Substrate water binding to the oxygen-evolving complex in photosystem II

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"Tron kan försätta berg
men tvivlet kan sätta tillbaks dem igen"

Tage Danielsson Eftertanke, Tankar från roten (1974)
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III. **Håkan Nilsson**, Fabrice Rappaport, Alain Boussac, Johannes Messinger. *Substrate-water exchange in photosystem II is arrested prior to dioxygen formation.* Submitted 2014

IV. **Håkan Nilsson**, Guangye Han, Dmitriy Shevela, Laurent Cournac, Alain Boussac, Fabrice Rappaport, Johannes Messinger and Jerome Lavergne. *Estimation of the equilibrium constant of the molecular oxygen generating S₄→S₀ (S₃⁺YZ→S₀YZ) transition in photosystem II.* Manuscript in preparation

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Abstract

Oxygenic photosynthesis in plants, algae and cyanobacteria converts sunlight into chemical energy. In this process electrons are transferred from water molecules to CO₂ leading to the assembly of carbohydrates, the building blocks of life. A cluster of four manganese ions and one calcium ion, linked together by five oxygen bridges, constitutes the catalyst for water oxidation in photosystem II (Mn₄CaO₅ cluster). This cluster stores up to four oxidizing equivalents (S₀,...,S₄ states), which are then used in a concerted reaction to convert two substrate water molecules into molecular oxygen. The reaction mechanism of this four-electron four-proton reaction is not settled yet and several hypotheses have been put forward. The work presented in this thesis aims at clarifying several aspects of the water oxidation reaction by analyzing the mode of substrate water binding to the Mn₄CaO₅ cluster.

Time-resolved membrane-inlet mass spectrometric detection of flash-induced O₂ production after fast H₂¹⁸O labelling was employed to study the exchange rates between substrate waters bound to the Mn₄CaO₅ cluster and the surrounding bulk water. By employing this approach to dimeric photosystem II core complexes of the red alga Cyanidioschyzon merolae it was demonstrated that both substrate water molecules are already bound in the S₂ state of the Mn₄CaO₅ cluster. This was confirmed with samples from the thermophilic cyanobacterium Thermosynechococcus elongatus. Addition of the water analogue ammonia, that is shown to bind to the Mn₄CaO₅ cluster by replacing the crystallographic water W₁, did not significantly affect the exchange rates of the two substrate waters. Thus, these experiments exclude that W₁ is a substrate water molecule.

The mechanism of O-O bond formation was studied by characterizing the substrate exchange in the S₃Y₂• state. For this the half-life time of this transient state into S₀ was extended from 1.1 ms to 45 ms by replacing the native cofactors Ca²⁺ and Cl⁻ by Sr²⁺ and I⁻. The data show that both substrate waters exchange significantly slower in the S₃Y₂• state than in the S₃ state. A detailed discussion of this finding lead to the conclusions that (i) the calcium ion in the Mn₄CaO₅ cluster is not a substrate binding site and (ii) O-O bond formation occurs via the direct coupling between two Mn-bound water-derived oxygens, which were assigned to be the terminal water/hydroxy ligand W₂ and the central oxo-bridging O₅.

The driving force for the O₂ producing S₄→S₀ transition was studied by comparing the effects of N₂ and O₂ pressures of about 20 bar on the flash-
induced O₂ production of photosystem II samples containing either the native cofactors Ca²⁺ and Cl⁻ or the surrogates Sr²⁺ and Br⁻. While for the Ca/Cl-PSII samples no product inhibition was observed, a kinetic limitation of O₂ production was found for the Sr/Br-PSII samples under O₂ pressure. This was tentatively assigned to a significant slowdown of the O₂ release in the Sr/Br-PSII samples. In addition, the equilibrium between the S₀ state and the early intermediates of the S₄ state family was studied under ¹⁸O₂ atmosphere in photosystem II centers devoid of tyrosine YD. Water-exchange in the transiently formed early S₄ states would have led to ¹⁶,¹⁸O₂ release, but none was observed during a three day incubation time. Both experiments thus indicate that the S₄→S₀ transition has a large driving force. Thus, photosynthesis is not limited by the O₂ partial pressure in the atmosphere.
Abbreviations

α Miss parameter
β Double hit parameter
ADP Adenosine diphosphate
ATP Adenosine triphosphate
DFT Density functional theory
EDNMR ELDOR-detected nuclear magnetic resonance
EPR Electron paramagnetic resonance
ESEEM Electron spin echo envelope modulation
EXAFS Extended X-ray absorption fine structure
FIOPs Flash-induced oxygen evolution patterns
Fd Ferredoxin
FNR Ferredoxin – NADP+ reductase
FTIR Fourier transform infrared spectroscopy
MIMS Membrane-inlet mass spectrometry
NADPH Nicotinamide adenine dinucleotide phosphate
OEC Oxygen evolving complex
OWIE Oxygen water isotope exchange
PSI Photosystem I
PSII Photosystem II
PSIIcc Photosystem II core complex
TIC Translocon at the inner envelope membrane of chloroplasts
TOC Translocon at the outer envelope membrane of chloroplasts
UV-Vis Ultraviolet – visible spectroscopy
Wf Fast exchanging substrate water in the Mn$_4$CaO$_5$ cluster
Ws Slowly exchanging substrate water in the Mn$_4$CaO$_5$ cluster
XAS X-ray absorption spectroscopy
$Y_D$ Tyrosine 160 on the D2 protein
$Y_Z$ Tyrosine 161 on the D1 protein
Introduction

The origin of Earth

Planet Earth was formed over 4.5 billion years ago from a disk of gas orbiting the sun [1, 2]. Biological life on the young earth was impossible due to high surface temperature (~1000°C) and the heavy bombardment with metiorites. A giant impact occurred when the earth was only 30 million years old [3, 4]. The force was enough to eject part of Earth’s material into orbit around the earth, and this material eventually coalesced to form the moon [5, 6]. At this point, the surface of Earth was probably covered with magma [7]. It took 100-200 million years for the earth to become cool enough to retain water [8, 9] and another 200-400 million years before ocean waves were hitting the first shore lines [10]. It was likely during this period (4.4-4.0 billion years ago) that crucial events towards the origin of life occurred.

The origin of life and oxygenic photosynthesis

The earliest evidence for biological life on Earth are 3.8 billion years old carbon traces found in Greenland rocks [11]. These traces were assigned to early organisms, which might have been phototrophs, utilizing the energy from sunlight and electrons from inorganic molecules such as H₂, H₂S or 2Fe(OH)₂ to fix CO₂ [12]. The first phototrophs that emerged were anoxygenic as no oxygen was released in the energy conversion reactions [13]. However, the evolution of oxygenic photosynthesis that followed introduced a reaction mechanism for water oxidation to obtain reductive electrons and protons [14]. The byproduct of this reaction was molecular oxygen and the gradual accumulation of this molecule in the atmosphere was likely poisonous to most of the early organisms [15]. The high oxygen content in the atmosphere of today (~21%) was not present 3.8 billion years ago. The atmosphere was then rather highly reducing and contained CH₄ and NH₃, or weakly reducing by containing CO₂, N₂ and H₂ [16]. The first clear sign for oxygenic photosynthesis are geochemical chromium data of South African rocks, suggesting that appreciable levels of atmospheric oxygen were present 3.0 billion years ago [17]. However, 3.5 billion year old microfossils indicate that already by that time organisms similar to modern cyanobacteria existed that had two coupled photosystems, which is typical for oxygenic photosynthesis [18].

Photosystem I and II absorb light energy and convert it into an electrochemical potential that is used to fix CO₂ and build carbohydrates. However, only photosystem II possess a catalyst for water oxidation. Its chair-like structure is made up by four manganese atoms and one calcium
atom that are connected via five \( \mu \)-oxo bridges [19]. The remarkable chemistry of this Mn\(_4\)CaO\(_5\) cluster links the one-electron photochemistry of the reaction center to the four-electron oxidation of two water molecules [20]. The Mn\(_4\)CaO\(_5\) cluster did not exist in the anoxygenic predecessor that only carried one photosystem. One intriguing suggestion how manganese atoms became constituents of photosystem II is related to a hypothesis that a gene duplication event gave rise to two ancestral photosystems within the same genome [21]. If these photosystems were expressed in absence of their natural electron donor, aqueous Mn cations that were exposed to ultraviolet radiation could have given off electrons to them, which reduce the oxidative stress and was thus an evolutionary advantage. Finally, the risk of aggregating surplus electrons in the photosystem II like reaction center was bypassed by redirecting the electrons to the ancestral photosystem I [21]. This brief outline describes a plausible sequence of events that may have led to the linear flow of electrons from photosystem II to photosystem I upon flash induced water oxidation.

