siliconized glass or plastic cover slips (MD4-06, MD4-14, Molecular Dimensions) in hand-prepared 24-well plates; and using 4 μl drops in dialysis buttons (HR3-314, Hampton Research) covered with two layers of 12-14 kDa MWCO Visking dialysis membrane (DTV.12000.02, Medicell Membranes Ltd) with 1-2 ml of buffer.

Crystallisation plates were stored in a thermostatic cabinet at 17 °C in the dark. Crystals generally appeared after 1 day and grew for up to 1 week. Crystals were looped and in frozen

in liquid nitrogen, where necessary via well buffer supplemented with 23% (v/v) glycerol for cryoprotection.

2.5.10. **Crystal dehydration**

Crystals were dehydrated in order to reduce the size of the unit cell and improve the resolution of X-ray diffraction data (Krauss et al., 2012) using dialysis, in-situ buffer exchange and on-line vapour humidity control. For dialysis, which has been used successfully in other large membrane complexes (Sazanov et al., 2013), fully-grown crystals were looped into 4 μ l of crystallisation buffer in a dialysis button prepared as described above. The external buffer was replaced every 24 h, increasing the concentration of PEG-1450 (Sigma) to 30% (w/v) and decreasing the concentration of salt(s) to 50% of the initial concentration over 5 days before freezing. For in-situ buffer exchange, which was used successfully to produce the highest-resolution PSII structure (Umena et al., 2011), 2 μ l of well buffer was added to sitting drops containing fully grown crystals. Every 20-30 min 2 μ l of the buffer was removed and replaced with buffer with a 3% (w/v) higher concentration of PEG-1450 and a 5% lower salt concentration to 30% PEG-1450 and 50% salt before freezing. On-line vapour humidity control was achieved using the HC1 device (Sanchez-Weatherby et al., 2009) at Diamond's I03 beamline. Crystals were looped into a drop of well solution and mounted on the beamline on a mesh screen at room temperature. Excess mother liquor was then removed with a paper wick. Crystals were initially equilibrated at 92% RH which corresponded to PSII in the well buffer, followed by dehydration at a rate of $1\% \cdot \text{min}^{-1}$ with 5 min equilibration time before each data collection.

2.5.11. **X-ray diffraction measurements**

X-ray diffraction patterns were obtained using I03, I04 and I04-1 beam-lines equipped with Pilatus3 6M, Pilatus 6M-F and Pilatus 2M (Dectris) detectors respectively at the Diamond synchrotron. Anomalous scattering measurements to locate the bromine atoms in samples containing DBMU were made at the bromine k-edge (0.92 Å, 13.47 keV). Crystals were cooled in a 100 k nitrogen gas stream cryostat (Oxford Cryosystems) during measurements. Diffraction data were collected over 180° with 0.1° per frame. The structure of the PSII complex was solved by molecular replacement using PHASER software, using the 3ARC structure (Umena et al., 2011) as a search model. A model of DBMU was built in COOT

(Emsley and Cowtan, 2004) and aligned with anomalous difference maps. Data processing using xia2, XDS and programs of the CCP4 suite were carried out by Dr James Murray (Imperial College, London).

2.5.12. Fluorescence yield measurements

A temperature-controlled dual-modulation kinetic fluorometer (FL-3000 with TR-2000, Photon Systems Instruments (Trtílek et al., 1997)) was used to make time-resolved measurements of chlorophyll fluorescence. A 1 ml sample of *T. elongatus* cells from growing cultures ($0.5 < OD_{730} < 1$) in DTN or purified active PSII dimers (pool 3, see section 2.5.3) diluted to $5 \mu\text{g}(\text{chl}) \cdot \text{ml}^{-1}$ with buffer (20 mM MES-NaOH (pH 6.5), 5 mM MgSO_4 , 2.5 mM MgCl_2 , 2.5 mM CaCl_2 , 0.5 M mannitol, 0.03% β -DDM) was loaded into the cuvette. Where indicated an appropriate amount of ferricyanide was added to the buffer before diluting the sample to give a final concentration of 0.6 or 6 μM . Samples containing ferricyanide were incubated for >2 h at 4°C before measurements were made. A $50 \mu\text{s}$ 620 nm LED flash was used to excite PSII and weak $3 \mu\text{s}$ red LED flashes were used to induce measured fluorescence. In order to assess connectivity, a Joliot-type pump-probe spectrometer (JTS-10, Bio-Logic) was used with a single actinic laser flash (5 ns, 532 nm, 1.5 mJ; Minilite II Nd:YAG, Continuum) followed by $10 \mu\text{s}$ blue LED measuring flashes. DBMU inhibition was measured using the same actinic flash and $10 \mu\text{s}$ white LED measuring flashes passed through a narrow-bandpass interference filter (543 nm, 3 nm FWHM; Laser 2000) angled to give a transmission peak at 540 nm. Measurements were carried out at 21°C or at 45°C in complete darkness. In order to block forward electron transfer from Q_A to Q_B , $1 \mu\text{l}$ of 100 mM DCMU dissolved in ethanol was added to the samples.

The actinic pulse chosen to be non-saturating in order to minimise the effect of excitonic connectivity between PSII reaction centres which can cause a non-linear relationship between fluorescence and the proportion of Q_A that is reduced (Lavorel and Joliot, 1972; Stirbet, 2013). This also reduces the impact of artefacts arising from triplet states of chlorophyll.

The decay of variable fluorescence F_V arising from centres containing Q_A^- was normalised using the following equation:

$$F_V = \frac{(F_t - F_0')}{(F_M' - F_0')} \quad \text{Equation 4}$$

where F_t is the measured fluorescence at time t , F_0' is the fluorescence measured before the actinic flash, and F_M' is the fluorescence after the actinic flash.

2.5.13. Absorbance measurements

Time-resolved measurements were made using a lab-built dual-laser spectrophotometer (Béal et al., 1999). Crude PSII sample ($20 \mu\text{g}(\text{chl}) \cdot \text{ml}^{-1}$, $0.64 \mu\text{M}$ PSII, in Buffer 1) was loaded into the temperature-controlled 5 ml sample chamber and circulated with a peristaltic pump through the cuvette (2.5 mm path length; 25°C). Measuring flashes were provided by an optical parametric oscillator pumped by a frequency-tripled Nd:YAG (355 nm), which produces monochromatic flashes (1 nm full-width at half-maximum) with a duration of 5 ns at 10 Hz. Excitation flashes were provided by a dye laser (685 nm, 10–15 mJ) pumped by the second harmonic of a Nd:YAG laser (532 nm) at 5 Hz. Two data points were collected after each saturating flash with each sample before the sample was refreshed and the flash spacing changed to measure a new set of points on the kinetic.

2.5.14. Fitting decay data

The fluorescence and absorbance decays showed multiphasic kinetics which were fit to multi-component decay functions by user-generated scripts for use with the MATLAB coding environment. The components included in each fit function depended on the nature of the decay for the duration of the data modelled and nature of the sample. This analysis enabled the deconvolution of different decay components, giving half-times of the reactions they represent as well as their relative amplitudes.

Exponential decay functions of the form $Ae^{(-t/T)}$ were used to model decay reactions where T is the time constant for the reaction ($t_{1/2} = T \cdot \ln(2)$) and A is the amplitude of the component. These components, however, may represent a combination of reactions occurring in the same time range, depending on the sample. Hyperbolic decay functions of the form $A/(1 + t/T)$ were used to empirically model broader decays representing a distribution of first-order rates. The amplitudes of each component are given as the percentage of the decay

over the specified time, from a value extrapolated from the fit function at $t = 0$ to the final Y_0 value, that they account for. Y_0 is also given as a percentage of this decay value. Normalised fluorescence curves were calculated by dividing the fluorescence data and fits by the sum of the amplitudes of the decay components, i.e. setting the fit decay function to 1 at $t = 0$.

Decay kinetics were fit to functions containing multiple exponential components (see Equation 2-5) a combination of exponential and hyperbolic components (see Equation 2-6), depending on their duration and the quality of the fit, using custom scripts in MATLAB (MathWorks).

$$A(t) = \sum_{i=1}^n A_i e^{-t/T_i} + Y_0 \quad \text{Equation 2-5}$$

$$A(t) = A_1 e^{-t/T_1} + A_2 e^{-t/T_2} + \frac{A_3}{1 + t/T_3} + Y_0 \quad \text{Equation 2-6}$$

Where $A(t)$ is the absorbance yield at time = t , $A_1 - A_3$ are the amplitudes of each phase with time constants $T_1 - T_3$ and Y_0 is the proportion of the signal which had not decayed before the experiment was completed. The half-times for the hyperbolic component is T and for the exponential decay components can be calculated as:

$$t_{1/2} = \ln(2) \cdot T \quad \text{Equation 2-7}$$

The parameters generated were used to calculate amplitudes and half-times for a set of first-order reactions which describe the observed decay and correspond to Q_A^- oxidation reactions in the protein.

2.5.15. 77 K fluorescence spectra

PSII samples diluted to a chlorophyll concentration of 5-20 $\mu\text{g}\cdot\text{ml}^{-1}$ with Buffer 1 (see 2.5.2) and glycerol (20% (w/v) final) were injected into quartz tubes and flash-frozen in liquid nitrogen. The fluorescence was measured over a 600-800 nm range after PSII excitation at

435 nm (10 nm FWHM) with a 515 nm short-pass filter to reduce noise using a luminescence spectrophotometer (LS 50, Perkin Elmer).

2.5.16. Low-temperature optical spectroscopy

Low-temperature (<130 K) light-minus-dark difference absorbance spectra of concentrated PSII samples (>8 mg(chl)·ml⁻¹, 9 mM chl, 0.256 mM PSII) in Buffer 1 (see 2.5.2) supplemented with 20% glycerol for cryoprotection and 5 mM FeCN to oxidise Q_A and Q_B were measured using a CCD-based spectrophotometer (OceanOptics). Small (~1 µl) drops of sample were held in 500 µm diameter loops mounted on a positioning goniometer, cooled by a nitrogen gas stream generated by an X-Stream 2000 (Rigaku) to <130 K. The spectra were measured using white light and photoexcitation generated using 40 mW 473 nm diode laser (Thorlabs) for 170 s after 20 s of dark measurements. Measurements were made at 1 Hz and averaged to improve the signal:noise ratio.

2.5.17. Thermoluminescence measurements

Thermoluminescence measurements of the stability of charge separated states were made using a non-commercial device made in-house. A 200-250 µl sample of dark-adapted thylakoids or pure PSII diluted to 2-50 µg(chl)·ml⁻¹ was placed on a metal sample plate in the dark and cooled to 1 °C for 30 s before applying a saturating <1 µs flash from a xenon flashtube. The sample was immediately cooled to -15 °C over ~10 s and stabilised for a further 5 s before heating to 80 °C at either 20 or 60 °C·min⁻¹ using a Peltier element. Photons emitted by luminescence arising from charge recombination in PSII were detected using a photomultiplier (Hamamatsu) and recorded with the sample temperature in a lab-built setup with a 1 Hz detection rate.

2.5.18. EPR measurements of tyrosine radicals

Purified active dimeric PSII purified as described in sections 2.5.2 and 2.5.3. All subsequent procedures were performed in complete darkness. Frozen samples were thawed on ice, diluted with Mn-depletion Buffer (5 mM MES-NaOH (pH 6.5), 2.5 mM MgCl₂, 2.5 mM CaCl₂, 0.5 M mannitol, 0.03% β-DDM, 0.5 mM EDTA-2Na, 10 mM NH₂OH) to 0.5 mg(chl)·ml⁻¹ and incubated for 30 min at room temperature in order to remove the Mn₄CaO₅ cluster (Sugiura and Inoue, 1999). The samples were then washed five times and

diluted to $1.14 \text{ mg(chl)} \cdot \text{ml}^{-1}$ with Wash Buffer (2.5 mM MgCl_2 , 2.5 mM CaCl_2 , 0.5 M mannitol, 0.03% β -DDM). Samples were diluted with Wash Buffer containing CAPSO (Acros Organics), Tris (Melford), HEPES (Melford) or MES (Sigma) adjusted to pH 9.5, 8.6, 7.7 or 6.8 respectively with NaOH or HCl to a final buffer concentration of 100 mM. After incubation at room temperature for 1 h 100 μl of each sample was loaded into a quartz EPR tubes using a flexible tube attached to a 100 μl Hamilton syringe and flash-frozen in an ethanol bath cooled with liquid nitrogen before further cooling in liquid nitrogen and storage in a cooled dry nitrogen shipper.

EPR spectra were measured between 3250 and 3450 G using an EleXsys E500 EPR spectrometer (Bruker). Samples were measured (1) after loading in the dark, (2) during illumination in the cavity with a 1 mW 532 nm laser until the resulting signal was saturated, (3) after subsequent thawing, incubation at room temperature for 60 s and re-freezing, and (4) after illumination for 60 s at room temperature with a 1 mW 532 nm laser, incubation at room temperature for 60 s and re-freezing,. Spectra were recorded at 15 K using the following settings: microwave power=0.05 mW, microwave frequency = 9.4 GHz, field modulation amplitude=2 G, field modulation amplitude=100 KHz, conversion time=40.96 ms, time constant=40.96 ms. The signal was quantified by calculating the area between the curve and a baseline modelled as a binomial quadratic function fit to the signal-free data. The proportion of the Y_D signal that was generated by illumination at 15 K was obtained by subtracting signal (1) from signal (3) and dividing by signal (4).

Chapter 3: Crystallographic analysis of PsbA3-PSII

X-ray crystallography can be used to produce atomic-resolution structural data of complete protein complexes and has been used to generate a 1.9 Å model of PsbA1-PSII (Umena et al., 2011). In order to extend this work, it would be useful to be able to make comparisons with the structure of PSII containing different subunit isoforms which are known to affect the function of the complex. This would allow a more detailed understanding of the design of the enzyme mechanics and its limitations, potentially informing the design of modified PSII. This technique is also ideally suited to determine the binding mode of inhibitors including DCMU. A more complete understanding of this binding site could contribute towards the development of herbicide-resistant PSII, as well as improving our understanding of the energetic effects of herbicides on PSII.

3.1. Generating mutants in *T. elongatus* expressing only a single gene for D1 or D2

In order to study the effect of modifications in D1 (*psbA*) and D2 (*psbD*) it is necessary to produce single knockout mutant strains expressing only a single isoform each protein. This enables homogenous PSII complexes to be obtained of different isoforms, as well as of modified proteins made by modifying the remaining gene. This section describes the creation of gene knockout mutants in *T. elongatus* containing a single *psbA* or *psbD* gene. Both *psbD* genes were targeted for deletion whereas only the region containing *psbA1* and *psbA2* were targeted for D1 due to the difficulty of multiple transformations required to leave only *psbA1* or *psbA2*, as well as the focus on the high-light form.

3.2. Cloning strategy

In order to generate transformation vectors, overlap-extension PCR (OE-PCR) was used with purified genomic DNA as a template to clone the required targets, plus ~1 kb of up- and down-stream sequence in order to improve recombination frequency.

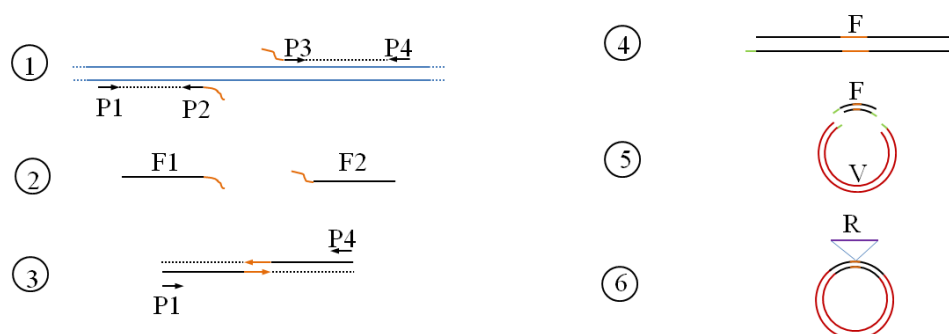


Figure 3-1: Cartoon showing the PE-PCR fragment assembly method used to generate mutagenesis vectors. Primary fragments F1 and F2 are amplified from genomic DNA (blue) using primers P1-4. Primers P3 and P4 contain overlapping complementary sequence with unique blunt restriction sites (orange). The secondary fragment F is generated using the overlapping F1 and F2 as a template with primers P1 and P4. Fragment F is ligated into the vector V and transformed into *E. coli* before sub-cloning an antibiotic resistance marker (R) into the unique restriction site to complete the plasmid.

For deletion vectors, the transcribed region was omitted from the primary fragments, while for mutagenesis vectors the overlapping primers were designed to introduce the unique restriction sites downstream of the stop codon. These fragments were then ligated using TA-cloning into a pGEM-T Easy vector backbone (Promega). The vectors were then blunt-digested at unique restriction sites added during OE-PCR into which a blunt-digested resistance cassette was ligated. The resulting plasmids were then purified, desalted and used for electroporation.

3.3. Transformation, selection, segregation

While the exact mechanisms by which electroporation assist in transporting DNA across cell membranes is not fully understood there are a number of theories in the literature, many involving electrostatic generation of transmembrane pores (Chen et al., 2006; Shigekawa and Dower, 1988). This technique has been used to translocate DNA in a number of cyanobacteria, including *T. elongatus* and relatives (Mühlenhoff and Chauvat, 1996; Thiel and Poo, 1989; Toyomizu et al., 2001).



Figure 3-2: Photograph of a selection plate growing after a successful transformation experiment showing the distribution of colonies embedded in top agar from high antibiotic concentration on the left to lower concentration on the right.

3.4. Generating $\Delta psbD1$ and $\Delta psbD2$ strains

3.4.1. PCR and OE-PCR amplification of D2 fragments

Primary fragments D1-FD1/2 and D2-FD1/2 were amplified from the *psbD1* and *psbD2* regions respectively from *T. elongatus* genomic DNA using Phusion® polymerase (NEB). Amplicons of the correct size (Figure 3-3 (A) and (B)) were gel-purified to remove products of non-specific primer binding. These fragments were then used as the template for OE-PCR reactions to produce secondary fragments (Figure 3-3 (C) and (D)) for ligation into pGEM-T Easy.

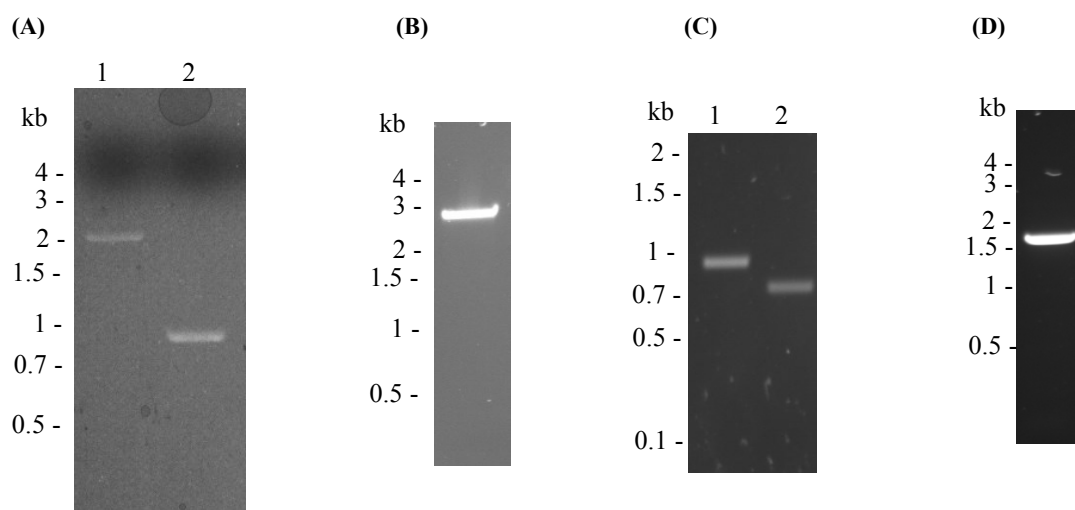


Figure 3-3: Agarose gels showing primary fragments for *psbD* deletion of (A) D1 (lane 1: D1-FD1 (2006 bp); lane 2: D1-FD2 (948 bp)) and (C) D2 (lane 1: D2-FD1 (913 bp); lane 2: D2-FD2

(757 bp)). Secondary fragments are shown in **(B)** (D1-FD (2954 bp)) and **(C)** (D2-FD 1652 bp). Lengths given are for the correct sequences.

3.4.2. Ligation of *psbD* gene fragments into pGEM-T Easy

After gel purification of the secondary fragments, a single adenine overhang was added to their 3' ends in a Taq PCR reaction. The resulting fragments were purified before ligation into pGEM-T Easy and transformation into *E. coli*. Plasmid DNA purified from ampicillin-resistant colonies was screened by single and double digests to identify plasmids of the correct size. Correct plasmids were confirmed by sequencing, named pD1D and pD2D for *psbD1* and *psbD2* respectively.

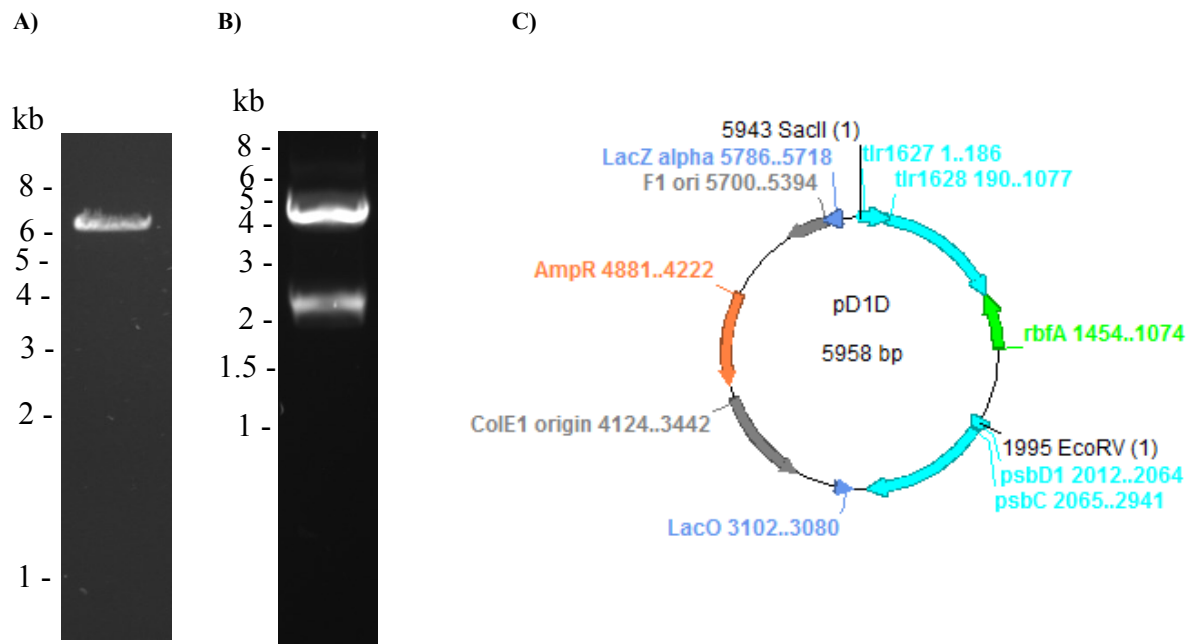


Figure 3-4: Map and agarose gels showing pD1D digested with **(A)** SacII, single cut (~5958 bp) and **(B)** SacII + EcoRV, double cut (3948 bp, 2010 bp). **(C)** Map of pD1D showing the restriction sites used to determine insertion orientation.

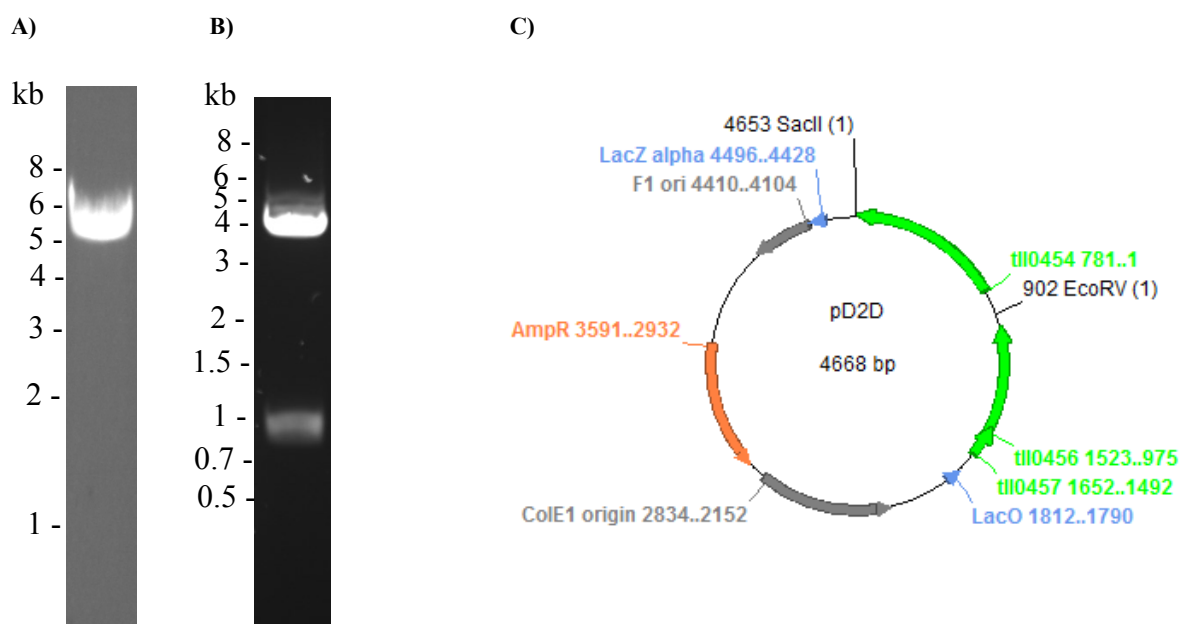


Figure 3-5: Map and agarose gels showing pD2D digested with (A) SacII, single cut (~4668 bp) and (B) SacII + EcoRV, double cut (3751 bp, 900 bp). (C) Map of pD2D showing the restriction sites used to determine insertion orientation.

3.4.3. Insertion of resistance cassettes into pD1D and pD2D vectors

Sequenced plasmids were linearized at the introduced EcoRV restriction sites and dephosphorylated before ligation with a phosphorylated kanamycin (km) resistance cassette (1252 bp). After transformation into *E. coli*, plasmid DNA purified from kanamycin-resistant colonies were screened by gel electrophoresis to identify plasmids of the correct size. As a further test, restriction digests were carried out on potential plasmids before they were sequenced to confirm and determine the direction of the insertion.

