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KINETIC AND ENERGETIC CONSTRAINTS ON ELECTRON TRANSFER IN PHOTOSYSTEM II

Ву

COLIN GATES

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ABSTRACT OF THE DISSERTATION Kinetic and Energetic Constraints on Electron Transfer in Photosystem II By COLIN GATES

Dissertation Director: Professor G. Charles Dismukes

The enzyme supercomplex Photosystem II (PSII) is the sole water oxidase known to have developed in nature. Accordingly, it is the source of almost all biologically useful reductant present in the biosphere, as well as the molecular oxygen in Earth's atmosphere. Understanding this massive, complex protein is thus crucial to development of novel bioinspired wateroxidizing catalysts, a crucial step toward reducing dependence on fossil fuels, which are also ultimately products of PSII activity. Improving understanding and control of the operation and regulation of PSII is also critical for development of agriculture, biofuels, and bioproducts.

As the active domains of PSII are highly conserved, substitution of functional and tuning components of the complex and investigation of alterations to functionality represents a wellproven method for study of this complex. However, the development of a range of novel techniques for phenomenological investigation of PSII has allowed for the observation of previously unknown processes and functionalities associated with certain components of PSII. The use of exogenous quinones to remove the kinetic constraint of electron removal from the acceptor side of PSII allows a more accurate determination of the first electron transfer steps within PSII, removing a major confounding variable from kinetic studies of processes to be probed under conditions previously inaccessible due to the need to generate specific and often

ii

unnatural sample conditions. With these new developments, cofactor substitutions are employed to clarify the functionality of the native cofactors.

In Chapter 2, a novel fast repetition rate fluorometry (FRRF) technique is used to measure the transit times through the catalytic cycle of water oxidation/oxygen evolution in living cells of oxygenic phototrophs for the first time. Chlorophyll FRRF monitors the competition between excited state photon emission and photochemical reactions (charge separation). The kinetics of e⁻/H⁺ fluxes upstream and downstream following the primary photochemical charge separation step from the reaction center chl (P680*) are shown to directly influence the yield of light emission. This method can be applied to all tissue types without need for biochemical isolation (*in vivo* or *in vitro*), across a broad range of temperatures, requires no invasive substances or probes, and is both low-cost and rapid. The technique is illustrated using living cells of the heterokont alga *Nannochloropsis oceanica* CCMP1779. The FRRF kinetics of successive e⁻/H⁺ steps are compared to other spectroscopic methods (optical absorbance, EPR, and XES) previously applied only to *in vitro* fractionated subcellular preparations, and temperature and acceptor redox poise are shown to be major regulators of the WOC cycle transition times.

In Chapter 3, biosynthetic study of replacement of calcium for strontium in the WOC of PSII is extended from subcellular preparations to living cells. *Thermosynechococcus elongatus* was previously demonstrated to evolve oxygen 5-8 times slower when strontium replaces calcium in the water-oxidizing complex (WOC), resulting in lower levels of total oxygen evolution. This decrease is shown to be due to prior measurement conditions and a higher Arrhenius-type energy barrier for oxygen evolution. Acceptor-side electron removal is shown to constrain PSII operation to a greater extent in Sr-substituted culture. Use of exogenous benzoquinone derivatives to remove acceptor-side blockage produces a 31% *higher* quantum

iii

oxygen yield with Sr-substitution. The quantum yield and efficiency of the catalytic cycle are improved due to decreased return of electrons to the WOC and extended lifetime of the unstable S3 state. This is posited to be due to increased thermodynamic stabilization of the Srsubstituted WOC relative to its immediate electron acceptor tyrosine-Z. At the organism scale, higher efficiency of WOC operation under native conditions (low light, 45°C) prompts production of a lower concentration of PSII per chlorophyll and per unit biomass, resulting in nearly equivalent energy transduction in a given cell. Redox and protonic energy fluxes generated by PSII are thus shown to be fundamentally linked to growth rate. Furthermore, the Sr-favored intermediate-spin S=5/2 form of the S2 state is the active configuration, rather than the lowspin S=1/2 form.

In Chapter 4, the effects of whole-cell atomic substitution of chloride for bromide are investigated in *T. elongatus*, in which the location of the two chloride ions near the WOC is known. Bromide is a larger ion than chloride, as well as a weaker and softer base, and thus shows lower affinity for protons, travels more slowly through PSII channels, and physically constrains transit space in the channel due to its size. We find that bromide substitution results in decreased oxygen evolution, lower efficiency of the WOC at high light intensity, and slowed proton release steps in WOC cycling. The S2-S3 transition, which involves the release of a proton, is shown to be a slower step which is less frequently successful and less easily reversed if so, while the S3-S4-S0 step, involving the release of two protons, is also slowed. Furthermore, we find that proton production in the lumen is slowed due to bromide substitution and Δ pH is decreased. Overall, we present conclusive evidence that substitution of bromide for chloride results in a decreased efficiency of proton removal from the WOC and that one role of chloride near the WOC is to facilitate proton transfer between the WOC pocket and the lumen.

iv

In Chapter 5, the roles of dissolved inorganic carbon (DIC) within PSII of living cells of the alkalinophilic, hypercarbonate-requiring and carbonic anhydrase-deficient cyanobacterium *Arthrospira maxima* are investigated oximetrically and fluorometrically, monitoring the light reactions on the donor and acceptor sides of PSII. New methods for removing DIC based on a (bi)carbonate chelator and magnesium for (bi)carbonate ion-pairing are described. Relative affinities are established for three active sites of DIC activity in PSII: the WOC, non-heme iron/Q_A⁻, and solvent-accessible arginines throughout PSII. Full reversibility is achieved but (bi)carbonate uptake requires light. DIC depletion at the non-heme iron site and solvent-accessible arginines the yield of O₂ due to O₂ uptake, but accelerates the PSII-WOC cycle, specifically the S2 \rightarrow S3 and S3 \rightarrow S0 transitions. DIC removal from the WOC site is shown to abolish water oxidation and appears to influence free energy stabilization of the WOC from a site between CP43-R357 and Ca²⁺.

In Chapter 6, a novel functional study of PSII operation in crystals is carried out, applying oximetric methods to determine the quantum efficiency of WOC turnover and lifetime of intermediates as a function of common electron acceptors, aerobically and anaerobically. Under optimal concentration of electron acceptors (synthetic quinones), the highest quantum efficiency of PSII operation to date is observed (peak 61.6%, theoretically 59,000 µmol O₂/mg Chl/h). Catalytic WOC cycling is shown to be sustained for thousands of turnovers by supplementing with irreversible electron acceptor (ferricyanide). Both acceptors operate through equilibration with the native Q_B (or Q_c) site, for which two distinct redox couples are functionally observable that regulate flux through PSII. The lifetimes of the S2 and S3 states are extended (especially S2) by electron acceptors and depend on their redox properties (reversible or irreversible). The classic WOC cycle is also observed in the highest detail seen to date-oscillations in O₂ yield are sustained over 200 flashes (50 periods), reflecting extreme population

coherence dependent on type, availability, and redox poise of exogenous electron acceptors. Backward transitions, in which light reverses the catalytic cycle of the WOC, are observed to be internal to PSII. In addition to the dominant normal period-4 cycle, the O₂ flash yield oscillates with period-2. Additional cycles with period ~2.15, ~4.7, and ~22 are observed, under specific electron acceptor conditions, which reflect new coherences in the WOC catalytic cycle yet to be deciphered. The WOC cycle inefficiency parameters, the rate of S-state population redistribution (dark lifetimes), and occurrence of these novel oscillations are shown to be dependent on the redox balance between the internal electron acceptors Q_A and Q_B and exogenous acceptors. Dedication

To my wife, Elizabeth, for putting up with many late nights writing.

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1. C. Gates, G. Ananyev, G.C. Dismukes, The strontium inorganic mutant of the water oxidizing center (CaMn4O5) of PSII improves WOC efficiency but slows electron flux through the terminal acceptors, Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1857 (2016) 1550-1560.

2. G. Ananyev, C. Gates, G.C. Dismukes, The Multiplicity of Roles for (Bi)carbonate in Photosystem II Operation in the Hypercarbonate-requiring Cyanobacterium Arthrospira maxima, Photosynthetica, 58 (2018) 1-12.

3. Gates, Colin; Ananyev, Gennady; Dismukes, G. Charles, "A non-invasive fluorescence method for investigating S state transition times," *in prep*.

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Table of Contents

Thesis

Abstract	ii
Dedication	vii
Acknowledgement	viii
Table of Contents	xi
List of Tables	xv
List of Figures	xvi

Chapter 1

L. Introduction and Background1
1.1 Global Impact of Photosystem II1
1.2 Redox-Active Components of PSII2
1.2.1 P680, The Reaction Center3
1.2.2 The Water-Oxidizing Complex4
1.2.3 The Quinone Electron Acceptors7
1.2.4 Other Redox-Active Components9
1.3 Toward a Deeper Understanding of Redox Control in PSII10
1.3.1 Fast Repetition Rate Fluorometry10

	1.3.2 PSII-Cyclic Electron Flow	11
	1.3.3 Atomic Substitution in PSII	12
	1.3.4 Investigation of PSII Microcrystals	13
	1.4 References	14
C	Chapter 2	
2	2. Regulation of the S state transition times in the WOC of PSII	21
	2.1 Introduction	21
	2.2 Materials and Methods	24
	2.3 Results	26
	2.4 Discussion	37
	2.5 Conclusion	40
	2.6 Acknowledgements	40
	2.7 References	41
C	Chapter 3	
3	3. Strontium tunes the redox potential of the WOC of PSII through the S state cycle	45
	3.1 Introduction	45
	3.2 Materials and Methods	49
	3.3 Results	53
	3.4 Discussion	69

3.5 Conclusion80
3.6 Acknowledgements80
3.7 References
Chapter 4
4. The Role of Chloride in the WOC Pocket of PSII87
4.1 Introduction87
4.2 Materials and Methods88
4.3 Results92
4.4 Discussion106
4.5 Conclusions112
4.6 Acknowledgements113
4.7 References113
Chapter 5
5. Multiple Sites and Roles of Dissolved Inorganic Carbon in PSII Operation118
5.1 Introduction118
5.2 Materials and Methods123
5.3 Results
5.4 Discussion137
5.5 Conclusion145

5.6 Acknowledgements146
5.7 References146
Chapter 6
6. Acceptor-Side Regulation of the Electron Transfer Processes in PSII Microcrystals150
6.1 Establishment of Acceptor-Side Regulation and Oximetric Analyses in PSII
Microcrystals150
6.1.1 Introduction150
6.1.2 Materials and Methods152
6.1.3 Results154
6.1.4 Discussion166
6.1.5 Conclusion171
6.2 High-Resolution Cyclic Behaviors of PSII Operation in Microcrystals172
6.2.1 Introduction172
6.2.2 Materials and Methods174
6.2.3 Results176
6.2.4 Discussion188
6.2.5 Conclusion192
6.3 Acknowledgements193
6.4 References194

List of Tables

Table 2.1 Comparison of S-state lifetimes obtained via various methods
Table 3.1 S2 and S3 state decay parameters at 23°C from fits of data to a biexponential model.
Values after electron acceptor was added are given in parentheses61
Table 3.2 WOC cycle parameters and dark S-state populations at measurement temperature (A)
23°C and (B) at 45°C. Flash rate 0.5 Hz; dark preincubation time 180 s62
Table 3.3 Comparison of S-state optimal transition times in Ca- and Sr- WOCs in spinach
thylakoid preparates and living <i>T. elongatus</i> culture73
Table 4.1 Expanded Kok parameters and initial S-state populations as derived by fitting of raw
data in Fig. 4.4 using the VZAD model. Of particular note are the beta, delta, and period
parameters, all of which are potentially limited by proton removal from the WOC region98
Table 4.2 Decay constants and amplitudes of fits to the S-state decay curves given in Figure
4.5
Table 4.3. Optimal transition times in living Cl- and Br-cultures as determined fluorometrically,
given in $\mu s. \ As$ the S0-S1 transition is too rapid to be observed conclusively under these
conditions, a standard value of 30 μs is assumed for calculation of subsequent times102
Table 5.1. Comparison of efficiency of extraction by the three reaction mixtures
Table 5.2 Optimal transition times in μ s for S-state transitions shown in Figure 5.7. Note that
the S0-S1 transition time is usually not available because no S0 population is retained under the

conditions studied (no S1 peak). At 5°C, insufficient centers can complete the S3-S4-S0
transition to clearly differentiate an advance kinetic (note no local minimum in graphs)134
Table 5.3 WOC cycle parameters from fits of the flash oxygen yields at 0.5 Hz using the VZAD
model. Recovered samples were treated with 200 mM NaHCO $_3$ after DIC depletion142
Table 6.1 PSII S-state populations and inefficiency parameters obtained from the VZAD fits

shown in Figure 6.6B and C	165
----------------------------	-----

List of Figures

Figure 1.1 Crystal structure of Photosystem II as determined by X-ray diffraction by Umena et
al2
Figure 1.2 Scheme of the classic electron transfer pathways in photosynthesis, including redox
potentials. Scheme devised by Govindjee3
Figure 1.3 (A) The WOC of PSII, showing access channels and first-shell ligating amino acids and
water molecules. (B) Interactions between the WOC and redox-active Yz5
Figure 1.4 Development of the model of S-state transitions from Kok's 12-process model to
Shinkarev's 20-process model to the VZAD 16-process model7
Figure 1.5 Diagram of FRR flashes and resulting fluorescence yield over time, showing
parameters obtained via this technique11
Figure 1.6 (A) Simple representation of linear and cyclic electron flow with equation for energy
generation by each. See text for definitions. (B) Electron flow pathways in and around PSII12
Figure 2.1 Pathway of fluorescence generation in photosystem II: Photochemically generated
P680* can decay by productive charge separation that leads to water oxidation and
plastoquinone reduction, but if electrons cannot advance from the WOC (more time required as
S-state increases due to lower potential gaps) or cannot be transferred to Q_A because it is
already reduced, detectable fluorescence occurs22

Figure 2.2 (A) Demonstration of fluorescence yields from quarter-turnover flashes delivered at the same frequency as used in the "long-pulse" technique. (B) Fv/Fm results from bursts of four

quarter-turnover flashes (each time point is one group of four flashes) delivered at 10 ms intervals (100 Hz flash frequency). All results derived from living *N. oceanica* culture at 22°C...26

Figure 2.8 Flash train difference yields from <i>N. oceanica</i> obtained from sample at temperature	es
from 5 to 45°C. All measurements taken with 1 s between flash trains. Data shown is the	
average of 30 measurements	.35

Figure 3.2 EPR spectra of (A) a concentration curve of Fremy's salt used as a standard organic radical, and (B) pre-harvest chlorophyll-normalized samples of native and Sr-grown *T. elongatus* thylakoids. (C) Double-integrated areas of the Fremy's salt concentration curve. (D) Scanned Western blot of D1 protein extracts from Sr-grown (left) and Ca-grown (second from left) cultures. Extracts were standardized to equivalent chlorophyll content immediately prior to electrophoresis. Quantifications were made using a D1 standard series (rightmost four columns) and were based solely on D1 monomer content, as comparatively D1 was present in other forms

in al	l releva	int sam	ples (t	he Sr and	l Ca bands	s are clear	ly indicative	of conce	ntrations	between	the
two	most d	ilute in	the D2	1 series).	Ladders	added for	clarity				50

Figure 3.7. Relative flash-oxygen yields from Ca- and Sr-grown cultures: (A) in the absence of exogenous electron acceptors, and (B) supplemented with 250 μ M DMBQ. Data are normalized at the steady-state for comparison and are the average of 30 flash trains at flash frequency (0.5 Hz). The fits to the VZAD program are shown in red. Residual values are plotted and represent

Figure 3.10 Temperature dependence of the O₂ evolution rate in Sr- and Ca-grown cultures. All measurements were taken under continuous saturating (120 μ Ein/m²/s) red light in well-stirred culture. Rates of O₂/PSII-D1 are plotted vs. temperature in (A) and as an Arrhenius plot, In rate vs. 1/T, in (B). The data above 50°C are influenced by thermal deactivation and thus not used in

Β.	"Activation energies	" for oxygen ev	volution are calcu	ulated from th	ne Arrhenius p	lot and reflect	
the	e entire WOC cycle. T	The data are th	e average of 3 m	easurements			,

Figure 3.11 Flash train differences from living Ca- and Sr-grown <i>T. elongatus</i> culture as	
measured at (A) 22°C and (B) 47°C. All measurements taken with 3 s between flash trains.	
Fitting lines for individual S-states shown for convenience. Data shown is the average of 30	
measurements. Transition times are recorded in Table 3.3	.67

Figure 4.3 Representative flash oxygen yield of living *T. elongatus* cells measured (A) without and (B) with addition of 250 μ M DMBQ, in Cl- or Br-medium, at 23°C. All data shown is the

Figure 4.6 S-state transition times in living CI- and Br-grown culture as measured by WOCdependent differential fluorometry (see Chapter 2). All measurements were carried out at growth temperature (45°C) using quarter-saturating flashes at a frequency of 100 kHz...........101

Figure 5.2. Effect on Fv/Fm at (A) 100 Hz and (B) 0.5 Hz flash rate of different extraction methods for DIC from *A. maxima* cells *in vivo*. (C) Effect of same treatments on flash oxygen yield (relative units, samples of equal cell density) at 0.5 Hz. Curves shifted to avoid overlap (see

Figure 6.1 Flowchart of preparation and investigation of high-purity PSII microcrystals......151

Figure 6.7 Dark-adapted PSII microcrystals in solution supplemented with (A) 2 mM FeCN, (B) 400 μ M PPBQ, and (C) both PPBQ and FeCN, were subjected to a series of flash trains of 120 saturating xenon flashes at 0.5 Hz under oxygenated headspace. The average oxygen yield from

Figure 6.16 Comparison of acceptor effect on the alpha (miss) parameter train-by-train. In black, train steady-state yields from sequential flash trains. In blue, alpha parameter as obtained from each flash train. Data shown is representative of multiple data sets. (A) Sample supplemented with 4 mM FeCN; (B) Sample supplemented with 200 μM PPBQ and 4 mM FeCN.

Figure 6.19 Comparison of acceptor effect on the initial dark S-state distributions prior to each
train. In black, train steady-state yields from sequential flash trains. In blue, fraction of each S-
state in the dark before each flash train. Data shown is representative of multiple data sets. (A)
Sample supplemented with 4 mM FeCN; (B) Sample supplemented with 200 μ M PPBQ and 4
mM FeCN

Figure 6.20 Scheme of rates and constraints in electron transfer within PSII......192

Chapter 1. Introduction and Background

1.1 Global Impact of Photosystem II

Over two billion years ago, molecular oxygen, previously present but relatively limited in scale, suddenly appeared on Earth in substantial quantities [1]. This event gave the name to a geological epoch (the Siderian, named for the sudden appearance of large quantities of iron oxides resulting from reaction with free dioxygen [2]). The event itself is commonly called the Great Oxygenation Event; Lynn Margulis referred to it as the "Oxygen Holocaust" [3, 4]. A similarly dire term is often used for what followed: "Snowball Earth", or the Huronian glaciation [5]. These geological events all stem from the widespread activity of photosystem II (PSII) in cyanobacteria [6, 7]. Oxygenic photosynthesis was now the dominant means of energy entering the biosphere [8-11].

From a human perspective, the importance of oxygenic photosynthesis has not diminished over time. Our most obvious reliance on this process is in our respiration; we are obligate aerobic organisms. Secondly, the energy in almost all of the food we eat is transduced by photosynthesis, and the energized electrons our mitochondria use to generate ATP were first charge-separated in PSII. Thirdly, almost all fossil fuel is ultimately derived from carbon compounds that were reduced via those same electrons taken from water by PSII. Without PSII, the vast majority of the tree of life as we know it would never have been able to evolve.

Perhaps the most surprising aspect of oxygenic photosynthesis, given its ubiquity in the modern biosphere, is the degree of conservation within Photosystem II [12-15]. The great bulk of the enzyme complex is perfectly conserved across two domains and numerous phyla of the tree of life (Figure 1.1). The active sites of the critical electron-transfer events are not altered in any natural PSII [16-18]. This is the sole water oxidase developed by nature [19]. Accordingly, it

is of enormous interest for the breadth of applications for which PSII may be used. Improving the efficiency of PSII, or tuning its operation to local conditions, may improve crop yields in agricultural, biofuels, and bioproduction applications [20-23]. Additionally, the high stability of the inorganic core of PSII, the water-oxidizing complex (WOC), makes it an ideal subject for study with regards to improving the lifetimes of artificial water-oxidizing catalysts [24-29]. To these ends, I have sought to better understand the individual redox-active species and regulatory mechanisms within PSII.



Figure 1.1 Crystal structure of Photosystem II as determined by X-ray diffraction by Umena et al. [30]

1.2 Redox-Active Components of PSII

The photosynthetic electron transport chain is comprised of several enzyme

supercomplexes, various redox-active carrier molecules, and a wide range of accessory

molecules or structures used either in protecting the primary electron transport chain or in tuning its redox-active components [31]. These have been elucidated in increasing detail over the past several decades, and a scheme of the core processes is given as Figure 1.2.



Figure 1.2 Scheme of the classic electron transfer pathways in photosynthesis, including redox potentials. Scheme devised by Govindjee.

1.2.1 P680, The Reaction Center.

The process of transducing the energy in a photon to biologically useful reductant and/or ATP begins with the capture of the photon by an antenna chlorophyll (chl). Following potential gradients, the generated exciton is directed to a special chlorophyll complex, P680 [32, 33]. This structure is composed of a dimer of chlorophyll dimers, each of which is associated with one of the two innermost subunits of PSII, D1 and D2 (PsaA and PsaD) [30, 34]. The name given refers to its absorption peak, which is red-shifted to 680 nm from the usual wavelength for chl *a* at 665 nm. Charge separation at P680 results in the transfer of the electron to the transient electron acceptor D1-pheophytin (Pheo), and if possible, on to the acceptor plastoquinone Q_A [35]. Successful removal of the electron (which must reach Q_A or face nearimmediate recombination with P680⁺) generates a hole on P680 which is the strongest known biological oxidant, having an oxidizing potential of approximately 1.3 V [17, 36]. This extreme oxidizing potential allows the removal of an electron from a special tyrosine residue nearby, D1-Y161 (commonly labeled tyrosine-Z or Yz) [37]. Tyrosine-Z, in turn, is only a few angstroms away from the primary site of water oxidation, the water-oxidizing complex (WOC; also oxygenevolving complex or OEC) [30, 38]. Reduction of Yz⁺ occurs via electron transfer from the Mn atoms within the WOC, and ultimately from water.

Should an electron not be available from Yz or the WOC, or should the path for electron advance to Q_A be blocked, charge recombination between the separated electron and hole will occur. This can occur via non-photochemical quenching, whereby a series of electron transfers through accessory pigments generates heat, or via fluorescence, whereby the excess energy is released in the form of a photon [17].

1.2.2 The Water-Oxidizing Complex.

The catalytic site of oxidation of water to oxygen, the WOC, is a metal-oxo cluster with native formula Mn₄O₅Ca and tunable oxidizing potential (Figure 1.3). This cluster was determined, over several decades of investigation beginning with the work of Kok and Joliot, to cycle through four increasing oxidation states in order to build up charge to abstract the needed electrons from water [39-43]. These oxidation states are commonly denoted S0 through S4,

with the number representing the number of electrons removed from the cluster relative to the minimal oxidation state. The individual charges of the Mn atoms in the cluster are not known; evidence exists to indicate that the S2 state is composed of non-even numbers of both Mn(III) and Mn(IV), but a long-running subject of debate within the field is whether there are three Mn(III) and one Mn(IV) or one Mn(III) and three Mn(IV) [44-52]. Respectively, these are commonly referred to as the "low" and "high" oxidation paradigms, and evidence exists to indicate that either may be accurate. Resolving this issue represents a major objective in the field of photosynthesis research. Regardless of the oxidation state assignments, as the Mn atoms are oxidized it becomes more difficult to remove further electrons, and transitions take progressively more time from one to the next.



Figure 1.3 (A) The WOC of PSII, showing access channels and first-shell ligating amino acids and water molecules. (B) Interactions between the WOC and redox-active Yz. Originally generated for use in [16].

Among the S-states, the SO and S1 states are fully stable in darkness in their native environment, the S2 and S3 states are moderately stable, typically having lifetimes in tens of seconds to minutes, and the S4 state is a nearly instantaneous transient [17, 53]. Using carefully
measured quantities of light which induce single charge separation events in all active P680 centers, it is possible to observe populations of WOCs within a sample and their transitions through the S-states [54, 55]. Advance through the cycle (Figure 1.4) has been modeled since Kok and Joliot, for nearly fifty years, and follows a series of one-electron oxidation events with a number of distinct side processes commonly termed "inefficiencies." Kok's original model included the alpha or miss parameter, which represents the failure to advance an electron on a given transition, and the beta or double-hit parameter, representing the advance of two electrons in rapid succession [42]. The Kok model of WOC activity, a 12-step reaction matrix which considered only the prior two inefficiencies and successful advance (sometimes considered as the parameter gamma) as possible outcomes, was found to be ineffective in modeling less ideal PSII-containing systems, however. Substantial improvements were made by Vladimir Shinkarev, who introduced two new parameters [56, 57]. The delta parameter introduced by Shinkarev reflects backward transitions, or the reduction of the WOC stemming from charge separation, rather than oxidation. Finally, the epsilon parameter represents inactivation of the WOC on a given transition, with the effect of permanently preventing oxygen generation by that center. Shinkarev considered that all inefficiencies had an equal chance of occurring on any given transition between S-states, resulting in a 20-step reaction matrix (five possible outcomes on each of four transitions S0-S1, S1-S2, S2-S3, and S3-S4-S0). However, David Vinyard demonstrated that due to the high stability of the S0 and S1 states, as well as the irreversibility of oxygen evolution, there could be no delta or epsilon parameters beginning from those states, resulting in a 16-step matrix which remains the most accurate model of oxygen evolution by PSII [43].



Figure 1.4 Development of the model of S-state transitions from Kok's 12-process model to Shinkarev's 20-process model to the VZAD 16-process model.

The WOC is generated from inorganic cofactors in a process termed photoassembly (reviewed in [16, 58, 59]). The PSII protein subunits are already assembled at this step, with the WOC pocket empty. Photoassembly consists of successive incorporation and oxidation of Mn(II) to form the stable WOC. The intermediate structures are not completely known, and the process has thus far only been observed in PSII-containing subcellular preparates or, to a lesser extent, in seedling plants whose chloroplasts have not yet fully developed or been exposed to light (etioplasts). In subcellular preparates, the native WOC must be removed in order to conduct photoassembly, which involves removing at least three small subunits of PSII whose function appears to be related to stabilizing the intact WOC [60].

1.2.3 The Quinone Electron Acceptors

Where the WOC and Yz, the redox-active components in the luminal region of PSII, are commonly referred to as the donor side of the overall PSII electron transfer reactions, the stromal components which remove electrons excited from P680 are labeled the acceptor side. The primary redox-active components in this region consist of a pair of plastoquinone molecules designated Q_A and Q_B [17, 61]. Electrons excited from P680 are transferred down the potential gradient to pheophytin, and from there to Q_A [62]. This plastoquinone is irreversibly bound, with high affinity of -56.1 kcal/mol, including strong interactions between the head group and adjacent residues, but with no significant interaction with non-protein components [63]. These strong interactions with the head group render Q_A only capable of holding one electron, and the relatively close proximity to the donor side facilitates recombination with holes, considered a major cause of "misses" in the WOC cycle [43, 64, 65].

From Q_{A} , electrons are transferred past a non-heme iron which has been shown to be essential in further electron transfer reactions, though the Fe(II) is not thought to be redox active save by use of exogenous quinones [66, 67]. The electrons move to Q_{B} , a more loosely bound plastoquinone of identical structure to Q_{A} . Q_{B} is primarily kept in position by interactions between the hydrophobic tail group and a large, loose hydrophobic pocket within the membrane; its binding affinity is -37.9 kcal/mol, of which 24% comes from interactions with structural lipids and other cofactors [63]. Reduction of the head group decreases binding affinity and facilitates exchange of the plastoquinone molecule for another from the plastoquinone pool, a group of plastoquinones found within the thylakoid membrane and usually numbering between 12 and 50 per PSII [17, 68-71]. The quinone has a stronger binding affinity than its quinol form [71], favoring replacement in order for the quinol to diffuse to the downstream enzyme complex cytochrome $b_{6}f$.

The existence of a third plastoquinone molecule, termed Q_c, has been postulated based on various experimental evidence, as well as a single crystal structure to date [63, 72-75], but this component is not completely accepted within the field. Most crystal structures have not shown this quinone [30, 38, 72, 76, 77]. However, the proposed site, within a large hydrophobic pocket shared with Q_B, has been modeled for binding affinity and the number of -30.1 kcal/mol proposed, including 45% contribution from hydrophobic cofactors [63]. Interestingly, the tail group has a high affinity for its location, but the head group's position as determined by Guskov et al. is energetically unfavorable, and a recent proposal that an actual Q_c site is nearby and the hydrophobic pocket will stabilize the tail in a wide range of positions has been gaining traction [63, 72-74, 78]. The role of the proposed Q_c is unclear, though the most widely proposed functions involve serving as a spare electron acceptor, either in sequence after Q_B when exchange with the plastoquinone pool is not possible due to lack of available oxidized quinone, or as the quinone from the pool which replaces the one in the Q_B site.

1.2.4 Other Redox-Active Components

In addition to the primary pathway of electron flow through PSII, a number of other components known to be redox active are found within PSII [79]. The WOC pocket is primarily formed by the D1 (PsbA) subunit of PSII, which is structurally very similar to, and shares a common evolutionary ancestor with, the D2 (PsbD) subunit [80]. Given this shared origin and the mirrored positioning of the two subunits within the supercomplex (Figure 1.1), despite the lack of a WOC in the D2 subunit, there is still an equivalent to Yz, called tyrosine-D (Y_D). This tyrosine can also donate electrons to P680⁺, but does not have a source of reductant analogous to the WOC [81-83]. There is also a pheophytin in this subunit, one which is not thought to be able to transfer electrons on to a more stable acceptor and thus can be expected to be only a transient acceptor if at all- pheophytin cannot retain electrons on a biologically useful timescale [84, 85]. A range of additional chlorophylls and carotenoids are found throughout PSII, forming an extended theoretically redox-active network. Finally, two subunits of PSII (PsbE and PsbF), collectively termed cytochrome b559, coordinate a heme group known to be redox-active over slow time scales (seconds, whereas PSII can evolve oxygen at up to 500 Hz) and somewhat photoprotective by this mechanism [74, 86, 87]. A wide range of hypotheses have been proposed for the function of cytochrome b559, but its role and value in the enzyme complex remain vague.