**Journey into a leaf**

We are now going to make a journey into a leaf to explore in more detail where different components of the photosynthetic machinery, that convert sunlight into chemical (or free) energy, are located (Figure 1). The inner tissue of a plant is called mesophyll and it is made up of cells specialized in photosynthesis. These cells range from 20 to 40 \( \mu \)m in diameter and 100 to 200 \( \mu \)m in length [22], which is enough to embrace the various plasids of the cell. Plastids are units that have specific functions in the cell and they are shaped as compartments by their enclosing membrane. Their specific functions are executed by different proteins that reside both in the membrane and the inner compartments of the plastids. We will now zoom in further and investigate a plastid known as the chloroplast (Figure 1). The amount of chloroplasts in a cell varies among species, but the plant *Arabidopsis thaliana* that contains on average \(~80\) chloroplasts per cell may serve as an example here [23]. The chloroplasts range from 5 to 10 \( \mu \)m in diameter [24] and their complex structure is shaped by an intricate system of three different membrane enclosures: the outer, the inner and the thylakoids membrane. More than 95\% of chloroplast’s proteins, i.e. nearly 3000 different proteins, are synthesized in the cellular cytosol and thus have to be imported into the chloroplast through a double membrane (outer and inner), which
**Figure 1.** Overview of the main photosynthetic components inside a plant leaf. Image of protein complexes and Mn₆CaO₅ cluster were prepared from PDB.
is a consequence of the endosymbiotic origin of the chloroplast. This protein import occurs via two import machineries in the outer and inner membrane, the Tic and Toc translocons [25]. This import pathway is also responsible for translocating the most abundant protein in nature into the chloroplast, Rubisco [26]. This enzyme accumulates at the stromal side of the inner membrane and catalyzes the conversion of carbon dioxide into carbohydrates in a reaction cycle known as the Calvin-Benson cycle [27]. The overall process of carbon dioxide fixation in the stroma can be summarized by a simple chemical equation:

**Equation 1:**

\[
6 \text{CO}_2 + 12 \text{NADPH}_2 + 18 \text{ATP} \xrightarrow{\text{enzymes}} \text{C}_6\text{H}_{12}\text{O}_6 + 12 \text{NADP} + 18 \text{ADP} + 18 \text{Pi} + 6 \text{H}_2\text{O}
\]

The annual net production of fixed carbon from carbon dioxide via photosynthesis is \(\sim 112000\) billion kilogram \((112 \times 10^{12}\) kg\) [28] and this production is energy demanding. It can be seen from equation 1 that for every carbon dioxide that is fixed to a growing chain of carbohydrates two NADPH molecules are oxidized and three ATP molecules are hydrolyzed. NADPH provides the reducing power to the reaction by giving off two electrons whereas ATP provides energy for the work to be done by giving off a phosphate \((\text{Pi})\). This phosphate binds to a three carbon intermediate, making it more reactive and ready to accept electrons from NADPH. The reactions in the Calvin cycle are not directly dependent on the presence of light and are therefore described as the dark reactions of photosynthesis. The NADP and ADP molecules formed during the dark reactions need to be regenerated into NADPH and ATP by a series of reactions that are known as the light reactions (Figure 2).

**Figure 2.** Schematic view of the light and dark reactions that occur inside the chloroplasts of photosystem II
We are now going to continue to explore the chloroplast and take a look at the protein complexes that carry out the light reactions by zooming into the very special shape of thylakoid membranes (Figure 1). The thylakoid membrane encloses the lumen. In higher plants the thylakoid membrane folds to disk shaped, stacked bodies known as grana which are connected with each other via the unstacked stroma lamellae. The first stage of the light reactions takes place in photosystem II that is localized in the grana. The cytochrome b$_{6f}$ complex is distributed in both grana and stroma lamellae, whereas photosystem I and the ATP synthase are present in the edges of the grana and in the stroma lamellae [29]. The cooperation between photosystem II and photosystem I generates an electron current from H$_2$O to NADP, producing reducing power in the form of NADPH, and the byproduct O$_2$.

It is photosystem II that contains the catalytic center for water oxidation, which is also known as the oxygen evolving center (OEC). The height of photosystem II is ~ $115 \text{ ångström}$ ($115 \times 10^{-10} \text{ meter}$) and in order to be able to observe the OEC, we have to further zoom in until we reach the atomic scale (Figure 1). The OEC harbours the chair shaped Mn$_4$CaO$_5$ cluster, which measures $5.4 \text{ ångström}$ across ($5.4 \times 10^{-10} \text{ meter}$) [19]. Oxygenic autotrophs such as cyanobacteria, algae and plants all contain the Mn$_4$CaO$_5$ cluster and the annual net production of O$_2$ has been estimated to be $\sim 2.7 \times 10^{14} \text{ kilogram}$ [30]. In the following section oxygen evolution and light reactions are described by following the path of an electron as it is transferred from one cofactor to another, starting with water and photosystem II, and ending with FNR and NADPH.

**Light absorption and charge separation**

The first step in photosynthesis is the absorption of light. About 81000 Tera watt ($1 \text{TW} = 10^{12} \text{W}$) of the incoming solar radiation is absorbed every year by the hydrosphere and continental surfaces [31]. Less than 0.1 % of this light energy is converted via photosynthesis to chemical energy by primary producers [32]. Despite the low conversion percentage, the annual primary production of fixed carbon exceeds 100000 billion kilograms ($100 \times 10^{12} \text{ kg}$) [28, 33], which constitutes the raw material for synthesis of organic molecules such as carbohydrates and aminoacids. This energy conversion process is performed by plants, algae and photosynthetic bacteria by absorbing the photons from sunlight.

The photons from the sun are absorbed by pigment molecules that are assembled in light harvesting systems inside photosystem I and II, and in special antenna complexes associated with the photosystems (e.g. LHC I, LHC II or phycobilisoms). The main pigments in higher plants are chlorophyll $a$ and $b$, and the light harvesting system connected with each
photosystem contains normally around two-to-three hundred pigments of this type. The absorption of one photon leads to the excitation of one electron into an excited state of the chlorophyll molecule. This excitation energy is transferred through the whole antenna to a reaction center that traps the energy by charge separation. In photosystem II the reaction center contains four chlorophyll \(a\) molecules \(P_{D1}, P_{D2} \text{ Chl}_{D1} \text{ and Chl}_{D2},\) which are collectively known as \(P680,\) and a pheophytin molecule. The \(P\) denotes pigment and \(680\) is the maximum of the low-energy absorption peak expressed in nanometers. The arrival of excitation energy from one photon at \(P680\) expels one electron from \(P680\) to the nearby pheophytin molecule. The radical cation \(P680^{**}\) formed has a potential of around \(+1.25\text{V}\) and it is the strongest oxidant known for a biological system\([34, 35]\). We will see in a coming section that this voltage is insufficient to extract electrons from a water molecule directly, but that it is high enough to drive water oxidation via tyrosine \(Z\) and the \(\text{Mn}_4\text{CaO}_5\) cluster.

Photosystem I has a light harvesting system that is similar to that of photosystem II, but the reaction center and the redox cofactor differ. The reaction center in photosystem I is known as \(P700\) due to its further red shifted absorption maximum. It is formed by a pair of chlorophylls \((\_C-A1/\_C-B1)\) that act as the primary electron donor to a nearby chlorophyll pair known as \(A_0\) \((\_C-A/\_B-3)\). The \(P700/ P700^{**}\) midpoint potential has a value of about \(+500\text{mV}\)\([32, 36]\).

The absorption of light by photosystem II and photosystem I drives a linear flow of electrons from \(\text{H}_2\text{O}\) to \(\text{NADP}\) that involves a number of other electron transfer cofactors and complexes. Having two light driven photosystems that are coupled was proposed in the 1960s\([37]\). The authors depicted this in a redox potential diagram that later became known as the \(Z\)-scheme of photosynthesis due to its likeness with the letter \(Z\) (if rotated by \(90^\circ\)). The \(Z\)-scheme (Figure 3) marks the different reduction potentials of the molecules that transport the excited electron from water to \(\text{NADP}\). With the exception of the two charge separations in the reaction centers, these electron transfer events are always connected with the loss of potential energy. While wasteful, this arrangement leads to the exceptional high quantum yield of photosynthesis, since the loss of potential energy difference and the increase in spatial distance connected with the linear electron flow reduces the likelihood of potentially harmful charge separations even under low light conditions\([38]\). As such the excited electron from \(P680^*\) moves from a negative to positive redox potential until it reaches \(P700^{**}\) where it gets excited a second time. Part of the potential loss between the two photosystems is recovered by coupling these electron transfer steps with the ‘pumping’ of protons from the stroma into the lumen.
electron transfer reactions in photosystem II and photosystem I will be described in the following section.

**Figure 3.** The electron flow from H₂O to NADP in photosystem II. The main components in this transport chain are: the oxygen evolving complex (OEC or Mn₄CaO₅ cluster), tyrosine Yₜ (Y₂), a tetrad of chlorophyll molecules (P₆₈₀), pheophytine (Pheo), plastoquinone (Qₐ) (Q₈), cytochrome b₆f (Cytb₆f), plastocyanin (Pc), a pair of chlorophyll molecules (P₇₀₀), a pair of chlorophyll molecules (A₀), a pair of phylloquinones (A₁), an iron-sulfur complex (4Fe-4S), ferredoxin (Fd), ferredoxin-NADP reductase (Fd-NADP reductase).

