Oxidation and reduction reactions of the water-oxidizing complex in photosystem II

Vo Pham Long
To my beloved wife - Nguyen Le Ninh Thai, our future children (Tigon), my cherished family and family in law
Table of Contents

Table of Contents  i
Abstract ii
Abbreviations iv
List of Publications v
Introduction 1
  The dawn of life 1
  Natural oxygenic photosynthesis 2
  Water splitting reaction 4
  The “heart” of photosystem II complex 7
  Chemistry of the Mn_4CaO_5 cluster 9
  Developments in deconvolution of FIOP data 10
Materials and methods 13
  Chemicals and buffer solutions 13
  Preparations of biological samples 13
  Identification of chlorophyll concentration 15
  Catalase inactivation 15
  Measurement of oxygen evolution activity 17
  Joliot-type electrode experiment 17
  Data analysis of FIOP 20
Results and discussions 27
  Does the formation of H_2O_2 take place during Joliot-electrode measurement? 27
  Does the very slow phases of S_2 and S_3 decays really exist in S-state lifetime measurements? 32
  How dependent are miss parameters on S-state of Mn_4O_5Ca cluster and on the oxidation state of Y_D? 35
  What is the least efficient S-state transition of the Mn_4O_5Ca cluster, S_2→S_3 or S_3→S_0 transition? 37
  The effect of hydrogen/deuterium exchange on miss parameters 43
Conclusions 45
Acknowledgements 47
References 49
Abstract

The oxygen that we breathe and food that we eat are products of the natural photosynthesis. Molecular oxygen is crucial for life on Earth owing to its role in the glycolysis and citric acid pathways that yield in aerobic organisms the energy-rich ATP molecules. Photosynthetic water oxidation, which produces molecular oxygen from water and sunlight, is performed by higher plants, algae and cyanobacteria. Within the molecular structure of a plant cell, photosynthesis is performed by a specific intracellular organelle – the chloroplast. Chloroplasts contain a membrane system, the thylakoid membrane, which comprises lipids, quinones and a very high content of protein complexes. The unique photosynthetic oxidation of water into molecular oxygen, protons and electrons is performed by the Mn₄CaO₅ cluster in photosystem II (PSII) complex. Understanding the mechanism of water oxidation by Mn₄CaO₅ cluster is one of the great challenges in science nowadays. When the mechanism of this process is fully understood, artificial photosynthetic systems can be designed that have high efficiencies of solar energy conversion by imitating the fundamental principle of natural system. These systems can be used in future for generation of fuels from sunlight.

In this thesis, the efficiency of water-splitting process in natural photosynthetic preparations was studied by measuring the flash-induced oxygen evolution pattern (FIOP). The overall aim is to achieve a deeper understanding of oxygen evolving mechanism of the Mn₄O₅Ca cluster via developing a complete kinetic and energetic model of the light-induced redox reactions within PSII complex. On the way to reach this goal, the hydrogen peroxide that is electrochemically generated on surface of Pt-cathode was discovered. The chemical effect of electrochemically produced H₂O₂ that can interfere in the oxygen evolution pathway or change the observed FIOP data was demonstrated. Therefore, in order to record the clean FIOP data that are further characterized by global fitting program (GFP), H₂O₂ has to be abolished by catalase addition and by purging the flow buffer of the Joliot-type electrode with nitrogen gas.

After FIOPs free of H₂O₂-induced effects were achieved, these clean data were then applied to a global fitting approach (GFP) in order to (i) result a comprehensive figure of all S-state decays whose kinetic rates were simultaneously analyzed in a high reliability and consistency, (ii) the dependence of miss parameter on S-state transitions and the oxidation state of tyrosine D (Y₅₃) can be tested, (iii) how dependent of all S-state re-combinations (to S₁ state) on the various pH/pD values can be also determined in case of using Cyanidioschyzon merolae (C. merolae) thylakoids. Our data support previous suggestions that the S₀ → S₁ and S₁ → S₂ transitions involve low or no misses, while high misses occur in the S₂ → S₃ transition or the S₃ → S₀ transitions. 
transition. Moreover, the appearance of very slow $S_2$ decay was clearly observed by using the GFP analysis, while there are no evidences of very slow $S_3$ decay were recorded under all circumstances. The unknown electron donor for the very slow $S_2$ decay which can be one of the substances of PSII-protective branch (i.e. cytochrome $b_{559}$, carotenoid or Chl$Z$) will be determined in further researches.
### Abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>Miss parameter</td>
</tr>
<tr>
<td>β</td>
<td>Double hit probability</td>
</tr>
<tr>
<td>β₁</td>
<td>Double hit after the first flash</td>
</tr>
<tr>
<td>γ</td>
<td>Single hit parameter</td>
</tr>
<tr>
<td>δ</td>
<td>Fast backward S-state transition during flash train</td>
</tr>
<tr>
<td>d</td>
<td>Damping parameter</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Chl, [Chl]</td>
<td>Chlorophyll, concentration of chlorophyll</td>
</tr>
<tr>
<td>Cyt</td>
<td>Cytochrome</td>
</tr>
<tr>
<td>DCBQ</td>
<td>2,6-Dichloro-1,4-benzoquinone</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
</tr>
<tr>
<td>EXAFS</td>
<td>Extended X-ray absorption fine structure</td>
</tr>
<tr>
<td>FIOPs</td>
<td>Flash-induced oxygen evolution patterns</td>
</tr>
<tr>
<td>Fd</td>
<td>Ferredoxin</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MIMS</td>
<td>Membrane-inlet mass spectrometry</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-morpholinopropane-1-sulfonic acid</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>OEC</td>
<td>Oxygen-evolving complex</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly (methyl methacrylate)</td>
</tr>
<tr>
<td>PPBQ</td>
<td>Phenyl-1,4-benzoquinone (phenyl-p-benzoquinone)</td>
</tr>
<tr>
<td>PSI</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>PSII</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>P₆₈₀</td>
<td>Primary electron donor chlorophyll molecule in PSII</td>
</tr>
<tr>
<td>QA</td>
<td>Primary plastoquinone electron acceptor in PSII</td>
</tr>
<tr>
<td>QB</td>
<td>Secondary plastoquinone electron acceptor in PSII</td>
</tr>
<tr>
<td>RC</td>
<td>Reaction center</td>
</tr>
<tr>
<td>Sᵢ-state</td>
<td>Oxidation state of Mn₄O₅Ca cluster, where “i” represents for the number of stored oxidizing equivalents.</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet – visible spectroscopy</td>
</tr>
<tr>
<td>WOC</td>
<td>Water oxidation complex</td>
</tr>
<tr>
<td>XANES</td>
<td>X-ray absorption near edge structure</td>
</tr>
<tr>
<td>XAS</td>
<td>X-ray absorption spectroscopy</td>
</tr>
<tr>
<td>Y_D</td>
<td>Tyrosine 160 of D2 protein</td>
</tr>
<tr>
<td>Y_Z</td>
<td>Tyrosine 161 of D1 protein</td>
</tr>
</tbody>
</table>
List of Publications

1. Long Vo Pham and Johannes Messinger (2014) Electrochemically produced hydrogen peroxide affects Joliot-type oxygen-evolution measurements of photosystem II, Biochimica et Biophysica Acta 1837, 1411-1416
   Reprinted with permission from licensed content publisher Elsevier with license number 3747030614969

2. Long Vo Pham and Johannes Messinger (2015) Probing S-state advancements and recombination pathways in photosystem II with a global fitting program for flash-induced oxygen evolution yields, manuscript

3. Long Vo Pham, Tomasz Krupnik, Joanna Kargul, Johannes Messinger (2015) Effects of hydrogen/deuterium exchange and pH on the S_i state dependent miss parameters of flash-induced oxygen evolution of the extremophilic red alga Cyanidioschyzon merolae, manuscript

4. Håkan Nilsson, Long Vo Pham, Tomasz Krupnik, Joanna Kargul, Johannes Messinger (2016) Absence of a substrate water ‘flip’ during the S_2 → S_3 transition of the oxygen-evolving complex in photosystem II, manuscript
Introduction

The dawn of life

The formation of our planet, the Earth, started approximately 4.5 billion years ago (Dalrymple, 2001) by the accretion of solar nebula (Woolfson, 1993). There was no living organisms that could be survived on the Earth at this time because of very high surface temperature (approximately 1000°C) and strong bombardments by meteorites from outer space (Gomes et al., 2005). Another reason for no biological life on the young Earth was the primordial atmosphere which was created by volcanic outgassing did not contain oxygen and would have been toxic to humans and most modern lives. At the time point of the “Moon generation” event, which a few theories support the Moon was initially a part of the Earth (Halliday, 2012), the Earth’s surface was still covered by molten magma (Sleep et al., 2001). When the magma ocean was solidified and the surface temperature of Earth was significantly cooled down, the water could be retained and the first coast was created about 4.2-4.1 billion years ago (Valley et al., 2002).

The biological life forms whose fossils in metasedimentary rocks were earliest discovered in Western Greenland appeared on the Earth about 3.8 billion years ago (Rosing, 1999). At this time, only anoxygenic photosynthetic processes were performed by the primordial organisms (phototrophs). They utilized the solar energy to oxidize the inorganic reductants (e.g. H₂, H₂S, CH₄, etc) and use released electrons for carbon fixation (Olson, 2006). Furthermore, the prokaryotic cyanobacteria developed the ability to split water into molecular oxygen and metabolically bound hydrogen, using sunlight as energy source (Buick, 1992, Xiong and Bauer, 2002). Due to the evolution of photosynthesis which used water as initial electron source, carbon dioxide was reduced and converted to carbohydrate and molecular oxygen was generated via water oxidation process as a byproduct. The current atmosphere with high amount of oxygen (21%) which was rapidly formed and maintained after “Cambrian explosion” event (around 2.3 billion years ago) was an inevitable result of oxygenic photosynthesis (Barber, 2004, Messinger and Shevela, 2012). This led to the formation of the protective ozone layer that absorbs most of the ultraviolet (UV) radiation from the Sun. However, oxygen molecule is very toxic to a lot of organisms and the ones that survived had to develop or evolve their protective mechanism to oxygenic exposure (e.g. employing oxygen for efficiently burning the nutrient compounds within mitochondria). Nowadays, the photosynthetic water oxidation, which is performed by cyanobacteria, algae and higher plants, is one of the most important biological processes on Earth for conversion of solar energy to chemical stored energy in form of carbohydrate or fuels.
**Natural oxygenic photosynthesis**

Within the molecular structure of a plant cell, oxygenic photosynthesis is performed by a specific intracellular organelle – the chloroplast. Chloroplast is considered as a food factory of the cell because its function is to produce carbohydrate and use this compound to support energy for cell’s machinery. The chloroplast which is covered by a double membrane (outer and inner) encloses many stacks of thylakoid (grana). The grana are surrounded by chloroplast fluid (stroma) where the carbon dioxide fixation takes place. Thylakoid disk consists of an aqueous phase (lumen) which is encased by a lipid (thylakoid) membrane. **Figure A1** shows that thylakoid membrane comprises lipids, quinones and a high content of protein complexes: photosystem II (PSII), photosystem I (PSI), light harvesting complexes of PSII and PSI (LHC-II and LHC-I), cytochrome b_{6f}, electron carriers ferredoxin (Fd) and ferredoxin-NADP-reductase (FNR) at stromal side, plastocyanin (PC) at luminal side, and adenosine-5’-triphosphate synthase (ATP synthase).

**Figure A1**: The graphic representation of major protein complexes involved in oxygenic photosynthesis in thylakoid membrane. Reproduced from Shevela D. (Shevela, 2008).

The overall chemical reaction of natural photosynthesis can be expressed as:

\[ nH_2O + nCO_2 \xrightarrow{\text{sunlight}} C_nH_{2n}O_n + nO_2 \quad (A1) \]

**Reaction A1** hides the underlying complexity of photosynthesis, which involves light-dependent reactions leading to the production of oxygen, NADPH and protons (coupled to ATP synthesis via the ATPase), and light-independent reactions that utilize NADPH and ATP for the reduction of carbon dioxide to carbohydrates. The light reactions occur in the thylakoid membranes of chloroplasts and cyanobacteria. Three pigment-protein complexes, photosystem II, cytochrome b_{6f} and photosystem I, promote a linear sequence of electron
transfer reactions. Two mobile electron carrier’s, plastoquinone (PQ) and plastocyanin (PC), are responsible for the electron transfers between these three complexes (Heineke and Scheibe, 2009, Ort and Kramer, 2009, Messinger and Shevela, 2012).

PSII is at the beginning of this linear electron transfer chain (Govindjee et al., 2010). It catalyzes the oxidation of water to molecular oxygen (O₂) and thereby liberates the electrons and protons required for CO₂-fixation. PSII acquires the energy required for water oxidation by the absorption of visible light by chlorophyll molecules in its antenna. The light energy is then used to perform four photochemical reactions which are necessary to oxidize two water molecules and release one molecular oxygen, four protons and four electrons. Protons are released into the lumen (donor side of PSII) and electrons would be transferred to plastoquinone (Q_B) binding side. After Q_B molecule was fully reduced to Q_B⁻², the plastohydroquinone (PQH₂) is formed via protonation of Q_B⁻². The low binding affinity of PQH₂ to Q_B pocket causes its replacement by another oxidized plastoquinone molecule (PQ) from PQ pool and this compound then diffuses in thylakoid membrane to cytochrome b₆f complex (the detail information of water oxidation reaction will be further described in the next section).

The cytochrome b₆f complex, which functions as a redox link between PSII and PSI, consists of two b-type hemes (cyt b₅), one c-type heme (cyt f) and a Rieske iron-sulfur protein (FeS) (Berry et al., 2000). When PQH₂ successfully binds to cytochrome b₆f, it is oxidized and deprotonated by FeS protein. Protons from PQH₂ deprotonation, which were taken up from stroma are delivered into lumen. One of two electrons from PQH₂ oxidation, which is inserted to the linear electron transport chain passes through Cyt f to copper-containing protein plastocyanin (PC), which further carries electron toward PSI. The second electron participates in the reduction of one free PQ molecule to PQ⁻ (semiquinone). The next oxidation of a new PQH₂ supports one more electron to continuously reduce PQ⁻ to PQ²⁻, which will pick up two protons from stroma to regenerate PQH₂. This process is known as Q cycle which takes place in thylakoid membrane between PSI and cytochrome b₆f in order to facilitate proton from stroma to lumen. The cytochrome b₆f is therefore considered as an electron transfer and proton translocating membrane enzyme (Shevela et al., 2013).

After leaving the cytochrome b₆f complex, the reduced PC diffuses to PSI where it will be oxidized at the luminal side of the thylakoid membrane. Similar to PSII, PSI also captures and utilizes the solar energy to drive the redox reactions of the electron transfer chain. The photochemical excitation of the reaction center chlorophyll a molecule of PSI, P700, generates a positive hole as P700⁺
which will be electronically neutralized by electron donation from reduced PC (Brettel and Leibl, 2001, Melkozernov, 2001). The released electron from this charge separation process is firstly located in chlorophyll A₀, is continuously transferred to phylloquinone A₁, then to electron carrier ferredoxin (Fd) via [Fe₄-S₄] cluster and finally to ferredoxin-NADP-reductase (FNR) where NADP⁺ is protonated and reduced to NADPH molecule (Chitnis, 2001).

