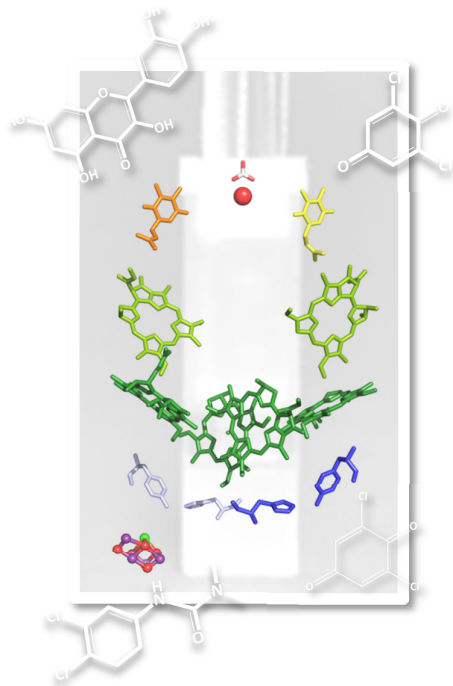


Protein film voltammetry and spectroelectrochemistry of the electron acceptor site in Photosystem II



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Thesis submitted for the degree of Doctor of Philosophy of
Imperial College London

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My parents

*The world would be a much better place
if there were more people like you.*

*"But would do sciences and art have in common? [...]
I think that the big challenges in the world can only be met
and overcome by people, who consider all available
disciplines to understand them."*

Bertolt Brecht (1957)

Declaration of originality

I hereby declare that this thesis, submitted in fulfilment of the requirements for the degree of Doctor of Philosophy of the Imperial College London, represents my own work and has not previously submitted to this or any other institute for any degree, diploma or other qualification. All other work has been appropriately referenced.



Katharina Brinkert

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Abstract

The key reaction of oxygenic photosynthesis, the light-driven oxidation of water, is carried out by Photosystem II (PSII), a light-driven water-plastoquinone oxidoreductase. Photosystem II is a multi-cofactor protein and its energetic characterization is quite a difficult undertaking. Electrochemical approaches play an important role as a tool for completing the energetic picture and for carrying out fundamental studies of the enzyme. In this work, two electrochemical methods have been applied to study the electron acceptor site of the Photosystem II: spectroelectrochemistry and protein film voltammetry.

The first part focuses on the re-measurement of the midpoint potential of the primary quinone electron acceptor, Q_A , in PSII core complexes isolated from *Thermosynechococcus elongatus* and in PSII enriched membranes from spinach using an optical transparent thin layer (OTTLE) cell. The obtained results show that the bicarbonate anion, ligated to the non-heme iron at the electron acceptor site in close proximity to Q_A , plays a significant role in controlling the redox properties of the $Q_A/Q_A^{\cdot-}$ -couple. This finding explains various controversies about existing literature values of the $Q_A/Q_A^{\cdot-}$ midpoint potential.

The second part describes investigations of photocurrents generated by Photosystem II in metal oxide hybrid systems. PSII isolated from *T. elongatus* was immobilized onto nanostructured titanium dioxide/indium tin oxide electrodes (TiO_2/ITO) and the origin of photocurrent upon illumination was studied. Using conditions in which PSII was immobilized as a monolayer, it was demonstrated that direct electron transfer occurs from the redox cofactor Q_A to the electrode surface, but that the electron transfer through the metal oxide is the rate-limiting step. Redox mediators enhance the photocurrent by taking electrons from the TiO_2 surface to the ITO and not from PSII.

Furthermore, the origin of cathodic photocurrents (i.e. electron flow from the electrode) was investigated. The results indicate that a one-electron reduction of oxygen to the superoxide anion radical ($O_2^{\cdot-}$) occurs at the ITO surface in darkness when an external bias lower than +300 mV vs NHE was applied. The findings can explain the light-driven and catalytic nature of this current by the fact that the reduction of $O_2^{\cdot-}$ occurs at the non-heme iron, which is driven by the photo-reduction of Q_A .

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Nānā na moe. - Look at your dreams.

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Mama, Papa, ich danke Euch für Euer Vertrauen, Eure Unterstützung und Liebe und dessen Selbstverständlichkeit. Die Welt wäre ein ganz großes Stück besser, wenn es mehr Menschen wie Euch geben würde. Danke - für alles.

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Abbreviations

<i>AC</i>	alternating current
<i>ATO</i>	antimony-doped tin oxide
<i>ATP</i>	adenosine triphosphate
<i>BOD</i>	bilirubin oxidase
<i>BBY membranes</i>	PSII enriched membranes
<i>Chl</i>	chlorophyll (if not stated otherwise: chlorophyll a)
<i>CPE</i>	constant phase element
<i>cytb₅₅₉</i>	cytochrome <i>b₅₅₉</i>
<i>DCBQ</i>	2,6-dichloro-1,4-benzoquinone
<i>DCMU</i>	3-(3,4-dichlorophenyl)-1,1-dimethylurea
<i>DF</i>	diformazan
<i>DET</i>	direct electron transfer
<i>E_c</i>	energetic position of the conduction band
<i>EDTA</i>	ethylenediaminetetraacetic acid
<i>E_f</i>	energetic position of the Fermi level
<i>E_m</i>	midpoint potential
<i>E_{redox}</i>	electrochemical level of the electrolyte
<i>E_v</i>	energetic position of the valence band
<i>ET</i>	electron transfer
<i>FNR</i>	ferredoxin-NADP reductase
<i>FTIR</i>	Fourier transform infrared
<i>FTO</i>	fluorine-doped tin-oxide
<i>HEPES</i>	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
<i>HP</i>	high potential
<i>IET</i>	interfacial electron transfer
<i>IP</i>	intermediate potential
<i>K_d</i>	binding constant
<i>K_m</i>	Michaelis constant
<i>LHC II</i>	light harvesting complexes II
<i>LP</i>	low potential

<i>MES</i>	2-(N-morpholino)ethane-sulphonic acid
<i>mesoITO</i>	mesoporous indium tin oxide
<i>MF</i>	monoformazan
<i>MM</i>	molecular mechanics
<i>Mn-depleted</i>	Photosystem II depleted of the Mn ₄ CaO ₅ cluster
<i>NADP⁺</i>	nicotinamide adenine dinucleotide phosphate (oxidized)
<i>NADPH</i>	nicotinamide adenine dinucleotide phosphate (reduced)
<i>NBT</i>	nitro blue tetrazolium
<i>NiNTA</i>	Ni ²⁺ -nitrilotriacetic acid
<i>OEC</i>	oxygen evolving complex
<i>ORR</i>	oxygen reduction reaction
<i>OTTLE</i>	optical transparent thin layer electrode
<i>PAM</i>	pulse amplitude modulation
<i>polySBQ</i>	poly-mercapto-p-benzoquinone
<i>P_{D1}</i>	chlorophyll molecule (D1 protein), primary oxidant in Photosystem II
<i>Pheo_{D1}, Ph_{D1}</i>	pheophytin molecule (D1 protein)
<i>Psb</i>	Photosystem b
<i>PSI</i>	Photosystem I
<i>PSII</i>	Photosystem II
<i>PT</i>	proton transfer
<i>PVF</i>	protein film voltammetry
<i>Q_A</i>	plastoquinone molecule associated with the Q _A site
<i>Q_B</i>	plastoquinone molecule associated with the Q _B site
<i>QM</i>	quantum mechanics
<i>RC</i>	reaction center
<i>ROS</i>	reactive oxygen species
<i>SHE</i>	standard hydrogen electrode
<i>TCO</i>	transparent conductive oxide
<i>TMPD</i>	N,N,N',N'-tetramethyl-p-phenylenediamine
<i>Tyr-Z, Y_Z</i>	tyrosine Z
<i>v/v</i>	volume/volume, volume concentration
<i>V_{FB}</i>	flat band potential
<i>w/v</i>	mass/volume, mass concentration

Chapter 1.

Introduction

1.1 Oxygenic photosynthesis

Oxygenic photosynthesis is the process in which oxygen and carbohydrates are produced from water and carbon dioxide utilizing sunlight. With the ability of converting solar energy into chemical energy, oxygenic photosynthesis remains one of the most important biological processes on earth, producing directly or indirectly the building blocks of all living organisms and also of oil, gas and coal. In contrast to some anaerobic photosynthetic organisms, which use H_2S as a reducing agent and produce sulphur as a major product, oxygenic photosynthesis utilizes H_2O as the reducing agent.

This chapter outlines the fundamental principles of photosynthesis focusing on the electron transport between the two major photosynthetic complexes, Photosystem I (PSI) and Photosystem II (PSII) and the structure, function and energetics of the PSII reaction center, which is of major interest for the research described in this thesis. Furthermore, it provides an overview about methods and ways to electrochemically investigate PSII to introduce the research objectives of this thesis work.

1.1.1 The architecture of thylakoid membranes and linear electron transport in photosynthesis

Oxygenic photosynthesis is accomplished by a series of reactions which occur in the chloroplast of photosynthetic eukaryotes and cyanobacteria. It is characterized by two main sets of reactions: the 'light' and the 'dark' reactions. Early biochemical studies demonstrated that chloroplast thylakoid membranes oxidize H_2O , reduce NADP to NADPH and synthesize ATP during the 'light' reactions (Vernon and Avron 1965). These reactions were shown to be catalyzed by two photosystems, Photosystem I (PSI) and Photosystem II (PSII), an ATP synthase, which produces the energy source ATP through a proton gradient formed by light-driven electron transfer reactions and the cytochrome

(cyt) b_6f complex, mediating the electron transport between PSII and PSI and partly converting the redox energy into the proton gradient used for ATP formation (Nelson and Yocum 2006). The 'dark' reactions utilize the reducing equivalents NADPH and ATP generated by the 'light' reactions to reduce atmospheric CO_2 into carbohydrates, which act as the respiratory source. The four protein complexes required for the light-driven reactions of photosynthesis reside in thylakoids, a membrane continuum of flattened sacs in the inner membrane component of the chloroplast (Nelson and Ben-Shem 2004). They form a three-dimensional network enclosing an aqueous space called the lumen and are differentiated into cylindrical stacked structures, the grana, and interconnecting single membrane regions, the stromal lamellae. The protein complexes catalyzing the electron transfer and energy transduction are distributed in the thylakoid membrane: PSI is located in the stromal lamellae, PSII is found in the grana, the F-ATPase is located mainly in the stromal lamellae, and the cyt b_6f complex can be found in the grana and grana margins (Albertsson 2001; Anderson 2002). The outer membrane of the chloroplast encloses the stromal compartment, containing all soluble enzymes required for the 'dark' reactions in addition to enzymes that synthesize proteins and the genome.