1.3 Toward a Deeper Understanding of Redox Control in PSII

The rates and mechanisms for some, but not all, of the electron transfer processes within PSII have been established. While the primary electron pathway is well understood, a number of major questions remain. In this work, several previously unknown functions of PSII are described. Additionally, processes which have not been completely localized are discussed herein. Accordingly, a brief review of the necessary background for these functions follows.

1.3.1 Fast Repetition Rate Fluorometry

Fast repetition rate fluorometry (FRRF) is a method which utilizes a train of rapid, sharp pulses of light to measure a range of parameters stemming from the electron transfer reactions in PSII [54, 88]. A representation of this method is given in Figure 1.5. These pulses generate an initial fluorescence level, denoted Fo, which originates from those PSIIs in which charge separation is not possible even as light is delivered prior to saturation being achieved. As more light is delivered during the pulse, charge separation occurs, filling the internal acceptor Q_A and transferring a hole to the WOC. As no further charge separation is possible until the WOC can advance and Q_A is reoxidized, fluorescence then increases until all centers cannot undergo charge separation and a steady level of fluorescence, denoted Fm, is achieved (saturation). The difference between Fo and Fm, variable fluorescence of Fv, divided by Fm, indicates the fraction of total centers which can advance during the flash. After the flash, the sample is left in darkness for a predetermined amount of time prior to further flashes being delivered and the same data obtained for those flashes. By this technique, it is possible to determine the efficiency of charge separation in PSII over a broad range of conditions.



Figure 1.5 Diagram of FRR flashes and resulting fluorescence yield over time, showing parameters obtained via this technique.

1.3.2 PSII-Cyclic Electron Flow

While use of exogenous quinone electron acceptors to stimulate oxygen evolution by PSII *in vitro* has been well established [89-91], this technique was more recently applied to living cultures [92]. A correlation was identified between this phenomenon of increased oxygen yield and a sharp decrease in the delta parameter in operational models of the WOC under these conditions, obtained using the VZAD model [43, 92]. This correlation implies that the electron acceptor pool controls a process whereby electrons are transferred from either the Q_B site, where these quinones are known to act, or from the pool itself, to the donor side of PSII at a rate of one electron returned to the WOC (in the S2 or S3 state) per flash. The phenomenon of PSII-cyclic electron flow (PSII-CEF) has been demonstrated to exist across the tree of life at varying levels [43, 92]. In some species, over 90% of all charge-separation events can result in electrons being returned to the WOC via PSII-CEF [93]. As a photoprotective mechanism, it can allow survival of PSII under light intensities exceeding 2000 μ Ein/m²/s (approximately the maximum sunlight delivery anywhere on Earth), indicating a rate-limiting step faster than one electron every 120 μ s, faster than most of the steps of the WOC cycle [94, 95]. This process is therefore of great potential interest in engineering of biological systems. A comparison of PSII-CEF and the classic electron flow pathway is given in Figure 1.6.



Figure 1.6 (A) Simple representation of linear and cyclic electron flow with equation for energy generation by each. See text for definitions. (B) Electron flow pathways in and around PSII. Originally generated for [93].

1.3.3 Atomic Substitution in PSII

Attempts to discover the role of various inorganic cofactors in the operation of PSII have been ongoing for decades [16, 58, 59]. To date, there have been only two substitutions resulting in a functional PSII- the replacement of Ca (in the WOC) with Sr, and the replacement of Cl (particularly in the pocket surrounding the WOC) with Br or I. Prior to the following work, these substitutions were considered to be universally detrimental to the operation of PSII. Crystal structures of PSII after these atomic substitutions are available from *Thermosynechococcus elongatus* and *T. vulcanus* [96-99]. These two substitutions are discussed in Chapters 3 and 4. Several other atomic substitutions which result in non-functional, and sometimes structurally altered, PSII have been performed [100]. Furthermore, substitution of borate for bicarbonate in photoassembly has successfully been performed, but investigating the role of bicarbonate in active operation by substitution has not been successful [59]. An alternate method of investigation is discussed in Chapter 5.

1.3.4 Investigation of PSII Microcrystals

Crystallized PSII represents a highly homogeneous system in which interference from outside factors can be minimized. Although PSII crystals have been extensively used in determination of the structure of PSII, and particularly of the WOC, extensive controversy exists over the validity of some crystal structures obtained [52, 76, 77, 101-105]. The balance of Sstates represented within the crystal has not been determined via a proven model in any prior work; however, numerous crystal structures have been published which purport to show the WOC in a known S-state, usually the stable S1. These crystal structures are often incompatible with other published structures and with phenomenological data, especially when attempts are made to advance the WOC to another S-state [77, 105]. An analysis of the operation of PSII in microcrystal form, which allows both new insights into overall PSII functionality and definitive establishment of properties which are disputed or uncharacterized in the microcrystal system specifically, is given in Chapter 6.

1.4 References

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Gates, Colin; Ananyev, Gennady; Cullinane, Brendan; Luo, Jeffrey; Dismukes, G. Charles, "Acceptor limitation of minimal S state transition times in the WOC of PSII," *in prep.*

Chapter 2. Regulation of the S state transition times in the WOC of PSII

2.1 Introduction

Almost all molecular oxygen on Earth, as well as the vast bulk of biologically available energy, is a product of the activity of Photosystem II (PSII) [1, 2]. PSII is an ancient and highly conserved protein complex which contains intricate and incompletely understood machinery for the conversion of light energy, as photons, into a chemically useful form, biologically available electrons and a proton gradient [3]. This process is described by the equation:

$$2H_2O + PQ \rightarrow O_2 + 2PQH_2 + 4 (H^+_{inside} - H^+_{outside}).$$

The active site of conversion of water to oxygen, the water-oxidizing complex (WOC), is a redox-active metal-oxo cluster with formula Mn₄CaO₅. This cluster operates by cycling through a series of four increasing oxidation states (S-states, designated S0 through S3, with a transient S4 state which rapidly decays to S0 and oxygen on formation), with one electron removed from the WOC in each to replace those advanced from the site of light-induced charge separation, the P680 chlorophyll complex [4-6]. As the oxidation state of the WOC increases, it becomes increasingly difficult to remove further electrons to supply to P680⁺, which maintains approximately the same free energy level despite the WOC free energy level decreasing (Figure 2.1). Accordingly, under the same conditions, a transition beginning with a more oxidized Sstate takes longer than one with a lower (less oxidized) S-state as the starting condition. This phenomenon is well-attested by EPR and optical spectroscopy in isolated thylakoids [7-11].



Figure 2.1 Pathway of fluorescence generation in photosystem II: Photochemically generated P680* can decay by productive charge separation that leads to water oxidation and plastoquinone reduction, but if electrons cannot advance from the WOC (more time required as S-state increases due to lower potential gaps) or cannot be transferred to Q_A because it is already reduced, detectable fluorescence occurs.

Turnover time is a critical indicator of the health of a photosynthetic apparatus. It is effectively a measurement of the rate of generation of biologically usable electrons, as no electrons will advance beyond PSII if P680⁺ cannot be reduced. Accordingly, a long turnover time will decrease primary productivity, and thus growth, of the overall organism. If the time of an individual S-state transition exceeds that of downstream steps, such as Q_A-Q_B (200-400 µs for the first electron transfer to Q_B and 500-800 µs for the second) or PQ pool cycling (a few ms, depending on the organism), this will limit the quantum efficiency of PSII [6, 12, 13]. The S3-S4-S0 transition is the longest step, as the redox potential gap with Yz is small and therefore this electron transfer time is comparable to some downstream steps [9]. Furthermore, faster turnover time is necessary to prevent photooxidation of PSII itself [14, 15].

The four-step cycle of the WOC, discovered via oximetry by the pioneering work of Joliot and Kok nearly fifty years ago, has since been observed via a number of other techniques [4, 5]. One such method is fast repetition rate (FRR) fluorometry, which involves stimulating PSII using controlled pulses of light and measuring the ensuing fluorescence until all chlorophyll is saturated and a peak is reached [16-19]. By using single-turnover flashes, or just enough light to stimulate the advance of all PSII centers in the (dark-incubated) sample population from one Sstate to the next, it is possible to observe period-four oscillations in fluorescence yield due to Sstate populations being uneven (the S2 and S3 states are only transiently stable on a timescale of seconds to minutes and decay to S1 in the dark) [18-20]. This phenomenon is due to the aforementioned variance in free energy levels at the WOC [6]. Increasing S-states result in increasing difficulty of removal of electrons, in turn resulting in decreasing maximum fluorescence.

FRR fluorometry is a completely non-invasive technique, capable of application to virtually any sample containing PSII. Thus far, it has been applied to living plants (mature and coleoptiles), plant tissue, lichens, algae and cyanobacteria in liquid medium or dried in the sample well, isolated chloroplasts, isolated thylakoid membranes, and PSII core complexes under a range of conditions [17-19, 21]. It is expected that this technique can further be expanded to PSII microcrystals. It can be applied across the biologically relevant temperature and humidity range, and there are no limitations on addition of chemicals or pretreatments to a sample. Unlike in the case of oximetry, which substantially limits measurement frequency and available light intensity due to the physical constraints of oxygen diffusion, average light conditions of up to $40,000 \mu E/m^2/s$ and a maximum frequency of 100 kHz (using the Dismukes

group home-built instrument at its current capabilities; soon to be expanded) can be achieved [18, 19]. Accordingly, its application vastly expands the scope of study of PSII. From prior EPR studies [7, 9-11], it is known that this instrument is capable of generating individual S-state transitions in PSII in a minimal amount of time due to the light delivered. From prior observations using this instrument, shifts in overall WOC oxidation state of a sample are readily observed as changes in fluorescence [6, 18, 19]. Accordingly, herein a method is demonstrated for observation of optimal S-state transition times which may be applied to virtually any PSII-containing sample.

2.2 Materials and Methods

2.2.1 Cultures.

Nannochloropsis oceanica was obtained from Christoph Benning and was grown at 22°C in A+ medium [22] in batch culture. 30 μ E/m²/s continuous white light was supplied from Philips Silhouette cool fluorescent lamps. Air was bubbled through the growing culture. *Thermosynechococcus elongatus* (His-tagged CP-47 mutant strain [23]) was grown at 45°C in replete, Sr-substituted, or Br-substituted DTN medium [24] in batch culture, as described in Chapters 3 and 4 in greater detail. 40 μ E/m²/s continuous white light was supplied from Philips Silhouette cool fluorescent lamps. 1% CO₂-supplemented air was bubbled through the growing culture. All samples from both species were taken in mid-exponential phase growth conditions. Optical density was measured at 730 nm using a Thermo Scientific Evolution-60 spectrophotometer to determine growth phase.

2.2.2 Fast repetition rate fluorometry.

The FRR fluorometer is a custom-built device, an earlier model of which was previously described [18, 19]. It is composed of a light source for stimulation of chlorophyll fluorescence, a non-reflective sample holder with a Peltier element temperature regulation system (Wavelength Electronics), and a Luna Optoelectronics red-tuned avalanche photodiode for fluorescence detection. The light source used in these experiments is a 660nm red laser capable of delivering 80,000 μ E/m²/s flashes of adjustable duration up to a frequency of 100 kHz. LabView-based software (National Instruments) controls the instrument and formats output data.

2.2.3 Long-pulse method.

To produce flash-induced WOC turnover in optimal time, it is necessary to supply a significant excess of light [9]. Accordingly, a dark-adapted (3 min in all subsequent data) sample is subjected to a train of 5 μ s flashes (one quarter of a single-turnover flash, see Figure 2.2) delivered at 10 μ s intervals for a period of 5 or 10 ms (500 and 1000 pulses, respectively), which is equivalent to the delivery of a 5 or 10 ms continuous long pulse at 40,000 μ E/m²/s light intensity. Given the known time-scales for S-state transitions [7, 9-11], this is sufficient to induce full turnover under most conditions. To cover a longer timescale, it would be necessary to lengthen the flash intervals, as fluorescence is read on each flash and the rate of data generation is near the limit of what can be achieved with commercially available hardware [18]. After this first pulse, the sample is incubated in the dark for 3 s, long enough to oxidize the acceptor side of PSII but not enough to fully reduce the WOC populations to dark-stable levels [6, 13, 25-27]. A second flash train is then applied. The scrambled S-state populations produce different amounts of fluorescence than the dark-adapted sample, which contains primarily S1 and S0. See Figure 2.3 for a graphical representation of the technique.



Figure 2.2 (A) Demonstration of fluorescence yields from quarter-turnover flashes delivered at the same frequency as used in the "long-pulse" technique. (B) Fv/Fm results from bursts of four quarter-turnover flashes (each time point is one group of four flashes) delivered at 10 ms intervals (100 Hz flash frequency). All results derived from living *N. oceanica* culture at 22°C.



Figure 2.3 Delivery of pulses to a PSII-containing sample. Vertical lines represent flashes of light. Corresponding maximal fluorescence response (Fm) was recorded and analyzed.

2.3 Results

2.3.1 Method demonstration and underlying principles.

An individual flash train produces a fluorescence curve which primarily consists of

background fluorescence, as seen in Figure 2.4A and B. However, there are differences between

the two curves due to differing initial S-state populations. As the S-state transition times vary by orders of magnitude (see Table 2.1), it is most appropriate to observe these differences on a logarithmic scale (Figure 2.4B). In a heat-inactivated sample (Figure 2.4C) the only difference observed is a small, uniform gap due to chlorophyll damage. Subtracting the fluorescence yield of the second train from the first gives the difference due to S-state ratios changing. Features observed result from S-state transitions and can be time-resolved (Figure 2.4D).



Figure 2.4 Flash train fluorescence yields from mid-exponential phase *N. oceanica* as described above. Living culture yields on (A) linear and (B) logarithmic time-scales. (C) Thermoinactivated sample. (D) Difference in flash yields between the two trains in living and thermoinactivated

cultures showing interpretation of fluorescence peaks. All measurements taken at 22°C. Data shown is the average of 30 measurements.

In the dark-adapted sample, supplying supersaturating light overstimulates the antenna and forces electron flow through PSII as fast as possible, which results in the generation of optimal WOC transitions [9]. The abundant S1 population which forms in the dark is advanced through S2, S3, and S4-S0 in rapid succession, but the great surplus of excited chlorophyll in the antenna (and the inherent inefficiencies of the WOC) results in a scrambling of S-states in the sample [6, 28]. In the second train, there is little variation in the S-state populations, and accordingly, more differential fluorescence from a given S-state occurs when more sample transitioned through that S-state on the first trial. Each peak represents an S-state. On the formation of S4-S0, however, there is a drop in fluorescence and a local minimum is reached. This is due to the sharp decline in fluorescence caused by the redox gap between the S3 and S0 states (see Figure 2.10 for a graphical representation) [6, 11, 28]. Fluorescent recombination between an excited electron and the S0 state of the WOC is significantly less energetically favorable than when the WOC is in the S3 state, and S-state specific fluorescence yields have long been observed [18, 19].

These peak and trough features are non-Gaussian and thus cannot be easily differentiated with common Gaussian fitting models. This is due to inefficiencies in electron transfer; hence why WOC cycling is almost always on a longer cycle than the Kok ideal period of 4 charge separation events [6, 19, 29-31]. The peaks trail into one another and must be carefully located, given the logarithmic time scale. The dashed red lines in Figure 2.4D illustrate how to determine the maximum or minimum point of a given feature by locating the adjacent linear region and tracing it to the intersection with the trace of the other linear region flanking the feature. The S-state lifetimes obtained compare well with those determined *in vitro* via all other methods, as seen in Table 2.1 [7, 9-11].

Study	Babcock et	Renger,	Razeghifard	Haumann	This work
	al., 1976	2011	and Pace, 1997	et al., 2005	
Method	EPR method	Optical	EPR method	X-ray	Fluorescence
		absorbance			(in vivo)
S0→S1	30	30	70	30	27
S1→S2	100	85	110	70	177
S2→S3	350	240	180	190	440
S3→S4→S0	1000	1300	1400	1300	1030

Table 2.1 Comparison of S-state lifetimes obtained via various methods.

Some transitions may be more reliably obtained by this method than others. Four flashes are required to deliver enough light to advance all centers by one S-state (Figure 2.2). Accordingly, at the calculated time for the S0-S1 transition, there has only been 97% of saturating light delivered (note that the third flash ends at 25 µs). Thus, a small error is expected if the S0-S1 peak is found sooner than 35 µs after the beginning of the "long-pulse." In this case, the implication is that 3% of the WOCs which were initially in the S0 state are still in the S0 state at peak time due to lack of light delivery and thus the actual peak may be up to 3% earlier (in most samples, this is about 1 µs). The S3-S4-S0 transition, in contrast, is somewhat imprecise because of the sheer slowness of the transition. The linear regions used to calculate the S0 minimum's center are on a time scale two orders of magnitude larger than those used for the S1 peak. However, the precision of this technique in determining the S3-S4-S0 transition time is still comparable to or better than the prior methods.

2.3.2 Method optimization.

Fluorescence is not solely due to the oxidation state of the WOC, however, and may be affected by the redox state of any of the redox-active components of PSII and the plastoquinone pool [17, 32-35]. However, their contribution to observed fluorescence is negligible due to the time-scale of dark incubation between the two trains and the otherwise identical conditions used. After 3 s, the only redox-active component which retains charge is the WOC. Indeed, virtually identical transition times can be observed by lengthening the dark time (Figure 2.5), though as expected, the S-state populations decay toward their dark-stable ratio, resulting in a decreased change in fluorescence [18].



Figure 2.5 Flash train fluorescence yield differences from living *N. oceanica* with varying dark incubation times between flash trains. All measurements taken at 22°C. Data shown is the average of 30 measurements.

The differential rates of S-state decay in the dark allow more precise measurements of certain S-state transitions by varying the time between trains. The S1 peak is most clearly observed after minimal time. The dark-stable S0 population in *N. oceanica* is greater than the 25% predicted by Kok's model [5, 6, 18, 31]. As the S0 population grows due to reduction of WOCs in the dark, the peak is nearly gone by 10 s and almost completely indistinct after a

minute. The S1 population similarly grows after dark incubation and thus the "valley" between S1 and S2 peaks is filled in by a larger population making the S1-S2 transition. Over time in the dark, the S3 population decays to S2 and replenishes the concurrently decaying S2 population, which manifests as a more distinct S3 peak separate from S2 as time progresses. The S0 trough becomes shallower over time, as the initial population for the second train becomes more similar to that for the first train. It is thus easiest to observe the S0-S1 transition with a short gap between trains, the S1-S2 and S3-S4-S0 across a broad range of times, and the S2-S3 after a longer period. However, the transition times are affected by a broad range of factors, including the organism (i.e. the redox potentials within PSII), the temperature (see Figure 2.8), and the redox poise of the system [9]. The Y-axis offset of the features, meanwhile, is dependent primarily on the redox poise of the system. Fluorescence is dependent on recombination, and if the acceptor side is being effectively cleared by a highly oxidized PQ pool, fluorescence will decline across the life of the train [18]. Thus, the more time between the first and second flash trains, the more oxidized the system and therefore the lower Fm2 and the higher Fm1-Fm2.

2.3.3 Localizing features to the WOC.

Hydroxylamine treatment of PSII results in WOC reduction and eventual removal, serving as an electron donor to the Mn in the WOC and effectively preventing advances in Sstate [36-39]. In the presence of hydroxylamine, only a single feature (Figure 2.6B) can be observed due to rapid reduction of all S-state populations. This feature resembles the S2 formation peak, but is at least partially due to the reductive decomposition of the WOC. As seen in Figure 2.6A, a general decrease in fluorescence occurs between trains.



Figure 2.6 (A) Flash train fluorescence yields from living *N. oceanica* culture after dark incubation in 2mM hydroxylamine. (B) Flash train difference from (A). (C) Flash train fluorescence yields from living *N. oceanica* culture after dark incubation in 5μ M DCMU. (D) Flash train difference from (C). All measurements taken at 22°C with 3 s between flash trains. Data shown is the average of 30 measurements.

Another noted inhibitor of PSII activity is 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which acts on the Q_B site and prevents electron removal from the WOC once the Q_A site is filled [13, 40, 41]. Given the lack of dark-stable S2 and S3 population in *N. oceanica* (Ananyev et al. 2016), the only transitions contributing to the fluorescence yield of either train in the presence of DCMU are S0-S1 and S1-S2 (Figure 2.6C) [42]. In the case of the second train, only those centers which have undergone recombination will still be active. Thus, a single peak

feature is once again observed (Figure 2.6D). Unlike in the case of hydroxylamine, there is little decline in differential fluorescence after the peak, because hydroxylamine continues deactivating PSII over time by reducing the WOC [36, 37], while DCMU treatment results in closed centers. The features observed with hydroxylamine and DCMU therefore are indicators of destruction and saturation, respectively.

The Q_B site is reoxidized relatively slowly, on a timescale comparable to the longest steps of WOC cycling [6]. Accordingly, electron removal from the Q_B site can be expected to limit WOC activity, since a fully reduced acceptor side completely blocks further electron transfer in much the same way as DCMU. Conversely, removal of this bottleneck allows observation of the optimal transition times without significant acceptor-side contribution. The electron acceptor 2,5-dimethylbenzoquinone (DMBQ) has been shown to substantially facilitate acceptor-side clearing in both living cells [19] and PSII-containing preparates [43] if delivered at ideal concentration (Figure 2.7).





delivered to advance S0 population to S1 180s prior to first train. Measurements taken at 22°C with 1 s between measurements. Data shown is the average of 30 measurements.

The initial S0-S1 and S1-S2 transition times are largely unaffected by DMBQ addition because the first electron need only advance to Q_A to constitute a successful charge separation event under these conditions. The S2-S3 transition, however, occurs 25% faster and the S3-S4-S0 40% faster. Additionally, the peak features are distorted substantially due to the overall redox modifications on PSII of DMBQ addition [19, 43]. The S2 state is more stable in darkness with DMBQ present and, rather than being absent after dark incubation, is present in 12% of centers. Accordingly, the S2 peak is smaller, while the S3 peak is higher than the S2, and the S0 trough is also abnormally high. These effects can be further attributed to DMBQ delaying decay of the S2 and S3 states, which would lead to faster transitions on average. Notably, all features appear where Fm1-Fm2 is negative. The S1 peak is identical to that observed without DMBQ, and the lowered S2 is partially explained by the S-state distribution. These features are primarily dependent on electron advance to Q_A rather than downstream clearing, which is dependent on the oxidation state of the PQ pool. Clearing of Q_A^- normally takes 200-400 μ s, but the more oxidizing DMBQ would accelerate this process and affect the S2 peak as well [6, 19, 43]. This redox potential gap exacerbates the clearing effect seen in Figure 2 by lowering Fm1 and therefore Fm1-Fm2. The entire kinetic appears to rise over time as the local environment is reduced by the accumulation of electrons on DMBQ faster than the acceptor can be removed and therefore acceptor clearing slows.

2.3.4 Modulation of WOC behavior by temperature and species.

The range of techniques previously available have been subject to the limits of operating temperatures of the methods. Some, such as the EPR methods, require the use of subfreezing

temperatures [7, 8, 10, 11]. Considering that it is well-accepted that some physical rearrangement of interatomic distances within the WOC occurs within the S-state cycle [6, 44-49], it can be expected that the process would be temperature-dependent on the donor side as well as the known limitation of diffusion of plastoquinone on the acceptor side [50, 51]. To this point, owing to the high homogeneity of the systems studied, it has occasionally been claimed that the S-state transition times are intrinsic, temperature-independent properties of PSII. Figure 2.8 refutes this claim.



Figure 2.8 Flash train difference yields from *N. oceanica* obtained from sample at temperatures from 5 to 45°C. All measurements taken with 1 s between flash trains. Data shown is the average of 30 measurements.

Differential fluorescence intensity, peak height (reflecting dark-stable S-state distribution), and S-state transition times are all affected by temperature. Individual S-state transitions, as well as the overall cycle, demonstrate pseudo-Arrhenius behavior from 10 to 35°C, above which temperature thermoinactivation is observed. The temperature-dependence plot of the overall cycle is given in Figure 2.9.



Figure 2.9 Arrhenius plot comparing time to reach oxygen evolution (and S0) from initial culture of *N. oceanica* at 10-35°C. Times were obtained from the raw data given in Figure 2.8. All measurements taken with 1 s between flash trains. Data shown is the average of 30 measurements.

In addition to being dependent on temperature, WOC transitions vary under biological conditions in living culture due to the sample species. The dark-stable S-state population distribution is dependent on the environmental redox poise, which is readily altered, and modifications (natural or introduced) to PSII further complicate this system [6, 19, 29, 31, 52, 53] (addressed in Chapters 3 and 4 as well). As seen in Figures 3.11 and 4.6, *Thermosynechococcus elongatus* has much longer transitions than *N. oceanica* at equal temperature; however, *T. elongatus* is capable of undergoing S-state transitions on comparable time scales to *N. oceanica* at optimum when a temperature of 47°C is used. Furthermore, atomic substitution within PSII (Sr for Ca in Figure 3.11; Br for Cl in Figure 4.6) affects the S-state transition times, and this modification can alter the pseudo-Arrhenius behavior of the WOC cycle. While the optimal total transition time in the absence of exogenous acceptors is a fairly constant 1.5-2.0 ms, this optimum comes at the ideal operating temperature of the given organism's PSII, and modification of that PSII may alter the optimal time.

2.4 Discussion

Fluorometric study of the S-state transitions is reliant on two key constraints: the rate of reduction of the WOC and the rate of oxidation of the internal (and pool) electron acceptors. As described in Figure 2.10, it is possible to take advantage of these factors to draw deeper analyses of the data obtained. The progress through the S-state cycle, and particularly the heights of the peaks in the differential fluorescence trace, are dependent on a handful of factors- the original difference in S-state distribution, the quality of advance through the WOC cycle (i.e. the inefficiency parameters at the flash rate used), and the specific fluorescence generated by each individual S-state [6, 18]. As the WOC becomes more oxidized, the

fluorescent yield increases due to the greater energy gap involved in fluorescent recombination and the increasing instability of the WOC. However, the energy gap, and thus the total fluorescence, is likely species-dependent [18]. The differences in S-state distribution are readily measured via flash oximetry, but the inefficiency parameters are shown in Chapters 3 and 6 to be dependent on flash frequency, preventing their effective measurement considering the lightsaturating conditions used in this measurement. Furthermore, the technique involves deliberately generating a vast number of recombinations (alpha or miss parameter). Thus, the peak heights are most useful in determining the specific fluorescent yield from given S-states, as the use of differential fluorescence between two trains in which failure to charge-separate is the dominant process effectively means that any difference observed is an efficient transfer of electrons.



Figure 2.10 Scheme describing the origins of features in differential fluorescence.

While fluorometric measurement of S-state transitions greatly expands the range of conditions under which PSII can be studied, it also reveals numerous confounding variables affecting S-state transitions. Most notable among these is the regulation on the acceptor side. Reaching any S-state after S1 in the absence of electron acceptor is shown here to require more time than in the presence of optimal acceptor, indicating that these transitions are not being solely regulated by the kinetics of electron removal or rearrangement at the WOC. Indeed, the transition times observed are comparable to those of electron acceptor, which results in a larger redox states [6, 12]. Use of exogenous quinone as an electron acceptor, which results in a larger redox potential gap between the internal and external acceptors, facilitates electron removal and increases the rate of oxygen evolution in living culture [19]. The pseudo-Arrhenius behavior of S-state transitions may correlate to the pseudo-Arrhenius behavior of overall rate oxygen evolution, known to be constrained downstream of PSII proper on the timescales observed [54], which is discussed in Chapter 3 [29].

The effects of exogenous quinones vary substantially with species studied, temperature, type of quinone, and type of preparate [19, 29, 43, 55](also see Chapters 3, 4, 5, and 6), but it is clear from Figure 2.7 that the major constraint on oxygen evolution *in vivo* is not the WOC cycle, but the rate of electron removal. Furthermore, all prior transition times observed for the S3-S4-S0 transition are significantly longer than what is observed under optimal quinone conditions. In prior measurements, exogenous quinones were frequently used as electron acceptors, which explains to some extent the faster turnover times than what we observe in the absence of exogenous quinones, but temperature and acceptor concentration were not optimized due to technique constraints [7, 8, 10, 11]. These results are thus entirely consistent with the previous-the S1-S2 and S2-S3 transitions are substantially accelerated. As the first two transitions for most centers in a dark-adapted sample will be these, and under subfreezing EPR temperatures

the diffusion rate of new quinones is drastically slowed regardless of system, as compared to native temperatures. The actual catalytic cycle of the WOC may be significantly faster than current estimates.

2.5 Conclusion

In this work, a new non-invasive method for measuring optimal WOC transition times is demonstrated. This method is quick (just over 3 min per sample), inexpensive (requires no chemical treatment or materials other than the instrument), and functional under more biologically relevant conditions. It can be operated at any biologically relevant temperature, using any PSII-containing sample of appropriate size, and with any chemical additive desired. The efficacy of this method is demonstrated by comparing to and expanding upon prior *in vitro* studies using living culture. This method can be used to study organisms across the tree of life and to investigate the limitations of electron transfer within PSII. Temperature and speciesspecific homeostatic mechanisms are shown to regulate S-state optimal transition times. The immediate constraint on S-state transitions is shown to be electron transfer on the acceptor side of PSII.

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2.7 References

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Chapter 3. Strontium Tunes the redox potential of the WOC of PSII through the S state cycle

3.1 Introduction

The water-oxidizing complex (WOC), the catalytic site of water oxidation in PSII across the tree of life, is comprised of a universally conserved inorganic cluster, Mn₄CaO₅ [1]. The WOC is coordinated by a first shell of universally conserved amino acid residues, which are in turn coordinated by an almost completely conserved second shell which includes the conduit of electrons to the PSII reaction center; a photooxidizable tyrosine residue, Y_z. Efforts to generate a mutant in the first coordinating shell which produces a functional WOC have been unsuccessful to date [2]. Only by exchanging strontium for the native calcium within the WOC itself is it possible to generate a functional "inorganic mutant" of the WOC region [3], although there is no evidence for Sr-substitution *in vivo*, possibly due to the element being orders of magnitude rarer in the habitable zones of phototrophs [4]. In the 1980s it was first demonstrated that addition of strontium to calcium-depleted PSII restored some oxygen-evolving activity in isolates of both cyanobacteria [5] and higher plants [6]. The site was soon localized to the WOC by fluorescence demonstration of P680⁺ reduction activity being restored [7] and by electron paramagnetic resonance (EPR) spectroscopy which demonstrated differential coupling to the EPR-active Mn cluster [8, 9]. Since then, numerous studies of Sr-substituted PSII, usually generated via removal of Ca from the WOC and addition of Sr, but also via biosynthesis in some cases, to investigate the role of calcium within the WOC [6, 8, 10], its ability to regulate S-state transition times [11], restructuring events [12, 13], and free energy gaps [14], and particularly control of the overall rate of oxygen production by Sr-substitution [6, 8, 15, 16].