The accumulation of protons in thylakoid lumen via water splitting process at donor side of PSII and Q cycle of PQ molecule at cytochrome b₆f complex creates a proton gradient and a difference of electrochemical potential between stroma and lumen. The generated potential energy is employed by ATP synthase for synthesis of ATP from ADP. The phosphorylation of ADP takes place when protons are pumped from lumen to stroma through the CFₒ/CF₁ protein complexes of ATP synthase (McCarty et al., 2000, Junge and Nelson, 2005). In short, the energy-rich compounds NADPH and ATP, which are generated by FRN and ATP synthase, respectively, are the final consequences after a long light-driven electron transport in thylakoid membrane. The carbon dioxide reduction, which utilizes the chemical energy stored in NADPH and ATP, is performed via cyclic metabolic pathway known as Calvin-Benson-Bassham cycle in the chloroplast stroma (Calvin et al., 1950).

**Water splitting reactions**

Photosystem II is a unique multi-subunit chlorophyll/protein complex that can oxidize water and produce molecular oxygen (O₂) in nature. It is also known as light-driven water-plastoquinone oxidoreductase. This large homo-dimeric complex (700 kDa) is embedded in the thylakoid membranes of many photosynthetic organisms such as higher plants, algae and cyanobacteria. Based on crystal structure from thermophilic cyanobacterium *Thermosynechococcus vulcanus* (*T. vulcanus*) at 1.9Å resolution (Umena et al., 2011), a single PSII core complex contains 35 chlorophylls, 11 beta-carotenes, 2 pheophytins, more than 20 lipids, 2 plastoquinones, 2 heme irons, 1 non-heme iron, 4 manganese atoms, 3 or 4 calcium atoms (one of which is in Mn₄CaO₅ cluster), 3 chloride ions, 1 hydrogen-carbonate ions, approximately 20 protein subunits (no PsbY found in this research) and more than 1300 water molecules.

All cofactors that are involved in photosynthetic water oxidation and electron transport chain are located in the PSII core proteins D₁ (PsbA) and D₂ (PsbD) (Debus, 1992). The excitation energy, which is attained by light absorption of LHC-II, is transported to the PSII reaction center by the inner chlorophyll-binding proteins CP43 (PsbC) and CP47 (PsbB) (Bricker and Frankel, 2002). In addition, the extrinsic proteins PsbO (33 kDa, known as manganese-stabilizing protein), PsbP (23 kDa), PsbQ (17 kDa), which are located at luminal side of PSII, work as enhancers of oxygen evolution. These proteins play a very
important role in protection of Mn₄CaO₅ cluster from outer oxidation or reduction agents and maintenance of water oxidation process (Seidler, 1996). Combination of the Mn₄O₅Ca together with its co-factors and functionally surrounding protein environment generate the oxygen evolving complex (OEC) or water oxidation complex (WOC) (Yano et al., 2006, Siegbahn, 2009, Service et al., 2014, Vogt et al., 2015).

The energy from sunlight, which is captured by many chlorophyll molecules of LHC-II complex, is transferred from the antenna system to the primary electron donor of the PSII reaction center, P680. Once the excitation energy reaches P₆₈₀⁰, a special tetrameric chlorophyll arrangement, the exited state of P₆₈₀, P₆₈₀*, forms and donates an electron to the nearby pheophytin molecule (PheoD₁), resulting in the P₆₈₀⁺⁺Pheo⁻ state (Figure A2, center). The primary charge separation is stabilized by electron transfer from Pheo⁻ to the primary plastoquinone, QA, and then to the secondary plastoquinone QB. A second successive light-induced charge separation leads on the acceptor side to the formation of QB²⁻, which is protonated by protons at stromal side and leaves as QBH₂. The vacant QB site is filled by a platoquinone molecule from the PQ pool in the thylakoid membrane (Figure A2, top). (Messinger and Shevela, 2012).

On the donor side, the positively charged P₆₈₀⁺⁺ radical, whose oxidation potential is the greatest one (around 1.2-1.3V) in nature, is not stable and needs to be neutralized by another reductant (Rappaport et al., 2002). The P₆₈₀⁺⁺ is reduced by tyrosine Z (YZ; D1-Tyr161), which forms a neutral YZ* radical by proton transfer to its hydrogen-bonding partner His190. The YZ*His190⁺ pair is signified in the following as YZox. The reduction of YZox by the Mn₄CaO₅ cluster concludes this reaction sequence (Lubitz et al., 2008, Dau et al., 2010, Govindjee et al., 2010, Siegbahn, 2013). Due to very high potential of P₆₈₀⁺⁺ radical, PSII protected itself against P₆₈₀⁺⁺ oxidation in case of no electron donation from YZ and the Mn₄CaO₅ cluster by the other substances, for instances, carotenoids or cytochrome b559 (PsbE and PsbF protein complex) (Lubitz et al., 2008).

Four successive oxidation reactions in WOC lead to formation of one oxygen molecule. The quantum efficiency of overall PSII chemistry included water oxidation and plastoquinone reduction is approximately 90% under optimal light conditions (Dau et al., 2012, Cox and Messinger, 2013).
Figure A2: Electron transfer cofactors and pathways in photosystem II (center). The $S_i$ state (Kok) cycle describing the reaction sequence of the OEC is shown at the bottom, and that of the $Q_B$ site on the top. $\alpha$ and $\beta$ symbolize the miss and double hit probabilities of the light-driven ‘forward’ reactions of the OEC, while $\delta$ is a shorthand for the various ‘back’ reactions to $S_i$ that occur during the dark-times between flashes. Figure was provided by Dmitry Shevela
The “heart” of the photosystem II complex

The structure of a 700 kDa homodimeric photosystem II complex was solved by X-ray diffraction (XRD) at 1.9Å resolution, which revealed that the WOC is a Mn₄CaO₅ cluster coordinated by a well functional protein environment (Umena et al., 2011). This research showed that five oxygen atoms bridge four manganese atoms and one calcium atom in the cluster. The shape of the whole cluster generally resembles a distorted chair, with the cubane as the chair base and the outside Mn₄ as the back of the chair (Figure A3). The “chair base” part is a distorted cubance, which is constructed by three Mn ions (Mn1, Mn2 and Mn3), four oxygen atoms (O1, O2, O3 and O5) and one Ca²⁺ ion. The Mn₄ ion is located outside the cubane and connected to the cubane core by the two µ-oxo-bridges O4 and O5 (Shen, 2015). The cubane is often depicted as being open on the basis of EXAFS results and DFT calculations (Figure A3) (Yano et al., 2006, Grundmeier and Dau, 2012, Siegbahn, 2013).

In addition to the oxo-bridged oxygen atoms, four closest water molecules which are designated as W1, W2, W3 and W4 are directly linked to the Mn₄CaO₅ cluster as terminal ligands. Two of these four water molecules (W1 and W2) are associated with Mn₄, and the other two (W3 and W4) are connected to the Ca²⁺ ion (Figure A3). In the most stable S₁-state of the Mn₄O₅Ca cluster, no direct water ligands have been found in association with the remaining three manganese ions, indicating that some of these four waters may act as the substrate for water oxidation. In addition, an extra substrate water molecule (or OH group) is bound to the S₃ state (Cox and Messinger, 2013, Siegbahn, 2013, Cox et al., 2014).

A question raised from the discovery is which two water molecules participate in oxygen formation process and structure maintenance of the Mn₄CaO₅ complex. Interestingly, the chemical bond lengths between oxygen atom O5 and its surrounding metal atoms of the Mn₄CaO₅ cluster (2.4-2.7Å) are longer than the other ones (1.8-2.1Å). The results imply these bonds are weak and O5 is one of the oxygen substrates for generation of molecular oxygen. In fact, the experimental results from MIMS technique, in which the highly-enriched ¹⁸O water (H₂¹⁸O) is used to identify two separated water exchange processes (one fast and one slow), do not only eliminate the nucleophilic attack pathway of O-O bond formation, but also show that W1 and W3 are not a substrate water (Nilsson, 2014). In addition, O5 or a position equivalent to O5 was first pointed out to be slow substrate “water” (Wₛ) by Messinger J. (Messinger, 2004). This assignment was subsequently confirmed by Siegbahn and his colleague based on consequences of DFT calculations (Siegbahn and Lundberg, 2005). The W₄ molecule whose position is quite similar to W₃ with a direct association to Ca²⁺ ion forms a hydrogen bond to tyrosine Z (Y₂), thus, it is probably not a substrate water mostly due to geometric reasons (i.e. its direction is pointing away from
the Mn$_4$O$_5$Ca cluster, see Figure A3). Besides, the results of Shen and his co-workers suggested that O-O bond generation occurs in two of three species O$_5$, W$_2$ and W$_3$ (Umena et al., 2011).

**Figure A3**: Structural model of Mn$_4$CaO$_5$ cluster with its surrounding protein residues based on DFT calculation (Krewald et al., 2015), Mn$^{III/IV}$, purple; Ca$^{2+}$, yellow; oxo-bridge, red; water-oxygen, blue; chloride ion, green; amino acid backbone, gray; carboxyl oxygen, red and His-nitrogen, dark blue. Reproduced from Nilsson H. (Nilsson et al., 2014)

Due to the easy reduction of Mn$^{III/IV}$ ions to Mn$^{II}$ by X-ray irradiation and slight discrepancies of the Mn-Mn bond lengths between different techniques as XRD (Umena et al., 2011), EXAFS (Yano et al., 2006, Dau and Haumann, 2008) and theoretical studies (Grundmeier and Dau, 2012, Pantazis et al., 2012, Blomberg et al., 2014), the recent studies have been shown a “radiation-damage-free” structure of PSII complex in S$_1$ state at 1.95Å resolution employing femtosecond
X-ray pulses (Suga et al., 2015). The WOC in the new structure has Mn-Mn distances and bond lengths of oxo-bridged oxygen, O5 to its surrounding metal atoms that are shorter by 0.1-0.2Å compared with the previous results from Umena et al. The observations suggest that O5 is a hydroxide ion instead of a normal oxygen dianion and may serve as one the substrate oxygen in water oxidation. This research also assigned the valences of each manganese atom as Mn1D(III), Mn2C (IV), Mn3B(IV) and Mn4A(III).

**Chemistry of the Mn₄CaO₅ cluster**

In the late 1960’s, Pierre Joliot and his co-workers built an electrode, now referred to as Joliot-type electrode, that allowed measuring the oxygen evolution yields per flash induced in algae by a series of single-turnover light flashes. In this way, he made the fundamental discovery that photosynthetic oxygen evolution occurs with a periodicity of four, in which the first maximum is observed after the third flash, and the next maxima are then seen after flashes 7 and 11 (Figure A4, left side) (Joliot and Joliot, 1968, Renger and Hanssum, 2009). This periodicity disappeared after several cycles, and a small oxygen yield was already observed after the second flash.

The detected FIOP that implies the complexity of water oxidation mechanism is explained by Kok and his co-workers within an elegant model (Figure A4, right side) (Kok et al., 1970, Joliot and Kok, 1975). The idea from Kok model assumes that each PSII reaction center, which generates a positive and negative charge via light absorption, is connected to only one water oxidation complex (WOC). The photochemical reaction in PSII reaction center is a one-electron reaction, which means the capture of one photon leads to extract one electron. The WOC, whose electrons are withdrawn one-by-one in each single-turnover flash via charge separation, stores four oxidizing equivalents before reacting with two water molecules for oxygen formation (Messinger and Renger, 2008). Hence, the water oxidation process of each WOC is taken place through five intermediate oxidation states, which were denoted S-state (i = 0, 1, 2, 3 and 4). The index “i” signifies the number of electrons extracted from the WOC by P₆₈₀⁺⁺ via YD₂ox (Kok et al., 1970, Forbush et al., 1971). The S₁ state is the most stable oxidation state of dark-adapted PSII sample, while the S₂ and S₃ states are reduced back to S₁ by electron transfer from acceptor site Qb (Diner, 1977, Rutherford et al., 1982, Robinson and Crofts, 1983, Rutherford and Inoue, 1984) or from reduced tyrosine D (YD; D₂-Tyr160) (Vermaas et al., 1984, Nugent et al., 1987, Vermaas et al., 1988). The S₀ state is slowly oxidized to S₁ by the long-lived radical YD₂ox (Styring and Rutherford, 1987). The S₄ state is a highly reactive intermediate (or set of intermediate states such as S₃Y₄; (Haumann et al., 2005, Nilsson et al., 2014, Nilsson et al., 2016)) oxidizes two water molecules within 1 ms. This transition liberates one molecular oxygen, four protons to luminal side, injects four electrons into the Mn₄CaO₅ cluster and
thereby completes the Kok cycle by setting the WOC back into the \( S_0 \) state (see Figure A4; right side).

**Figure A4**: The flash-induced oxygen evolution pattern (FIOP) of dark-adapted thylakoid sample (left) and extended Kok-model consisted of S-state dependent misses (\( \alpha \)), proton releases and substrate water binding (right). Figure is provided by Dmitry Shevela

The lower oscillations after the first cycle and appearance of a small oxygen yield after the second flash in FIOP is expressed in the classic Kok model using two important parameters: misses and double hits (Forbush et al., 1971, Messinger and Renger, 2008). The miss parameter (\( \alpha \)) provides the probability that an OEC does not advance to the next higher \( S_i \) state after a saturating flash excitation (\( S_i + \text{hv} \rightarrow S_i \)). The miss parameter is affected by many different redox equilibria between the cofactors at the donor and acceptor sides of PSII, for example, the presence of \( Q_A^- \) destabilizes the primary charge separation \( \text{P680}^*+\text{Pheo}^* \), thus resulting a miss (Renger and Hanssum, 1988, Shinkarev and Wraight, 1993, de Wijn and van Gorkom, 2002). In addition, the origins of misses may come from a kinetic competition between \( Y_Z \) and \( Q_A^- \) in reducing of \( \text{P680}^* \) (Lavergne and Rappaport, 1998, Christen and Renger, 1999, Christen et al., 1999, de Wijn and van Gorkom, 2002, Han et al., 2012). The double hit parameter (\( \beta \)) gives the percentage of PSII reaction centers that perform twice advancements in one flash (\( S_i + \text{hv} \rightarrow S_{i+2} \)). The double hit parameter strongly depends on the flash profile of the light source and can be avoided by using nanosecond laser flashes (Jursinic, 1981, Hillier and Messinger, 2005).