**Electron transfer by redox active cofactors**

Six different protein complexes participate in the electron transport chain: Photosystem II, Cytochrome b₆f, Plastocyanin, Photosystem I, Ferredoxin, Ferredoxin-NADP reductase and ATP synthase (Figure 4). The light reactions of the electron transport chain produce reducing power (NADPH) and a proton motive force to be used for the synthesis of ATP. Oxygen is released as a byproduct in the electron abstraction reaction that takes place in photosystem II.
Figure 4. Linear electron transport chain through the main photosynthetic components. The figures of the protein complexes were prepared from PDB PSII (3ARC), Cyt b6f (1VF5), Pc (2GIM), PSI (1JBO), Fd (1OFF), Fd-NADP+ reductase (2B5O), ATPsynth (1e79/1yce). In addition, cyclic electron flow events occur, which are not shown for the clarity of presentation.

Absorbtion of one photon by the chlorophylls (Chl) in the light harvesting complex of photosystem II leads via excitation energy transfer to the reaction center to the excitation of an of P680. The primary charge separation is assumed to occur between the ChlD1 of P680 and a nearby pheophytine (PheoD1). The transfer of one electron from ChlD1 to PheoD1 creates the radical cation P680●+ in which the subsequently formed P D1●+ is the strong oxidant that is required to oxidize water. The oxidizing power of P D1 is around +1.25 V [34, 35]. To avoid charge recombination between P680●+ and Pheo●- the electron is rapidly transferred (200-500 ps) [39-41] from Pheo●- to a tightly bound plastoquinone (QA) that is located on the stromal side of the thylakoid membrane. Due to its special protein environment it can only act as one-electron acceptor. The electron is thereafter transferred to the mobile secondary plastoquinone (QB). After the arrival of a second electron from the next charge separation in the reaction center, it takes up two protons from the stroma. The thus formed plastoquinol molecule (QH2) leaves its binding pocket at the acceptor side of photosystem II and the empty QB binding pocket is quickly occupied with a new plastquinone molecule. QH2 diffuses into the thylakoid membrane where it isoxidized by the Cytb6f complex. The released electrons flow one at a time through a Fe-S protein that reduces platocyanin, a soluble electron carrying protein that transfer the electron to photosystem I. The two protons that were taken up from the stroma are released into the thylakoid lumen together with two additional protons from the stroma that are pumped by the electron transfer
reactions occurring at the Cytb6f complex. Protons from the water oxidation reaction also contribute to creating a proton concentration difference across the thylakoids membrane. This results in a proton motive force that drives the ATP synthesis by sending protons through the ATP synthase back into stroma. The electron that was delivered by plastocyanin to photosystem I is excited a second time by P700. The electron is then transferred to a pair of chlorophylls (A0) and further to a pair of phylloquinones (A1) that reduce a 4Fe-4S cluster. A soluble protein called ferredoxin transports the electron from photosystem I to the ferredoxin-NADP reductase that catalyzes the formation of NADPH.

After this general description of photosynthesis and the photosystems, we will now focus on the main topic of this thesis, the chemistry of water oxidation in the oxygen evolving complex, and the components that are directly involved in this process.

**Kok’s S-state model of photosynthetic water oxidation**

A new paradigm in photosynthesis research was established in the early 1970’s when Kok and coworkers presented a model [42] explaining the observation of Joliot that well dark-adapted photosystem II (PSII) containing samples release molecular oxygen with a periodicity of four [43] upon illumination with short (µs), saturating light flashes (Figure 5). Joliot and coworkers also found that the first maximum of oxygen release occurs after the third flash, and that this period-four oscillation gets damped over time. Kok realized that the period-four oscillation and the good synchronization after dark-adaptation provide direct evidence for the existence of one stable and three meta-stable oxidation states of the oxygen-evolving complex. In addition, at least one transient state must be postulated. These states are referred to as S states, where the index (i = 0, 1, 2, 3, 4) gives the number of oxidizing equivalents stored in the OEC. Since (i) four oxidizing equivalents are required to oxidize two water molecules to O2, (ii) the first maximum of water oxidation occurs already after three flashes and (iii) each flash removes one electron at a time from the OEC via charge separation in the reaction center, the dark-stable state is the S1 state. It also follows that the other states are meta-stable (S0, S2, S3) and decay to S1 during extended dark-periods. O2 is liberated in this light-driven reaction cycle within 1 ms after the transient S4 state is reached. In this light-independent reaction sequence, a complexed peroxide is a postulated intermediate. After the release of O2 the OEC attains its lowest oxidation state, S0. From here it takes another four flashes to the next burst of O2 release.
The damping of the oscillation was explained to be the result of an increased S state desynchronisation among PSII centers in the sample [44]. A miss parameter (\(\alpha\)) and double hit parameter (\(\beta\)) were introduced and represent a lack of S state transition and a double advancement, respectively. It is now known that most S state transitions also involve the release of a proton, and that water-binding likely occurs during the \(S_4 \rightarrow S_0\) and \(S_2 \rightarrow S_3\) transitions (for review see: Cox Messinger 2013 [45]). Below we look in detail on the structures that allow this reaction cycle to take place in the OEC.

![Figure 5](image)

**Figure 5.** The left panel shows a flash induced \(O_2\) release pattern (FIOP) measured with a membrane-inlet mass spectrometer. The right panel depicts a modern version of the Kok model that also indicates in which transitions substrate water molecules bind to the Mn\(_4\)CaO\(_5\) cluster, and in which transitions protons are released to avoid a charge build up on the cluster.

**Proteins and oxygen evolution**

In higher plants and cyanobacteria at least seven intrinsic proteins are needed for oxygenic photosynthesis. These proteins are D1, D2, CP47, Cp 43, the \(\alpha\) and \(\beta\) subunits of cytochrome \(b_{559}\) and the 4.8 kDa \(psbl\) gene product. In addition to these proteins there are extrinsic proteins required to maximize oxygen evolution rates. Notable extrinsic proteins in higher plants are: PsbO, PsbP and PsbQ. In cyanobacteria are CyanoQ, CyanoP, PsbU and PsbV are present. The function of the PsbO protein may be to regulate calcium and chloride demand in photosynthesis. PsbO is also known as the manganese stabilizing protein since it prevents loss of manganese atoms from the Mn\(_4\)CaO\(_5\) cluster. PsbP and PsbQ are also regulating calcium och chloride demands for photosynthesis. For more information see recent reviews [46, 47]
The oxygen evolving complex

The ‘heart’ of the oxygen evolving complex (OEC) is the Mn$_4$CaO$_5$ cluster, but this functional unit also includes the protein environment and structural water molecules surrounding the Mn$_4$CaO$_5$ cluster since they are required for the intricate sequence of electron and proton transfer events that allow photosystem II to perform water oxidation at low overpotential. The ten atoms of the Mn$_4$CaO$_5$ cluster are arranged in a chairlike structure (Figure 6) [19]. The five oxygen atoms link the calcium and the four manganese atoms via µ-oxo bridges. The average bond length between Mn atoms and a bridging oxygen is ~ 2.1 ångströms in the crystal structure, while it is about 1.9 Å in extended X-ray absorption fine structure (EXAFS) measurements. This difference indicates that the Mn$_4$CaO$_5$ cluster gets reduced during the collection of the X-ray diffraction data. The bond length referred to here was determined in the dark-stable S$_1$ state. It is noted that the bond length and geometry of the Mn$_4$CaO$_5$ cluster changes when electrons are removed from the cluster during the reaction cycle [48, 49].

Manganese atoms have a wide range of available oxidation states and three of them are observed in natural systems such as the Mn$_4$CaO$_5$ cluster: Mn$^{II}$, Mn$^{III}$ and Mn$^{IV}$ [50]. The many oxidation states are a necessity, not only for the light induced water oxidation by the Mn$_4$CaO$_5$ cluster, but also during photoactivation when the Mn$_4$CaO$_5$ cluster is assembled [51]. This later process incorporates Mn$^{II}$ in an intermediate structure that is stabilized via light induced charge separations in the reaction center that in turn oxidizes Mn$^{II}$ to Mn$^{III}$ [52]. At high light intensities (2000 µmol photons m$^{-2}$ S$^{-1}$) the D1 subunit is replaced by a functional copy every 20 minutes [53] and these replacements require that the Mn$_4$CaO$_5$ cluster is reassembled. Thus, a functional Mn$_4$CaO$_5$ cluster contains manganese ions that in principle have oxidation numbers higher than +2. Pulsed electron nuclear double resonance (55Mn-ENDOR) have shown that the lowest oxidation state of the Mn$_4$CaO$_5$ cluster (S$_0$ state) is devoid of Mn$^{II}$ ions [54]. The proposed oxidation states in the S$_0$ state are (Mn$^{III}$)$_3$Mn$^{IV}$.