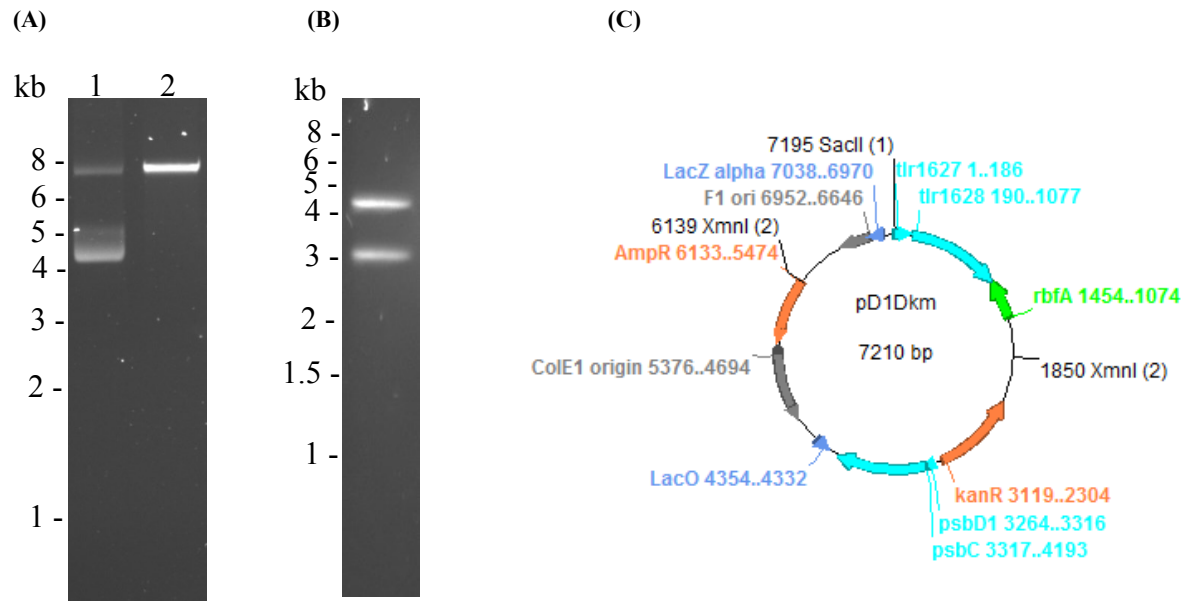


Figure 3-6: Map and agarose gels showing pD1Dkm (A) uncut (lane 1) and linearized with SacII (lane 2, 7210 bp), as well as (B) double-cut XmnI fragments (4289 bp, 2921 bp). (C) Map of pD1Dkm showing the restriction sites used to determine insertion orientation.

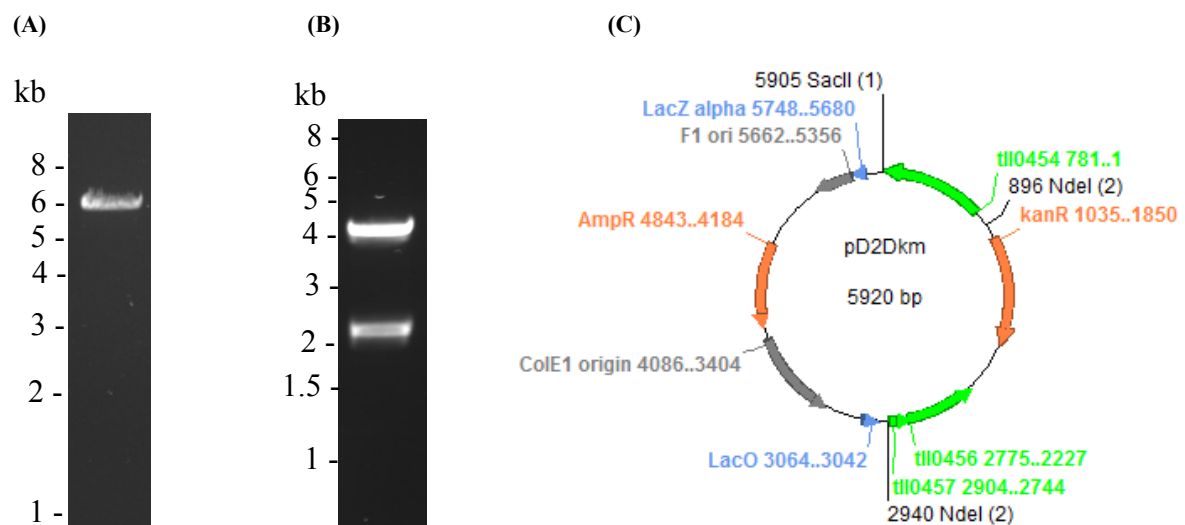


Figure 3-7: Map and agarose gel showing pD2Dkm (A) linearized with SacII (5920 bp), as well as (B) double-cut NdeI fragments (3876 bp, 2044 bp). (C) Map of pD2Dkm showing the restriction sites used to determine insertion orientation.

3.4.4. Screening transformants for *ΔpsbD* strains

The pD1Dkm and pD2Dkm plasmids were used in parallel electrotransformation trials. Cells were picked from individual colonies showing fast growth under antibiotic stress from a significant but slower-growing background on plates containing $12.5 \mu\text{g}\cdot\text{ml}^{-1}$ kanamycin two

weeks after transformation. These colonies were re-streaked twice onto plates with increasing antibiotic concentration to 50 $\mu\text{g}\cdot\text{ml}^{-1}$ kanamycin in order to achieve segregation.

For pD1Dkm transformants, colony PCR was then carried out using a crude cell lysates as the template in order to determine 1) if the antibiotic resistance cassette had integrated into the genome using primers to amplify an 805 bp fragment within the resistance cassette (Figure 3-8 (A)), and 2) if the antibiotic resistance cassette had integrated into the target locus using a forward primer annealing within the cassette and a reverse primer annealing 1515 bp downstream, at the end of the inserted region (Figure 3-8 (B)). These results show that the plasmid successfully recombined in to the genome by double-crossover, although wild-type sequence could still be detected in other PCRs and this strain is still under selection. The complete segregation of this ΔpsbD1 strain is, however, no longer a priority; both D2 sequences are identical and so the ΔpsbD2 strain is sufficient for studying modified D2 through future mutagenesis of *psbD1*.

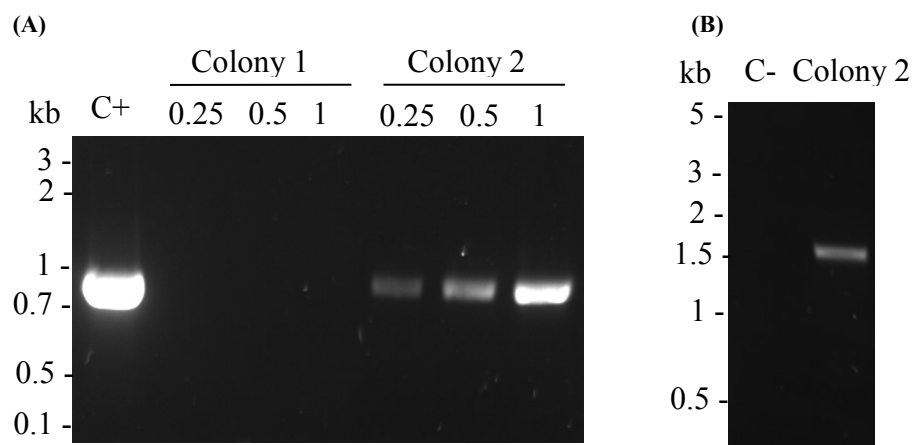


Figure 3-8: Screening colonies transformed with pD1Dkm. (A) PCR amplification of an 805bp fragment within the kanamycin resistance cassette from dilutions of crude cell lysates from two colonies transformed with the pD1Dkm showing high antibiotic resistance. The amount of template was varied in case the lysate was inhibiting the PCR reaction. The first lane is a positive control showing amplification from the purified pD1Dkm plasmid. (B) PCR amplification of a 1515 bp fragment from within the kanamycin resistance cassette to the end of the inserted region from dilutions of crude lysate of antibiotic resistant *T. elongatus*. WT DNA template was used as a negative control.

Similar screening was used for pD2Dkm transformants, which led to the isolation of a fully segregated ΔpsbD2 strain. This was confirmed by the absence of an amplicon in PCR reactions using primers binding to the transcribed region of *psbD2* (Figure 3-9). The

genotype of the selected strain was further confirmed by sequencing PCR amplicons of the modified region.

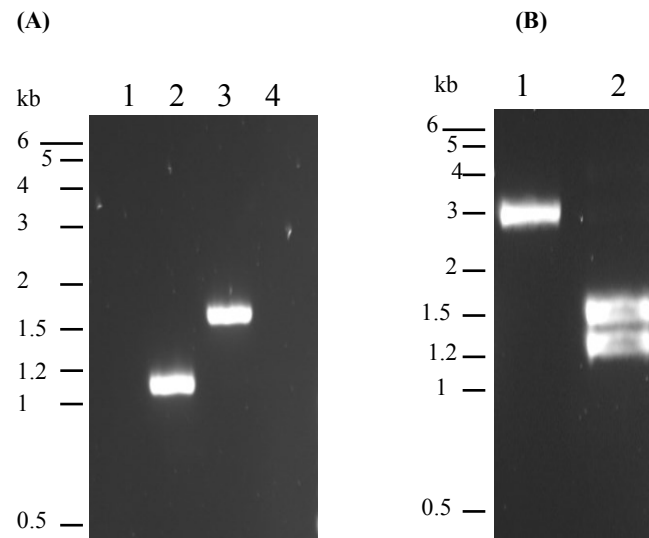


Figure 3-9: Screening colonies transformed with pD2Dkm **(A)** Gel showing fragments amplified from $\Delta psbD2$ (lanes 1 and 3) and WT (2 and 4) genomic DNA using a reverse primer annealing downstream of the inserted region and a forward primer binding either within the resistance cassette (lanes 3 and 4; 1676 bp) or within *psbD2* (lanes 1 and 2; 1118 bp). The absence of a band in lane 1 shows that there is no detectible wild-type sequence. **(B)** Gel showing a PCR product containing the modified region from $\Delta psbD2$ (lane 1) and a single-cut digest with *Sma*I. The WT amplicon is a similar length but lacks the *Sma*I site; the complete digestion of the product shows that there is no wild-type sequence.

3.5. Generating $\Delta psbA1/psbA2$ strain

In order to enable easy purification of PSII from the mutant strain by affinity chromatography, the 47H strain expressing a His₆-tagged CP47 was used to generate a strain lacking *psbA1* and *psbA2*. A chloramphenicol resistance cassette was used as a selectable marker due to the presence of a kanamycin resistance cassette associated with the His₆ tag.

3.5.1. PCR and OE-PCR fragments

Primary fragments A1,2-FD1/2 were amplified from *T. elongatus* genomic DNA using Phusion® polymerase (NEB). Amplicons of the correct size were gel-purified to remove products of non-specific primer binding. These fragments were then used as the template for OE-PCR reactions to produce secondary fragments for ligation into pGEM-T Easy.

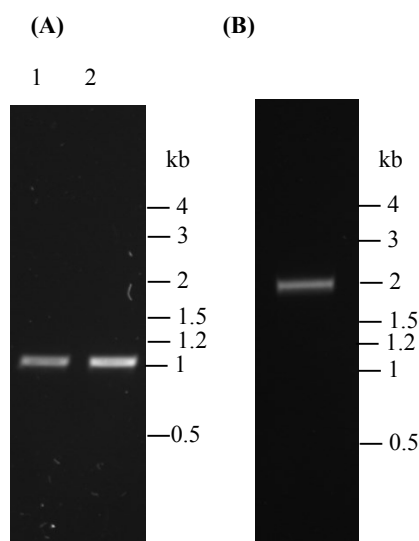


Figure 3-10: Agarose gels showing: A1,2FD construction. **(A)** the two overlapping PCR products A1,2-FD1 (lane 1, 1020 bp) and A1,2-FD2 (lane 2, 1010 bp); and **(B)** the OE-PCR product A1,2FD (2002 bp).

Secondary fragment A1,2FD was ligated into pGEM-T Easy as in section 3.4.2.

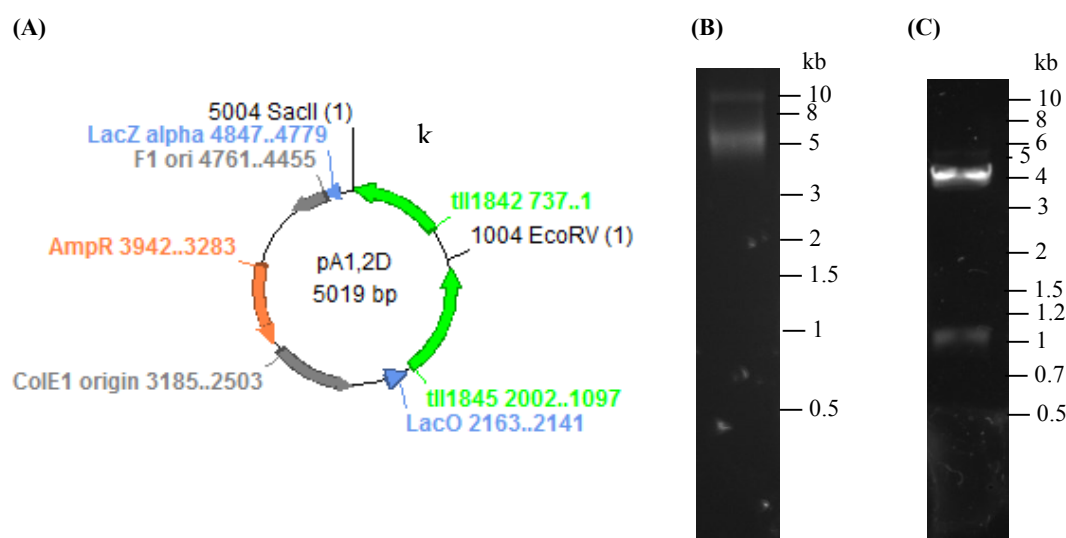


Figure 3-11: Map and agarose gels showing: pA1,2D construction. **(A)** Diagram of pA1,2D showing the unique SacII and EcoRV recognition sites used to verify the orientation of the inserted region. Agarose gels showing the lengths of the **(B)** SacII single digest (5019 bp) and **(C)** the SacII + EcoRV double digest (4000 bp and 1019 bp) of pA1,2D.

Sequenced plasmids were linearized at the EcoRV restriction site and dephosphorylated before ligation with a phosphorylated 1486 bp chloramphenicol resistance cassette (cm). After transformation into *E. coli*, chloramphenicol resistant colonies were screened by gel electrophoresis of DNA samples obtained by miniprep to identify pA1,2Dcm plasmids of the

correct size. As a further test, restriction digests were carried out on potential plasmids before they were sequenced to confirm and determine the direction of the insertion.

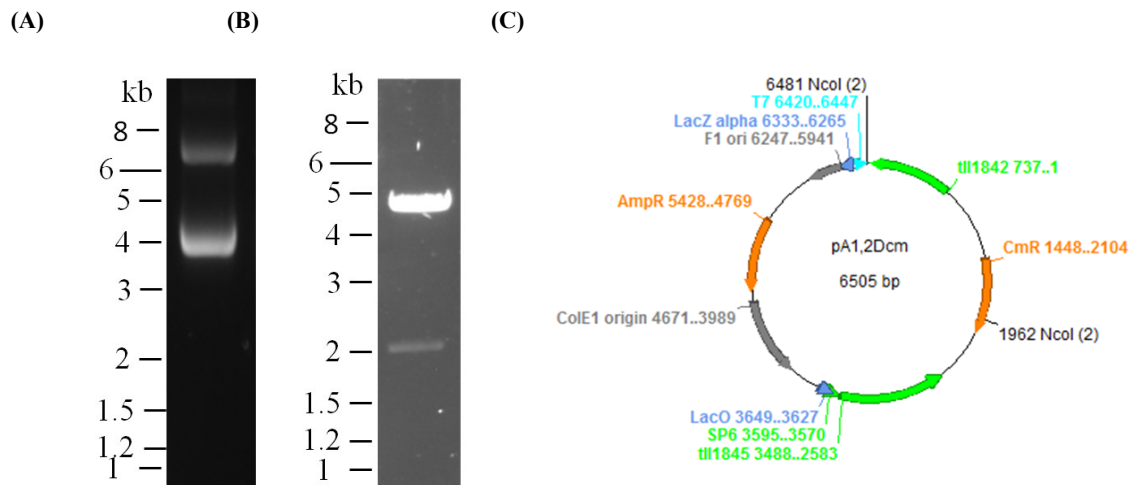


Figure 3-12: Map and agarose gels showing: pA1,2Dcm construction. Gel showing (A) uncut supercoiled and linear (~6.5 kb) bands from purified pA1,2Dcm and (B) double-digested pA1,2Dcm cut with NcoI (1962 bp and 4519 bp) as indicated in (C) the diagram of the construct.

The plasmid sequence was confirmed by sequencing before transformation. Screening of chloramphenicol resistant transformants using colony PCR yielded a $\Delta psbA1/psbA2$ strain which had no detectable wild-type sequence (Figure 3-13).

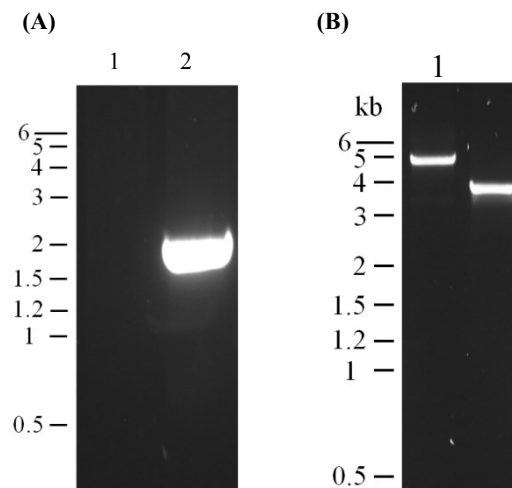


Figure 3-13: Screening colonies transformed with pA1,2Dcm. DNA gels showing fragment lengths generated from PCR reactions using 47H (lane 1) and $\Delta psbA1/psbA2$ (lane 2) genomic template DNA with primers binding (A) within the chloramphenicol resistance cassette and downstream of *psbA2*; and (B) upstream and downstream of the modified region to demonstrate the absence of a 4536 bp wild-type band in the $\Delta psbA1/psbA2$ strain (3560 bp).

3.5.2. Phenotypic effects of $\Delta psbA1/psbA2$ mutation

Multiple studies have not seen any significant difference in steady-state growth rate between the WT and $\Delta psbA1/psbA2$ *T. elongatus* strains under low, normal and high light conditions (Ogami et al., 2012; Sander et al., 2010). This is not unexpected as WT cells can adaptively express *psbA3* when it is favourable, although it does indicate that PsbA1 is not significantly better than PsbA3 under any of the conditions tested. Under normal-light ($50\text{--}60\ \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) conditions, removing *psbA3* has only been reported to have an effect when *psbA2* is also removed, although mRNA levels indicate that *psbA2* is not transcribed under these conditions (Ogami et al., 2012; Sander et al., 2010). Under high-light ($500\ \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) conditions, WT or *psbA3*-only strains were reported to grow significantly faster than strains lacking *psbA3* (Sander et al., 2010). It has been shown that expression of *psbA3* is strongly up-regulated under these conditions (Kós et al., 2008) and so it is likely that active PSII in the WT contain PsbA3 under these conditions. Under low-light ($10\ \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) conditions, Ogami et al. (2012) observed a difference only in the duration of the lag phase between WT and a $\Delta psbA1/psbA2$ strain. In contrast, competition assays using *Chlamydomonas reinhardtii* strains expressing heterologous analogues of PsbA1 or PsbA3 from *Synechococcus* 7952 showed that the PsbA1 analogue supported faster growth under very-low-light ($3\ \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) conditions whereas the PsbA3 analogue produced favourable growth under high-light ($290\ \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) light (Vinyard et al., 2013). In order to test these results I measured the growth rate of my $\Delta psbA1/psbA2$ strain and the 47H strain under low ($10\ \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and normal low ($50\ \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) light conditions.

No significant difference was observed under normal-light ($50\ \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) conditions compared to 47H strain before the stationary phase (Figure 3-14). Both strains showed an exponential increase in OD_{720} with a doubling time of 1.3 days for the first two days to $\text{OD}_{720}=0.5$, after which the growth rate decreased steadily. The $\Delta psbA1/psbA2$ strain, however, reached a significantly higher stationary phase cell density over the final day of the experiment ($\text{OD}_{720}=1.26\pm0.02$) than 47H ($\text{OD}_{720}=1.20\pm0.03$) (mean $\pm$ standard deviation over 24h, $p=0.013$), although this difference is small. As cell density increases, the difference in illumination intensity at different parts of the culture increases as more cells shade those further from the light source, and the difference between this condition and one with a similar illumination per cell (i.e. lower cell density, lower illumination intensity). This could indicate that the $\Delta psbA1/psbA2$ strain is better able to make use of light with a rapid variation

in intensity. This interpretation relies on the WT being unable to adapt to these relatively unnatural conditions and hence involves regulatory and associated costs, rather than different properties of PsbA1-PSII and PsbA3-PSII alone and so was not investigated further.

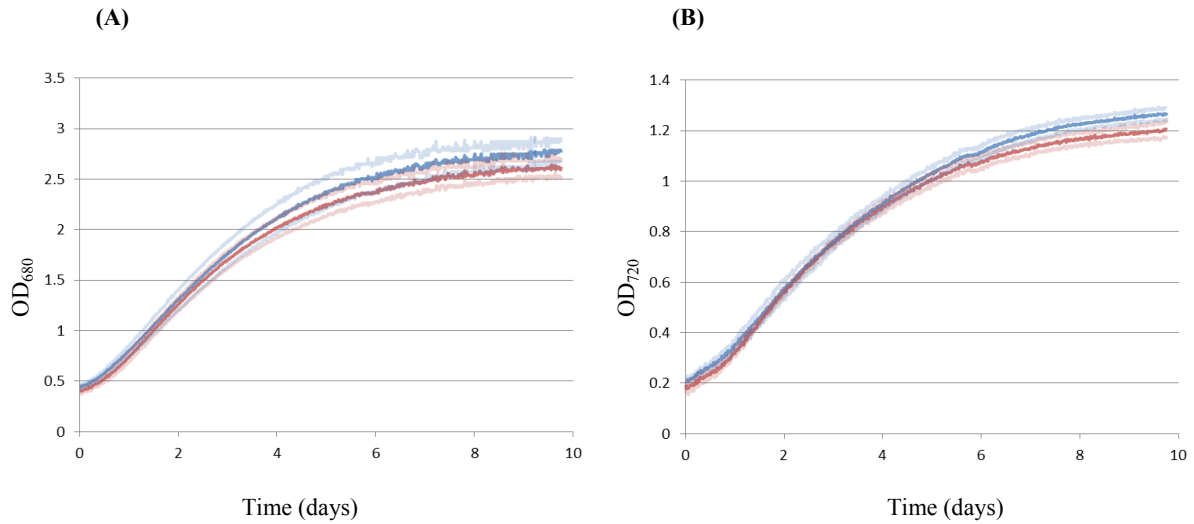


Figure 3-14: Growth assay under normal light conditions. Graphs showing the average change in optical density (dark lines) at (A) 680 nm and (B) 720 nm corresponding to chlorophyll concentration and cell density respectively of 47H (red) and $\Delta psbA1/psbA2$ (blue) cultures grown under low light ($50 \mu E \cdot m^{-2} \cdot s^{-1}$) and the standard deviation (lighter lines) from four biological replicates.

No difference in growth rate was also found under low light ($10 \mu E \cdot m^{-2} \cdot s^{-1}$) between the $\Delta psbA1/psbA2$ and 47H strains, similar to reports discussed above. The OD_{720} curves for both strains are linear (Figure 3-15 (B)) which indicates that the availability of photons is limiting growth rate under these conditions, even at low cell density. The data reported by Ogami et al. (2012) showed a non-linear increase in optical density below $OD_{730}=0.3$. While it is difficult to compare optical densities in the literature directly due to the influence of spectrophotometer design on scattered light, it is possible that my measurements did not start at sufficiently low cell density to observe this non-light-limited growth. These results show that the regulatory mechanisms which control *psbA3* expression are able to maintain a similar level of PSII activity to the wild-type even under conditions where *psbA3* would not normally be expressed. It also indicates that there is no significant difference in the photon use efficiency in these two strains. A shorter (by ~ 2 days) stationary phase observed by Ogami et al. (2012) was not seen in this study, although the pre-culture conditions that were used in their study are not clear.

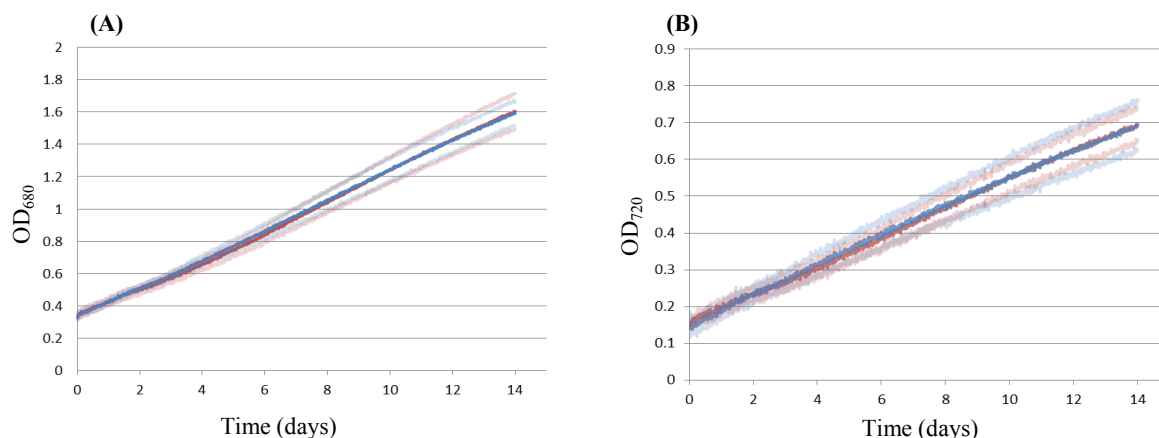


Figure 3-15: Growth assay under low light conditions. Graphs showing the change in optical density (dark lines) at (A) 680 nm and (B) 720 nm corresponding to chlorophyll absorbance and cell density respectively of 47H (red) and $\Delta psbA1/psbA2$ (blue) cultures grown under low light ($10 \mu E \cdot m^{-2} \cdot s^{-1}$). Lighter lines show the standard deviation from four replicates.