**Developments in deconvolution of FIOP data**

The classic Kok model is a revolutionary idea not only to explain experimental results of Joliot electrode measurements (i.e. FIOPs), but also to present a fundamental knowledge of photosynthetic water oxidation. However, many different observations from photosynthetic experiments cannot be solved by this model and the extension of Kok’s model is therefore necessary. There are
four additional points that have been added into Kok’s model during recent years. The first extension includes super-reduced $S_i$ states below the $S_0$-state ($S_1$, $S_2$, $S_3$, $S_4$ and $S_5$) due to the chemical treatments of the $\text{Mn}_4\text{CaO}_5$ cluster with reductants like hydroxylamine ($\text{NH}_2\text{OH}$) or/and hydrazine ($\text{NH}_2\text{NH}_2$) (Bouges, 1971, Meunier et al., 1996, Messinger et al., 1997). Secondly, an increased double hit after the first flash observed in $K_5[\text{Fe(CN)}_6]$-treated samples with oxidized non-heme iron (Jursinic, 1981, Zimmermann and Rutherford, 1986, Petrooupleas and Diner, 1987). Another phenomenon in Joliot electrode measurements is the loss of active PSII reaction center during a flash train, which can be demonstrated by comparison of total oxygen yield between first cycle to the next cycles. For instances, the sum of oxygen yields of flash 7\textsuperscript{th}, 8\textsuperscript{th}, 9\textsuperscript{th} and 10\textsuperscript{th} is typically smaller than the total oxygen yield of flash 3\textsuperscript{rd}, 4\textsuperscript{th}, 5\textsuperscript{th} and 6\textsuperscript{th}, depending on which kind of sample and experimental conditions. Hence, the third addition to Kok’s model is the introduction of inactivated state $S_\varepsilon$ (accessed with the probability $\varepsilon$ from all $S_i$ states) (Delrieu and Rosengard, 1987, Meunier and Popovicic, 1991) or by an activity (damping) parameter $d$ (Messinger et al., 1997, Isgandarova et al., 2003). The last one is the inclusion of the fast phase of back reactions of $S_2$ and $S_3$ with $Y_D$ during the dark-times between flashes, either by explicitly including the reactions (Vass and Styring, 1991, Isgandarova et al., 2003), or by introducing an empiric $\delta$ parameter (Packham et al., 1988).

Each of $S$-state transitions is connected to a specific kinetic rate, structural change of the $\text{Mn}_4\text{O}_5\text{Ca}$ cluster and alteration of oxidation state of a cofactor or protein residue insight PSII complex, for example, secondary quinone acceptor ($Q_B$) and tyrosine D ($Y_D$). Thus, the miss probabilities that depend on a lot of various redox equilibria in PSII complex should be $S_i$ state and $Q_B$ state dependent (Shinkarev and Wraight, 1993). However, the small amount of independent data points of a single FIOP (typically 16 oxygen flash yield per FIOP) does not allow a reliable identification of the individual $\alpha_i$ values for all $S_i \rightarrow S_j$ transitions ($j = i+1$ for $i = 0$, 1, 2 and $j = 0$ for $i = 3$) (Delrieu, 1983). A suggestion of $S$-state dependent miss parameters from Isgandarova S. (Isgandarova et al., 2003) with $\alpha_0=\alpha_1=0$, $\alpha_2=\alpha_3>0$ resulted in a small improvement of fit quality, but was not a unique solution. Some more attempts to determine the efficiency of the $S$-state turnover were done by employing many techniques such as chlorophyll fluorescence (de Wijn and van Gorkom, 2002), electron paramagnetic resonance (EPR) spectroscopy (Han et al., 2012), and Fourier transform infrared (FTIR) spectroscopy (Suzuki et al., 2012). All these researches support the $S$-state dependences of the miss probabilities, a definite solution was nevertheless not found yet.

Aiming to analyze the important parameters of miss, double hit and the $S$-state populations of PSII sample from FIOPs data, the classic and extended Kok
models were programmed for example as spreadsheets. After applying the normalized oxygen yields from FIOP data, these parameters are identified by minimizing the total squared deviations between experimental and calculated data points.

Alternatively, efforts were made to obtain analytical solutions that are based on a sigma (Delrieu, 1974, Lavorel, 1976) or eigenvalue analysis (Meunier et al., 1996). A fit program which was based on the standard Kok model was created by Shinkarev (Shinkarev, 2003). One more program was furthermore developed for extended Kok model, which consists of the above mentioned $S_e$ state and $\delta$ parameter for all $S_i$-states ($i = 0, 1, 2$ and $3$) (Shinkarev, 2005). These two analytical solutions for data deconvolution of FIOPs are programmed by using circulant matrices of transition probabilities (symmetric Markov models), resulting the eigenvalues and eigenvectors readily accessible. However, this mathematical approach leads to the generation of a perfectly symmetric Kok model, while the back reactions in PSII are not symmetric (e.g. no experimental evidences of the $S_1 \rightarrow S_0$ and $S_0 \rightarrow S_3$ back transitions was recorded). In order to surmount the disadvantage in Shinkarev’s models, Dismukes and co-workers developed recently the STEAMM (S-state Transition Eigenvalues of Asymmetric Markov Models) algorithm (Vinyard et al., 2013). Although many attempts have been done to build up an “excellent” model that is closely corresponding with all observations for determination of the miss and double hit parameters, all methods have so far been applied to individual FIOPs.

The Joliot oxygen-type electrode can be used to measure the kinetic rates of the $S_2$, $S_3$ and $S_0$ states turn back to the dark-stable $S_1$ state (see more in Figure B4, “Materials and methods” section). According to traditional way, the FIOPs which were recorded at various delay times were analyzed individually to extract the $S_i$ state populations for each time point (using the pre-determined $S_i$ state independent miss and double hit probabilities). The kinetic equations which were used to fit the S-state population of interest as a function of dark time were written by employing one (for $S_0$ decay) or two (for $S_2$ and $S_3$ decays) exponential function(s). Moreover, using this traditional approach for data analysis of S-state lifetimes means that the other inattentive S-states are ignored and the number of independent data points were quite small in order to fully investigate the S-state dependence of the miss parameters. In this thesis, a global fit approach was programmed in order to simultaneously analyze all oxygen yields of a standard FIOP (2Hz flash frequency) of a well dark-adapted sample and of all $O_2$-yields of all FIOPs taken during the time course of $S_0$ (11 FIOPs), $S_2$ and $S_3$ state (18 FIOPs each) lifetime measurements.
Materials and methods

Chemicals and buffer solutions

All of chemicals used in this research were purchased from Sigma, Aldrich, Fluka, Merck, VWR Chemicals, Cambridge Isotope Laboratories (CIL), etc with very high purity grade (>98%). Besides, the aqueous solutions were prepared by using deionized, filtrated water (Millipore quality).

Table B1: The composition of all buffers used in this study

<table>
<thead>
<tr>
<th>Buffer’s name</th>
<th>Composition</th>
<th>pH/pD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 1 (Grinding buffer)</td>
<td>1mM EDTA, 0.4M NaCl, 4mM MgCl₂, 50mM HEPES/NaOH, 5mM Sodium Ascorbate*, 2mg/ml BSA*</td>
<td>7.5</td>
</tr>
<tr>
<td>Buffer 2 (Washing buffer)</td>
<td>150mM NaCl, 8mM MgCl₂, 50mM MES/NaOH</td>
<td>6.0</td>
</tr>
<tr>
<td>Buffer 3 (Resuspension or incubation buffer)</td>
<td>10mM MgCl₂, 50mM MES/NaOH</td>
<td>6.0</td>
</tr>
<tr>
<td>Buffer 4 (Sucrose buffer)</td>
<td>15mM NaCl, 5mM MgCl₂, 5mM CaCl₂, 0.4M Sucrose</td>
<td>6.0</td>
</tr>
<tr>
<td>Buffer 5 [Chl] buffer</td>
<td>5mM MgCl₂, 10mM CaCl₂, 40mM MES/KOH, 25% (w/v) glycerol</td>
<td>6.1</td>
</tr>
<tr>
<td>Measuring buffer I</td>
<td>20mM NaCl, 5mM MgCl₂, 50mM MOPS/NaOH</td>
<td>7.0</td>
</tr>
<tr>
<td>Measuring buffer II</td>
<td>10mM CaCl₂, 5mM MgCl₂, 40mM succinic acid/NaOH, 1M Betaine</td>
<td>5.0</td>
</tr>
<tr>
<td>Measuring buffer III</td>
<td>10mM CaCl₂, 5mM MgCl₂, 40mM MES/NaOH, 1M Betaine</td>
<td>6.1</td>
</tr>
<tr>
<td>Measuring buffer IV</td>
<td>10mM CaCl₂, 5mM MgCl₂, 40mM HEPES/NaOH, 1M Betaine</td>
<td>8.0</td>
</tr>
<tr>
<td>Measuring buffer V</td>
<td>10mM CaCl₂, 5mM MgCl₂, 40mM MES/NaOD, 1M Betaine</td>
<td>6.1</td>
</tr>
</tbody>
</table>

* Sodium Ascorbate and BSA were added to Buffer 1 shortly before grinding spinach leaves.

Preparations of biological samples

The thylakoid membranes isolated from Spinacia oleracea (spinach) and Cyanidioschyzon merolae were used in this present research.
**Spinacia oleracea** (spinach, higher plant)

Fresh spinach leaves were purchased from the local market and were then kept in dark at room temperature for a few hours before starting sample preparation. All of isolation steps were performed in a cold room (4-5°C) under very dim green light. Spinach thylakoid samples that were used in this research were isolated from spinach leaves as previous description (Winget et al., 1965) with slight modifications (Messinger and Renger, 1993). Footstalks of spinach leaves were carefully removed to avoid destroying the remaining parts of the leaves, since they contain small amount of chloroplasts and in addition are hard to grind. Then, these leaves were washed by distilled water (at least 5 times) to eliminate most of soil and chemicals that cling on the surfaces of leaves. Cleaned leaves were ground in grinding buffer (buffer 1) by washed blender (Waring Blender, USA) to damage the plant cell membrane (cellulose membrane) and supply advantageous condition for extraction of chloroplasts organelle. Then, the mixture of ground leaves and buffer was filtered by several layers of cheesecloth (about 20 μm pore length) to exclude the unnecessary plant materials and get most of the chloroplast organelle in the extracted solution. The extraction was centrifuged (Avanti centrifuge, Beckmann Coulter™) for 10 minutes at 8,000 rpm using a JA-10 rotor. The pellet was collected and re-suspended with washing buffer and centrifuged once more. The supernatant was discarded and the pellet was re-suspended in buffer 3. The aim of a two-step centrifugation was not only to collect and concentrate chloroplast and thylakoid membranes but also to remove a part of other protein complexes from the stroma and of proteases that can destroy photosystem II. Chlorophyll concentration was measured by UV-VIS spectrophotometer (see below) for several times and got the average value. After the final isolation step, spinach thylakoid membranes (3.184 mg Chl/ml) in the mixture with buffer 4 were frozen as small beads in liquid nitrogen and then stored at -80°C until using for measurement.

**Cyanidioschyzon merolae** (extremophilic red microalga)

*C. merolae* cells were grown in the laboratory of Prof. Joanna Kargul (Poland). The experimental conditions of cell culturing and procedure of thylakoid isolation was described in detail by Krupnik T. and his collaborators (Krupnik et al., 2013). The cells whose culture media reached OD_{680}≈2.5 were harvested and concentrated by centrifugation at 5000rpm, 4°C for 10min. Cell pellets were washed with 50–100 ml of buffer 5 and then re-suspended in 50 ml of buffer 5 in addition of 50 μg/ml DNase I and the Complete™ protease inhibitor mixture (Roche Applied Science). *C. merolae* cells were breached by vigorous shaking with 0.1 mm glass beads in a BeadBeater (BioSpec) using 13 cycles of 10 sec and interim 4-min cooling off periods. Cell lysate and glass beads were separated by vacuum filtration. Thylakoids were pelleted by centrifugation at 104200 x g for
30 min at 4°C and washed once with buffer 5. Final thylakoid pellets were re-suspended in buffer 5 at [Chl] of 2−2.5 mg/ml, flash frozen in liquid nitrogen, and stored at -80 °C prior to use.

**Identification of chlorophyll concentration**

Chlorophyll concentration of three different plant leaves were determined by spectrophotometric technique according to Porra R.J. and his co-workers (Porra et al., 1989). The chlorophyll molecules bound in the PSII and PSI complexes can be extracted by diluting 10µl of Chl-containing photosynthetic sample (e.g. BBY, thylakoid, etc) to 10ml of “[Chl] buffer” which contains high amount of acetone (80%, v/v). The mixture was then divided into 8-10 tubes (1ml/tube) and these aliquots were centrifuged at 10,000xg for 1min. The supernatants were continuously transferred to 1x1cm cuvette (polystyrene or PMMA) for absorption measurements at 646.6nm, 663.6nm and 750nm. The concentration of chlorophyll was calculated by the following equations:

\[
[\text{Chl } a] = \left[ 12.25 \times (A_{663.6} - A_{750}) - 2.25 \times (A_{646.6} - A_{750}) \right] \times k
\]

\[
[\text{Chl } b] = \left[ 20.31 \times (A_{646.6} - A_{750}) - 4.91 \times (A_{663.6} - A_{750}) \right] \times k
\]

\[
[\text{Chl } a + \text{Chl } b] = \left[ 17.76 \times (A_{646.6} - A_{750}) + 7.34 \times (A_{663.6} - A_{750}) \right] \times k
\]

Where k is dilution factor, \( k = \frac{\mu l\text{ solution}}{\mu l\text{ sample}} \)

**Catalase inactivation**

Catalase from bovine liver (CAS: 9001-05-2, product number C9322-5G, 3809U/mg) which is a commercial product from Sigma-Aldrich was either used directly, or when needed, would be inactivated with the inhibitor NaN₃ in the presence of H₂O₂ (Lardinois and Rouxhet, 1996). All of experimental steps for catalase inactivation were summarized in Figure B1. In details, the catalase powder was dissolved in “measuring buffer I” (pH=7.0) to a final concentration of 40,000 U/ml (10.5mg/ml). Then, 1M NaN₃ (final concentration) was added and the mixture was incubated for 10 minutes. Finally, 1M H₂O₂ (final concentration) was added to the solution, which was incubated for 4 hours at room temperature. The inactivated catalase was collected and separated from these inhibitors by centrifugation in a molecular weight cut-off tube (500 µl, pore size 100.000 kDa; 30-40min centrifugation at 10.000 x g). The retained solution (50 µl) was further purified by repeated washing steps (4-5 times) with measuring buffer I. After the last centrifugation, the protein concentration was determined using the Bradford assay (Bradford, 1976, Sedmak and Grossberg, 1977). The residual enzymatic activity was probed by Beers assay (Beers and Sizer, 1952, Aebi, 1984).
In order to generally compare the secondary structures between active and inactive catalase, the CD (circular dichroism) measurements were performed by Jasco J-810 spectropolarimeter with scanned wavelength from 200-240nm and experimental CD data were deconvoluted by “K2D2” software. The results from CD data analysis (see Figure B2) indicated that the secondary structures of active and inactive catalase are quite similar to its crystal structure (32% alpha helixes, 17% beta strands, protein data bank source) with a small difference (only 2%) of alpha helixes and identical percentage of beta strands. The solution of inactive catalase was flash frozen by liquid nitrogen and stored at -20°C until used.