The calcium ion in the Mn$_4$CaO$_5$ cluster is also essential for its function. It is known that the catalytic cycle of calcium-deprived manganese clusters is blocked beyond the the S$_2$YZ$^\cdot$ state, which means that no oxygen is released [55]. No significant change to the Mn$_4$CaO$_5$ structure appears to take place after Ca/Sr substitution or Ca$^{2+}$ depletion [56]. However, electrochemical measurements in structural analogues to the the Mn$_4$CaO$_5$ cluster showed that one of the functions of Ca$^{2+}$ might be to modulating the redox potentials of manganese centers via µ-oxido ligands [57, 58].
Important questions for understanding the mechanism of water-oxidation are where and how the substrate water molecules bind to the Mn$_4$CaO$_5$ cluster during the reaction cycle. This will be addressed in a separate section below. Here we just note four terminal water ligands, W$_1$, W$_2$, W$_3$ and W$_4$ that are bound to Mn$_4$ and calcium.

![Figure 6](image_url)

**Figure 6.** The Mn$_4$CaO$_5$ cluster (oxygen evolving complex - OEC) with its surrounding amino acids are shown in the left panel. The right panel shows its geometry of the structure in more detail. Four manganese ions denoted Mn$_1$, Mn$_2$, Mn$_3$ and Mn$_4$ (purple) and one calcium denoted Ca (green) are connected by five oxygen atoms (red). Four water molecules denoted W$_1$, W$_2$, W$_3$ and W$_4$ (blue) bind to the Mn$_4$CaO$_5$ cluster. Mn$_4$ ligates W$_1$ and W$_2$ whereas calcium ligates W$_3$ and W$_4$. The distal chloride atoms denoted Cl$^-$ (yellow) is also shown. The image was prepared from PDB (3ARC).

The Mn$_4$CaO$_5$ cluster is ligated to the D1 and CP43 subunit of photosystem II by one histidine and six carboxylate ligands (D1-His-332, D1-Glu-189, D1-Asp-170, D1-Glu 333, D1-Asp-342, D1-Ala 344 and CP43-Glu354) (Figure 6) [19]. The geometry of the Mn$_4$CaO$_5$ cluster is likely additionally maintained by second sphere amino acids (CP43-Arg 357, D1-Asp61, D1-His 337, CP43-Arg 357 and D1-Val 185) that also interact with water molecules from the vast pool present in PSII. Mutational studies of second sphere amino acids and their influence to the catalytic cycle of the Mn$_4$CaO$_5$ cluster indicate that they may affect the hydrogen bonding network or even the protonation state of the substrate waters [59, 60]. Thus, the oxidation of two substrate water molecules is not confined to the Mn$_4$CaO$_5$ cluster, but is also affected by surrounding components of PSII.
Tyrosine Z and D

PSII contains two redox active tyrosines denoted Y_Z (D1-Tyr 160) and Y_D (D2-Tyr 161). Tyrosine Y_Z mediates the electron transfer from the Mn_4CaO_5 cluster to P680^++. The first step in this electron transfer is the oxidation of Y_Z by the highly reactive cation radical P680^++. This electron transfer creates a neutral tyrosine radical (Y_Z^•) [61] due to a coupled proton transfer from the tyrosine to a nearby histidine (His-190) [62-64]. According to the proton-rocking mechanism this proton returns to Y_Z upon its reduction by the Mn_4CaO_5 cluster [65]. Tyrosine Z is located ~ 7 ångströms away from the Mn_4CaO_5 cluster [19, 66] and the spatial separation between P680 and the Mn_4CaO_5 cluster together with the rapid reduction of P680 by Y_Z have at least two consequences for the quantum yield of water oxidation. First, the rapid reduction of P680^++ stabilizes the charge separation by preventing charge recombination between P680^++ and QA^- [67]. Secondly, the spatial separation avoids reversed electron transfer from P680 to the Mn_4CaO_5 cluster [68]. The rate of electron transfer from tyrosine Y_Z to P680^++ depends on the redox state of the Mn_4CaO_5 cluster (S state dependence) [69, 70]. Two different rates that lead to reduction of P680^++ by Y_Z occur in the nano second time scale: half times of 20 – 50 ns (S_0 → S_1) and half times of 300 – 600 ns (S_1 → S_2) [71, 72]. The reduction of the tyrosine radical Y_Z^• by Mn_4CaO_5 cluster is also S state dependent: ~ 30 µs (S_0 → S_1), ~ 70 µs (S_1 → S_2), ~ 190 µs (S_2 → S_3) and ~ 1.5 ms (S_0 → S_1) [73, 74].

The other tyrosine (Y_D) that is present in photosystem II is not mediating linear electron transport from the Mn_4CaO_5 cluster to P680^++. In fact, a mutational study has shown that PSII is still producing O_2 after replacement of tyrosine Y_D by phenylalanine [75]. However, an EPR study [76] revealed that tyrosine Y_D in its reduced form is able to reduce the S2 and S3 states of the Mn_4CaO_5 cluster, and that Y_D in its oxidized form (Y_D^ox) can oxidize the Mn_4CaO_5 clusters from the S_0 to the S_1 state. Y_D thus contributes to the synchronization of the OEC in the S_1 state during dark-adaptation. Tyrosine Y_D may also participate in the photoactivation process when Mn^{II} ions are oxidized and assembled to a functional Mn_4CaO_5 cluster [77]. Further support for this notion comes from a study showing that Y_D^ox can oxidize overreduced states of the Mn_4CaO_5 cluster [78].

Effect of cofactor replacement

Calcium and chloride are cofactors for water-oxidation, i.e. they have both to be present in the OEC for allowing water oxidation to proceed. Up to four calcium and three chloride ions have been localized within each PSII monomer, however only one Ca^{2+} and one or two Cl^- are essential for water oxidation.
The critical Ca$^{2+}$ is part of the Mn$_4$CaO$_5$ cluster (see below), and the two important chlorides are positioned $\sim$ 6.7 and $\sim$ 7.4 ångströms away from the Mn$_4$CaO$_5$ cluster (Figure 6) [19, 79]. It has been proposed that the two chloride ions stabilize some amino acids in a position required for activity [80, 81]. Also, a number of studies suggest that the two chloride ions are needed to maintain the structure of proton exit channels [82]. Depletion of chloride from PSII inhibits $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions [83].

Calcium is a constituent of the Mn$_4$CaO$_5$ cluster. As such it is ligated for example to oxygen 5, which, in different studies, has been ascribed to be one of the two substrate water molecules [20]. Depletion of calcium also inhibits $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions [55, 84], and it was recently shown that calcium depletion induces rearrangements of an H-bond network that extends to one of the chloride binding sites [85].

Calcium and chloride ions can be functionally replaced by surrogate ions. While Cl$^-$ can be replaced by Br$^-$, I$^-$ and some other anions, only Sr$^{2+}$ can functionally replace Ca$^{2+}$. Biosynthetic exchange of strontium for calcium and bromide for chloride in PSII of *Thermosynechococcus elongatus* resulted in fully functional Mn$_4$CaO$_5$ clusters, which were however somewhat kinetically retarded in the S state transitions [86]. For example, Sr/Br samples exhibited a $\sim$ 7 times slower $S_3Y_Z^\bullet \rightarrow S_0Y_Z$ transition compared to the native Ca/Cl sample. An even stonger effect was demonstrated for Sr/I PSII samples [87]. By this exchange, the halftime of the $S_3Y_Z^\bullet \rightarrow S_0Y_Z$ transition is slowed down from $\sim$1.1 ms (Ca/Cl) to $\sim$ 45 ms (Sr/I). In this thesis two experiments are presented that employ this slowed kinetic of the O$_2$ formation to study properties of transient intermediates that occur between $S_3Y_Z^\bullet$ and $S_0Y_Z$ (Paper III).