3.6. PSII purification

In order to produce PSII samples of sufficient purity for protein crystallography I used sequential nickel-affinity and anion-exchange chromatographies. For these methods I used a strain of *T. elongatus* whose CP47 protein has a C-terminal His₆-tag, facilitating the separation of complexes containing CP47. While the active form of the complex is dominant under the growth conditions when the cells are harvested, various other CP47-containing sub-complexes involved in PSII assembly, degradation and repair (Nixon et al., 2010) are also present in the eluate. These were separated by anion-exchange chromatography, improving the homogeneity and per-chlorophyll oxygen evolution activity of the sample. These samples are also useful for biochemical characterisation under controlled conditions.

The complexes present after each stage of purification become less representative of those *in vivo* due to the selective nature of the methods as well as degradation. For example, membrane solubilisation is likely incomplete when using detergent concentrations which leave PSII intact, although this is less likely to have a large effect in cyanobacteria (Mullineaux, 1999) than in eukaryotes which have a greater degree of lateral heterogeneity in the thylakoid membrane (Danielsson et al., 2006). Another consideration is the effect of the detergent DDM (Privé, 2007) on complex stability and oligomeric state, although detergent concentrations are kept low after the brief initial solubilisation step. While direct evidence for detergent-induced dimerization has not been reported, β -DDM molecules present in monomeric PSII crystals at the dimer interface suggests that they could inhibit dimerization

(Broser et al., 2010). The similar monomer:dimer ratios observed before and after the anion exchange step indicates that this purification step does not alter the complex structure. β -DDM is gentle enough to preserve high activity in purified samples. In order to improve stability, betaine was included in chromatography buffers (Papageorgiou and Murata, 1995) where possible and mannitol was used as its replacement stabilising solute where not.

3.6.1. Nickel-affinity purification of CP47-containing complexes

The His₆-tag nickel-affinity chromatography protocol consistently yielded a chlorophyll-enriched extract (Figure 3-16) with an oxygen evolution activity between 2200-3000 $\mu\text{mol}\cdot\text{mg}(\text{chl})^{-1}\cdot\text{h}^{-1}$ containing around 2% of the initial chlorophyll in the culture (data not shown). Figure 3-17 shows the room-temperature absorbance spectrum of this sample, confirming the enrichment of PSII. PSII samples prepared in this way are herein referred to as ‘crude PSII’ in order to differentiate them from samples which have been purified using additional anion exchange chromatography to separate different CP47-containing complexes.

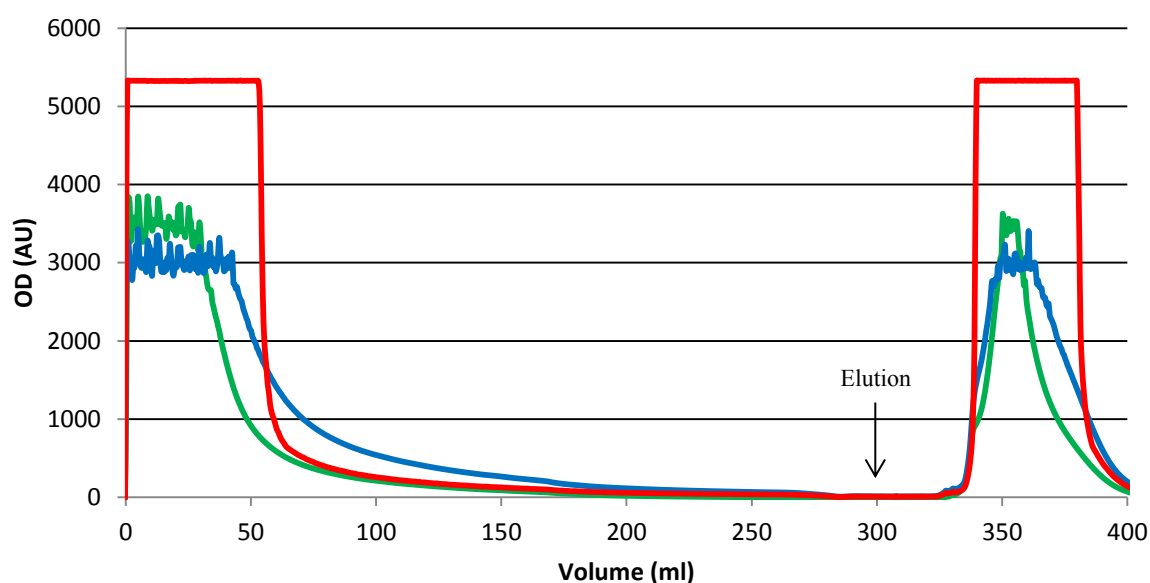


Figure 3-16: Chromatograph showing typical nickel column wash and elution from WT PSII bound to ~30 ml of resin. Absorbance was measured at 670 nm (red), 280 nm (green) and 440 nm (blue). Elution was started at 300 ml.

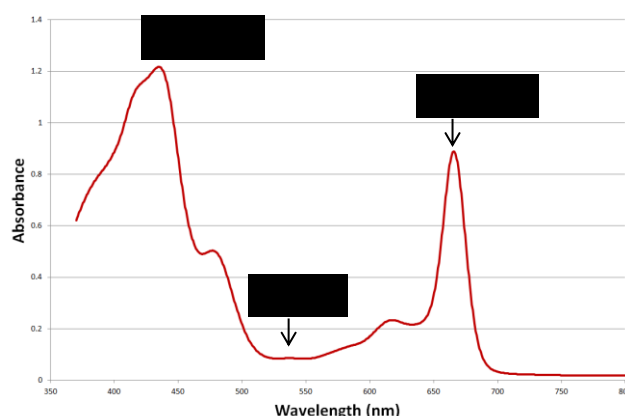


Figure 3-17: Room temperature absorbance spectra of the crude WT PSII sample, similar to those reported previously, showing peaks characteristic of chlorophyll *a* in PSII at 674.5 nm and 430 nm, and of pheophytin *a* in PSII at 545 nm (Müh and Zouni, 2005; Sugiura and Inoue, 1999).

A typical size-exclusion chromatograph of a crude PSII sample is shown in Figure 3-18. This composition of ~46% dimeric and ~54% monomeric PSII is similar to results reported previously (M. M. Nowaczyk et al., 2006), although the *in vivo* composition is still contested (Boekema et al., 1995; Danielsson et al., 2006; Hankamer et al., 1997; Rögner et al., 1996; T. Takahashi et al., 2009).

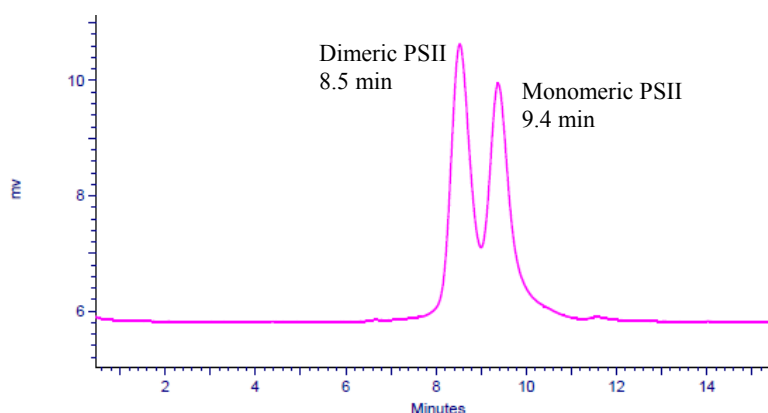


Figure 3-18: Size exclusion chromatograph of crude WT PSII sample, measured at the 674 nm chlorophyll peak. Peaks at 8.5 and 9.4 min correspond to dimeric and monomeric forms of PSII respectively.

The roughly 50:50 monomer-dimer ratio was also confirmed using CN-PAGE, shown in Figure 3-19.

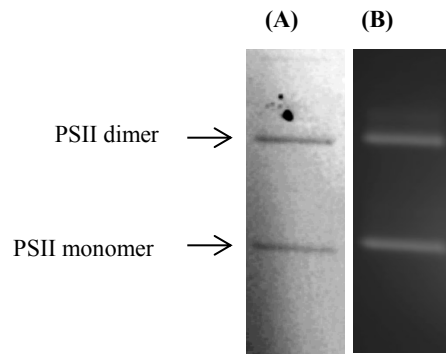


Figure 3-19: CN-PAGE of crude WT PSII imaged using (A) Coomassie-stained and (B) fluorescence-emission imaging from 47H showing the roughly equal proportion of monomeric and dimeric complexes.

In order to determine the purity of the PSII sample its low-temperature fluorescence spectrum was measured (Figure 3-20). The emission peak at ~693 nm is likely a combination of 695 nm and 685 nm fluorescence peaks, partially resolved in the blue trace, characteristic of chlorophyll associated with CP47 and P₆₈₀ respectively (Andrizhiyevskaya et al., 2005; Nakatani et al., 1984). More importantly there is no significant emission peak at 725 nm which would be expected if PSI were present, indicating that all of the chlorophyll present is bound to PSII. Although the excitation wavelength is not optimised to excite phycobilisomes, low emissions between 635 nm and 655 nm suggest low phycobilisome contamination. The poor resolution of this measurement is likely due to technical issues and the deterioration of the equipment but is sufficient to demonstrate the purity of the sample.

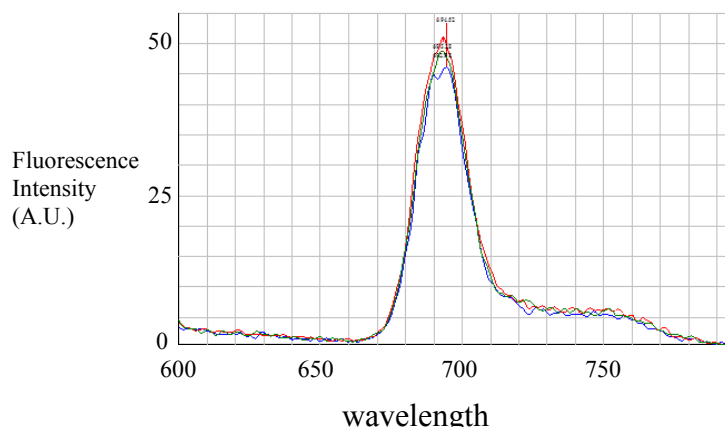


Figure 3-20: Fluorescence spectrum of crude WT PSII sample after excitation with 435 nm light, exciting the Soret band of chlorophyll *a*, at 77 K. This spectrum, showing a peak at 693 nm with a low shoulder extending to 760 nm, is similar to others in the literature for PSII from *Synechocystis* (Mimuro et al., 2007) and shows that contamination by PSI (720 nm emission) is low enough to be undetectable. The slight low-wavelength skew could indicate minor phycobilisome contamination (684 nm emission).

3.6.2. Separating different CP47-containing complex populations from crude PSII by anion exchange chromatography

While the active reaction centre complex constitutes the dominant fraction of CP47-containing complexes in the cell under the growth conditions used, a minority of complexes which are undergoing assembly, degradation or repair (Nixon et al., 2010) will be present in the purified sample. Notably, a minor pool of monomeric complexes which can be separated by anion-exchange chromatography (pool 1) has been identified which contains Psb27, lacks luminal subunits PsbO, PsbU and PsbV, and show only marginal oxygen evolution activity (M. M. Nowaczyk et al., 2006).

In order to prepare a more homogenous sample, the crude PSII sample was separated using a ~7 CV 5-120 mM MgSO_4 gradient consistently yielding four fractions, similar to reports in the literature (Kuhl et al., 2000; M. M. Nowaczyk et al., 2006).

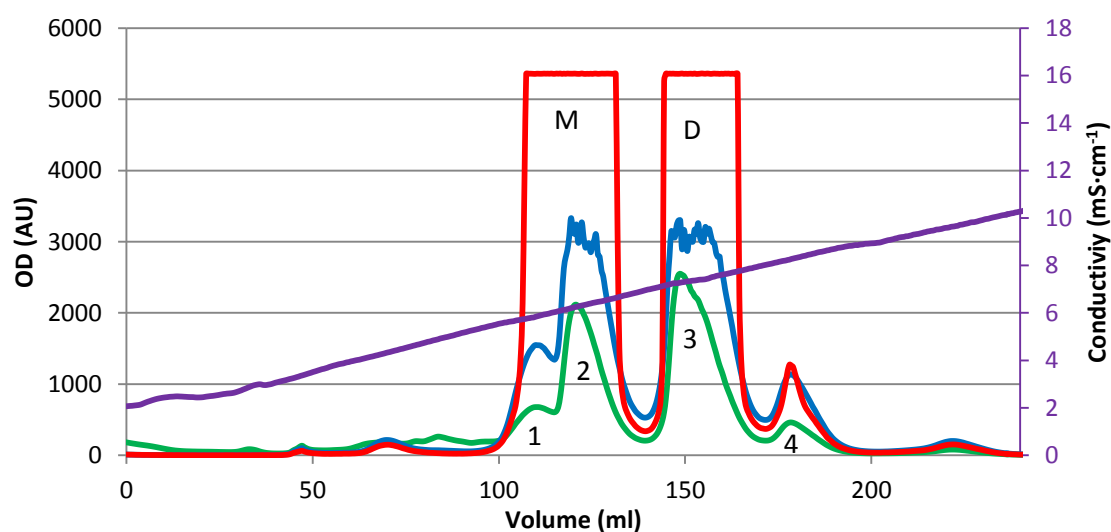


Figure 3-21: Anion-exchange chromatograph separating 47H PSII showing the four major fractions (numbered). M: Monomeric fraction; D: Dimeric fraction; Absorbance was measured at 670 nm (red), 280 nm (green) and 440 nm (blue). Conductivity ($\text{mS}\cdot\text{cm}^{-1}$) during the elution gradient is shown in purple.

Figure 3-21 shows a typical elution profile from the anion exchange column. The fractions were pooled according to their position on the chromatograph and their activity. Peaks two and three constitute the active monomer and dimer fractions respectively. These pools exhibit the highest oxygen evolution activities of $\sim 2400 \mu\text{mol}\cdot\text{mg}(\text{chl})^{-1}\cdot\text{h}^{-1}$ and $\sim 3000 \mu\text{mol}\cdot\text{mg}(\text{chl})^{-1}\cdot\text{h}^{-1}$. Pool 1 has low oxygen evolving activity ($100\text{-}200 \mu\text{mol}\cdot\text{mg}(\text{chl})^{-1}\cdot\text{h}^{-1}$) and has previously been reported to bind Psb27, a protein

implicated in biogenesis and repair of PSII (M. M. Nowaczyk et al., 2006). Pool 4 evolves less oxygen than the active pools two and three ($\sim 800\text{--}1000 \mu\text{mol}\cdot\text{mg}(\text{chl})^{-1}\cdot\text{h}^{-1}$). It has been suggested that this fraction represents dimer wherein one monomer is damaged while the other is active (M. M. Nowaczyk et al., 2006). A minor peak is also visible in Figure 3-21 at ~ 225 ml. This may correspond to the low-activity pool 5 reported by Grasse et al. (2011) but has not been studied further here.

SEC and CN-PAGE were again used to confirm the oligomeric state of each pool. Fluorescence imaging, a more sensitive method than Coomassie stain for detecting chlorophyll-containing proteins, shows that the monomer/dimer separation is incomplete, and that there is some PSI trimer contamination in pool 2. In order to minimise contamination between the pools, fractions between pools were discarded.

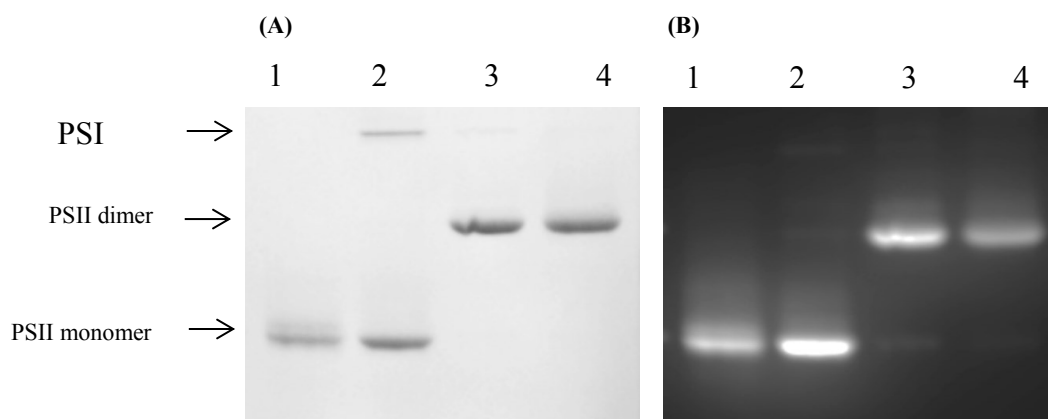


Figure 3-22: CN-PAGE of 47H PSII pools after anion-exchange separation imaged with (A) Coomassie-stained and (B) fluorescence-imaging showing the oligomeric state of each pool.

3.6.3. PSII purification from the $\Delta psbA1/psbA2$ strain

PSII was purified from the $\Delta psbA1/psbA2$ strain described in section 2.5.3. SDS-PAGE (Figure 3-23) showed the same subunit composition for the different pools purified from the WT and $\Delta psbA1/psbA2$ mutant strains. There was consistently a larger proportion of monomeric compared to dimeric PSII resulting in a large overlap between pools 1 and 2. This can be seen in the chromatograph shown in Figure 3-24 (A) and by the presence of PsbV and PsbU in the pool 1 shown in Figure 3-24 (B). This overlap could be reduced by loading smaller samples on the column for each fractionation or by increasing the salt concentration in the elution buffer more slowly. This work, however, is only concerned with the active dimer fraction and so methods to more fully resolve pools 1 and 2 were not developed.

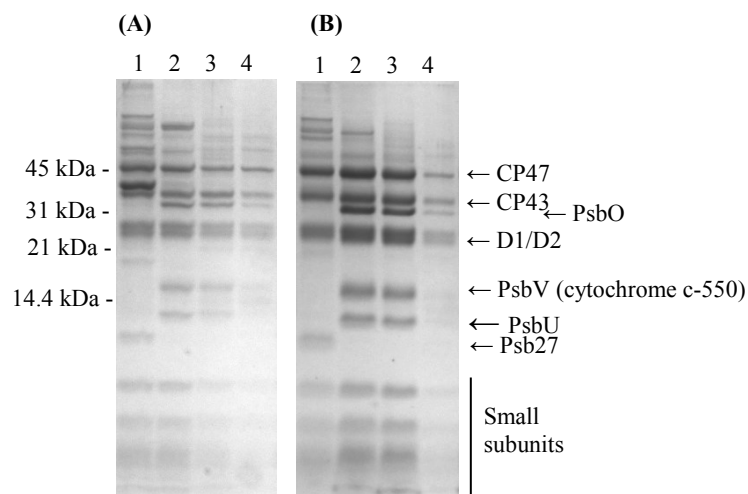


Figure 3-23: SDS-PAGE of anion-exchange fractions showing the subunit composition of fractions of pools 1-4 in lanes 1-4 from **(A)** WT and **(B)** $\Delta psbA1/psbA2$ strains.

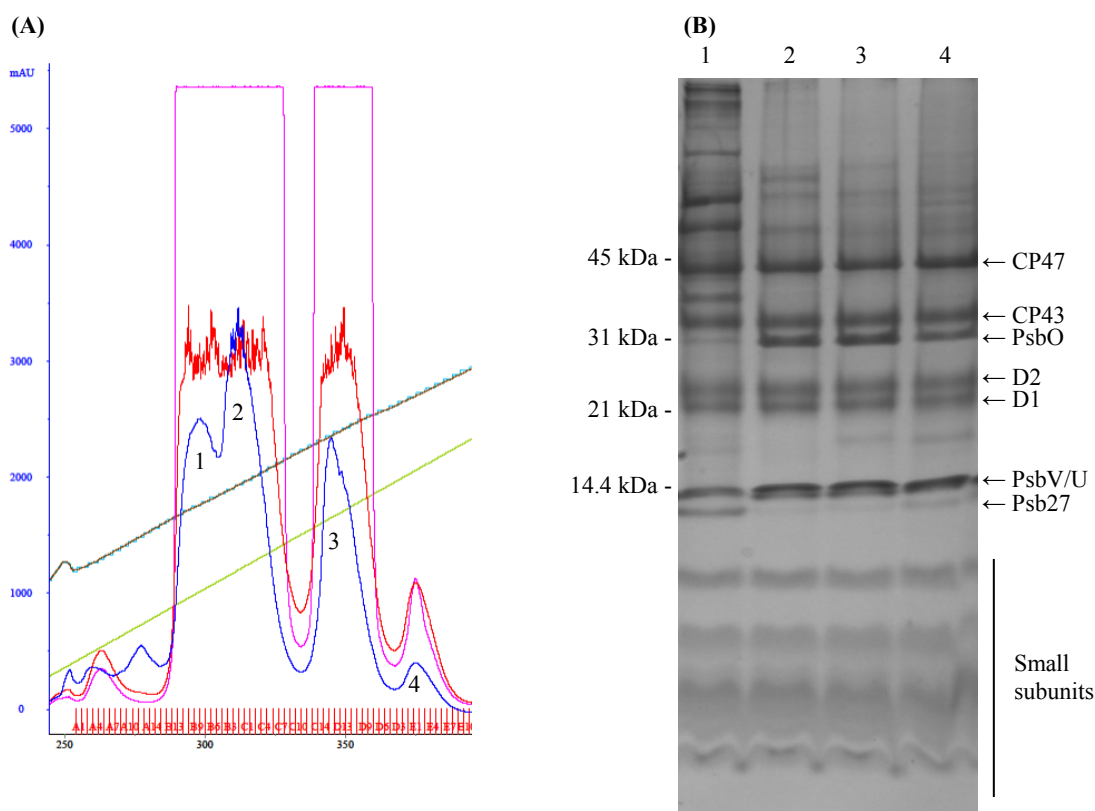


Figure 3-24: Purification of PsbA3-PSII. **(A)** Chromatogram showing the elution profile of PsbA3-PSII from the anion exchange column. Absorbance was measured at 670 nm (pink), 280 nm (blue) and 440 nm (red). Conductivity ($\text{mS} \cdot \text{cm}^{-1}$) during the elution gradient is shown in grey. **(B)** SDS-PAGE gel showing the subunit composition of fractions obtained from this preparation. The pool 3 sample from this preparation and others was used for crystallographic studies.

In order to assess the state of the manganese cluster in each pool, samples were sent to a collaborator (Sidsel Birkelund Schmidt, Research Assistant, University of Copenhagen) for

LC-ICP-MS analysis. While the results cannot be considered fully quantitative due to aspects of the sample preparation, pools 2 and 3 had 5-fold higher concentration of manganese. This tentatively supports the hypothesis that the decreased activity of pools 1 and 4 is related to damage of the Mn_4CaO_5 cluster.

The pool containing active dimeric PSII (pool 3) was used for crystallographic and biochemical characterisation as described in subsequent sections.

3.6.4. Biochemical confirmation PsbA3 expression

The oxygen evolution activity of the crude PSII, as well as the anion-exchange pools, was found to be similar for PsbA1-PSII and PsbA3-PSII. While PsbA3-PSII has been reported to have a higher oxygen evolution activity than PsbA1-PSII by Sugiura et al. (2008) Sander et al. (2010) also found there to be no difference. The increase by a factor of 1.3 above PsbA1-PSII activity observed by Sugiura et al. has been attributed to changes in the rate-limiting quinone exchange at the QB site, although structural differences at this site in PsbA3 are more conserved in the PsbA2 isoform which was found to have only 75% of the PsbA1 activity (Sugiura and Boussac, 2014a). This suggests either that QB exchange is not rate-limiting in PsbA2-PSII or that the difference in activity between the isoforms is also dependent on their treatment during purification. Details of the purification methods used by Sander et al. indicate that activities as high as those reported by Sugiura et al. for PsbA3-PSII can be obtained from WT *T. elongatus* in conditions likely producing mostly PsbA1-PSII (Kuhl et al., 2000). It is possible that differences observed arise from the effect of the different purification methods used on the native lipid environment around the QB site and the quinone exchange pathway, and that this effect is different for some methods in PsbA1- and PsbA3-PSII, although likely causes are not clear from the published methods.

In order to confirm the expression of PsbA3 in active PSII, PSII was purified from this strain and characterised using known biochemical characteristics which differ between PsbA1-PSII and PsbA3-PSII. Low temperature spectroscopic measurements of the C550 shift were made in crude PSII preparations which has been shown to be affected by the D1-Glu130Gln substitution in PsbA3 (Hughes et al., 2010; Sugiura, Azami, et al., 2014). The data shown in Figure 3-25 demonstrate the difference in wavelengths of this shift in crude PSII from 47H (PsbA1) and the $\Delta psbA1/psbA2$ strain, with the signal shifted to longer wavelengths in the

mutant sample, as reported previously (Hughes et al., 2010), confirming that the mutant is expressing PsbA3 (Figure 3-25).

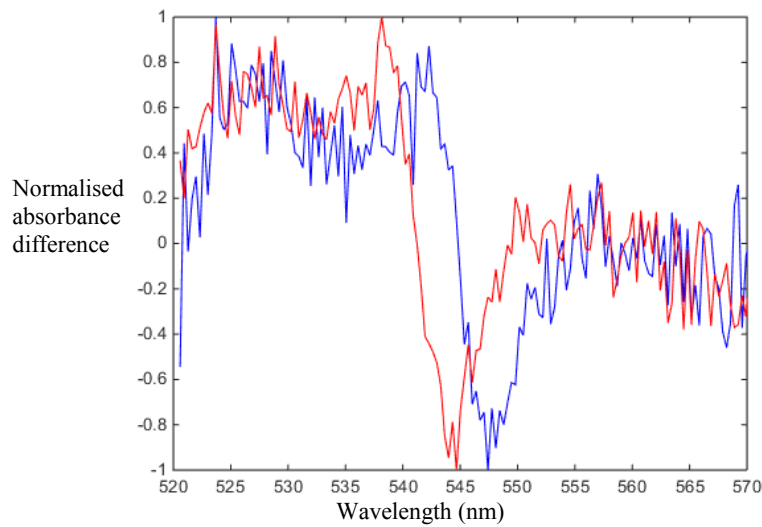


Figure 3-25: Light-minus-dark ΔA difference spectra measured during low-temperature (<130 K) laser illumination showing the C550 shift, as well as the weaker peak at 525 nm associated with oxidation of the Cyt b_{559} , in crude PSII containing PsbA1 (red) or PsbA3 (blue).

Thermoluminescence measurements of the stability of the $S_2Q_B^-$ state in these crude PSII samples were also made which showed that the $\Delta psbA1/psbA2$ strain is producing PsbA3-PSII. A single flash from a xenon lamp was used to generate $S_2Q_B^-$ in dark adapted samples which were then cooled to -15 °C before heating at 20 °C $\cdot$ min $^{-1}$ to 80 °C. The lower peak temperature (43 °C) of the PsbA3 sample compared to the PsbA1 sample (54 °C) is similar to previous reports in the literature (Sander et al., 2010; Sugiura, Azami, et al., 2014; Sugiura, Kato, et al., 2010), indicating a less stable charge-separated state.