The accepted model of photosynthetic electron transport is illustrated in **Figure 1.1**, following a so-called "Z-scheme": Photosystem II absorbs light energy through an assembly of light-harvesting chlorophylls, which funnel the trapped light energy to the central chlorophylls in the reaction center. This induces the formation of an excited electronic state over the central pigments, which are known as P_{680} according to the wavelength of their lowest-energy absorption band. The excited P_{680}^* is a strong reducing agent and rapidly loses an electron to a nearby electron acceptor (A). A cascade of fast electron transfer reactions stabilizes this charge separation by increasing the distance between the electron donor and acceptor, which reduces recombination reactions. The electron is passed along an electron transport chain via the cyt b_6f complex to Photosystem I (PSI). PSI simultaneously absorbs light energy through light-harvesting chlorophylls and generates an electron for the reduction of NADP to NADPH by the ferredoxin-NADP reductase (FNR). It is reduced again by the electron originating from the light absorption process in PSII. In turn, the remaining cation in PSII is able to oxidize a tyrosine residue and subsequently, a pentanuclear tetramanganese-calcium cluster (Mn_4CaO_5), commonly referred to as the oxygen-evolving complex (OEC). The OEC cycles through five different oxidation states (S_i , $i=0-4$) to generate electrons, protons and oxygen from the oxidation of water (Kok et al. 1970):

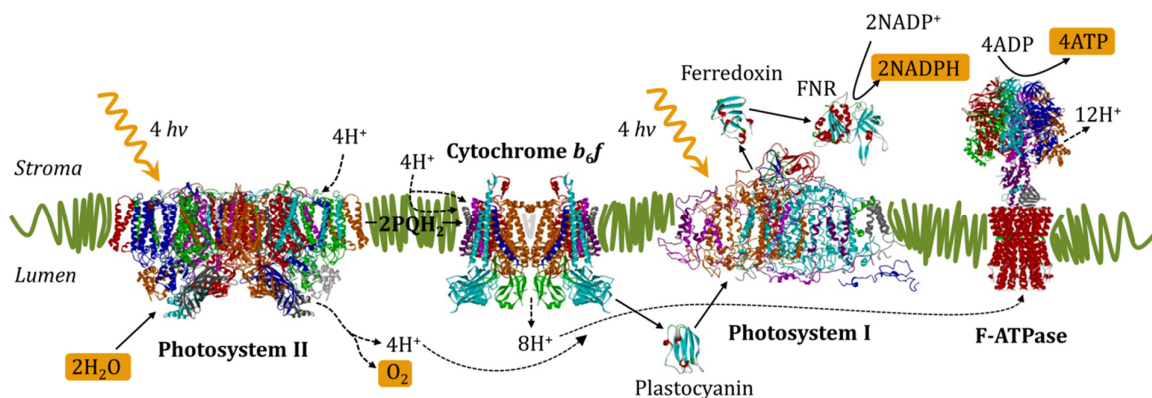


Figure 1.1 Schematic overview of the electron transport chain of the photosynthetic light reaction.

1.1 Structure and function of Photosystem II

Photosystem II is a light-driven water-plastoquinone oxidoreductase, which carries out the key reaction of oxygenic photosynthesis: the light-driven oxidation of water (Cardona et al. 2012; Rappaport and Diner 2008). It is a large, multisubunit transmembrane protein complex found in the photosynthetic membranes of cyanobacteria and photosynthetic eukaryotes and is composed of 17 transmembrane subunits, three extrinsic proteins and several cofactors with a total molecular weight of 350 kDa (**Figure 1.2**, Umena et al. 2011). A crystal structure is currently only available from cyanobacteria at a resolution of 1.9 Å (Umena et al. 2011), whereas for plant PSII, only a three-dimensional model of the supercomplex exists, which was obtained by cryoelectron microscopy and single particle analysis (Nield et al. 2000). Crystal structures are only available of the PsbP and PsbQ extrinsic subunits at a resolution of 1.6 and 1.95 Å, respectively (Ifuku et al. 2004; Calderone et al. 2003).

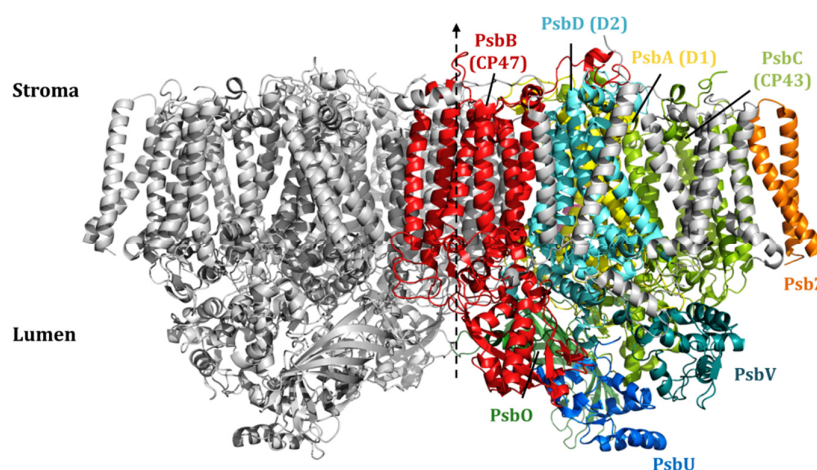


Figure 1.2 X-ray crystal structure of the PSII dimer from *T. vulcanus* at a resolution of 1.9 Å, indicating the position of the extrinsic polypeptides, the intrinsic subunits PsbA - D and the small accessory subunit PsbZ (pdb reference 3ARC, Umena et al. 2011).

The primary processes of the photosynthetic light reactions are initiated by the photoexcitation of the pigments P₆₈₀, which are formed by the chlorophyll a pair, P_{D1} and P_{D2} and the chlorophylls Chl_{D1} and Chl_{D2} in the PSII reaction center. Within picoseconds, an initial charge separation reaction occurs, involving the formation of P_{D1}⁺P_{D2}⁻, P_{D1}⁺Chl_{D1}⁻ and Chl_{D1}⁺Pheo_{D1}⁻ (with P, Chl and Pheo standing for pair of chlorophylls, monomeric chlorophyll and pheophytin, respectively, **Figure 1.3 (A)**; Dau et al. 2012; Cardona et al. 2012), leading to the formation of the charge pair P_{D1}⁺Pheo_{D1}⁻ **(1)**, which in turn reduces another subsequent electron acceptor, the plastoquinone Q_A **(2)**. The electron hole at P_{D1}⁺ is able to abstract electrons on the time scale of microseconds from an active tyrosine residue Tyr-Z **(3)**, which in turn reduces the OEC located at the luminal (donor) side **(4)**. Q_A is located close to the stromal (acceptor) side of the protein and reduces in approximately 0.2 - 0.4 ms the terminal electron acceptor Q_B **(5)**, which is, once doubly reduced (0.8 ms), released into a membrane quinone pool (Dau et al. 2012).

The catalytic center of the OEC is composed of four Mn-ions and one Ca²⁺-ion coordinated by μ-oxo bridges and amino acid residues. Four successive light reactions drive the four successive oxidations between the S_i and S_{i+1} states (Dau & Haumann, 2008). Upon the formation of the S₄ state, one molecule of oxygen is produced and released, which regenerates the S₀ state. Although the Kok cycle (Joliot et al. 1969; Kok

et al. 1970) rationalizes already the period-four oscillation of flash-induced oxygen evolution and succeeds in formally describing the function of the enzyme, it does not contain information about the chemical nature of the involved transient states nor the precise mechanisms underlying the interconversion of the S states and the absolute oxidation states of the individual Mn ions in the different S states (Cox et al. 2013). Recent computational analyses support the widely accepted hypothesis of the “high-valence scheme”, where the Mn oxidation states are assigned as III, IV, IV, IV in the S₂ state (Krewald et al. 2015). **Figure 1.3 (B)** provides an overview about the current view of the extended Kok cycle, indicating the oxidation and proton release events at each transition upon photon absorption by P₆₈₀ and the formal oxidation states of the four Mn-ions of the OEC in the S₀ - S₃ states according to the high-valence scheme.