**Substrate water binding**

The only direct way to study how the Mn$_4$CaO$_5$ cluster interacts with the two substrates waters is to exchange them against isotopically labeled water molecules (H$_2$,$^{18}$O) and measure the amount of isotopically labeled oxygen that is produced by photosynthesis. This method has been used by researchers in the photosynthetic field for over seven decades. Isotopically labeled water (H$_2$,$^{18}$O) was used already in 1941 to show that oxygen from photosynthesis derives from water molecules [88, 89] rather than carbon dioxide, which had been the prevailing idea (although due to the rapid equilibration between oxygen atoms from water and hydrogen carbonate catalyzed by carbonic anhydrases this conclusion may be less unique than thought then). In 1963 a mass spectrometer inlet system was devised which
allowed the mass spectrometer to detect gases dissolved in a liquid phase [90]. A teflon membrane was used to separate the liquid sample phase from the high vacuum of the mass spectrometer. This type of inlet system was used for the first time in the 1980’s to study substrate water interactions with dark-adapted oxygen evolving centers (S₁ state) by mixing chloroplasts with labeled water [89]. However, the time resolution of the experimental set-up [91] was limited to ~ 30 seconds due to slow mixing of sample and labeled water. Thus, when the first flashes were given to induce oxygen production all substrate was already completely exchanged. Therefore, the authors concluded that the oxygen evolving complex does not contain any non-exchangeable substrate water in the S₃ state [92], and that water oxidation does occur in a concerted four-electron step rather than via stable water-oxidation intermediates. A similar conclusion was reached in a subsequent study by Bader et al [93]. The major breakthrough came in 1995 when one exchanging substrate water was observed in the S₃ state [94]. This breakthrough was a result of several improvements made to the experimental set-up. First of all was the rate of labeled water injection to the sample increased, which reduced the mixing by a factor of 1000 (from ~ 30 seconds to ~ 30 milliseconds). Important were also the development of a closed system and the removal of background oxygen from the H₂¹⁸O by using enzymes, which greatly improved the signal to noise ratio. These improvements made the membrane-inlet mass spectrometer usable for studying substrate water exchange. Several follow-up studies, which used the same basic set-up that was developed in 1995 [94], have given invaluable information about substrate water interactions with the oxygen evolving complex. Substrate water exchange has been measured in the S₀, S₁, S₂, and S₃ states [95-100] and the method has recently been reviewed [20, 101]. The main findings from these substrate water exchange measurements are: 1) in the S₃ state both substrate waters are bound in chemically different environments; 2) In the S₂ state also both substrate waters are bound, the exchange in the S₂ state is only slightly faster than in the S₃ state (Paper I); 3) In the S₀ and S₁ state at least one substrate water is bound; 4) the slowly exchanging substrate water is likely bound in a bridging position between Ca and Mn; 5) the fast exchanging substrate water is likely a terminal Mn ligand. However, the water-exchange in the final S state transition (S₃Yₐ*- → S₀ transition) just before O-O bond formation was until now unresolved because of its short half time (~ 1.1 ms) [86]. One of the papers in this thesis demonstrates, for the first time, substrate water exchange measurements in the S₃Yₐ*- state (Paper III). These measurements were made possible by improving the mixing time of labeled waters and sample in the sample chamber to 8 ms (Paper III), and by using photosystem II samples with exchanged cofactors that extended the half time for the S₃Yₐ*- → S₀ transition to ~ 45 ms [87].
Materials and Methods

An isotope ratio mass spectrometer was used in the substrate water exchange experiments to detect gases dissolved in a liquid sample. The types of gases studied were either based on a single element (argon) or a compound of elements such as molecular oxygen (O₂). These elements naturally occur in different variants known as isotopes. Isotopes are atoms of the same element carrying different numbers of neutrons in the atomic nucleus. The mass number (A) of an element (E) states the sum of protons and neutrons of the element (4E), for example stable isotopes of oxygen exist with mass numbers 16 (16O; 99.76%), 17 (17O; 0.04%) and 18 (18O; 0.21%). Two different oxygen isotopes in three different combinations (isotopolouges) were analysed in the substrate water exchange experiments: (16O16O, m/z=32) (18O16O, m/z=34) (18O18O, m/z=36). The abbreviation m/z denotes a dimensionless quantity that is obtained by dividing the mass number (m) of an ion with its charge number (z). The m/z notation is utilized in mass spectrometry to discriminate and detect ions. Thus, for example, an ion with mass number 50 that carries 2 charges will be observed at m/z = 25.

The four main components in the mass spectrometer used are: 1) the sample chamber with membrane inlet system; 2) the ion source (ionization); 3) the mass analyser (separation); and 4) the collectors (detection) (Figure 7). The gas-inlet of the mass spectrometer is described in detail further below. In short, the gas is pulled from the sample chamber into the mass spectrometer through a semipermeable membrane by the high vacuum in the mass spectrometer (3 x 10⁻⁸ mbars). Separation and detection of the gas molecules requires that each atom or molecule carries a positive charge (z). This is achieved in the ion source by a beam of electrons that collide with the incoming gas. The ions are thereafter accelerated by an electric field into the mass analyser and separated by a magnetic field into trajectories according to their mass to charge ratio (m/z). A Faraday collectors array is used to detect ions simultaneously at seven different mass to charge ratios: (m/z=32, 16O16O) (m/z=34, 18O16O) (m/z=36 18O18O; 36Ar) (m/z=40, 40Ar) (m/z=44, 12C16O16O) (m/z=46 12C18O16O) (m/z=48 12C18O18O). However, the main objective in this work was to study flash induced oxygen evolution.
The four main components in the mass spectrometer used are: 1) sample inlet (not shown, see below); 2) the ion source (ionization); 3) the mass analyser (separation); and 4) the collectors (detection).

The sample chamber is made of stainless steel with a quartz window mounted at one side (Figure 8). Gas from the liquid sample was led from the chamber and into the ion source by the high vacuum of the mass spectrometer. A cooling trap that either was filled with liquid nitrogen (-196°C) or dry ice with ethanol (-78°C) was used to protect the ion source from liquid contamination. The liquid sample phase of 165 µL was separated from the high inlet vacuum (3 × 10⁻⁸ mbars) by a gas permeable silicon membrane, ~ 25 µm thickness (Mempro MEM-213) with support from a porous Teflon disc, Ø 10 mm (Bel-Art Products).
Figure 8. Sample chamber (cross section) that was used in substrate water exchange measurements.

**Substrate water exchange**

Prior to loading the sample into sample chamber an aliquot (40µL) of $^{18}$O-labelled water (~98% enrich.) was prepared and loaded into a gas tight syringe (Hamilton CR-700-50) This was done in a glove box saturated with nitrogen to reduce the argon content in the water aliquot. Removal of dissolved oxygen in the water aliquot was achieved by adding a mix of glucose oxidase ($8 \times 10^{-6}$ g/mL), glucose ($5 \times 10^{-5}$ g/mL) and catalase ($3 \times 10^{-6}$ g/mL). The glucose oxidase reduced the oxygen content to a non-detectable concentration during a 20 minute time period. Loading of the sample into the sample chamber was made, in the dark, through an inlet at the sample chamber top.
The syringe containing the $^{18}$O-labelled water was mounted to a stand over the chamber inlet and carefully lowered until the syringe needle was immersed in the pre-filled sample chamber. Dissolved gases in the sample entered the ion source by pervaporation through the semipermeable membrane. Prior to the substrate water exchange measurement was the sample degassed under continuous stirring for 20 minutes.

**Glucose oxidase reaction:**  
$\beta$-D-Glucose + O$_2$ + H$_2$O $\rightarrow$ glucono-6-lactone + H$_2$O$_2$

**Catalase reaction:**  
H$_2$O$_2$ $\rightarrow$ 2 H$_2$O + O$_2$

After these preparations, the substrate water exchange measurements can be outlined in four main steps: 1) presetting of the S state to be studied by flash illumination; 2) rapid injection of $^{18}$O-labelled water to a photosynthetic sample; 3) pause for exchange between $^{18}$O-labelled waters and bound $^{16}$O-substrates; 4) flash induce photosynthetic oxygen production; 5) analysis of the isotopic ratio of oxygen produced. It is important to note that the time between preflashe(s) and the detecting flash sequence was kept constant while the H$_2^{18}$O-exchange time was varied. This minimizes any possible effects of S state decay on the water-exchange data (Figure 9).

Substrate water exchange was probed in different S states (S$_1$, S$_2$, S$_3$ and S$_3$$^+$$Y_2^+$$^\ast$). S state transitions were induced by a series of xenon flashes (~ 6 µs FWHM). After presetting the S state the $^{18}$O-labeled water was injected into the sample, and the final turnover flash(es) that induced photosynthetic water oxidation and production of molecular oxygen were given in fast sequence to minimize further water exchange in the subsequent S states (Figure 9). The rapid injection of $^{18}$O-labelled water and the subsequent sample flashes were timed via a Labview program. The syringe injection was powered from by pressurized nitrogen gas (8 bars) via a fast switching valve (FESTO MHE2-MS1H 3/2G-M7-K) that pushed a pneumatic piston (Festo AEVC-12-10-A-P) connected to the syringe plunger.
Figure 9. Scheme of the flash and injection sequences that were used to measure substrate water exchange in the Mn$_4$CaO$_5$ cluster of photosystem II.
Several measurements in one particular S state were made by varying the time point for injection of $^{18}$O-labeled water, which gave $^{18}$O-labeled oxygen yields corresponding to different durations of $^{18}$O-labeled water exchange. These oxygen yields were plotted as a function of exchange time (Figure 10). Notably in the left panel of figure 10 is the biphasic pattern of the plotted oxygen yields that is typical at mass to charge ratio 34. The two phases of the exchange pattern represent two unique binding sites for the substrate water at the catalytic Mn$_4$CaO$_5$ cluster, one with low substrate affinity (fast exchange) and another with high substrate affinity (slow exchange). Two rate constants can be derived from the biphasic m/z=34 pattern by fitting the sum of two first order exponential functions (equation 2). Note that m/z=34 only represents O$_2$ with one exchanged substrate water (at fast or slow binding site). The right panel shows the monoexponential pattern at m/z = 36 when both the fast and the slow substrate water were exchanged. The exchange at m/z = 36 is fitted with a single exponential function (equation 3). Observe that the left and right panel in figure 10 show m/z = 34 and m/z = 36 results from the same measurements.