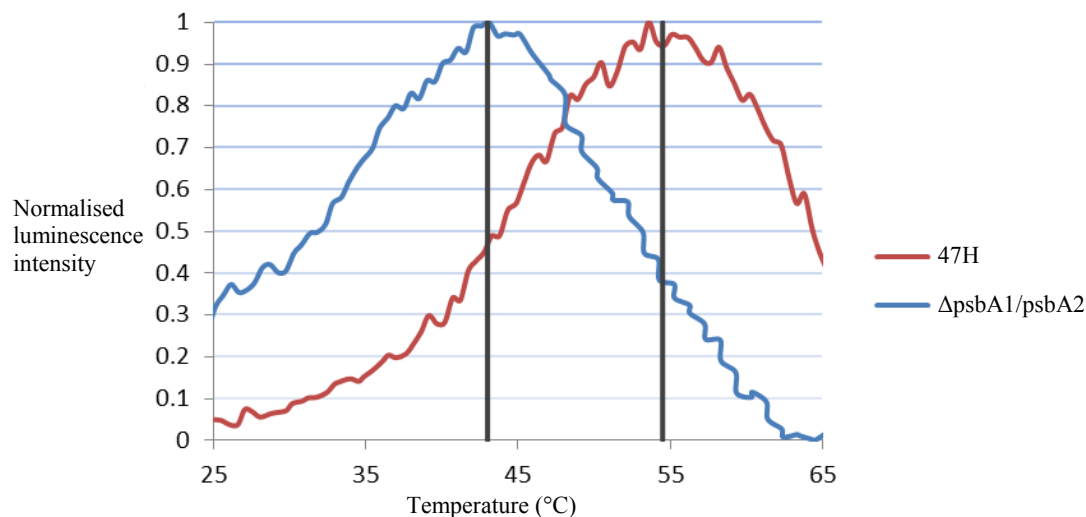


Figure 3-26: Thermoluminescence bands arising from $S_2Q_B^-$ recombination in crude PSII samples from 47H and the $\Delta psbA1/psbA2$ strain showing the lower temperature in the sample containing PsbA3.

Using these techniques, no PsbA1 could be detected. Further analysis of protein samples by mass spectrometry could be used as a more sensitive means of detecting PsbA1 but was not thought necessary or carried out here.

3.7. Crystallographic Studies

This section describes the crystallisation studies using highly-active dimeric His₆-tagged PSII complexes purified from *T. elongatus* by anion-exchange chromatography. The main aims are to provide supporting evidence for zinc binding to a cation-binding site identified previously (Murray and Barber, 2006), to determine the structure of the high-light-induced D1 homologue PsbA3 in the cyanobacterial PSII reaction centre and to identify the binding mode of the inhibitor DCMU. Differences between the structures of these modified reaction centres and the published reaction centre structures can provide insight into the origins of differences in their function.

3.7.1. Developing crystallography protocols and improving diffraction resolution

Crystallization trials and optimisation were carried out in order to develop a method for consistently producing large, strongly-diffracting PSII crystals. Highly-active pure PSII monomers and dimers were used prepared as described in section 2.5.3, flash-frozen in liquid

nitrogen and stored at -80 °C until used. From initial trials at the nanolitre scale, one buffer was chosen (MD1-41-F5: 0.1 M NaCl, 0.325 M Sodium Acetate, 0.1 M Tris, 21% (v/v) PEG 400, pH 8) that reproducibly produced nice-looking (~1 mm, single, sharp edges, rhombic shape) crystals from dimer samples. While crystals of monomeric PSII have been grown and used successfully for x-ray diffraction measurements (Broser et al., 2010) initial screening did not give any conditions which reliably yielded crystals using my monomer preparation and so only dimers were used for the rest of the work. These samples have the highest activity per chlorophyll and are suggested to be the dominant active form *in vivo* (Watanabe et al., 2009) which makes them the most relevant to this study. Sitting and hanging drop vapour diffusion crystal trials were then carried out in order to scale-up the crystallisation and identify optimal condition for our PSII preparations under which large, single crystals can be grown. These trials at the microliter scale determined an optimal PEG 400 concentration in this buffer to be 17% (v/v), generating a small number of crystals within 2 days followed by crystal growth over several days.

X-ray diffraction patterns from crystals produced under these conditions were collected using the I02 beamline at the Diamond Light Source synchrotron. The best of the diffraction datasets produced extended to 4 Å, from which a 6 Å electron density map could be generated. These data showed that the crystals have the same lattice structure, the same crystal form, and a similar unit cell size as previously reported crystals, and that they are composed of homogenous PSII complex dimers containing intact Mn_4CaO_5 clusters. In order to improve purity and hopefully diffraction strength, protein samples were subjected to batch pre-crystallisation in 30% (w/v) PEG 400 and pelleted in a microcentrifuge, a procedure which was used to obtain the highest-resolution PSII structure (Umena et al., 2011). The samples were then re-solubilised in Crystallisation Buffer and used as described. No consistent improvement, however, was seen when compared to crystals prepared without the pre-crystallisation treatment.

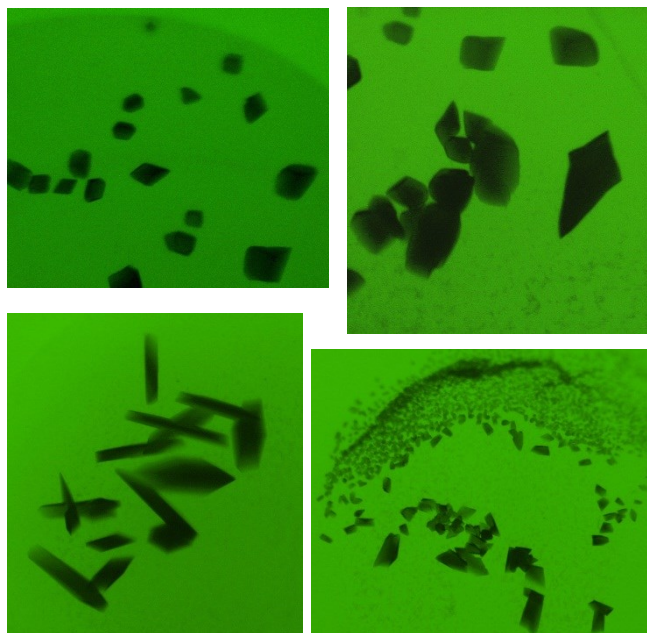


Figure 3-27: Pictures of PSII crystals taken using a dissection microscope illuminated with green light, showing some of the variation in morphology between the larger, more single crystals obtained.

Controlled crystal dehydration is sometimes used to improve the resolution of X-ray diffraction data. A variety of approaches have been characterised, including the addition of salt to the well buffer (Douangamath et al., 2013), adding a dehydrating buffer to the crystal's mother liquor in situ, equilibration against a dehydrating buffer in the well (Heras et al., 2003) and others (Heras and Martin, 2005; Krauss et al., 2012). A survey of the reported PSII crystals, many of which employed some form of dehydration, shows a positive correlation between a crystal's unit cell volume and the resolution limit of the diffraction data obtained from it (Figure 3-29). A number of different dehydration techniques were tested on fully-grown crystals (see 2.5.9) with the aim to improve the crystal packing and hence resolution. While small improvements in X-ray diffraction were seen, they were not sufficient to generate atomic-resolution data.

A new crystallisation screen using low molecular weight poly- γ -glutamic acid (PGA-LM) as a precipitant (Hu et al., 2008) was tested when it became available (MD1-51, Molecular Dimensions). This screen yielded many more suitable conditions for crystal growth, of which a few at different pH were chosen for optimisation. Crystals obtained from one of these conditions (MD1-51-A4: 0.6 M sodium formate, 0.1 M sodium acetate, 8% (w/v) PGA-LM, pH 5) which were gradually dehydrated by in-situ buffer exchange yielded the highest resolution dataset, diffracting to 3.44 Å. A second condition (MD1-51-A4, 0.3 M sodium

malonate, 0.1 M Tris, 8% (^{w/v}) PGA-LM, pH 7.8) which reliably produced large crystals was also used as its pH is closer to that on the acceptor side of the complex (Belkin et al., 1987).

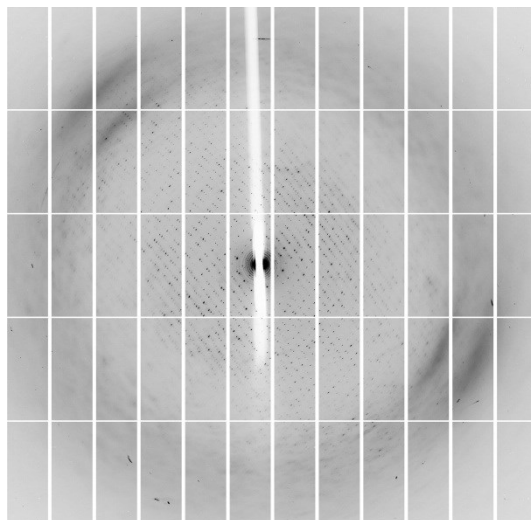


Figure 3-28: Picture of a diffraction pattern from PSII crystals, showing spots out to 3.44 Å.

These initial trials demonstrated the ability to reproducibly make PSII dimer crystals which diffract to a sufficient resolution to identify structural modifications.

3.7.2. Controlled dehydration using humidified vapour stream

In order to better understand the dehydration process in PSII more controlled study was undertaken using the on-line humidity control device HC1 (Sanchez-Weatherby et al., 2009), described in section 3.7.2. While the diffraction strength of a protein crystal is dependent on the number of unit cells contributing to the pattern, the intensity of the peaks is also dependent on the degree of order exhibited by the crystal. This is affected on the largest scale by the mosaicity of the crystal lattice. Small phase errors arising from dislocations, inclusions or asymmetrical protein orientation in the unit cells will broaden the interference spots, reducing the accessible resolution range. Reducing the degree of crystal solvation leads to more tightly-packed proteins and can increase their degree of order. Figure 3-29 shows the relationship between the unit cell volume of and the resolution of x-ray diffraction data obtained from PSII crystals reported in the literature, all having an orthorhombic space group $P2_12_12_1$. Alternative crystal forms have been obtained (Broser et al., 2010; Hellmich et al., 2014) and are omitted here. The small unit cell size of the best-diffracting crystals reflects their high degree of order in the crystal packing and low solvation.

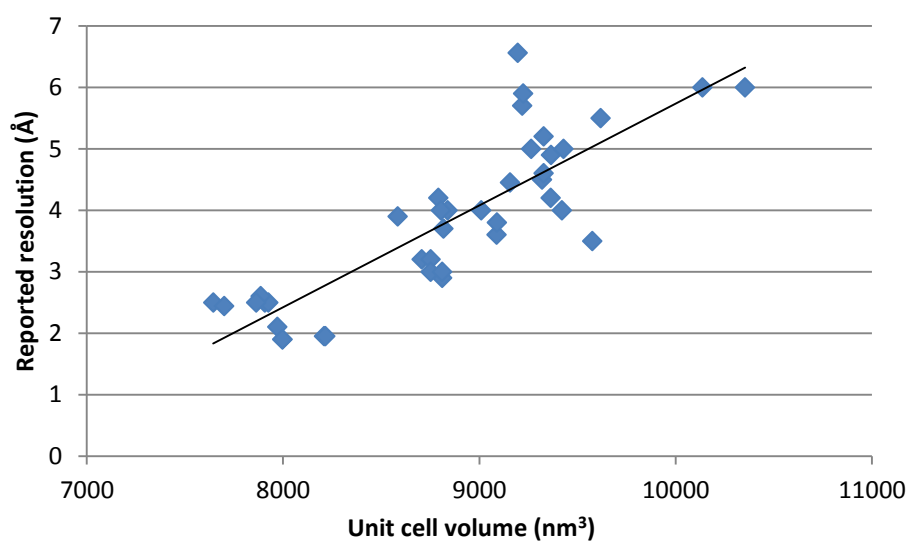


Figure 3-29: Correlation between unit-cell size and reported X-ray diffraction resolution for PSII crystals reported in the literature, with a linear regression trend line. References and data are shown in Table 3-1.

Table 3-1: Survey of X-ray diffraction datasets published in the literature for rhombic dimer crystals with space group $P2_12_12_1$.

Citation	Resolution (Å)	a	b	c	PDB
(Broser, 2010)	3.6	119.9	224.7	337.3	
(Ferreira et al., 2004)	3.5	135	228.9	309.9	1S5L
(Gabdulkhakov et al., 2011)	3.2	126.9	225	304.9	4V82
(Guskov et al., 2009)	2.9	127.7	225.4	306.1	4V62
	4.2	127.6	224.8	306.5	
	3.9	126.1	223.7	304.3	
	4	132.9	229.4	309	
(Hellmich, 2014)	6	141	232	309.9	
	6	140	230.9	320.3	
	2.5	116.4	218.4	300.7	
(Hellmich et al., 2014)	2.44	116.4	218.9	302.2	4PJ0
(Kawakami et al., 2009)	4	130	225.6	307.2	3A0B 3A0H
	3.7	129.6	224.6	302.9	
	4	128.6	225.7	304.5	
	4	128.5	224.7	304.9	
(Kern et al., 2005)	3.2	127.5	224.6	305.6	1W5C
(Kern et al., 2012)	6.56	130.8	227.8	308.6	4FBY
(Kern et al., 2013)	5.7	131.9	227.5	307.2	4IXQ
	5.9	132	227.6	307	4IXR
(Kern et al., 2014)	4.9	132.9	229	307.7	4TNH
	4.5	132.3	228.7	308	4TNJ
	5.2	132.6	229.3	306.8	4TNK
	4.6	132.4	228.8	307.9	4TNI
(Koua et al., 2013)	2.1	121.9	228.8	285.8	4IL6
	2.5	121.2	228.2	285.9	
	2.5	121.3	228.5	285.9	
	2.6	121.1	227.7	286	
(Kupitz, Basu, et al., 2014)	5	133.3	226.3	307.1	4PBU
	5.5	136.6	228.1	308.7	4Q54
(Loll et al., 2005)	3	127.7	225.4	306.1	2AXT
(Murray et al., 2008)	4.45	131.7	226.7	306.7	
	4.2	133.4	226.8	309.5	
(Suga et al., 2014)	1.9	122.2	228.5	286.4	4UB6 4UB8
	1.95	123.8	230	288.3	
	1.95	123.8	230	288.5	
(Umena et al., 2011)	1.9	122.2	228.5	286.4	3WU2
	2.5	121.4	225.6	287.1	
(Zouni et al., 2000)	5	134	227	310	
(Zouni, Witt, et al., 2001)	3.8	130	227	308	

In order to study the effect of solvation on the unit cell size, and to see if further improvements to diffraction strength could be achieved, crystals looped directly from the mother liquor were equilibrated with a humidity-controlled air stream generated by the HC1 device (Sanchez-Weatherby et al., 2009) on the I04-1 beamline at the Diamond synchrotron. This allowed the direct measurement of changes in the crystal packing and diffraction power in individual crystals during controlled dehydration. Crystals were mounted on a mesh loop and the surrounding mother liquor was gently removed with a tissue. A relative humidity of 92% was found to correspond to the water pressure of the mother liquor by measuring changes in the volume of a loop-mounted drop. Crystals were slowly dehydrated from 92% with two diffraction images taken 90° apart after a period of equilibration at set humidity

levels. Dehydration was continued until a significant deterioration in the diffraction pattern was seen.

The data collected (Figure 3-30) show a strong linear positive correlation between relative humidity and the unit cell volume in all crystals measured. While all of the crystals measured were prepared from the same PSII sample and grown under the same conditions, they did not all shrink at the same rate as the relative humidity was increased. Crystals with a smaller initial unit cell shrank more than those with a larger unit cell as the relative humidity was decreased. Using this method a large proportion of the range of unit cell sizes reported in the literature could be achieved. While modest improvements in diffraction quality were seen as the crystals dehydrated, none of the measurements gave spots beyond 6.5 Å. The quality of the diffraction data decreased before the crystals shrank to the size reported for the highest resolution crystals. Re-hydration of desiccated crystals resulted in an increase in their unit cell size, returning to values similar to those measured before dehydration as well as a decrease in diffraction strength.

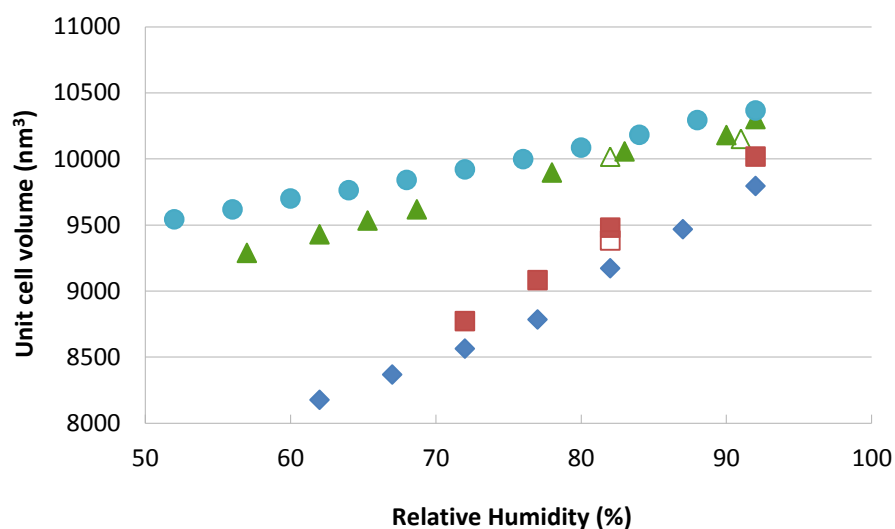


Figure 3-30: Graph showing the effect of controlled dehydration (closed symbols) and subsequent rehydration (open symbols) using an on-line vapour-stream device as described above. Different colours correspond to different crystals measured. None of the measurements gave spots beyond 6.5 Å.

These results suggest that there is significant heterogeneity among crystals prepared using this method, independent of sample purity and buffer composition. This likely arises from the stochastic nature of crystal nucleation and growth. It also demonstrates that there are significant differences between these and crystals with higher diffraction strength beyond solvation. Trials using crystallisation conditions identical to those reported which produced

the highest-resolution data (Shen et al., 2011), as well as attempts to optimise these conditions for my sample, did not consistently yield large crystals. The differences between the PSII purification methods used stem from my use of a His-affinity tag to purify crude PSII. Firstly, they use solubilisation with low concentrations of LDAO in order to remove thylakoid regions which do not contain PSII and precipitation after increasing the concentration of LDAO to further remove non-PSII components, before exchanging the detergent with β -DDM. LDAO is, however, not present in the final crystal. The relatively gentle detergent β -DDM was used throughout and a Ni-affinity purification step to purify crude PSII as this resulted in less contamination by PSI than using other methods. Their subsequent anion exchange used a NaCl gradient in order to separate PSII monomers and dimers whereas MgSO_4 was used here, which has been shown to further separate monomer and dimer fractions into high- and low-activity peaks. While it is possible that the different salt used affects the complex differently, SDS gels and the crystal data collected show that the complex is intact in my samples. After anion exchange the samples were treated similarly.

3.7.3. Zinc binding sites in PSII related to their functional effects

The inhibitory effect of zinc on plant PSII activity has been studied in plants and related to its effect on the binding of subunits which stabilise the Mn_4CaO_5 cluster, particularly PsbP and PsbQ (23 kDa and 17 kDa proteins respectively), in competition with calcium (Rashid et al., 1991). The cyanobacterial homologues of these proteins, however, are not retained in the purified samples used for crystallisation. Zinc, like manganese or calcium, forms a divalent cation in solution. The chemical similarities between these ions results in similar binding patterns, while the higher number of electrons in zinc atoms results in stronger diffraction. More importantly, the K-edge of zinc (9660.7 eV) is close to the X-ray energies used in these measurements resulting in easily-detected anomalous scattering. This makes zinc a useful probe for possible calcium and manganese binding site, the two elements which form the catalytic centre of the enzyme as well as having other proposed functional roles.

Crystals incubated with 50 mM zinc (~800 per PSII) showed that it is able to replace the non-heme iron as well as the calcium at the interface between PsbO and the D1-D2 core (James Murray, personal communication). Zinc exchange with the non-heme iron has been detected previously as the absence of the EPR signal arising from Q_A^- interacting with the high-spin iron in PSII-enriched membrane fragments from spinach. These experiments reported a

concentration dependence of the exchange with full inhibition of the signal at 24mM (~10000 Zn^{2+} per PSII) and only 10-20% inhibition at 2.4 mM (Jegerschöld and MacMillan, 1999). In this study, in order to address the physiological relevance of this exchange, PSII crystals were made from samples incubated with lower concentrations of zinc.

The anomalous signal map generated from crystals incubated with 5 mM zinc (~80 per PSII) show the same exchange with a calcium at the interface between PsbO and the reaction centre with high occupancy, generating a signal stronger than that from the heme bound to cytochrome b_{559} . This binding site is located at the mouth of a proposed water/proton channel leading from the luminal bulk solution to the Mn_4CO_5 cluster (Rutherford and Faller, 2001). These results suggests that it is possible to differentiate between acceptor-side effects and donor-side effects on enzyme kinetics using different concentrations of zinc which is useful if acceptor- or donor-side effects are to be studied independently.

Replacement with zinc also provides an experimental tool with which to study the role of the non-heme iron in Q_A to Q_B electron transfer, which is currently not well understood (Petrouleas and Crofts, 2005). The non-heme iron is involved in Q_A to Q_B electron transfer, and has been implicated in the herbicide effects on the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ midpoint potential (Krieger-Liszka and Rutherford, 1998) as well as the effect of bicarbonate depletion (Petrouleas et al., 1994). In purple bacteria, however, studies show that this electron transfer rate is relatively unaffected by exchange with other divalent metals (Debus et al., 1986).

There is another proposed cation binding site on cyanobacterial PSII near Q_A , thought to mediate cation effects on Q_A redox potential and hence electron transfer kinetics (Khan et al., 2015). This is similar to the cation binding site reported in reaction centre crystals from *Rhodobacter sphaeroides* (Paddock et al., 1999; Utschig et al., 1998). None of the PSII crystals measured in this work showed evidence for such a site, which is supported by a similar absence in other published cation-bound structures (Zouni, Kern, et al., 2001).

3.7.4. Structural analysis of PsbA3-containing PSII complexes

Existing crystal structures of PSII in the literature were made from material from complexes containing PsbA1, the D1 protein isoform expressed under normal growth conditions (Table 3-1). The high-light-induced isoform PsbA3, however, shares important structural similarity with PSII from higher plants and is therefore an important model to study. It also provides a

useful comparison to PsbA1 in terms of yields, energetics, kinetics and efficiency (Sugiura and Boussac, 2014b; Vinyard et al., 2013). Important modification include (1) an increase in the E_m of the $\text{Pheo}_{D1}/\text{Pheo}_{D1}^-$ couple by 17 mV due in part to a D1-Gln130Glu substitution (Giorgi et al., 1996; Kato et al., 2009; Sugiura, Azami, et al., 2014), (2) an increase in the E_m of the Q_A/Q_A^- couple by 38 mV (Kato et al., 2012), (3) altered intra-complex interactions (Sugiura, Iwai, et al., 2010) and (4) more efficient repair (Tichý et al., 2003). Computational models have also been used to predict structural differences based on primary sequence (Loll et al., 2008).

Using crystallographic procedures described in section 3.7.1 and PSII purified from the $\Delta\text{psbA1}/\text{psbA2}$ strain described in section 3.6.3, X-ray diffraction data was collected from PSII crystals (Figure 3-31) containing the D1 isoform PsbA3, summarised in Table 3-2. Diffraction spots could be seen up to $3.2 \text{ \AA}$ (Figure 3-32) and yielded a dataset with a low-resolution limit of $3.7 \text{ \AA}$ summarised in Table 3-2.

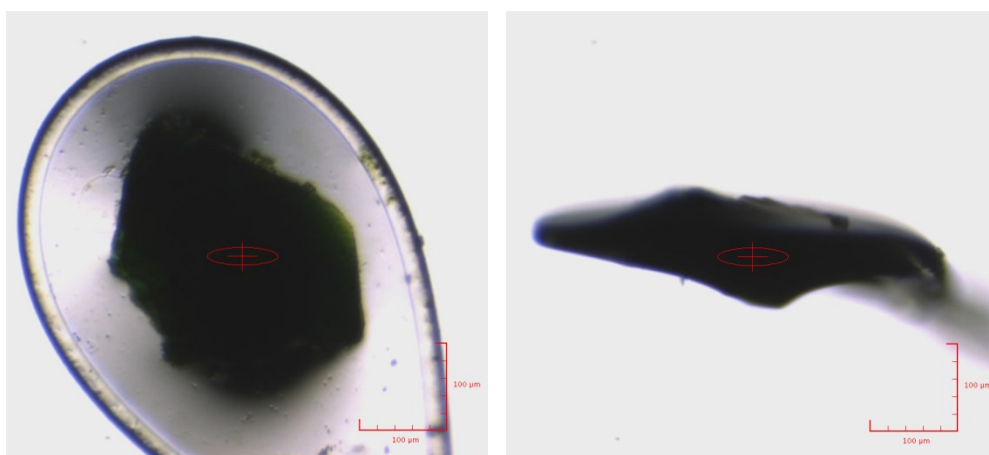


Figure 3-31: Pictures of mounted PSII crystal from which the $3.7 \text{ \AA}$ dataset was obtained.

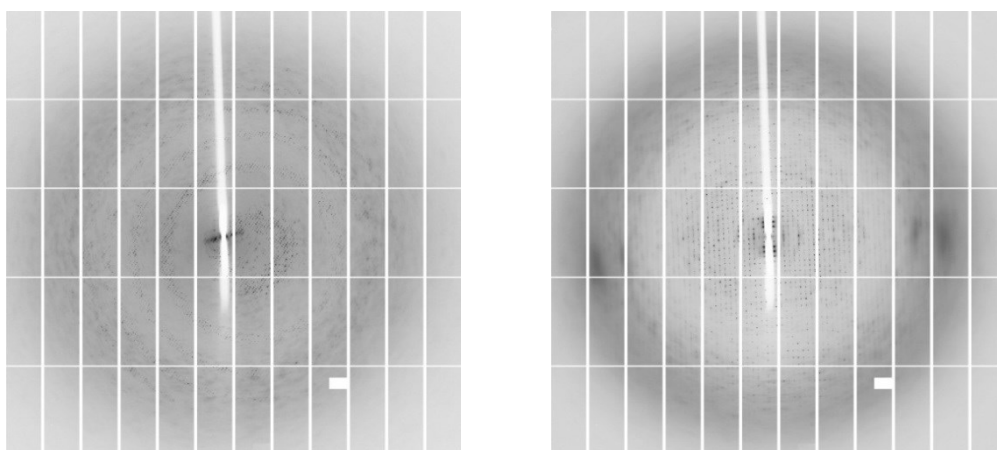


Figure 3-32: Two diffraction patterns measured 90° apart showing spots as far as $3.2 \text{ \AA}$.