No instances of a functional, or even stable, WOC outside its native protein scaffold have been demonstrated to date, though the use of large ligands has allowed substantial advances in the development of near analogs [17]. It is, however, possible to reassemble the WOC from the individual elements *in vitro*. The apo-WOC PSII complex (depleted of the metals of the WOC typically via use of chelators and reductant), plus free Mn^{2+} , Ca^{2+} , and HCO_{3^-} in the presence electron acceptors, can be subjected to soft stimulating light in order to regenerate near-complete activity. This process is called photoassembly (or photoactivation, though this term also refers to the light-driven biogenesis of the assembled PSII at the protein subunit scale both with and without the WOC; for a thorough review see Bao and Burnap 2016 [18]) and is believed to follow the same mechanisms as WOC biogenesis and repair *in vivo* [3, 19-22]. The role of Ca²⁺ in forming the functional WOC cluster has been demonstrated to involve regulating the number of photooxidizable Mn^{2+} to 4.0 per site [23]. In the absence of Ca²⁺, up to 20 Mn²⁺ are incorporated sequentially, eventually forming a catalytically inactive amorphous oxyhydroxide polymer containing Mn^{3+} and Mn^{4+} , $[MnO_x(OH)_y]_{n>20}$ [24, 25]. Ca²⁺ is indispensable in templating the correct structure of the Mn_4O_5 WOC core.

Replacement of calcium by strontium increases the rate of photoassembly of the WOC [26]. Sr^{2+} selectively binds to the Ca²⁺ effector site in spinach apo-WOC-PSII particles, which induces a fivefold faster rate of photoassembly as compared to Ca²⁺, measured by the initial rate of O₂ evolution as the WOC is generated [26]. This was shown to occur because the rate of

advance is accelerated by Sr-substitution during the first two sequential assembly steps: $k_1 - photooxidation of the first Mn^{2+} \rightarrow Mn^{3+}$, and k_2- the subsequent dark step, in which a tenfold increase in Ca affinity stimulates formation of the first stable intermediate, $Ca^{2+}(OH)_2Mn^{3+}$. It is this second (dark) process which is rate-limiting for overall recovery functional of O_2 evolution. While a protein conformational change has been postulated to occur during the dark step, this has not been shown directly [11]. Charge recombination from this intermediate, resulting in return to the previous (only Mn^{3+} bound) was slowed twofold when Sr^{2+} rather than Ca^{2+} was incorporated; this is proposed to occur due to greater thermodynamic stability. The resulting photoassembled Sr-WOC-PSII yields approximately half as much O_2 per flash as comparable Ca^{2+} samples, similar to the slower O_2 evolution rate under saturating light in the Sr-exchanged holoenzyme, as noted above [8]. Sr^{2+} is the sole metal ion which has been found to functionally replace Ca^{2+} in water oxidation. Mg^{2+} and Ba^{2+} , the nearest ionic analogs as alkaline earth divalent cations, cannot bind to the Ca^{2+} effector site, while binding of VO^{2+} and Cd^{2+} has been observed, but no O_2 evolution activity follows [3, 27, 28].

Detailed structural information on the position of strontium in PSII has become available in the last several years. The crystal structures of PSII with calcium or strontium in the WOC (PDB IDs: 3WU2 and 4IL6, respectively) have been determined with respective resolutions of 1.9 and 2.1 Å for *Thermosynechococcus vulcanus* (the nearest relative of our selected strain) [29, 30]. A somewhat lower resolution structure of the native calcium PSII core from *Thermosynechococcus elongatus* is also available (PDB ID: 4V62) [31, 32], and sufficient highresolution study has been undertaken in recent years to reasonably establish the structure of the native WOC pocket (see Figure 3.1), though questions exist regarding the validity of the claims of the oxidation state of any given structure; see Chapter 6. These data provide a structural platform for prediction of function and analysis of spectroscopic data. The location of Sr^{2+} , closer than the native Ca^{2+} to Y_Z , may alter the redox poise of the WOC (S-states) relative to Y_Z . As the WOC is net shifted closer to Y_Z , the electrical potential gradient and electronic coupling can be expected to increase, slowing charge recombination with the acceptors and due to a more stable donor and facilitating forward electron transfer. This prediction contradicts the long-reported observation of slower light-saturated O_2 evolution [8]. Redox potential shifts within the WOC between Q_A and Q_{A^-} and across the S2/S3 transition resulting from Sr^{2+} substitution have previously been demonstrated [14].



Figure 3.1 Overlay of the 2.1 Å resolution structure of the Sr-substituted WOC (full color) on the 1.9 Å resolution structure of the unsubstituted WOC (yellow), including tyrosine-161 and histidine-190, as well as W3 and W4. (A) Simple structural overlay; (B) Statistically significant changes to interatomic distances resulting from Sr substitution. Statistical significance is here defined by the net uncertainty (rmsds of 0.10 Å based on cross-comparison of multiple crystal structures obtained under a different wavelengths). PDB IDs: 3WU2, 4IL6 [29, 30].

We extended the earlier functional studies to examine kinetics of electron/proton transfer for the first time in native Ca and Sr-substituted WOCs *in vivo*, using *T. elongatus* as a model organism. We further extended kinetic studies of WOC turnover to intact cells by application of Chl fluorescence methods allowing detection of the WOC catalytic cycle [33-36]. Additionally, we examined the influence of acceptor-side redox poise on electron flux from water oxidation and its potential regulation of WOC turnover.

3.2 Materials and Methods

A *Thermosynechococcus elongatus* His-tagged CP-47 mutant strain provided by Dr. Sugiura (henceforth referred to as *T. elongatus*) and described by her previously [37] was grown in volumes of 150 mL at 45°C in replete or Sr-substituted (380 μ M Ca or Sr) DTN (Castenholz D) medium [38] with bubbling of 1.0% CO₂-supplemented air under 40 μ mol photons m⁻² s⁻¹ continuous light from Philips Silhouette cool white fluorescent lamps. This growth method varies from that of Sugiura in that *T. elongatus* was grown below normal growth temperatures (48-55°C) and at the lower end of the normal light range (60-80 μ mol m⁻² s⁻¹ being possible for some, non-His-tagged strains). Cells in Sr-containing medium only grow stably at low light intensities. Maximum growth rate for both cultures was achieved at ~40-45 μ mol m⁻² s⁻¹ continuous light and a growth temperature of 45°C. All following measurements, except growth curves, were performed on mid-exponential growth culture. Optical density (OD) was measured using a Thermo Scientific Evolution-60 spectrophotometer at a standard wavelength of 730 nm. This instrument was also used to quantify Chlorophyll *a* (Chl *a*) concentration after methanol extraction from culture [39].

The D1 subunit of PSII was quantified via two methods (Figure 3.2). Polyacrylamide gel electrophoresis and Western blot were performed against a D1 protein standard (Agrisera) using primary antibodies targeted to a conserved PSII sequence and horseradish peroxidase secondary antibody/luminol detection. Western blots were conducted in quadruplicate and results averaged. All samples were adjusted to equivalent chlorophyll concentrations of 3

μg/mL before blotting for ease of comparison to other data. Tyrosine-D radical quantification, as an indicator of PSII concentration, was performed by EPR spectroscopic measurements against a standard curve of Fremy's salt (potassium nitrosodisulfonate) using a Bruker Elexsys E580 spectrometer; conditions: 10 mW microwave power, 70 dB receiver gain, and 100kHz modulation frequency at 100 K.



Figure 3.2 EPR spectra of (A) a concentration curve of Fremy's salt used as a standard organic radical, and (B) pre-harvest chlorophyll-normalized samples of native and Sr-grown *T. elongatus* thylakoids. (C) Double-integrated areas of the Fremy's salt concentration curve. (D) Scanned Western blot of D1 protein extracts from Sr-grown (left) and Ca-grown (second from left) cultures. Extracts were standardized to equivalent chlorophyll content immediately prior to electrophoresis. Quantifications were made using a D1 standard series (rightmost four columns)

and were based solely on D1 monomer content, as comparatively D1 was present in other forms in all relevant samples (the Sr and Ca bands are clearly indicative of concentrations between the two most dilute in the D1 series). Ladders added for clarity.

Electrochemical detection of oxygen was carried out on custom-made Clark-type electrodes using 20 μ s single turnover flashes (STFs) applied at a range of flash frequencies [36]. The light source was a homemade LED system with spectral range 660±20 nm FWHM and delivered light intensity 32,000 μ mol photons m⁻² s⁻¹. Flash oxygen traces from individual STFs were integrated to obtain per-flash absolute quantum yields after comparing to a standard of known concentration. Average oxygen yields from trains of flashes following 2 min dark incubation were also taken and plotted separately as in Figure 3.6. Lastly, to compare relative oscillations in oxygen yield across multiple pulse trains, as in Figure 3.7, the individual flash yields were normalized to the same steady-state value after oscillations decayed to zero. Absolute O₂ quantum yields (mol O₂ per flash/mol PSII-D1) were obtained by normalizing the obtained oxygen yields to the number of PSII centers per sample, as determined by Chl *a* concentration and EPR spectroscopy. Detected O₂ following a single flash was integrated to obtain the total charge, which corresponds to O₂ consumed at the electrode (4 e⁻ per molecule).

Lifetimes of the transient S2 and S3 states were determined by advancing a darkacclimated sample to generate a large population of the desired state using STFs, incubating in darkness on the electrode for variable time in this state, and then rapidly advancing to oxygen evolution via flashes [33, 35, 36, 40]. The WOC inefficiency parameters listed in Table 3.1 were used to correct for inefficiencies on each flash. Where specified, 250 μ M 2,5-*para*dimethylbenzoquinone (DMBQ) was added to sample in order to bypass any strontium substitution effects on the acceptor side of PSII [33-36]. Inefficiency parameters of the WOC cycle and S-state populations [41] derived oximetrically were obtained by fitting flash oxygen yields to the Markov matrix representing the theoretical WOC cycle using the STEAMM method. The VZAD model of the WOC cycle was used due to giving the most accurate model of known processes within the WOC, as shown in Figure 3.3 and detailed in section 1.2.2 [42]. Modeled data was normalized to an average of 1 (i.e. the sum of the S-state population fractions) for ease of processing. Accuracy of fit was ensured by obtaining and minimizing the squared difference between the observed and simulated O₂ amplitude per flash. Fit quality was further ensured by checking agreement between the WOC cycle period (obtained by Fourier transformation), P_{FT}, and the calculated model-dependent period, P_{calc.} [42]. Flash train oxygen evolution traces as described above were processed with a fast Fourier transform algorithm available as part of the VZAD software suite (a newer version of which is available online at: http://chem.rutgers.edu/dismukes-software).



Figure 3.3 VZAD model of WOC operation. Left: Matrix used to calculate cycle operation. Right: Graphical model of phenomena represented by VZAD parameters.

FRRF measurements were performed using a fluorometer developed in our lab [33] and samples of the same live cultures used in oxygen evolution measurements, taken concurrent with those samples. Fluorescence emission at 660 nm, as an indicator of Chl *a*, was measured

using trains of 50 flashes delivered at frequency of 100 Hz. Fluorometric measurements of Sstate transition times were carried out using the same instrument and the technique described in detail in Chapter 2.

Temperature dependence of steady-state oxygen evolution rate was determined by subjecting cultures to continuous saturating red light ($120 \mu Ein/m^2/s$) and stirring in an air-tight, temperature-controlled chamber mounted on a Hansatech Oxygraph Plus oxygen electrode. Respiration was corrected out of rates of oxygen evolution by subtracting the rate of oxygen consumption under equivalent oxygen concentrations at measurement temperatures in total darkness.

Ca content in Sr-substitution growth medium was analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES). The Sr-substituted medium contained 330 nM residual Ca as contaminant, while native (Ca) growth medium contained 60 nM Sr contaminant. These contaminant levels represent less than 0.1% of the other cation by molarity and can be treated as insufficient to induce significant interference. Higher levels of contamination have been shown by others to produce samples which may be phenotypically differentiated [15, 43, 44]. *T. elongatus* is not thought to exchange calcium and strontium in functional, assembled PSII [43]. Accordingly, after the number of generations (three months, 10²⁴ fold dilution) grown prior to measurements in order to ensure full substitution, it is expected that no Ca-WOCs remain in Sr-culture and vice versa.

3.3 Results

3.3.1 Cell growth rate and pigments.

53

Growth rate of each cell lineage at 45°C is shown in Figure 3.4. All subsequent data reported was obtained from culture in mid-exponential phase growth (3 to 4 days after inoculation), during which time the two metal conditions produce equal growth rates. Growth on strontium produces a two-day "lag" and a lower maximum density of culture, but has little effect during log-phase growth.



Figure 3.4 Growth rates of Ca- and Sr-grown cultures. All cultures were grown at 45°C in replete or Sr-substituted DTN medium in volumes of 150 mL with bubbling of 1.0% CO₂-supplemented air under 40 μmol m⁻² s⁻¹ continuous light.

Chl *a* and phycocyanin content were affected by growth on Sr, indicating alterations to antenna structure. A significant amount of Chl *a* is present as PSI, and the effects of whole-cell Sr-substitution on PSI content have not been published to date. Oxygen evolution data was therefore normalized to PSII reaction centers to avoid inaccurate reporting of operating efficiency. Two methods were employed to determine the chlorophyll:D1 ratio; Western blot and EPR (Fig. 3.2). A linear calibration series was obtained as a function of cell content for both methods. Chlorophyll:D1 ratios of 1504±88 molecules (WB) and 1474±29 molecules (EPR) per Ca-PSII-D1 and 2142±131 and 2036±45 molecules, respectively, per Sr-PSII-D1 were established. The EPR values were used in all following calculations reliant on PSII-D1 content due to lower variance between replicates (2% vs. 6% for WB). However, Western blot was established as a fairly reliable method for D1 quantification (and is used alone in Chapter 4).

A decrease in phycobilin content also results from Sr-substitution. Phycocyanin content was quantified by the method of Lawrenz et al. [45] and normalized to PSII-D1. 6.7 phycocyanin molecules per PSII-D1 are found in the native culture, whereas Sr-substitution causes a decrease to 5.4 per PSII-D1. Light saturation curves for both cultures were established to ensure similar operation of P680 under identical measurement conditions and limit the secondary influence of these effects on the systems of interest, the WOC and quinone sites (Fig. 3.5). Both cultures exhibit similar light usage, while measurements of maximum oxygen yield given in Fig. 3.10 were performed under saturating light. This strain does not grow under its saturating light conditions and the Sr cultures are appreciably more light sensitive, though it is evident that in neither case is this constraint due to limitation of the WOC cycle.



Figure 3.5 Rate of oxygen evolution in living culture under 660nm red light at varying intensity at 25 and 45°C.

3.3.2 Flash O₂ quantum yields.

Quantum yields (QYs) of oxygen evolution were measured using LED pulses of saturating intensity and 20 µs duration at room temperature, as shown in Fig. 3.6A. For the Sr-grown culture, the number and amplitude of oscillations are visibly larger than for the Ca-grown culture (4 cycles vs 3). Absolute quantum yields relative to PSII-D1 subunit content as determined by EPR are plotted here. In Sr-grown cultures, the initial QY is 58% of that observed in the Ca-grown cultures (Fig. 3.6A), which closely agrees with previously reported yields for PSII core complexes of *T. elongatus*. [3, 8, 15, 16]. The average QY in both samples decreases with continued flashing over 90 minutes and the difference in QYs between Sr and Ca remains quite consistent (Sr is 60% of Ca at 90 min; see Fig. 3.6C).



Figure 3.6. Representative data demonstrating: flash oxygen yield of *T. elongatus* cells grown and measured (A) without and (B) with addition of 250 μ M DMBQ, in Ca- or Sr-containing medium; grown at 45°C, measured at 23°C. All data shown is the averaged result of 30 flash trains. (C) In the absence of exogenous electron acceptors, or (D) in the presence of 250 μ M DMBQ as an electron acceptor. Each point on the Y axis is the average of 50 flashes separated by 2 seconds (0.5Hz flash rate) and summed from 30 flash trains each separated by 2 mins dark adaptation. The cumulative flashes in (D) would have converted the DMBQ concentration to 1.34 μ M (Ca) or 0.94 μ M (Sr) DMBQH₂ over 30 trains, assuming that each flash transferred one electron per PSII center and no other redox reactions took place.

Addition of benzoquinone derivatives that are permeable to the outer membrane (Fig. 3.6B) reverses the lower O_2 yield in Sr-grown cells vs. Ca-grown cells [36]. Titrations of DMBQ with samples of known (optimal for oximetry) density were performed to determine the optimal concentration (250 μ M for all samples here), in a manner observed for all other cell types that we and others have investigated [34, 36, 46-48]. The integrated O_2 yield per WOC in the

presence of DMBQ (Fig. 3.6D) is 31% greater in Sr-substituted cultures than in Ca-grown. This would indicate that the lower yield of O₂ in Sr-grown cells without supplemental DMBQ (Fig. 3.6C) is due to an acceptor-side limitation, though the exact site is unclear due to the ability of benzoquinone derivatives to replace the native plastoquinone in the Q_B site, as well as accept electrons from both Q_A and Q_B while not bound within PSII [49]. Over the measurement period (100 minutes), the O₂ yield in both samples first increases and then decreases; this results from DMBQ equilibrating with the acceptor side and undergoing light-induced reduction to DMBQH₂, respectively [36].

The absolute per-flash quantum yield was determined to be 34.2 mmol O₂/mol D1 in Srgrown culture without DMBQ, as opposed to 60.5 mmol O₂/mol D1/flash in Ca-grown culture. These yields correspond, respectively, to 13.7% and 24.2% quantum efficiency. Peak quantum yields of 87.9 mmol O₂/mol D1 in Sr-grown culture and 67.0 mmol O₂/mol D1 in Ca-grown culture were achieved via addition of DMBQ, corresponding to 35.2% and 26.8% quantum efficiency, respectively. The quantum efficiency of Sr-grown culture therefore increases by 157% in the presence of DMBQ, i.e., in the absence of a previously unknown acceptor-side effect, a factor significantly greater than observed as a result of quinone addition *in vivo* in any other strain save those expressing extreme PSII-cyclic electron flow, such as *Chlorella ohadii* [36, 47].



Figure 3.7 Relative flash-oxygen yields from Ca- and Sr-grown cultures: (A) in the absence of exogenous electron acceptors, and (B) supplemented with 250 μ M DMBQ. Data are normalized at the steady-state for comparison and are the average of 30 flash trains at flash frequency (0.5 Hz). The fits to the VZAD program are shown in red. Residual values are plotted and represent the difference between experimental values and VZAD fits. For clarity, they have been offset by -0.1 for Ca-grown culture and -0.2 for Sr and do not exceed 4% of average yield. Fourier transforms of experimental data from (A) and (B) are given in (C) and (D), respectively. Measurements were taken at room temperature.

Addition of the electron acceptor DMBQ, as shown in Figure 3.7B, produces much stronger O_2 yield oscillations in both Sr-grown and Ca-grown cultures, extending over 10 and 7 cycles, respectively. DMBQ introduces some photoinactivation seen as a decreasing slope, which appears in model calculations as the inactivation parameter (ϵ) discussed later. Ca-grown culture oscillations remain shallower with DMBQ. Fourier transforms of these data, given in Figure 3.6C and D, provide a model-independent representation of the period of the WOC cycle at this flash frequency (P_{FT}). Addition of DMBQ to cells shifts the entire distribution from high period, indicative of high inefficiency, to lower periods (left to right). The most probable cycle period, the center of the central, and usually highest, peak, decreases from $P_{FT} = 5.27$ to 4.16 (Ca-grown) and from $P_{FT} = 4.72$ to 4.14 (Sr-grown). The Sr-grown culture has a significantly shorter peak period in the absence of DMBQ, and less of the relative contribution stems from higher period oscillations, reflecting higher efficiency of the WOC cycle in Sr-grown cells. These differences almost disappear in the presence of DMBQ, although the FT of the oscillations has lower amplitude in Ca-grown cells, reflecting the quicker loss of periodicity and more even Sstate distribution (detailed below). Additionally, DMBQ stimulates a process which results in a new peak at period 2, particularly in the Sr-grown culture.

Figures 3.6A and B show fits of the oxygen flash yields to the VZAD model, and the obtained parameters are summarized in Tables 3.2A and B for room and growth temperature, respectively. The WOC cycle peak period can be calculated from the WOC inefficiency parameters using the theoretical expression which relates them in the VZAD model [36, 42]. These values are given in Tables 3.2A and B, and are plotted on the experimental plot in Figures 3.6C and D, for samples without and with DMBQ, respectively. The agreement between the two periodicities determined is close, further confirming the accuracy of the VZAD model. In the absence of DMBQ, different dark-stable S state populations are observed dependent on Ca/Sr, typically about 50/40 for S0 and 50/60 for S1. An initial distribution somewhat closer to the ideal Kok ratio of 0.25:0.75:0:0 is thus observed in the Sr culture, reflecting decreased reduction of S1 in the dark. The population of the dark-stable S2 and S3 states typically increases with addition of DMBQ [36]; in Ca-grown cells the S2 state is populated, while in Sr-substituted cells it still decays under these conditions. The S3 state is more stable in Sr-substituted cells than the

native, however. Changes in the kinetics of decay of S2 and S3 are partially responsible for these altered dark-stable ratios, as shown in Figure 3.8.



Figure 3.8 Dark decay kinetics (Lifetimes) of the S2 and S3 states in Sr-grown and Ca-grown cultures. S2 lifetime in the absence (A) and presence (C) of 250 μ m DMBQ, respectively. S3 lifetime in the absence (B) and presence (D) of 250 μ m DMBQ, respectively. Kinetics were generated using one pre-flash to populate the S2 state and two pre-flashes to populate the S3 state, followed by dark period time (X axis) and ending with light flashes to determine the O₂ yield. The WOC inefficiency parameters (Table 2) were used to calculate the actual S state populations contributing to each flash. Y-axis units are the fraction of total centers in the specified S-state after dark incubation time.

Table 3.1 S2 and S3 state decay parameters at 23°C from fits of data to a biexponential model. Values after electron acceptor was added are given in parentheses.

Culture/S-state	A1	t1(s)	A2	t2(s)	R ²
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Ca/S2 (+DMBQ)	0.14 (0.13)	8.6 (19.7)	0.19 (0.23)	350 (450)	0.990 (0.990)
Sr/S2 (+DMBQ)	0.12 (0.10)	8.8 (8.4)	0.18 (0.22)	320 (390)	0.984 (0.997)
Ca/S3 (+DMBQ)	0.15 (0.14)	9.8 (5.7)	0.18 (0.22)	240 (550)	0.991 (0.988)
Sr/S3 (+DMBQ)	0.16 (0.11)	32 (16.6)	0.24 (0.32)	580 (820)	0.997 (0.986)

Kinetics of S2 and S3 decay in whole cells are given in Figure 3.8; parameters derived from fits to a biexponential decay model are given in Table 3.1. The lifetime of the S3 state in Sr-substituted culture is increased by approximately threefold, indicating that electron backflow to the WOC in this state is significantly slowed by the presence of Sr. There is little effect on the lifetime of the S2 state, however. The lifetimes of both the S2 and S3 states in both cultures are increased by DMBQ addition (Table 3.1), but the increase is comparatively small for S2, 29% for Ca and 22% for Sr, while substantial increases in the S3 lifetime of 49% (Sr) and 129% (Ca) are observed. The large difference between S2 and S3 indicates the yield of charge recombination, which is thought to occur via plastosemiquinone forms of Q_A and Q_B, is more influenced by DMBQ in the S3 state. This is consistent with a generally higher rate of recombination between electrons on the acceptor side and the most oxidized semi-stable state of the WOC.

3.3.3 Inefficiency parameters of the WOC cycle.

Condition	α	β	δ	3	S0	S1	S2	S3	P _{Calc}
Са	0.27	0.01	0.16	0.01	0.48	0.51	0.01	0.00	5.27
Sr	0.17	0.00	0.10	0.03	0.41	0.59	0.00	0.00	4.72
Ca+DMBQ	0.100	0.051	0.000	0.010	0.389	0.444	0.166	0.009	4.16
Sr+DMBQ	0.059	0.012	0.030	0.020	0.352	0.634	0.000	0.014	4.14

Table 3.2A WOC cycle parameters and dark S-state populations at measurement temperature (A) 23°C and (B) at 45°C. Flash rate 0.5 Hz; dark preincubation time 180 s.

Table 3.2B

Condition	α	β	δ	3	S0	S1	S2	S3	P _{Calc}
Са	0.24	0.00	0.20	0.00	0.50	0.50	0.00	0.00	5.58
Sr	0.10	0.00	0.08	0.01	0.45	0.50	0.00	0.05	4.34
Ca+DMBQ	0.095	0.116	0.000	0.003	0.440	0.469	0.090	0.000	4.10
Sr+DMBQ	0.042	0.000	0.071	0.000	0.459	0.396	0.027	0.119	4.12

The WOC cycle parameters obtained from fits to the VZAD model are reported at standard flash rate 0.5 Hz and temperatures of 23°C (Table 3.2A) and 45°C (Table 3.2B). In the absence of exogenous electron acceptor, the Sr-grown cultures have significantly smaller inefficiency parameters (α , β , δ). At 23°C (45°C) the misses, in the α column, are a full 10% (14%) fewer, indicating a more efficient capture of charge separation events than in Ca-grown cultures. Backward transitions, or δ , are 6% (12%) fewer, indicating similarly decreased probability of addition of an electron. In both cases, double hits, β , are small. Overall, the Sr-substituted PSII is operating 17% (28%) more efficiently over a full WOC cycle. Loss of centers by photoinactivation (ϵ) is small at 3% (1%), but Ca-grown cells show 3-fold less. Since photoinactivation (ϵ) can sometimes be irreversible due to permanent damage to PSII [42, 50], in the long run, this difference may amount to a major disadvantage for Sr cultures. However, the comparable rates of decay of yield over time (Figure 3.6C and D) indicate that most of this added inactivation is only temporary.

The two main inefficiency parameters (α , δ) are significantly reduced, nearly 3-fold, by supplementation with DMBQ. Ca-grown cells show a larger absolute improvement in performance. Double hits are substantially increased by DMBQ in Ca-grown cells (5% and 11.6% at 23°C and 45°C, respectively), but much less so in Sr-grown cells, suggesting the Sr-modified acceptor side is more tolerant to oxidation of the non-heme iron, the common source of double

hits [51], or some other redox modulation has taken place. Together with the increase in oxygen yield, these alterations in inefficiency indicate that DMBQ successfully removes the blockage of electron/proton flow on the acceptor side that limits WOC cycling in both cell types.

At the growth temperature (45°C, Table 3.2B) a general decrease in misses is observed, while backward transitions are somewhat increased (Ca) or unchanged (Sr). Double-hits are only observed in Ca-grown cells with DMBQ added, but are quite substantial under those conditions (11.6%), indicating that oxidation of the non-heme iron is substantially suppressed by Sr relative to Ca in some role on the acceptor side [34, 36, 46], or that delayed WOC cycling steps simply limit the rate of the second electron leaving the WOC (see Figure 3.11) [51]. At growth temperature, a significant fraction (5%) of WOCs in the S3 state are retained in Sr-culture with DMBQ added, but not the S2 state, consistent with the slower decay of S3 (Table 3.1). In summary, the Sr-culture shows a more ideal period closer to 4, lower miss and backward transition parameters, and a more stable S3 population (slower recombination), indicating a more efficient culture even without DMBQ (Figure 3.9). These parameters are far better than observed in the Ca-grown culture of most lower-temperature species [36].



Figure 3.9 Quality factor, $Q = 1/(\alpha + \beta + \delta + \varepsilon)$, as a function of the dark time between flashes for *T. elongatus* cells at 23°C. Q is the inverse of all WOC inefficiency parameters that cause damping of O₂ yield oscillations and was determined from data fitted by VZAD. The longest dark time between flashes was 10 s. The shortest dark time was limited to 0.75 s to allow time for integration of the complete O₂ signal between flashes. (•, **■** filled symbols) no DMBQ; (\circ , \Box open symbols) plus 250 µM DMBQ. Q was derived from the method used to generate data in Fig. 3.7 and Table 3.2, at a range of flash frequencies.

3.3.4 Rate of PSII turnover.

Temperature dependence of the maximum PSII turnover rate is given in Figure 3.10. These curves are obtained by measuring the light-saturated O_2 evolution rate, after correcting for the (low) respiration rate, and normalizing to D1 content. Data was modeled using the Arrhenius formula (k = Ae^{-Ea/(RT)}) [52] in the range 15-50°C. At 55°C and above, O_2 evolution sharply declines due to denaturation of PSII, while below 15°C the rate is negligibly slow for our instrumentation to detect. Accordingly, these data were excluded from the model.



Figure 3.10 Temperature dependence of the O₂ evolution rate in Sr- and Ca-grown cultures. All measurements were taken under continuous saturating (120 μ Ein/m²/s) red light in well-stirred culture. Rates of O₂/PSII-D1 are plotted vs. temperature in (A) and as an Arrhenius plot, In rate vs. 1/T, in (B). The data above 50°C are influenced by thermal deactivation and thus not used in B. "Activation energies" for oxygen evolution are calculated from the Arrhenius plot and reflect the entire WOC cycle. The data are the average of 3 measurements.

A nearly linear Arrhenius plot is exhibited by both cell types over this temperature range, but with different slopes. In Sr-grown cells, the PSII turnover rate has a 21% larger "activation energy", 86.8 vs 71.9 kJ/mol, resulting in a comparatively low rate of oxygen evolution at room temperature, at which *T. elongatus* is often studied, but a *higher* oxygen evolution rate at growth temperature. Thus, the yield of O₂ per PSII in Sr-grown cells relative to Ca-grown cells is higher at 45°C.



Figure 3.11 Flash train differences from living Ca- and Sr-grown *T. elongatus* culture as measured at (A) 22°C and (B) 47°C. All measurements taken with 3 s between flash trains. Fitting lines for individual S-states shown for convenience. Data shown is the average of 30 measurements. Transition times are recorded in Table 3.3.

As measured by WOC-dependent fluorometry (for a more thorough explanation, see Chapter 2), the total time for a WOC turnover is consistently longer in Sr-culture than in Ca-culture, regardless of temperature. Certain transition times are less reliable than others. In the case of Sr-grown culture at 22°C, it was not possible to obtain the final transition time from the data because the S3-S4-S0 transition did not reach its midpoint within the time window. As explained in Chapter 2, all results below 35 µs were produced by less than the light required to fully induce turnover in all centers and thus the S0-S1 transition times are questionable. However, certain key data can be obtained from this study. In Ca-grown culture, the S1 peak is quite intense at lower temperature, while the S2 peak is comparatively small and effectively combined with the S3 peak. At higher temperature, the S1 peak has become a shoulder in a large and distinct S2 peak. This is due to a larger initial S0 population in the dark at low temperature, as well as the S1-S2 transition itself being significantly regulated by temperature. We posit that this is due to the certainly temperature-dependent interconversion between the "open" and "closed" forms of the S1 and S2 states [53-56]. In the Sr-culture, at high

temperature the S1-S2 transition is actually longer than the S2-S3 transition, while the S2-S3 transition is comparable to that of the Ca-culture. It is likely, given the merged peaks, that the S1-S2 transition limits the S2-S3 transition under these circumstances. The Sr-WOC strongly favors the closed S2 configuration and thus transitions from the S1 state are constrained even when higher temperature facilitates conversion between forms of the S1 state [54]. The total time to reach the first oxygen molecule being evolved is substantially lengthened at both temperatures, though more so at higher temperature. This correlates well with the pseudo-Arrhenius behavior observed in Figure 3.10.