**Equation 2:**

$$^{34}Y = 0.66(1-e^{-34k_f \cdot t}) + 0.34(1-e^{-34k_s \cdot t})$$

**Equation 3:**

$$^{36}Y = (1-e^{-36k \cdot t})$$

**Figure 10.** The left and right graphs show several substrate exchange measurements made in the $S_3$ state (dots) that are fitted (black line) to determine the rate of substrate water exchange. Each dot represents the yield of molecular oxygen for either $^{16}$O$^{18}$O (left graph) and $^{16}$O$^{18}$O (right graph) that was determined for a certain exchange time with labeled water (H$_2^{18}$O). Measurements at m/z=34 give a characteristic biphasic exchange that corresponds to one exchanged substrate water molecule (either the fast or slow exchanging water). At m/z= 36 is the bisphasic exchange not visible as the slow exchanging water is rate limiting.
Determination of H$_2$O-injection rate

The rate of H$_2$O injection into the sample chamber and time for complete mixing with sample was determined in separate experiments by measuring fluorescence as a function of time after fluorescein injection (Figure 11a & 11b). Fluorescein solution (30 μM, 40 μL) was injected with a modified syringe (Hamilton CR-700-50) into the sample chamber (Figure 11b) that was pre-filled with a buffer solution (0.1 M Tris pH 8.0, 165 μL). The fluorescein was excited by a led lamp (Luxeon V-Star blue, 1W) via a band pass filter (Newport 470 nm – 495 nm) and a fiber optic tube. The resulting fluorescence (fluorescein - λex 490 nm; λem 525 nm) was emitted through a branch of the optical fiber and a second band pass filter (Newport 520 nm – 547 nm). A photodiode (Hamamatsu S-2281/C9329) converted the fluorescence emission into an electrical signal, which was measured with an oscilloscope. Figure 12 shows a typical fluorescence trace (8 ms mixing time, rate constant = 233 s$^{-1}$).

**Figure 11a.** Rate of injection into sample chamber and subsequent mixing time was determined from fluorescence measurements
Figure 11b.

Figure 12. The green trace shows the increasing fluorescence after injecting fluorescein into the sample chamber that was prefilled with buffer media. The rate of injection and subsequent time for mixing inside the cell was determined from the fluorescein trace.
Flash-induced oxygen oscillation pattern

Flash induced oxygen patterns (FIOPs) (Figure 5) were measured to estimate the percentage of catalytic Mn$_4$CaO$_5$ clusters that undergo a flash induced S state transition twice ($\beta$) and not at all ($\alpha$). This phenomenon is denoted double hit ($\beta$) and miss ($\alpha$). The distribution of $\alpha$ and $\beta$ was assumed to be equal for all S states. The data analysis program for FIOP oscillations were based on the following equations:

**Equation 4:** \[ Y_n = (1 - \alpha)[S_3]_{n-1} + \beta[S_2]_{n-1} \]

The oxygen yield by a given flash ($Y_n$) reflects the double hit transitions in $S_2$ and the sum of single and double transitions in $S_3$. The complete S state distribution, before ($S_{n-1}$) and after a flash ($S_n$), can be described in a matrix ($K$) that includes the probabilities for miss ($\alpha$), double hit ($\beta$), single hit ($\gamma = 1 - \alpha - \beta$) and zero transition.

**Equation 5:** \[ S_n = K \times S_{n-1} \]

\[
[S]_n = \begin{bmatrix}
S_0 \\
S_1 \\
S_2 \\
S_3
\end{bmatrix}
\]

and

\[
K = \begin{bmatrix}
\alpha & 0 & \beta & 1 - \alpha \\
1 - \alpha - \beta & \alpha & 0 & 0 \\
\beta & 1 - \alpha - \beta & \alpha & 0 \\
0 & \beta & 1 - \alpha - \beta & \alpha
\end{bmatrix}
\]

Oxygen evolution at elevated pressure

Oxygen evolution was also studied at elevated pressure. A MIMS sample chamber designed to withstand high pressures was used in these measurements (Figure 13). A silicon membrane, reinforced with a metal grid, separated the liquid sample phase from the high inlet vacuum of the mass spectrometer. A cooling trap filled with liquid nitrogen (-196°C) was used to protect the ion source from liquid contamination. A pressure gauge was attached to the sample chamber to monitor pressure inside the sample chamber.
Oxygen evolution at elevated nitrogen or oxygen pressure (~ 20 bars) was studied in *T. elongatus* photosystem II core particles that either contained Ca/Cl or Sr/Br cofactors. The sample was continuously stirred in the dark and the applied pressure slowly increased during a 40 minute period. When the sample reached full pressure (~ 20 bars) oxygen evolution was induced by illuminating the sample through the sample chamber window. Samples were either illuminated with continues light or a group of saturating Xe-flashes at 50 Hz or 2 Hz.

![Sample chamber (cross section)](image)

**Figure 13.** Sample chamber (cross section) that was used in measurements at elevated pressure.
Results and discussion

The experiments in this thesis probe the mechanism of water oxidation in photosystem II by measuring the exchange rates of the two substrate ‘water’ molecules in the various S2, S3 and the S3YZ• by membrane inlet mass spectrometry (MIMS). Specifically the following questions are addressed:

1) Do both substrate molecules bind already in the S2 state? (Paper I)
2) Is W1 a substrate water? (Paper II)
3) Is Ca a substrate binding site? (Paper III)
4) Is the O-O bond formed via nucleophilic attack or via direct coupling of two Mn-bound water-derived ligands? (Paper III)
5) What is the driving force for the S4 → S0 transition? (Paper IV)

As will be detailed below, the results of this study help to eliminate the nucleophilic attack of both free water and Ca-bound water/hydroxo (Figure 14) as possible reaction pathways for O-O bond formation in photosystem II. They further exclude that the water that likely binds during the S2 → S3 transition to the Mn4CaO5 cluster is the fast exchanging substrate water. Thus a mechanism is proposed in which W2 and O5 are the two substrates (Figure 14D), and which uses a structure akin to the closed cube (g = 4.1) S2 state structure of the Mn4CaO5 cluster for O-O bond formation during the S4 → S0 transition. The driving force for this reaction is likely large.

Figure 14. Proposed mechanisms for O-O bond formation. (A) nucleophilic attack of a free water onto a MnV = O or a MnIV-oxyl radical; (B) nucleophilic attack of a Ca-bound water onto a MnV = O or a MnIV-oxyl radical; (C) coupling of a Ca-oxyl radical with a Mn-bound oxyl radical; (D) coupling of a Mn-oxyl radical and a Mn-oxo (bridge). The substrate waters are denoted according to their exchange rates, fast (Wf) and slow (Ws).
Are both substrate molecules bound in the S₂ state? (Paper I)

Three different binding sites for the fast exchanging water (Wᶠ) can be conceived if the structural flexibility between the open and closed cube configuration in the S₂+YZ state [102] is taken into account: Mnₐ₄, Mnₐ₁ and the Ca²⁺ ion. Tightly related to this question is another one: Is the fast exchanging Wᶠ already bound to the Mn₄CaO₅ cluster in the S₂ state, or does it bind during the S₂ → S₃ transition?

Previous substrate water exchange measurements in spinach thylakoids did not resolve the fast exchanging water in the S₂ state, possibly because of limited time-resolution of the instrumentation (≤ 100 s⁻¹) [94, 96]. Two follow up studies managed to resolve Wᶠ exchange in the S₂ state (~ 120 s⁻¹) only if extrinsic proteins were removed (17 kDa and 23 kDa) [99] or at reduced temperature (10°C) [98]. In both cases the rate of exchange was rather close to the time-resolution given by the mixing rate, so that some doubts remained, especially because (i) FTIR experiments [103, 104] suggest that a water binds during the S₂ → S₃ transition, and (ii) the mechanism computed by Siegbahn involves the binding of the fast substrate to the Mnₐ₁ ion during the S₂ → S₃ transition. The good time-resolution of our instrumentation (175 s⁻¹) plus the use of PSII core complexes from an extremophile allowed to clearly demonstrate that Wᶠ is already bound to the Mn₄CaO₅ cluster in the S₂ state in a very similar way as in the S₃ state. This excludes Mnₐ₁ as binding site for the substrate water (in the S₂ and S₃ states).

Substrate water exchange measurements in the S₂ and S₃ state of the extremophilic red alga *Cyanidioschyzon merolae* are shown in Figure 15. The exchange rates of Wᶠ (see fast rise of the m/z = 34 peaks, inserts Figure 15) in the S₂ and S₃ state were found to be similar and well resolved (Table 1). This shows that the interaction between Wᶠ and the Mn₄CaO₅ cluster in *C. merolae* is similar in both S states. Since the data demonstrate beyond doubt that Wᶠ is already bound in the S₂ state, the water that likely binds during the S₂ → S₃ transition cannot be a substrate water during the present reaction cycle. It may rather be a prebound substrate for the next cycle.
Figure 15. Substrate water exchange from *Cyanidioschyzon merolae* PSII core complexes measured in the S3 state (upper panels) and in the S2 state (lower panels). The left panel shows the biphasic exchange pattern of fast and slow exchanging substrate waters measured at m/z = 34. The inserts show the early phase of the fast exchanging substrate water. The right panel shows a mono exponential exchange pattern that corresponds to the slowly exchanging substrate water measured at m/z = 36. The solid lines are kinetic fits to the biphasic respective monophasic exchange pattern.
Table 1 Substrate water H$_2^{18}$O/H$_2^{16}$O exchange rate constants for PSII core complexes from *Cyanidioschyzon merolae*. H$_2^{18}$O/H$_2^{16}$O exchange was measured in the S$_2$ and S$_3$ states at 20°C, pH = 6.1, and the rise of the labeled $^{16,18}$O$_2$ ($^{34}k$) and $^{18,18}$O$_2$ ($^{36}k$).