Figure B1: The experimental procedure of catalase inactivation

<table>
<thead>
<tr>
<th>Predicted secondary structure percentages</th>
<th>Predicted secondary structure percentages</th>
</tr>
</thead>
<tbody>
<tr>
<td>α helix: 34.49%</td>
<td>α helix: 29.91%</td>
</tr>
<tr>
<td>β strand: 17.8%</td>
<td>β strand: 17.55%</td>
</tr>
</tbody>
</table>

Spectra plot

Figure B2: The fitted (green) and experimental (red) CD data of active and inactivated catalases after data deconvolution.
**Measurement of oxygen evolution activity**

Oxygen electrode is one of the most commonly used devices for measuring the dissolved oxygen in solution or partial pressure of oxygen (sometimes referred to as “oxygen tension”) in the gas phase. The “oxygen electrode” is one of the technical terms often used to describe the electrochemical sensor for oxygen. Another name of this device is “Clark-type electrode” due to its generally recognized inventor Leyland C. Clark (Clark et al., 1953).

The oxygen evolution rate of photosynthetic samples were measured by using Clark-type oxygen electrode (Rank Brothers Ltd, digital model 10 and Qubit Systems Inc.) at 20°C for 1min after switching on a white light projector at saturating light intensity. The measurements were performed by employing [Chl]=10-20µg/ml (final concentration) in 1-2ml desired buffer (often “measuring buffer 1”) in the presence of 200µM PPBQ (or DCBQ) and 1mM K₃[Fe(CN)₆] as artificial electron acceptors. Clark-type electrode was calibrated by using air-saturated water at atmospheric pressure (1atm). Oxygen evolution rate was calculated based on subtraction of oxygen evolution slope (after turning on the light) to baseline slope (before switching on the light), and rate’s unit was expressed in µmol (O₂)*[mg (Chl)]⁻¹*h⁻¹. The oxygen evolution rates of all thylakoid samples used in this study were higher than 250 µmol (O₂)*[mg (Chl)]⁻¹*h⁻¹.

**Joliot-type electrode experiments**

The oscillation patterns of oxygen yields per flash (known as FIOPs) were obtained with a home-built Joliot-type oxygen electrode (Joliot and Joliot, 1968) with a modification by Messinger, J. (Messinger et al., 1993). The thylakoid samples used in Joliot electrode measurements were kept in constant temperature within ±0.2°C (Figure B3) and in the absence of exogenous electron acceptors. 10µl aliquots of the thylakoid suspension were transferred to the surface of bare Pt-cathode in very dim green light, and the sample was kept insight electrode for 2-3min in order to reach desired temperature and sedimentation. The polarization voltage of -750 mV was switched on 40 seconds (or longer, if specified) before exposing the sample to a series of 16 Xe-flashes (2 Hz; Perkin Elmer, LS-1130-4). A LabView (National Instrument) routine was used to trigger the flash lamp and to record the data at a sampling rate of 3600 points/second.

Moreover, the working mechanism of Clark-type and Joliot-type electrodes are based on polarographic principle. When the Pt cathode is polarized at -750mV with respect to the Ag anode, every oxygen molecule that reaches its surface from the experimental solution (gas-permeable membrane is needed in Clark electrode measurements) is reduced through the following reaction:
Pt-cathode: $O_2 + 2H_2O + 2e^- \rightarrow H_2O_2 + 2OH^-$

$H_2O_2 + 2e^- \rightarrow 2OH^-$

Totally: $O_2 + 2H_2O + 4e^- \rightarrow 4OH^-$

The reduction and oxidation are two parallel processes in every redox reactions. Therefore, another oxidation reaction must be coincidently occurred at Ag anode as follows:

$4Ag \rightarrow 4Ag^+ + 4e^-$

$4Ag^+ + 4Cl^- \rightarrow 4AgCl$

Totally: $4Ag + 4Cl^- \rightarrow 4AgCl \downarrow +4e^-$

The overall electrochemical process that occurs in an “oxygen electrode” can be expressed in below reaction:

---

**Figure B3:** The schematic structure of home-built Joliot-type electrode. Reproduced from Shevela D. (Shevela, 2008)
When the Joliot electrode (or Clark electrode) is repeatedly used, the bright surface of Ag anode rapidly become “tarnished” due to the accumulation of solid AgCl. This coat of silver chloride on silver anode should be removed by using ammonia solution (10-15%, w/w) and toothpaste.

**Dark adapted and pre-flash samples**

FIOP experiments were performed using either pre-flashed or dark-adapted thylakoid samples, respectively. S₁Y₀ thylakoid samples contained a high percentage (>80%) of the reduced form of tyrosine D (Y₀D) due to long-term (several months or years) dark-storage at -80°C (Messinger and Renger, 1990, Messinger and Renger, 1993). S₁Y₀D⁰X thylakoids with approximately 90% oxidized tyrosine D, were prepared by giving one saturating flash to an aliquot of the S₁Y₀ sample (pH 7.0, 20°C), followed by a 10-15 minutes dark-incubation at room temperature (Messinger and Renger, 1990). Before the FIOP experiments were performed, the frozen stock solution of thylakoid sample was defrosted in the dark on ice and diluted to [Chl]=0.5mg/ml with desired buffers.

**S-state lifetime measurements**

**Figure B4**: The scheme explaining the flash/variable dark-time (Δtd) protocol employed for the S₃, S₂ and S₀ lifetime measurements, and the color code for flash-induced oxygen yields used in Figures C4, C5 and C7. The short vertical lines symbolize the time points of the flashes.

The S₂, S₃ and S₀ state lifetimes of both non-preflash (S₁Y₀D) and preflushed (S₁Y₀D°X) thylakoid samples were measured with the Joliot-type oxygen electrode by exciting dark adapted samples with one (S₂), two (S₃) or three (S₀) preflash(es) while they were resting on the Pt cathode surface (Figure B4).
After the desired dark times (Δtd), a flash train of 16 flashes (2 Hz) was given to PSII samples and the resulting FIOPs were recorded and deconvoluted by employing two analytical approaches as described below (Isgandarova et al., 2003, Shevela et al., 2006, Shevela et al., 2007). Approximately 11 or 18 time points were collected per S0 or S2 (and S3) state decays, respectively.

Data analysis of FIOPs

Individual analysis (paper 1)

The oxygen yields of the first 16 flashes of each FIOP were analyzed within the framework of an extended Kok model that was programmed within an Excel spreadsheet. This model included the reduced S-1 state of Mn4CaO5 cluster and a damping parameter (d) that accounts for the loss of active PSII centers during the flash train (Messinger et al., 1997, Isgandarova et al., 2003, Shevela et al., 2006). The program is based on the formulas:

\[
\begin{bmatrix}
[S_{-1}]_n \\
[S_0]_n \\
[S_{1,n}]_n \\
[S_{2,n}]_n \\
[S_{3,n}]_n
\end{bmatrix} =
\begin{bmatrix}
\alpha_{-1} & 0 & 0 & 0 & 0 \\
\gamma_{-1,n} & \alpha_0 & \beta_n & \gamma_{30} \\
\beta_n & \gamma_{0,n} & \alpha_1 & \beta_n \\
0 & \beta_n & \gamma_{1,n} & \alpha_2 & 0 \\
0 & 0 & \beta_n & \gamma_{2,n} & \alpha_3
\end{bmatrix} \times
\begin{bmatrix}
[S_{-1}]_{n-1} \\
[S_0]_{n-1} \\
[S_{1,n-1}]_n \\
[S_{2,n-1}]_n \\
[S_{3,n-1}]_n
\end{bmatrix} \times d \quad (1)
\]

and

\[
Y_{n,fit} = (1 - \alpha_3) \times [S_{3,n-1}]_n + \beta_n \times [S_{2,n-1}]_n \quad (2)
\]

Where, \([S_i]_{n-1}\) and \([S_i]_n\) are the S_i state populations before and after the n-th flash

- \(\alpha_i\) is the S_i state dependent miss probability
- \(\beta_n\) is the flash number dependent double hit probability (\(\beta_n\) can be higher on the first flash under certain circumstances)
- \(\gamma\) is the single hit probability (e.g. \(\gamma_{1,n}=1-\alpha_1-\beta_n\))
- d is the damping parameter (see above)

\(Y_{n,fit}\) is the oxygen yield generated after the n-th flash.

All fits related to this part are based on S_i state independent (equal) miss and double hit parameters, since these are most commonly used and we found our conclusions to be invariant towards various possible S_i state dependent approaches. The fits were performed within a spreadsheet program (Microsoft Excel) using the ‘GRG nonlinear’ method of the ‘Solver’ subroutine of Excel to minimize the deviation \(dy^2\) between the experimental and calculated oxygen yields by varying a specified set of parameters:
\[ dy_n^2 = \sum_{n=1}^{F} \left[ Y_n^{exp} - Y_n^{fit} \left( \frac{\sum_{n=1}^{F} Y_n^{exp}}{\sum_{n=1}^{F} Y_n^{fit}} \right) \right]^2 \] (3)

Here, \( Y_n^{exp} \) is the experimental oxygen yield of the \( n \)th flash and \( F \) stands for the number of analyzed flashes (or independent data points). The \( S_i \)-state populations were normalized according to equation 4, and the fit quality was determined by equation 5.

\[ \sum_{i=1}^{3} [S_i] = 1 \] (4)

and

\[ FQ = \frac{dy_n^2}{F_{total} - P} \] (5)

Where, \( P \) is the number of free parameter used in the fit procedure.

In this traditional way of analyzing FIOPs and determining \( S_i \) state lifetimes, a systematic fit approach identifies miss and double hit parameters from the FIOP taken at 2 Hz flash frequency. The parameters were then used for determinations of the \( S_i \) state populations under investigation from every FIOP of each \( S_i \) state lifetime measurement. Then, the respective \( S_i \) state population is plotted against the dark-time between the preflash(es) and the main flash group, and the resulting time dependence of the \( S_i \) state population is fit by one or the sum of two mono-exponential decays, while all other \( S_i \) state populations are generally ignored, excepted a treatment of all \( S \)-states during the \( S_3 \) state decay from Boussac A. (Boussac et al., 2004).

In detail, the kinetic rates of \( S_2 \) and \( S_3 \) state decay were identified by fitting the \( S_2 \) or \( S_3 \) state population as a function of dark time (sec). The mathematical equation was written as the sum of two first order decay reactions, where the amplitude of the fast phase reflects the amount of \( Y_D \) (6). In some cases, also fits with three phases (with one more additional very slow phase) were tested.

\[ S_i(t_d) = A_{i,fast} \exp^{-k_{ij,fast}t_d} + A_{i,slow} \exp^{-k_{ij,slow}t_d} \left( +A_{i,vs} \exp^{-k_{ij,vs}t_d} \right) \] (6)

Where, \( k_{ij,fast} \), \( k_{ij,slow} \) and \( k_{ij,vs} \) are the rate constants
\( A_{i,fast} \), \( A_{i,slow} \) and \( A_{i,vs} \) are the amplitudes of the fast, slow and very slow phase of decay \( (A_{i,fast} + A_{i,slow} + A_{i,vs} = 1) \).

In contrast, the \( S_0 \) oxidation to \( S_1 \) was described well by a mono exponential reaction: \( S_0 Y_D^{OX} \rightarrow S_1 Y_D \) (7).

\[ S_0(t_d) = S_0 Y_D^{OX} (initial) \exp^{-K_{01}t_d} + C \] (7)

Where, \( C \) is a constant that reflects the \( S_0 Y_D \) fraction.
Global fitting approach (paper 2 and 3)

In this different approach of FIOP deconvolution, all of FIOPs from three S-state lifetime measurements (S₂, S₃ and S₀ decay reactions to S₁) are used for further data analysis and two sets of data were recorded. The first set was performed with PSII samples having a large percentage of reduced tyrosine D (YD) due to long-term storage at -80°C (Nugent et al., 1987, Messinger and Renger, 1990, Vass et al., 1990, Messinger and Renger, 1993), and the second set was recorded with PSII samples in which YD was mostly oxidized due to a pre-flash treatment (see above for sample preparations). In total 48 individual FIOPs of each set were analyzed simultaneously. This allows testing the comprehensive figure of how the Sᵢ states decay in PSII, and to test the dependence of the miss parameters on S-state transitions. In addition, the most consistent rates of all Sᵢ state decays in both data sets, which were collected on the same preparation within one week, were tried to figure out.

Moreover, the influences of both reduced and oxidized forms of tyrosine D (YD and YD^{OX}) on the miss parameters were previously pondered. For instances, the high concentration of YD^{OX} in preflash PSII sample is able to destabilize the primary charge separation of P680^+/Q₅^- because the radical YD^{OX} has the similar positive charge to P680^+ molecule (Rutherford et al., 2004). Vice versa, the reduced form YD of dark-adapted sample that possibly donate electron to the P680^+ instead of YZ also results a higher miss probability for the working cycle of the Mn₄O₅Ca cluster although the oxidation rate of YD is much slower compared with oxidation of YZ. These convincing speculations are the reasons why the current version of global fitting program was built by using extended Kok model of the quaternary cycle of the PSII donor side in addition to the binary cycle of Q₅/Q₅^- transitions and influences of redox state of tyrosine D (YD/YD^{OX}) to miss parameters of all Sᵢ-state transitions (Figure A2). This means that the current miss parameters are not only Sᵢ-state dependent, but also additionally dependent on the oxidation state of YD. In general, the global fitting program consists of two parts: (i) the Sᵢ state transition matrices in addition of the YD and YD^{OX} for describing the flash-induced forward reactions, (ii) a set of differential equations describing the decay reactions occurring during the variable dark-time t_d during Sᵢ state lifetime measurements and in the 500 ms between flashes of the FIOP and (iii) an additional model for the acceptor side with Q₅/Q₅^- transitions. In detail, the mathematical matrix (1) was converted into 2 matrixes (8) and (9) included all “super” reduced Sᵢ states (i = 1, 2, 3, 4 and 5) of the Mn₄CaO₅ cluster. Besides, the equations (2) and (4) were slightly modified to (10) and (11), while equations (3) and (5) were kept as in traditional approach.
\[
\begin{pmatrix}
\alpha_{y_{ox}} & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\gamma_{s,n} & \alpha_{y_{ox}} & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\beta_{s} & \gamma_{s,n} & \alpha_{y_{ox}} & 0 & 0 & 0 & 0 & 0 & 0 \\
\gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \alpha_{y_{ox}} & 0 & 0 & 0 & 0 & 0 \\
\beta_{s} & \gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \alpha_{y_{ox}} & 0 & 0 & 0 & 0 \\
\gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \alpha_{y_{ox}} & 0 & 0 & 0 \\
\beta_{s} & \gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \alpha_{y_{ox}} & 0 & 0 \\
\gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \beta_{s} & \gamma_{s,n} \\
\end{pmatrix}
\]