Table 3-2: Data collection statistics for PsbA3-PSII crystal structure.

	Sweep 1	Sweep 2	Sweep 3
High resolution limit (Å)	3.71	16.59	3.71
Low resolution limit (Å)	110.34	110.34	3.81
Completeness (%)	95.9	97.6	70.9
Multiplicity	3.7	3.3	2.6
I/sigma	13.3	40.9	2.1
Rmerge	0.054	0.017	0.378
Anomalous completeness (%)	88.3	93.5	49.3
Anomalous multiplicity	2.0	1.9	1.7
Beamline	I04		
Wavelength:	0.97625 Å		
a, b, c (Å)	126.75, 224.21, 305.20		
α , β , γ (°)	90, 90, 90		
Spacegroup:	P2 ₁ 2 ₁ 2 ₁		

This is the first report of a crystal structure of the high-light-induced PsbA3-PSII. These data demonstrates that the differences between PsbA3 and PsbA1 do not alter the backbone structure of the protein in complex to a large degree. This demonstrates the relevance of models using PsbA3 primary structure and PsbA1 electron density maps (Loll et al., 2008).

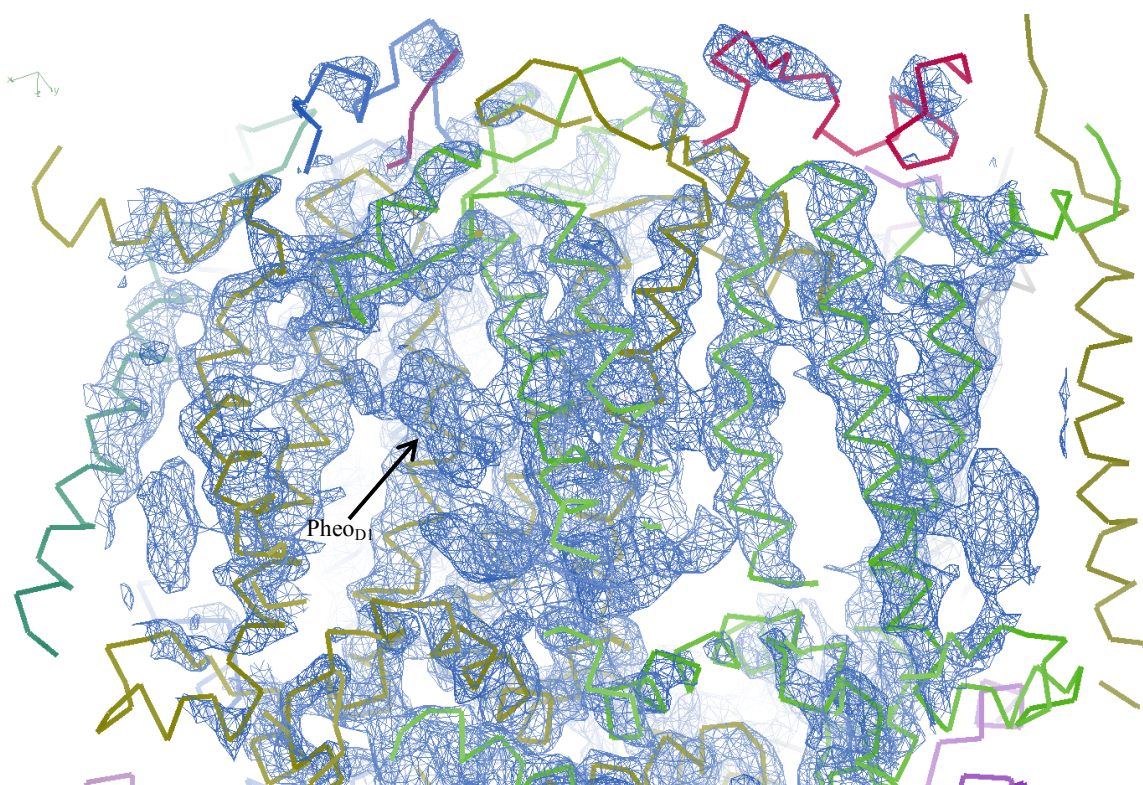


Figure 3-33: Cartoon of the generated PsbA3-PSII model showing the fit between the electron density obtained from these crystals (blue mesh, 1.55 rmsd) and the backbone carbons of the 1.9 Å structure for D1 (tan) and D2 (green). Helices from other subunits are also visible. Electron density difference

in the region near Pheo_{D1} (arrow) were not well resolved enough to confidently assign side chain positions.

While significant difference density was observed in the region surrounding Pheo_{D1} (4.2 rmsd), known to be modified in PsbA3 with an effect on the midpoint potential of Pheo_{D1}, the resolution of the data generated was not sufficiently high to accurately assign this to specific changes in the arrangement of side-chains. This result limits the structural variation between PsbA1 and PsbA3 and supports previous models which assume similarity with PsbA1 (Loll et al., 2008).

3.7.5. Determining the binding mode of DCMU using its analogue DBMU

DCMU is a common PSII inhibitor for photosynthetic studies relating to quinone exchange and acceptor-side electron transfer. This section presents crystallographic data that contributes to our understanding of its interaction with PSII. Understanding better its mode of action could inform the design of novel synthetic herbicides (Šeršeň et al., 2015).

DCMU was shown to compete with the terminal electron acceptor quinone for binding at the Q_B site (Lavergne, 1982). Referred to as a urea-type inhibitor, it differs from other Q_B-site-binding triazine-type (e.g. atrazine, terbutryn) and phenolic-type (e.g. ioxynil, bromoxynil) inhibitors in its effects and mutant cross-resistance, and therefore in its presumed mode of action (Nakajima et al., 1996; Sinning et al., 1989; Sinning, 1992). It was shown to block forward electron transfer in plant PSII by competing with quinone and semiquinone for binding in the Q_B site (Lavergne, 1982), as predicted previously (Velthuys, 1981).

Early binding niche predictions were based on primary sequence and resistant mutants (Trebst, 1987). DCMU-containing bRC crystals were reported (De la Rosa, 1991; Sinning, 1992) but not deposited in the PDB. The inhibitor was thought to bind to L-His190, homologous to D2-His215 in *T. elongatus* which is one of four histidine ligands to the non-heme iron. Two competing binding models have it bound to D2-His215 (by analogy to bRC data) or D2-Ser264 and D2-Phe265 (suggested by computational models) (Mackay and O'Malley, 1993), while more recent studies support the latter binding mode (R. Takahashi et al., 2010). This scheme is also supported by mutagenesis studies (Brusslan and Haselkorn,

1989; Trebst, 1987). This is similar to the terbutryn-bound structure published recently (Broser et al., 2011).

DCMU binds strongly with a dissociation constant of 0.76 μM measured in monomeric PSII from *T. elongatus* (K. Zimmermann et al., 2006), although it is not clear how the ~ 20 -fold difference between binding in centres containing Q_B versus Q_B^- observed in PSII from spinach would have affected these measurements (Lavergne, 1982). Small molecules binding to the Q_B site are known to affect the conformation of the D1 protein (Trebst et al., 1988). DCMU binding raises the E_m of the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ couple by +50 mV, stabilising the semiquinone, whereas phenolic herbicides shift the potential by -45 mV. This shift has been measured potentiometrically (Krieger-Liszkay and Rutherford, 1998) and related to FTIR-measured changes in bond lengths propagated via the non-heme iron, D1-His215 and D2-His214 (Takano et al., 2008). This contributes to protection against photodamage by energetically favouring direct recombination triplet formation (Keren et al., 1997; Kirilovsky et al., 1994).

In order to get good structural information from relatively low-resolution crystals a DCMU analogue was used in which the chlorine atoms attached to the phenyl ring are replaced with bromine, referred to here as DBMU (see Figure 3-34). This has the advantage that the K-edge energy of the heavier bromine atom is higher than that of chlorine (Cl K-edge 2822.4 eV, 4.4 Å; Br 13473.7 eV, 0.92 Å) resulting in both a stronger X-ray absorbance and a large anomalous signal when measured at accessible X-ray energies. The single-wavelength anomalous diffraction dataset generated can then be used with the high-resolution model (Umena et al., 2011) as a source of phases to more accurately locate the heavy atoms.

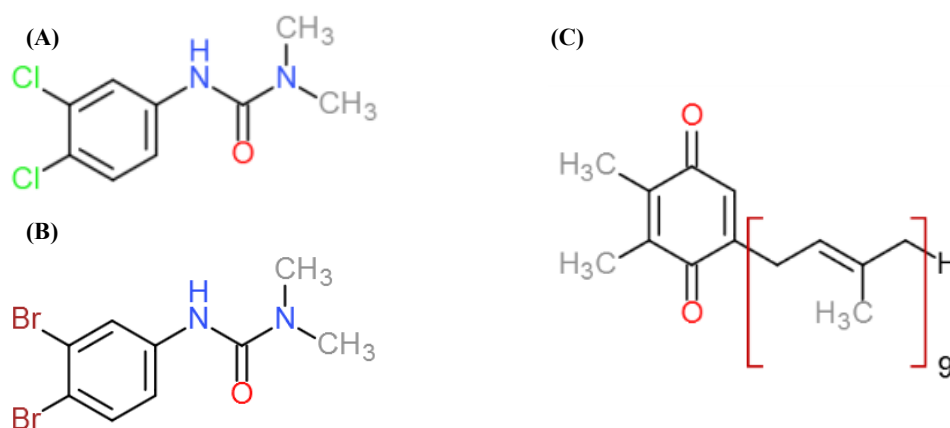


Figure 3-34: Cartoons of Q_B ligands (A) DCMU, (B) DBMU and (C) native PL9 showing the structural similarities in the head groups. Generated using <http://web.chemdoodle.com/demos/sketcher>

Figure 3-35 shows the inhibitory effect of DBMU on fast Q_A^- oxidation after photo-reduction by a single saturating flash. The sample without inhibitor shows normal kinetics for Q_A^- oxidation by Q_B ($\sim 300 \mu\text{s}$ phase), with exchanging Q_B ($\sim 9 \text{ ms}$ phase) and recombination with the manganese cluster ($\sim 7 \text{ s}$ phase). The forwards electron transfer components occurring in the 0.1-100 ms range have been reduced to 10% of the variable fluorescence, modelled here as a single decay component due to the small amplitude. This is consistent with DBMU blocking forward electron transfer in the majority of centres. The half-time of the slow Q_A^-/S_2 decay was not significantly different from samples inhibited by DCMU, indicating that they have a similar effect on acceptor-side energetics.

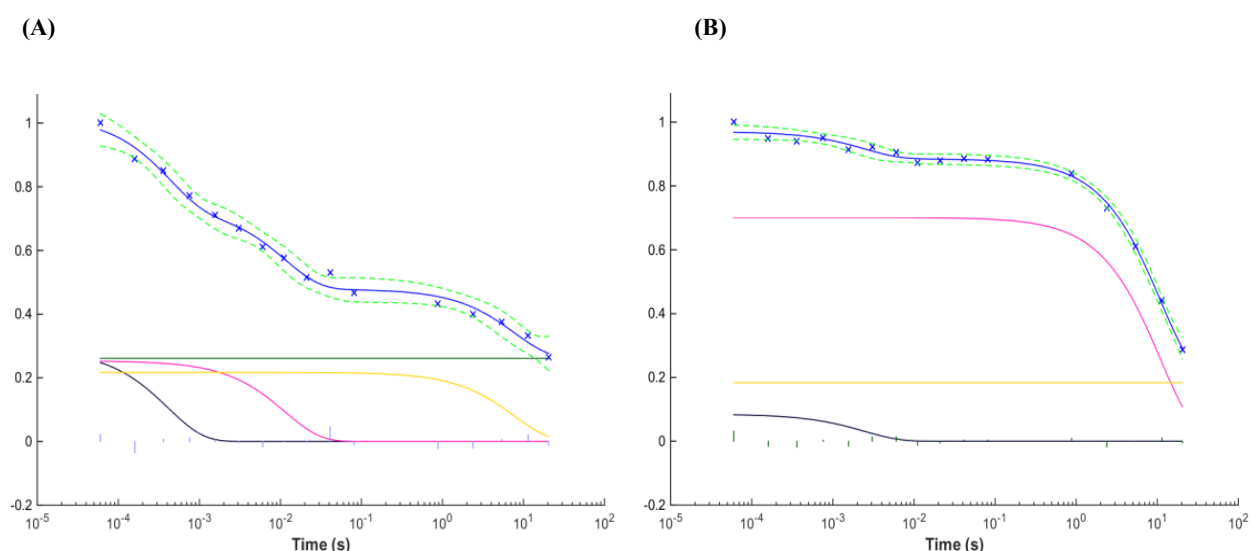


Figure 3-35: Graphs demonstrating DBMU inhibition of Q_A^- oxidation by fast forward electron transfer. The kinetics of an absorbance changes at 540 nm was measured using a JTS-10 after photo-reduction by a saturating laser flash in PsbA3-PSII samples (A) without and (B) with 5 mM DBMU. The uninhibited decay (blue crosses) is fit to a function (blue line) consisting of three exponential components (black, pink, yellow lines) and a linear baseline (dark green line). The inhibited decay is fit with two exponential components (black, pink lines) and a constant baseline (yellow line). The 95% confidence interval for the fits (light green dashed lines) and the residuals for each data point (vertical lines) are also shown. These samples were subsequently used to generate crystals for X-ray diffraction studies.

With this sample, data summarised in Table 3-3 was collected and used to produce the first structural model of PSII in complex with a urea-type structure at a resolution of 7.6 Å from crystals grown using a sitting drop method.

Table 3-3: Data collection parameters for crystals containing DBMU.

	Sweep 1	Sweep 2	Sweep 3
High resolution limit	7.58	33.9	7.58
Low resolution limit	137.26	137.26	7.78
Completeness	99.8	82.1	100
Multiplicity	38.5	25.2	30.4
I/sigma	33	126.6	4.6
Rmerge	0.095	0.025	0.911
Anomalous completeness (%)	99.8	78.3	100
Anomalous multiplicity	20.8	18.5	16
Beamline	I03		
Wavelength	0.91976 Å		
a, b, c (Å)	126.75, 224.21, 305.20		
α , β , γ (°)	90, 90, 90		
Spacegroup	P2 ₁ 2 ₁ 2 ₁		

Crystallographic models were made by Dr James Murray as described in section 2.5.11. Figure 3-36 shows the position of the anomalous signal from bromine atoms in the Q_B site. This is similar to the position of the chloride atoms in the FTIR-based model generated using AutoDock and the 2.9 Å structure (3BZ1, now 4V62) (R. Takahashi et al., 2010) (Figure 3-37). The results presented here support this model of binding and the assignment of D2-Ser264 and D2-Phe265 as binding partners, both conserved residues in PsbA1 and PsbA3. In this conformation, binding is expected to the C=O group of DCMU and the effect of replacing Cl with Br is likely to be negligible.

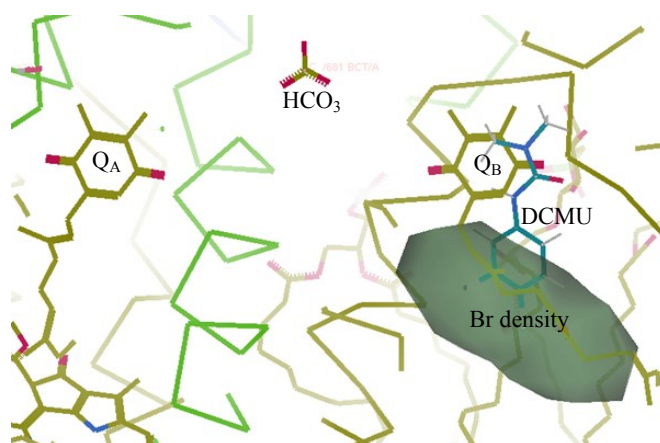


Figure 3-36: Cartoon showing the location of Br atoms in the Q_B site. Protein backbone structure was refined using X-ray diffraction data and the existing high-resolution structure (Umena et al., 2011) as a search model. A superimposed model of DCMU (blue sticks) is shown positioned in the Q_B site according to the anomalous scattering signal (shaded area) measured in this study. Backbone structure of D1 and D2 are shown in yellow and green respectively.

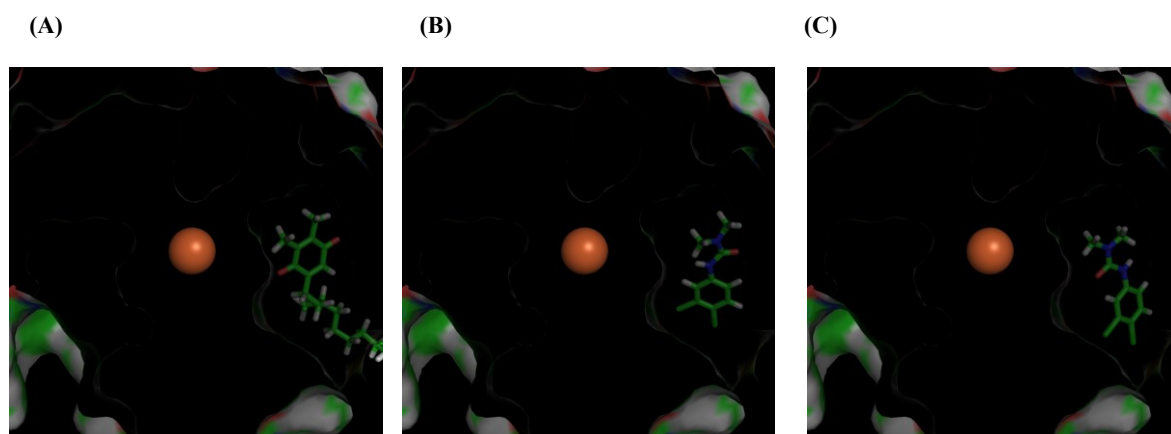


Figure 3-37 Cartoons showing the internal surface of the Q_B site containing (A) native plastoquinone, (B) DCMU oriented according to the FTIR-based model of Takahashi et al. (2010), or (C) DCMU oriented according to DBMU anomalous diffraction data generated in this study. Protein surface, native plastoquinone and non-heme iron (orange sphere) modelled using the high-resolution structure presented by Umena et al. (2011).

3.7.6. Conclusions

In order to study PsbA3-PSII, a mutant strain of *T. elongatus* lacking *psbA1* and *psbA2* was generated, similar to others reported in the literature (Sander et al., 2010; Sugiura et al., 2008). This strain also expresses a His₆-tag at the C-terminal tail of CP47 introduced previously (Iwai et al., 2010), facilitating purification. This also now enables the study of different sub-complexes containing CP47 or CP43 involved in PSII degradation and turnover (Nixon et al., 2010), which has been shown to be affected by D1 isoform present (Komenda et al., 2000; Tichý et al., 2003) and related to differences in the D1 N-terminal sequence (Komenda et al., 2007). Growth assays confirm the negligible phenotypic effects of removing *psbA1* and *psbA2* on growth under laboratory conditions under light-limited conditions, when *psbA1* is normally expressed (Ogami et al., 2012). This result is at odds with experimental results *in vitro* which show an increase in the rate of direct charge recombination between P_{680}^{+} and Q_A^{-} (Sugiura and Boussac, 2014a), which would decrease the yield of quinone reduction. This energetic cost, however, may be too small to detect in these assays, or possibly offset by the associated reduction in ³Chl yield and resulting photodamage by ¹O₂.

Presented here are the first reported crystal structures of the high-light-induced PsbA3 isoform of D1 in PSII, as well as PSII in complex with a urea-type inhibitor analogue of DCMU. While the low resolution of the data does not enable accurate designation of side-chain positions, these data demonstrate the similarities with the PsbA1 isoform (Umena et al.,

2011) in terms of the D1 backbone structure and complex formation, and the orientation of DCMU in the Q_B site.

Chapter 4: Investigating the fast kinetics of Q_A^- oxidation in purified active PSII dimers from *T. elongatus*

The aims of the work presented in this chapter are to determine the rates of electron transfer from Q_A^- to subsequent electron acceptors in PSII from the thermophilic cyanobacterium *T. elongatus*. Values currently reported in the literature are inconsistent with our understanding of these reactions in plant samples. In order to obtain more reliable numbers, the kinetics of fluorescence yield decay, corresponding to Q_A^- oxidation, was measured in cells and active PSII dimers in different oxidation states after photo-excitation. Flash-induced absorbance change kinetics were also measured which gives information specifically about Q_A^- oxidation by Q_B^- . These measurements provide a more accurate and consistent picture of acceptor-side electron transfer in these samples (Müh et al., 2012). This information is also important for biophysical and structural studies on PSII using this material, commonly used because of their stability.

4.1. Aligning S-states of the Mn_4CaO_5 cluster

In order to study the catalytic cycle of PSII it is useful to be able to generate homogenous samples of reaction intermediates. Long dark adaptation has been shown to synchronise the state of the Mn_4CaO_5 cluster in its S_1 state (Forbush et al., 1971; Vermaas et al., 1984). Over a series of flashes, centres containing reduced Y_D in the dark will miss one oxidation of the cluster when Y_D is oxidised. Thus, the dark-stable state is often modelled as 75% S_1 , 25% S_0 . In experiments much shorter in duration than the rate of Y_D equilibration with the Mn_4CaO_5 cluster (1-1.5 s) this effect is not important. Measurements can be made on samples poised in advanced S-states by using low temperature to block forward reactions and trap unstable states. While the homogenous S_2 state can be easily generated by illumination at 77 K (Brudvig et al., 1983), forming the S_3 state is much more difficult and requires subsequent annealing at room temperature in order to oxidise photo-reduced electron acceptors (Guiles et al., 1990; Ioannidis et al., 2002; Ioannidis and Petrouleas, 2000; Matsukawa et al., 1999; Nugent et al., 1997). These methods have limited use due to the relatively low yield of S_3

generated. It is therefore necessary to develop methods for generating advanced S-states with high yield.

The introduction of X-ray free electron laser (XFEL) crystallography has enabled the study of protein structures in real time, free of X-ray damage (Kern et al., 2012). A recent high-profile study using this technique reported structural data obtained from PSII from *T. elongatus* after treatment intended to generate the S₃ state of the Mn₄CaO₅ cluster (Kupitz, Basu, et al., 2014). This was the first crystallographic data reported for this state, following on from S₂ measured using similar methods (Kern et al., 2013) and the near-atomic resolution structure of dark-stable S₁ from the closely related *T. vulcanus* (Umena et al., 2011). In these experiments, two sequential saturating laser flashes were used to advance the catalytic cycle of the Mn₄CaO₅ cluster from the dark-stable S₁. S₃ is the last state before water oxidation and its structure would provide valuable information about the contested reaction mechanism (Cox et al., 2013).

Saturating laser flashes applied at room temperature provide a method for generating intermediate states with high yield (Boussac et al., 2015, 2008). Assuming all of the centres are active and open, the proportion of S₂ generated by the first flash depends on the quantum yield of charge separation which is ~90% (J.-M. Hou et al., 2001; Vasil'ev et al., 2004). In order to achieve a high turnover yield on the second flash, it is necessary to allow Q_A⁻ reduced by the first flash to be oxidised by other acceptor-side oxidants. In the presence of Q_A⁻, the charge-pair P₆₈₀⁺/PheO_{D1}⁻ is generated with a low yield at room temperature and is unstable, recombining in the nanoseconds time scale (Gibasiewicz et al., 2001; Schlodder and Brettel, 1988; van Miegheem et al., 1995). The rate of forward electron transfer from Q_A⁻ is in the milliseconds timescale and therefore studies on intermediate S-states are generally done with flashes separated by >500 ms.

In the XFEL study it was reported that the flash separation was 210 μs (Kupitz, Basu, et al., 2014). This is rationalised by the fast rate of the S₁ to S₂ transition and limited by other experimental factors. An increase in the size of the crystals' unit cell after the second flash, which reverted after a subsequent flash, was given as supporting evidence to show the transition between S-states in their samples, although the effect of a single flash was not measured. The timing of the second flash spacing is short compared to the oxidation rates of Q_A⁻ by Q_B in the literature (see below). Given this information, it is likely that the proportion of S₃ generated by the second flash will be limited by the proportion of centres in which the

photo-reduced Q_A^- is still present, and that only a small fraction of S_3 is expected to be formed with this treatment. This would imply that the structural changes they report would have to be reassigned. Detailed studies of Q_A^- oxidation were, however, carried out in PSII particles isolated from plants (de Wijn and van Gorkom, 2001) and it is therefore possible that in this specific material, active dimeric PSII cores purified from *T. elongatus*, faster oxidation could occur.

Given the potential importance of the XFEL results, it is necessary to investigate the Q_A^- oxidation kinetics under different redox conditions. The present work looks at the rates of fast Q_A^- oxidation in purified PSII active dimers from *T. elongatus* and the effect of the redox state of electron acceptors. Given the flash spacing used and rates reported in the literature the hypothesis that the rate of Q_A^- oxidation in these samples after the first flash is too slow to achieve a high yield of charge separation on the second flash is tested. Results in this chapter support this hypothesis and show how external oxidants can improve the yield. These results should not only be useful to the field in general but inform the interpretation of the XFEL results in terms of the measured distribution of S-states, and suggest changes in the method which could optimise turnover yield.

4.2. Fluorescence yield measurements of Q_A^- oxidation rates

Fluorescence measurements are well suited to address this issue as they are a direct probe of the effects of photochemistry on the yield of subsequent excitation. The rapid kinetics of Q_A^- oxidation have been characterised using variable fluorescence in thylakoids isolated from spinach, with multiple phases assigned to oxidation by quinone in the Q_B site ($t_{1/2} = 200\text{--}400\ \mu\text{s}$), by semiquinone in the Q_B site ($t_{1/2} = 600\text{--}800\ \mu\text{s}$), by quinone exchanging with an empty Q_B site ($t_{1/2} = 2\text{--}3\ \text{ms}$) and by quinone exchanging with a Q_B site containing hydroquinone ($t_{1/2} \approx 0.1\ \text{s}$) (de Wijn and van Gorkom, 2001). This set of reactions corresponds to electron transfer 5 in Figure 1-3. In centres lacking an electron acceptor or in the presence of DCMU, Q_A^- recombination with the Mn_4CaO_5 cluster on the seconds timescale takes place (e.g. Rappaport et al., 1994). These reactions are not well studied in cyanobacterial PSII.