<table>
<thead>
<tr>
<th>S state</th>
<th>$k_f$, s$^{-1}$</th>
<th>$k_s$, s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>S$_2$</td>
<td>52 ± 8</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>S$_3$</td>
<td>42 ± 2</td>
<td>1.2 ± 0.3</td>
</tr>
</tbody>
</table>
Is W1 a substrate water? (Paper II)

To this project I contributed substrate water exchange measurements (MIMS) in the S\textsubscript{2} state in presence of the water analog ammonia. In combination with EPR, ESEEM and EDNMR measurements carried out at the Max Planck institute in Mülheim, Germany, this study provided results showing that (i) the rapidly exchanging µ-oxo bridge is O5 (which makes O5 to a very likely candidate for W\textsubscript{s}) \cite{56, 105} and (ii) that W1 is not a substrate water.

Ammonia is a water analogue known to bind to PSII at two different sites. One of these sites (SYII) was previously shown to be a Mn coordination site \cite{106}. In Paper II it is demonstrated that ammonia binding to the oxygen evolving complex causes changes to the \textsuperscript{17}O EDNMR signal of the H\textsubscript{2}\textsuperscript{17}O enriched S\textsubscript{2} state. On the basis of the EPR data analysis and DFT calculations it was concluded that ammonia displaces W\textsubscript{1} from its binding site at Mn\textsubscript{A4} and thus perturbs via a trans effect the binding of the \textsuperscript{17}O-labeled O5.

The remaining question now was if W\textsubscript{1} is one of the two substrate waters bound to the Mn\textsubscript{4}CaO\textsubscript{5} cluster. The most direct way to probe this is to use a time resolved mass spectrometer and to measure the exchange of bound substrate waters with isotopically labeled water (H\textsubscript{2}\textsuperscript{18}O). A large deceleration to one of the exchange rates is expected if ammonia is displacing a substrate water molecule. Figure 16 shows the results from the exchange measurements that were made in the S\textsubscript{2} state at pH 7.6 in either the presence of 100 mM NH\textsubscript{4}Cl (red) or 100 mM NaCl (black). The rise of the m/z = 34 peak is biphasic, showing that in \textit{T. elongatus} PSIIcc, as observed in Paper I for \textit{C. merolae} PSIIcc, both substrate waters are already bound in the S\textsubscript{2} state. There is clearly no strong effect on the two exchange rates upon ammonia addition, which means that W\textsubscript{1} is not a substrate.
These findings thus exclude W1 as a substrate, which further reduces the number of possible reaction mechanisms for O-O bond formation. For example, it excludes the proposal by Kusunoki that the terminal water ligands W1 and W2 at MnA4 form the O-O bond via germinal coupling [107].

Figure 16. Substrate water exchange from *Thermosynechococcus elongatus* core complexes measured in the S2 state at pH 7.6 either in the presence of 100 mM NH₄Cl (red triangles) or 100mM NaCl (black squares). The left panel show the biphasic exchange pattern of fast and slow exchanging substrate waters measured at m/z = 34. The right panel shows a mono exponential exchange pattern that corresponds to the slowly exchanging substrate water measured at m/z = 36. The solid lines are kinetic fits to the biphasic respective monophasic exchange pattern. (reproduced with permission from *Proceedings of the National Academy of Sciences USA.*)

**Is Ca a substrate binding site? (Paper III)**

On the basis of Papers I and II, and on the likely but not fully proven assumption that O5 is Ws (reviewed in Cox and Messinger 2013), only W2 on MnA4 and W3 bound to Ca remain likely candidates for Wf. If W3 is the fast exchanging substrate water, then it would react in the S4 state via nucleophilic attack with O5. In addition, one should expect that its exchange rate would be affected by Ca/Sr exchange. The data presented in Paper III demonstrate that (i) the exchange rate of Wf is basically unaffected by biosynthetic Ca/Sr exchange and (ii) that both substrate oxygens are tightly bound in the last transient state (S3Y2) prior to O-O bond formation.
Since nucleophilic attack naturally involves one tightly bound (slowly exchanging) and one loosely bound (slowly) exchanging reaction partner, this results excludes the nucleophilic attack pathway for O-O bond formation and thus W3 on Ca as substrate water.

The final reactions that oxidize the two substrate water molecules and release molecular oxygen from the Mn₄CaO₅ cluster are still elusive. The onset of the S₃ → ’S₄’ → S₀ transition is the flash-induced formation of the YZ*, which is followed by a ~ 200 µs delay before the begin of molecular oxygen release. This delay has been probed by optical spectroscopy, EPR, XAS and mutational studies [73, 74, 108, 109]. On that basis this lag phase was ascribed to a deprotonation reaction of the Mn₄CaO₅ cluster. This deprotonation is depicted in the reaction formula S³⁺YZ● → S₃YZ● where the plus sign denotes the presence of a positive charge at the Mn₄CaO₅ cluster that is removed by the deprotonation reaction. The dot denotes the neutral tyrosine YZ radical.

The question addressed in the experiments in paper III is if the two substrate waters exchange with the same rate in the S₃YZ● state as in the S₃⁺YZ state. A similar exchange rate may be expected, since the Mn oxidation states are identical in both states. Previously the short half-lifetime of 1-2 ms of the transient S₃YZ● state prevented the study of the binding affinity of the two substrate waters, since this time is shorter than the half-lifetime of 3 ms for mixing the sample with H₂¹⁸O. In Paper III the half-lifetime of the S₃YZ● state was extended from ~ 1.1 ms to ~ 7.2 ms and even ~45 ms by replacing the native cofactors Ca/Cl with Sr/Br and Sr/I in PSII core samples from Thermosynechococcus elongatus [86, 110], which opened a time window for probing the exchange rates of both substrate waters.

First the effect of the cofactor replacement on the binding affinities of the two substrate water molecules was determined in the S₃⁺YZ state. The data in Paper III show that the exchange rate of W₄ increases about 10-fold if Ca²⁺ is replaced by Sr²⁺, while the exchange of Cl⁻ against Br⁻ or I⁻ has no effect. In contrast, only a very small (20-30%) deceleration of the exchange of W₅ is observed upon Ca/Sr substitution. This confirms previous data obtained with spinach samples using biochemical Ca/Sr substitution, and thus supports that Ws is connected with Ca and makes a binding of W₅ to Ca rather unlikely.
Simulations using these exchange rates obtained in the $S_3^+YZ$ state show that the yields of $^{16,18}\text{O}_2$ that can be obtained by injecting $\text{H}_2^{18}\text{O}$ into the samples directly after the third flash that forms the $S_3YZ^*$ state are $51(\pm6)\%$ for Sr/I-PSII and $18(\pm2)\%$ for Sr/Br-PSII of the maximum amplitude in the $S_3^+YZ$ state. Thus these yields would be easily detectable. The data presented in Paper III show that the isotope ratio of the molecular oxygen produced did not deviate from natural enrichment. Further simulations demonstrated that the exchange rates of both substrate waters need to decrease significantly to be consistent with the measured data. As discussed in detail in Paper III this demonstrates that both substrate waters are rather tightly bound to the Mn$_4$CaO$_5$ cluster directly before the O-O bond formation. This finding is inconsistent with nucleophilic attack mechanisms, but expected for direct coupling mechanisms involving two Mn-bound substrate water molecules (Figure 14D).

**Possible mechanism of O-O bond formation**

By excluding other possibilities, the results from Papers I-III indicate strongly that the fast exchanging water ($W_f$) is the terminal water $W_2$ that is bound to Mn$_{A4}$. This assignment is also consistent with the dramatic deceleration of the exchange rate of $W_f$ in the $S_1 \rightarrow S_2$ transition [98] since according to present DFT calculations it is the Mn$_{A4}$ ion that is oxidized from Mn$^{III}$ to Mn$^{IV}$ in this transition.