And

\[
\begin{pmatrix}
\alpha_{y_{d}} & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\gamma_{s,n} & \alpha_{y_{d}} & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\beta_{s} & \gamma_{s,n} & \alpha_{y_{d}} & 0 & 0 & 0 & 0 & 0 & 0 \\
\gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \alpha_{y_{d}} & 0 & 0 & 0 & 0 & 0 \\
\beta_{s} & \gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \alpha_{y_{d}} & 0 & 0 & 0 & 0 \\
\gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \alpha_{y_{d}} & 0 & 0 & 0 \\
\beta_{s} & \gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \alpha_{y_{d}} & 0 & 0 \\
\gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \beta_{s} & \gamma_{s,n} & \beta_{s} & \gamma_{s,n} \\
\end{pmatrix}
\]

\[
Y_{n}^{\text{fit}} = (1 - \alpha_{3}Y_{d}) * [S_{3}Y_{D}]_{n-1} + (1 - \alpha_{3}Y_{d}^{\text{OX}}) * [S_{3}Y_{D}^{\text{OX}}]_{n-1} + \beta_{n} * ([S_{2}Y_{D}]_{n-1} + [S_{2}Y_{D}^{\text{OX}}]_{n-1})
\]

And

\[
\sum_{i=3}^{3} [S_{i}Y_{D}] + \sum_{i=3}^{3} [S_{i}Y_{D}^{\text{OX}}] = 1
\]

Where, \([S_{i}Y_{D}]_{n}\) and \([S_{i}Y_{D}]_{n}\) are the \(S_{i}\) state populations of \(Y_{D}\)-contained PSII complex before and after the \(n\)th flash

\([S_{i}Y_{D}^{\text{OX}}]_{n}\) and \([S_{i}Y_{D}^{\text{OX}}]_{n}\) are the \(S_{i}\) state populations of \(Y_{D}^{\text{OX}}\)-contained PSII complex before and after the \(n\)th flash

\(\alpha_{i}Y_{d}\) and \(\alpha_{i}Y_{d}^{\text{OX}}\) are the \(S_{i}Y_{D}\) and \(S_{i}Y_{D}^{\text{OX}}\) dependent miss probabilities, respectively.
δ is a shorthand for the S₁ state changes during dark times between pre-flashes and flash train as well as between the flashes of a regular sequence, e.g. \( \delta_{2t} = \exp(-k_{s2}t_d) \), \( \delta_{3t} = \exp(-k_{s3}t_d) \) (see Figure B5).

The double hit parameter whose calculated percentage is often small (typically 1-2%) was simply treated as being independent of S₁ state transitions. This parameter can be expectedly lower in case of the S₂ \( \rightarrow \) S₀ and S₃ \( \rightarrow \) S₁ double transitions because their turnover rates are limited by the 1-2 ms kinetics of O₂ formation and by acceptor side reactions. Additionally, the smaller double hit can be observed when acceptor side is fully reduced (i.e. 100% Q₈⁻). This means the \( \beta \) probability should depend on the oxidation state of Q₈. Nevertheless, also this effect was neglected. The Q₈ and Q₈⁻ percentages were calculated employing matrixes 12 and 13.

\[
\begin{bmatrix}
[Q₈]_{n,0} & [Q₈]_{n,i} & [Q₈]_{n,2} & [Q₈]_{n,3}
\end{bmatrix} = \begin{bmatrix}
(a_1 + \beta) & 0 & 0 & 0 \\
0 & (a_1 + \beta) & 0 & 0 \\
0 & 0 & (a_2 + \beta) & 0 \\
0 & 0 & 0 & (a_0 + \beta)
\end{bmatrix} \begin{bmatrix}
\gamma_1 & 0 & 0 & 0 \\
0 & \gamma_2 & 0 & 0 \\
0 & 0 & \gamma_3 & 0 \\
0 & 0 & 0 & \gamma_4
\end{bmatrix} \begin{bmatrix}
[Q₈]_{1,0} & [Q₈]_{1,i} & [Q₈]_{1,2} & [Q₈]_{1,3}
\end{bmatrix} + d (12)
\]

\[
\begin{bmatrix}
[Q₈]_{n,0} & [Q₈]_{n,i} & [Q₈]_{n,2} & [Q₈]_{n,3}
\end{bmatrix} = \begin{bmatrix}
(a_1 + \beta) & 0 & 0 & 0 \\
0 & (a_1 + \beta) & 0 & 0 \\
0 & 0 & (a_2 + \beta) & 0 \\
0 & 0 & 0 & (a_0 + \beta)
\end{bmatrix} \begin{bmatrix}
\gamma_1 & 0 & 0 & 0 \\
0 & \gamma_2 & 0 & 0 \\
0 & 0 & \gamma_3 & 0 \\
0 & 0 & 0 & \gamma_4
\end{bmatrix} \begin{bmatrix}
[Q₈]_{1,0} & [Q₈]_{1,i} & [Q₈]_{1,2} & [Q₈]_{1,3}
\end{bmatrix} + d (13)
\]

The above equations give the Q₈ and Q₈⁻ populations for the first 4 flashes; for subsequent flash numbers this repeats with a period of four with a few applied simplifications. Because dark-adapted thylakoid samples have 100% of S₁ state population (Vermaas et al., 1984, Isgandarova et al., 2003), it is assumed that the number of electrons arriving to PSII acceptor side should follow the S₁ state dependent miss parameters. The double hit parameters were assumed to be independent on flash number, S₁ state and Q₈/Q₈⁻ transitions. Empty Q₈ sites were not considered, because 500 ms dark times between 2Hz-flashes is long enough for rebinding of free plastoquinone from the pool in the thylakoid membrane, if available. Therefore, double hit on the acceptor side leads to the same oxidation state of Q₈ as before flash excitation. In addition, permanently empty acceptor sides whose cause is the lack of oxidized plastoquinone in thylakoid membrane contribute to the decline of oxygen yields during the flash sequence and thus decrease the activity parameter d. Figure B5 summarizes the redox backward reactions of the S₁ state that were included in the above extended Kok model. Due to the new experimental conditions in FIOP’s measurements (i.e. catalase addition in PSII sample and nitrogen saturated buffers), the chemical effects of electrochemically produced H₂O₂ which
originating the fast reductions of $S_2 \rightarrow S_0$ and $S_3 \rightarrow S_1$ (gray transitions in Figure B5) could be negligible (Pham and Messinger, 2014).

**Figure B5:** Graphical representation of all considered $S_i$ state decays that occur in the dark-times between flashes or the delay time between preflash(es) and the main flash group. The fast phases of $S_3$ and $S_2$ decays (red arrows) were also applied for 500ms dark time between 2Hz flashes of main flash train, and minor $S$-state transition due to chemical effects of $H_2O_2$ (gray arrows) were ignored. For simplicity, all reactions were treated as mono-exponential and irreversible. $k_{(vs)}$ is the rate constant for the very slow $S_2$ or $S_3$ decay.

Due to a long time-consuming and applying many complicated equations, an analytical solution for this complicated network comprising parallel and consecutive reactions in Figure B5 was not programmed. Alternatively, a numerical approach was applied in which each individual $S_i$ state decay was calculated using equations of parallel reactions. This method was done in many calculation steps, in which each step represented a very short fraction of the total decay time. The amount of calculation step could be adjustable to optimize either the speed of calculation or the accuracy. The equations used for this process are given below (Equations 14-21).

\[
\begin{align*}
[S_3 Y_D]_m &= [S_3 Y_D]_{m-1} \ast \exp\left(-\left(k_{31(slow)} + k_{32(vs)}\right)dt\right) \\
[S_2 Y_D]_m &= [S_2 Y_D]_{m-1} \ast \exp\left(-\left(k_{21(slow)} + k_{21(vs)}\right)dt\right) + [S_3 Y_D]_{m-1} \ast \exp\left(-\left(k_{31(slow)} + k_{32(vs)}\right)dt\right) \\
[S_1 Y_D]_m &= [S_1 Y_D]_{m-1} + \left(\frac{k_{32(fast)}}{k_{32(fast)} + k_{32(vs)} + k_{31(slow)}}\right) \ast \left(1 - \exp\left(-\left(k_{31(slow)} + k_{32(vs)}\right)dt\right)\right)
\end{align*}
\]
\[
[S_{Y_D}^{(OX)}]_m = [S_{Y_D}^{(OX)}]_{m-1}^* + \left[ S_{2Y_D}^{(OX)} \right]_{m-1} \left( 1 - \exp\left( -\left( k_{2i_{(slow)}} + k_{2i_{(ox)}} \right) dt \right) \right) \\
+ [S_{Y_D}]_{m-1}^* \left( \frac{k_{2i_{(fast)}}}{k_{2i_{(fast)}} + k_{2i_{(slow)}} + k_{2i_{(ox)}}} \right) \left( 1 - \exp\left( -\left( k_{2i_{(fast)}} + k_{2i_{(slow)}} + k_{2i_{(ox)}} \right) dt \right) \right) 
\] (16)

\[
[S_{Y_D}^{(OX)}]_m = [S_{Y_D}^{(OX)}]_{m-1}^* \exp\left( -k_{m*} dt \right) 
\] (17)

\[
[S_{Y_D}^{(OX)}]_m = [S_{Y_D}^{(OX)}]_{m-1}^* \exp\left( -\left( k_{2i_{(fast)}} + k_{2i_{(slow)}} + k_{2i_{(ox)}} \right) dt \right) \\
+ [S_{Y_D}]_{m-1}^* \left( \frac{k_{2i_{(slow)}} + k_{2i_{(ox)}}}{k_{2i_{(slow)}} + k_{2i_{(slow)}} + k_{2i_{(fast)}}} \right) \left( 1 - \exp\left( -\left( k_{2i_{(fast)}} + k_{2i_{(slow)}} + k_{2i_{(ox)}} \right) dt \right) \right) 
\] (18)

\[
[S_{Y_D}]_m = [S_{Y_D}]_{m-1} + \left[ S_{2Y_D}^{(OX)} \right]_{m-1} \left( 1 - \exp\left( -k_{m*} dt \right) \right) \\
+ [S_{2Y_D}]_{m-1} \left( \frac{k_{2i_{(slow)}} + k_{2i_{(ox)}}}{k_{2i_{(fast)}} + k_{2i_{(slow)}} + k_{2i_{(ox)}}} \right) \left( 1 - \exp\left( -\left( k_{2i_{(fast)}} + k_{2i_{(slow)}} + k_{2i_{(ox)}} \right) dt \right) \right) 
\] (19)

\[
[S_{Y_D}]_m = [S_{Y_D}]_{(initial)} 
\] (20)

Where \([S_{Y_D}^{(OX)}]_m\) and \([S_{Y_D}^{(OX)}]_{m-1}\) are populations after and before the \(m\)th calculation step, and \(dt\) is the fraction of calculation time derived by dividing the actual dark time by \(m\) calculation steps. The percentage of \(Y_D\) and \(Y_D^{OX}\) in the dark-adapted samples is a fit parameter that is well constrained by the amplitude of fast phases \((k_{(fast)})(S_2\) and \(S_3\) decays. In contrast, the ratio of slow phase to very slow phase is derived from the fraction of centers with \(Q_B^-\) directly after the particular flash number \(n\) (see equations 12 and 13). It is thus assumed that the slow phase corresponds to the centers with reduced acceptor side \((Q_B^-)\), while the very slow decay occurs by an unknown donor in centers having oxidized \(Q_B\) \((Y_D/Y_D^{OX}\) being distributed equally about both types of centers).
Results and discussions

The investigations presented in this thesis aimed at obtaining a deeper understanding of the working mechanism of the water oxidation reaction in PSII complex by Joliot electrode measurements and results from FIOPs data analyses. The experiment performances and consequences will be shown and discussed in detail below. The research aimed specifically at:

1. Determination of the two-electron reductant causing the two-steps reduction of $S_1 \rightarrow S_{-1}$ and $S_2 \rightarrow S_0$. The data show that this substance is hydrogen peroxide which is electrochemically produced by the Joliot electrode. Two methods to minimize the chemical effect of $H_2O_2$ on S-states of the WOC were discovered and thus facilitated the further studies of the intrinsic PSII reactions.

2. Programing a global fitting approach that can be used to analyze FIOP data of all S-state decays at the same time in order to result a comprehensive figure of forward (oxidation) and backward (reduction) reactions of WOC in relations with the other components of PSII complex (e.g. tyrosine D, secondary quinone electron acceptor $Q_B$, etc)

3. Identifying the conversion efficiencies of the individual S-state transition of the OEC in spinach and in the extremophilic red microalga *Cyanidioschyzon merolae* at different experimental conditions (e.g. pH and hydrogen/deuterium exchange effect).

**Does the formation of $H_2O_2$ take place during Joliot electrode measurements? (paper 1)**

As described above, the Joliot-type electrode consists of a bare platinum cathode, onto which the PSII sample is placed directly (Figure B3). It is typically 40 sec before the measurement start, the Pt-cathode is polarized by about -750 mV against Ag/AgCl anode so that it reduces both the dissolved $O_2$ of flow buffer and the $O_2$ formed after flash illumination (Renger and Hanssum, 2009). Due to the direct exposure of PSII sample on surface of Pt-cathode without any gas-permeable membrane (as in design of Clark electrode), the produced oxygen from PSII sample is not only reduced to water with a fast respond and high sensitivity required for collecting FIOPs, but can also react with electrons on electrode’s surface and protons in solution to generate hydrogen peroxide as an intermediate in that process (Damjanov et al., 1966, Sanchez-Sanchez and Bard, 2009, Katsounaros et al., 2012). This is an important concern because the $Mn_4CaO_5$ cluster of the OEC is known as an oxidant, which can be reduced by reductants such as $NH_2OH$, $NH_2NH_2$, $H_2S$ and $H_2O_2$ to form one of the “super-reduced” S-states below the natural $S_0$ state (Bouges, 1971, Kok and Velthuys, 1977, Velthuys and Kok, 1978, Sivaraja et al., 1988, Guiles et al., 1990, Messinger et al., 1997). In fact, FIOPs recorded from the PSII sample which was chemically treated with 0.03% $H_2O_2$ at alkaline pH
(pH 8.8) indicated a two-electron reduction of the S₁ state into the S₋₁ state (Velthuys and Kok, 1978). Besides, when the chloroplasts were illuminated by a single flash to advance S₁ to S₂ state prior to the addition of H₂O₂, the FIOP (measured 5 minutes later when all hydrogen peroxide was gone) displays a maximum of oxygen yield after the fourth flash, indicating that most of the S₂ state was converted into the S₀ state (Velthuys and Kok, 1978, Frasch and Mei, 1987, Frasch and Mei, 1987).