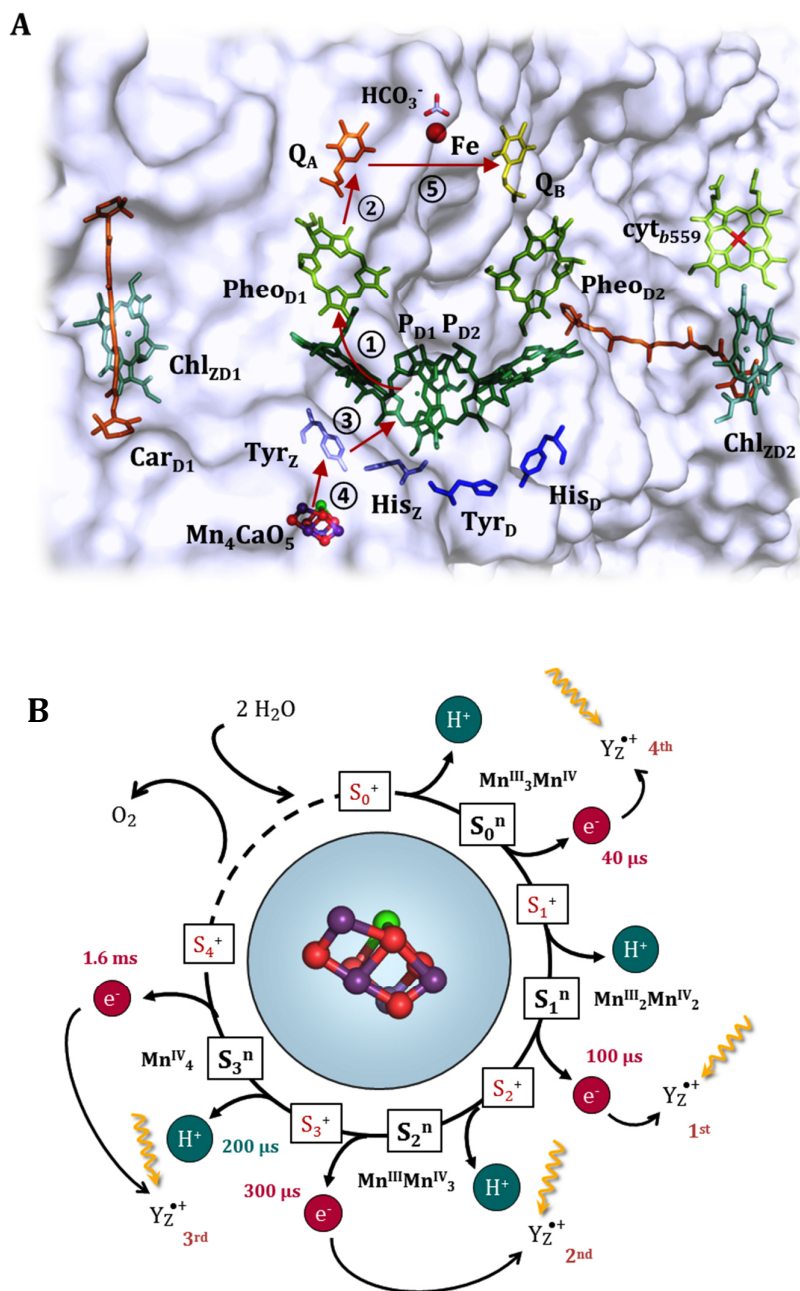


Figure 1.3 (A) Schematic representation of the arrangement of cofactors involved in the electron transfer chain in PSII according to the crystal structure (pdb reference 3ARC). The numbers represent the order of electron transfer steps after charge separation. Step 1 represents both, the charge separation and the first rapid stabilization step, leading to the formation of $P_{D1}^+P_{heoD1}^-$ (see text). (B) Model of the S-state cycle firstly proposed by Kok et al. (1970), showing the electron and proton release in the individual S-states (Haumann et al. 2005) and the net oxidation state of the respective S-state (high-valence model, Krewald et al. 2015). A complete cycle requires sequential absorption of four photons, whereas each photon leads within $<1 \mu s$ to the formation of $Y_Z^{\bullet+}$.

1.2.1 PSII in thermophilic cyanobacteria

The current crystal structure from *Thermosynechococcus vulcanus* (**Figure 1.2**) provides structural details regarding the placement of the individual subunits and the orientation of pigments associated with the reaction center and its antenna system. The major intrinsic subunits are PsbA (D1), PsaB (CP47), PsbC (CP43), PsbD (D2) and PsbE and F, the polypeptides which donate axial Histidine ligands to the heme iron of cyt *b₅₅₉* (Nelson and Yocum 2006). The membrane spanning helices of the PsbA and PsbD reaction center (RC) subunits are in proximity to one another and the chromophores comprise six chlorophylls (Chl), two pheophytins (Pheo), the plastoquinones Q_A and Q_B and the redox-active tyrosines Y_Z and Y_D. In proximity to PsbA is the subunit PsbC and PsbD is located close to PsbB. Both, PsbC and PsbD, consist of large extrinsic loops, which are readily visible as significant protrusions into the luminal space below their membrane-spanning α -helices. Biochemical and mutagenesis experiments predicted that these loops provide binding sides for the attachment of PsbO (Bricker and Frankel 2002), a nuclear encoded extrinsic protein required for the stabilization of the manganese cluster, which can be found in the crystal structure. Further extrinsic subunits are PsbV (cyt *c₅₅₀*) and PsbU, which, along with PsbO, form a structure facilitating the retention of the inorganic cofactors (Ca²⁺, Cl⁻) of the OEC (Seidler 1996). They appear to bind through interactions with PsbO and with one another (Nelson and Yocum 2006). Extraction of PsbO from PSII lowers the rate of O₂ evolution and to maintain its residual activity, unphysiologically high concentrations of Cl⁻ are required (Seidler 1996). A loop of PsbO extends in the direction of the Mn-cluster and is proposed to function as well as a hydrophilic pathway between the lumen and the OEC (Ferreira et al. 2004).

Accessory subunits such as PsbL, M and T are suggested to be involved in the formation of PSII dimers, PsbJ, K, N, and Z are clustered near PsbC and are presumably involved in carotenoid binding and PsbI and X are hypothesized to stabilize the binding of Chl_{ZD1} and Chl_{ZD2}, which are bound to PsbA and D (Ferreira et al. 2004). Every PSII monomer contains of 35 chlorophylls, 2 pheophytins (Pheo_{D1} and Pheo_{D2}), 2 heme irons, 1 non-heme iron, 2 plastoquinones (Q_A and Q_B), 1 Mn₄CaO₅ cluster, 2 Cl⁻, 12 carotenoids, one bicarbonate ion and 25 lipids (Umena et al. 2011).

1.2.2 PSII in higher plants

The extrinsic proteins of plant PSII are PsbO, PsbP and PsbQ (Seidler 1996). The latter ones replace PsbV and PsbU in cyanobacterial PSII, but bear substantial differences: the plant subunits contain either a four-helix bundle (PsbQ) or are rich in β -sheets (PsbP), which are absent in PsbU and V. Furthermore, they can be removed without significant loss of the Ca^{2+} -ion and are also thought to protect the manganese atoms from damage from reductants (Wincencjusz et al. 1997). Reconstitution studies and results from site-directed mutagenesis experiments on the binding of PsbO to PSII (Betts et al. 1997) support the hypothesis that two copies of PsbO are present in plant PSII (Xu and Bricker 1992). These two copies might be responsible for an additional PsbO-PsbB interaction observed in spinach (Odom and Bricker 1992), but not in cyanobacteria.

1.2.3 The non-heme iron and the bicarbonate ligand at the electron acceptor site

The electron acceptor site of Photosystem II contains a mononuclear non-heme iron (NHI) site, which is flanked between the two quinones, Q_A and Q_B (**Figure 1.2 (B)**, Muh and Zouni 2013). The NHI is a hexacoordinated Fe^{2+} -ion with a distorted octahedral geometry, coordinated by four histidine residues at the D1 protein (D1-H215, D1-H272) and D2 protein (D2-H214, D2-H268) and a bidentate bicarbonate ligand. There has always been an unresolved question concerning the function of the NHI, since it does not participate as an intermediate in the native electron transfer reaction from Q_A to Q_B , although it is suitably placed to function as a redox mediator (**Figure 1.4 (A)**). Beside structural and regulative roles, the non-heme iron was suggested to possess an enzymatic activity, which is of particular interest in view of the fact that PSII inevitably operates under aerobic conditions with O_2 being the product of the water-splitting reaction. Here, the potential production of harmful reactive oxygen species (ROS) becomes an important issue (Pospisil 2009, 2012, 2014; Pospisil et al. 2004). Various enzymes are known to possess a mononuclear NHI complex as the active site and catalyze various biochemical reactions (Solomon et al. 2000). In most cases they are oxidases or oxygenases, which are able to activate the relatively unreactive triplet O_2 (Costas et al. 2004; Kovaleva and Lipscomb 2008).

The non-heme iron and its ligand network not only facilitate a structural connection between Q_A and Q_B at the PSII acceptor site, but also one between the donor and acceptor sides across the PSII membrane: D1-H215 and D2-H214 of the NHI are on the same helix as the axial ligands D1-H198 and D2-H197 of P_{D1} and P_{D2} , respectively, which extends even further in the PsbA subunit down to the direct metal ligands of the OEC, such as D1-E189 and D1-D170 (**Figure 1.3 (B)**). This is of particular relevance with respect to the redox properties of Q_A in the absence and presence of the Mn_4CaO_5 cluster (see section 1.4.2).

An intriguing property of the NHI is the bidentate bicarbonate anion ligand, which is thought to have a regulatory role in the activity of PSII (Shevela et al. 2012) and which can be displaced by other molecules such as formate and acetate (Shevela et al. 2007). The bicarbonate ligand is also thought to be involved in the two-electron gate mechanism of the electron transfer (ET) from Q_A to Q_B : the first step of the electron transfer is not coupled to the protonation of Q_B , whereas the second step is a proton-coupled ET according to the PT/ET mechanism, i.e., the ET follows proton transfer (PT) to $Q_B^\bullet$, which receives a second proton once $Q_BH^\bullet$ is formed (Muh and Zouni 2013). Govindjee et al. (1976) performed fluorescence experiments on CO_2 -depleted chloroplasts and found that the $Q_A^\bullet$ decay was slowed down after three or more flashes (Govindjee et al. 1976). This implies that three electrons can be stored in the acceptor site, leading to the formation of $Q_A^\bullet Q_B^{2-}$ or $Q_A^\bullet Q_BH^\bullet$. Therefore, removal of the bicarbonate ligand inhibits the actual ET by disabling the exchange of Q_BH_2/Q_B with the quinone pool in the thylakoid membrane: since Q_BH_2 cannot leave the Q_B -site, a blockade for proton delivery to Q_B has to take place, which leads to the proposal of bicarbonate being involved in the PT to Q_B^{2-} (Govindjee and Eaton-Rye 1986). This hypothesis was supported by the 1.9 Å resolution structure, which shows that the Q_B -site is more or less devoid of water, but water molecules can mainly be found around D1-H252 and in the region of D1-Y246 and D1-E244 (**Figure 1.4 (A)**). Assuming that PT requires a network of protonatable amino acid site chains and water molecules (e.g., Bondar and Dau 2012), the water molecules indicate two possible PT pathways to Q_B : via D1-H252 and D1-S264 to the distal carbonyl group of Q_B and via D1-Y246, the bicarbonate and D1-H215 to the proximal carbonyl group. If bicarbonate is removed, the latter pathway is disturbed and the first proton likely takes the route via D1-S264 and $Q_A^\bullet Q_BH^\bullet$ is formed (Shevela et al. 2012), which is supported as well by recent QM/MM computational modelling approaches (Saito et al. 2013). The question remains, whether bicarbonate is

actually actively involved in PT as a protonatable group or supports the hydrogen bond network which promotes the PT.