Flash-induced fluorescence decay measurements are often not able to resolve all of the components of Q_A^- oxidation due to experimental limitations and the overlap between the different reactions. Decay kinetics in cells and protein samples are instead fit to three components for oxidation by (1) a quinone or semiquinone in the Q_B site being reduced

(herein called the Q_B phase), (2) a quinone entering an empty Q_B site or exchanging with Q_BH_2 and being reduced (herein called the exchange phase) and (3) recombination with the hole stabilised by the manganese cluster (herein called the recombination phase). A range of values for the half-time of these phases can be found in the literature for different samples: 0.39-1.1 ms, 6.4-22 ms and 1.5-10s for PSII membranes isolated from plants (Boisvert et al., 2007; Chernev et al., 2011; Sigfridsson et al., 2004; Vass et al., 1992, 2002); 550-660 μ s, 6.2-7 ms and 9.7-13.3 ms for *Synechocystis* cells (Cser and Vass, 2007; Deák et al., 2014; Sicora et al., 2008); $\sim$ 1 ms, $\sim$ 30 ms and $\sim$ 50 s for *T. elongatus* cells (although detailed deconvolutions have not been reported) (Deák et al., 2014; Deák and Vass, 2008; Kós et al., 2008); 0.78-1.5 ms, 9-90 ms and 5-10 s for PSII dimers purified from *T. elongatus* and 3.7-3.6 ms, 35-46 ms and 5-13 s for PSII monomers purified from *T. elongatus* (Kargul et al., 2007; Mamedov et al., 2007; K. Zimmermann et al., 2006). This work addresses the variation in these values reported for PSII cores. The kinetics of electron transfer from Q_A^- to Fe^{3+} have been report in the range 5-55 μ s (Boussac et al., 2011; Petrouleas and Crofts, 2005).

4.3. Analysis of fluorescence yield decay

Fluorescence yield decay gives a time-resolved measurement of the proportion of centres that are unable to stabilise charge-separated states following Q_A photo-reduction. In a dark-adapted sample of intact PSII particles, maximum photochemical quantum yield is achieved. In this ‘open’ state, Q_A is in its oxidised state and illumination results in Q_A reduction in $\sim$ 90% of excited centres (J.-M. Hou et al., 2001; Shuvalov and Dolgova, 2007), with higher yields in samples with smaller antennae (Wientjes et al., 2013). Excess energy is lost as radiative and non-radiative recombination. While Q_A is reduced, photochemical quenching by forward electron transfer is blocked. As a result, the quantum yield of fluorescence in these ‘closed’ centres is increased (Krause and Weis, 1991). As the proportion of Q_A that is reduced decays through forward and backward electron transfer reactions and direct recombination, the fluorescence yield decays with an equivalent kinetic.

4.4. Fluorescence yield decay after photoexcitation in cells

In order to determine the rates of electron transfer reaction in cells under physiological conditions, measurements were made using an FL-3000 fluorescence spectrometer (Photon Systems Instruments). A 1 ml sample was taken from cell cultures in log phase

($0.5 < OD_{730} < 0.7$), transferred into the cuvette in the sample chamber pre-heated to growth temperature (45 °C) and immediately measured. Using this method, half-times were measured for the electron transfer from Q_A^- to Q_B or Q_B^- and from Q_A^- to a quinone exchanging with the pool to be $341 \pm 124 \mu s$ and $4.60 \pm 1.5 ms$ respectively (Figure 4-1, Table 4-1). These two components contribute roughly equally to the decay. In these samples there was no significant residual variable fluorescence after $\sim 0.1 s$, indicating that the amount of centres lacking Q_B (in which a long-lived Q_A^- would be expected to recombine with S_2 in the seconds' timescale) is negligible.

In order to measure the rate of Q_A^- recombination with the donor-side, DCMU was added to block forward electron transfer (Cser and Vass, 2007). Inhibition by DCMU has also been shown to increase the midpoint potential of the Q_A/Q_A^- redox couple by 50 mV (Krieger-Liszkay and Rutherford, 1998). This affects the rate of back-reactions and direct recombination as the $P^+Q_A^-$ state has less driving-force compared to centres containing a quinone in the Q_B site. The DCMU-inhibited decay shown in Figure 4-1 **(B)** is dominated by a broad kinetic with a half-time of $767 \pm 97 ms$. This is faster than decays presented in the literature of $\sim 10 s$ (Kós et al., 2008) and $\sim 4 s$ (Sander et al., 2010), although explicit deconvolutions were not carried out.

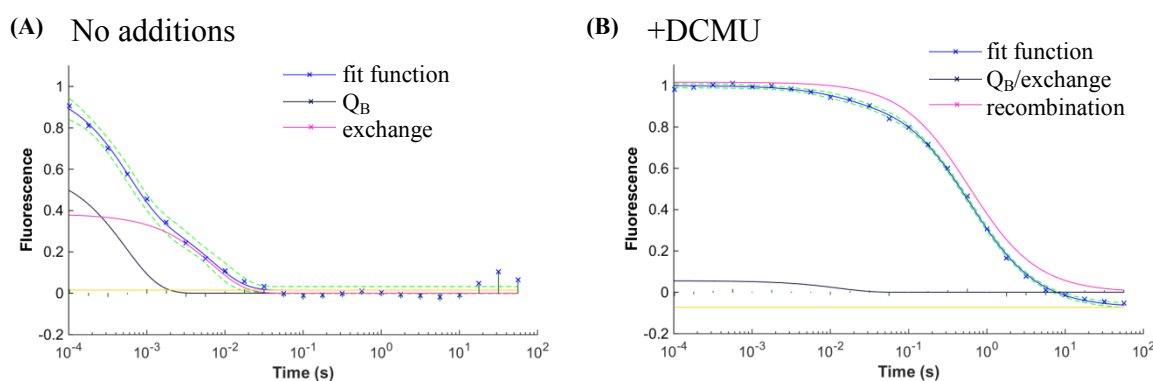


Figure 4-1: Kinetics of fluorescence decay in *T. elongatus* cells **(A)** under physiological conditions and **(B)** after 5 mins in the dark at 21 °C with the addition of DCMU. These figures show the data (blue crosses) and the fit function (blue line) as well as each component (black, pink lines), the offset (Y_0 ; yellow lines) and the residuals (green lines)

In order to compare samples at physiologically relevant temperature to conditions similar to those used for measurements on purified proteins, these rates were measured again after 3 min incubation in the dark at 21 °C. This treatment decreased the rate of the middle

component, attributed to quinone exchange, by 2.8 ms. This effect is likely due to decreased membrane fluidity, affecting the rates of quinone diffusion and exchange.

Table 4-1: Kinetic parameters for the multiphasic fluorescence decay in *T. elongatus* cells. Data are average $\pm$ standard deviation of three biological replicates. A_1 and A_2 are the amplitudes of components with half-times $t_{1/2}$ 1 and $t_{1/2}$ 2 respectively, as described in section 2.5.14.

	45 °C ^a		21 °C ^a		+DCMU ^b	
A_1 (%)	57	$\pm$ 4.7	42.4	$\pm$ 7.9	5.65	$\pm$ 0.41
A_2 (%)	43	$\pm$ 4.7	58	$\pm$ 7.9	94.3	$\pm$ 0.41
$t_{1/2}$ 1 (ms)	0.341	$\pm$ 0.12	0.527	$\pm$ 0.03	8.16	$\pm$ 2.1
$t_{1/2}$ 2 (ms)	4.60	$\pm$ 1.5	7.41	$\pm$ 0.56	767	$\pm$ 240
Y_0	0.642	$\pm$ 2.7	1.75	$\pm$ 1.0	-9.63	$\pm$ 5.7

^a Fit with two exponential components over 100 s.

^b Fit with one exponential and one hyperbolic component over 100 s.

Measurements made in cells are subject to a number of factors which make accurate determination of the rate of Q_A^- oxidation difficult. It is possible that there are more than two reactions contributing to the decay in the absence of DCMU resulting from the heterogeneity of reaction centre complexes present in a living membrane at different stages of assembly, repair and degradation. For example, purified monomeric and dimeric PSII have been shown to have significantly different forward electron transfer rates *in vitro* (K. Zimmermann et al., 2006) and a mixture may be present *in vivo*. While the dominant oligomeric state *in vivo* is contested, our crude preparation yields roughly 50:50 monomer:dimer. The number of different factors influencing Q_A^- oxidation *in vivo* makes interpretation of the data difficult. In order to remove these variables I use purified active dimers, allowing more controlled measurements under standard conditions.

4.5. Fluorescence yield decay in protein samples

In order to accurately measure the forward electron transfer reactions occurring at short times, active PSII dimer samples were prepared as described in section 2.5.3. These samples are similar to the isolated active PSII dimers used by Kupitz et al. (2014) for micro-crystallisation and subsequent XFEL measurements, also purified using anion-exchange chromatography.

4.5.1. Assessing the importance of connectivity in isolated PSII dimers

An uneven distribution of excitation among reaction centres affects the fluorescence yield in samples containing a mixture of open and closed centres. *In vivo*, shared antennae complexes transfer excitation energy to reaction centres allowing equilibration between them. Excitation which is equilibrated across multiple reaction centres is more likely to be quenched photochemically in open centres due to the short lifetimes of the excited state (Joliot and Joliot, 1964; Stirbet, 2013). This connectivity between reaction centres results in a non-linear correlation between Q_A redox state and fluorescence yield decay after a flash and can be corrected using a number of models.

Non-saturating actinic flashes reduce the effect of connectivity as the change in the ratio of open:closed centres over the course of the decay is smaller than with saturating flashes. This difference can be used to assess the effect of connectivity in a sample. Figure 4-2 shows the fluorescence during a decay after a saturating laser flash (doubled Nd:YAG, 532 nm) plot against a similar flash generating only 66% of the full fluorescence signal. A connected system would show a faster proportional decrease in fluorescence in the saturated case as the $Q_A:Q_A^-$ ratio change is greater, resulting in a deviation above a linear trend at intermediate fluorescence values. These data shows that the effect of connectivity between reaction centres in isolated PSII on the relationship between fluorescence decay and Q_A^- oxidation is negligible. Thus the data presented here were not corrected for this non-linearity effect and variable fluorescence was used as a direct correlate of Q_A^- concentration.

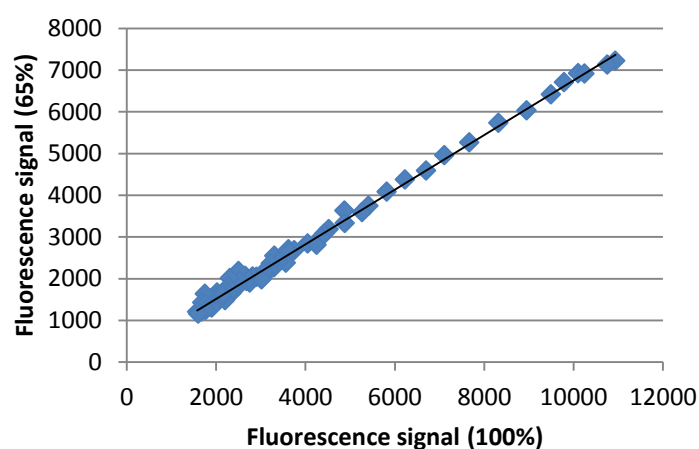


Figure 4-2: Graph showing a lack of connectivity in pool 3. Laser intensity was attenuated to generate saturating and 65% activation and the fluorescence signal at each time was plotted against each other. A linear trend is shown, which has the same R-squared value as a second-order polynomial fit

4.5.2. Fluorescence yield decay from active PSII dimers

Fluorescence decay measurements were made using isolated PSII after ~2 h incubation on ice, with the same non-saturating actinic flash used for measurements in cells. These data show that not all of the reaction centres are able to reduce Q_B . Figure 4-3 (A) shows the decay in the fluorescence yield over 100 s, fit with three exponential components and a constant. In the first second, the decay is similar to that seen in cells with two components with half-times of 612 μ s (60-70%) and 5.85 ms (20-25%), corresponding to the same forward electron transfer reactions. After 1 s there is a slow third exponential component with an 11 ± 1.7 s half-time which contributes 10-20% of the decay. A small proportion (0-10%) of the signal did not decay in the duration of the experiments and was fit as a constant. Figure 4-3 (B) shows the decay in the presence of DCMU, an inhibitor known to block forward electron transfer, fit with a single exponential component plus a constant. In these samples the Q_B and exchange phases are absent and the half-time of the recombination phase is reduced to 8 s.

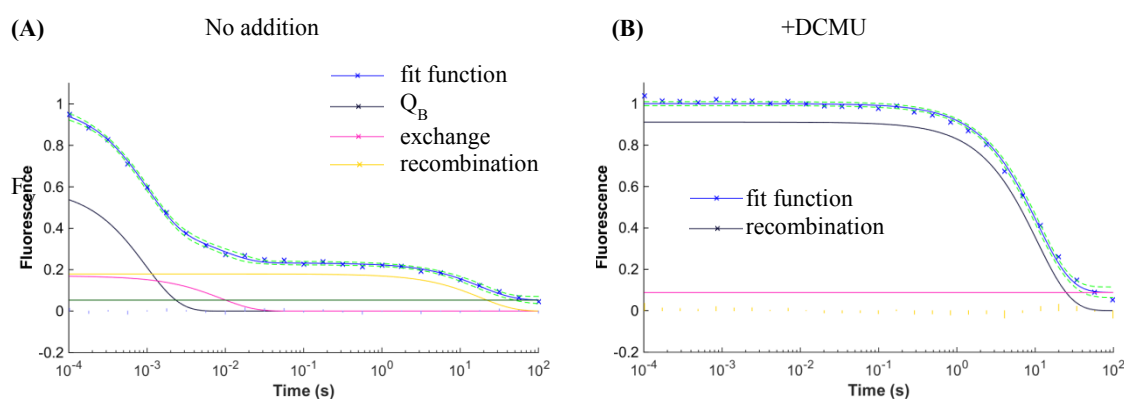


Figure 4-3: Fluorescence decay kinetics of active PSII dimers (pool 3) (A) before and (B) after the addition of DCMU. The data (blue crosses), fit (blue curve) and 95% confidence interval (green dotted lines) are shown in both kinetics. The uninhibited decay is fit with the sum of three exponential components (black, pink, yellow lines) plus a constant (green line) and the residuals are plotted in purple. The inhibited decay is fit with a single exponential decay (black line) plus a constant (pink line). These samples were thawed and incubated on ice for ~2h before measuring.

The rates of the fast and middle components are faster than other values reported in the literature (0.78, 1.5 ms and 9, 90 ms), while the rate of the slow component is within the range of reported values (Kargul et al., 2007; K. Zimmermann et al., 2006). These previous studies used a hyperbolic component to model the slow-decaying part of their kinetics, whereas a third exponential component was used in the present work. This difference, however, does not affect the fit at short times and cannot account for this difference.

The half-time for the fast component in these samples is around three times longer than the inter-flash delay used by Kupitz et al. (2014) to generate S_3 , and only constitutes ~60% of the decay. These data suggest that only ~10% of the photo-reduced Q_A^- in the sample was oxidised after 210 μ s and thus a second flash at this time would give only a very small yield of centres in the S_3 state.

The recombination phase of uninhibited isolated PSII samples, which was not seen in cell samples, corresponds to recombination of Q_A^- with the manganese cluster. This reaction can occur only in centres which have no available or exchangeable electron acceptor. The absence of an electron acceptor can also be detected by thermoluminescence, where a single flash at 1 °C given to a dark-adapted sample gives a low-temperature band arising from S_2/Q_A^- (Q-band), rather than a higher-temperature band arising from S_2/Q_B^- (B-band) (Ducruet and Vass, 2009; Rutherford et al., 1982). These bands appear around 20 °C and 45 °C in *T. elongatus* (Sugiura and Inoue, 1999).

Figure 4-4 (red line) shows the thermoluminescence measurements made on this sample with a large peak around 48 °C characteristic of S_2/Q_B^- recombination. There is, however, a significantly larger emission at lower temperatures than expected for a single-Gaussian fit for this curve (Figure 4-4, blue line). Reasonable parameters for a two-Gaussian fit, however, could not be obtained due to the sensitivity of the fitting to the initial parameters. The residual after subtraction of a single Gaussian (Figure 4-4, green line) was fit to a second Gaussian function ((Figure 4-4, purple line) whose peak at ~20 °C confirms that it corresponds to Q_A^- recombination with S_2 . This indicates that there is some recombination from Q_A^- generated by a single flash, supporting the conclusion that the sample contains a proportion of centres with a fully-reduced or absent Q_B .

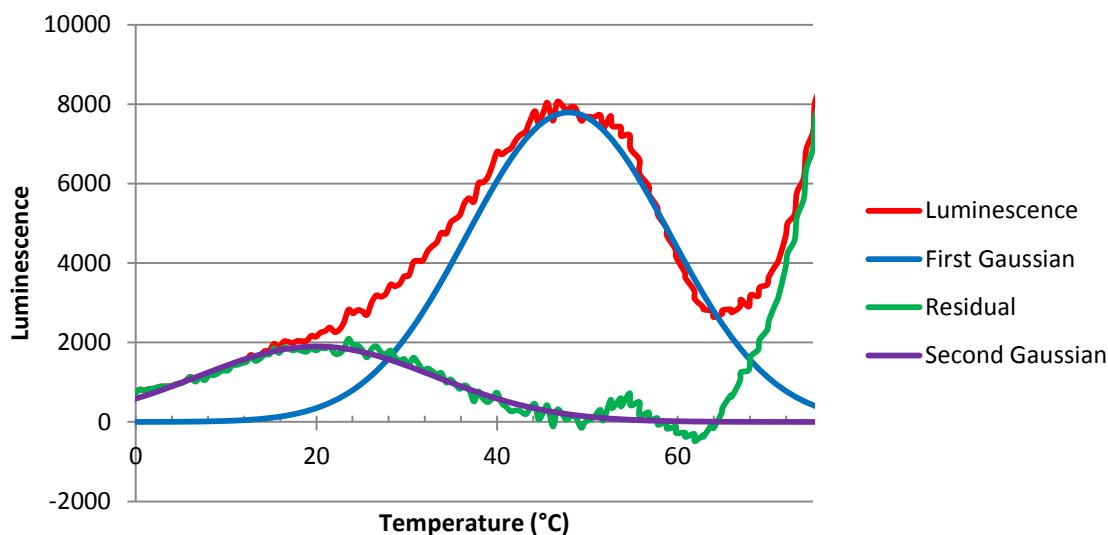


Figure 4-4: Thermoluminescence curves from pool 3 showing the data (red), the single Gaussian fit corresponding to Q_B^-/S_2 recombination (blue), the residual signal after subtraction corresponding to Q_A^-/S_2 recombination (green) and the single Gaussian fit corresponding to Q_A^-/S_2 recombination (purple).

These results suggest that there is still some $Q_B H_2$ present in the samples after several hours of dark adaptation, or some of the centres have no Q_B available. The centres which give rise to the $\sim 20^\circ C$ band in the thermoluminescence corresponds to those in which Q_A remains reduced for >1 s, giving rise to the recombination phase of the fluorescence yield decay. These illumination conditions are similar to those used prior to the XFEL experiment, which report crystal handling in dim green light and a period of dark adaptation, although the duration of this dark adaptation is not clear.

4.5.3. Fluorescence yield decay from long-dark-adapted active PSII dimers

In order to assess the potential effects of dark adaptation on Q_A^- oxidation, samples were stored overnight on ice. Figure 4-5 shows the decay of the fluorescence yield in PSII samples after overnight dark-adaptation. This treatment resulted in a significant reduction in the amplitude of the slow component (Q_A^-/S_2 recombination) to $\sim 7\%$ of the total decay.

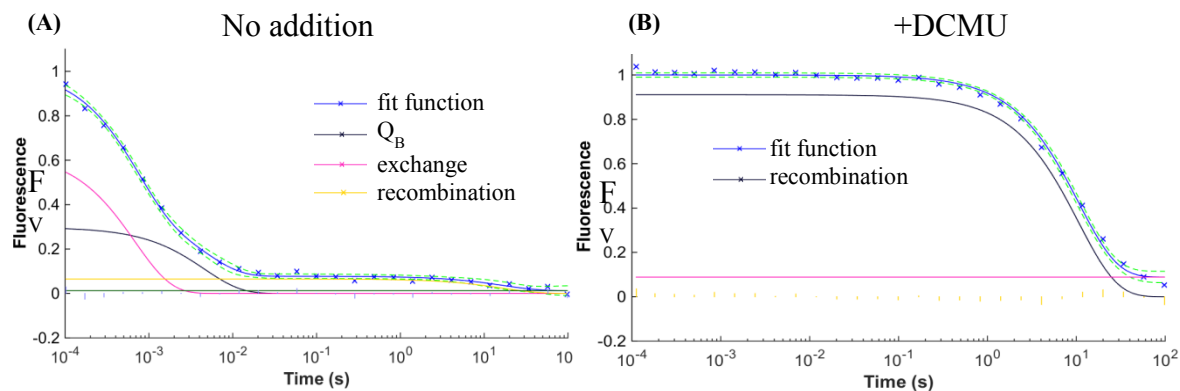


Figure 4-5: Fluorescence decay kinetics of active PSII dimers **(A)** after overnight dark adaptation and **(B)** after overnight dark adaptation plus DCMU. Fittings are the same as in Figure 4-3.

The half times of the Q_B and exchange phases of the fits were reduced to $404 \pm 80 \mu\text{s}$ and $2.9 \pm 0.5 \text{ ms}$ respectively by overnight dark adaptation, and were unchanged by subsequent 24 h dark adaptation (Table 4-2, Figure 4-7 **(A)**). The increase in the rate of the Q_B phase reflects a change in the redox state of Q_B as Q_A^- oxidation by Q_B is faster than by Q_B^- (de Wijn and van Gorkom, 2001). The observation that the rate does not change after a second overnight dark adaptation suggests that the sample is fully relaxed, i.e. Q_B^- had been fully oxidised by this treatment (Figure 4-7). This data also fits with the expectation that the rate of the exchange phase is dependent on the redox state of the exchangeable quinones, with a more oxidised sample giving a faster decay. There was no significant effect on the rate of the decay in the presence of DCMU.

The fluorescence yield decay was measured over a series of actinic flashes in order to assess the effect of photo-reduction. The flashes were spaced at 100 s intervals, by which time the Q_A^-/S_2 recombination is complete. Figure 4-6 **(A)** shows the variable fluorescence decay over 100 s after the first 11 flashes from a long-dark-adapted sample, fit to a three-exponential decay. These curves are shown normalised from 1 at time $t=0$ to 0 at 100 s in Figure 4-6 **(B)**. Figure 4-6 **(C)** shows the same data truncated to 1 s with a two-exponential decay fit, normalised from 1 at time $t=0$ to 0 at 1 s in Figure 4-6 **(D)**. For each decay, F_0' was taken as the as the fluorescence yield before the flash. The rate of the fast component of the decay increased from $310 \pm 143 \mu\text{s}$ on the first flash to $1.1 \pm 0.19 \text{ ms}$ after the third flash, after which it is stable for the remaining seven flashes. After multiple flashes the ratio of Q_B to Q_B^- will approach 50:50, similar to illuminated samples. Although the small amplitude of the exchange phase (around 10%) in these decays makes the fit highly sensitive to noise, a

decrease in the rate of the exchange phase can also be seen in the height of the signal between 1 ms and 10 ms (Figure 4-6 (D)) increasing after each flash.

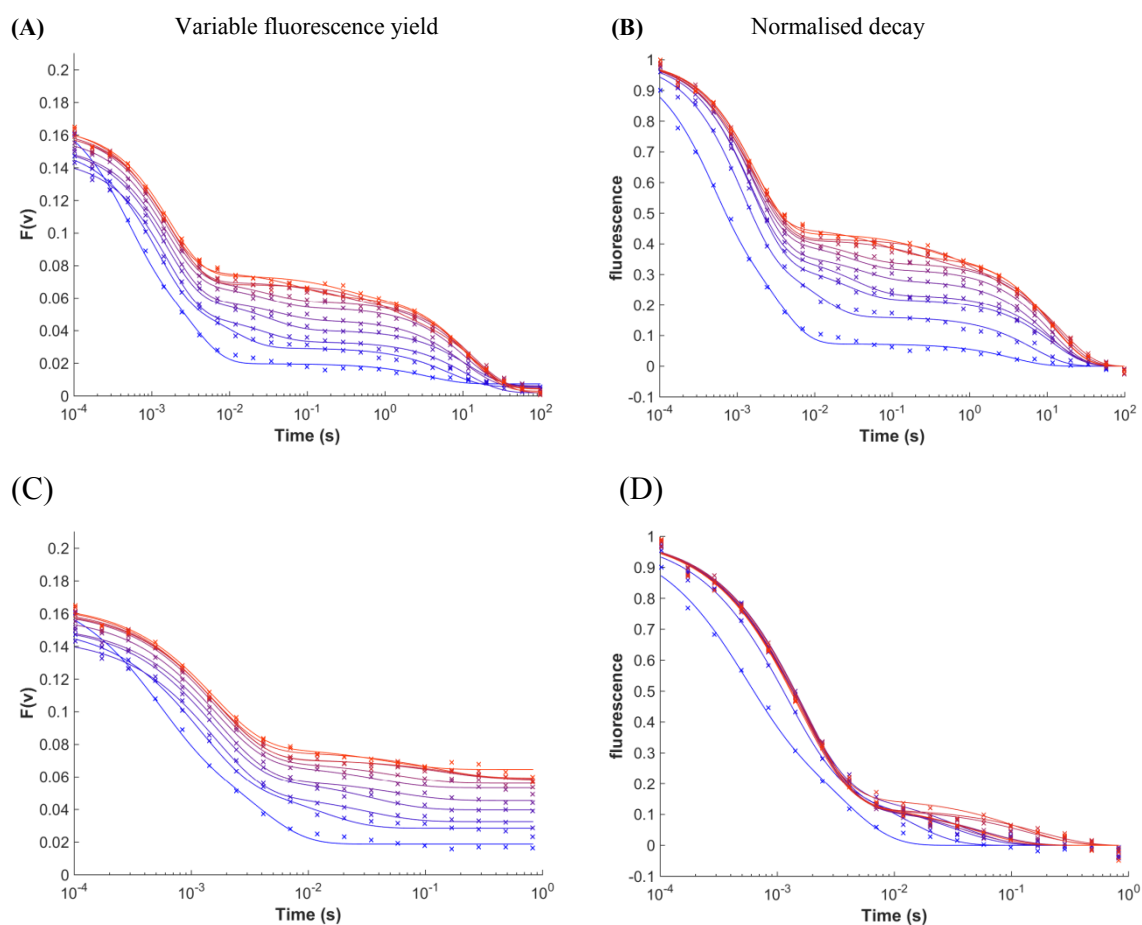


Figure 4-6: Variable fluorescence decay in isolated PSII dimers over a series of flashes separated by 100 s. A colour gradient is used from blue (first flash) to red (eleventh flash). Plots (A) and (B) show a three-exponential fit of each decay over the first 100 s whereas plots (C) and (D) show a two-exponential decay fit to the first 1 s of the decay. Plots (B) and (D) are normalised using the fits from 1 at $t=0$ to 0 at $t=100$ or 1 s.