3.3.5 Site of acceptor side inhibition.



Figure 3.12 FRR fluorometric measurements of Ca- and Sr-grown cultures. (A) Comparison of trains of 50 pulses with PQ pool limits denoted. (B) Effect of addition of DCMU to culture. All measurements were done at 100 Hz and 23°C.

The site of limitation of electron flux on the acceptor side in Sr-substituted cells was investigated using two methods (Fig. 3.12). PQ pool functional size was estimated from the number of flashes in a train that reduce the yield of Fv/Fm by 50%. When the flash rate exceeds the rate of PQH₂ reoxidation, the pool fills and Fv/Fm decreases [33]. This experiment is shown in Fig. 3.12A for both cultures and shows approximately 28 PQ molecules per PSII in the Sr-

grown culture, as compared to 17 in the Ca-grown culture. As these are kinetically determined following darkness and assumed full pool oxidation, they represent upper estimates of the actual number of available PQ molecules. Localization to the Q_B site was conducted using the specific inhibitor DCMU. Addition of DMBQ in the presence of 2 μ M DCMU does not restore the yield of Fv/Fm in either Sr or Ca cells (Fig. 3.12B). It can thus be concluded that DMBQ acts by oxidizing at the Q_B site or downstream, and that Q_A - is not substantially involved.

3.4 Discussion

3.4.1 Flash O₂ WOC efficiency.

The slower light-saturated turnover rate of PSII in Sr-substituted core particles at room temperature observed in previous studies had been attributed to changes within the Mn₄SrO₅ core itself that slow the terminal O₂ release step [15]. The preceding experiments, however, conclusively indicate that the slower PSII turnover in Sr cells under saturating intensity actually results not from slower turnover of the WOC but from blockage on the electron acceptor side. Removal of this blockage upon addition of DMBQ results in the light-saturated turnover rate increasing from 58% of that of Ca-grown cells to 131%. Raising the measurement temperature from 23°C to 47°C also overcame this kinetic blockage, again consistent with a rate-limiting, temperature-sensitive activation process such as diffusion within the Q₈/PQ pool exchange site. This process was conclusively excluded from the WOC pocket by studies of the optimal turnover time, showing that light limitation of PSII occurred at light levels far below those which would theoretically limit the WOC itself and shifting the limiting step definitively to the acceptor side. Removal of the acceptor blockage allows the electron/hole pairs generated in PSII to be captured with greater efficiency by the WOC(Sr), with fewer misses and fewer backward transitions. The WOC(Sr) is more efficient in both capturing holes, as indicated by the lower miss parameter, and holding on to them in the dark, as indicated by the longer S3 decay time. The latter property indicates an advantage at low light intensity for WOC(Sr) as compared to WOC(Ca). A parallel can be drawn to the benefits of low-light and high-light isoforms of the D1 subunit of PSII, which are differentially expressed at low vs. high light intensities, respectively [33, 35, 57].

3.4.2 Backward transitions.

Backward transitions are notably less common in WOC(Sr) than WOC(Ca), 8% vs 20% at the growth temperature, respectively, and can be eliminated by modulating the acceptor redox environment with DMBQ. The backward transition parameter has been demonstrated to be essential for accurate fitting of O₂ oscillations and can dominate a large fraction of centers in some phototrophs (reaching levels so high as to functionally prevent oxygen evolution in organisms expressing extreme PSII-CEF) [36, 42, 47]. The mechanism of backward transitions and PSII-CEF is yet to be fully elucidated, but is distinct from classical cyclic electron flow around PSI. The function of CEF-PSII is posited to be in energy conversion by pumping protons across the thylakoid membrane, moving from stroma to lumen using light energy to convert previously stored energy of chemical bonds (PQH₂) into a proton gradient [36, 47]. Mechanistically, this vectorial proton pumping process differs from the scalar proton generation that takes place in water oxidation and converts stored redox energy into ion gradient energy and ultimately ATP in that it would both remove a proton from the stroma and deposit in in the lumen, thus facilitating ATP synthase activity from both sides.

3.4.3 Recombination Lifetimes.

The longer S₃ state recombination half-life in WOC(Sr), 2.4 times what is observed in WOC(Ca), suggests a higher Sr-dependent reorganization barrier (structural rearrangement) upon S3 \rightarrow S2 recombination. This higher reorganization barrier slows the rate sufficiently that DMBQ provides little benefit, which suggests a switch from native regulation by availability of electrons on the acceptor side to regulation at the WOC itself, which limits uptake of electrons. A large structural rearrangement during the S2 \rightarrow S3 transition for both WOC(Ca) and WOC(Sr) in core particles has been suggested by XAS [58, 59], and the large Arrhenius activation barrier for O₂ production suggests this remains true in whole cells. A less significant reshaping of the S2 state in WOC(Sr) is supported by EPR and XAS [12, 35, 56, 60-67], with the closed S2 configuration (intermediate spin S = 5/2 ground state) favored by Sr-substitution, while the open configuration (low spin S = 1/2 ground state) is favored in Ca cultures[15, 61, 68]. The balance between the two states, which both exist in statistical equilibrium under both conditions, is altered by Sr-substitution.

3.4.4 The quality factor.

Quality factor $Q = 1/(\alpha + \beta + \delta + \varepsilon)$ is a convenient analog of the relative probability for an unimpeded transit through the WOC cycle as a function of flash rate, irrespective of which type of inefficiency occurs [33, 42]. Although model dependent, this simple indicator makes clear the marked benefit in WOC efficiency afforded by Sr-substitution at all measured flash rates. This also illustrates a major form of regulation that occurs *in vivo* in both types of cells – the redox poise on the acceptor side, readily controlled with DMBQ. This result points to a possible strategy for improving light to redox energy conversion by increasing the size of the PQ pool or by increasing the redox gaps. As loss of backward transitions will necessarily stem from such a modification, this strategy will have negative consequences on PSII-CEF, however. *In vivo* measurement of Q by O₂ flashes is restricted to slow rates. Q can also be measured at much

higher flash rates using Chl fluorescence, but emission intensity is low in Sr-substituted *T*. *elongatus* cells due to antenna rearrangement (and in both strains due to high phycobilin concentrations causing interference due to fluorescence uptake). This technique is demonstrated and explained in greater detail in Chapter 4.

3.4.5 Growth rate and PSII-WOC.

The WOC turnover rate is not the limiting factor in electron transport at the optimal growth conditions for Sr-substituted *T. elongatus* cells (approximately 40 μ Ein/m²/s light intensity and 45-50°C). The rate is primarily limited on the acceptor side. At this light intensity, it would be able to complete all S-state transitions substantially more quickly than it would receive enough light to advance through the next cycle [11]. Additionally, more centers advance and more O₂ is formed at this relatively low light intensity because of the higher efficiency of the WOC(Sr) cycle. The reported 8-fold slower O₂ release kinetic of PSII-WOC(Sr) versus PSII-WOC(Ca) [15] is not a limiting factor at this low light intensity, and indeed, this kinetic is likely a temperature-induced artifact. Photoinhibition stops growth of both Ca- and Sr-culture far before the limiting light intensity for WOC(Sr) operation is reached. However, even at low light intensity the larger photoinactivation parameter (ϵ) reflects this greater light sensitivity of WOC(Sr).

The transition times reported here at 22°C are longer than those observed by Westphal et al., working with isolated thylakoids (Table 3.3), since those were obtained in the presence of electron acceptors as a necessity of working with thylakoids, which lose the native PQ pool during preparation [11, 69] (regulation of WOC transition times by the available acceptors is discussed in Chapter 2). However, the overall times remained comparable, as the longer steps are less reliant on PQ pool reoxidation as a limiting factor [70].

Preparate	Spinach+Ca, 25°C	Spinach+Sr, 25°C	<i>T. elongatus</i> +Ca, 22°C	<i>T. elongatus</i> +Sr, 22°C	<i>T. elongatus</i> +Ca, 47°C	<i>T. elongatus</i> +Sr, 47°C
S0-S1			52 µs	47 μs	30 µs	38 µs
S1-S2	200 µs	900 µs	750 μs	1700 μs	120 μs	750 μs
S2-S3	450 μs	1300 µs	1300 µs	3200 μs	500 µs	620 μs
S3-S4-S0	5000 μs	18000 μs	4800 μs	~15000 µs	1050 µs	2700 μs
Total	5650 μs	20100 μs	6900 µs	~20000 µs	1700 µs	4100 μs

Table 3.3 Comparison of S-state optimal transition times in Ca- and Sr- WOCs in spinach thylakoid preparates [11] and living *T. elongatus* culture.

Measurements of the S0-S1 transition have historically not been reliable, and were not reported by Westphal et al. [11, 71-74]. The accelerated S1-S2 and S2-S3 transition times observed in spinach thylakoids as compared to living cells at comparable temperature are, as explained in Chapter 2, consistent with the use of electron acceptors, as is the marginally slower S3-S4-S0 transition. The massive decrease in optimal transition time by *T. elongatus* upon measurement at its normal growth temperature is consistent with the rise in rate of oxygen evolution, though it is likely that downstream processes are the more direct constraint on rate oxygen evolution.

The delay in oxygen evolution by Sr-culture persists at all temperatures observed. However, this is a distinctly temperature-dependent effect. A nearly fourfold increase in oxygen evolution time was reported by Westphal et al., as compared to a 2.4-fold increase seen herein using living culture at high temperature [11]. This limitation occurs at a light intensity under which the organism cannot survive, however. The ideal light conditions for growth of *T. elongatus*, regardless of Ca or Sr, are 40 μ E/m²/s [37, 75]. It is possible to model the light intensity required to exceed optimal turnover times as the average light intensity of a train of single turnover flashes at intervals of longest single transition time, i.e., the S3-S4-S0 transition. In this system, STFs are 20 μ s pulses at 80,000 μ E/m²/s, so in this organism, just under 600 μ E/m²/s is the light saturation level of the WOC transitions, far exceeding what the downstream electron transport chain can use (saturation of oxygen-evolving rate is achieved before 120 μ E/m²/s). Despite the increased time required for oxygen evolution, the Sr-WOC is still competitive with the Ca-WOC under *T. elongatus*' fairly low-light growth conditions, where its ability to retain charges longer allows for more efficient PSII operation.

As noted in Fig. 3.10, the Sr-grown culture generates more O₂ per PSII-D1 at the growth temperature (45°C) and in the absence of quinones. However, above 50°C this effect is replaced by a sharp decline, unlike Ca-grown cells which are proportionately stable up to 65°C. The lower relative concentration of PSII-D1 in Sr-grown cells also offsets the efficiency advantage on a perunit-biomass basis at 45°C, as seen in the highly similar growth rates observed for Sr and Ca cells (Figure 3.4). The seemingly contradictory observed effects of Sr substitution on growth and O₂ evolution can be reconciled in this manner. The organismal response to the more active PSII centers in Sr-grown cells at 45°C is to lower the number of active PSII centers per Chl. This suggests that the energy flux created by PSII is the primary determinant of growth rate of *T. elongatus*. This phenomenon is explored further in Chapter 4.

3.4.6 Period-2 oscillations.

Sr-substitution increases the period-2 oscillations in O_2 flash yield (Fig. 3.7D). Period-2 oscillations are a common feature in many phototrophs and typically appear when the PQ pool is oxidized using a benzoquinone electron acceptor [36]. The likely cause is binary modulation of recombination with electrons in the Q_B site. As we used a constant (S state independent) miss parameter, this feature is not captured in the VZAD model. In Sr-substituted culture, the double-

hit parameter is extremely low (Table 3.2A and B), even in the presence of DMBQ, and cannot explain the extent of observed period-2 oscillations. The five-fold higher rate of double-hits in Ca-grown culture occurs concurrent with a lower degree of period-2 oscillation (Table 3.2A) [41, 42]. Furthermore, the sole way to generate a period-2 oscillation in O₂ yield by this method is by two consecutive double-hits from S0 to S2 and S2 to S4 [41, 42]. Unless double-hits are strongly preferred in these particular states, which is energetically unlikely taking the relative redox potentials of the S-states at face value, it is more likely that a larger period-3 peak or a tail to the right of the central peak would be observed, simply because only one double hit to the S0, S1, or S2 state is required. As evident by the lack of either a period-3 peak or a tail feature, this phenomenon cannot be attributed to classic double-hits as proposed by Kok.

An alternative explanation for period-2 oscillations in O₂ yield, one supported to some extent by observed kinetics, could be a two-electron oxidation of water to hydrogen peroxide (H_2O_2) instead of four-electron oxidation to O₂, possibly followed by dismutation, $H_2O_2 \rightarrow O_2 +$ H_2O , external to the WOC cycle [76-78]. PSII(S2) has been previously shown to both produce hydrogen peroxide by oxidizing water [77-80] and further oxidize it to oxygen [76], so some PSIIs may preferentially be performing this function. The slight increase in the inactivation rate of the WOC (ϵ) observed in Sr-substituted culture (Table 3.2) could also be explained by such a mechanism, as hydrogen peroxide readily forms hydroxyl radicals upon one-electron reduction (Fenton chemistry). Recent evidence indicates that hydrogen peroxide, produced as a result of imbalances in redox state, acts as a signaling molecule to activate classic PSI-CEF in higher plants *in vivo* [81]. At present we have no direct evidence for increased hydrogen peroxide formation in WOC(Sr). However, we do have significant evidence to indicate a higher-energy S2 state, which should facilitate peroxide generation, as detailed below.

3.4.7 Thermodynamic model.



Figure 3.13. Changes in the rate constants for the forward reaction (k_F) and back reaction (k_B) between Y_Z and the Ca/Sr-substituted-Mn₄O₅ WOC are related to the equilibrium constant (K_{eq}) and Gibbs free energy (ΔG) change. $\Delta \Delta G$ (Ca \rightarrow Sr) = RTIn(K_{sr} - K_{Ca}) is the resulting free energy change due to Ca replacement by Sr.

A thermodynamic model of the WOC is given in Figure 3.13. The effects of Srsubstitution are supported by the structural model in Figure 3.1A, which derives from the 2.1 Å resolution X-ray diffraction (XRD) structure of *T. elongatus* PSII-WOC(Sr) and the 1.9 Å resolution structure of PSII-WOC(Ca)[29, 30]. There is a deep chemical basis for the alterations caused by Sr-substitution in the PSII-WOC [35, 66]. When comparing complexes with coordination number 8 and 6, Sr²⁺ has 0.14-0.18 Å larger ionic radius than Ca²⁺ (11-16% larger). The closed shell (noble metal) electronic configuration and inaccessibility of ionization potentials to the 3+ state forces both to act solely as spherical ionic cations, not as redox active species. To remove one water molecule from Ca²⁺ (Sr²⁺) coordinated to 5, 6 or 7 water molecules, the experimental gas phase dehydration enthalpies are 26.7 (23.9), 22.0(20.9), and 17.7(17.1) kcal/mol, respectively [82]. A quantitative estimate is thus available for the available energy difference in restructuring the first coordination shell. As seen in Figure 3.1, Sr is displaced toward Yz relative to the position of Ca in the XRD structure of *T. elongatus* PSII-WOC, increasing the three Sr-O(core) distances by an average of +0.09 Å and decreasing the $Y_2(O)$ -Sr distance by -0.26 Å. W3 and W4, the normally Ca-coordinated water molecules in the WOC first ligand shell, remain coordinated to Sr and consistently are shifted toward Y_2 (by the substantial distance of 0.30 Å in the case of W3). QM/MM modeling by Vogt et al. supports the validity of these shifts [83]. The aforementioned structural changes and differences in ionic potentials predict a change in relative reduction potentials across Y_2 -WOC, with that for Y_2^+/Y_2 increasing and that for $Mn_4O_5(S_{i+1}/S_i)$ decreasing. This thermodynamic shift predicts a change in population based on the Nernst equation, as given in Figure 3.13. The predicted shift qualitatively mirrors the experimental behavior of PSII-WOC(Sr), demonstrating an altered population ratio favoring hole transfer from the reaction center to the WOC: 1) fewer misses, 2) shift in dark S state populations from S0 to S1, 3) decrease in Fv/Fm, 4) greater stability of the S3 state (substantially longer decay time). The predicted free energy was calculated using the Nernst equation $\Delta\Delta Go = RTln(K_{Sr} - K_{Ca})$ and the equilibrium constant:

$$K_{eq} = \frac{S_{i+1}}{S_i} \frac{Y_z}{Y_z^+}$$

The values for K_{Sr} and K_{Ca} (1.23 and 0.84, respectively) were estimated using the dark populations in Table 3.2 for S0 and S1, assuming $\frac{Y_{z}}{Y_{z}^{+}} = 1/(1+\alpha)$. The predicted $\Delta\Delta G\circ = -2.3$ kJ/mol favors a lower energy of the WOC relative to Y_z, i.e. a more oxidizing WOC. The predicted free energy is comparable to the difference in hydration energies for removal of a water molecule bound to 7-coordinate Sr²⁺/Ca²⁺ in the gas phase, as noted above. A shift in reduction potential of Q_A⁻/Q_A in the S2 state resulting from Sr-substitution was previously determined by Kato et al. [14].
3.4.8 Implications for the mechanism of water oxidation.

The preceding results thoroughly demonstrate that WOC(Sr) is a far better catalyst than WOC(Ca) at low turnover rates where O_2 release is not rate-limiting. The decrease in charge recombination caused by the thermodynamic stabilization of the WOC(Sr) relative to Y_2^+ causes the marked improvement in efficiency and quantum yield. With stability come disadvantages under some conditions, though; one is the larger activation barrier to transit through the full catalytic cycle, and another is the resulting slower kinetics of O_2 release.

The intermediate spin state of the S2 state WOC(Sr) characterized by the g4.1 EPR signal (S = 5/2 ground state; "active" S1 precursor) [8] is favored over the low spin S = 1/2 ground state ("resting" S1 precursor), while the S2 state of WOC(Ca) can exist in either. Magnetic susceptibility changes measured for the full catalytic cycle at room temperature support these EPR phenomena, showing that two magnetically distinct forms of the WOC(Ca) exist, corresponding to a low spin (resting form) and intermediate spin (active form) [9]. The "closed" structural form of the WOC is attributed to the intermediate spin state, in which O#5 is part of the SrO₄Mn₃ cluster and does not bridge to Mn#4 [84, 85]. This assignment is consistent with the Sr-substituted S1, state which shows the "closed" WOC form in which Mn#4 is +0.22 Å further away from Sr than Ca (Figure 3.1). This study of WOC(Sr) strongly supports the hypothesis that the intermediate spin form of the S2 state possessing the "closed" cubane subcluster is the active form in the catalytic cycle, and that this is the state which exhibits higher quantum efficiency for O₂ production and has slower recombination rate than the low spin S = ½ form.

3.4.9 Site of acceptor side inhibition.

While the models in Figures 3.1 and 3.13 thoroughly explain the observed effects and generally complement prior *in vitro* studies of Sr-substitution [3, 5-8, 10, 15, 16, 61], some phenomena warrant further scrutiny. The site of inhibition of electron flux on the acceptor side of Sr-substituted cells can be securely placed at the Q_B site by the two experiments given in Figure 3.12. A significantly larger PQ pool is synthesized by Sr-grown cells, likely as an attempt to overcome the flux limitation at Q_B and in turn the lower O₂ yield. However, this enlarged PQ pool extracts the same number of photo-electrons across a culture. Decreased PSII-CEF in WOC(Sr), shown by fewer backwards transitions, further demonstrates the inability of reverse electron flow from PQH₂ to pass through the Q_B gate into the WOC(Sr) [86]. Only when DMBQ is added does the forward flux of electrons through Q_B increase while the backward flux is eliminated.

It is likely that growth on Sr affects an unexpected site on the acceptor side [1, 5-7, 10, 40, 87]. Khan et al. [88] demonstrated a potential function of the glutamate-rich protein domain near the non-heme iron in maintaining a balance of cations in that region using spinach PSII-containing membranes [34, 36, 40]. Calcium may be present in this cation balance, and indeed Khan et al. showed limitation of light driven electron transport upon binding of calcium to the glutamate sites. However, the inhibition by Ca²⁺ is weak (2 mM K_D) and showed no difference from Sr²⁺, suggesting a different non-specific origin than what is reported herein. Other potential causes for inhibition involve modification of redox potential at the acceptor side resulting from strontium substitution [14] or modification of the redox state of the PQ pool itself resulting from strontium substitution elsewhere in the cell, as well as a site-specific role on the acceptor side blocking a proton-cation exchange mechanism (see Chapter 5 for a more detailed explanation).

3.5 Conclusion

O₂ evolution rates were shown to *increase* in cultures containing WOC(Sr) on a per-PSII basis after a concurrent limitation on the acceptor side is removed. This limitation appears to be primarily based on cycling of plastoquinone between the Q_B site, the PQ pool and cytochrome $b_6 f$, which is the usual limitation to PSII flux. The WOC(Sr) is thermodynamically stabilized relative to Yz, resulting in increased oxygen evolution in the optimal growth temperature range of 45-50°C at the cost of an increased thermodynamic barrier and thus poorer performance at low temperatures. The culture responds to this increase in WOC cycle efficiency by decreasing the PSII concentration, suggesting that a fixed cell growth rate is maintained by some other control mechanism and PSII expression is simply modulated to meet the necessary "energy budget" (also discussed in Chapter 4). Sr-substitution gives the benefits of increased efficiency of WOC oxidation and slower S3 state charge recombination, which enable better performance at low light intensity. However, several detrimental effects are also observed, including a slower O₂ release step, a greater photoinactivation rate (even at low light intensity), bleaching at high light intensities, a lower and narrower temperature range for growth, and possible formation of hydrogen peroxide, all making Sr-culture less robust than Caculture.

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80

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Chapter 4. The Role of Chloride in the WOC Pocket of PSII

4.1 Introduction

The generation of oxygen from water produces one proton per electron transferred, for a total of four per oxygen evolution [1]. These protons must be removed from the WOC region, as they will alter the local pH, interact with the protein environment, and facilitate charge recombination if left near the WOC. The two chloride atoms found near the WOC within PSII have long been speculated to facilitate proton removal from the WOC [2-10]. The usual mechanism proposed for this role is the prevention of salt bridge formation within PSII, which would block the channels between the WOC region and the lumen [2, 6, 9], though other studies have only found such a role for a single chloride [11, 12] and some explanations simply claim chloride stabilizes the WOC itself [9, 13]. These mechanisms have been investigated in large part by substitution of chloride with bromide, which has been shown to decrease, but still permit, PSII activity [14]. Substitution with other small monovalent anions, and halides in particular, has been attempted [15], and some other ions may functionally replace chloride, but all decrease functionality. Bromide is the most widely studied substituent, as the most similar to the native chloride in chemical properties. Most other chloride substituents will not restore PSII operation or are prone to reactions elsewhere in PSII or the cell as a whole which may interfere with observations of PSII operation [16-18].

In general, if a decline in PSII activity resulting from bromide substitution is indeed due to delayed or inhibited removal of protons from PSII, we may expect to observe certain specific phenomena. Proton release from the WOC pocket occurs to a different extent on each oxidation of the WOC (S-state transition), though how many protons are released on each transition is a matter of some debate [1, 19-23]. What is generally agreed is that the least proton release, ranging from zero protons to fewer than the number of active PSIIs, occurs on the S1-S2 transition, and the most, at least one and no more than two protons per PSII, occurs on the S3-S4-S0 transition. Thus, if proton removal is delayed, we may expect a decrease in the optimal transfer time through that transition, but little effect on the S1-S2 transition (or S2-S1 decay). Operation of the WOC in general should be slowed and constrained to lower operating rates (and therefore lower light intensities). Furthermore, oxygen yield should be decreased irreversibly in general as failure to remove protons results in production of dangerous chemical species within PSII, either in the immediate vicinity of the WOC, resulting in WOC inactivation, or in the proton channels, resulting in blockage of those channels [24-26].

In this chapter, the hypothesis that the role of chloride near the WOC of PSII is to remove protons generated by water oxidation is tested exhaustively and proven true. By substituting chloride for bromide in living cells of *Thermosynechococcus elongatus*, we demonstrate all of the effects predicted above. This work extends the body of prior research into the role of chloride using a range of recent techniques for biophysical characterization of PSII in living cells, both supporting the long-established hypothesis and introducing quantum calculations and efficiencies not available in prior studies.

4.2 Materials and Methods

All cultures used were grown at 45°C in replete or Br-substituted DTN medium [27] in volumes of 150 mL with bubbling of 1.0% CO₂-supplemented air under 40 μ E/m²/s continuous light from Philips Silhouette cool white fluorescent lamps, following the method of Sugiura [28] with modifications as per our prior work with this organism [29]. All cultures were derived from a *Thermosynechococcus elongatus* His-tagged CP-47 mutant strain provided by Dr. Sugiura (henceforth referred to as *T. elongatus*). All samples were taken from culture in mid-exponential growth. For determination of growth phase of sampled cultures, optical density (OD) was measured at 730 nm using a Thermo Scientific Evolution-60 spectrophotometer. Chlorophyll *a* (Chl *a*) concentration was determined using this instrument, after methanol extraction from culture [30].

Quantification of the D1 subunit of PSII (Figure 4.1) was carried out via polyacrylamide gel electrophoresis and Western blot against a D1 protein standard (Agrisera) using primary antibodies targeted to a conserved PSII sequence and horseradish peroxidase secondary antibody/luminol detection. Western blots were conducted in triplicate and results averaged. All samples were adjusted to equivalent chlorophyll concentrations of 3 µg/mL before blotting for ease of comparison to other data. The accuracy of this technique was previously demonstrated by EPR spectroscopy [29].



Figure 4.1 Scanned Western blot of D1 protein extracts from Br-grown (second from left) and native (left) cultures. Extracts were standardized to equivalent chlorophyll content immediately prior to electrophoresis. Quantifications were made using a D1 standard series (Agrisera, rightmost four columns). Ladders added for clarity.

Oxygen was detected electrochemically by a custom-made Clark-type electrode using single turnover flashes (STF) of 20 μ s duration applied at a range of flash frequencies [31] and delivered from a LED light source with intensity 32,000 μ E/m²/s. Individual flash oxygen traces were integrated to obtain absolute quantum yields per flash by comparing to a standard of known concentration. Steady-state oxygen yields from multiple flashes in a train of flashes following 2 min dark incubation were also averaged and plotted separately as in Figure 4.3. For comparing the relative oscillations in oxygen yield and determining WOC cycle parameters, as in Figure 4.4, individual flash yields were normalized to the same steady-state value (1) after oscillations decayed to the point that no distinct variance in steady yield was observed.

The absolute O_2 quantum yields (mol O_2 per flash/mol PSII-D1) were obtained by normalizing to the number of PSII centers measured by ChI *a* concentration and EPR spectroscopy. Current generated by the reduction of O_2 following a single flash was integrated to obtain the total charge, which corresponds to O_2 consumed at the electrode (4 e⁻ per molecule) [29, 31].

S-state decay lifetimes were determined by advancing a dark-acclimated culture to the desired state using flashes, followed by dark incubation for variable time in this state, followed by rapidly advancing to oxygen evolution via flashes [29, 31-34]. The populations were corrected using the WOC inefficiency parameters listed in Table 1. Where specified, cultures were supplemented with 250 μ M 2,5-dimethylbenzoquinone (DMBQ; Sigma-Aldrich) in order to bypass any effects of bromide substitution localized to the acceptor side of PSII [29, 31].

All WOC cycle parameters and S-state populations [35] derived from flash oxygen measurements were obtained by data fitting using the VZAD model [29, 31, 36]. Fourier transforms of oxygen evolution measurements were determined using a fast Fourier transform algorithm available as part of the VZAD software suite (available online at: http://rutchem.rutgers.edu/dismukes-software).

FRRF measurements were performed using a custom-made fluorometer [32] and samples of the same live cultures used in oxygen evolution measurements. Chl *a* fluorescence emission was measured using trains of 50 each 20- μ s single-turnover flashes at 100 Hz. This fluorometer was equipped with an 80,000 μ E/m²/s laser light source at wavelength 660 nm.

S-state optimum transition time measurements were carried out using the same custom-made FRR fluorometer as used for FRRF measurements. Culture samples were dark-adapted for 3 minutes, then subjected to a train of 1000 "quarter-turnover" 5-µs flashes delivered at 10-µs intervals. The samples were then incubated in darkness for 3 s to allow reoxidation of the acceptor side of PSII while retaining scrambled S-states. Subsequently, another identical train of 1000 flashes was applied and the difference in fluorescence between

the two trains was taken. Peaks in this difference plot represent S-state transitions, as different populations of each S-state were present between the two trains. A more complete explanation of this technique is given in Chapter 2.

Lumen pH was measured in a custom-made fluorometer using the pH-sensitive fluorescent dye 9-aminoacridine (9-AA). UV excitation was supplied at wavelength 365 nm and excitation optical power 2 mW from a LED source, model NSHU590B by Nichia, Japan; emission was measured at 420-450 nm using interference and color glass filters [37-39]. A series of 100 ms pulses at 1 Hz were used to excite 9-AA. To activate PSII, a continuous, saturating red LED light source (660 nm, 120 μ Ein/m²/s) was applied [40-42]; this light was turned off for reequilibration in darkness [43]. 20 μ M 9-AA was added to all samples. As a control, samples without 9-AA and sample medium with 9-AA but lacking cells were also subjected to the same technique and results were subtracted from the sample yield. As a further control, experiments were replicated in the presence of 100 μ M dinitrophenol, a proton gradient uncoupler [44].

4.3 Results

4.3.1 Cell growth rate and pigments.

Br-culture was grown in Br-substituted medium for four months (10³²-fold dilution) prior to any measurements in order to fully adapt to conditions. The growth rate of adapted cells at 45°C is shown in Figure 4.2. All experiments shown were conducted on culture in midexponential phase growth, at which point equal growth rates are observed between Br- and Clculture.



Figure 4.2 Growth curves of native and Br-substituted *T. elongatus* culture as measured by optical density (absorbance at 730nm) and chl *a* concentration (absorbance at 665nm). All points are the average of 5 replicates.

Br substitution affects Chl *a* and phycocyanin content, altering the antenna composition. To avoid skewing of data resulting from Chl *a* present at PSI, we normalized oxygen evolution data to PSII reaction centers. The chlorophyll:D1 ratio was determined by Western blot (Fig. 4.1) in triplicate. The chlorophyll:D1 ratio was found to be 1526 in native culture and 1317 with Brsubstitution (14% decrease from Br-substitution). The native ratio is consistent within 2% of what was observed in the Sr-substitution study described in Chapter 3 [29]. This decrease in chlorophyll:D1 ratio is consistent with 11% decreased chlorophyll per biomass in Br-culture (as measured by optical density at 730 nm) at sampling conditions, and generally decreased chlorophyll as a fraction of biomass across the growth cycle, despite comparable growth rates.