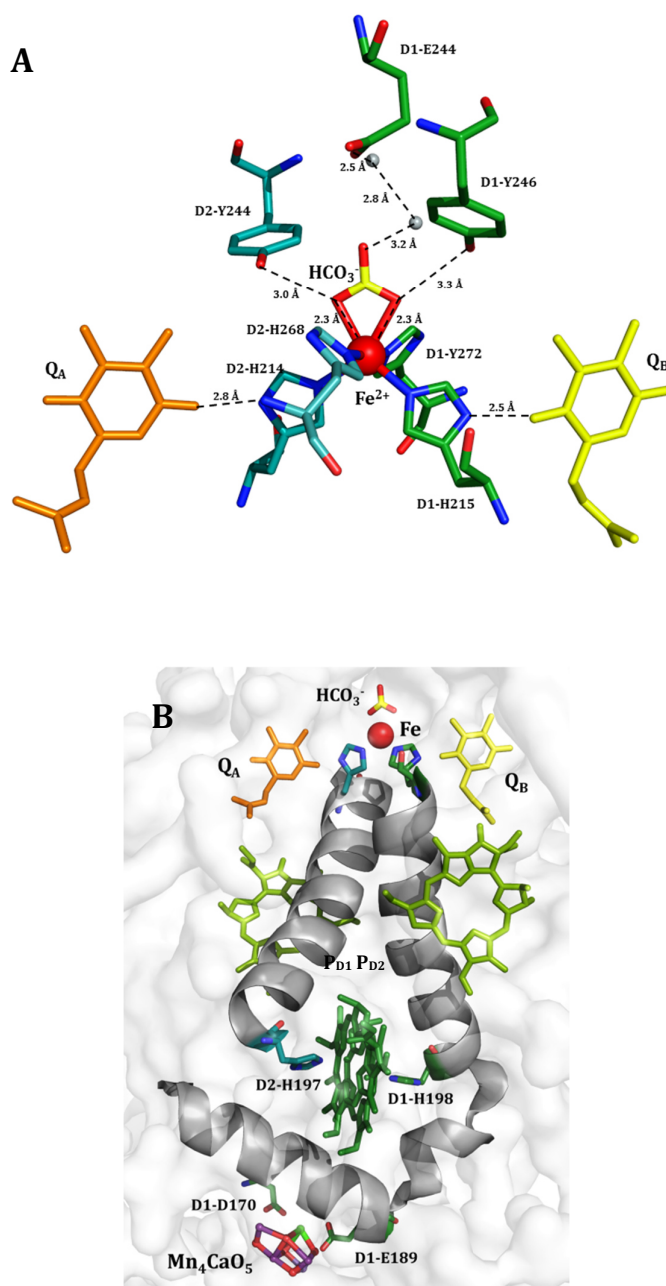


Figure 1.4 (A) Hydrogen-bond network around the non-heme iron and the bicarbonate ligand at the electron acceptor side of Photosystem II and involved amino acids and water molecules. The dashed lines indicate the hydrogen bonds. (B) Structural connection of the acceptor and donor site in Photosystem II via the D1-H215 and D2-H214 close to the NHI, which are linked to the axial ligands D1-H198 and D2-H197 of P_{D1} and P_{D2}, respectively. This connection extends even further in the PsbA subunit down to direct metal ligands of the OEC, such as D1-E189 and D1-D170 (pdb reference 3ARC, Umena et al. 2011).

1.2.4 The energetic picture of PSII based on thermodynamic and kinetic studies

Water is a very stable molecule and its oxidation as described in equation (1.1) requires the availability of a strong oxidizing species ($E_m(2H_2O/O_2) = +820\text{mV}$, pH 7). PSII is the only biological complex, which is able to split water by generating and stabilizing a sufficient high enough oxidizing power (Rappaport and Diner 2008).

When excitation arrives at the PSII reaction center, it is not localized on a specific pigment as in purple bacterial reaction centers, but a slew of different charge separations occur in the first few picoseconds, which leads to an electron transfer reaction resulting in the formation of the charge pair $P_{D1}^+Pheo_{D1}^-$ with the cation being mainly localized on P_{D1} (Groot et al. 2005; Holzwarth et al. 2006). Measurements of the initial charge separation process are complicated and multiphasic due to the difficulty in distinguishing between absorbance changes associated with excited states and radical pair states, which are nearly isoenergetic and coupled with inhomogeneous optical broadening. Femtosecond infrared and visible spectroscopy ($T \geq 4^\circ\text{C}$) provide quite different numbers for PSII reaction centers isolated from spinach: Groot et al. (2005) detect the formation of $Pheo_{D1}^-$ in 0.6 - 0.8 ps with P_{D1}^+ (often designated as “P680+” due its absorption maximum), whereas Holzwarth et al. (2006) find that the initial radical pair state containing $Pheo_{D1}^-$ appears in 3.2 ps and the state $P_{D1}^+Pheo_{D1}^-$ appears with a life time of 11 ps. Nevertheless, both groups agree that $Pheo_{D1}^-$ appears prior to P_{D1}^+ and attribute the oxidized donor to $Pheo_{D1}^-$ as Chl_{D1}^+ . Since Chl_{D1} is thought to be the longest wavelength pigment in PSII (Raszewski et al. 2008; Cox et al. 2009; Raszewski et al. 2005), the localization of the exciton on the pigment should be favored. The formation of the $Chl_{D1}^+Pheo_{D1}^-$ state is supported as well by Stark spectra, which indicate the existence of a $Chl_{D1}^{\delta+}Pheo_{D1}^{\delta-}$ charge transfer state (Frese et al. 2003). Electron transfer from $Pheo_{D1}^-$ to Q_A is reported to occur in the time scale of 200-500 ps (Renger and Holzwarth 2005).

Based on the kinetic studies of electron transfer dynamics in PSII (summarized in **Figure 1.5**), one can determine the relative free-energy level of various transition states formed during the turnover of PSII, which is essential for the understanding of the water-splitting mechanism. Additionally, the measurable midpoint potential of a limited number of titratable electron carriers allow a further completion of the energetic picture: if the free energy change associated with the electron transfer between one of

these titratable redox cofactors and another electron carrier is known, one can determine their relative reduction potentials.

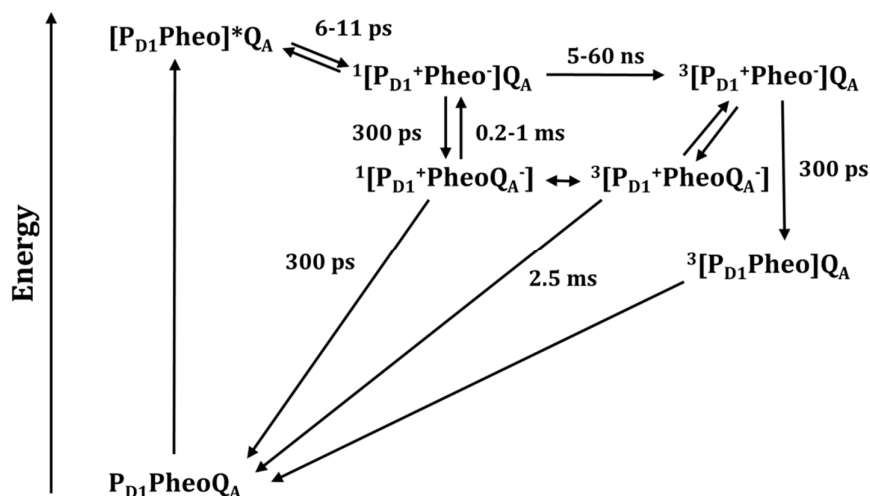


Figure 1.5 Electron transfer dynamics at the PSII acceptor side. Redrawn from Rappaport and Diner (2008).

Currently, the midpoint potentials of three electron carriers are directly measurable in redox titrations: Pheo_{D1}, Q_A and cyt_{b559}. Over the last 30 years, various groups have carried out these redox titrations, obtaining quite different results in their measurements: Klimov et al. (1979) and Rutherford et al. (1981) estimated the midpoint potential of the Pheo_{D1}/Pheo_{D1}⁻-couple to be -600 to -650 mV by measuring the amount of photo-accumulated Pheo_{D1}⁻. Recent measurements by Kato et al. (2009) on PSII core complexes isolated from *T. elongatus* using a spectroelectrochemical set-up in an optical thin-layer cell resulted in a potential of $E_m(\text{Pheo}_{D1}/\text{Pheo}_{D1}^-) = -505 \pm 6 \text{ mV vs SHE (pH 6.5)}$. A similar value was obtained by Allakhverdiev et al. (2010), who measured PSII complexes from *Synechocystis* sp. PCC 6803: $E_m(\text{Pheo}_{D1}/\text{Pheo}_{D1}^-) = -536 \pm 8 \text{ mV vs SHE (pH 7, 25}^\circ\text{C)}$. Additionally, they observed a significant influence of the commonly used PSII membrane stabilizer betaine on the potential: the absence of 1 M betaine in the titration buffer resulted in a negative shift of the redox potential to $-589 \pm 11 \text{ mV}$ (Allakhverdiev et al. 2010).

The midpoint potential of the Q_A/Q_A⁻-couple, the primary quinone electron acceptor in PSII, is as controversial as the one of the Pheo_{D1}/Pheo_{D1}⁻-couple: currently, two highly discussed midpoint potentials exist (see section 1.4.2 for a detailed discussion):

Shibamoto et al. (2009) obtained in spectroelectrochemical titrations of PSII core complexes from *T. elongatus* a value of $E_m (Q_A/Q_A^-) = -140 \pm 2$ mV, whereas Ido et al. (2011) could confirm in fluorescence titrations previously obtained values of PSII core complexes from *T. elongatus* of $E_m = -60$ mV $\pm$ 48 mV (Krieger et al. 1993, 1995; Johnson et al. 1995).

Considering these various references, different strategies may be followed to calculate the operating potentials of the other electron carriers. All of them rely on the determination of the free-energy change associated with electron transfer reaction and the assumption that the redox potential difference between the electron donor and acceptor corresponds to this change. Unfortunately, PSII bears contradictions between ΔG and ΔE_m , which have led to the distinction between the 'equilibrium redox potential' and the 'operating redox potential' (Rappaport and Diner 2008). This difference results, firstly, from different time domains involved in the equilibrium redox titrations and functional analysis, since redox titrations require the thermodynamic equilibrium between the sample and the solution and functional analysis allows the probing of transient states, where the free- energy may significantly differ from that of equilibrated states. Indeed, Armstrong et al. (2000) illustrated in fast-scan electrovoltammetry experiments that in response to the change in the redox state of a cofactor, the environment in the protein may undergo an energetic relaxation reaction, e.g., proton transfer or conformational changes, which potentially decreases the lifetime of the transient oxidized or reduced cofactor (Armstrong et al. 2000). Secondly, most of the membrane proteins involved in electron transfer reactions bind several cofactors which are usually located less than 15 Å from one another. This distance could result in a significant electrostatic interaction between the different cofactors, which is included in the determined free-energy level of a cofactor, illustrated e.g., by Gunner and Honig (1991) and Alric et al. (2004).