In these long-dark-adapted samples, only ~20% of Q_A^- oxidation had occurred after 210 μ s, again indicating that this treatment is not sufficient to enable significant photochemical yield if illuminated at this time.

4.5.4. Fluorescence yield decay from ferricyanide-oxidised dimers

In order to further increase the rate of Q_A^- oxidation, samples were incubated with ferricyanide. Ferricyanide ($E_m \approx 466$ mV at pH 6.5) acts as an electron acceptor and has been shown to oxidise quinones (Hoganson and Babcock, 1988) as well as the non-heme Fe^{2+} to Fe^{3+} ($E_m \approx 430$ mV at pH 6.5 in plant PSII) (Petrouleas and Diner, 1987). The addition of 0.6

and 6 μM (10 and 100 per PSII respectively) potassium ferricyanide in the dark to <2 h dark-adapted samples increased the rate of Q_A^- oxidation in the <1 s time range (Figure 4-7 (A)). The proportion of Q_A^- remaining after 1 s was similar to that in samples after overnight dark adaptation, indicating that both treatments are able to fully oxidise Q_B and that the majority of centres have access to an exchangeable quinone.

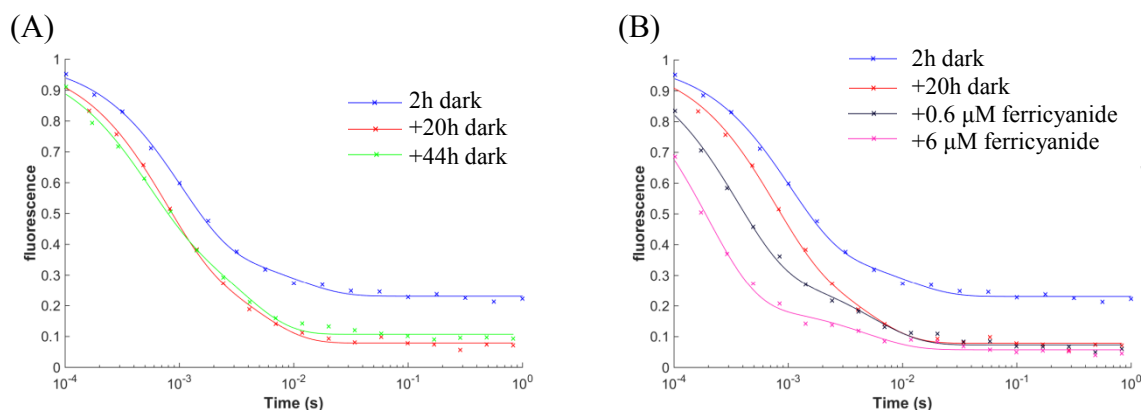


Figure 4-7: Fluorescence decay kinetics of active PSII dimers in oxidising conditions, showing the effects of (A) dark adaptation and (B) the addition of ferricyanide.

The addition of 6 μM ferricyanide to short-dark-adapted samples decreased the half-time of the fastest component to 120 μs . After >44 h dark-adaptation, 0.6 μM ferricyanide was sufficient to reduce the half time of the fastest component to $108 \pm 37 \mu\text{s}$. In these samples, ~65% of the fluorescence signal had decayed in the first 210 μs , indicating that this proportion of centres would be able to undergo a second excitation, charge separation and S-state turnover with a second actinic flash.

Table 4-2: Kinetic parameters for the bi-phasic fluorescence decay in pool 3 after dark adaptation with and without the addition of 0.6 μM ferricyanide. Data are average $\pm$ standard deviation of three replicates, both fit with two exponential components over 1 s.

	>44 h dark-adapted		>44 h dark-adapted +0.6 μM ferricyanide	
A_1 (%)	66	± 8.9	83	± 2.5
A_2 (%)	34	± 8.9	17	± 2.5
$t_{1/2} 1$ (μs)	404	± 81	108	± 37
$t_{1/2} 2$ (ms)	2.9	± 0.5	2.6	± 1.5
Y_0	10	± 2	6.4	± 0.57

4.6. Spectroscopic signal of Q_A^- oxidation by Q_B^- at 320 nm

While the fluorescence yield decay reflects Q_A^- oxidation by any oxidant, it is possible to distinguish between electron transfer from Q_A^- to Q_B and from Q_A^- to Q_B^- using optical

methods. The absorbance at 320 nm corresponds to a peak in the quinone reduced-minus-oxidised difference spectrum (Stiehl and Witt, 1969; van Gorkom, 1974). Thus, changes in absorbance at this wavelength are only affected when the concentration of semiquinone changes, i.e. when Q_A^- donates an electron to Q_B^- forming Q_A and Q_BH_2 .

This kinetic was measured in crude PSII using a dual-laser spectrophotometer (Béal et al., 1999) in collaboration with a colleague, Sven De Causmaecker. Saturating actinic laser flashes were used at a repetition rate of 5 Hz, each followed by a single detection laser flash at a variable time after the actinic flash, giving a single point on the decay. A fresh sample was used for each point of the decays. The first flash gave only a small decay as the majority of centres in the dark-adapted sample contained the oxidised Q_B . Figure 4-8 shows the absorbance decay after the second, third and fourth flashes from 10 μ s to 100 ms after the flash. The half-time of this decay was 1.0 ± 0.2 ms.

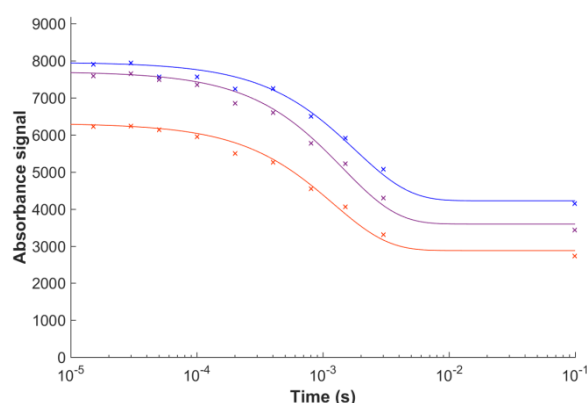


Figure 4-8: 295 nm absorbance change at in crude PSII after the second (blue), third (purple) and fourth (red) saturating laser flash at 5 Hz, corresponding to Q_A^- oxidation by Q_B^- .

4.7. Discussion

4.7.1. Accounting for Connectivity

Measurements described in section 4.5.1 indicate that the effect of connectivity between PSII reaction centres on the relationship between variable fluorescence yield and the ratio of centres containing Q_A and Q_A^- is negligible in these samples. Previous estimates of electron transfer rates in isolated PSII adjust the fluorescence decay data using a model proposed by Joliot and Joliot (1964) which introduces a parameter p corresponding to the probability of an

excitation being transferred from a closed reaction centre to another centre. The proportion of open centres q is calculated using the equation:

$$q = \frac{F_v'}{1 - p(1 - F)} \quad \text{Equation 8}$$

where F_v' is the variable fluorescence yield $\frac{F_t - F_0}{F_M - F_0}$. As p increases, the estimated rate of Q_A^- oxidation decreases relative to the measured rate of fluorescence decay. A value for p of 0.5 has been applied to decays from PSII dimers from *T. elongatus* (Kargul et al., 2007; Vass et al., 2002; K. Zimmermann et al., 2006), representing a 50% chance of excitation leaving a closed centre instead of decaying. This value, however, seems to be based on measurements made on membranes and membrane fragments from plants and therefore its relevance to purified PSII is unclear. The results presented here indicate that connectivity is low. Therefore, previously reported kinetic parameters which have been adjusted for connectivity are likely underestimates of the rate of Q_A^- oxidation in their samples (Kargul et al., 2007; Mamedov et al., 2007; K. Zimmermann et al., 2006). This adjustment is more relevant to measurements on larger photosynthetic systems such as PSII membranes, thylakoids, chloroplasts and whole cells where antennae can pass excitations between many reaction centres (Stirbet, 2013).

4.7.2. Q_A^- oxidation by Q_B and Q_B^-

The results show that significant dark-adaptation of purified PSII samples is required before they reach a true 'dark-stable' state, attributed to the slow oxidation of electron acceptors. The recombination phase arising from samples without access to a secondary acceptor quinone constitutes a significant proportion of the decay in short-dark-adapted samples. This component was absent after overnight dark-adaptation, with the majority of centres able to transfer an electron to Q_B . This indicates that >90% of centres have access to a secondary acceptor quinone, a fraction of which is fully-reduced in short-dark-adapted samples blocking forward electron transfer. This quinol population is likely generated by illumination during purification. The remaining small fraction of centres with long-lived (>1 s) Q_A^- may not have access to any quinone, it having been removed during purification.

The rate of the Q_B phase of fluorescence decay after overnight dark-adaptation, in which the dominant fast electron transfer from Q_A^- is to Q_B , indicates that the half-time of this electron

transfer is around 400 μ s. The half-time of the 320 nm absorbance decay, in which only the electron transfer from Q_A^- to Q_B^- is measured, is around 1 ms. Model data were prepared by combining different proportions of these two exponential decays and the best single-exponential fit was found using the same methods as were used to fit the experimental data. The ~ 600 μ s decay measured in short-dark-adapted samples corresponds to a 60:40 ratio of $Q_B:Q_B^-$ which is in agreement with other reports. Long-lived dark-stable Q_B^- has been seen previously (Demeter and Vass, 1984; Rutherford et al., 1982, 1984), with ~ 40 -50% remaining after short dark-adaptation in crude PSII from *T. elongatus* (Fufezan et al., 2005; Sedoud, Cox, et al., 2011) and around 22% in dark-adapted spinach thylakoids (de Wijn and van Gorkom, 2001). There is likely also a small contribution arising from equilibration with PQH₂ reduced during the purification procedure. The 50:50 $Q_B:Q_B^-$ ratio corresponds to an apparent half-time of 667 μ s. Thus, illumination or incomplete dark-adaptation of the sample cannot account for the longer half-times reported in the literature (Kargul et al., 2007; K. Zimmermann et al., 2006). It is likely that these values, as well as the 1.1 ms value measured in this work after three flashes, corresponds to an overlap between electron transfer and quinone exchange processes which are not well resolved in their samples.

While it is unlikely that dark adaptation alters the energetics of these reactions, it is possible that there are other reactions occurring in dark-adapted samples in the <10 ms time range which cannot be resolved with these measurements and contribute to the increase in the decay rate. One possibility is that the non-heme iron is oxidised in centres containing Q_B^- to Fe^{3+} , which then acts as a fast oxidiser of Q_A^- . A similar phenomenon has been demonstrated using EPR in spinach PSII membranes 1) in the presence of high-potential quinones (Petrouleas and Diner, 1987; J. Zimmermann and Rutherford, 1986) or 2) in the presence of carboxylate anions which lower the E_m of the iron reduction (Deligiannakis et al., 1994). While it was shown that the addition of DCMU induced Fe^{2+} oxidation in PsbA3-PSII but not PsbA1-PSII crude samples from *T. elongatus* containing Q_B^- , a small but unquantified proportion of Fe^{3+} has been reported in dark-adapted crude PsbA1-PSII (Boussac et al., 2011; Sedoud, 2011). Computational analysis suggests that the reduction of Q_A or Q_B would decrease the Fe^{2+}/Fe^{3+} midpoint potential to ~ 300 mV, making it more easily oxidised (Ishikita and Knapp, 2005b). This is, however, still far above the measured -80 mV and estimated 40 mV midpoint potentials of Q_A/Q_A^- and Q_B/Q_B^- respectively. It is therefore possible that the 400 μ s estimate of the half-time of Q_A^- to Q_B electron transfer is an underestimate, with an unresolved contribution from electron transfer to Fe^{2+} . In any case, this rate of electron transfer would

only be fast enough to enable a second photo-oxidation of the Mn_4CaO_5 cluster in ~20% of centres after 210 μs (Kupitz, Basu, et al., 2014).

4.7.3. Fe^{3+} as a fast electron acceptor

The addition of ferricyanide resulted in a significant increase in the rate of the decay, producing a 100 μs decay component. This is attributed to oxidation of (1) the non-heme iron (introducing a faster phase; Q_A^- to Fe^{3+}) and, to a lesser degree, (2) Q_B^- (increasing the rates of the Q_B and exchange phases).

The generation of a DCMU-insensitive electron acceptor (originally named R, a.k.a. Q_2 , Q_{400}) with a E_m around 360 mV at pH 7.8 by ferricyanide was first observed as a decrease in the rate of fluorescence induction (Ikegami and Kato, 1973). This secondary quencher was later identified as the non-heme iron (Fe in Figure 1-3) of the quinone-iron acceptor complex (Petrrouleas and Diner, 1986).

The fastest component of the ferricyanide-induced decay only accounted for ~80% of the decay, even in samples incubated with 6 μM ferricyanide. This result indicates that there is a proportion of centres in which the non-heme iron is not oxidised, possibly resulting from a modified E_m preventing oxidation by ferricyanide. Alternatively, the 80% could be an underestimation of the yield of Fe^{3+} due to double-turnover during the actinic flash. The amplitude of a $g=1.84$ EPR signal from $\text{Q}_\text{A}^-\text{Fe}^{2+}$ (Rutherford and Zimmermann, 1984) which is associated with the absence of bicarbonate, was shown to be pH dependent and inversely correlated with that of the fast ferricyanide-induced decay (Petrrouleas and Diner, 1987). The slow-decaying 20% in the presence of ferricyanide could arise from centres that lack bicarbonate, increasing the reduction potential of the non-heme iron to values closer to or above that of ferricyanide, thereby preventing its oxidation (Nugent et al., 1988). These centres will instead oxidise Q_A^- via Q_B as occurs in long-dark-adapted samples. The 2.6 ms half-time of this remaining decay indicates that it arises from centres which an empty Q_B site, although it is possible that there is an unresolved contribution from direct Q_A^- to Q_B electron transfer. The binding constant for bicarbonate is likely to be in the low micromolar range (Allakhverdiev et al., 1997), although higher values have been reported (Jursinic and Stemler, 1986; Stemler and Murphy, 1983), similar to the estimates that of air-saturated buffer of ~20 μM .

The fastest component of the ferricyanide-induced decay had a half-time of around 100 μ s. This is likely to be an overestimate due to the possibility of an overlap with Q_A^- oxidation by Q_B in centres with a reduced non-heme iron. The reduction of Fe^{3+} by Q_A^- has in chloroplasts has been attributed to a ~ 5 μ s effect on the flash-induced fluorescence rise at pH 6.5 (Bowes et al., 1979), a 7 μ s component of the C550 shift at pH 6.5 (Diner and Petrouleas, 1987), a 25 μ s component of the C550 shift at pH 7.5 (Petrouleas and Diner, 1987), a 50 μ s decay in 552 nm absorbance in PsbA3-PSII from *T. elongatus* in the presence of DCMU (Boussac et al., 2011) and a 140 μ s component of fluorescence decay in chloroplasts poised at 456 mV (Bowes and Crofts, 1980). Due to technical limitations, the first measurements in this study were made 100 μ s after the flash. If the half time of Q_A^- oxidation by Fe^{3+} were significantly shorter than this, its contribution to the fluorescence decay would not be observed. The fits obtained here estimate a similar maximum fluorescence at time $t=0$ for samples with and without the fast decay induced by ferricyanide. This indicates that ferricyanide does not induce a significant decay at short times that is not included in the fitting.

4.7.4. Implications for flash spacing

These results show that the 210 μ s flash spacing used by Kupitz et al. (2014) is unlikely to generate a significant proportion of centres in S_3 . Their flash spacing was chosen due to technical limitations of the measurements and the fast rate of the S_1 to S_2 transition (Dekker et al., 1984). The acceptor-side, however, is in practice the limitation. Assuming that their samples can be prepared in absolute darkness, a flash-spacing of 1 ms and 10 ms are expected to yield $\sim 60\%$ and 90% respectively. Instead of increasing the flash spacing, the rate of Q_A^- oxidation can be increased. Ferricyanide can increase the yield after 1 ms to 80% , which may be improved by further treatment to increase the yield of oxidised non-heme iron.

4.7.5. Q_A^- oxidation in Cells

The fluorescence decay kinetics *in vivo* at 21 °C show a rate for the Q_B phase consistent with Q_A^- oxidation by mixture by Q_B and Q_B^- , as expected. The large proportion of the exchange phase at $\sim 50\%$ is a result of the equilibrium between and empty Q_B site and reduced quinones in the membrane pool. In comparison, the lower yield and faster rate of this component in purified samples suggests that they have a higher effective concentration of oxidised quinone available on the first flash. The micellar structure of β -DDM-solubilised

PSII may constrain the available quinones closer to the Q_B site, rather than freely diffusing in the thylakoid membrane. The decreased rate of this phase at 21 °C compared to 45 °C likely results from a decrease in diffusion rates and fluidity of the membrane.

In all cell samples, the fluorescence had completely decayed to the F_0 value after 100 ms, similar to isolated PSII samples after overnight dark-adaptation. This shows that the availability of oxidised quinone electron acceptors is high. Previous reports have identified and characterised a transient fluorescence rise in cells after ~1 s under microanaerobic conditions which has been attributed to equilibration between Q_A and reduced quinone in the quinone pool via Q_B (Deák et al., 2014; Deák and Vass, 2008). The absence of this phenomenon in these samples is a good indication that the quinone pool was not highly reduced under the growth conditions used. Thus, neither the availability of oxidised quinones nor PSII activity is likely to be limiting the rate of photosynthesis under these conditions. Instead, it appears that quinone concentration, diffusion and exchange are limiting the rate of Q_A^- oxidation.

In cells in the presences of DCMU the slow decay is broader than a single exponential component, indicating either that this decay arises from a number of different reactions with similar half-times or heterogeneity of states with different half-times for the reaction. The driving force for this reaction (Q_A^-/S_2 recombination) *in vivo* will be dependent on the membrane potential $\Delta\psi$ as well as the cofactors' midpoint potential (Nicholls and Ferguson, 2013). This could explain the broadening of the apparent rate of this reaction, likely arises from differences in the culture conditions used, as well as heterogeneity within a culture. This is, however, unlikely to account for the range of values reported in the literature for reasonable $\Delta\psi$.

4.7.6. Measurement limitations

There are a number of factors that limit the use of fluorescence yield as a measure of Q_A^- oxidation. The rate at which measurements can be made is limited by a combination of detector sensitivity and the actinic effect of the detection flashes. Each detection flash generates a small amount of Q_A^- , a necessary side-effect of probing the fluorescence yield. The intensity, duration and frequency of these flashes is adjusted such that no increase in fluorescence can be seen in the presences of DCMU. In order to increase the detection rate while not significantly affecting the amount of Q_A^- , the detection flash intensity and duration

must be decreased, decreasing with it the signal to noise ratio. This limits the resolution of the data, which is particularly problematic when measuring fast decays at short times. This resolution limitation affects the interpretation of the parameters obtained from the functions fit to the data. Including an additional exponential component to the decays does not consistently or significantly increase the adjusted R-squared value for the fits (typically >0.98) which suggests that they are sufficient to describe the data. This could also arise from the scarcity of data, particularly when modelling multiple overlapping components; the degrees of freedom in the data may just be too low to confidently assign additional components.

Fluorescence measurements could not be made reliably at short times using a saturating laser flash as the actinic source due to artefacts arising from residual light in the system. The actinic flash used in this work was chosen to give a sufficiently large signal while allowing measurements to be made soon after the end of the flash without sample-independent signal. This limits the time range over which measurements can be made, depending on the intensity of the flash, such that a significant proportion of fast kinetics are lost in the presence of ferricyanide.

Fluorescence measurements at short times can also be affected by quenching species generated by the actinic flash. In particular, direct quenching by long-lived P_{680}^{+} (Bruce et al., 1997) and absorbance by triplet chlorophyll generated in the antennae by excess excitation would reduce the measured fluorescence. The absence of a rise in fluorescence at short times in the presence of DCMU (see Figure 4-3 and Figure 4-5), which would be the expected effect of the decay of a quencher while Q_A^{-} is stable, indicates that these measurements are not significantly affected by these phenomena. The non-saturating nature of the actinic flash used in this work and the small antennae size in this sample contributes to a low triplet chlorophyll yield. This triplet is rapidly quenched by carotenoid with a half time of 17 ns in cyanobacterial PSII (Schlödter and Brettel, 1988).

4.8. Conclusions

Specific electron transfer rates for Q_A^{-} reoxidation are reported here for the reduction of Q_B (400 μ s), Q_B^{-} (1 ms), and Fe^{3+} (100 μ s) for PSII cores. Additional estimates are made for the rates of Q_B exchange (2.7 ms) and $Q_B^{-}S_2$ recombination (11 s). These rates are similar to those reported for mesophilic plant species and supersede the existing values reported for this

species. The results show that the recent study done with two flashes separated by 210 μs would have generated very little of the S_3 state. The changes observed and attributed to the S_3 state must be attributed to other phenomena. The main result of the second flash would be to generate charge separation and recombination in a minority of the centres and prompt fluorescence in the rest. Changes in the size of the crystals' unit-cell size which were interpreted as demonstrating S-state turnover could also have alternative explanations. Changes in the volume of PSII measured using photoacoustics have been attributed to a number of charge transfer processes, with particular artefacts associated with high-energy laser excitation (Hou and Mauzerall, 2011; Hou and Sakmar, 2010). Photothermal beam deflection measurements show an apparent volume increase associated with the S_3 to S_0 transition of about 42 $\text{\AA}^3$ (Klauss et al., 2009; Krivanek et al., 2008), which conflicts with the larger unit-cell size reported for crystals in S_3 than in S_0 (Kupitz, Basu, et al., 2014). This could be tested by XFEL studies on the same samples but having blocked electron transfer by DCMU.

Here we also show that when the iron is oxidised Q_A^- is reduced much more rapidly (100 μs). Pre-oxidation of the non-heme iron provides a potential approach for reducing the inter-flash delay required for generating S_3 using two saturating laser flashes. Given the limitations in the XFEL experiments, biochemical conditions that would allow the microcrystals to be pre-treated with ferricyanide without significantly affecting diffraction resolution should be tested.

Chapter 5: Investigating the role of hydrogen bonding in Y_D oxidation

The functional role of the widely-conserved Y_D , as well as the structural features which modify its chemistry relative to the structurally symmetrical, redox-active Y_Z involved in forward electron transfer, are not well understood. The work presented in this chapter tests a hypothesis regarding the effect of a unique, local hydrogen bonding network on Y_D redox chemistry, contributing to our understanding of the ‘inactive’ electron transfer pathways with the potential to inform the design of modified or artificial systems.

5.1. Redox-active tyrosines in PSII

The PSII core protein subunits D1 and D2 each contain a symmetrical tyrosine, Y_Z and Y_D (D1-Tyr161 and D2-Tyr160 respectively), which can both be oxidised by the photo-generated $P_{680}^{+\bullet}$ cation radical. The proton-coupled oxidation and reduction of Y_Z is part of the forward electron transfer chain, linking the catalytic Mn_4CaO_5 cluster to the central chlorophylls. The role of Y_D , reviewed by Rutherford et al. (2004), has been related to the ability of its cation to oxidise the S_0 and lower oxidation states of the manganese cluster (Messinger and Renger, 1993; Styring and Rutherford, 1987) and the effect this has on photoassembly and stability of the manganese cluster in the dark. The electrostatic effect of its oxidation has also been proposed to affect the reduction potential of the $P_{680}^{+\bullet}$ cation (Ananyev et al., 2002; Malwina Szczepaniak et al., 2008) and promoting the localisation of the on P_{D1} (Faller et al., 2001), which is supported by computational studies (Saito, Ishida, et al., 2011). Phototrophic growth in mutants lacking Y_D from *Synechocystis* (Vermass et al., 1988) and *T. elongatus* (Sugiura et al., 2004), however, show that these roles are not essential under laboratory growth conditions.

The oxidation of Y_Z by $P_{680}^{+\bullet}$ is five orders of magnitude faster than that of Y_D (Rutherford et al., 2004). This has been related to differences in their hydrogen bonding structure: where Y_Z is deprotonated via a short-hydrogen-bonded histidine D1-His190 (Mamedov et al., 1998; Saito, Shen, et al., 2011), models suggest that Y_D is deprotonated by a nearby water molecule rather than the structurally symmetrical histidine D2-His189. The water then pivots about a

bound phenylalanine (D2-Phe169) into distal position, releasing the proton via a series of waters into the bulk (S. Nakamura and Noguchi, 2015; Saito et al., 2013b).

As PSII is likely to have evolved from a homodimeric ancestor, it is thought that Y_D represents a modified, slowed-down version of a formerly active, Y_Z -like residue (Williamson et al., 2011). Whereas Y_Z donates a short hydrogen bond to D1-His190, the equivalent bonding is reversed between Y_D and D2-His189 (K. A. Campbell et al., 1997). This is related to the structural replacement of D1-Asn298 with D2-Arg294 as the hydrogen bonding partner to D1-His190 and D2-His189 respectively, shown in Figure 5-1 (Saito et al., 2013b). Thus a model has been proposed for the involvement of a hydrogen bonding network from Y_D via D2-His189, D2-Arg294 and CP47-Glu364 in orienting the hydrogen bond between Y_D and D2-His189 (Ferreira et al., 2004). Results presented in this chapter test the hypothesis that the orientation of the hydrogen bond between Y_D and D2-His189 is determined by a hydrogen bonding network extending to CP47-Glu364.

A pH dependence of the Y_D oxidation yield after illumination at low-temperature has been measured using EPR (Babcock and Sauer, 1975) in active (Havelius and Styring, 2007) and Mn-depleted (Faller et al., 2002) PSII from *Synechocystis*, with pK_a at pH 8 and 7.6 respectively. The rate of Y_D oxidation in these samples has also been shown to be pH dependent (Vass and Styring, 1991), increasing to rates comparable to Y_Z with a pK_a of 7.7 (Faller et al., 2001). FTIR studies have shown a difference above pH 7.5 in the hydrogen bonding pattern between Y_D and a nearby D2-His189, while both residues remain protonated up to pH 10 (Hienerwadel et al., 2008). These data have been interpreted as a switch from Y_D as an H-bond acceptor to a strong H-bond donor, based on DFT (Takahashi 2007) and QM/MM calculations (Saito et al., 2013b). This switch at higher pH makes the environment of Y_D more like that of Y_Z which donates a short H-bond to D1-His190 (Umena et al., 2011). Differences in the kinetic isotope effect above and below pH 7 (Jenson et al., 2007) as well as evidence for multiple proton pathways near the pK_a (Jenson and Barry, 2009) also suggest different deprotonation pathways.