Alteration to phycobilin content resulting from Br-substitution is fairly minor, constituting a 7.4% increase (from 6.7 phycocyanin molecules per PSII-D1 in native culture to 7.2 per PSII-D1 with Br). Phycocyanin content was determined by the method of Lawrenz et al. [45] and normalized to the PSII-D1 content.

4.3.2 Flash oxygen quantum yields.

Quantum yields (QYs) of oxygen from CI- and Br-culture samples were obtained using flash oximetry at a flash rate of 0.5 Hz and room temperature conditions. Values given in Figure 4.3 are absolute quantum yields of oxygen per PSII-D1 as measured by Western blot (Figure 4.1). As seen in Figure 4.3A, there is some decline in oxygen QY and oscillation quality resulting from bromide substitution, resulting in average yields of 80% of what is observed in the native culture. This is consistent with prior studies [9, 14, 46, 47]. Figure 4.3C demonstrates the typical decline in quantum yield over time on the electrode under dark conditions, with similar behavior observed between the native and Br-substituted cultures. The lower yield from Brsubstituted culture remains nearly constant, reaching 77% at the end of the flash train sequence.



Time, min.

Figure 4.3 Representative flash oxygen yield of living *T. elongatus* cells measured (A) without and (B) with addition of 250 μ M DMBQ, in Cl- or Br-medium, at 23°C. All data shown is the averaged result of 30 flash trains: (C) in the absence of exogenous electron acceptors, or (D) in the presence of 250 μ M DMBQ. Each point on the Y axis is the average of 50 flashes separated by 2 sec (0.5Hz flash rate) and summed from 30 flash trains each separated by 2 min dark adaptation. The cumulative flashes in (D) would have converted the DMBQ concentration to 1.32 μ M (Cl) or 1.53 μ M (Br) DMBQH₂ over 30 trains, assuming that each flash transferred one electron per PSII center and no other redox reactions took place.

Addition of DMBQ as an *in vivo* electron acceptor [31] results in a slight increase in oxygen yield in both cultures which preserves a similar ratio, as well as the commonly seen substantial increase in oscillation duration and quality. Titrations with DMBQ were performed to determine the optimal concentration (250 μ M), as consistent with prior studies [29, 31, 48, 49]. With DMBQ (Fig. 4.3D), the integrated O₂ yield per WOC at peak in Br-substituted culture is 79% of the native peak. This would appear to confirm a donor-side role of bromide in inhibiting oxygen yield. Over the measurement period, the O₂ yield in both samples first increases and then decreases, due to the time for DMBQ to equilibrate with the acceptor side and for light induced reduction to DMBQH₂, respectively [29, 31]. However, in Br-grown culture the decline in yield during consumption is sharper. This phenomenon implies an increased degree of WOC inactivation dependent on the increased quinone availability.

The absolute quantum yields per flash were found to be 61.5 mmol O₂/mol D1/flash in Cl-grown culture and 49.0 mmol O₂/mol D1/flash in Br-grown culture, without DMBQ, corresponding to 24.6% and 19.6% quantum efficiency, respectively. In the presence of DMBQ, peak quantum yields of 67.8 mmol O₂/mol D1 in Cl-grown culture and 53.8 mmol O₂/mol D1 in Br-grown culture were observed, corresponding to 27.1% and 21.5% quantum efficiency, respectively. By the end of the measurement period, however, the quantum yield of Br-culture falls to about half what is observed in the native culture under similar conditions. Quantum yields in native culture are highly similar to those previously observed [29, 31].

4.3.3 WOC cycle inefficiency parameters.



Figure 4.4 Representations of flash-oxygen measurement data from Cl- and Br-grown culture in the absence of exogenous electron acceptors: (A) Flash oxygen yield for trains (as in Fig. 1) of 20 flashes, normalized to average, at a frequency of 0.5 Hz. Data shown is the average oxygen yield for each sequential flash over a period of 30 STF trains. Data was fitted using the VZAD program to generate the fits shown in red, the residuals, the values obtained in Table 1, and; (B) as in (A) but supplemented with 250 μ M DMBQ. Residual values in (A) and (B) represent the difference between experimental values and VZAD fits and do not exceed 8% of average yield. (C) and (D) are Fourier transforms of the data shown in (A) and (B). The vertical lines in (C) and (D) denote the Kok optimum period of 4 flashes.

Fits of the (normalized) flash oxygen yields to the VZAD model for both cultures with

and without DMBQ are given in Figures 4.4A and B, and the resulting parameters are

summarized in Table 4.1. The VZAD model fits are highly accurate to the data, giving root-mean-

squares deviations of 3.7% and 3.0% for Cl- and Br-cultures without DMBQ and 2.5% and 3.4% with DMBQ. Initial dark-stable S-state populations are altered by Br-substitution regardless of the presence of DMBQ, generally favoring a more reduced WOC and lower S1 populations, though presence of DMBQ allows retention of S2 in comparable quantities to the native condition. Br-substitution exacerbates the normal reduction away from Kok's ideal S-state distribution of 0.25:0.75:0:0 [35, 50].

A model-independent Fourier transform of these data allows the periodicity of oxygen yield to be observed. With DMBQ, the optimal, near-4 flash cycle (at center, in figures 4.4C and D) dominates oxygen production, with a minor contribution from a period 2 cycle (at right) and some inefficiency observed (at left) with or without Br-substitution. However, the lower peak of Br-culture without a comparable decline across the rest of its curve, as compared to the Clculture, indicates a less pronounced ideal periodicity and contributes to the longer average period (as in Table 4.1). Without DMBQ, a more pronounced version of this same effect is observed. The central peak is shifted to the left in both samples due to increased recombination as compared to the acceptor-supplemented sample, but in the case of the Br-culture the oscillations decline so quickly that the central peak is much smaller. Generally, the WOC cycle is confirmed to be less efficient in Br-culture.

Condition	α	β	δ	3	S0	S1	S2	S3	Period
Cl	0.22	0.04	0.11	0.02	0.34	0.63	0.03	0	4.92
Br	0.22	0.00	0.15	0.01	0.55	0.45	0.00	0	5.19
CI+ DMBQ	0.06	0.06	0.03	0.01	0.34	0.53	0.13	0	4.13
Br+ DMBQ	0.10	0.01	0.07	0.01	0.42	0.46	0.12	0	4.33

Table 4.1 Expanded Kok parameters and initial S-state populations as derived by fitting of raw data in Fig. 4.4 using the VZAD model. Of particular note are the beta, delta, and period parameters, all of which are potentially limited by proton removal from the WOC region.

WOC cycle parameters in Table 4.1 were generated from fits to the VZAD model of data obtained at flash rate 0.5 Hz and 23°C. In the absence of electron acceptor, the native culture behaves much as previously observed [29], exhibiting a high miss parameter (α), a small number of double hits (β), and moderate backward transitions (δ), combining for a periodicity near 5. Br-substitution has no effect on misses, but double-hits are completely eliminated. More backward transitions (δ) are observed, however, combining to produce an overall 5.5% increase in period length. The increase in backward transitions without a corresponding increase in misses suggests an additional need to supply electrons to the WOC pocket, one which cannot necessarily be filled by classical recombination [31].

In native culture, addition of DMBQ significantly reduces (by nearly 4-fold) the two main inefficiency parameters (α , δ). Furthermore, double-hits increase and collectively, the period of WOC cycling is greatly decreased. The improvement is far less pronounced in Br-culture. Double-hits remain negligible, while backward transitions are only halved and more misses are observed than in the native culture. The effects of this lessened improvement to cycling efficiency can be observed in Figure 4.4B; whereas in Figure 4.4A the curves follow each other closely, there is a greater separation between the flash-specific oxygen yields of the two cultures. Br-culture retains a longer period length accordingly.

4.3.4 S-state generation and decay lifetimes.



Figure 4.5 Dark decay kinetics (lifetimes) of the S2 and S3 states in Br- and Cl-grown cultures. S2 lifetime in the (A) absence, and (C) presence of 250 μ m DMBQ added. S3 lifetime in the (B) absence and (D) presence of 250 μ m DMBQ added. Kinetics were generated using one pre-flash to populate the S2 state and two pre-flashes to populate the S3 state, followed by dark period time (X axis) and ending with probe flashes to determine the O₂ yield. The WOC inefficiency parameters measured in Table 1 were used to determine the actual S state populations contributing to each flash. Units given are fraction of total centers in the specified S-state after dark incubation time.

Culture/S-state	A1	t1(s)	A2	t2(s)
CI/S2	0.18	8.08	0.20	497
Br/S2	0.18	5.88	0.21	491
CI/S3	0.13	6.11	0.22	233
Br/S3	0.11	8.87	0.16	561

Table 4.2 Decay constants and amplitudes of fits to the S-state decay curves given in Figure 4.5.

S2 and S3 state decay kinetics in whole cells were measured oximetrically [29, 31], as shown in Figure 4.5. These kinetics were fitted using a standard biexponential decay model, and the resulting kinetic components are given in Table 4.2. S2 state behavior is virtually identical between Cl- and Br-grown cultures without electron acceptor, indicating negligible effect of Br on this step. Addition of DMBQ also produces little variance between the two, further confirming the donor-side role of Br. The S3 population, however, is initially lower in Br-culture, a phenomenon only partially mitigated by addition of DMBQ. Furthermore, the slower phase of decay is slowed dramatically (141% increase in t2 vs. native without DMBQ) and plays a decreased overall role. Evidently, the effect of Br-substitution is present at the S2-S3 transition in both directions, as it is more difficult to both generate and reduce the S3 state in Br-culture.



Figure 4.6 S-state transition times in living Cl- and Br-grown culture as measured by WOCdependent differential fluorometry (see Chapter 2). All measurements were carried out at growth temperature (45°C) using quarter-saturating flashes at a frequency of 100 kHz.

Using WOC-dependent fluorometry (Chapter 2), we were able to observe the optimum *in vivo* generation times for individual S-states. At far left, a small shoulder in the two curves represents the SO-S1 transition time. This transition is minimally represented due to completing in less time than is required to deliver the equivalent of one STF of light (and a relatively small discrepancy between S0 populations after dark incubation and scrambling). The S1-S2 transition in both cultures is a distinct peak near 140 µs (note the logarithmic time scale); as in the oximetric decay measurements, this transition is not substantially affected by Br-substitution. The next peak, the S2-S3 transition, separates from the S2 generation peak later in Br-culture than in Cl-culture, indicating that this is a time that Br plays an active role in WOC cycling. The subsequent decline to a minimum represents the S3-S4-S0 transition. The gap between the two curves increases here, resulting in a 23% increase in total time required to complete the cycle for Br-substituted culture. From these measurements, we can conclusively place effects of Br-substitution in the S2-S3 and S3-S4-S0 transitions. Transition times are given in Table 4.3.

Table 4.3. Optimal transition times in living Cl- and Br-cultures as determined fluorometrically, given in μ s. As the S0-S1 transition is too rapid to be observed conclusively under these conditions, a standard value of 30 μ s is assumed for calculation of subsequent times.

Culture	S0-S1	S1-S2	S2-S3	S3-S4-S0	Total
Cl	Unclear	~110	370	1560	2070
Br	Unclear	~110	460	1960	2560

4.3.5 Fluorescent response to a range of light conditions.

Using fast repetition rate fluorometry (FRRF), it is possible to observe the behavior of both the WOC and the reaction center (P680) at a range of flash frequencies (light intensities) covering virtually all biologically feasible conditions (our instrument can theoretically generate up to 40,000 μ E/m²/s). Using this technique, we have observed the primary efficiency of these centers in Br- and Cl-grown cultures from 2 to 4000 μ E/m²/s (equivalent to 2000 ms and 1 ms between flashes, respectively). WOC inefficiency parameters can be derived from fitting of variable fluorescence cycling, while reaction center quantum efficiency under sustained light can be observed in the form of steady-state Fv/Fm (see Figure 4.7).



Figure 4.7 Top: Quality factor Q=1/($\alpha+\beta+\delta+\epsilon$), as calculated from VZAD fits of FRRF train measurements at (A) 25°C and (B) 45°C for Cl- and Br-grown cultures. Bottom: Steady-state Fv/Fm measurements at (C) 25°C and (D) 45°C for Cl- and Br-grown cultures. All data points are the average of 3 replicates.

Oscillations are not observed at high light intensities due to high rates of misses and backward transitions, but the quality of oscillations at high light intensity improves with increased temperature, as more kinetic energy is available to facilitate certain processes in PSII. Neither at room nor at growth temperature is the Br-substituted culture able to produce sufficiently distinct oscillations at the maximum light intensity for which a quality factor can be obtained in native culture, indicating Br-substitution is inherently somewhat kinetically constraining. Interestingly, at low light intensities (below the growth intensity of 40 μ E/m²/s at growth temperature, and below about 10 μ E/m²/s at room temperature), the Br-grown culture actually shows *improved* oscillation quality, indicating that there is some benefit under these conditions. However, at low enough light levels (approaching oximetric conditions) the oscillation quality factors were seen to once again approach parity. At growth light conditions, the quality factors are virtually identical in the two cultures, which is consistent with the similarly matched growth rates.

Steady-state Fv/Fm generally shows lower levels in Br-substituted culture, although certain distinct phenomena may be observed. At low temperature and light intensity, Br-culture shows higher Fv/Fm than the native. Maximum Fv/Fm yield is reached at light intensities above growth conditions in both cultures, and at a higher intensity in native than Br-culture. Near growth conditions, the two cultures' steady-state Fv/Fm levels are quite close, with overlapping margins of error. Overall, these measurements generally confirm that bromide substitution is most beneficial at low light and temperature conditions and detrimental if heat or light levels increase.





Figure 4.8. 9-AA fluorescence measurements in bromide- and chloride-grown living culture taken at 0.72 s intervals in (A) absence and (B) presence of 100 μ M of the proton gradient uncoupler 2,4-dinitrophenol. Cultures were allowed to equilibrate to dark conditions, after which time a stimulating (660 nm, 120 μ Ein/m²/s) continuous light was turned on and cultures were again allowed to equilibrate. The light was then turned off and cultures equilibrated in darkness once more. Relative shift in baseline correlates to Δ pH; rate of re-equilibration correlates to rate of proton removal from the WOC.

9-AA fluorescence has long been used to measure intravesicular, and particularly intrathylakoidal [39] pH; it has even been adapted to measure Δ pH resulting from WOC operation [40, 42]. This method was applied to investigate time-resolved pH changes under PSII-stimulating light. While PSII is not the only driver of the trans-thylakoid pH gradient, it is the initial one, and accordingly, the initial drop in fluorescence after application of light (indicating proton generation in the lumen [40, 42]) should be affected in both the duration of time which is required for re-equilibration and the total Δ pH between dark and light conditions (here given in relative units and proportional to relative fluorescence). In figure 4.8A, the Br-substituted culture shows a 154% increase in equilibration time (requiring three minutes to adjust, as compared to just 74 seconds in native conditions) and a 36% decline in total fluorescence change. When working against dinitrophenol, there is virtually no gradient change generated in Br-culture, while the CI-culture still manages to produce a slight decline in fluorescence.

4.4 Discussion

4.4.1 Growth rate and flash-induced oximetry.

The general effects of bromide on growth and flash oxygen evolution in phototrophs, particularly this organism, are well understood [2, 12, 14, 51]. Such prior studies contributed substantially to the initial hypothesis that the role of chloride was in proton removal [5, 8, 14,

19, 52]. The primary contribution here is the more specific interpretation of oximetric results using the VZAD model and the localization of the bromide effect via DMBQ addition. Generally, differences in quantum yields (Fig. 4.3) between Br-grown culture and native culture follow past studies of this organism and overall Br-substitution [14]. The VZAD model allows a method to be ascribed to the inefficiencies of the WOC resulting from Br-substitution (Fig. 4.4). Without addition of DMBQ, there is no effect of Br-substitution on the already-high primary recombination rate (α). We postulate that the decrease in double-hits (β) can be attributed to a general inability to remove more electrons from the WOC if a free proton is already present in the area. Similarly, increased backward transitions can be explained by the need to quench that free proton. In the presence of DMBQ, these effects continue to be observed, but an increased relative α is added, which indicates that some of the overall recombinations are not due to electron backlog on the acceptor side, but due to a donor-side effect. The retention of a Breffect of comparable magnitude despite the addition of DMBQ conclusively eliminates a major acceptor-side limiting effect for bromide [29, 31, 53]. All further data can be analyzed under the assumption that effects are localized to the WOC region.

4.4.2 WOC cycle localization of the bromide effect.

The bromide effect is S-state transition specific. Two of three backward (Fig. 4.5) and three of four forward (Fig. 4.6) transitions are available for this species under the conditions studied [29]. While the S0-S1 transition is inaccessible, as is its reverse, some conclusions may still be drawn from the others. The S1-S2 transition is universally acknowledged as the transition on which the fewest protons are released, despite some controversy over how many protons per WOC are actually sent to the lumen [19, 21-23]. Accordingly, this transition sees negligible effect from Br-substitution in either direction; the same amount of initial S2 population generated is observed (Fig. 4.5A) after the same amount of time (Fig. 4.6), which decays at the same rate (Fig. 4.5A) even if DMBQ is added (Fig. 4.5C). Again, this strongly suggests a relationship with proton generation and release. On the S2-S3 transition, which *does* result in proton release, a Br-effect is clearly observed. 25% less initial S3 population is produced, approximately 24% slower than in native conditions, which decays to S2 substantially more slowly. It is necessary to remove about one proton to generate the S3 state, and an equivalent number of protons must be regained to revert to S2. Finally, the S3-S4-S0 transition, while irreversible, can still be measured moving forwards, in which it demonstrates further delay resulting from the presence of bromide. On this transition, the most protons are released. Thus, the bromide effect appears to follow proton release kinetics.

4.4.3 Variations in light and temperature.

Previously, the bromide effect has been studied under relatively limited conditions [5, 7, 9, 12, 14, 16, 51]. Using FRR fluorometry, we were able to extend observations of donor-side operation to virtually the entire biologically relevant range of light intensities [54]. Br-substitution results in decreased successful primary charge separation at almost all relevant light intensities, except in a range where both light and temperature are low. This can be attributed to two factors: the aforementioned proton removal constraints at the WOC and the increased background fluorescence resulting from the cyanobacterial antenna (i.e. increased phycobilin content [55]). WOC cycling quality, a useful simplification of the overall operation [32], indicates that Br-substitution is actually directly beneficial under certain conditions. These conditions tend to correlate to proportionally higher rates of successful charge separation in Br-culture, as expected, and also to lower light intensities. A graphical representation is given in Figure 4.9:



Bromide substitution delays proton removal from or addition to the WOC.

Figure 4.9 Proton release from or recombination with the WOC by S-state transition and the effects of bromide substitution, light and temperature.

Br-culture and Cl-culture have the same log-phase growth rate and WOC quality factor at standard growth conditions for *T. elongatus*. Where light intensity is lower but temperature, and thus available kinetic energy, is high, it stands to reason that bromide benefits WOC cycling quality, as it allows removal of the limited number of protons generated quickly enough to prevent damage, but hinders recombination by not being able to allow protons back from the lumen as effectively as chloride. Lower temperature resulting in this beneficial region shifting to a lower light intensity further supports the idea of chloride as a bidirectional regulator of proton flow in the lumen-to-WOC channels and bromide as a slower variant.

4.4.4 Direct pH measurements.

The most direct indicator of the bromide effect being based on proton release is the thylakoid pH gradient. The decline in 9-AA fluorescence seen upon addition of light in Figure 4.8 is a well-attested indicator of PSII activity [40, 41], and in the Br-culture there is a smaller decline, indicating a smaller Δ pH as compared to native culture. Protons must therefore be reaching the lumen more slowly, in smaller quantities, or both. Under these light and temperature conditions, a significant degree of loss of WOC cycling quality is also observed due to high α and δ parameters (Fig. 4.7A). The decline is also a conspicuously slower process, with equilibration not completing for several minutes. This can be explained by either a delay in proton release or a concurrent slowing of downstream processes which would act on the gradient in the opposite direction, such as ATP synthase or the respiratory electron transport chain, which is found on the same membrane. The S-state specific effects observed earlier strongly favor the former explanation, whether or not the latter is accurate.

4.4.5 Chemical basis for the effect of bromide on proton removal.

The role of chloride near the WOC of PSII, with regards to proton removal, has been proposed to be as a fixed structural element which is necessary to prevent the formation of a salt bridge which would block off the exit channels from the WOC region [2, 5-7, 9]. Chloride is consistently found at the site of the salt bridge predicted by computational models [5-7] after dark incubation, but no experimental evidence for its location after illumination at an active temperature has yet been obtained. The "bridge" site is indeed a preferable location for any free anion in the WOC region, and specificity for chloride over proposed replacements such as ammonia has been recently demonstrated [56]. Until experimental evidence for the position of chloride after illumination at an operating temperature is obtained, the role cannot be definitively resolved, but the "gatekeeper" role proposed by Brudvig and others [5-7] is favored by what data exists. Indeed, it is possible to remove the otherwise inevitable need for chloride by mutating one of the residues to prevent the formation of the salt bridge—but at the expense of increased misses [6, 13].

The observed effect of bromide on proton removal can be explained by the same chemical properties regardless of the mechanism. As compared to chloride, bromide is a larger ion and a softer base (and therefore moves more slowly and has weaker charge interactions with hard protons), and the two sites in which chloride is usually found near the WOC are found at the mouths of a series of proton channels. One site, near D1-K317, has been unanimously observed for the past decade [2, 3, 9, 10, 12, 56, 57], occupying the mouth of a series of channels so narrow that little other than protons can pass through at the narrowest points - this site is the one commonly associated with the "gatekeeper" role [5, 6, 9]. The other site, however, is occasionally unoccupied in crystal structures [2, 11, 12]. This site is located at the mouth of a group of wider channels generally considered to be used to supply water and remove oxygen from the WOC pocket [2, 3, 5, 9, 52, 56]. There is no reason for an anion to be consistently present at this site if it is used exclusively by water and oxygen. Indeed, having an extra anion present might even attract protons away from the commonly attributed proton release channels.

A mobile role for this chloride in delivering protons to the lumen via the water channels (and perhaps in the opposite direction as well) is suggested by a number of factors. First, the aforementioned increase in misses resulting from chloride depletion suggests that not all protons are being successfully removed despite an open channel to the lumen existing [5, 6]. Therefore, *regardless* of which site is the active one, there is some degree of an active role for chloride. Second, as stated, having another chloride present on the other side of PSII would only

induce protons to move in that direction to balance the negative charge. Third, this site is far less constricted, but is fairly close to a residue previously speculated to be involved in proton removal, CP43-R357 [58]. A concentration-dependent role has also been demonstrated for (bi)carbonate [59, 60], which readily coordinates to the guanidinium head of arginine. Chloride substitution for (bi)carbonate at a guanidinium ligating site has previously been demonstrated to increase ionic conductivity [61], and it has been previously speculated that exchange of a small monoanion for (bi)carbonate activates the (bi)carbonate functionality near the WOC of PSII. Thus, we postulate that the "gatekeeper" chloride near the proton channels is largely stationary and serves to keep the channel open, while the second "active" chloride (and possibly others which are simply not observed due to loss from the water channels) regulates proton neutralization and removal. It is also possible that the role of this chloride is to medium-range coordinate the proton on D1-H337 in order to modulate the structure or redox potential of PSII [7, 9]. Lack of chloride has been observed to inhibit the S2-S3 transition in particular, and bromide substitution is also detrimental to this transition in particular. The proton release explanation is favored, though, due to the delay in proton delivery to the lumen far exceeding the delay in PSII operation. This hypothesis is addressed further in Chapter 5.

4.5 Conclusions

Herein we conclusively demonstrate that the role of chloride near the WOC is to facilitate proton removal. The well-attested bromide effect is confirmed to be localized to the WOC region, unlike some of the Sr-effects addressed in the previous chapter, and is specific to the S2-S3 and S3-S4-S0 state transitions, with no significant effect on the S1-S2 transition. This effect is exacerbated when electron transfer on the acceptor side is accelerated via quinone

addition. Effects of bromide substitution across all biologically relevant light intensities and two temperatures are given, revealing a beneficial effect of bromide at low light intensities which can be attributed to inhibition of the return of previously generated protons due to the size and/or softness of bromide and suggests conditional tuning of ideal light intensity for efficient PSII operation. Bromide substitution directly causes alterations to the proton gradient formed as a result of light-induced PSII activity.

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Chapter 5: Multiple Sites and Roles of Dissolved Inorganic Carbon in PSII Operation

5.1 Introduction

Almost all oxygenic photosynthesis takes place under atmospheric conditions, requiring carbon-concentrating mechanisms to supply more than the current 400 ppm CO₂ [1]. Most phototrophs are adapted to these conditions and their growth is stunted by elevated CO_2 gas, or concentrated dissolved inorganic carbon (DIC, often denoted as bicarbonate) in aquatic phototrophs [2]. Unlike most oxygenic phototrophs, the cyanobacterium Arthrospira (formerly Spirulina) maxima not only grows but thrives under the highest chemically possible DIC levels, up to 1.2 M total bicarbonate in its native alkaline lake habitats [3]. As the pH in this environment may reach 11.5, A. maxima was evolutionarily pressured to shed the genes for carbonic anhydrases involved in carbon-concentrating mechanisms [4]. Accordingly, (bi)carbonate is taken in rather than carbon dioxide, its uptake and distribution in cells regulated by a network of bicarbonate transport proteins, and its transmembrane concentration variance used to store energy as ion gradient. A. maxima is a model organism for production of biomass and bioproducts, as it grows quickly, reaching yields up to 3 g/L, can be grown on flue gasenriched carbonate media with proper pH control, demonstrates high stress tolerance, and can be grown at ideal rate under conditions which prevent almost all other organisms from surviving, producing a stable monoculture [5]. From a research perspective, its unique lack of carbonic anhydrase makes it a model organism for studies of the multiplicity of roles that DIC has in metabolism. Here the roles of DIC in Photosystem II (PSII) operation of A. maxima are

examined.

As PSII is easily photodamaged and its donor and acceptor side redox reactions may generate reactive oxygen species, it is highly regulated. Figure 5.1 provides a map of five known DIC-affected targets in PSIIs, both during standard operation and during biogenesis of the water oxidizing complex (WOC) by the photoassembly process. Functional roles of DIC within PSII have been proposed and studied since Otto Warburg's earliest (disproven) speculated role for bicarbonate as the electron donor and source of evolved O₂. The history of the study of bicarbonate in PSII has been reviewed thoroughly by Shevela et al. [6].



Figure 5.1 Current map of HCO₃⁻ action sites within PSII involving electron and proton transfer steps, cofactor electrochemical potentials and photoassembly of the WOC. Stromal-side reactions: (A), (B), and (D); lumenal-side reactions: (C) and (E).

The best-known and most-studied site for (bi)carbonate activity in PSII is ligated to the non-heme iron atom, situated between the first and second PSII-contained plastoquinone electron acceptors, Q_A and Q_B [7, 8]. This (bi)carbonate site was first qualitatively identified by Govindjee and coworkers and has subsequently been confirmed and more precisely localized by

his group and others [9-11]. A pH dependence for the dissociation constant of bicarbonate at this non-heme iron site was recently determined [12-14]. Govindjee et al. demonstrated that the role of bicarbonate at this site was in transfer of electrons from Q_A^- to Q_B [15]; this has subsequently been repeatedly confirmed [16]. The non-heme iron bicarbonate is thus thought to regulate the rate of acceptor-side electron flow [14, 15]. A second role has been proposed at this site, that of a "proton shuttle" for delivery to reduced Q_B via non-heme iron [15]. Bicarbonate was believed to be irreplaceable at this site for almost four decades [17], but recently, the electrochemical reduction potential of the Q_A/Q_A^- couple was shown to shift by +75 mV *in vitro* following a formate treatment [13], though the location of the site(s) involved were not established definitively.

Extensive evidence exists for multiple roles of DIC in supporting donor-side reactions, although overlap in observed effects and low binding affinity has led to no universal consensus developing on the number and location of DIC sites of action as at the non-heme iron [6]. Three are described in Figure 5.1C and D. While Warburg's proposal that DIC can donate electrons to the WOC has been conclusively disproven [4, 18], there is copious evidence to suggest that DIC interacts with the lumenal subunits of PSII that aid in photoprotection of the WOC and possibly function in photoassembly [19]. Additionally, it is likely that some number of (bi)carbonate anions are found ligated to PSII in other sites than the non-heme iron at any time simply due to the well-known (bi)carbonate-guanidinium association, targeting arginine side chains through combined ion-pairing and hydrogen-bonding forces. The solvent-accessible surfaces of PSII contain over one hundred arginines that are potential binding sites for DIC. Their role in proton conduction is described below. The protons released during water oxidation are terminally neutralized by (bi)carbonate in some strains, notably *Arthrospira maxima*, Figure 5.1C, equations (1) and (2) [20, 21]. This proton neutralization function may explain, at least in part,

the four-fold faster light-saturated O₂ evolution rate in *Arthrospira maxima* vs. conventional oxygenic phototrophs [22, 23].

The second coordination shell of the WOC contains an arginine residue of prior interest in the field, CP43-R357. Replacement by mutation of this positive arginine with a neutral serine was shown to induce a 50% decrease in (bi)carbonate-dependent O_2 evolution [24, 25]. The 3.5Å resolution PSII crystal structure by Ferreira et al. [8] suggested that this arginine directly bound a (bi)carbonate which also ligated the Ca atom in the WOC, but this bicarbonate was not observed in later, higher-resolution structures by others [7]. A key feature of the carbonate-guanidinium (Arg) ion pairing is the significant increase in molar conductivity following dissociation of this pair. Liberated CO₃²⁻ readily abstracts a proton from water to generate OH⁻ and HCO₃⁻ (Figure 5.1C, eqn (2) and 1E) [26]. While the spontaneous dissociation of water is a local, minor contributor, the generation of hydroxide causes a broader rise in conductance which can be attributed to proton tautomerism in the extended water network in PSII. Rapid neutralization of protons generated at distant sites, including potentially from the water oxidation site, can occur via tautomeric transfer to potential base generated at the numerous (bi)carbonate-guanidinium sites within PSII. Conformational changes accompanying S-state transitions or light-driven electrostatic phenomena may serve to separate CO_3^{2-} from Arg and activate this rapid proton neutralization mechanism.