Nevertheless, these observations have not prevented groups from compiling energy schemes for the electron transfer processes occurring in PSII, combining results from thermodynamic and kinetic analyses. **Figure 1.6** shows a recent energy scheme of the electron transfer steps in PSII.

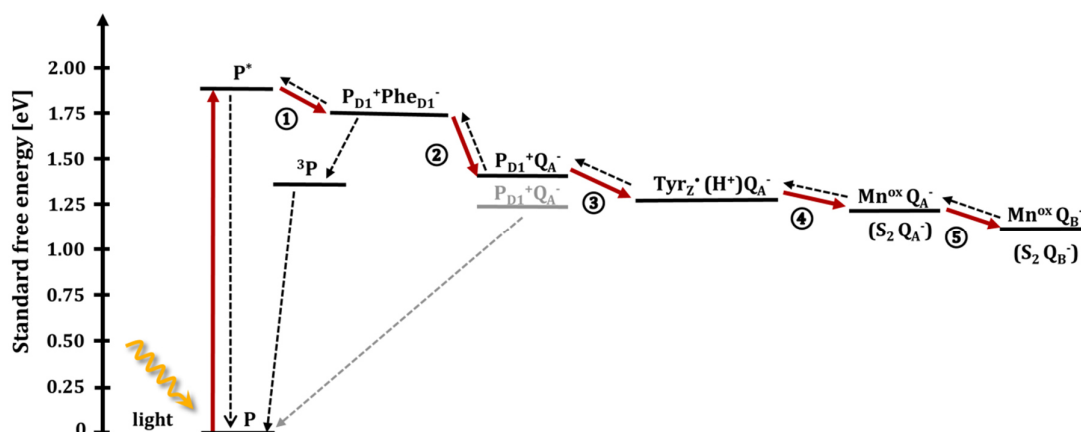


Figure 1.6 Energy scheme of the electron transfer steps according to **Figure 1.2 (B)** in PSII after the absorption of one red photon when all centers are in the dark adapted stable state, S_1 . The photon absorption results in the formation of the first excited singlet state, which is 1.8 eV above the ground state. **Section 1.2** and **Figure 1.3 (A)** describe the complexity of this process in more detail. Within 1 ms and a quantum yield of about 90 %, Q_B is reduced by one electron and the Mn_4CaO_5 cluster is oxidized by one electron. Energy is lost after each step, with the major loss occurring when the radical pair $P_{D1}^+Q_A^-$ is formed. Back reactions are indicated with dashed lines. Since the midpoint potential of the Q_A/Q_A^- couple changes in inactive PSII centers, the charge recombination pathway from the $P_{D1}^+Q_A^-$ state is different in these centers, which is indicated in grey. Redrawn from Cardona et al. (2012).

1.2.5 Charge recombination pathways and the challenge of dealing with oxygen

One of the main challenges encountered by Photosystem II is to find a way to favor energy-conversion processes over competing reactions in which the high energy intermediates decay to the ground state and lower the efficiency of the reaction. This requires the avoidance of back-reactions, short-circuits, by-passes, side-reactions, futile cycles and leaks. PSII mainly deals with this obstacle through kinetic control: forward reactions are faster than backward reactions (Rutherford et al. 2012). Cofactors are appropriately spaced within the protein to secure rapid vectorial electron transfer across the membrane, separating the positive and negative charges from each other. Direct recombination reactions of the radical pairs are strongly exergonic; the standard free energy gap is so big that the reactions are in the “Marcus inverted region” and are therefore relatively slow (Marcus, 1956). Additionally, the increased distance between the two charges of the radical pair slows down direct recombination (Rutherford et al. 2012).

Furthermore, a simple way to prevent energy loss is to actively slow-down the back-reaction by making it strongly uphill: the shortest route for the electrons to get back to $P_{D1}^{•+}$ from $Q_B^{•-}$ is e.g., via $Pheo_{D2}$, the pheophytin on the “non-functional” second branch of the reaction center (“B-side”). The distance is about 9 Å, but the energy gap is thought to be very large, since the $Pheo_{D2}$ midpoint potential is suggested to be more negative than the one of $Pheo_{D1}$ and the potential for the $Q_B/Q_B^{•-}$ couple is about 100 mV more positive than the one of the $Q_A/Q_A^{•-}$ couple (Wakeham et al. 2003; Rappaport and Diner 2008). Therefore, a back-reaction via this route is eliminated, contributing to a long life time of the $Q_B^{•-}$ state. A potential back-reaction route for the electron from $Q_B^{•-}$ is via Q_A and since both cofactors are similar in energy - they are already in equilibrium. The next step from $Q_A^{•-}$ to $Pheo_{D1}$ is the step requiring a significant amount of energy: their midpoint potentials differ by several hundred meV (Johnson et al., 1995; Rappaport et al. 2002). Nevertheless, PSII is able to undergo this route of charge recombination, where the exact pathway is determined by the size of the energy gap between Q_A and $Pheo_{D1}$: charge recombination can be ‘direct’, from $Q_A^{•-}$ to $P_{D1}^{•+}$ and ‘indirect’, via the formation of the $P_{D1}^{•+}Pheo_{D1}^{•-}$ radical pair. PSII is able to modulate the size of the energy gap and therefore, the yields of these pathways to mitigate protein damage and optimize function (Johnson et al. 1995).

During the ‘indirect’ pathway, the $P_{D1}^{+}\text{Pheo}_{D1}^{-}$ pair decays to the $^3P_{D1}$ triplet state (Rutherford et al. 1981; Keren et al. 1997; Johnson et al. 1995). This triplet state lies about 1.3 eV above the ground state and since PSII is far from operating anaerobically, but indeed, produces O_2 itself, the triplet is likely to react with $^3O_2$ to form singlet oxygen ($^1O_2$, 0.98 eV; Schweitzer and Schmidt 2003), a highly reactive and damaging species (Keren et al. 1997; Johnson et al. 1995). Most purple bacterial reaction centers are able to quench the ^3P state with carotenoids, which are in van der Waals contact with the bacteriochlorophylls of the reaction center, before it can react with $^3O_2$. In PSII, the chlorophylls in the core reaction center are too oxidizing that carotenoids in proximity cannot approach them without being oxidized themselves (van Gorkom and Schelvis 1993). In fully-functional PSII, electrons for the P_{D1}^{+} reduction are plentifully available due to the water-splitting and the indirect charge recombination route occurs infrequently. Nevertheless, e.g., during photoactivation processes, when PSII is already assembled but the Mn_4CaO_5 -cluster is still absent, this process is switched off due to the higher redox potential of the Q_A/Q_A^{-} couple (see section 1.4.2): in the absence of the Mn_4CaO_5 cluster, the structural surrounding of Q_A changes and the Q_A/Q_A^{-} midpoint potential is about 150 mV more positive than in the functional enzyme (Krieger et al. 1995; Johnson et al. 1995). This results in a larger free energy gap between $P_{D1}^{+}Q_A^{-}$ and $P_{D1}^{+}\text{Pheo}_{D1}^{-}$ and therefore, the other charge recombination route is favored: the ‘direct’ pathway, where the formation of the troublesome $^3P_{D1}$ state is avoided.

Another demonstration of the redox-tuning abilities is demonstrated to be caused by the binding of herbicides to PSII (Krieger-Liszkay and Rutherford 1998): when phenolic herbicides bind in the Q_B pocket, they not only block the electron transfer from Q_A to Q_B , but also change the structural surrounding of Q_A by binding via H-bonds to the imidazole which is a ligand to the non-heme iron. This is suggested to weaken the H-bond of the imidazole-Fe-imidazole motif to Q_A on the other side (Takahashi et al. 2010) and to cause a change of the Q_A midpoint potential in a way that the ‘indirect’ recombination route via $P_{D1}^{+}\text{Pheo}_{D1}^{-}$ is favored. Van Gorkom et al. (1993) suggested that the indirect route is the major recombination pathway at room temperature, which was shown by Rappaport et al. (2002).

Another (indirect) recombination pathway occurs with a yield of approximately 3 % (de Grooth and van Gorkom 1981): the reformation of P^* followed by an exciton decay to the ground state P via emitted fluorescence.

All processes are in an equilibrium electron transfer reaction with the S_2 state. Other recombination routes such as the electron transfer from $\text{Pheo}_{D1}^{\bullet-}$ to the Mn_4CaO_5 cluster (S_2 state) or $\text{Q}_A^{\bullet-}$ to Y_Z^{ox} can rather be disregarded due to the distances and involved equilibrium constants.

1.2.5.1 Chlorophyll fluorescence

When the chlorophyll a molecules in the PSII reaction centre absorb light energy and are excited, i.e., electrons are moved to a higher energy state, they drive the primary photochemical reactions involving charge separation and electron transfer. This photochemical pathway is, however, not the only way in which the exciton energy is consumed: the other two competing pathways are i) thermal dissipation, the non-radiative de-excitation of the excited states of the chlorophyll molecule to heat, and ii) chlorophyll fluorescence, the emission of photons by the radiative de-excitation of excited molecule (Rohacek and Bartak 1999). The three energy pathways are in competition to each other and the path with the most rapid rate constant predominates (Campbell et al. 1998).

In higher plants, chlorophyll fluorescence originates almost exclusively from chlorophyll a, because the energy of the excited states of chlorophyll b is transferred with a high efficiency to the chlorophyll a molecules. Furthermore, chlorophyll fluorescence is predominantly emitted from PSII at room temperature (about 90 %; Govindjee 1995). This is a consequence of the requirement for the efficient operation of the electron transport between PSII and PSI, which requires that the populations of PSII and PSI work in series and their reaction centers transfer electrons at similar rates: PSI has a higher proportion of chlorophyll a compared to PSII, with chlorophyll a/b ratios of 9 for PSI compared to 2.5 for PSII (Ben-Shem et al. 2003; Umena et al. 2011). Therefore, the probabilities of light absorption by PSII and PSI change depending on the spectral properties of the incoming light and its temporal and spatial dynamics (Porcar-Castell et al. 2014).