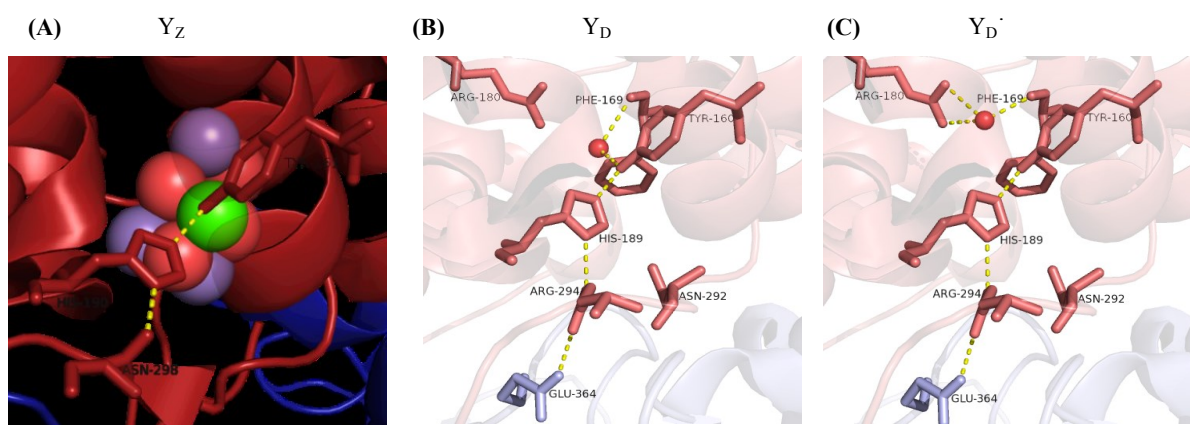


Figure 5-1: Diagrams showing residues surrounding Y_Z and Y_D from D1 (dark red), D2 (rose) and CP47 (blue). Yellow dotted lines represent hydrogen bonding at low pH. Cartoon (A) shows the environment of Y_Z including the Mn_4CaO_5 cluster (large spheres). The two cartoons (B) and (C) show the proximal and distal sites occupied by water (small spheres) in the presence of protonated and deprotonated Y_D respectively. These models were made using the 1.9Å crystal structure (Umena et al., 2011).

Mutants containing substitutions designed to disrupt the H-bonding network surrounding Y_D have been made in order to test their effect on these pH effects. These strains of *T. elongatus*, which were generated by Sabah El Alaoui and Diana Kirilovsky at Saclay in France, contain a point mutation at CP47-Glu364 which is linked to D2-His189 via D2-Arg294 (Umena et al., 2011). In these mutants, the negatively charged glutamate residue is replaced with an uncharged glutamine or a hydrophobic leucine. The yield of photo-induced tyrosine radical formation at 15 K was measured using its EPR signal (Blankenship et al., 1975) in purified active PSII dimers as a function of pH in order to assess the functional and structural impact of these mutations on the environment of Y_D .

5.2. EPR-detected pH-dependence of low-temperature Y_D oxidation

The X-band (9.4 GHz) EPR tyrosine signal was measured in samples of Mn-depleted 47H PSII dimers (pool 3) poised at a range of pH values after a series of illuminations and relaxation. PSII was isolated from 47H, CP47-Glu364Gln and CP47-Glu364Leu as described in section 2.5.4 yielding similar pools of active and less-active monomers and dimers. The oxygen evolution activity from these samples was not significantly different from the wild-type. NH_2OH and EDTA were used to remove the Mn_4CaO_5 cluster and reduce any $Y_D^\cdot$ that was oxidised during the purification (Sugiura and Inoue, 1999). Samples were first measured in the dark to obtain a baseline signal corresponding to the amount of $Y_D^\cdot$ formed during preparation and loading ($Y_D^\cdot$ [dark]). Continuous scans were then made during illumination of

the sample in the cavity with a 1 mW green laser until the resulting signal was saturated, typically 2-3 mins. The sample was then removed from the cavity, thawed and dark-adapted at room temperature for 30 s in order to allow short-lived chlorophyll and carotenoid radicals ($g=2.0025$) to relax before re-freezing and measurement of the low-temperature-illuminated signal ($Y_D \cdot$ [15 K hv]). The sample was then removed from the cavity and thawed, illuminated with the same light source for 60 s at room temperature and dark-adapted for a further 30 s before re-freezing and measurement of the room-temperature-illuminated $Y_D \cdot$ signal ($Y_D \cdot$ [294 K hv]).

Figure 5-2 shows the EPR spectra of the mutant samples in the dark, after illumination at 15 K and after illumination at room temperature at pH 8.6, showing the relative size of the signals generated by each treatment. The size of each $Y_D \cdot$ signal was quantified as the area between the curve and a binomial quadratic baseline between 3320 and 3380 G. This was done in order to minimise the influence of narrower residual radical signals on the quantification. The proportion of Y_D oxidised by illumination at 15 K in each sample was calculated using the following equation:

$$\text{yield} = \frac{Y_D [15 \text{ K hv}] - Y_D [\text{dark}]}{Y_D [294 \text{ K hv}] - Y_D [\text{dark}]} \quad \text{Equation 9}$$

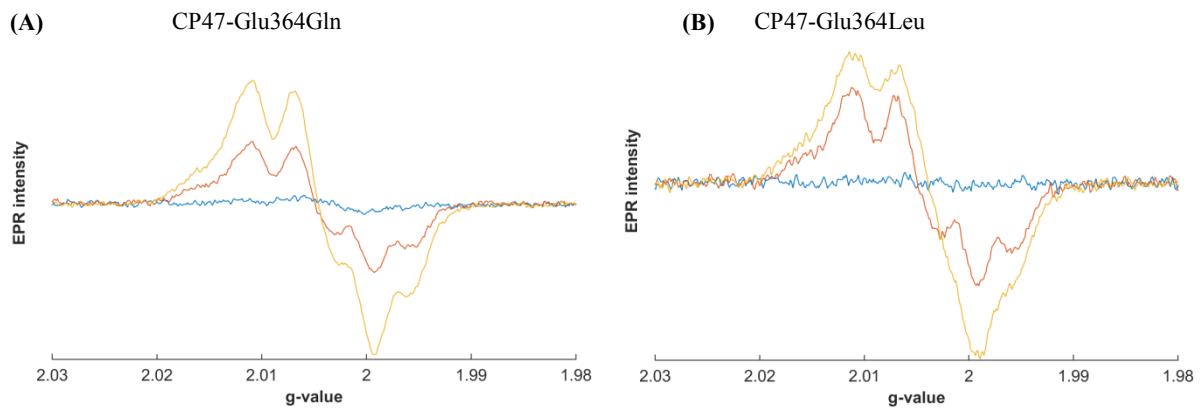


Figure 5-2: $Y_D \cdot$ signals from Mn-depleted PSII dimers purified from CP47-Glu364Gln and CP47-Glu364Leu at pH 8.6 in the dark (blue), after illumination at 15 K and relaxation at room temperature (orange) and after illumination and relaxation at room temperature (yellow).

The dark signal in the mutant samples was consistently low, $2.5 \pm 1.7\%$ of the full signal for all samples and $4.0 \pm 1.2\%$ of the full signal in the samples at pH 8.6 or 9.5. This shows that

the NH_2OH treatment was able to reduce the majority of $\text{Y}_\text{D}^\cdot$ and that illumination on the samples prior to measurements was negligible. Dark 47H samples contained a significant amount of $\text{Y}_\text{D}^\cdot$ even at low pH indicating that they had been exposed to light at room temperature during sample preparation and so only the shape of the $\text{Y}_\text{D}^\cdot$ $_{[294\text{ K hv}]}$ signal could be used.

Illumination at 15 K produced a mixed signal containing contributions from $\text{Y}_\text{D}^\cdot$ as well as narrow un-interacted chlorophyll and carotenoid radicals ($g=2.0026$ and $g=2.0024$ respectively (Hanley et al., 1999)) (not shown). These radicals are known to be formed in centres where Y_D cannot be oxidised. Subsequent relaxation at room temperature reduced the chlorophyll and carotenoid radical signals, leaving only the typical EPR signal from $\text{Y}_\text{D}^\cdot$ (characterised by its g -value ($g=2.0048$) and partially-resolved proton hyperfine splitting (Babcock et al., 1989; Blankenship et al., 1975)). $\text{Y}_\text{D}^\cdot$ is known to be relatively stable in mesophilic organisms and is therefore not expected to be significantly reduced by the relatively short incubation at room temperature (Babcock et al., 1989; Vass and Styring, 1991). The illumination at room temperature followed by the short dark relaxation generated a large signal corresponding to 100% $\text{Y}_\text{D}^\cdot$.

Figure 5-3 shows the effect of pH on the $\text{Y}_\text{D}^\cdot$ of low-temperature illumination. The proportion of Y_D oxidised by this treatment increased with pH to around 44% in CP47-Glu364Gln and 66% in CP47-Glu364Leu). The $\text{p}K_a$ of the signal was derived from a single proton titration fit of the data using the following equation:

$$\text{low - temperature yield} = \frac{a}{1 + 10^{-(\text{pH} + \text{p}K_a)}} \quad \text{Equation 10}$$

where the low-temperature yield is calculated using Equation 9 at each pH value and a is a constant that arises from the fact that only a proportion of the tyrosines are oxidised at high pH (Figure 5-3). This analysis gave a $\text{p}K_a$ at pH 8 for both samples.

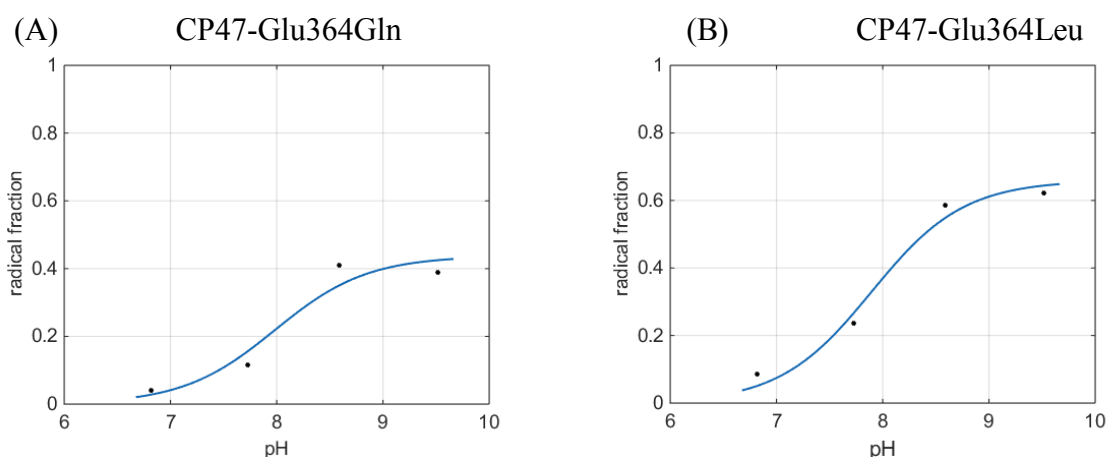


Figure 5-3: pH-dependence of the tyrosine oxidation at 15 K in Mn-depleted PSII dimers from *T. elongatus* strains (A) CP47-Glu364Gln and (B) CP47-Glu364Leu. Each point shows the relative amount of $Y_D^{\cdot}$ signal formed at low temperature.

Figure 5-4 shows the g-values of the $Y_D^{\cdot}$ $_{[15\text{ K hv}]}$ and $Y_D^{\cdot}$ $_{[294\text{ K hv}]}$ signals from mutant and wild-type samples, determined by fitting the data between $g=2.003$ and $g=2.006$ to a binomial quadratic and calculating the point at which the curve crosses the baseline. There is no difference in the $Y_D^{\cdot}$ $_{[15\text{ K hv}]}$ signal at high pH, with variation at low pH arising from the low signal:noise ratio. This estimate of the g-value of the radical signal formed at room temperature in the CP47-Glu364Leu mutant is lower than in the 47H and CP47-Glu364Gln mutants.

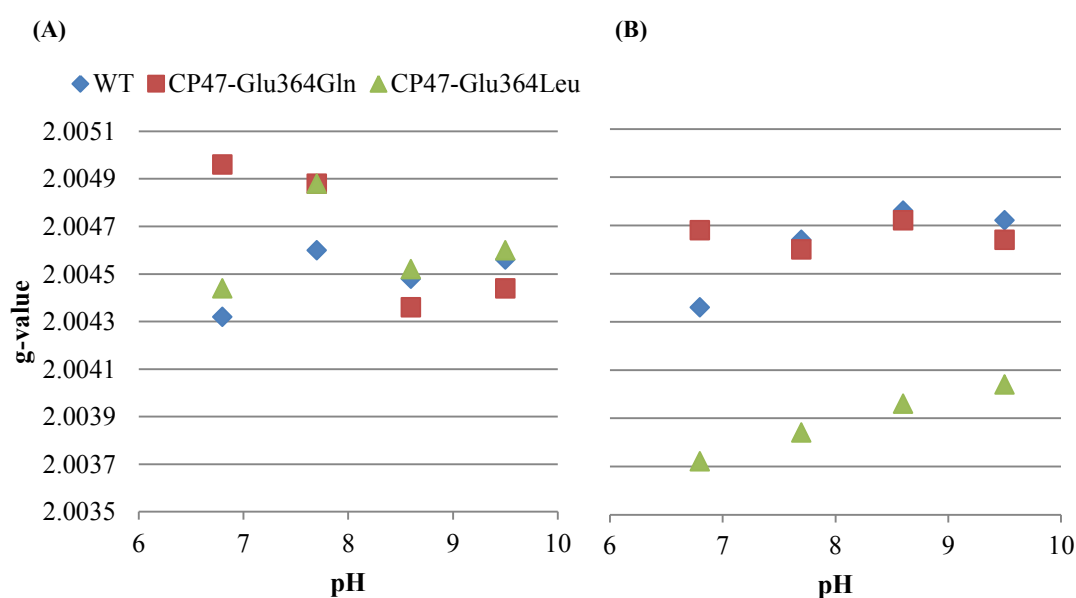


Figure 5-4: Graph showing the g-values of the $Y_D^{\cdot}$ $_{[15\text{ K hv}]}$ and $Y_D^{\cdot}$ $_{[294\text{ K hv}]}$ signals in Mn-depleted PSII dimers from 47H (blue diamonds), CP47-Glu364Gln (red squares) and CP47-Glu364Leu (green triangles) strains of *T. elongatus*.

5.3. Discussion

This work confirms the conclusions of Faller et al. (2002) regarding the effect of pH on Y_D oxidation by P_{680}^+ at cryogenic temperatures in mesophilic species (*Synechocystis* and spinach). Here it is shown in the thermophilic cyanobacterium *T. elongatus* with significant yield of low-temperature Y_D oxidation occurring at pH >7.8). This pK_a is thought to involve a deprotonation reaction resulting in the re-orientation of the hydrogen bond between D2-His189 and Y_D to resemble the short, single-well hydrogen bond between D1-His190 and Y_Z in active centres (Faller et al., 2002).

Preliminary data collected from wild-type *T. elongatus* samples showed a pK_a at around pH 8, not significantly different from wild-type *Synechocystis*, although these data are less reliable and need to be repeated. Replacement of the negatively-charged CP47-Glu364 with Gln or Leu is expected to affect the hydrogen bonding network, changing its strength and distribution. Surprisingly, the CP47-Glu364Gln and -Glu364Leu mutations did not significantly modify the pK_a of low-temperature Y_D oxidation compared to wild-type *Synechocystis*. This seems to suggest that the hydrogen bonding network involving CP47-Glu364 is not directly involved in the deprotonation reaction that triggers low-temperature Y_D oxidation.

The yield of low-temperature-illuminated $Y_D^{\cdot-}$ was found to be less than 100%, even at high pH. This effect was also seen earlier by Faller et al. (2002) and was attributed to heterogeneity in the sample; the distribution of H-bond strengths at high pH being wide enough to make a fraction of them unable to undergo the low-temperature proton movement. It seemed clear that the difference between the rates of $Y_D^{\cdot-}$ and $Car^{+ \cdot}/Chl_Z^{+ \cdot}$ accumulation during low temperature illumination was too great for the observed difference in yield to result from competing reactions; when Y_D is able to donate it does so with high quantum yield, otherwise the carotenoid pathway can donate in competition with the back-reaction between P_{680}^+ and Q_A^- . These phenomena were also seen in this study. Whereas Faller et al. (2002) used crude PSII, this study used isolated PSII dimers, significantly reducing the heterogeneity of the sample in terms of oligomeric state and subunit composition of the complexes. While modification of a fraction of the complexes during sample preparation cannot be excluded, these results indicate that the heterogeneity arises from a distribution of states in active, complete complexes. The higher yield of low-temperature $Y_D^{\cdot-}$ formation at

high pH in CP47-Glu364Leu than CP47-Glu364Gln can be interpreted as the formation of a more homogenous, short, single-well hydrogen bond (Saito et al., 2013b).

The CP47-Glu364Leu mutation also resulted in a change in the g -value of the $Y_D^{\cdot}$ $_{[294\text{ K hv}]}$ signal but not the $Y_D^{\cdot}$ $_{[15\text{ K hv}]}$ signal. The change in the signal could be due to (1) the presence of an additional overlapping radical signal, or (2) modification of the hyperfine structure of the tyrosine radical signal.

Modification of the protein environment of Y_D has been shown to affect the g -value and line-width of its radical signal (Tang et al., 1993; Tommos et al., 1993), with a lower g -value induced by a more positive environment (Mezzetti et al., 1999; Stone, 1963; Un et al., 1995). An unusually low g_x -value has been reported for the Y_D radical signal formed at low-temperature when measured without allowing the sample to warm. This reflects the more positive environment of the tyrosine in its deprotonated state where the charge is trapped close to the unpaired spin. These changes were resolved using higher-resolution high-field EPR studies (Faller et al., 2003). According to (1), the absence of a difference after illumination at 15 K indicates that this difference arises only from centres in a low-pH-type state. This effect would therefore be expected to be less in samples at higher pH, corresponding to the higher $Y_D^{\cdot}$ yield at 15 K. While the difference does decrease with increasing pH, it is not proportional to the increase in the low-temperature yield.

The presence of an overlapping un-interacted radical signal with a lower g -value could explain the difference between the signals induced at high temperature. P_{680}^{+} can be reduced by a number of side-path donors proceeding via carotenoid including Chl_Z and Cyt *b*₅₅₉ (Faller et al., 2005). These radicals are unstable at higher temperature and decay at least in part by recombining with reduced quinones (Hanley et al., 1999). Incubation at room temperature was sufficient to remove this component of the signal in wild-type samples, as reported previously (Faller et al., 2002). It is possible that the CP47-Glu364Leu mutation increases the lifetime or yield of these radicals, resulting in a larger residual signal. The blockage of electron transfer from $Q_A^{\cdot}$ to Q_B at low-temperature limits the yield of oxidising equivalents to one per reaction centre, minimising this effect on samples illuminated at 15 K. The presence of an additional overlapping absorbance would also affect the quantification of the signal, resulting in an underestimation of the low-temperature yield of $Y_D^{\cdot}$.

5.4. Conclusions

Y_D undergoes a change in its oxidation rate by more than 5 orders of magnitude as the pH is increased (Rutherford et al., 2004). This represents a deprotonation event with a pK_a near pH 8. It was suggested that this could be due to a pK_a on a group involved in the hydrogen bonding chain around Y_D (i.e. D2-Glu364, D2-Arg294, D2-His189, Y_D , H_2O) (Saito et al., 2013b). The association between D2-Glu364 and D2-Arg294 may also involve an electrostatic interaction, or even an ion pair (Arg^+Gln^-). Replacement of D2-Glu364 with neutral (Gln) and hydrophobic (Leu) amino acids was expected to perturb the electrostatic environment in the hydrogen bonding chain, resulting in a change in the value of the pK_a . Here it was shown that this did not occur. Thus result then casts doubt on the idea that the protonation event occurs among the components of this hydrogen bonding chain. The modified signal seen in the CP47-Glu364Leu mutant PSII is consistent with a long-lived radical, suggesting an alteration in radical lifetime or yield at room temperature. It also showed increased yield of $Y_D^\cdot$ at high pH at 15 K, indicating a more well-ordered hydrogen bond between Y_D and D2-His189.

Saito et al. (2013b) also suggested an alternative model in which the deprotonation event led to a conformational change around the hydrogen bonding chain that resulting in the D2-Asn298, which is only a short distance (3.4 Å) from D2-His189, to approach the site and displace the D2-Arg294, thereby reversing the hydrogen direction, changing it from the usual Y_D orientation to one that is typical of Y_Z . This alternative model is consistent with the present result; however it does not provide much further details concerning the origin of the group undergoing deprotonation.

Outcomes and future work

6.1. Crystallographic studies of PsbA3-PSII

Mutant strains of *T. elongatus* were generated in which one of the two genes coding for D2, either *psbD1* or *psbD2*, is replaced with a kanamycin resistance cassette, enabling future site-directed mutagenesis studies in the remaining gene. A new mutant strain lacking two of the three D1 genes, *psbA1* and *psbA2*, was also generated which expresses *psbA3* as the sole D1 isoform. Growth assays did not show a significant effect of this mutation under light-limited conditions, in contrast to expectations from the yields and energetics measured on isolated PSII *in vitro* (Sugiura and Boussac, 2014a). Anion-exchange chromatography showed an altered distribution of complexes and oligomeric state in PSII samples purified using His₆-tag affinity chromatography, with a higher yield of the Psb27-containing monomer pool. The first X-ray crystallographic structure of PSII containing PsbA3 as the D1 isoform is presented, obtained from active PSII dimers isolated from this strain. This demonstrates its similarity to existing structures for PsbA1-PSII. Zinc-binding experiments showed that while exchange with the acceptor-side non-heme iron is possible it may not be relevant *in vivo*. They demonstrate conditions under which it is possible to distinguish between donor-side and acceptor-side effects of zinc, a useful experimental tool in studying the role of cations in the complex. The first crystallographic evidence for the binding mode of a urea-type DCMU analogue inhibitor to Q_B is also presented, supporting existing models of binding to the Q_B site in competition with the native quinone electron acceptor (R. Takahashi et al., 2010). In order to develop the inhibitor-binding work, computational modelling of the molecular dynamics of the Q_B site is being carried out in collaboration with Ilyas Yildirim at the University of Cambridge, Department of Chemistry.

In order to improve the resolution of structural information obtained from these samples, further optimisation is required. Dehydration experiments were not able to improve diffraction quality in line with reports in the literature. It is possible that purification procedures used are not conducive to producing highly-diffracting crystals. While the His₆-tag and anion exchange chromatography methods used here yield complete PSII complexes and appear to separate additional PSII complex fractions, hence improving the purity of the final sample, other key factors such as the detergent or lipid composition could be adversely affected. It is also possible that the fractions separated by the anion-exchange

chromatography used here, particularly pool 4 (less-active dimers), are in part artefacts of the purification procedure and are not present in crude PSII or *in vivo*. Subsequent work is needed to identify the effects of these methods on the state of the complex in comparison to others reported in the literature (Umena et al., 2011). Now with access to an ÄKTA FPLC system, improvements may also be achieved by implementing the methods reported by Shen et al. (2011).

6.2. Kinetic studies of Q_A^- oxidation

The rates of Q_A^- oxidation reactions in purified PSII dimers from *T. elongatus* have been characterised using fluorescence decay kinetics, separating the half-times of Q_A^- oxidation by Fe^{3+} (100 μ s), by Q_B (400 μ s) and by Q_B^- (1 ms) and by an exchanging quinone (2.7 ms). These results demonstrate the importance of sample treatment and proper dark-adaptation in determining the functional state of the acceptor-side of the complex. While these results conflict with existing values in the literature for similar samples (Kargul et al., 2007; K. Zimmermann et al., 2006) they are in agreement with more detailed studies using plant samples (de Wijn and van Gorkom, 2001). It has been reported that dark-adaptation generates Fe^{3+} in a minority of centres. Complementary methods for quantifying the $Fe^{2+}:Fe^{3+}$ ratio such as using the EPR signal associated with Fe^{3+} (Nugent and Evans, 1980; J. Zimmermann and Rutherford, 1986) would be valuable to assess the impact on the kinetics.

The rates of Q_A^- oxidation reactions have been reported to be much slower in purified PSII monomers than in dimers (Mamedov et al., 2007; K. Zimmermann et al., 2006). A more detailed study such as presented here using monomers could be valuable in determining the functional relevance and energetic implications of dimerization.

Given these results it is concluded that the 210 μ s flash spacing used by Kupitz et al. (2014) is too short to yield a significant fraction of centres in the S_3 state of the Mn_4CaO_5 cluster. It is shown that it is possible to increase the photochemical yield to 60% of a second flash fired 210 μ s after the first flash when the non-heme Fe^{2+} is oxidised as an alternative electron acceptor. Fe^{2+} oxidation is found to be incomplete in the samples measured, indicating that further improvements in yield could be achieved. The dissociation of bicarbonate is suggested to inhibit Fe^{2+} oxidation and thus supplementing the buffer could be useful. This needs further investigation. An alternative approach could involve the addition of glycolate, which is known to lower the E_m of Fe^{2+}/Fe^{3+} (Petrouleas et al., 1994).

Fluorescence decay kinetics measured in cell samples are consistent with values measured in isolated PSII. They also demonstrate the limitation of quinone exchange on PSII activity *in vivo* during the exponential growth phase.

6.3. Functional studies of Y_D

The low-temperature oxidation of Y_D in inactive PSII dimers from *T. elongatus* was shown to have a pK_a around pH 8, similar to values reported for *Synechocystis* (Faller et al., 2002). Measurements on 47H samples need to be repeated in order to confirm their similarity to *Synechocystis*. Differences in low-temperature yields between the mutants indicate modifications to the high-pH conformation affecting the yield of Y_D oxidation. High-field studies could be carried out in order to differentiate these signals and analyse at higher resolution the effects on the hyperfine structure of the signal arising from Y_D (Un et al., 1995, 1996). The orientation of the tyrosines in the protein could also be measured using ENDOR measurements in oriented samples such as crystals or 1-dimensionally ordered membranes (Dorlet et al., 2000; Teutloff et al., 2009). Ideally, the crystal structure of PSII could be determined at high pH, although new crystallisation conditions would have to be found. High pH results in the loss of water splitting activity so crystallisation trials under these conditions have received little attention up to now. Alternatively, the replacement of other members of the hydrogen bonding system that are more closely associated with Y_D could be carried out. Of particular interest is the replacement of D2-Arg294 with Asn by site-directed mutagenesis, thereby potentially generating a Y_Z -like hydrogen bonding situation. Mutants of this kind have been generated in the group in *Synechocystis* and should be characterised in the near future.

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