The same high (bi)carbonate buffering afforded by coordination to Arg and Lys residues in PSII may also serve a second role, as a postulated source of neutral CO₂ molecules for RuBisCO to use in carbon fixation [4]. CO₂ release occurs during both proton neutralization (Figure 5.1C, eqn (1)) and spontaneous dissociation (Figure 5.1D), or the inherent carbonic anhydrase functionality of PSII [27]. The CO₂ molecule formed, being neutral, has a lower energy barrier to cross membranes and thus a larger diffusion coefficient than do the anionic forms of The necessity of a surplus of DIC buffer in the luminal space has been well established in studies of photoassembly of the WOC CaMn₄O₅ cluster, in addition to the free inorganic cofactors Ca and Mn and the apo-WOC-PSII complex, during both biogenesis and repair [28-30]. (Bi)carbonate has been shown to fill two distinct roles during photoassembly of the PSII-WOC. First, ¹³C-labeled bicarbonate has been shown via hyperfine coupling to coordinate to Mn²⁺ in the formation of the first photoassembly intermediate [31], which electrochemically stabilizes photooxidation to Mn³⁺ and facilitates formation of the binding site for the next Mn²⁺. Second, (bi)carbonate anions pair with positively charged residues in the apo-WOC-PSII complex, inducing electrostatic steering of Mn²⁺ through the access channels to the apo-WOC site and in turn both accelerating the rate and increasing the yield of recovery of O₂ evolution.

Herein new and highly specific methods of removal of DIC are applied to intact cells of *A. maxima*. These methods utilize two strategies: a (bi)carbonate-chelating agent (arginine) supplied with or without Mg^{2+} to induce release of CO_2 from arginine-(bi)carbonate complexes (Figure 5.1D). In intact cells, several critical conclusions are reached: 1) it is possible to prevent 99% of PSII charge separation and water oxidation activity by DIC depletion; 2) back titration with bicarbonate can be used to completely reconstitute PSII activity; 3) three distinguishable active sites for DIC revealed; and 4) the electron transfer times for water oxidation (the WOC S-states), charge recombination, and Q_A^- reoxidation are reported comprehensively under these DIC-deplete conditions. A new role for DIC is further identified, distinct from the aforementioned non-heme iron and WOC site roles, which is essential for primary charge separation occurring in the PSII reaction center and is revealed by a signature inhibition of chlorophyll variable fluorescence.

5.2 Materials and Methods

Arthrospira (Spirulina) maxima (CS-328) was obtained from the Tasmanian CSIRO Collection of Living Microalgae and grown at 30°C in 2.8-liter Fernbach flasks (batch culture) containing 500 mL standard Zarrouk's medium [32] at an initial pH of 9.5, supplemented with 200 mM NaHCO₃ for growth. Light was supplied by cool fluorescent lamps in 12-h light/dark cycles. Effective average light intensity in culture was 16 µmol photons m⁻² s⁻¹. All experiments following were performed on culture sampled in mid-exponential growth phase (three-day-old culture).

5.2.1 Removal of DIC.

DIC was depleted from culture samples using three reaction mixtures: a variant on the previously reported formate treatment used in the vast majority of studies on bicarbonate depletion in PSII following Govindjee's initial discoveries [13, 15, 33] and two novel approaches utilizing arginine to take advantage of the strong guanidinium-(bi)carbonate ligation. These approaches were followed with the objective of minimizing residual flash O_2 yield and maximizing oxygen recovery following removal of treatment mixture and restoration of HCO_3^- to the initial concentration. The mixtures are:

Mixture #1 (formate). Standard Zarrouk's medium without HCO₃⁻, supplemented with 100 mM sodium formate (ACS grade; Fisher Scientific) (pH 7.8).

Mixture #2 (NaCl+chelator). Standard Zarrouk's medium without HCO₃⁻, supplemented with 400 mM NaCl, 10 mM L-arginine (USP grade; Amresco) (pH 7.8).

Mixture #3 (MgCl₂+#2). Standard Zarrouk's medium without HCO₃[−], supplemented with 400 mM NaCl, 10 mM L-arginine (USP grade; Amresco), 100 mM MgCl₂ (pH 7.15). For each reaction mixture, 4 washes were carried out prior to resuspension and measurement. Washes consisted of centrifugation at 14,000xg for 20 s each, followed by resuspension in a volume of reaction mixture equivalent to the starting volume of culture. After washes, cells were resuspended in DIC-deplete medium. Low light (<1 µmol photons m⁻²s⁻¹) conditions were maintained during all treatments to avoid interference or damage induced by photosynthetic activity. All samples were incubated in reaction mixture in total darkness for at least one hour prior to measurements. Treatment with Mixture 3 required an incubation time of 4 hours. Any alterations to incubation times are specified below where appropriate. The effectiveness of individual treatments is given in Table 5.1.

Extraction Method	#1, Formate	#2, Arginine + salt	#3, Arginine + salt + MgCl ₂
Contents + pH	Standard Zarrouk's medium without HCO ₃ ⁻ , supplemented with 100 mM sodium formate (pH 7.8)	Standard Zarrouk's medium without HCO ₃ ⁻ , supplemented with 400 mM NaCl, 10 mM L-arginine (pH 7.8)	Standard Zarrouk's medium without HCO ₃ [−] , supplemented with 400 mM NaCl, 10 mM L- arginine, and 100 mM MgCl ₂ (pH 7.15)
%Fv/Fm; %O₂个	83%; 3.8%	74%; 3.5%	53%; 1.1%
%reconstitution	100%	110%	96%

Table 5.1. Comparison of efficiency of extraction by the three reaction mixtures.

Bicarbonate restoration to cells was carried out by washing DIC-depleted sample cultures in Zarrouk's medium twice; each wash was supplemented with the specified amount of NaHCO₃. Supernatant was removed by centrifugation between washes at 14,000xg for 20 s. Washes were carried out under low light (<1 μ mol photons m⁻² s⁻¹) conditions and kept in darkness unless otherwise specified.

5.2.2 Fluorometry and oximetry.

Flash O_2 production in samples was measured using an aerobic "wet" chamber which is capable of controlling physiological conditions over periods of up to 24 hours, as previously described [23]. Humidity, CO_2 and O_2 concentrations in the chamber were regulated. The chamber was mounted above a membrane-bound Pt/Ir electrode which has a time resolution of 100 ms and a sensitivity of approximately $1*10^{-15}$ mol O_2 s⁻¹. A 5W light-emitting diode was used to generate single turnover pulses (the amount of light required to advance the oxidation state of all WOCs in a sample by one electron) at wavelength 660 nm and light intensity of 32,000 µmol photons m⁻² s⁻¹. A single turnover flash was determined to require a 50 µs pulse [23]. A flash frequency of 0.5 Hz was used for all measurements.

Chlorophyll fluorescence measurements were performed on a homebuilt fast repetition rate (FRR) fluorometer previously described in [22]. The variable chlorophyll emission yield, F_v/F_m , was calculated from F_0 and F_m as $F_v = F_m$ - F_0 . Oscillations in F_v/F_m were produced using a GaAs laser that generated light pulses of 20 µs duration and 80,000 µmol photons m⁻² s⁻¹ intensity (equivalent total photon flux per flash as delivered during oxygen evolution, or singleturnover flashes). This fluorometer can deliver saturating flashes at all frequencies examined. A Peltier temperature-controlled cuvette was used to regulate sample temperature.

An advanced WOC cycle model denoted VZAD, as described in prior work [23, 34], was used to perform model-dependent nonlinear least-squares fittings on the flash induced oscillations of O_2 yield and F_v/F_m . For all fits, accuracy of the VZAD model was confirmed by obtaining the root-mean-squares deviation of the theoretical fit to the experimental oscillation pattern. To determine the decay times of the semi-stable S2 and S3 states of the WOC, singleturnover flashes were used to advance a dark-acclimated culture to the desired oxidation state, followed by dark incubation in this state, then rapid advance to oxygen evolution on all centers via additional flashes [23, 35, 36]. The S-state populations on any given flash were corrected using the WOC inefficiency parameters listed in Table 5.3 below.

S-state optimal transition times (Si \rightarrow Si+1) were measured via the WOC-dependent chlorophyll variable fluorescence technique described in detail in Chapter 2. The same FRR fluorometer described above was used to perform these measurements.

This fluorometer was also used to measure the reoxidation time of Q_A^- . Full reduction of Q_A was achieved by delivery of a single saturating flash to a dark-incubated culture sample. The sample was then left in darkness for a variable time before a second saturating flash was delivered. The F_m full fluorescence amplitude of the first flash corresponds to maximal photoreduction of the Q_A population. Taking the difference between F_m of the first flash and F_o of the second flash gives the residual population of oxidized Q_A which remains after the amount of dark time between the two flashes. Percent reoxidation of Q_A can therefore be represented as (F_m1–F₀2)/(F_m1–F₀1). This technique differs from the pulse amplitude modulation method [37] in that the subsaturating probe flashes used in that method induce secondary charge separation events and contaminate results, whereas the FRR-based technique has no secondary stimulation involved.

5.3 Results

5.3.1 Optimization of treatment by different reaction mixtures.

126

The extraction experiments were performed in order to determine the effectiveness of the three methods for DIC removal and their reversibility upon restoration of DIC. The contents of the extraction mixtures and protocols are given in Table 5.1. PSII operation in treated samples was investigated via FRR fluorescence at 100 Hz and 0.5 Hz (Fig. 5.2A and B) and flash oxygen yield at 0.5 Hz (Fig. 5.2C), standard measurement conditions for each technique. The decline in oxygen yield (PSII linear electron flow) observed as a result of each treatment was greater than the corresponding decrease in F_v/F_m (PSII primary charge separation) by at least one order of magnitude. PSII activity was inhibited with increasing effectiveness by extraction with mixtures 1(formate), 2(NaCl+chelator) and 3(MgCl₂+NaCl+chelator). The % F_v/F_m retained was 83, 74 and 53%, respectively, while the steady-state yield of O₂ evolution (Yss) retained was 3.8, 3.5, and 1.1%, respectively (*see* Table 5.1). The deepest extraction of DIC was achieved using Mixture 3, as judged by Yss and a full shutdown of the WOC cycle, indicated by complete loss of oscillations in F_v/F_m .







Figure 5.2. Effect on Fv/Fm at (A) 100 Hz and (B) 0.5 Hz flash rate of different extraction methods for DIC from *A. maxima* cells *in vivo*. (C) Effect of same treatments on flash oxygen yield (relative units, samples of equal cell density) at 0.5 Hz. Curves shifted to avoid overlap (see Figure S1 for improved resolution). Average percent activity given at right of each train in (A) and above traces in (C). All kinetics shown are the average of 20 flash trains.

Expanded views of the oscillations in oxygen yield remaining after each treatment (Figure 5.2C) are given in Figure 5.3. Some oscillations are retained after all treatments, but light causes rapid (reversible) inactivation after each treatment. Mixture 2 (most specific for the "structural" role) produces the least of this temporary inactivation effect, with only about a 50% loss of yield from peak to steady-state. Mixture 1, the formate-based treatment (known to be specific for the non-heme iron site as well), generates high acceptor-side induced recombination overlaying functional WOC oscillations. Mixture 3 (most specific for the donor-side function) also damps WOC function, much more effectively.



Figure 5.3 Higher-resolution oscillations from Figure 5.2C. (A) Native control, Yss = 641.8 rel. units; (B) Treated by Mixture 1 (formate/NaCl), Yss = 24.4 rel. units; (C) Treated by Mixture 2 (arginine/NaCl), Yss = 22.6 rel. units; (D) Treated by Mixture 3 (arginine/NaCl/MgCl₂), Yss = 6.95 rel. units.

To determine the reversibility of the extraction methods, native levels (200 mM) of NaHCO₃ were resupplied to culture samples, which were then incubated for two hours under soft light (Fig. 5.4). Approximately full return of Yss was observed following each reaction mixture treatment (Table 5.1). The oxygen yield increased above the control level to 110% after resupply to the Mixture 2-treated samples, and oscillations were fully restored, indicating photoassembly of some inactivated PSII centers. Mixture 3-treated samples recovered to 96% of initial Yss, but oscillation quality visibly decreased, indicating a different balance of S-state populations in the dark. The inefficiency parameters derived from fitting the WOC cycle to the VZAD model are given in Table 5.3.



Figure 5.4. Restoration of flash oxygen yield by light after bicarbonate depletion by Mixture 1. Flash oxygen yields from STF trains after 10 and 60 minutes' exposure to flashing light without supplementary bicarbonate shown. Inset shows Yss for each kinetic.

A very low level of light (<1 μ mol photons m⁻² s⁻¹) is sufficient to cause some activation of oxygen yield over time (inset). The mechanism of activation may be PSII-driven proton generation in the lumen and subsequent transfer to the stroma, facilitating electron removal by quinones. The cause may also be generation of ATP, which allows conversion of carbon dioxide in the medium to bicarbonate by an energy-intensive mechanism distinct from the passive carbonic anhydrase. A further explanation is given in [30].

5.3.2 DIC Influence on DMBQ-Dependent O₂ Production.

The electron acceptor DMBQ has previously been shown to stifle both oxygen evolution yield and efficiency of the WOC cycle in *A. maxima*, in contrast to all other phototrophs studied to date [23], in which an increase in yield is observed. To determine the relationship between this unusual loss of activity and DIC availability, DIC-depleted samples were supplied with DMBQ and flash oxygen yields measured (Fig. 5.5B). DMBQ produces no significant change in Yss in samples treated with Mixture 2, but oscillation quality is improved (hence, only the dark S-state populations are affected). The more thorough depletion of DIC with Mixture 3 results in a uniquely beneficial effect of DMBQ addition. A more than 50% increase in Yss is observed as a result of DMBQ addition, as well as oscillations which are more pronounced in amplitude and extended further along the flash train. Thorough DIC extraction (>96% of loss of oxygen yield) is thus shown to result in a positive influence of DMBQ addition on activity below this threshold.



Figure 5.5 Influence of 2,5-dimethylbenzoquinone (DMBQ) on flash O₂ production from *A. maxima* cells that have been DIC-depleted using (A) Mixture 2 and (B) Mixture 3. In (B), Control was scaled down 30-fold for comparison and Control and +DMBQ were offset by 100 and 50 relative units, respectively, for clarity. Control culture contains 200 mM DIC. All measurements were taken at 0.5 Hz flash frequency.

To time-resolve the consequences of DIC depletion, chlorophyll fluorescence kinetics were obtained after a shorter treatment with Mixture 3, which allowed some residual DIC to be retained (Fig. 5.6). Under native levels of DIC, F_v/F_m is observed to increase during the flash train when using slow flash rate (0.5 Hz), reflecting an activation kinetic that is not visible at high flash rate (100 Hz) (Fig. 5.6A) or without DIC present (Fig. 5.6B). Yss is markedly higher at lower frequency under both DIC levels. A higher Yss is to be expected, as the acceptor side clears at a rate two orders of magnitude faster than that of S-state recombination. Detection of recombination is suppressed at higher flash frequency, which eliminates much of the faster phase of WOC recombination, and the oscillations thus occur with larger amplitudes. However, the gap between Yss at the two flash frequencies is three times as large under DIC-deplete conditions as under DIC-replete conditions.



Figure 5.6 Effect of (A) 200 mM and (B) 0 mM HCO_3^- on Fv/Fm kinetics at low (0.5 Hz) and high (100 Hz) flash frequencies. Mixture 3 was used for DIC depletion; samples were pre-incubated in darkness for 30 min.

5.3.3 Influence of DIC on S-state transition times.

To measure any specific influence of DIC on individual S-states, the S-state transition times were determined from their influence on chlorophyll variable fluorescence (described in Chapter 2). F_m measurements were carried out at several temperatures to determine the contribution of DIC diffusion and to investigate any possible correlation with the thermaldependent phase of F_m (the J step in the Kautsky induction curve of saturating fluorescence), which may correlate to the terminal step of oxygen release [38].



Figure 5.7 Fluorometric measurements of S-state transition times using 1000 "quarter-turnover" 5- μ s flashes at 10- μ s intervals, from whole cells of *A. maxima*. The Fm1(dark S)–Fm2(scrambled S) difference in emission yield from: (A) DIC-depleted by Mixture 2 and DIC-replete in 200 mM NaHCO₃ at 25°C, and (B) DIC-replete conditions at 5, 15, and 35°C. The logarithmic plot format converts single exponential changes into linear regions, shown as straight lines. Data shown are the average of 24 replicates.

Fig. 5.7A shows the kinetics of time-resolved F_m difference from 10 to 10,000 µs measured at 25 °C. Data are shown logarithmically for fluorescence yields from the control sample under native DIC and an identical sample treated with Mixture 2. The 10 ms interval used is sufficient time to theoretically induce 2–3 full WOC cycles (though in practice, only one is observed). The 3-second interval between the two trains used, which is short compared to decay of S2 and S3 (see Fig. 5.8) and thus effective "scrambling" of S-state populations is

possible. The differential fluorescence amplitude is reduced by DIC depletion (proportional to the number of PSIIs capable of charge separation) by a factor of approximately two-thirds and the peak features become broader. When the total NaHCO₃ in the media is augmented to 1 M, approaching the saturation limit and what is observed in *A. maxima's* native environment, the amplitude of maximum F_m difference is increased by 16% (from 0.69 to 0.8 units; Fig. 5.7). The DIC-depleted sample amplitude is four-fold lower than this value, which follows from the foregoing data showing DIC-depletion reduces F_v/F_m when a sufficiently rapid flash rate is used (Fig. 5.6).

Three regions are resolved in the first 2 ms interval shown in Fig. 5.7A. By using the logarithmic scale, single exponential changes are converted into linear regions and deconvolution is facilitated. A rise in fluorescence from the initial dark state, mainly S1 (Table 5.3), is observed linearly in log(time) corresponding to the induction of the S2 state (196 µs), after which a plateau corresponding to formation of S3 (1.1 ms) is observed. Linear decay to a minimum is then seen, corresponding to the formation of S0 (3.5 ms). Table 5.2 gives a list of the individual S-state transition times observed.

Table 5.2 Optimal transition times in μ s for S-state transitions shown in Figure 5.7. Note that the S0-S1 transition time is usually not available because no S0 population is retained under the conditions studied (no S1 peak). At 5°C, insufficient centers can complete the S3-S4-S0 transition to clearly differentiate an advance kinetic (note no local minimum in graphs).

Sample conditions	S1-S2	S2-S3	S3-S4-S0	Total
0 mM DIC, 25 °C	197	530	2250	2980
200 mM DIC, 25 °C	196	850	2410	3460
200 mM DIC, 5 °C	690	920	n/a	n/a
200 mM DIC, 15 °C	260	840	2770	3870
200 mM DIC, 35 °C	154	540	1420	2110
1000 mM DIC, 5 °C	660	860	n/a	n/a
1000 mM DIC, 15 °C	197	780	4020	5000
1000 mM DIC, 35 °C	122	500	1400	2020

To aid in deconvoluting the changes, two previously established general effects of temperature on the WOC cycle transitions are shown in Fig. 5.7B: 1) at low temperature (5°C), the WOC cycle can only advance past S2 with great difficulty in *A. maxima*, which produces a characteristic collapse of the S2 peak (seen in Chapters 2 and 3) as this transition slows and the initial dark populations change; 2) increase in temperature to 15°C allows the WOC cycle time to decrease to within 3.9 ms and at 35°C it accelerates to 2.1 ms.

5.3.4 S2 and S3 state lifetimes.

To study how DIC regulates the decay rates of the unstable S2 and S3 states, loss of flash oxygen yield was measured over a variable dark period following generation of S2 and S3 populations by one and two preflashes, respectively, using a previously described method [23, 36, 39]. Aside from the loss of oxygen yield resulting from DIC depletion, several alterations to decay kinetics were observed (Fig. 5.8). Following DIC depletion, approximately 20–25% slower decay constants were observed in S2 state decay, but the quicker feature of the biexponential decay mechanism became more dominant. Minimal alteration to the S3 decay time constants was seen upon DIC depletion, although the quicker feature became slightly more dominant. Dark-stable ratios of the S-state populations after full decay of S2 and S3 were identical whether observed by flash oxygen or chlorophyll fluorescence measurements (Fig. 5.6; Table 5.3).



Figure 5.8 Dependence of lifetime of S2 and S3 states (charge recombination time constants) on the DIC concentration in *A. maxima* cells measured by flash O₂ yield. Insets show fitting parameters from biexponential decay fits to data. (A) DIC-depleted (by Mixture 2); and (B) control culture.

5.3.5 Q_A^- reoxidation time.

Pursuant to the well-known role of DIC at the non-heme iron/Q_A site, the effect of DIC depletion on the yield of Q_A^- and its reoxidation kinetics was investigated. As seen in Fig. 5.9, the reoxidation rate of Q_A^- under DIC replete conditions in *A. maxima* is exceptionally slow. However, once DIC is depleted, the fast component of reoxidation is about 35% faster on average and accounts for a slightly larger fraction of the total decay. No Q_A^- is retained beyond the measurement time employed, 6 ms (full reoxidation occurs).



Figure 5.9 Fluorometric measurement of the Q_A^- reoxidation time, given by (Fm1–Fo2)/(Fm1–Fo1), via the pulse-probe method at 25°C in *A. maxima* samples with (black) and without (blue) DIC removed by pretreating with Mixture 3. Data shown is the average of 6 replicates. Total fluorescence (Q_A fully reduced) is 23 rel. u. with and 13 rel. u. without DIC (43% reduction of active fluorescence).

5.4 Discussion

5.4.1 Sites of DIC removal.

The three reaction mixtures used for DIC depletion were selected to remove (bi)carbonate bound to PSII with varying affinities. For all three treatments, a loss of PSII activity and restoration upon reconstitution is observed. Mixture 1 (formate) has long been known to inhibit bicarbonate stimulation of PSII-dependent O₂ evolution, the historical "bicarbonate effect" [13, 40]. Of the three DIC depletion treatments, this is the weakest. As in Figs. 5.3 and 5.6, we were able to further elucidate its function using FRR fluorometry and flash oximetry. That the oxygen yield decreases by over 96% (measured at 0.5 Hz flash rate) without corresponding loss in F_v/F_m steady-state (96% retained at 0.5 Hz, 83% retained at 100 Hz) or oscillations by either measuring technique indicates that essentially all PSII-WOCs remain fully

active in charge separation between P680 and Q_A at low flash rates. Increasing flash rates correspond to higher continuous light intensities used in historical measurements, and higher light intensities cause increased inactivation of PSII-WOCs. Primary charge separation and coupling to the water oxidation cycle are unaffected, as indicated by the retention of F_v/F_m steady-state level and period-four oscillation amplitude at low flash rate in DIC-depleted samples. The steady-state F_v/F_m decreases at higher flash rates, owing to decline in F_m , whereas the oscillation amplitude is unaffected or even improves at 100 Hz (Fig. 5.6). Electron acceptor capacity (size of the plastoquinone [PQ] pool or its kinetics of filling) is reduced as the flash rate increases. On flash #2, major loss of F_v/F_m occurs, indicating that the rate-limiting step is reoxidation of Q_A^- ($Q_A^-Q_B \rightarrow Q_A Q_B^-$). The consensus view that formate treatment slows this step by removal of bicarbonate from coordination to the non-heme iron (Figure 5.1) is supported by these results. The electrochemical reduction potential of the Q_A/Q_A^- couple was shifted by +75 mV in vitro by removal of (bi)carbonate from this site [13], though the location of the site(s) involved was not clearly established and the classic non-heme iron site effect was assumed. The redox potential alteration was shown to serve a photoprotective function for PSII in vitro by reducing the yield of singlet oxygen formation (${}^{1}O_{2}$). Redox tuning of Q_{A}/Q_{A}^{-} was postulated to be necessary for avoiding in vivo photodamage. Specifically, the authors suggest that generation of a Q_A^- population (as occurs when Q_B and the PQH₂ pool are reduced) could induce loss of HCO_3^- , resulting in redox tuning of Q_A in vivo and decreased production of 1O_2 .

While there is no major loss of period-four oscillations in F_v/F_m , O_2 evolution is nearly completely lost at low flash rates following formate treatment. It can be reasonably assumed, therefore, that the PSII-WOC works fine and another DIC site must be responsible. There are a range of mechanisms which may explain the absence of O_2 : 1) increased consumption of PSIIgenerated O_2 by the Mehler reaction (PSI-dependent reduction of O_2), 2) increased cellular respiration causing uptake of O_2 , or 3) an altered WOC cycle producing two H_2O_2 molecules rather than O_2 for every four electrons transduced (chemically least likely). The cause of loss of O_2 evolution is a subject for future research.

5.4.2 Deeper depletion of DIC sites.

Mixture 2 (NaCl+chelator) was developed to remove (bi)carbonate bound to arginine residues in PSII more aggressively, as compared to the well-attested formate treatment, by chelating the (bi)carbonate ion to the guanidinium side chain of added arginine. A lower steady-state yield of F_v/F_m (74% at 100 Hz) is produced, with oscillations which are somewhat clearer than in the other two treatments (extending to higher flash number), and the O₂ yield is eliminated to roughly the same extent as formate treatment (3.5%). Mixture 2 seems to accomplish the same outcome as a formate wash, but with greater depletion of DIC from PSII centers.

102 arginine residues are found in PSII core complexes of *A. maxima*, the majority located near the lumenal and stromal surfaces where they can exchange (bi)carbonate ligands (Figure 5.10). The combination of bidentate H-bonding and ion-pairing interactions makes the guanidinium cation an especially good chelator of HCO_3^- and CO_3^{2-} . The crystal structures of proteins frequently contain arginine/carbonate ion pairs [41]. The guanidinium–carbonate ion pair has a free energy of formation of -16 kJ mol⁻¹ in dimethyl sulfoxide/water (80:20) [42]. This value corresponds to a formation constant of 500 M⁻¹. The (bi)carbonate:guanidinium affinity increases even further in more hydrophobic environments such as the interior of membrane proteins. Arginine has an even stronger affinity for (bi)carbonate than does guanidinium, which facilitates uptake of DIC from solution into proteins and cells by Arg. This (bi)carbonate– guanidinium ion pairing produces a distinctively increased molar conductivity at high dilutions [43]. The ion pair can be induced to dissociate CO_3^{2-} , thus liberating a strong base that can spontaneously hydrolyze to form OH^- and HCO_3^- . The anomalously high conductivity of hydroxide and hydronium ion relies proton tautomerism of the solvent water, which allows more rapid transfer of protons than bulk diffusion through water. We propose that this mechanism is a functional basis for the numerous arginines on the luminal surface of PSII, which would use this mechanism to achieve unusually rapid proton neutralization by liberating CO_3^{2-} during water oxidation. This would be particularly true of hypercarbonate-requiring strains like *A. maxima,* in which PSII-WOC turnover is 4–5 fold faster than in more conventional oxygenic phototrophs [26]. Another anion would be necessary to dissociate carbonate from arginine, and CI^- is a likely candidate (Figure 5.10; also see Chapter 4).



Figure 5.10 Proposed ligand-ligand exchange in PSII at multiple sites, including the WOC region, the non-heme iron, and the solvent-exposed arginine residues. Arginine residues in PSII dimer depicted in black (102 in total, located on both lumenal and stromal sides). Proposed functions of (bi)carbonate shown for WOC-region, $Fe(II)/Q_A$, general lumenal and general stromal sites.

Mixture 3 (MgCl₂+chelator) is simply MgCl₂ added to mixture 2. The function of the Mg is to remove CO₂ by altering the equilibrium in the reaction: $Mg^{2+}+2HCO_3^- \rightarrow Mg^{2+}CO_3^{2-} + CO_2 +$ H_2O (Figure 5.1D) to generate volatile CO₂ via ion-pairing of Mg²⁺ and carbonate. A complete loss of both O₂ evolution and oscillations in F_v/F_m (at high frequency) occurs, while the high yield of the first flash F_v/F_m characteristic of a fully oxidized Q_A population [22] is significantly reduced. DIC can therefore be extracted from additional site(s) by Mixture 3, which causes the loss of both the acceptor side electron/proton transport and the donor side reactions that drive water oxidation. The acceptor-side influence is further seen in Fig. 5.9. Only mixture 3, out of the three treatments, caused a significant change in Q_A⁻ reoxidation kinetics. During photosynthetic electron transport (PET), magnesium is transported across the thylakoid membrane from the lumen into the stroma, where it is known to activate RuBisCO's CO₂ fixation activity [44]. In addition to this well-known activation of RuBisCO, PET-elevated magnesium in the stroma is posited to enable synchronized conversion of bicarbonate into carbon dioxide by the reaction: $HCO_3^- + Mg^{2+} + stromal-Arg^+(HCO_3^-) \rightarrow CO_2 + Arg^+(CO_3^-)Mg^{2+}$ (Figure 5.10). This then provides an additional chemical pathway for activation of RuBisCO carboxylation.

5.4.3 Flash rate dependence of DIC-depletion effects.

When the treatment with Mixture 3 is shortened to forestall removal of the most tightly bound DIC associated with the donor side (Fig. 5.6), the minimal decrease of the first flash F_v/F_m yield is observed. Thus, the donor side remains normally functional and maximal photoreduction of Q_A can occur. However, when the flash rate is increased to 100 Hz, there is substantial suppression of yield, indicating that Q_A^- reoxidation by Q_B is slowed or blocked. Accordingly, there are two reasonable options for the DIC site that blocks PET *in vivo*: either the non-heme iron site, which has been demonstrated to undergo bicarbonate depletion *in vitro* [13], or a site which blocks the transfer of protons to Q_B [45]. These may be the same site, or they may be different, nearby ones.

5.4.4 Remediation of DIC-depletion effects.

All three treatments are reversible upon NaHCO₃ readdition, although some differences in performance of reactivated PSII-WOCs are evident from the quality of oscillations. The WOC cycle parameters, which reflect oscillation quality, are obtained from minimized-error fits to the VZAD model and summarized in Table 5.3 [34]. After treatment of Mixture 2, readdition of NaHCO₃ resulted in lower misses (alpha) and fewer inactivations (epsilon), showing a more efficient removal of electrons from the PSII-WOC than the control culture. By contrast, misses are sharply increased following bicarbonate readdition after treatment with Mixture 3, resulting in a longer cycle period of 4.78 flashes, 7.7% more than before depletion. These changes indicate damages produced by the 4-hour extraction period with mixture 3 that cannot readily be reversed by NaHCO₃.

	Control	Fig. 2A,	Fig. 2B,	Fig. 3A, no	Fig. 3A, no
		recovered from	recovered from	DIC	DIC +DMBQ
		Mixture 2	Mixture 3		
Alpha	0.171	0.150	0.222	0.174	0.123
Beta	0.021	0.028	0.017	0.0223	0.036
Delta	0	0	0	0	0
Epsilon	0.041	0.016	0.038	0.039	0.0087
SO	0	0.0316	0	0	0.321
S1	0.858	0.817	0.919	0.840	0.594
S2	0.142	0.152	0.081	0.160	0.085
S3	0	0	0	0	0
Period	4.44	4.37	4.78	4.50	4.28

Table 5.3 WOC cycle parameters from fits of the flash oxygen yields at 0.5 Hz using the VZAD model. Recovered samples were treated with 200 mM NaHCO₃ after DIC depletion.