Measuring chlorophyll fluorescence has become a very useful technique to rapidly obtain qualitative and quantitative information on photosynthesis, e.g., the organization, function and acclimation of the photosynthetic apparatus at the subcellular and leaf levels (Baker 2008; Govindjee 1995; Maxwell and Johnson 2000). Various types of fluorimeters have been developed. Among them, fluorimeters working on the principle

of *pulse amplitude modulation* (PAM) of the chlorophyll fluorescence emission are widely used (Schreiber et al. 1986). The main feature of the PAM is that it produces two different light signals, a measuring beam and a saturation pulse, through the fiber optic probe. Firstly, the red-light measuring beam is rapidly switched on and off with pulse widths in the microsecond range, a constant pulse amplitude and pulse frequencies in the range of 10 Hz to 200 Hz (Porcar-Castell et al. 2014). This modulated beam is in principle not strong enough to stimulate photosynthesis, but does generate a fluorescence signal. The fluorescence value obtained by the measuring light is termed F_0 in dark-adapted tissue and F_t (or just F) when the tissue is illuminated by actinic or background light and it is not in the dark. In other words: the fluorescence signal from the measuring light is representative of the reaction centers which are 'open': they are oxidized (with Q_A being oxidized in PSII) and the RC is available for photochemistry. Secondly, a very intense pulse of white light (0.6 s - 1 s) saturates all reaction centers such that they become 'closed' (Porcar-Castell et al. 2014). Closed reaction centers are fully reduced (with Q_A^- being present in PSII) and are therefore temporarily unavailable for photochemistry. At this point, fluorescence becomes maximal, and the value is noted as either F_m in dark-adapted plants or as F_m' in samples under actinic light. Due to the weakness of the modulated measuring light, the fluorescence quantum yield (Φ_F) of dark-adapted samples can be determined and the fluorescence quantum yield at different intensity levels of the actinic light. It is defined as the ratio of n_F , the total number of emitted photons and n_A , the total number of photons absorbed:

$$\Phi_F = \frac{n_F}{n_A} = \frac{k_F}{k_F + k_D + k_T + k_P}. \quad (1.2)$$

Another way of expressing Φ_F is the use of the rate constants of the de-excitation processes (Govindjee 1995), where k_F is the rate constant of the chlorophyll a fluorescence, k_D the thermal deactivation, k_T the excitation energy transfer to non-fluorescent pigments and k_P the rate constant for photochemical process.

1.3 Electrochemistry of membrane proteins

Integral membrane proteins account for approximately one-third of the coding capacity of the genes in an average organism and are critically important for a significant amount of central functions in the cell (Melin and Hellwig 2013). However, they are extremely difficult to study due to their hydrophobic and amphiphilic nature, but since a significant amount of their reactions are redox reactions, electrochemical methods can play a considerable role in studying their mechanisms. Dynamic electrochemical techniques have been successfully applied to investigate the thermodynamic and kinetic properties of (membrane) proteins which are electrochemically connected or “wired” to an electrode surface. The common approach is to immobilize the protein on an electrode surface or solubilize it in the presence of i) a detergent in order to disperse the protein in the medium and ii) appropriate, soluble redox mediators, which mediate the electron transfer between the electrode and the respective cofactor in the protein. In the latter studies, electrochemistry is usually coupled with spectroscopy [ultraviolet (UV)-visible (VIS), infrared, electron paramagnetic response (EPR)] to determine the midpoint potential of a cofactor and monitor changes in the protein structure or the cofactor itself upon oxidation or reduction. When the protein is studied immobilized onto an electrode surface, the main challenge is to adsorb the protein in the native state while efficiently exchanging electrons.

1.3.1 Spectroelectrochemistry

Spectroelectrochemistry allows the recording of a specific molecular response as a consequence of a chosen electrode potential (Melin and Hellwig 2013). The incremental application of a potential gradually changes the spectral profile of a system corresponding to the changes in the population of oxidized and reduced species. This allows the determination of various redox properties, including the midpoint potential (E_m) and the number of electrons n transferred (Dhungana and Crumbliss 2008). Electrode materials are adjusted to the cell-type which is chosen for the respective study, including the cuvette type, the material of the cuvette window and/or the solvent. Their selection is based on the effective electrochemical window determined by the overpotential range which is exhibited by the electrode material for solvent reactivity; e.g., a gold mesh working electrode provides a window to study systems with more

negative redox potentials compared to a platinum mesh electrode. The surface of the working electrode needs to be modified in order to avoid denaturation of the protein during the experiment and has to be carefully cleaned between each experiment to obtain accurate and reproducible results (Dhungana and Crumbliss 2008).

UV/VIS or fluorescence spectra are usually specific for the redox state of a cofactor and the combination with electrochemistry allows the identification of compounds which undergo heterogeneous redox conversion, e.g., cytochrome c (Taniguchi et al. 1980; Moss et al. 1990) and hemoglobin (Song and Dong 1988) has been studied with UV/VIS spectroelectrochemistry. The use of an optically transparent thin-layer electrode (OTTLE) cell is ideal for the UV/VIS spectral monitoring of redox profiles. It requires only a small sample volume and allows for maximum exposure of the sample to the electrode surface. In fact, electrochemistry takes place in a chamber with a volume of about 30-60 μL (with a pathlength of 0.02 - 0.04 cm), resulting in a very thin diffusion layer between the working electrode and the bulk solution and a decrease in the ohmic-level drop. This is ideal to rapidly obtain an equilibrium after each applied potential, thereby cutting down the overall time necessary to complete the experiment and to avoid the exposure of the protein to an electrical potential for extended periods (Dhungana and Crumbliss 2008). Experiments in an OTTLE cell are usually carried out using a three electrode system (working, reference and auxiliary) and by flushing the solution with Ar or N₂ to maintain anaerobic conditions.

A main challenge in protein (spectro-)electrochemistry is the efficient facilitation of heterogeneous electron transfer between the protein and the electrode, which is usually quite slow and often requires the longer application of a specific potential or a significant overpotential. Long exposure times can cause the denaturation and/or aggregation of the protein, which disrupts the electron transfer and results in an inaccurate redox profile. To avoid these problems, electrochemical mediators are used which rapidly diffuse and penetrate the protein structure, facilitating the electron transfer between the electrode and the redox site. Dutton (1978) and Dhungana and Crumbliss (2008) summarize the necessary requirements for a mediator to serve its function:

- i) The redox mediator has to react effectively and reversibly with the electrode and the redox cofactor without altering the redox profile of the protein.

- ii) It should not decompose upon oxidation or reduction.
- iii) The redox potential has to be close to that expected of the protein cofactor and can be estimated using the following equation:

$$|E_m^0 - E_p^0| \leq \frac{2RT}{n_m F} \quad (1.3)$$

where E_m^0 is the redox potential of the mediator, E_p^0 is the redox potential of the protein, n_m is the number of electrons transferred for the mediator, R is the gas constant ($R = 8.3145 \text{ J}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$), T is the temperature in Kelvin and F is the Faraday constant ($F = 96485.34 \text{ C}\cdot\text{mol}^{-1}$).

- iv) The mediator should undergo rapid reversible electron transfer (heterogeneous and homogenous) at the electrode surface and the redox-active site of the protein with its concentration being in excess over the protein concentration to facilitate favorable electron-transfer kinetics.

The recorded changes in absorbance or fluorescence represent the changes in absolute concentration of the oxidized and reduced species at various applied electrode potentials and the spectroscopic data from a spectroelectrochemical experiment can ideally be fitted to the Nernst equation (Dutton 1978; Dong et al. 1995): the fraction of reduced protein, which is proportional to the spectral intensity according to Beer's law can be plotted against the applied potential and the data can be fitted according to the converted Nernst equation (1.4):

$$E = E_m + \left(\frac{RT}{nF}\right) \ln\left(\frac{[ox]}{[red]}\right) \quad (1.4)$$

$$\Rightarrow \frac{[red]}{[tot]} = \frac{1}{\left(1 + e^{\left(\frac{E - E_m}{\frac{RT}{nF}}\right)}\right)} \quad (1.5)$$

With $n = 1$ (for a one-electron system), $R = 8.3145 \text{ J}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$, $F = 96485.34 \text{ C}\cdot\text{mol}^{-1}$ and the temperature, T (298.15 K), the following equation is obtained to calculate the fraction of reduced protein:

$$\frac{[red]}{[tot]} = \frac{1}{\left(1 + e^{\left(\frac{E - E_m}{25.6mV}\right)}\right)} \quad (1.6)$$

1.3.2 Protein film voltammetry

Protein film voltammetry (PFV) consists of the direct electrochemical characterization of thin films of redox proteins immobilized on the surface of electrodes (Armstrong 1990; Hirst 2006; Leger and Bertrand 2008; Armstrong et al. 2000; Gulaboski et al. 2012). Adsorbed proteins are usually investigated by cyclic voltammetry, square wave voltammetry or differential pulse voltammetry, three dynamic methods based on the measurement of the current or differences of the current upon the application of a time-dependent potential, providing so-called current-potential curves (Bard and Faulkner 2001). Another method is chronoamperometry, which consists of current vs time measurements at a constant potential after applying a perturbation. In this set-up, the electrode is meant to act as a redox partner of the enzyme, allowing the control of the redox states of the cofactors and consequently, of the catalytic activity by the application of a potential. This approach offers a significant number of advantages (Melin and Hellwig 2013):

- Only a small amount of sample is required; usually about 10^{-12} - 10^{-11} mol/cm² is sufficient enough to obtain a protein monolayer on the electrode surface.
- With only one modified electrode, various conditions such as temperature, pH or inhibitor concentration can be studied.
- The protein is “wired” to the electrode surface; therefore, obstacles occurring due to protein diffusion to the electrode surface are suppressed.
- The voltammograms provide thermodynamic and kinetic information of the protein simultaneously.
- Potential scan rates can be finely tuned from 1 mV/s to 1000 V/s, allowing the investigation of slow and fast chemical reactions coupled to electron transfer.