![Figure C1](image)

**Figure C1:** The FIOPs obtained after 40sec (black, square) and 20min (red, round) polarization of -750mV in absence of catalase. The data are normalized to the sum of the oxygen yields obtained by flashes 3-6.

The dark (square) data points of **Figure C1** show the oscillation pattern of oxygen yield of S₁Y₃D°X spinach thylakoids obtained after 40 seconds polarization of the electrode, which is the minimum time required for achieving a stable polarization at -750 mV. This FIOP presents a deep oscillation with maxima at the 3rd, 7th and 11th flash, and low oxygen yields after the 5th and 6th flashes. Extension of the polarization time up to 20 min decreased the maximum at the 3rd flash and led to a corresponding increase of the yields after the 5th and 6th flashes (red sphere FIOP, **Figure C1**). Analysis of these two patterns within the standard Kok model resulted in very similar miss and double hit parameters for both data sets, but indicated a significant increase in the S₋₁ population while the S₁ state population is reduced. This influence is largely independent of the
polarization voltage in the typical range of -600 mV to -750 mV (Pham and Messinger, 2014). Only a small and constant $S_0$ population which is approximately 2-3% is found. These calculated results suggest that the $S_1$ state is reduced to the $S_{1-1}$ state during the polarization time by a two electron reductant, which could also reduce the $S_2$ state into $S_0$ during the dark-time between the first and second flash (see more evidences below). Based on the previous data in the literature, the two-electron reductant candidate is $H_2O_2$, which can be formed at the electrode surface by partial reduction of oxygen and causes the two-steps reduction of $S_1$ to $S_{1-1}$ state.

![Graph](image.png)

**Figure C2**: Percentage of $S_{1-1}$ population as a function of polarization time (sec) in spinach $S_YD^{ox}$ thylakoids with (red line and full circles) or without (blue line and open squares) addition of catalase (10,000 U/ml = 2.63 mg/ml). Triangles and open circles indicate the $S_{1-1}$ population generated in presence of BSA (2.63 mg/ml) or inactivated catalase (2.63 mg/ml), respectively. All experiments were repeated 3 times and fitted individually (error bars give the standard deviations). Other conditions: 20°C, pH 7.0, [Chl]= 0.5 mg/ml, -750 mV (from 40 sec to 20 min), $t_{sed} = 3$ min.

Measurements in the presence or absence of catalase, bovine serum albumin (BSA) or inactivated catalase (IC) at different polarization time were done. The characterized results in **Figure C2** show that the increase of the $S_{1-1}$ population in PSII complex without any additions is linear with the polarization time, demonstrating that the reductant is indeed formed during the time of
polarization at -750mV. The same phenomena were observed in case of thylakoid sample with addition of inactivated catalase or BSA, revealing these additional proteins had no effects on the percentage of S₁ state population. In contrast, addition of active catalase almost completely suppressed the reduction of S₁ to S₀. Another result shows that using the nitrogen-saturated flow buffer, which strongly reduces the dissolved O₂ concentration, had a similar effect as adding catalase. Therefore, the results conclusively identify H₂O₂, formed from the reduction of dissolved oxygen from the flow buffer, as two-electron reductant that generates the S₁ state by S₀ reduction during prolonged polarization times. Interestingly, the linear extrapolation of all data sets in Figure C2 to zero polarization time indicate that approximately 4% OEC of dark-adapted thylakoid exists in S₁ state. This observation may be an artifact of the equal miss fit approach, as suggested in a previous study using thylakoids isolated from Thermosynechococcus elongatus (Isgandarova et al., 2003), or an existence of real S₁ state population under these conditions.

In addition, the S₂ state of the Mn₄O₅Ca is especially reactive with exogenous reductants (Messinger and Renger, 1990, Messinger et al., 1991). Hence, the S₂ lifetime measurements are possibly affected by the formation of H₂O₂ on the Pt electrode surface. The transient increase of the S₀ population in Figure C3-A (blue inverted triangles) shows that even at the shortest polarization time (40 sec) a significant two-electron reduction of S₂ to S₀ state took place. The addition of catalase completely prevents this effect (Figure C3-B), confirming that this is caused by electrochemically produced H₂O₂.

Moreover, the S₃→S₂ and S₀→S₁ state decays are affected by electrochemically produced H₂O₂ similar to the S₂→S₁ decay, but less noticeable. The half-life times of the fast S₂ and S₃ decays (reduction by Y_D) and the slow S₃ decay (recombination with acceptor side electrons, Q_B) are almost invariant in the presence or absence of active catalase. The result is expected, because the fast S₂Y_D and S₃Y_D re-combinations are competitive with the H₂O₂ reduction of S₂/S₃, but the S₃ state is known to react especially slowly with exogenous reductants such as NH₂OH or NH₂NH₂ (Messinger et al., 1991). In contrast, the rate constants of both the slow S₂ and the S₀ states are about 1.3 times smaller in absence of H₂O₂ (presence of catalase). This trend is consistent with the above proposal, since catalase addition removes a competing pathway for S₁ state decay. In case of S₀, the formation of a small percentage (up to 5%) of both the S₂ and of S₂ state population was observed in experiments when catalase was absent (data not shown). This confirms earlier suggestions that the S₀ state can be oxidized to S₂ by H₂O₂ (Frasch and Mei, 1987) or be reduced by reductants to S₂ (Messinger et al., 1991), i.e. that H₂O₂ can react with the Mn₄CaO₅ cluster as oxidant or reductant.
Figure C3: $S_2$ state decay measurements in preflashed spinach thylakoids ($S_Y Y_{D^{ox}}$). Both panels show the change in $S_i$ state populations (square’s, $S_2$; triangles, $S_1$; inverted triangles, $S_0$; circles, $S_{-1}$) as function of dark-time between the $S_2$ state generating flash and the subsequent flash train. Red lines and purple dashed line: fits of $S_2$ decay; other lines simply connect data points. A: no protein additions and air-saturated flow buffer, B: with added catalase ([catalase] = 10.000U/ml) and nitrogen saturated flow buffer. A constant polarization time of 40 s prior to the flash train was used. Other conditions: [Chl] = 0.5 mg/ml, 20°C, pH 7.0, -750mV, $t_{sed} = 3$ min.

Furthermore, while the data of the $S_2$ state decay which is traditionally fitted by sum of two exponential equations is well described in absence of catalase (red line in Figure C3-A), a systematic deviation is observed if H$_2$O$_2$ was removed by catalase (dashed line in Figure C3-B). The red line in Figure C3-B
therefore present a three-phase fit. The fit approach with 3 distinguishable phases, which was independently applied for S\textsubscript{2} lifetime measurements of both S\textsubscript{1}Y\textsubscript{D} and S\textsubscript{1}Y\textsubscript{D}\textsuperscript{OX} thylakoids, resulted the expectedly different amplitudes for the three phases and basically the same decay rates. Nevertheless, for the S\textsubscript{3} state in all cases one or two phases (fast, Y\textsubscript{D}; and slow) were sufficient for describing its decay to S\textsubscript{2}. Further studies will be required for a firm assign of the slow and very slow phases in the S\textsubscript{2} state decay to specific intrinsic electron donors; an attractive possibility would be Q\textsubscript{B} and an unknown electron donor (cytochrome b\textsubscript{559}, carotenoid, Chl\textsubscript{Z}), respectively. Alternatively, Q\textsubscript{B}H\textsubscript{2} may account for the very slow decay.

**Does the very slow phase of S\textsubscript{2} and S\textsubscript{3} decays really exist in S-state lifetime measurements?**

As the above mentions about identification of S-state decay rates following the traditional way, the interested S-state population which was determined by employing miss and double hit parameters from the 2Hz-FIOP is plotted against the dark time between the preflash(es) and main flash train in its respective lifetime measurements, while all others S-state populations are ignored. In this way usually the rate of S\textsubscript{2} decay during an S\textsubscript{3} lifetime experiments is usually not analyzed. In order to fix this problem and improve the traditional way of FIOP data analysis, the next investigation is hence to program a global fitting approach that simultaneously analyzes all oxygen yields of a standard FIOP (2Hz flash) of two datasets each of S\textsubscript{0}, S\textsubscript{2} and S\textsubscript{3} state lifetime measurements that were recorded from well dark-adapted S\textsubscript{1}Y\textsubscript{D} and S\textsubscript{1}Y\textsubscript{D}\textsuperscript{OX} samples.

The starting values for the kinetic fit approach were obtained by analyzing the dataset in the traditional way, i.e. by deconvoluting each FIOP individually. Continuously, the S\textsubscript{1}Y\textsubscript{D} and S\textsubscript{1}Y\textsubscript{D}\textsuperscript{OX} datasets were analyzed separately with the extended Kok model described above (“global fitting approach” section) under the assumption of equal misses on all S\textsubscript{i} state transitions in order to find the best common rate constants for the two data sets. After running this procedure several rounds, the fast decay can be best determined in Y\textsubscript{D}-thylakoids and the slower phases are better resolved in the Y\textsubscript{D}\textsuperscript{OX}-thylakoid measurements. The symbols in **Figure C4** show how the experimental flash-induced O\textsubscript{2} yields varied as a function of t\textsubscript{d} during the three lifetime measurements. The normalized oxygen flash yields Y\textsubscript{2}-Y\textsubscript{5} (S\textsubscript{2} state decay) or Y\textsubscript{1}-Y\textsubscript{4} (S\textsubscript{3} and S\textsubscript{0} state decay) were plotted as a function of dark time. The S\textsubscript{2} population can be qualitatively followed by the decay of Y\textsubscript{2} (red spheres), while Y\textsubscript{1} represents the S\textsubscript{3} state population (black squares). The changes in the S\textsubscript{1} and S\textsubscript{0} populations can be estimated by amplitude changes of Y\textsubscript{3} (green triangles) and Y\textsubscript{4} (blue inverted triangles), respectively. **Figure C4** give the best fits with (panel B) and without (panel A) including a very slow decay of S\textsubscript{2} and S\textsubscript{3} in addition to the well-established fast decay (Y\textsubscript{D}) and slow decay (Q\textsubscript{B}\textsuperscript{-}) (Isgandarova et al., 2003).
Figure C4: The normalized oxygen yields induced by the 1st (black), 2nd (red), 3rd (green) and 4th (blue) of the flash group as a function of dark time between pre-flashes and flash group in the $S_3$ lifetime measurement of $S_YD$ thylakoids allowing two (A) or three (B) phases of $S_2$ decay in the data analysis. The data were normalized to the sum of 16 oxygen yields in every FIOP. All lines represent fit results.
The \( S_1Y_D^{ox} \) data set was well fitted by employing the simple two-phase decay model. Vice versa, a significant improvement with two-fold better fit quality was observed with three phases in case of the \( S_1Y_D \) thylakoids. **Figure C4** shows that the two-fold worse fit quality that was obtained by using two-phase decay fit is clearly observed by plotting of oxygen flash yields after the 2\(^{nd}\) and 3\(^{rd}\) flashes during the \( S_3 \) decay at delay times longer than 25 seconds. This corresponds to the transition of the \( S_2 \) state, which is formed by the fast \( S_3 \) reduction. The discrepancy shows that the \( S_2 \) decay in the \( S_3 \) state lifetime measurements is slower than in the simultaneously fitted \( S_2 \) decay of \( S_1Y_D^{ox} \) samples in \( S_2 \) state lifetime measurements. A very good agreement between all measured data points and the fit was achieved by introducing an additional very slow \( S_2 \) decay rate for PSII centers with oxidized acceptor side (\( Q_B \)).

The prominent appearance of the very slow \( S_2 \) decay during the \( S_3 \) lifetime measurements of the \( S_1Y_D \) sample can be explained by the lack of reduced quinone (\( Q_B^- \)) molecules in PSII acceptor side. During a long period of dark-storage at \(-80^\circ\)C, all \( Q_B \) was oxidized due to electron transfer from \( Q_B^- \) to \( Y_D^{ox} \) (Nugent et al., 1987). After the two given preflashes, the current PSII type is thus highly enriched in the \( S_3Y_DQ_B \) state, which rapidly converts to \( S_2Y_D^{ox}Q_B \). For further decay to \( S_1 \), this \( S_2Y_D^{ox}Q_B \) state needs one electron from another donor than \( Q_B^- \), maybe from rebinding \( Q_BH_2 \) in thylakoid membrane, or from a member of the protective branch in PSII, for example cytochrome \( b_{559} \). Besides, the similar situation should occur in \( S_1Y_D^{ox} \) thylakoids, but here the sample contains 40-50\% \( Q_B^- \) due to the pre-flash protocol, thus resulting a description of the \( S_2 \) decay with a single average rate sufficiently good (since \( k_{2(s)}/k_{2(vs)} \approx 4 \)). The calculated rate constant of the very slow \( S_2 \) decay in this research is very similar to the value (\( k_{21(vs)} \approx 0.0035 \text{ s}^{-1} \) at \( 18^\circ\)C), which was described previously when the \( S_2 \) decay in PSII membrane fragments was studied by EPR in presence of artificial electron acceptors (Styring and Rutherford, 1988). Our data strongly reinforce this earlier report and present that the very slow \( S_2 \) decay can take place in untreated thylakoid samples.

Nevertheless, no conclusive evidence for a very slow decay of the \( S_3 \) state was observed and under all circumstances, the rates of the slow and the very slow \( S_3 \) decay are indistinguishable. The difference between our observation and the data from Styring and Rutherford (Styring and Rutherford, 1988) was likely due to experimental constraints. The disadvantage of our thylakoid sample in this research is (i) the \( S_1Y_D \) samples lead to the fast reduction of \( S_3 \) by \( Y_D \), making it difficult to accurately determine the very slow \( S_3 \) decay rate and (ii) the \( S_1Y_D^{ox} \) samples have an equal amount of \( Q_B \) and \( Q_B^- \), resulting the observation of a single decay phase with averaged rate.
How dependent are miss parameters on the S-state of the \( \text{Mn}_4\text{O}_5\text{Ca} \) cluster and on the oxidation state of \( \text{Y}_D \)? (paper 2)

As the above results, the very good fit quality that was used \( \text{S}_1 \) state independent misses is a challenge to explore the \( \text{S}_1 \) state dependent misses as expected. Thus, an initial attempt is using of \( \text{S}_1 \) state dependencies suggested in three recent accounts (de Wijn and van Gorkom, 2002, Isgandarova et al., 2003, Han et al., 2012). Due to slight differences in PSII material, temperature and pH it seemed not to be realistic to apply simply the suggested miss parameters directly. Alternatively, the trends of miss parameters that were proposed in these earlier studies are employed to set starting values and define restrictions to the fits. For example, the trend of misses as (i) \( \alpha_0 = 0, \alpha_1 = \alpha_2 < \alpha_3 \) are simulated by the idea of de Wijn based on the competition between \( \text{Y}_Z \) and \( \text{Q}_A^- \) in \( \text{P}_680^{+*} \) reduction via chlorophyll fluorescence measurements (de Wijn and van Gorkom, 2002); (ii) the second option of misses \( \alpha_0 = \alpha_1 = 0 \) and \( \alpha_2 = \alpha_3 \) were followed the suggestion by Isgandarova et al via FIO\'s experiments (Isgandarova et al., 2003); and (iii) the last suggestion for misses \( \alpha_3 < \alpha_0 = \alpha_1 < \alpha_2 \) were a copy of the recent proposal by Han et al. that is based on the analysis of the flash number dependence of several EPR signals.