In Paper III two possible variants of for the O-O bond formation between $W_2$ and O$_5$ are discussed. They build on the recently described structural flexibility in the $S_2^+YZ$ state of the Mn$_4$CaO$_5$ cluster [111]. In the open cube structure (lower energy isomer) O$_5$ forms a $\mu_3$-oxo linkage between the Mn$_{B3}$, Mn$_{A4}$ and Ca. In the closed cube structure (higher energy isomer) O$_5$ links Mn$_{D1}$, Mn$_{B3}$ and the Ca$^{2+}$ ion. Due to this structural flexibility in the $S_2^+YZ$ state, it is a priori not clear which of the two structures can advance into the $S_3^+YZ$ state. Obviously, each of the structures suggests other mechanisms of O-O bond formation. Since the combined data of Papers I-III make it highly likely that $W_2$ is $W_f$, the closed cube structure most likely proceeds to the $S_3^+YZ$ state and is involved in O-O bond formation. Figure 17 thus shows a radical coupling between $W_2$ on Mn$_{A4}$ and O$_5$ that employs the closed cube structure.
What is the driving force for the $S_4 \rightarrow S_0$ transition? (Paper IV)

Another controversial question about the mechanism of water oxidation is the driving force of the $S_4 \rightarrow S_0$ transition. Experiments by Clausen and Junge [112] suggested that the O-O bond formation occurs near the thermodynamic equilibrium. In contrast, more recent studies did not find any inhibition of $O_2$ formation by $O_2$ pressures up to 20 bar [113-115]. In Paper IV, two new approaches are described to further explore the possible reversibility of this reaction.

The oxygen water isotope exchange (OWIE) (Paper IV)

In these experiments, the equilibrium constant of the $S_4 \rightarrow S_0$ transition is probed, i.e., the frequency with which PSII centers in the $S_0$ state can bind $O_2$ and return to the $S_3^+Y_z^-$ or $S_3Y_z^-$ states. The idea of this experiment is summarized in Figure 18. If equilibrium does exist between the $S_0$ state and an earlier $S_4$ state, then the incubation of $S_0$ in the presence of labeled oxygen ($^{18}O_2$) would lead to the formation of two $^{18}$O-labeled substrate ligands. If any of the two substrate water molecules would exchange with bulk $^{16}$O-water during the lifetime of this state then $^{16,18}O_2$ would be formed and released upon return to the $S_0$ state. The release of single labeled molecular oxygen ($^{16,18}O_2$) can then be detected at m/z = 34 by a membrane-inlet mass spectrometer.

$$S_0 + ^{18}O_2 \xrightarrow{K} S_4(H_2^{18}O)_2 \xrightarrow{k_{fast}} S_4(H_2^{18}O, H_2^{16}O) \xrightarrow{S_0 + ^{16,18}O_2}$$

Figure 18: Schematic representation of the OWIE experiment. Protons are omitted for simplicity.
A challenge in these experiments is to stabilize the S₀ state for long enough to be able to probe even the occurrences of rare exchange events. Therefore, a Y_D-less mutant of Thermosynechococcus elongatus was employed, which excludes the oxidation of S₀ to S₁ by Y_Dox. In this way, the S₀ state was stable for at least 3 days. Another challenge is to prevent any entry of molecular oxygen from air into the reaction volume. To minimize this, the experiments were performed inside an N₂ filled glove box.

The results from the OWIE measurements show no ¹⁶,¹⁸O₂ formation in the S₀ samples within 3 days. They are thus consistent with the results of Paper III that the substrate molecules in the S₃Y₂⁻ state are very tightly bound, and/or they indicate that the S₄ → S₀ transition is basically irreversible.

**O₂ overpressure (Paper IV)**

The idea behind these experiments is that with a high enough external O₂ pressure one should be able to prevent the light-induced production of molecular oxygen by PSII. The pressure required to achieve this product inhibition would then be a measure of the driving force of the S₄ → S₀ transition.

These measurements are related to a previous study that used UV-Vis spectroscopy in an attempted to stabilize the postulated intermediate during the S₄ → S₀ transition by increasing oxygen pressure [112]. The main conclusion from that study was that an over pressure of 2.3 bars leads to half-inhibition of the oxygen release. This result was proven wrong in three later studies [113-115]. One of them made use of a membrane-inlet mass spectrometer to measure ¹⁸O₂ evolution from H₂¹⁸O at elevated ¹⁶O₂ pressure in Synechocystis sp PCC6803. That study showed that the photosynthetic O₂ release was unperturbed up to pressures of 20 bar O₂ [115].

In Paper IV these experiments are repeated with PSII preparations containing the cofactors Sr²⁺ and Br⁻ the native cofactors Ca²⁺ and Cl⁻. A previous study found that the potential of the Mn₄CaO₅ cluster was 60 mV lower in the Sr/Br-PSII as compared to the Ca/Cl-PSII [86, 116]. This was estimated from the position of the thermoluminescence blow curve of the S₃O₅⁻ recombination, and suggested that the driving force for the photosynthetic O₂ release might be also reduced. In turn the Sr/Br-PSII samples might be more susceptible to elevated O₂ pressures.
To test this hypothesis oxygen evolution of Ca/Cl-PSIIcc and Sr/Br-PSIIcc was measured at elevated pressures of O₂ and N₂ in presence of a high enrichment of H₂¹⁸O. Figure 19 shows the photosynthetic oxygen yields that was produced by a sequence of 20 flashes that were given either at 2 Hz or 50 Hz. Panel A and C confirm the previous findings that samples containing the native Ca²⁺ and Cl cofactors maintain full photosynthetic oxygen production at 20 bar O₂ and 17 bar N₂. This is not observed for the samples containing Sr²⁺ and Br⁻ cofactors. Panel B and D show that the photogenerated oxygen yields are reduced at elevated O₂ pressure as compared to elevated N₂ pressure. This effect was stronger at higher flash frequencies. Consistent with this, the flash induced oxygen evolution patterns (FIOPs) that were measured at elevated O₂ respective N₂ pressure with a flash frequency of 0.04 s are similar to each other, which shows that even in the Sr/Br-PSIIcc's the miss (α) and double hit (β) parameters are unaffected by elevated oxygen pressure (Figure 20).

**Figure 19.** Flash induced oxygen yields from *Thermosynechococcus elongatus* core complexes at elevated O₂ and N₂ pressures. Flashes were given at 2 Hz (upper panels) or 50 Hz (lower panels). Photosystem II containing native Ca²⁺ and Cl⁻ cofactors (left panels) and replaced Sr²⁺ and Br⁻ cofactors (right panels). Blue traces show measurements made at elevated N₂ pressure. Black traces show measurements made at elevated O₂ pressure.
Figure 20. Flash induced oxygen evolution patterns (FIOPs) from *Thermosynechococcus elongatus* core complexes at elevated O$_2$ and N$_2$ pressures. Flashes were separated by 25 seconds dark periods. Photosystem II containing native Ca$^{2+}$ and Cl$^-$ cofactors (blue trace) and replaced Sr$^{2+}$ and Br$^-$ cofactors (black trace). Left panel show measurements made at elevated O$_2$ pressure. Right panel show measurements made at elevated N$_2$ pressure.

The frequency dependence of the O$_2$ pressure inhibition of the Sr/Br PSIIcc shows that this is caused by an O$_2$-induced kinetic limitation rather than the expected thermodynamic limitation. A possible explanation for that could be a slowed release of O$_2$ in Sr/Br-PSIIcc at 20 bar O$_2$. 
Acknowledgements

During my years as PhD student at the Department of Chemistry at Umea University I have not only learned tremendously about photosynthesis and how to conduct proper science, but I also got to know people devoted to science. The skills of these people have contributed immensely to my work and therefore I take this opportunity to express my gratitude.

**Prof. Johannes Messinger**, my supervisor who has shared his great scientific knowledge with me, giving me in-depth knowledge about photosynthesis and invaluable knowledge of the scientific method. The well thought out experiments of yours was challenging but truly inspiring!

**Dr. Alain Boussac**, providing me with excellent samples for several of the experiments I have conducted during my time as a PhD student. Your visit at the Umeå Renewable Energy Meeting in 2011 coincided with the very first substrate water exchange measurements made in the $S_3Y_{Z'}$ state. It was a pleasure for me to meet you and be able to demonstrate our lab during a real experiment!

**Lars Lundmark**. Technical engineer at Umeå University. Your expertise in electronics and instrumentation helped me more than once. Thanks!

**Dr. Dmitriy Shevela**, when I first arrived, you guided me through the lab, sharing tips and tricks for our mass spectrometer. Besides being a great researcher, you possess skills in graphics that are outstanding, which is evident in paper II and III. Thanks!

**PhD stud. Sergey Koroidov**, always giving a helping hand when necessary! I especially remem bers when you managed to write a LabVIEW program for me in due time for an important experiment. Thanks!

**PhD stud. Long Vo Pham**, one of the most friendliest persons I have ever met! Always kindly sharing your knowledge of lab procedures when asked. Thanks for being such a great friend in the lab!

**Dr. Guangye Han**. We worked together on the very first experiment of mine. Since you had many years of experience from working in the lab, I got a lot of valuable tips from you during this time. It was great getting to know you!
Members of the Artificial Leaf Project:

Dr. Hans-Martin Beerends; PhD stud. Hasna Bourajoini; Dr. Eduardo Gracia; Dr. Iqbal Javed; Jan Forsgren; PhD stud. Srikanth Revoju; PhD stud. Thung Pham Ngoc; Dr. Yongqi Liang

During our seminars you taught me a lot about artificial photosynthesis. Some of you have been working in our labs, which gave me the opportunity to get to know you better.

Ylva, Alma and Elvira – my family!
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