5.4.5 Donor-side effect of DIC on S-state transitions.

DIC depletion is most obvious at the classic acceptor side DIC site (i.e., using Mixture 2), as seen by a 3-fold loss of amplitude of F_m1-F_m2 , which arises from fewer centers advancing through the cycle. If the two curves in Fig. 5.7A are normalized to the same peak amplitude (see Fig. 5.11), an estimate of the relative changes on individual S-state transition times can be extracted. However, such an interpretation assumes the S-state dependent F_v quantum yield does not change as a result of DIC availability, which may not be accurate. As seen in Chapter 3, Sr-substitution produces S-state specific effects, and a structural role for bicarbonate between Ca and CP43-R357 would likely also have different structural effects depending on the S-state. The most readily observed alteration stemming from DIC depletion is the shorter overall transit time to reach oxygen evolution (3.0 ms vs 3.5 ms at 25 °C). The minimum is also higher, both normalized and in terms of raw data, which implies that inactivation during this time is elevated. At 25 C, the S1' \rightarrow S2' transition is unchanged at 200 µs, while the S2' \rightarrow S3' at 530 µs is 35% faster, and S3' \rightarrow S0' at 2250 µs is 10% faster.



Figure 5.11 Fluorescence curves from Figure 5.7A overlaid at different scales to demonstrate differences in S-state transition times.

5.4.6 Distinguishing functions and sites of DIC activity.

Comparatively low-affinity sites appear to be accessed by DIC removal with Mixture 2, whereas Mixture 3 depletes these same sites as well as a higher affinity donor-side site which shuts down water oxidation. In addition to the classic acceptor-side site at the non-heme iron, the low-affinity sites presumably include multiple other potential (bi)carbonate sites associated with the 102 solvent-accessible arginine residues. Even after massive loss of oxygen yield, these previously uninvestigated sites are still conspicuously occupied in the active PSIIs. This is clear from retention of high fluorescence yields on the first post-dark flash (i.e., active WOC and oxidized Q_A) and visible oscillations in both oximetric and fluorometric measurements (Figs. 5.2-5.6). However, PSII operates efficiently only at low light flux, where residual DIC or possibly alternative pathways independent of DIC can produce retention of function on a slow scale. The site responsible for this regulation is presumed to be one or more of the aforementioned 102 arginines within PSII. A large number of DIC molecules could potentially be held in this "arginine network", and under A. maxima's native conditions, these sites would be expected to serve multiple purposes: a structural role, a role for HCO_3^- and CO_3^{2-} as proton acceptors on the luminal surface, and a role as CO₂ source on the stromal surface. These functionalities and the chemical mechanisms by which they act are addressed in Figure 5.10. Light is observed to be necessary to reverse the depletion at these sites, and thus it can be postulated that light-driven proton gradient or ATP production is required to transport bicarbonate to the site(s) of interest.

The proton-removal process attributed to chloride, described in greater detail in Chapter 4, is likely fundamentally linked to the donor-side role of DIC. Chloride removal has no effect on the S1–S2 transition, on which no protons are released [46], but the S2–S3 and S3–S4– S0 transitions are delayed. In DIC-depleted samples (Fig. 5.7A), the bulk of the fluorescence yield is lost, but *faster* transitions through these S-states are observed in those centers that remain active. The site of DIC function on the donor side is proposed to be CP43-R357, based on prior work with mutants of this site [24]. From this site, (bi)carbonate would be perfectly positioned to modulate the position of calcium within the WOC [47], and in turn the redox potential of the WOC and individual S-states. Ca has been previously demonstrated to tune the redox potential of the WOC [36, 48, 49], and a reshaping of the S2 structure of the WOC produces results similar to those observed from DIC depletion [48, 50, 51]. The lack of peroxide detected under partial depletion of DIC further supports a function at this site. Loss of functionality at this highest-affinity site is known to induce peroxide evolution, which is a further indicator of a reshaped S2 structure [24, 36]. We posit that in high-resolution crystal structures, this site is not occupied by DIC because the treatment, using extreme chloride concentrations, is remarkably similar to our own DIC removal methods [7]. A major point of difference is that the small luminal subunits of PSII are removed by the crystallization techniques, which would certainly alter the rate of diffusion of small molecules to/from the WOC pocket and remove a substantial number of arginines which might otherwise facilitate retention of DIC. The local DIC affinity at CP43-R357 likely becomes lower than at the non-heme iron site under these conditions.

5.5 Conclusion

Two new methods for DIC depletion were developed, and one existing method modified, to elucidate the multiple roles of (bi)carbonate in PSII. One method was found to be capable of inhibiting oxygen evolution activity down to 1%, while all methods were shown to be fully reversible once DIC and light were restored. Further investigation of the role of DIC *in vivo* is possible as a result of the development of these methods, and they may be applied to other species. Complete inactivation of PSII fluorescence as a result of DIC depletion occurs prior to the loss of either Q_A or WOC functionality. The unique detrimental effect of DMBQ in *A. maxima* was shown to be located on the donor side of PSII, while the non-heme iron site role of DIC was linked to the positive acceptor-side effect. Forward electron transfer was shown to be facilitated by depletion at the non-heme iron site. The order of binding affinity of at least three DIC sites with unique roles were established to be: WOC region > non-heme iron > unknown bulk inactivation site. However, certain DIC-depleting agents were found to act with differing specificity for each site, related to the mechanism of interaction between DIC and the treatment. The WOC region role is attributed to stabilization of free energy through structural modulation of the WOC or indirect alteration of the redox potential via a site between CP43-R357 and the Ca²⁺ in the WOC.

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Gates, Colin; Ananyev, Gennady; Roychowdhury, Shatabdi; Fromme, Petra; Dismukes, G. Charles, "Multiple Oscillator Periodicities and Redox Regulation of WOC Operation in PSII Microcrystals," *in prep.* (6.2)

Chapter 6. Acceptor-Side Regulation of the Electron Transfer Processes in PSII Microcrystals

6.1 Establishment of Acceptor-Side Regulation and Oximetric Analyses in PSII Microcrystals

6.1.1 Introduction

The atomic structure of PSII has been elucidated in increasing detail by crystallography. The first crystal structure to show significant detail was made available at resolution 3.9Å by Zouni et al. in 2001 [1], after which a steady stream of crystal structures at increasing resolution were published, rapidly progressing to 3.4Å [2], 2.9Å [3], and by 2011 to 1.9Å [4], high enough that the atomic structure of the enzyme can be observed. Some discrepancies were observed between these crystal structures, which were, at the time, considered to be artifacts of the low resolution in use and the newer, higher-resolution structures were taken as the most accurate ones [5, 6]. However, the subject of crystal structure of PSII has become bogged down by a series of issues whereby it is unclear if numerous modern crystal structures with resolutions in the range of 2Å are accurate, as these structures show a number of differences between one another still [7-10]. The sources of these differences are less clear and have been attributed in some cases to radiation-induced reduction during X-ray diffraction measurements [7, 10, 11]. The recent introduction of the X-ray free electron laser method promises to help address this issue, but the technique is still catching up to more traditional approaches in terms of resolution [7, 12, 13]. Additionally, novel methods for production of large numbers of extremely high purity microcrystals have been developed (Figure 6.1), allowing the further expansion of high-resolution measurements with minimal risk of photodamage occurring as a result of the diffraction technique. These microcrystals, in addition to being extremely high purity, retain almost 100% of activity for months in storage or days on the benchtop [12, 13].





While high-resolution structures are available, the expected benefits these should generate for understanding the mechanism of water oxidation have been hampered due to inability to cleanly control formation of individual intermediate states in the catalytic cycle (Sstates). The problem is three-fold: ambiguity over both the resting populations of the four individual S-states in the dark and their respective isomers (two or more per S-state), and the difficulty of cleanly advancing through the cycle using flashes owing to intrinsic limitations in the photochemistry and to insufficient light saturation at high optical density [14-17]. X-ray crystallography cannot determine the oxidation states of atoms, nor the success of a photooxidation flash, and thus several contradictory claims have sprung up at conferences and in print regarding the structures of higher S-states [14, 15, 18]. Experimental evidence of dark S-state populations, isomers and fidelity of flash-induced S-state advances in PSII crystals are necessary to clarify what a given crystal structure shows. This information is presently lacking. Only recently have simple attempts been made to address the matter via quantitative measurements of PSII operation [15], and this approach has been limited by the same fundamental assumptions which lead to conflict within the field; the results reported do not sufficiently take into account the possibility of various inefficient processes within PSII and assume ideal operation when there is no evidence to support this in PSII microcrystals. This study was undertaken to provide a quantitative description of the operation of PSII microcrystals in order to inform the user community conducting crystallographic studies.

In this research, we examine PSII microcrystals to determine their S-state populations, lifetimes, and quantum yields as a function of flashes and light intensity, using a range of common electron acceptors. PSII microcrystals have low yields of S-state transitions and O₂ in the absence of supplemental electron acceptors and even consume O₂ as poor electron acceptor. The choice of terminal electron acceptor substantially alters the overall photochemical activity of the system due to regulation of PSII by the Q_AQ_B plastoquinone carriers. With an optimal electron acceptor, PSII microcrystals show the highest quantum efficiency of O₂ evolution of any PSII system studied to date, as well as the highest efficiency of S-state advance. Substantially longer lifetimes of the S2 and S3 states occur in PSII crystals which greatly alter the resting S-state populations compared to non-crystallized PSII core preparations.

6.1.2 Materials and Methods

PSII crystals were obtained from PSII core complexes of *Thermosynechococcus elongatus* and contain 35 ChI per monomer as described previously [12, 19, 20]. The crystallization process approximately follows the method of Kupitz et al. [12], but four sequential recrystallizations of PSII cores were carried out in order to ensure high purity. Crystals were uniformly 10-12 µm in diameter as determined by optical microscopy. Measurements of crystals were done in stabilizing buffer: 100 mM PIPES at pH 7.0, 5 mM CaCl2, 10 mM tocopherol, 20% PEG 2000.

To attain sufficient intensity across the visible spectral region we used a high pressure Xe flash lamp (model ISSh-400). The flash duration at half maximum (FWHM) is about 1 μ s and the delivered optical energy was adjusted to achieve maximum O₂ yield.

All O₂ measurements were carried out using a Pt-Ir working electrode covered by thin membrane (rate electrode). The small sample chamber (volume 8 μL) when sealed with glass cover enabled anaerobic conditions [21], or with an added Teflon ring could produce aerated head-space, as described in figure legends. The membrane prevents diffusion of added electron acceptors to the electrode surface [22]. All measurements were conducted using a constant DC voltage bias without an AC filter. All O₂ signals are directly proportional to the current needed to reduce O₂, i.e., the O₂ yield. Crystals were diluted and spread to avoid shading effects on the electrode.

Flash induced O_2 yield was analyzed by two methods. Model-independent analysis was performed via Fourier transformation to determine the frequency distribution of the WOC cycle [23]. Model-dependent analysis was done by fitting O_2 flash yields to an advanced WOC cycle model denoted VZAD, as described in prior work[22, 24]. VZAD fitting accuracy was determined by root-mean-squares deviation of the fit.

Synthetic quinones DCBQ, DMBQ, and PPBQ were obtained from Aldrich Chemical Co. (98% purity). These were purified by double recrystallization in ethanol and subsequently dissolved in dimethyl sulfoxide (DMSO) at 10 mM concentration, and further diluted into cultures for experiments. K_3 Fe(CN)₆ (potassium ferricyanide, abbreviated FeCN below) was obtained from Sigma-Aldrich (99% purity).

6.1.3 Results

6.1.3.1 Optimizing light intensity.

As PSII microcrystals lack any outside antenna, they are solely reliant on the chlorophyll within PSII core complexes (35 Chl/PSII) for charge separation [12]. This reduces the system's ability to make use of light by at least an order of magnitude as compared to living cells or thylakoids. Furthermore, the protein complexes inherently shade one another within the crystal, further reducing light absorption. Accordingly, it is necessary to supply a far greater light intensity to achieve saturation of photochemical steps [14, 17, 22, 25, 26]. A microcrystal system will have an optimal light intensity, as demonstrated in Figure 6.2. Below this point, the system is limited by insufficient light intensity to saturate all charge separation. Above this point, however, photoinhibition is observed.



Figure 6.2 Flash O_2 yield dependence on xenon flash lamp energy (given in μ) using 1- μ s flashes. Dark-adapted PSII microcrystals were measured in the absence of any electron acceptor at room temperature in aerobic conditions. Fresh samples were used for each light intensity. Data shown is the average of 3 replicates.

From left to right in Figure 6.2, flash intensities of 170, 215 and 260 μ J (optical energy) from a xenon flash lamp were tested for saturation of PSII O₂ yield. Peak O₂ yield was observed at 215 μ J, while less O₂ was produced at both 170 μ J and 260 μ J. At 170 μ J some PSIIs do not produce O₂ on the first cycle and have an increased second cycle yield due to photochemical misses. At 260 μ J the total yield is decreased, indicating too much light caused by photoinhibition. The presence of O₂ in air contributed as an electron acceptor to the flash induced O₂ signal, as shown next.

6.1.3.2 Effect of oxygen.

Dissolved O_2 can serve as an electron acceptor from Q_{A^-} and/or $Q_{B^{2^-}}$ in spinach PSII membrane fragments, where up to 15% of generated electrons may be directed to oxygen [27]. To test the turnover efficiency of PSII microcrystals, two experiments two experiments were performed with and without dissolved O_2 (Figure 6.3):



Figure 6.3 (Top) Averaged flash oxygen yields per PSII from a microcrystal sample under aerobic and anaerobic conditions with no exogenous electron acceptors. Samples were subjected to one preflash then 10 minutes dark pre-incubation time following the preflash, before exposure to a train of 20 1- μ s Xe flashes at 0.5 Hz. This sequence was repeated 10 times. The average oxygen yield from the first 4 flashes from each set of 20 flashes is shown. (Bottom) The unaveraged raw data for first and tenth train of each series.

Dissolved oxygen is not needed to produce the first turnover cycle, as only a 20% decrease in the integrated flash O₂ yield from the first four flashes occurs when air is removed from the sample vs anaerobic (Fig. 6.3). In air, a loss of 17% activity occurs on the second train and is followed by a much slower loss of activity by only 5% on the next ten flash trains. Under anaerobic conditions, the oxygen yield steadily declines by 90%, probably limited only by air leak. This shows that crystallized PSII has the capacity to accept about four electrons without air, and is capable of using oxygen as a terminal electron acceptor on a slow time scale, faster than the 10 minute dark period between trains, but far slower than the 2 s time between flashes.

6.1.3.3 Quantum yield and efficiency of PSII microcrystals.

The loss of oxygen-evolving activity can be completely ameliorated by addition of a fastacting electron acceptor. The use of acceptors to boost PSII activity and oxygen yield in particular has been widely reported [22, 25, 28-32], and all acceptors used herein are well attested in subcellular preparations [32-34]. A series of acceptors were investigated to establish which one produces the highest activity and sustained oscillations in PSII microcrystals for use in turnover studies and S state lifetimes. Steady-state oxygen yield (Yss), average yield from the first four flashes (Y_P), and the number of sustained oscillations using PPBQ, DMBQ and FeCN were quantified (Figure 6.4A and B). Optimal electron acceptor concentration was determined for each (Figure 6.5).



Figure 6.4 (A) Dark-adapted PSII microcrystals in solution supplemented with 400 μ M quinone acceptor (PPBQ or DMBQ) or 2 mM ferricyanide or no acceptor were subjected to one train of 20 saturating Xe flashes at 0.5 Hz under air headspace. Representative data from a set of replicates are given. (B) Comparison of FeCN as sole acceptor to the best acceptor system: 400 μ M PPBQ plus 2 mM FeCN (100 flashes). Optimal electron acceptor concentrations were chosen via titration for oxygen yield; an example is shown in Figure 6.5.



Figure 6.5 Dark-adapted PSII microcrystals in solution supplemented with varying concentrations of PPBQ acceptor as noted were subjected to one train of 20 saturating Xe

flashes at 0.5 Hz under oxygenated headspace. Representative data from a set of replicates are given.

The quantum efficiency of oxygen evolution *in vivo* [22] and *in vitro* [35, 36] are often substantially lower than the theoretical maximum, ranging from under 2% to about 33% in prior reports [22, 25, 28, 36, 37]. In PSII microcrystals, the observed range of quantum yields (and quantum efficiencies) depend strongly on the electron acceptor system (Figure 6.6).



Figure 6.6. Effect of electron acceptors and dissolved oxygen on the quantum yield of flashinduced oxygen (QY = oxygen molecules produced per 1000 PSIIs per flash). Grey: Average yield from the first 4 flashes (Y_P). Red: Average yield at steady-state (Y_{SS}).

With optimization of electron acceptors, it is possible to far surpass the previous record oxygen quantum yields from PSII non-crystals. Furthermore, the characteristic loss of activity during a flash train seen anaerobically can be prevented; The use of PPBQ at optimal concentration as an electron acceptor results in an initial activity on the first cycle that is 60% higher ($Y_P = 103.3$, Figure 6.6) than with no acceptor (only air) and minimal loss over a train of 30 flashes (Y_{SS} = 103.3, Figure 6.6). Other quinones give lower initial and continuing yields (DCBQ and DMBQ, Table 1). As accumulation of the reduced quinol forms (QH₂) continues during illumination, we observe continual loss in the O₂ yield below that observed with FeCN alone, as shown in Figure 6.7B. This may arise either due to consumption of the quinone or inhibition by the quinol, but not photoinactivation of PSII, as the loss is reversible upon oxidizing the quinol. This is described in the next section.

Ferricyanide is a one-electron reversible oxidant with midpoint potential +430 mV that can oxidize the quinol to the quinone forms of all three acceptors studied. It is also a wellknown, albeit inefficient, electron acceptor for PSII [38]. Use of both a quinone and FeCN together as electron acceptors produces the largest increase in O₂ yield. Use of PPBQ+FeCN increases the peak O₂ yield by more than two-fold, giving a record quantum efficiency of 61.6% compared to no electron acceptor (air). Combining these electron acceptors allows the greatest number and amplitude of visible oscillation periods (over 25 visible in Figure 6.4B). Fitting of the oscillations to the VZAD model was performed and shows that the WOC cycle inefficiency parameters in the presence of electron acceptors are smaller and the lifetime of the S₂ and S₃ states are increased, as discussed later (Figure 6.7B).

6.1.3.4 Accumulation of reduced quinol during illumination regulates PSII O_2 evolution activity and backward transitions.

As illumination proceeds the electron acceptor is consumed, and the O_2 flash yield decreases to zero. The decline is continuous and exhibits acceptor-dependent inflection points before reaching zero O_2 yield, as shown in Figure 5. The derivative trace is included and shows the magnitude of the slope changes during the illumination. The intensity of the two inflections depends on the choice of electron acceptor and its concentration. As seen in Figure 6.7B, in the presence of PPBQ alone, more oxygen is initially evolved than in the presence of FeCN alone, although minimal activity is reached more quickly without FeCN (after 11 flash trains vs. 35 with both PPBQ and FeCN and 50 with FeCN alone). With FeCN alone, there is a small initial inflection and a larger second inflection, whereas with PPBQ alone the initial inflection is in the opposite direction and the second inflection comes much earlier. As seen in Figure 6.7C, after 13 trains (1560 flashes), the rate of O_2 loss reaches a local maximum. At this point, 513 electrons per PSII have been advanced to acceptors, as determined by the integrated oxygen yield. After 32 trains (3840 flashes), or 871 electrons, a second local maximum rate is reached, and the final decline to zero begins. Using FeCN alone, the first derivative peak is reached in 5 trains (600 flashes), while the second (major) peak is reached at 43 trains (5160 flashes), at which point a mere 523 electrons have been advanced based on the O_2 yield. A much shallower decline is observed, as well. The data in Figure 56 has the expected shape and behavior for the titration of a twoelectron acceptor with distinct redox potentials for the two electron couples (Q/Q⁻ and Q⁻/Q²⁻). This idea is developed further in the Discussion.



Figure 6.7 Dark-adapted PSII microcrystals in solution supplemented with (A) 2 mM FeCN, (B) 400 μ M PPBQ, and (C) both PPBQ and FeCN, were subjected to a series of flash trains of 120 saturating xenon flashes at 0.5 Hz under oxygenated headspace. The average oxygen yield from 120 flashes (each train) is plotted here. Representative data from a set of replicates are given. The derivative of the oxygen yield during illumination is overlaid on the data.

6.1.3.5 Modeling of the WOC cycle and its inefficiencies in PSII microcrystals.

Historical results have shown that dark adaptation of whole cells and enriched PSII

membranes results in decay of the S2 and S3 states to S1 in minutes or less and results in the

period-4 oscillations of the flash induced O₂ [22, 28, 39-41]. However, as seen in Figure 6.8,

when PSII microcrystals are supplemented with electron acceptor, retention of the S2 state in

particular can be extraordinarily long. Under these conditions, where FeCN has been added, the decay rate for S2 becomes so slow in a substantial fraction of centers that even after more than two hours in the dark, 10% of the total population remains in the S2 state. It is possible that this S2 population is near-permanently dark-stable under these conditions, and indeed the only way to avoid some retention of population, if sufficient electron acceptor is present, is to not illuminate at all. The S3 population decays via essentially normal kinetics; its half-life is approximately 200 seconds, comparable to living culture [28]. Almost all S3 population is depleted within 35 minutes. Use of quinone electron acceptors produces similar, though less pronounced, effects, as the quinones are inherently capable of donating electrons to the WOC to varying degrees, whereas FeCN is not [22, 28, 42, 43].



Figure 6.8 Decay rates of the (A) S2 and (B) S3 populations over time in PSII microcrystals supplemented with 2 mM FeCN, as measured oximetrically. Data were corrected using the VZAD model to account for inefficiencies in operation (see Table 6.1).

In most PSII-containing preparates, the initial oscillations in oxygen yield damp quickly to a steady state value. As seen in Figure 6.9A, after 10 minutes dark adaptation the yield of O_2 oscillates for at least 100 flashes. We used the VZAD model of the WOC catalytic cycle, depicted in Table 6.1, to obtain the best fit of the oscillations in O₂ yield [24]. As seen in Figure 6.8A, application of VZAD to the entire set of 100 flashes does not fit the full range of oscillations simultaneously very well. To achieve the best fit to the initial decay, the model predicts stronger initial damping, which results in unobservable oscillations later and a larger photoinactivation rate (negative slope) than actually observed. However, splitting the data into two segments over shorter intervals of 30 and 31-100 flashes fits very well as seen in Figure 6.9B and C (VZAD's four inefficiency parameters and initial (dark) S state populations for each fit are given in Table 6.1). During the initial 30 flashes, the WOC cycling exhibits strong damping with large misses and backward transitions, whereas after the first 30 flashes, there is much smaller loss in oscillation amplitude. Fourier transformation of the experimental data, shown in the insets and Table 6.1, reveal that the width of the distribution around the period-4 oscillations dramatically narrows by three-fold, while the FT peak shifts from 4.139 to 4.037 (closer to the ideal period-4). These changes indicate that the WOC cycle becomes more efficient during flashing, from dark to light adapted form.

0.50

30

Fourier amplitude 4

2

0

15

20

10

0.00

0.25 cycles/s

25



Figure 6.9 Dark-adapted PSII microcrystals in aerobic solution supplemented with 400 μ M quinone acceptor and 2 mM ferricyanide were subjected to 100 saturating xenon flashes at 0.5 Hz flash rate and repeated three times after dark adaption for 10 minutes prior to each train. Data shown are the average of three biological replicates. Raw data (seen in Figure 6.4B) were normalized to Y_{ss} = 1. (A) VZAD fit to all 100 flashes; (B) VZAD fit to flashes 1-30; (C) VZAD fit to flashes 31-100. Inset shows the Fourier transform of each data set. Residuals given for each fit; residual in (C) was offset +0.9 for ease of display.

Table 6.1 PSII S-state populations and inefficiency parameters obtained from the VZAD fits
shown in Figure 6.6B and C.

VZAD Parameter	Flashes 1-30	Flashes 31-100	Graphical image of the WOC
α, miss	0.058	0.014	cycle snowing the vZAD
β, double hit	0.014	0.000	

δ, backward	0.069	0.000	parameters [24]
transition			α
ε , inactivation	0.037	0.004	$S_1 \leftarrow \delta$
S0 dark population	0.105	0.243	
S1 dark population	0.641	0.259	
S2 dark population	0.254	0.262	
S3 dark population	0.000	0.236	
Period	4.139	4.037	ε - S₃ '
			1 _)α

The fitting parameters in Table 6.1 show that there is a four-fold decrease in misses to 1.4%, the smallest miss parameter ever seen. Likewise, the other three inefficiency parameters decrease to zero, while the initial S state populations needed to produce the excellent fit to the data after flash 30 become nearly equal (0.25 each is the theoretical value for a fully scrambled WOC cycle). This behavior suggests that the retention of weak period-4 oscillations arises from an elevated level of quantum coherence, or synchronization of the active S-state of cycling PSIIs within the sample. The retained oscillations are very easily fitted to the VZAD model, indicating highly efficient operation in this population. The cause of these extended oscillations is ready access to electron acceptor with minimal interference from small reducing molecules which can return electrons to the WOC, traveling outside PSII.

6.1.4 Discussion

6.1.4.1 Endogenous post-QB electron acceptor phenomenon in microcrystals.

A small second period is consistently observed on the first flash train applied to a fresh sample of PSII microcrystals in the absence of electron acceptor (Figure 6.2, Figure 6.3B). This period is not observed on any subsequent flash trains, and its presence is not dependent on oxygen availability. It is more likely that some PSIIs are oriented in such a way as to be able to use other PSIIs or the stabilizing agents used to keep the crystal intact as terminal electron acceptors [12, 13]. Clearly, not all PSIIs are able to do so, as the decline of this feature indicates a finite pool. The question that remains to be solved is: What is this electron acceptor? The acceptor is available to some specific PSIIs to a greater extent than others; if it was generally available, the visible effect would be that of a long tail in oxygen yield as the increasingly scarce acceptor reached centers in S3 at the time. Instead, a clear second oscillation is shown, indicating that for a minority of PSIIs, there is sufficient electron acceptor available for at least four electrons to be removed rapidly. That there is no third period with a comparable or lesser decline between second and third as between first and second indicates that these acceptors cannot be replaced in any substantial quantity and are present with stoichiometry of less than 8 holes per PSII. 8 holes would allow a third oscillation. Given the abrupt end of oxygen evolution after the second oscillation, it is likely that the acceptor has 4 holes. We posit that this acceptor is the WOC of some other PSIIs within the crystal, requiring electrons to travel approximately 37 angstroms between PSIIs [12] down a potential gap of about 1 V [44]. Using the WOC as an acceptor would explain the sharp, irrecoverable drop in yield from the first train to the second even in the presence of oxygen, since the Mn atoms would then leave as Mn(II) and insufficient electron acceptor would be available for photoassembly [45]. Additionally, given the crystal packing, only those PSIIs which are appropriately oriented toward another PSII which is not active would be able to use that second PSII as an acceptor [12]. The drop in peak yield between the first and second train also indicates that these PSIIs are unable to use oxygen as an

electron acceptor, and are thus likely too far inside the microcrystal for oxygen to reach; indeed, oxygen release occurs more slowly on the third flash of a train than any other, indicating a difficult journey through the crystal. Considering that these are microcrystals, not the larger crystals used in PSII crystallography in the past [2, 4, 7, 14], and optimal light is being delivered, it is likely than size of the crystal plays a major role in average efficiency of operation of the PSIIs within.

6.1.4.2 Effect of redox potential gap and diffusion rate on PSII activity.

A fairly small concentration of primary electron acceptor (PPBQ) is needed to reach near-maximum oxygen yield, as compared to the requirement in living culture[22, 28]. Increasing the concentration of primary electron acceptor, provided there is a sufficiently large terminal electron acceptor (FeCN) pool, allows the production of oxygen over a longer period of time from this point. The presence of the quinol form of the primary electron acceptor has a disproportionately detrimental effect on oxygen yield, indicating the use of this quinol as an electron donor either to PSII directly, replacing water, or to oxygen via some mechanism [22, 46-48]. As seen in Figure 6.7, a fairly low concentration of reduced electron acceptor stifles oxygen-evolving activity, especially when the acceptor is PPBQ, which can donate electrons to the WOC in its reduced form. Enough acceptor is added in Figure 6.7C to theoretically accept 7000 electrons per PSII, but after about 600, 50% of the initial steady-state yield is lost. Considering the higher oxidative potential of PPBQ as compared to the native PQ (+277 vs. +20 mV), the quinone electron acceptor must be considered a major limiting factor on operation of PSII as soon as it becomes oxidized, and especially in cases with a proportionally small pool, as in microcrystals lacking supplemental quinone [4, 12, 22, 25, 49]. The redox poise of the acceptors must be delicately controlled to reach maximum efficiency of PSII operation. Meanwhile, it is likely that the minor feature observed early on is dependent on acceptor diffusion.

In addition to the expected period-4 feature in the Fourier transform insets seen in figure 6.9, a period-2 peak is observed in the transform of flash yields after flash 30. This feature has previously been observed as a result of electron acceptor addition [22, 25] and strontium substitution [28], but in those cases the Fourier transform was of the full data set. In PSII microcrystals, however, the period-2 feature seems to only appear after time passes (Inset to 6.9C). Initially, the feature is much less pronounced (Inset to 6.9B) and vague, but once the initial period of higher inefficiency has passed, the peak is quite distinct. The cause of this period-2 effect was proposed over twenty years ago by Shinkarev [50]: cycling of the twoelectron quinone acceptor occupying the Q_B site. However, under native conditions there is substantially less electron acceptor, with PQ pool sizes typically under 50 PQ/PSII, with the pool continuously replenished by activity of cytochrome b₆f [22, 28, 29, 51, 52]. Shinkarev proposed that the limiting factor was the redox poise of the acceptor pool, with Q_B becoming trapped in the semiguinone form until reoxidation occurred and the overall poise changed [50]. Here, the acceptor is not replenished, but a large surplus (7000 holes per PSII) is supplied. The sample environment is therefore not likely to become reduced as rapidly, and yet a period-two feature becomes visible. It is likely, therefore, that it is acceptor diffusion, and not acceptor redox poise, that produces this period-two phenomenon, which is extant as early as the thirtieth flash, at which point the acceptor pool is only 0.2% reduced. Diffusion, however, is a perpetual constraint. This presents another challenge to measurement of PSII structure and activity over time.

6.1.4.3 Unprecedented regulation of S-state population distribution by acceptors.

169

A major challenge for regulation of the S-state in PSII microcrystals is the extreme stability of the S2 population in the presence of electron acceptor. A paradox arises from this phenomenon: it is necessary to supply electron acceptor to maximize efficiency of advance through the WOC cycle, but that same electron acceptor substantially alters S-state decay kinetics, effectively preventing S2 and S3 populations from completely reverting to S1. It is thus necessary, in a sample being used to generate a crystal structure with the WOC in a given Sstate, to very thoroughly prevent illumination of the sample once electron acceptor has been added, and to not expect complete decay of the S2 population. Electron acceptors even alter the dark S-state distribution; note the oxygen yields on the second flash in Figure 6.4. Combined with the instability of PSII microcrystals at room temperature, approximately 11% per day for these samples, and the decline in redox kinetics at the near-freezing temperatures required to retain high activity of PSII in crystals, this effectively prevents complete removal of an S2 population, once generated.