Based on the results from GFP-analysis, the fit qualities of the \( \text{S}_1\text{Y}_D^{\text{ox}} \) and \( \text{S}_1\text{Y}_D \) thylakoids were only slightly improved compared with the equal miss approach if the constraints based on de Wijn and Isgandarova were applied. Using the constraints based on the suggestions by Han et al., gave rise to a further small improvement of the fit quality. This may be due to including a small miss factor connected with the oxidation of \( \text{S}_0 \) and by having the highest miss in the \( \text{S}_2 \rightarrow \text{S}_3 \) transition. The next fits where all \( \alpha_i \) values were free variables were performed. By varying the starting conditions for the fits, two stable solutions were found as Fit 1 and Fit 2. The parameters obtained in Fit 1 were very similar to those of Han et al. (Han et al., 2012) with a small improvement of fit quality. Fit 2 presents that an almost equally good fit quality can be obtained if the highest miss factor is assigned to the \( \text{S}_3 \rightarrow \text{S}_0 \) transition. Starting conditions where \( \alpha_0 \) or \( \alpha_i \) are set to be dominating, or with \( \alpha_2 = \alpha_3 \) were not stable and converted in either Fit 1 or Fit 2.

The achieved results support the models where the highest miss occurs in spinach thylakoids at pH 7.0/20°C in either the \( \text{S}_2 \rightarrow \text{S}_3 \) or the \( \text{S}_3 \rightarrow \text{S}_0 \) transition. It indicates that using the global fit approach resulted a much higher selectivity and reliability than fitting individual pattern, where many more options result the same fit quality. It is known that the \( \text{S}_2 \rightarrow \text{S}_3 \) transition involves a structural change, which likely includes the uptake of a water molecule by the \( \text{Mn}_4\text{CaO}_5 \) cluster (Suzuki et al., 2008, Bovi et al., 2013, Cox and Messinger, 2013, Siegbahn, 2013, Capone et al., 2015, Shoji et al., 2015) and which is also highly sensitive to changes in the H-bonding network (Klauss et
al., 2012, Shoji et al., 2013, Service et al., 2014, Debus, 2015). Similarly, the $S_3 \rightarrow S_0$ transition is very complex, but is split into a relatively straightforward light-induced $S_3Y_Z \rightarrow S_3Y_Z^{ox}$ ($S_3$) transition and the demanding $O_2$ formation, which however has a large driving force (Rappaport et al., 1994, Haumann et al., 2005, Han et al., 2012, Nilsson et al., 2014, Nilsson et al., 2016).

![Figure C5](image)

**Figure C5:** Normalized oxygen yields as a function of dark time in three different $S_i$-state ($i = 2$, 3 and 0) lifetime measurements obtained with dark-adapted $S_iY_D$ (left) and $S_iY_D^{ox}$ (right) thylakoid samples. All lines are fit results.

Alternatively, the high miss for the $S_3 \rightarrow S_0$ transition was explained by presents of state $S_2Y_Z^{ox}$ in Boltzmann equilibrium with $S_3Y_Z$, which can lead to an increased reduction of $P680^{+}$ by $Q_A^-$ (de Wijn and van Gorkom, 2002). However, EPR data suggest that the $S_2Y_Z^{ox}$ population becomes only significant
at pH > 8.5 (Geijer et al., 2001). Considering the study of Han et al. (Han et al., 2012) that has provided constraints for the S_i state dependent miss parameters by following the EPR signals of all S_i states within a flash pattern, we favor the analytical solutions with the highest miss (20-30%) in the S_2 → S_3 transition.

Nevertheless, the adjustment of additional free parameters in a fit model is often expected that the fit quality is 50% or less in the more complicated model. Despite the very clear trends described above, the improvements in fit quality when using S_i state dependent misses were even in our global fit approach on the borderline of being conclusive. Importantly, the S_i state lifetimes are nearly constant between the different fits. The rate of the fast S_3 state decay showed the largest deviations, but the result was still invariant within ± 10%. Figure C5 shows the fit results for all S_i state decays for both S_iY_D and S_iY_D^{ox} thylakoids obtained with the fit in which the S_2 → S_3 transition is the least efficient S-state advancement. Further data analysis showed that the total miss of all S_i state transitions of the S_iY_D^{ox} sample was a few percent larger than for S_iY_D sample in all cases, while in an earlier study, the opposite trend was observed (Isgandarova et al., 2003). It has been suggested previously that the oxidation state of Y_D may affect the miss parameter. In its reduced form Y_D may donate with low probability an electron to P_{680}^•+ (Faller et al., 2001, Havelius and Styring, 2007), while in its oxidized form the positive charge may affect the efficiency of primary charge separation (Rutherford et al., 2004). Further experiments will be required to clarify this point.

**What is the least efficient S-state transition of the Mn_4O_5Ca cluster, S_2 → S_3 or S_3 → S_0 transition?**

The question is arisen due to the unclear results from all of the S-state lifetime measurements of spinach thylakoid at neutral pH. This question was thus further analyzed via FIOP experiments with isolated thylakoids from the extremophilic red micro-alga *Cyanidioschyzon merolae* (C. merolae) at different pH values and after hydrogen/deuterium (H/D) exchange. Similar to measurements with spinach thylakoid, for each pH or pD value a full set of data was recorded, comprising of one 2 Hz FIOP and 11-18 FIOPs each recorded at different time points during the S_3, S_2 and S_0 lifetime measurements. Employing the global fitting program (GFP), the 8 first O_2-yields of each FIOP were simultaneously analyzed instead of using all 16 oxygen yields as with spinach thylakoids due to the significant loss of many active PSII reaction centers or very slow oxidation, diffusion of reduced plastoquinone on the acceptor side. Fits with different numbers of free parameters were performed. Based on the consequences of several fitting cycles, the inclusion of the very slow S_2 and S_3 decays generally resulted in an improvement of the fit quality by only 10% over fits with only one slow phase of S_2 and S_3 decays. That is why the global fitting program in these cases is simplified by ignoring both very slow S_2 and S_3 decays.
In addition, the *C. merolae* thylakoids are directly used after thawing without any preflash treatments as spinach thylakoids since the amount of Y\(_D\) in this sample is quite small (about 10%). Hence, it was difficult to obtain systematic fits results using both the percentage of Y\(_D\) and the two rate constants for the fast phase of S\(_2\) and S\(_3\) decays as free parameters. The percentage of Y\(_D\) was thus fixed to 10%, which generally gave the best fit results. Because of this small percentage, \(k_{32(\text{fast})}\) and \(k_{21(\text{fast})}\) are mostly identified by the S\(_0\) state lifetime measurements. In the process, an increasing amount of Y\(_D\)\(_{OX}\) is reduced to Y\(_D\) and thus affects the Y\(_3\)/Y\(_4\) ratio via back reactions in the 500 ms dark times between flashes of the FIOP. This in turn results in an interdependence of these parameters with \(k_{01}\). For all fits 100% S\(_1\) population is assumed for the dark-adapted state, because no improvement of fit quality could be found when including S\(_0\) or S\(_4\) state. Despite the GFP fits the miss parameters and on the S\(_i\) state lifetimes simultaneously, the effects of pH and H/D exchange on these PSII properties will be discussed separately.

**Figure C6**: FIOPs of dark-adapted *C. merolae* thylakoids obtained at 20\(^\circ\)C and pH 5.0 (black squares), 6.1 (red spheres) or pH 8.0 (blue triangles). The data were normalized to the oxygen yield induced by the third flash. The flash frequency was 2 Hz. The lines connect data points.

The ratio of oxygen flash yields (Y) induced by the 3\(^{rd}\) flash (Y\(_3\)) to that of the 4\(^{th}\) flash (Y\(_4\)) is often used to evaluate generally the miss parameter. It is clearly seen that the ratio Y\(_3\)/Y\(_4\) of the FIOP obtained at pH 6.1 (red spheres in **Figure**
**C6** is the greatest value compared with that of the alkaline (pH 8.0) or acidic region (pH 5.0), indicating a low average miss probability. **Figure C6** also shows that the overall oscillation amplitude of the third cycle (flashes 11 and onwards) was untypically low. This phenomenon was previously observed in the FIOPs of many spinach BBY samples, implying a limited pool size and/or lacking of plastoquinone acceptor per PSII complex. After washing the samples by pH 5.0 buffer via a several repeated cycle of centrifugation (about 10 min), a FIOP with an increased miss parameter was obtained (black square in **Figure C6**), corresponding with a small \( Y_3/Y_4 \) ratio. Interestingly, the average \( O_2 \) flash yield did not decrease as much at higher flash numbers as at pH 6.1. This can be explained by the total \( O_2 \) yield was only about 25% compared with one at neutral pH. The decreased amount of active PSII complexes thus increased the number of plastoquinone molecules available for the active once. The strong decline in the number of active PSII centers was surprising, maybe because the washing procedure with desired buffers (10 min) is long enough to damage a significant amount of active PSII reaction centers prior to the measurements.

The first 6 oxygen flash yields of the FIOPs obtained at acidic and alkaline (blue triangle in **Figure C6**) region are quite similar. The impressive point between these two FIOPs are the clear discrepancy of the oxygen yield ratio \( Y_7/Y_8 \) which was specifically inverted. This observation expressed a significantly different distribution of the miss parameters over the S-state transitions at pH 5.0 and pH 8.0 values. Total oxygen evolution per flash at pH 8.0 was approximately 45% compared to that at pH 6.1, confirming a larger platoquinone pool per active PSII complex. The above discussions are however the relative estimation of miss parameters at various pH values, no reliable conclusions can be made only based on individual FIOP analysis. These overall observations of miss parameters were quantitatively confirmed by employing GFP-analysis.

At pH 6.1 an equal miss fit results in a miss parameter \( \alpha \) of about 11%, while the in the best unequal miss fit all misses (35%) occur in the \( S_2 \rightarrow S_3 \) transition. Importantly, the fit quality of the S-state dependent (unequal) miss fit is twice as good as that of the equal miss fit, strongly supporting the fit where \( \alpha_2 \) parameter is greatest one. This is further favored by the finding that this fit result is systematically found once releasing the equal miss constrain. Another fitting options in which the starting values are 35% \( \alpha_1 \) or \( \alpha_3 \) (all other \( \alpha_i = 0 \)) for thylakoid at pH=6.1 also result a similar fit qualities (FQ) compared with that of the fit where the \( S_2 \rightarrow S_3 \) transition is the least transition. Forcing most misses into \( \alpha_0 \) resulted in unsatisfactory fits with very bad FQ. One more time, no clear fit results were obtained at neutral pH (pH 6.1 for *C. merolae* and pH 7.0 for spinach thylakoids) to answer the topic question.
The FIOP dataset obtained at pH 5.0 was continuously characterized by GFP-analysis. The fits at acidic pH present the identical trends, except that the miss fits were higher, i.e. 18% for equal miss fits and 54% for the $S_2 \rightarrow S_3$ transition. Additionally, the unequal miss fit was nearly 3 times better compared to the equal miss fit. The fit where $\alpha_3$ is dominating has a nearly as good FQ as the one with $\alpha_2 = 54\%$, however the value of $k_{21(fast)}$ equals $k_{21(slow)}$, i.e. was hitting the lower fit limit. These fit options where the $\alpha_1$ or $\alpha_3$ are the largest value were considered as unrealistic. In addition, the fit where $\alpha_i$ is forced to be maximum results in a fit that is nearly as poor as the equal miss fit. The large difference between the equal and unequal miss approach comes from the – relative to $Y_3/Y_4$ – large $Y_7/Y_8$ ratio, which can only be modeled correctly with unequal misses where $\alpha_2$ dominates.

In contrast to pH 5.0 and 6.1, the FIOPs obtained at pH 8.0 cannot be fit well with only an assumption that all misses occur in the $S_2 \rightarrow S_3$ transition or the $S_3 \rightarrow S_0$ transition. For this alkaline pH, an almost equally large percentage of misses surprisingly occurred in the $S_0 \rightarrow S_1$ transition. The best fit for thylakoid at pH 8.0 was uniquely obtained by dominating of both $\alpha_0$ and $\alpha_2$. The quantitative GFP-analysis of the C. merolae FIOPs obtained at three different pH values thus strongly supports unequal misses where nearly all of the misses occur in the $S_2 \rightarrow S_3$ transition at acidic and neutral pH, while at alkaline pH also the $S_0 \rightarrow S_1$ transition becomes less efficient.

According to proton liberations through S-state transitions that could be the limiting factor for the efficiency of a Si state transition, one would expect that for such transitions the miss probability would decrease with increasing pH. While this trend was observed for $\alpha_2$ when comparing pH 5.0 and pH 6.1, this trend did not hold for pH 8.0. Moreover, the recent pH jump experiments from Dau H. group showed that the largest pK value for acidic inhibition is 4.6 for $S_3 \rightarrow S_0$ transition (Zaharieva et al., 2011). Nevertheless, none of the S-state transitions showed increased inefficiencies at alkaline pH region. Furthermore, the $S_0 \rightarrow S_1$ transition was found to be pH independent between pH 3 and pH 9. Based on the found results, one would expect that the $S_3 \rightarrow S_0$ transition is the only one S-state conversion that is affected by the pH values tested in this research. While the fits with large $\alpha_3$ generally gave reasonable results, although their FQ were consistently worse than those with large $\alpha_2$. The inconsistency between our data and results of Zaharieva et al causes by the different time scales of incubation at the indicated pH - 1.5 sec versa >10 min – are responsible for the deviating results. Our preferred assignment for the acidic to neutral pH ($\alpha_2 >> \alpha_3, \alpha_1, \alpha_0$) achieved a good agreement with results by Han et al. who reported a similar result by following the Si state populations during a flash train via Si state specific EPR signals (Han et al., 2012). In addition, since the overall fit quality obtained from C. merolae samples is not as good as with spinach thylakoids and
many miss parameters go to zero (i.e. hitting the lower limits), the latter efforts have to perform with the insufficient constraints for the fast $S_2$ and $S_3$ decays in these experiments.

The reason why the greatest miss parameter is connected to the $S_2 \rightarrow S_3$ transition is not only the known structural changes of the Mn$_4$CaO$_5$ cluster, the binding of one additional substrate (possibly in form of hydroxide) and the small driving force of this transition (Cox et al., 2014, Shen, 2015). The increase of the miss probability of the $S_0 \rightarrow S_1$ and $S_2 \rightarrow S_3$ transitions at alkaline pH region cannot be easily explained by any of the above proposals. Because no artificial electron acceptors were used in this study, the period two oscillation of the miss parameter at PSII acceptor side may be taken as indication that the protonation of $Q_B$ became a limiting factor between pH 7 and pH 8. Further studies will be necessary to untangle the donor and acceptor side contributions at alkaline pH.