The obtained current-potential curves contain in general three principal contributions: i) the inherent catalytic properties of the protein, ii) the kinetics of interfacial electron transfer (IET) from the protein to the electrode and iii) the diffusion properties of the substrate and products, which can be avoided by using rotating electrodes, allowing a constant flux of substrate to diffuse to the enzyme. If the most accessible cofactor of the enzyme can be brought within a distance of 14 Å to the electrode surface, direct electron transfer (DET) from the protein to the electrode can occur (Page et al. 1999). In this

case of DET, the enzyme can exhibit its catalytic activity directly, e.g., in cyclic voltammograms by a sigmoidal wave at a potential corresponding to the reduction or oxidation of the cofactors. Furthermore, the Michaelis-Menten constant K_m can be determined in this way by varying the substrate concentration in the electrolyte. If direct electron transfer is too slow or cannot occur because the cofactors are too far away from the protein surface, redox mediators or smaller proteins can be added to the electrolyte or co-immobilized to improve the communication between protein and electrode.

1.4 Electrochemistry of Photosystem II

Photosystem II is a multi-cofactor protein and as described above, its energetic characterization is quite a difficult undertaking. Recently, electrochemical approaches have been investigated as a tool for completing this picture and for carrying out fundamental studies of the enzyme. A number of reports describe e.g., the immobilization of PSII on electrodes and the observation of photocurrents in the absence and presence of external redox mediators (Badura et al. 2008; Terasaki et al. 2008), the use of this set-up for the detection of herbicides (Varsamis et al. 2008; Bettazzi et al. 2007), explosives (Bhalla and Zazubovich 2011) or to determine in cyclic voltammetry experiments the midpoint potential of cofactors (Zhang et al. 2014; Alcantara et al. 2006). In other systems, PSII preparations from cyanobacteria and higher plants have been used as electron sources in hybrid systems with metal oxides for water oxidation and photocurrent generation (Kato et al. 2012, 2013) and photo-biofuel cells have been designed with PSII photoanodes (Yehezkeli et al. 2014), which were used e.g., in combination with a bilirubin oxidase/ carbon nanotube cathode to convert solar energy into electrical energy (Yehezkeli et al. 2012).

Recently, it has become of interest to study PSII as well spectroelectrochemically, e.g., in the described OTTLE cell. This approach has been primarily used for the determination of the midpoint potential of cofactors: Shibamoto et al. 2008 introduced this technique to determine the redox potential of cyt b_{559} in the D1-D2-cyt b_{559} complex from spinach. They applied this set-up as well to measure the redox potential of the Q_A/Q_A^- couple in various cyanobacteria and higher plants (Shibamoto et al. 2009; 2010), which was shortly afterwards verified by Allakhverdiev et al. (2011) using a standard potentiometric setup. The group studied as well the influence of Ca^{2+} , Sr^{2+} , Cl^- and Br^- ion

exchange on the Q_A/Q_A^- redox potential in PsbA1 and PsbA3 mutants in *T. elongatus*, which are the genes encoding for the D1 protein under normal and high light conditions, respectively (Kato et al. 2012c). Furthermore, the redox potential of the Pheo_{D1}/Pheo_{D1}⁻ couple has been determined by using a spectroelectrochemical approach: in various cyanobacteria (Kato et al. 2009) including the chlorophyll d containing species *Acaryochloris marina* (Allakhverdiev et al. 2010) and in the PsbA3 mutant of *T. elongatus* (Sugiura et al. 2014).

1.4.1 The Q_A/Q_A^- redox potential

The midpoint potential of the Q_A/Q_A^- -couple has been studied for several decades and as indicated above, it turned out to be quite a complex undertaking: Krieger et al. reported in a literature survey from 1995 over 35 different values for the Q_A/Q_A^- couple (Krieger et al. 1995). The reason for this variation was found to be dependent on the titrated species, the titration method (fluorescence, spectroscopic, EPR) and the conditions used during the titration (pH, temperature, presence/absence of redox mediators and inhibitors) and led to values which are not necessarily comparable. Studies by Krieger et al. (1993) showed for the first time the influence of the functional and structural state of the enzyme on the potential: steady state redox titrations of the fluorescence yield performed at pH 6.5 revealed that the midpoint redox potential was shifted towards positive direction from $E_m = -80$ mV to $E_m = +40$ mV after incubation of PSII particles at pH 4.2 for 15 min in the presence of a Ca^{2+} chelator (Krieger et al. 1993). The original midpoint potential was restored after the addition of $CaCl_2$. In 1995, Krieger et al. could confirm the existence of these high and low potential values for the Q_A/Q_A^- redox potential and showed that the low potential form existed in PSII complexes in which the Mn_4CaO_5 cluster was absent. Both potentials were independent of the pH from pH 5.5 to pH 7.5. Further on, Johnson et al. (1995) associated the existence of the high and low potential form with photoactivation: a treatment of PSII enriched membranes from spinach with hydroxylamine caused the release of the Mn_4CaO_5 cluster and resulted in the upshift of the Q_A redox potential. When oxygen evolution was reconstituted by incubation of the sample in light in the presence of $MnCl_2$ and $CaCl_2$, the midpoint potential of the Q_A/Q_A^- -couple was shifted back from +55 mV to lower potentials (-80 mV), which were obtained in intact samples. The possibility for PSII, to “switch” between these two potential forms was suggested to play a significant role in

protecting PSII during the assembly of the Mn_4CaO_5 cluster (Johnson et al., 1995): the difference ΔE_m was suggested to determine the route of charge recombination from $\text{Q}_\text{A}^\bullet$, being either 'direct' or 'indirect', as discussed in section 1.2.5: the high potential form of the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ -couple in the absence of the Mn_4CaO_5 cluster leads to a larger energy difference between $\text{P}_{\text{D1}}^{\bullet+}\text{Q}_\text{A}^\bullet$ and $\text{P}_{\text{D1}}^{\bullet+}\text{PheOD1}^\bullet$, which favors the 'direct' charge recombination pathway and avoids the formation of the $^3\text{P}_{\text{D1}}$ state.

Shibamoto et al. (2009, 2010) could confirm the existence of the two redox potentials for the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ -couple and obtained a similar value for ΔE_m (+160 mV), but determined much lower overall potentials: they obtained a potential of $E_m(\text{Q}_\text{A}/\text{Q}_\text{A}^-) = -140$ mV for intact PSII and +20 mV for Mn-depleted PSII isolated from *T. elongatus*. After the publication of a value for the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ -couple of $E_m = -162 \text{ mV} \pm 3 \text{ mV}$ for PSII enriched membranes from spinach in the presence of redox mediators determined again by spectroelectrochemistry (Shibamoto et al. 2010), Krieger et al. subsequently re-measured and verified their previously determined midpoint potentials and obtained values of $E_m = -60 \text{ mV} \pm 48 \text{ mV}$ for intact PSII and $E_m = +60 \text{ mV} \pm 25 \text{ mV}$ for Mn-depleted PSII (Ido et al. 2011).

The magnitude of the redox potential shift from the low to the high potential form was recently verified as well by Allakhverdiev et al., although the absolute values for the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ -couples were found to be 60 mV lower (Allakhverdiev et al. 2011) and are in agreement with Shibamoto et al. (2010).

Beside studies of the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ redox potential in various organisms and PsbA mutants in cyanobacteria (see above), the influence of herbicide binding to PSII has been investigated: urea-type herbicides such as DCMU cause an upshift of the redox potential of the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ couple to $E_m(\text{Q}_\text{A}/\text{Q}_\text{A}^-) = -28$ mV, whereas phenolic herbicides such as ioxynil and bromoxynil were demonstrated to cause a downshift of the midpoint potential to $E_m(\text{Q}_\text{A}/\text{Q}_\text{A}^-) = -113$ mV and -125 mV, respectively (Krieger-Liszkay and Rutherford 1998). It is suggested that phenolic herbicides interact with the D1-His215 residue, which coordinates the non-heme iron: Fourier transform infrared (FTIR) difference spectra indicate the formation of a strong hydrogen bond between the carbonyl group of the phenolate form of the ioxynil and the nitrogen atom of the D1-His215 (**Figure 1.7**). This hydrogen bond is proposed to cause a change in the hydrogen bond network around the non-heme iron, which affects as well the hydrogen bond between the carbonyl group of Q_A^- and the nitrogen atom of the D2-H214, another ligand to the non-heme iron. This change is suggested to lower the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ redox potential

(Takano et al. 2008), which is proposed to subsequently lead to charge recombination favoring the 'direct' route via the formation of the $^3P_{D1}$ state and thus increasing the efficiency of photodamage (Rutherford and Krieger-Liszky 2001).

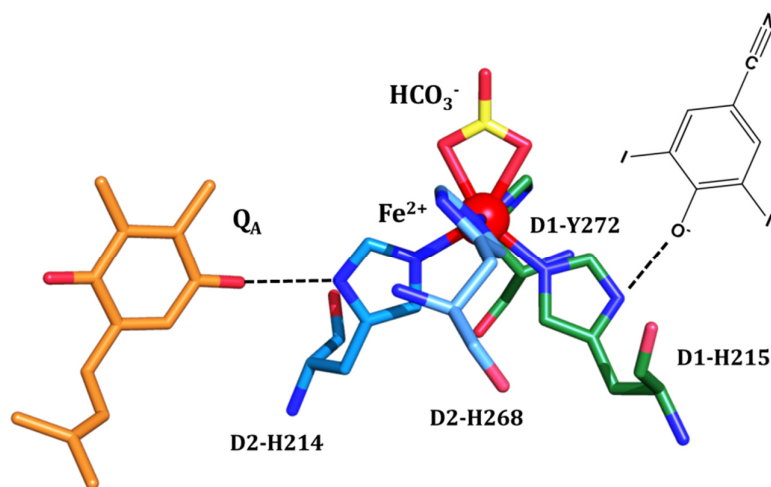


Figure 1.7 Interaction of the phenolic herbicide ioxylnil with the non-heme iron ligand D1-H215 via the formation of a strong hydrogen bond (dashed line) according to Takano et al. (2008), which is likely to affect the hydrogen bond (dashed line) between Q_A and the other non-heme iron ligand D2-H214 in close proximity, resulting in a redox potential change of the Q_A/Q_A^- -couple (pdb reference 3ARC, Umena et al. 2011).