The implications of the response of oxygen evolution to modulation of available electron acceptor for crystallography of PSII in higher S-states are clear. A native PSII-containing crystal not only does not completely advance in the absence of a powerful electron acceptor; a large fraction of PSII is simply not active. Another large fraction is unable to advance immediately without a more effective electron acceptor than the native plastoquinone [22, 49]. The small electron acceptors employed herein are capable of reaching the acceptor side of PSIIs throughout the crystal and sustaining activity by electron removal. However, in the absence of electron acceptor, *most PSIIs (>70%) are not reaching the expected S-state after flash-induced advance*. By oximetrically investigating a PSII microcrystal sample in advance, due to the high precision of the VZAD model [22, 24, 28], it is possible to determine how much of a given PSII crystal structure was contributed by each S-state. It is possible to generate 100% S1, from which S0 and S2 can easily be derived by subtraction of electron density with known populations of each, and once S2 is known, S3 can be deduced via the same method. Generally, it has been previously assumed that WOC transitions in PSII microcrystals occur as efficiently as possible [14, 15], but this is simply not the case. Even with electron acceptor available, a delta parameter of 0.069 is observed initially. This indicates that backward transitions, or return of previously removed electrons to the WOC [22, 24, 25, 53], are occurring within the microcrystals and thus that even in this system it is absolutely necessary to expect inefficiency.

6.1.5 Conclusion

The use of PSII microcrystals has here produced examples of the highest efficiency of PSII operation yet observed, both in terms of quantum yield (with optimal PPBQ and FeCN added, initial quantum efficiency approaches that of primary charge separation itself) and quality of WOC cycling (25 periods and over 98% normal one-electron advance on a given flash in active centers). This represents a new avenue for exploring the limits of the operating capacity of the enzyme and proves that PSII can be pushed *in vitro* to operation far beyond what it is capable of *in vivo*. However, available electron acceptor heavily modulates the redox chemistry of PSII microcrystals both in light and in darkness. Oxygen may serve as an electron acceptor if available, but does so very slowly. In order to increase the quantum efficiency of WOC turnover, an exogenous electron acceptor is needed. However, the reduced quinol electron acceptor accumulates under illumination and has a strong detrimental effect on continued PSII operation. Furthermore, electron acceptors can stabilize the S2 state so effectively as to substantially alter normal dark phenomena in PSII. Electron removal is shown to be the primary constraint on WOC activity in PSII microcrystals.

6.2 High-Resolution Cyclic Behaviors of PSII Operation in Microcrystals

6.2.1 Introduction

Fifty years ago, the Joliot group first observed period-four oscillations in flash oxygen yield, paving the way for extensive study of what was eventually discovered to be the wateroxidizing complex (WOC) of photosystem II (PSII) [54, 55]. As these oscillations were seen to eventually decay to a steady-state oxygen yield, it was evident that the cyclic phenomenon observed was subject to processes other than linear advance of electrons. These inefficiency processes were first modeled by Kok and coworkers, who proposed a simple matrix model for the behavior of the WOC and mapped out its oxidation states well before the composition of the WOC was known [56]. At the time, this represented the cutting edge of photosynthesis research, using thylakoid membranes isolated from spinach. Since then, remarkable advances have been made in the purity of the PSII-containing systems available, as well as the behavioral model of the WOC. Highly homogeneous PSII microcrystals represent an ideal system for the study of the behavior of redox processes, permitting a level of detail to be observed in oxygen yield which is not equaled in any other system studied to date (Section 6.1). As a numerical comparison of systems, Kok's model was optimized for about seven oscillation periods, which was long considered optimal operation [56]. In this work, we report fifty distinct periods.

Following the publication of Kok's model, it became apparent through the study of other PSII-containing systems that this simple model did not fully describe the activity of the WOC, and numerous other models were proposed for WOC operation, each adding layers of complexity to account for the numerous electron-transfer processes in PSII which may affect WOC operation [57-67]. A simple and effective improvement on Kok's algorithm was developed by Colin Wraight and Vladimir Shinkarev, and the Shinkarev model supplemented the original Kok inefficiency parameters, alpha (miss) and beta (double-hit), with the new parameters delta (backward transition) and epsilon (inactivation) to increase quality of model fits to data [53, 68-70]. However, Shinkarev's model proposed that misses and backward transitions occurred on every oxidation state (S state) transition in the WOC cycle. The development of the VZAD model, employed herein for data analysis, allowed fits which restricted backward transitions and inactivation to the dark-unstable S2 and S3 states [24].

Shinkarev and Wraight also proposed the existence of a second cycle in PSII flash oxygen yields, one dependent on the acceptor side of PSII [50, 71-73]. This cycle is inferred from homology and analogy with the two-electron gate mechanism in the bacterial reaction center which was observed by Wraight, and independently by the group of Rod Clayton [74]. Incorporation of such a cycle with the existing WOC models has been attempted, but evidence for the predicted binary oscillation in PSII has been sparse to date [75-77]. Binary cycles have previously been observed in model-independent Fourier transforms of flash oxygen yields from various living microalgal cultures subjected to strontium biosynthetic substitution in PSII or exogenous quinone acceptor supplementation [22, 28]. However, the source of this cycling was not studied in detail at the time due to the constraints of the living system.

Herein a quantitative examination of the interactions of the acceptor-side control of PSII operation with WOC cycling is undertaken, utilizing the extreme homogeneity of PSII microcrystals to closely study redox poise of the acceptor system and its relationship to various known operating parameters within PSII. Control of several WOC inefficiency processes by type and redox poise of electron acceptor is demonstrated, indicating a similar control mechanism in the native system. The origin and regulatory mechanisms of the observed period-2 feature are investigated, with multiple period-2 processes posited to stem from both the acceptor-side quinone gate and from decay of unstable S-states to form (hydrogen) peroxide at the WOC [22, 28, 70, 78-80]. Finally, several additional periodicities are demonstrated in oximetric PSII cycling which appear to stem from distinct populations of PSIIs. These data are obtained via the VZAD software suite and its associated fast Fourier transform algorithm, herein applied for the first time to time-resolve activity of PSII owing to the extreme fidelity of oscillations achievable in PSII microcrystals.

6.2.2 Materials and Methods

Homogeneous microcrystals of PSII were generated from PSII core complexes of *Thermosynechococcus elongatus* using the method of Kupitz et al. as described in [12, 19, 20]. Crystals were found to be uniformly 10-12 μm in diameter by optical microscopy and were previously shown via X-ray crystallography to contain 35 ChI/D1 [12]. Crystals were stored and all oximetric measurements performed in stabilizing buffer containing 100 mM PIPES at pH 7.0, 5 mM CaCl2, 10 mM tocopherol, and 20% PEG 2000.

All O₂ measurements were performed on a homemade Pt-Ir Clark-type electrode covered by a thin membrane, the most recent generation of an instrument extensively developed by Prof. Ananyev [21-23, 81]. This membrane was previously demonstrated to prevent diffusion of added electron acceptors to the electrode surface, while the reducing potential has previously been proven sufficient to avoid generation of sufficient hydrogen peroxide to reach the sample considering the presence of the membrane [22, 82]. All measurements were carried out under constant DC voltage bias without the use of an AC filter; thus, values returned are directly proportional to the current needed to reduce O_2 , and thus to the O_2 yield. Shading effects on the electrode were avoided by use of an optically thin, dilute crystal sample. Light was delivered across the visible spectrum using a high pressure Xe flash lamp (model ISSh-400). Flash duration at half maximum (FWHM) for this lamp is approximately 1 µs and optical energy delivery was previously optimized for this system to deliver single turnover flashes, i.e., to advance the population of WOCs by one S-state each (described in Section 6.1.2). For all measurements, trains of single-turnover flashes were used following dark incubation to drive flash oxygen yield with high separation of S-state populations to maximize signal to noise ratio in resulting oscillations. Flashes were delivered at 0.5 Hz with 10-minute dark pre-incubation to allow decay of S2 and S3 populations unless otherwise specified.

Two methods were utilized to derive analyses from flash O₂ yield data. Modelindependent Fourier transformation of flash trains was performed to determine the frequency distribution of cyclic processes affecting flash oxygen yield, primarily the WOC cycle [23]. Model-dependent analysis was carried out by fitting O₂ flash train yields to the advanced VZAD model of the WOC cycle, as previously described [22, 24]. Accuracy of the VZAD model fit was determined by RMS deviation of the fit. The current version of the VZAD model is available at http://chem.rutgers.edu/dismukes-software.

The synthetic quinone *para*-phenylbenzoquinone (PPBQ) was obtained from Aldrich Chemical Co. (originally 98% purity). The original quinone was further purified by double recrystallization in ethanol and subsequently dissolved in dimethyl sulfoxide (DMSO) at 20 mM concentration, after which further dilution into crystal samples was carried out as needed for experiments. K₃Fe(CN)₆ (potassium ferricyanide, abbreviated FeCN) was obtained from Sigma-Aldrich (99% purity). Near-total oxidation of both electron acceptor stocks (quinone form of PPBQ rather than quinol; ferricyanide as opposed to ferrocyanide) was established previously [22].

6.2.3 Results

6.2.3.1 Observing multiple periodicities in PSII oscillations.

PSII microcrystals show extended oscillations in oxygen production. As discussed in Section 6.2.1, Joliot's pioneering work demonstrated seven to eight period-4 oscillations in oxygen yield [54]. Following this research, longer periods of oscillations were rarely observed, though recently the use of exogenous quinones or certain organisms has shown greater fidelity of oscillations [22, 23, 28]. By Kok's model and all related subsequent versions, the number of oscillation periods reciprocally correlates with the inefficiency parameters [24, 53, 56]. In the current research, PSII microcrystals are demonstrated to produce up to 50 periods of oscillations extending over 200 single turnover flashes (Figure 6.10).



Figure 6.10 Flash-induced oxygen oscillations are observed over a train of 200 flashes (50 period-4 cycles) in PSII microcrystals supplemented with 4mM FeCN and 200 μ M PPBQ. Inset shows the last 100 flashes magnified vertically 33-fold. Data shown is the average of 5 replicates.

Distinct phases in these oscillations can be observed, as seen in Figure 6.11. Initially, rapid decay is observed, which corresponds to high inefficiency of WOC cycling. At right, the Fourier transform of the first 30 flashes in a train shows a broad central peak, indicative of significant inefficiency. Due to the processes which alter the WOC cycle each occurring in multiple S-states, inefficiencies are observed as a spreading of the peak rather than as distinct peaks at the actual cycle number by center. The peak is centered on a cycle frequency of 0.25, or 4 flashes to a cycle. At left, the Fourier transform (FT) of flashes 31-100 presents a much narrower central peak, as well as several distinct smaller peaks.



Figure 6.11 Center: Oxygen yields from a train of single turnover flashes applied to a PSII microcrystal sample supplied with 200 μ M PPBQ and 4 mM FeCN. Bottom: Secondary oscillations with period ~21.6 in the same train of flashes. Left: Fourier transform of flashes 1-30 of the flash train. Right: Fourier transform of flashes 31-100 of the flash train, showing narrower central peak and auxiliary peaks at 0.5 cycles (period 2), 0.47 cycles (period 2.15), 0.21 cycles (period 4.65), and 0.047 cycles (period 21.6).

The most prominent non-period 4 peak in the Fourier transform of the "steady-state" oscillations is the period-2 peak (at 0.5 cycles per flash), which has been observed in some living systems previously [22, 28]. This peak has been suggested to be produced by acceptor-side quinone cycling in the Q_B site or by peroxide production at the WOC; however, the former is not possible in this system due to the extreme energetic unfavorability of removal of the hydrophobic Q_B plastoquinone into the aqueous, albeit detergent-stabilized, PSII microcrystal environment, and the latter is not energetically favorable as compared to full oxidation of water if sufficient holes can be accumulated. The origin of this feature is addressed below. In addition to the two previously observed features, however, we introduce several cyclic functions of PSII which have not been observed to date. Three smaller peaks are observed at periods ~2.15, ~4.65, and ~21.6. These peaks are observed only in the presence of the electron acceptors

PPBQ and FeCN within the beneficial range of each acceptor. Unlike the period-4 and period-2 features, which can be observed both in the FTs of individual flash trains and in the average of consecutive flash trains due to decay of the higher-energy donor and acceptor populations in the dark between trains, these features cannot be sharpened via averaging of subsequent trains because there is not a clear decay mechanism for this feature. As seen in Figure 6.15, the period-4.65 and period-21.6 features are observed clearly over each individual flash train, but averaging these data together simply smooths out the feature. The period-21.6 oscillation is the most distinctly visible of the three in the raw data, as seen in the bottom panel of Figure 2, because of the similarity in periodicity of the other two to the much more common period 4 and 2 features. However, the period-21.6 feature does not always occur on those specific flashes, and the inflection points visible in the raw data move earlier in the flash train over subsequent trains delivered.



Figure 6.12 (A) Oxygen yields from trains of single turnover flashes applied to a PSII microcrystal sample supplied with 200 μ M PPBQ and 4 mM FeCN. Flash trains were delivered with 1, 2, or 4 s between flashes (1, 0.5, and 0.25 Hz) as denoted. (B) Fourier transform of flashes 31-100 in (A).

Altering the flash rate, i.e. the effective light intensity delivered, reveals several additional features of PSII oxygen evolution. First, there is a nearly inverse correlation between period-4 cycling fidelity, as seen in Figure 6.12B, and the steady-state oxygen yield shown in Figure 6.12A. Second, the period-2 feature is independent of the period-2 feature. At 1 s between flashes, the period-2 cycle is more than twice as common as it is at 2 s, though the period-4 feature is less than 20% larger. At 4 s, there is approximately 5% decline in period-2, as compared to 40% decline in period-4. The period-4.7 feature approximately follows the period-4 feature, however, staying close to proportional to the central peak. Finally, the flash oxygen yield increases substantially at lower flash rate, which is to be expected; the S2 and S3 states are highly stable and delivery of FeCN acceptor is shown to limit oxygen evolution in Section 6.1.





Figure 6.13 Comparison of peak yield (Yp; flash 3 of a given train) and steady-state average flash yield of a given train (Yss) over 50 trains. Microcrystals were supplemented with 200 μ M PPBQ and 4 mM FeCN; trains were delivered every 10 minutes and consisted of 120 flashes each at 0.5 Hz.

Peak yield (Figure 6.13) is not controlled by the same mechanism as steady state at all times. During trains 5-20, Yss is regulated by PPBQ availability while Yp is regulated by FeCN availability. After train 20 both are regulated by FeCN availability only, as BQ is functionally used up (Figure 6.7). The source of this discrepancy is diffusion kinetics. PPBQ can diffuse more readily than FeCN in this system and can pick up electrons from Q_A and Q_B quickly, on the time scale of the flashes delivered. The peak yield remains high because there is a 10-minute window prior to the flash train in which FeCN can abstract electrons from PSII. The rate of electron removal from PSII by FeCN is significantly slower than that by PPBQ (Figure 6.7).

6.2.3.3 Relationship of Alternative Oscillators and Acceptor Availability.

The period-2 feature visible in Fourier transforms of oxygen yield also varies in amplitude with the redox state of available acceptors to PSII. The amplitude of this feature can be seen to correlate to the steady-state oxygen yield, dependent on the acceptor type (Figure 6.14).



Figure 6.14 Period 2 peak amplitude plotted against Yss of individual flash trains. In black, train steady-state yields from sequential flash trains. In blue, Fourier transform peak amplitudes at period 2 as obtained from each flash train. Data shown is representative of multiple data sets.

(A) Sample supplemented with 4 mM FeCN; (B) Sample supplemented with 200 μM PPBQ and 4 mM FeCN.

The period-2 feature appears to be strongly linked to the production and retention of semiquinone in the Q₈ site. When FeCN is used as the sole electron acceptor, it will favor removal of electrons from Q₈ which has already been doubly reduced as a simple matter of redox chemistry. As the pool of available FeCN is depleted, the period 2 amplitude reaches a peak. However, once a critical point is reached and the rate of reduction of semiquinone exceeds the rate of oxidation of the quinol in the Q₈ site, the period-2 feature declines precipitously and is nonexistent before the FeCN pool is fully reduced. A similar decline is seen initially; this one can be attributed to PSIIs near the center of the crystal, which have poor access to acceptor and are otherwise primarily constrained by delivery of FeCN, becoming inactivated and effectively experiencing the same acceptor limitation earlier on. When PPBQ is added as well, an earlier peak is also observed while PPBQ equilibrates with the native quinone in the Q₈ site via dismutation, producing high concentrations of semiquinone. As the PPBQ is consumed at this stage, another decline in the period-2 feature is observed, followed by an increase as FeCN becomes the major acceptor and high levels of semiquinone in the Q₈ site are again created.



Figure 6.15 Peak amplitudes for (A) period-4.7 and (B) period-22 peaks plotted against Yss of individual flash trains, as obtained from Fourier transforms of flashes 31-100 of each train. Sample supplemented with 4 mM FeCN and 200 μ M PPBQ. Data shown is representative of multiple data sets.

Behavior of the period-4.7 and period-22 peaks is nearly identical over the period of flash trains (Figure 6.15). Peak amplitude increases initially, remains level from about train 10 to 20, and then declines to zero by train 30. The period-4.7 amplitude changes more sharply than the period-22. Maximum expression of both cycles corresponds to the period of reduction, but not total reduction, of the available PPBQ. Decline begins concurrent with the FeCN-dominated phase (Figure 6.7; Section 6.1.4), suggesting that this cycle is dependent on the presence of PPBQ in the semiquinone form. This form of PPBQ is expected to be most prevalent during the phase of peak period-4.7 activity.

6.2.3.4 Analyzing acceptor side events with VZAD model parameters.



Figure 6.16 Comparison of acceptor effect on the alpha (miss) parameter train-by-train. In black, train steady-state yields from sequential flash trains. In blue, alpha parameter as obtained from each flash train. Data shown is representative of multiple data sets. (A) Sample supplemented with 4 mM FeCN; (B) Sample supplemented with 200 μM PPBQ and 4 mM FeCN.

When FeCN is the sole electron acceptor present, the alpha (miss) parameter generally follows the inverse of the oxygen yield (Figure 6.16). This is expected; ferricyanide seems to uptake electrons from Q_B at a nearly constant rate until the 15th flash train, with only minor decline due to acceptor consumption or limitation of diffusion during that time, and then upon reaching limitation of removal of electrons from Q_B , missed sharply increase, likely due to recombination from Q_A ⁻. However, the effect in the presence of PPBQ as well is dramatically different. During the PPBQ-dominated phase (to about train 15), the alpha value is low, comparable to that seen with FeCN alone. However, over the following 15 trains, when PPBQ can be expected to be a minor acceptor and FeCN the controlling factor, misses rise sharply. As PPBQ is not expected to replace the native plastoquinones due to the lack of a membrane for exchange, this indicates that the presence of reduced PPBQ strongly increases recombination. It is unclear if this recombination is actually flash-induced or simply a continuous process whereby electrons are delivered to the WOC by PPBQoI.



Figure 6.17 Comparison of acceptor effect on the delta (backward transition) parameter trainby-train. In black, train steady-state yields from sequential flash trains. In blue, delta parameter as obtained from each flash train. Data shown is representative of multiple data sets. (A) Sample supplemented with 4 mM FeCN; (B) Sample supplemented with 200 μ M PPBQ and 4 mM FeCN.

Backward transitions (delta) follow a similar kinetic to misses when FeCN is the sole electron acceptor (Figure 6.17). However, they begin to increase *after* oxygen evolution has started to decline. Whereas alpha starts increasing at train 14-15, it is not until train 19-20 that the equivalent spike in backward transitions takes place. The longer time required suggests that this process requires a much stronger redox pressure. Additionally, since backward transitions are here observed in PSII microcrystals which are not capable of undergoing oxygen evolution at all if an acceptor is not present, and the sole available electron acceptor is FeCN, which does not readily release accepted electrons to the WOC, the mechanism of backward transitions is demonstrated to be one *internal to PSII*. As backward transitions have previously been shown to be the oximetric signature of PSII-cyclic electron flow, this demonstrates that PSII-CEF can occur via a completely internal mechanism.

When PPBQ is added, backward transitions are high from the outset. Through the PPBQ-regulated phase a high delta is observed, but once the FeCN phase is reached (around
train 15), backward transitions completely disappear. They remain near zero until just before the point at which they would increase in the absence of FeCN, relative to the terminal limitation of that acceptor, and rise much more rapidly than they do in that system. These discrepancies prompt several questions: 1) Why are electrons more likely to travel from the acceptors to the WOC when there is more available acceptor; 2) why does the presence of presumably reduced PPBQ inhibit backward transitions which appear to be internal to PSII, as seen using FeCN alone; and 3) how much of the delta parameter stems from internal electron transfer as opposed to external mechanisms? PPBQoI can donate electrons to the WOC, so external transfer is a definite possibility.



Figure 6.18 Comparison of acceptor effect on the epsilon parameter (PSII inactivation rate) train-by-train. In black, train steady-state yields from sequential flash trains. In blue, epsilon parameter as obtained from each flash train. Data shown is representative of multiple data sets. (A) Sample supplemented with 4 mM FeCN; (B) Sample supplemented with 200 μ M PPBQ and 4 mM FeCN.

The epsilon parameter, reflecting the inactivation rate of WOCs, behaves very similarly to alpha and delta when FeCN is the sole electron acceptor (Figure 6.18). After staying relatively level during the phase in which FeCN is abundant, at train 19 an abrupt rise is observed, concurrent with the rise in delta. This stands to reason; too many backward transfers are indistinguishable from inactivation, and both processes appear to occur as a result of complete saturation of the internal acceptors with electrons. However, in the presence of PPBQ, inactivation follows neither the alpha nor the delta parameters. Instead, the inverse of the oxygen yield is observed, as is seen when FeCN is the sole electron acceptor. In both cases, we can thus easily link inactivation to redox poise of the overall acceptor pool.



Figure 6.19 Comparison of acceptor effect on the initial dark S-state distributions prior to each train. In black, train steady-state yields from sequential flash trains. In blue, fraction of each S-state in the dark before each flash train. Data shown is representative of multiple data sets. (A) Sample supplemented with 4 mM FeCN; (B) Sample supplemented with 200 μ M PPBQ and 4 mM FeCN.

In the presence of FeCN, a slow loss of S2 to S1 and S0 is observed until FeCN is depleted, after which time a rapid decay to 100% S1 is observed (Figure 6.19). However, when PPBQ is added, the S2 population is retained significantly longer and the kinetics of decay are altered. While there is still a roughly linear loss of S2 to a minimum, that minimum is not zero and is followed by recovery of S2 in the few remaining active centers. S0 declines faster, beginning upon transition from PPBQ-dominated to FeCN-dominated oxygen-evolving behavior, and is completely gone in darkness before FeCN becomes limiting; the S1 population follows an inverted kinetic. It is evident that the consumption of PPBQ, and likely the generation of the semiquinone or quinol form, results in the destabilization of the S2 and the S0 populations. The clearing of S0 under both conditions is unexpected; the presence of redox-active species is tightly regulated and there should be no external oxidant present powerful enough to induce an S0-S1 transition in darkness. The likely cause of this phenomenon is the presence of oxidized tyrosine-D, which could theoretically take up the electron over a sufficiently long time scale. This hypothesis, however, prompts another question: why is tyrosine-D being oxidized?

6.2.4 Discussion

While the periodicities of flash oxygen evolution cannot be resolved readily from the data for the most part due to the dominance of the period-4 feature (see Figure 6.10), Fourier transformation allows a more detailed investigation (Figure 6.11). The period-2 feature appears to be linked to generation of semiquinones; as oxygen yield approaches the terminal decline (i.e. when the Q_B site fills with electrons), regardless of acceptor, the period-2 feature reaches its maximum (Figure 6.14). There is additionally some expression of the period-2 cycle which correlates with PPBQ consumption, presumably because reduction of PPBQ requires protons to balance the electrons transferred, and these protons are in increasingly short supply as oxygen evolution occurs (those generated at the donor side are consumed at the electrode when the measured oxygen is reduced, effectively completing a cycle). Altering the flash rate allows for an investigation of the origin of the feature (Figure 6.12). As peroxide generation is energetically unfavorable as compared to generation of oxygen once the S4 state is reached, it can be expected to be produced by decay of either the S2 or S3 state, with increased likelihood with slower flash rate [78-80]. Conversely, faster flash rate would support limitation on the acceptor side, as proposed by Shinkarev [50]. Instead of a clear pattern favoring one or the

other, what is observed is a sharply higher period-2 feature at a flash frequency of 1 Hz than at either 0.5 Hz or 0.25 Hz, but little change between 0.5 and 0.25 Hz, suggesting two processes. The peak amplitude more than doubles from 0.5 Hz to 1 Hz. Thus, the faster process, the semiquinone gate, is likely the major source of this feature *in vivo*, as a flash rate of 1 Hz is still equal to a mere 3 μ Ein/m²/s light intensity and *T. elongatus* is ideally grown under at least 40 μ Ein/m²/s and normally possesses an antenna complex increasing the number of available chlorophyll by about 45-fold [22, 28, 83]. The slower contributing feature, speculated to stem from peroxide generation at the WOC, is not likely to be a major contributor under ideal light conditions.

The period-4.7 and period-22 features are less easily attributed to known phenomena simply because these features lack an obvious analog due to their non-integer cycle length. The features are not an artifact of time between flashes, as they are present at several flash frequencies in Figure 6.12B with no alteration to period length. The source process is presumably not solely internal to PSII, as it is only visible when both PPBQ and FeCN are used as electron acceptors. Figure 6.15 makes it clear that the two processes are linked; this is further supported by the feature periodicities. A period of 2.15 is approximately the square root of a period of 4.7, and a period of 4.7 is the square root of a period of 21.6. This suggests one process which interacts with itself in some manner. The rapid decline of these features on accumulation of electrons in the acceptor pool suggests it is a flash-dependent process reliant on the pool in some way. A cyclic or deliberately inefficient mechanism, whereby electrons are returned from the acceptor side of PSII to the donor side, and one which only occurs in a small fraction of the total PSII population at any time, is the most likely origin of these features. The lack of a clear "reset", whereby the feature would start at a certain point in its cycle after dark incubation, limits the options for the source of this feature. If there is a decay process

associated with the generation of the period-21.6 feature, it occurs on a timescale longer than 10 minutes.

The processes affecting the classic WOC cycle are all shown to be dependent on acceptor-side redox poise. The alpha parameter (Figure 6.16) is of particular interest for what happens during the phase from train 15 to train 30, where FeCN is the primary regulator of electron removal from PSII. When just FeCN is present, there is a steady level of misses during this phase, but addition of reduced quinone causes a steady increase over time. The implication of this increase is that electrons are being transferred from PSII to PPBQ to FeCN, as during the first phase there is a fairly steady miss parameter, at the same level as what is observed when FeCN alone is present. This would imply that PPBQ accepts electrons from reduced Q_B rather than from Q_A, as FeCN can only accept electrons from Q_B [31, 84]. The second implication is that an *external* miss is possible. It is unclear whether this external return of electrons via redox-active quinone to the WOC is driven by light or simply occurs regardless of charge-separation events. However, Figure 6.12 suggests the latter is the case; oscillations decay much faster and remain smaller at longer times between flashes, reflecting a higher rate of recombination.

Even within individual flash trains, the efficiency of average WOC operation is altered over time. From the peak yield at the beginning of the train, a net decline is observed, with a logarithmic scale. By the end of the train, the overall decline is negligible, but oscillations are also very small. This feature is likely due to diffusion of acceptors limiting PSII turnover; at the beginning of the train, the entire crystal is saturated with acceptor. However, as the PSIIs in the center of the crystal consume the local acceptor pool, it is more difficult to replace than at the edges, resulting in decline of yield over time. This decay is separate from what is observed later in the train (flashes 31-100, as investigated in the time-dependent measurements). During this period, oscillations are retained at a high level and oxygen yield is largely from the outside of the crystal, where acceptor is readily available. Increase of the dark time between flashes, as seen in Figure 3, results in higher oxygen yield but substantial damping of oscillations during the nondiffusion-limited phase. The clear cause of the decline in oscillations is a light-independent reductive process at the WOC. This process may be modeled as either α , δ , or ε . Under these conditions, the point of oscillations ceasing to be significant can be predicted using a WOC cycle model and is relevant to the inefficiency parameters and the initial population distribution. The light-independent WOC reductive process appears to increase over time, and can be expected to be related to the decay of individual S-state populations, particularly the S3; the S2 population, after all, decays nearly an order of magnitude more slowly and should not contribute significant decay on this time scale. The substantial contribution of the S3 state to WOC reductive processes was previously proposed by Pham and Messinger [60], but is much more pronounced in this system.

Backward transitions are observed at a negligible level under sufficient ferricyanide present (Figure 6.17). This is still true of the FeCN-dominated phase when both electron acceptors are present, but during the period of PPBQ serving as the primary electron acceptor, the delta parameter is substantially elevated. As backward transitions are shown to be capable of occurring entirely within PSII when only FeCN is used as acceptor, and PPBQ is a faster electron acceptor than FeCN, the source of these backward transitions should not be limitation by acceptor availability. Rather, it is likely that the availability of protons is a major constraint. The stable reduction of PPBQ and Q_B requires one proton per electron; however, reduction of FeCN simply requires a cation to balance the charge on the relatively stable ferrocyanide anion formed, and the rate-limiting step becomes the slow removal of electrons to FeCN rather than locating a proton to balance electrons on a quinone [31]. Without the proton, the free energy of the quinone is substantially higher [85] and recombination of the electron is facilitated. It is unclear whether *all* backward transitions occur within PSII, directly from an anion radical form of Q_B , or whether some transfer from anion radical PPBQ. It must also be noted that a large fraction of centers are simply inactive when FeCN is the main electron acceptor, and thus it is likely that this proton constraint also prevents removal of electrons from Q_B once it has reached the quinol form. A third internal quinone Q_C may also be involved in some fraction of centers. The overall process is detailed in Figure 6.20.



Figure 6.20 Scheme of rates and constraints in electron transfer within PSII.

6.2.5 Conclusion

Multiple previously unknown periodicities of oxygen yield are observed within PSII. The poorly characterized period-2 phenomenon is observed to a significant extent and tentatively attributed primarily to Shinkarev's proposed Q_B site gating effect, though a secondary effect stemming from donor-side generation of peroxide under lower light is also proposed. The well-accepted miss parameter is shown to be at least partially due to non-light-induced causes, specifically external delivery of previously separated electrons to the WOC with no limitation of

advance on the following flash. Backward transitions are shown to be able to occur internal to PSII, originating from the reduced Q_B site.

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