**Figure C7**: $S_2$, $S_3$ and $S_0$ state lifetime measurements at pH 5.0, pH 6.1 and pH 8.0. The normalized flash-induced oxygen yields of flashes 2-5 ($S_2$) or 1-4 ($S_3$, $S_0$) are plotted as a function of dark-time between the preflash(es) and the flash train. The color code is given in the respective inserts. Symbols are the experimental amplitudes, while the lines connect the amplitudes calculated by the global fitting program. All lines represent fit results.
In S₁ state lifetime experiments, after exciting the dark-adapted PSII sample (S₁ state) to the desired S-state by 1-3 flash(es), the dark time, t₀, to the subsequent FIOP flash train (2Hz) was varied in suitable steps to probe the fast and slow decay components (Figure C7). The symbols and color codes in Figure C7 which are similarly designed to the previous data of spinach thylakoid (Figure C4) show how the experimental flash-induced O₂ yields varied as a function of t₀ during the three lifetime measurements. In the top row of Figure C7 the pH dependence of the reaction S₂ → S₁ was studied. The simultaneous decay of Y₂ and the corresponding increase of Y₃ demonstrated for all three pH values a clean one-electron reduction process. A small rise of Y₄ and Y₅ was observed due to miss and double hit. Only a very small fast phase was visible, which was corresponding to 10% of Y₀ in C. merolae thylakoids. There were not so many data points for reliable characterization of the fast phase at various pH values, it is seen that the slow phase of S₂ decay is about 2-3 times faster at pH 5.0 than at the two other pH values.

Moreover, the data at center row of Figure C7 displayed the strong pH dependence of the S₃ decay. Due to the rapid S₃ decay at pH 5.0, the sequential decay of S₃ (Y₃) → S₂ (Y₂) → S₁ (Y₃) could be clearly discerned. Y₄ increased nearly in parallel to Y₃ due to misses. For the S₃ decay a strong retardation of the slow decay was observed at the higher pH values. For the S₀ → S₁ reaction a qualitative analysis was more complicated, because the expected decrease of Y₄ was compensated by additional misses coming from the increase of S₁ state population via the parallel reaction of S₀ oxidation and the S₃ and S₂ reductions. In addition, the increasing Y₀ population which is caused by the S₀ oxidation to the S₁ state further increases Y₄ by fast reduction of fractions of S₂ and S₃ between 500ms dark time of flashes of the FIOP. Nevertheless, it appeared that the S₀ oxidation possessed a complicated pH dependence with the slowest rate near neutral pH. Unfortunately, no previous data for the S₀ → S₁ reaction were published at pH 5 or pH 8. Despite some small deviations, overall a satisfactory agreement with the data was achieved. The fits confirm the trends discussed above for the slow S₂ and S₃ state decays at the three pH values. While the S₂ state decay is relatively pH independent (within a factor of 2), the rate of S₃ decay which has a good agreement with the previous data on spinach thylakoid (Messinger and Renger, 1994) slowed by more than a factor of 10 between pH 5 and pH 8.

The most surprising finding is the very strong pH dependence of the rates for the S₀ → S₁ transition. While the rate of this transition at pH 6.1 was well reproduced under all tested fit conditions and agreed within a factor of four with previous estimates in spinach thylakoids (Messinger and Renger, 1994) and was practically identical with that determined with T. elongatus thylakoids.
(Isgandarova et al., 2003), the much faster oxidation rates at pH 5 and pH 8 came as a surprise. Due to the present experimental conditions with a small amount of $Y_D$, it was unable to obtain strong constraints for the fast decay kinetics, the values for $k_{01}$ varied at the two extreme pH values quite considerably depending on the fit scenario. At pH 5.0 values between 0.21 s$^{-1}$ and 2.7 s$^{-1}$ were obtained, while $k_{01}$ was found to be in the range of 0.00007 s$^{-1}$ and 0.035 s$^{-1}$ at pH 8.0. Possible reasons for a faster $S_0$ oxidation could be connected to the recently described two different positions of a water molecule near $Y_D$ that appears to influence the redox potential of $Y_D/Y_0^{OX}$, and other pathways for $S_0$ to $S_1$ conversion may be possible under extreme pH conditions. At alkaline pH also the easier removal of a proton may increase the rate of conversion, since the $S_0 \rightarrow S_1$ transition is coupled to a proton release (Rappaport and Lavergne, 1991, Siegbahn, 2013, Klauss et al., 2015).

The effects of hydrogen/deuterium exchange on the miss parameters

Another FIOP dataset, which was recorded by performing three typical S-state lifetime experiments after hydrogen/deuterium (H/D) exchange at pH 6.1, was also characterized by GFP analysis. The results reveal that nearly all fit parameters were identical in $H_2O$ and $D_2O$. The only clear exceptions are the two-fold increase of the rate of $S_3 Q_0^-$ decay ($k_{32(\text{slow})}$) and a six-fold increase of the rate for $S_0 Y_D^{OX}$ oxidation to $S_1 Y_D$. In addition, a minor increase of $\alpha_2$ from 35% to 37 % was observed. The higher value of $\alpha_2$ in $D_2O$ should be explained with the idea that proton release during the $S_2\rightarrow S_3$ transition is required, because a slowdown of that rate due to the greater O-D bond strength will increase the miss parameter by increasing recombination reactions between $Q_{A^-}$ and $Y_{Z^{OX}}$ (Zaharieva et al., 2011). However, the small magnitude of this effect indicates that this can only be one of several factors contributing to the low efficiency of this transition.

Similarly, the acceleration of the slow $S_3$ decay in $D_2O$ and the much shorter $S_3$ state lifetimes at high proton concentration are consistent with the demand to take up a proton when returning to the $S_2$ state. In line with this idea, no clear H/D effect and a much weaker pH effect is seen on the rate of $S_2$ decay, which is not coupled to a proton uptake. This suggests that the pH has a strong effect on the potential of the $S_3$ state, while the effect on $Q_{B^-}$ is less important for the $S_1$ state lifetimes. The six-fold acceleration of the $S_0$ oxidation to $S_1$ by H/D exchange supports indirectly the surprising finding that low pH values induce the same effect (but even much stronger). The $S_0 Y_D^{OX} \rightarrow S_1 Y_D$ reaction involves the following two half reactions:

$$S_0 \rightarrow S_1^- + e^- \rightarrow S_1 + H^+$$

$$Y_D^{OX} + e^- + H^+ \rightarrow Y_D$$
So while the formation of the $S_1$ state should occur faster at high pH, the reduction of $Y_D^{OX}$ may occur faster at low pH. Indeed, a strong H/D effect was observed for the reactions $S_2Y_D \rightarrow S_1Y_D^{OX}$ and $S_3Y_D \rightarrow S_2Y_D^{OX}$ (Isgandarova, 2004) and was recently linked to the position of a water molecule near $Y_D$ (Styring and Rutherford, 1987, Vass and Styring, 1991). Zadarieva et al observed a rapid inactivation of centers in the $S_0$ state at low pH that occurs with a pKa of 4.6. Our data were thus checked if a specific slower phase of $S_0$ state inhibition could affect the $S_i$ state quantitation. However, no indications for that could be found.
Conclusions

The main consequences from the first exploration help to clearly observe the formation of hydrogen peroxide on the surface of Pt cathode of Joliot electrode during experiment. Besides, the reason why $\text{H}_2\text{O}_2$ production can take place was also found out. Catalase addition and using $\text{N}_2$-saturated buffer in Joliot electrode measurements are the ways to minimize the chemical effects of electrochemically produce $\text{H}_2\text{O}_2$ on $S_0$ and $S_2$ lifetime measurements. These new experimental conditions also result a reliable determination of the $S_i$-state distribution in dark-adapted PSII samples. In absence of $\text{H}_2\text{O}_2$-induced effects, three distinguishable phases in the $S_2$ state decay and a residual $S_{-1}$ state population in dark-adapted spinach thylakoids were observed.

Three different kinetic phases in the $S_2$ decay of spinach thylakoid was confirmed by the second study in which the global fitting approach was programmed and used, which included an acceptor side cycle to calculate the ratio of slow to very slow phase. Under our conditions, i.e. in absence of added electron acceptors and at neutral pH, the very slow phase of $S_2$ decay was most prominent in the $S_3$ state lifetime measurements of $S_1Y_D$ thylakoids, i.e. when a significant population of the $S_2Y_D^{\text{ox}}Q_B$ state formed quickly by electron donation of $Y_D$ to $S_3Q_B$. Likely candidates for the very slow electron donor are cofactors of the ‘protective’ branch of PSII (Barber and Rivas, 1993). Further experiments are required to clarify the nature of the electron donor responsible for the very slow phase of $S_2$ state decay.

The systematic improvement of the fit quality was achieved by using $S_i$ state dependent misses. In the best fits, practically all misses occur during the $S_2 \rightarrow S_3$ or the $S_3 \rightarrow S_0$ transition. This is in line with several previous suggestions (de Wijn and van Gorkom, 2002, Isgandarova et al., 2003, Han et al., 2012, Suzuki et al., 2012), and reinforces the view that one of these transitions is the most challenging one during the reaction cycle of the OEC in PSII. Considering the study of Han et al. (Han et al., 2012) that has provided constraints for the $S_i$ state dependent miss parameters by following the EPR signals of all $S_i$ states within a flash pattern, the analytical solutions with the highest miss (20-30%) in the $S_2 \rightarrow S_3$ transition are the most favorable fit option as our thought.

In short, the developed GFP-analysis for the fits of $S_3$, $S_2$ and $S_1$ state lifetime measurements provide a powerful tool for studying the efficiency of photosystem II under various conditions, species or mutants. Despite the highly acidic environmental conditions $C. \text{merolae}$ thrives in, photosystem II in isolated thylakoids behaves very similar to spinach and $T. \text{elongatus}$. The data indicate that the redox potential of $Q_A/Q_A^-$ plays only a minor role for the miss parameter and the $S$ state lifetimes, and support the idea that the $S_2 \rightarrow S_3$
transition is the least efficient step during the oxidation of water to molecular oxygen in photosystem II.
Acknowledgements

The time when I have been as a PhD student at Chemistry department, Umeå University is long enough for me not only to study and perform researches in natural photosynthesis, but also to make friends with many of kindhearted people who always support and encourage me.

**Prof. Johannes Messinger**, my principle supervisor who has not given me only many invaluable experiences in experimental methods and lab skills, but also a lot of scientific knowledge in natural photosynthesis. I really appreciate your excellent supervision, which is highly patient and enthusiastic.

**Lars Lundmark**, technical engineer and honorary doctor at Umeå University, I really don’t know how to precisely express my appreciation to you, a conscientious teacher and a “second father” in the hearts of many Vietnamese students and me. Thank you so much for everything you did for Vietnam, Cambodia and many other countries, R.I.P.

**Dmitry Shevela**, you are a very good artist in drawing of scientific models that I have ever seen. I would like to thank you a lot for your efforts in creating many beautiful figures that I have used in my paper and this thesis, and many interesting discussion between us.

**Sergey Koroidov**, thank you for your attempt in developing a computational software based on LabView platform that have been used to control the Xenon flash lamp and record data from Joliot electrode measurement.

**Håkan Nilsson**, my first impression that you are really quiet and it lets me a bit nervous in talking to you. And now I know you are very nice person and are willing to share your knowledge in the lab, especially the training time when I learn how to work with mass spectrometer. Thank you so much

**Lisa Henriksson**, the best beautiful wing walker that I firstly met in my life (until now). Although the time you had been in Umeå is not so long, I had learnt many things from you, and I knew what the “wing walking” art is. Many thanks to you and wish you all the best.

**Casper De Lichtenberg**, I expectedly think that you are a badminton player before I met you (since many Danish people play badminton quite well). And now I knew you are a saxophonist, a bit disappointed (just kidding). Thank you for being a nice, funny friend in the office with me.
Members of the Artificial Leaf project:
Hans-Martin Berends, Hasna Bourajoini, Eduardo Gracia, Jan Forsgren, Srikanth Revoju, Tung Pham Ngoc, Yongqi Liang, Anurag Kawde, Wai Ling Kwong (Canny), Manuel Boniolo.

Additional acknowledgements: (Vietnamese and English)
Con/cháu/anh/mình muốn gửi đến ông bà ngoại (Phạm Văn Bay và Nguyễn Thị Tư), ông bà nội (Võ Văn Thiệt và Lâm Thị Túc), cha mẹ (Võ Thanh Hải và Phạm Cửu Huyễn), mẹ vợ (Nguyễn Văn Hồng Phát và Lê Thị Loan Phượng), vợ yêu (Nguyên Lê Ninh Thái), em trai (Võ Phạm Lân), cô, dì, chú, bác, đường, thím, cấu, mẹ hai bên gia đình noi ngoại bên gia đình của mình và gia đình bên vợ, cùng như đến tất cả những người thân yêu, bạn bè một lời cảm ơn chân thành và sâu sắc nhất. Như tất cả những sự giúp đỡ về vật chất cùng như có vẻ vế tình thân mà con/cháu/anh/mình mới có đủ sức mạnh và nghị lực để có gang làm việc hết mình và đạt được những thành công nhất định như ngày hôm nay. Bên cạnh đó cũng không thể nào quên được những sự sệ chia, giúp đỡ dù ít hay nhiều của tất cả những cô chú, anh chị, bạn bè mà không tâ nào có thể nói ra hết ở đây, từ khi em/mình đặt chân đến xứ sở Bắc Âu này (Umeå, Thụy Điển). Một lần nữa, xin được tri ân tất cả mọi người.

I would love to send my deepest appreciation to my grandparents on my mother’s side (Pham Van Bay and Nguyen Thi Tu), my grandparents on my father’s side (Vo Van Thiet and Lam Thi Tuc), my parents (Vo Thanh Hai and Pham Cuu Huyen), my parents in law (Nguyen Van Hong Phat and Le Thi Loan Phung), my beloved wife (Nguyen Le Ninh Thai), my younger brother (Vo Pham Lan), all of my uncles and my aunts in both father’s side and mother’s side of my family and my family in law. Due to all of their financial supports and spirit encouragements, I have enough energy to study, to work hard and achieve a few certain successes at now. In addition, I never ever forget the helps from all of very nice people I have met from the first time when I arrived to this Nordic country (Umeå, Sweden). One more time, thank you so much.
References


Debus, R. J. (2015). "FTIR studies of metal ligands, networks of hydrogen bonds, and water molecules near the active site Mn₄CaO₅ cluster in photosystem II." Biochimica Et Biophysica Acta-Bioenergetics 1847(1), 19-34.


Styring, S. and A. W. Rutherford (1987). "In the oxygen evolving complex of photosystem II the S$_0$ state is oxidized to the S$_1$ state by D$^+$ (signal II$_{slow}$)." Biochemistry 26(9), 2401-2405.