1.4.1.1 Potentiometric vs spectroelectrochemical redox titrations

The potentiometric redox titrations carried out by Krieger et al. (1995, 1998) and Ido et al. (2011) are based on the gradual addition of either the reductant sodium dithionite for the reductive or the oxidant potassium ferricyanide for the oxidative titrations while recording the chlorophyll a fluorescence as an indication of the reduction state of Q_A with a PAM fluorimeter: the reduction of Q_A blocks the electron transfer from $Pheo_{D1}$ to Q_A and the absorbed photon energy is dissipated as fluorescence from the chlorophyll a molecules in the PSII reaction center and its antenna (Cramer and Butler 1969). In the spectroelectrochemical approach by Shibamoto et al. (2009, 2010), the fluorescence level of chlorophyll a was recorded in a spectrofluorimeter while reducing Q_A electrochemically in the described OTTLE cell set-up. The redox titrations were carried out in the presence of external redox mediators, anthraquinone-2-sulfonate and

N,N,N',N'-tetramethyl- p-phenylenediamine (TMPD, $E_m = +300$ mV, pH 6.5), which influenced the Q_A/Q_A^- midpoint potential significantly in measurements carried out by Ido et al. (2011): the group reported a potential shift of $\Delta 80$ mV towards lower potentials in the presence of redox mediators when titrating inactive, manganese-depleted PSII samples from spinach, which could account for the different value obtained by Shibamoto and coworkers. However, a specific redox mediator, which influences the Q_A redox potential, could not be identified. The question has remained open about which values are the relevant E_m values for the Q_A/Q_A^- -couple.

1.4.2 Protein film voltammetry of PSII

In the quest for alternative energy conversion systems and the production of biofuels, the interest in the integration of PSII in photoelectrochemical devices has rapidly grown (Esper et al. 2006). Aiming at increasing the photocurrents for potential application in bio-photoelectrochemical devices, investigators have explored different electrode materials and their chemical modification as described above. Three main variables have been identified to play a role in controlling the magnitude of the photocurrent: the electronic coupling between the immobilized PSII and the electrode, the orientation of the protein complex with respect to the electrode surface and the protein concentration on the surface. Electronic coupling between the protein and the electrode is the one of the key issues for the construction of efficient electroactive devices, which is only poorly understood in the currently discussed PSII metal oxide hybrid systems (e.g., Kato et al. 2014).

1.4.2.1 Electron transfer studies of PSII with protein film voltammetry

Electron transfer from the protein to the electrode was suggested to occur with respect to the redox properties from two separate locations (**Figure 1.8**), both on the PSII acceptor side: Q_A and Q_B (Kato et al. 2012). Direct electron transfer from Q_A is theoretically possible, as it is located in proximity of the solvent-exposed surface of the protein. The functional role of Q_B is on the other hand to shuttle the electrons by exiting the protein and using a channel open to the membrane.

The availability of such a channel allows the use of external, mobile and artificial electron acceptors. When PSII is confined onto an electrode surface, two different ways of conveying the electrons to the electrode are therefore available: direct and mediated. Both have been exploited in the search for stable and efficient photocurrents. Enhanced non-mediated photocurrent was for example observed, when His-tagged PSII was immobilized onto Ni^{2+} -nitrilotriacetic acid (NiNTA) modified gold surfaces with the acceptor side facing the electrode surface (Zhao et al. 2002; Maly et al. 2005; Terasaki et al. 2008). The photocurrent was increased in this configuration, when the electrode surface was increasingly roughened with the deposition of Ni^{2+} -NTA-nanoparticles, resulting in a higher amount of immobilized PSII (Terasaki et al. 2008). Another system, where an increase in the protein concentration on the metal oxide resulted in an increase in photocurrent was recently described by Kato et al., where PSII was immobilized on mesoporous indium-tin oxide (*meso*ITO) electrode (Kato et al. 2012). The three-dimensional metal oxide environment allowed for a higher protein coverage and direct (mediator-free) electron transfer from PSII to *meso*ITO.

Another attempt to increase the photocurrent generated by PSII was made by using confined mediators. Examples are the immobilization of PSII in polymer hydrogels consisting of conductive Os(II/III)-polypyridine complexes as electron transfer relay units (Badura et al. 2008) and the immobilization of PSII on electrodeposited conductive layers of poly-mercapto-p-benzoquinone (polySBQ) on gold (Maly et al. 2005). The latter system was further developed by the Willner group with the construction of a water/ O_2 powered photo-bioelectrochemical cell with an anode consisting of PSII assembled on the pSBQ layer and a cathode of glassy carbon, modified with the oxygen reducing enzyme bilirubin oxidase (BOD, Yehezkeli et al. 2012).

While focusing on methods to increase the overall generation of photocurrent, only a few groups have so far explored the photophysical origin of the current generated by PSII immobilized onto metal oxide electrodes and made use of the possibility to investigate the protein mechanistically via direct electron transfer from PSII to the metal oxide.

PSII was reported to deliver electrons from Q_A to ferricyanide (Sugiura and Inoue 1999) and cytochrome c (Larom et al. 2010) and although it has not been explicitly demonstrated yet, Q_A was already suggested to be the donor for electron injection from PSII into metal oxides: a negatively charged region on the surface of PSII was recently identified in proximity of Q_A by Ulas et al. (2011), functioning as a docking site for

cationic exogenous electron acceptors. The group reported in mechanistic studies the full recovery of PSII oxygen evolution activity in solution in the presence of the herbicide

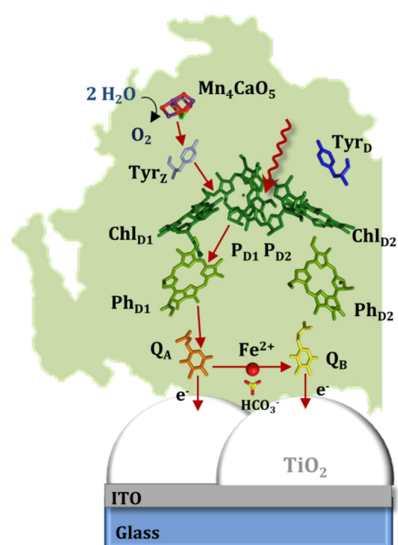


Figure 1.8 Scheme of the orientation of PSII on the TiO_2/ITO electrode and indication of the electron transfer steps after charge separation. The two possibilities of electron transfer from the enzyme to the electrode are indicated.

DCMU when Co^{III} complexes were added. Because of the inhibition effect of DCMU on Q_B and the electron transfer from Q_A to Q_B (Trebst 2007), they concluded the existence of an electron-transfer pathway from Q_A^- to Co^{III} . This observation is consistent as well with studies performed by Kato et al., where PSII immobilized on *meso*ITO gave rise to a significant level of photocurrent in the presence of DCMU (Kato et al. 2012). These results seem to indicate as well the occurrence of interfacial electron transfer not only from Q_B , but also from Q_A through an alternative electron pathway (**Figure 1.8**). The interpretation of these results was further complicated, when photocurrent was recorded in the presence of DCMU and 2,6-dichloro-1,4-benzoquinone (DCBQ) as an artificial electron mediator: DCBQ is known to bind into the Q_B binding pocket, accept electrons from Q_A and is exchanged against fresh acceptor molecules (Shevela

and Messinger 2012). Therefore, its presence leads to a higher catalytic turn-over rate of the OEC and to significantly enhanced photocurrents due to its function of transferring electrons directly from Q_A to the electrode. DCMU in turn inhibits the electron transfer from Q_A to DCBQ in solution by binding more strongly to the Q_B binding pocket and replacing DCBQ. The observation of a significant remaining photocurrent in a system, where DCBQ and DCMU are present, complicated the determination of the origin of photocurrent; Q_A and Q_B could both operate as electron donors.

1.5 Objectives of the research project

The midpoint potential of the redox cofactor Q_A in Photosystem II, $E_m(Q_A/Q_A^-)$, is one of the key parameters to determine the energetics within the enzyme and its exact value in plants and cyanobacteria is vitally important. The first part of the research project described in this thesis addresses the question of the discrepancy between the two existing midpoint potentials of the Q_A/Q_A^- couple obtained in potentiometric and spectroelectrochemical redox titrations: firstly, a set-up was designed, which allowed the reproduction of the conditions used by Shibamoto et al. (2009, 2010). It was evaluated by spectroelectrochemical titrations of an inorganic molecule (potassium ferricyanide) and an enzyme (horse-heart cytochrome c) in the presence of redox mediators to determine the error range of the obtained midpoint potentials in the set-up. Secondly, the midpoint potential of the Q_A/Q_A^- couple was measured in PSII enriched membranes isolated from spinach and in PSII core complexes isolated from *T. elongatus* by using the same conditions applied by Shibamoto et al. (2009, 2010). The obtained values were compared to the ones reported in the literature and further studies were carried out by specifically modifying PSII to identify the origin of the reported potential differences and complete its energetic picture.

The determination of the redox potential of the Q_A/Q_A^- couple resolved the problem of the discrepancy between the existing values and verified the picture of the energetic properties of the PSII electron acceptor side. The enzyme was then mechanistically and electrochemically investigated in a second part of the research project in a metal oxide hybrid system via protein film voltammetry: PSII was immobilized in form of mono- and multilayers onto metal oxide surfaces and the electron transfer from the protein into the metal oxide was studied when an external potentials was applied. The aim was to understand the fundamental principles of electron transfer from the protein into the metal oxide by studying the photocurrent generated by PSII upon illumination in this type of metal oxide hybrid system. Firstly, on the basis of the measured photocurrent, the source of electrons was identified and the anomalous effects of electron mediators and herbicides such as DCMU on the photocurrent were investigated. A model was developed to rationalise the discrepancies, allowing the reassessment of the utility of such systems for PSII investigations. Secondly, different semiconductor materials were tested as target materials in the PVF set-up for protein electron transfer studies and thirdly, the effect of oxygen on the photocurrents generated by PSII was investigated.

Chapter 2.

Materials and methods

2.1 Materials

3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 2,6-dichloro-1,4-benzoquinone (DCBQ; $E_m = +310$ mV, pH 7, determined via cyclic voltammetry in a three electrode system with a platinum mesh working electrode, a platinum counter electrode and an Ag/AgCl reference), 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one (quercetin; $E_m = +331$ mV, pH 7), p-benzoquinone ($E_m = +293$ mV, pH 7), 2,5-dimethyl-p-benzoquinone ($E_m = +176$ mV, pH 7), 1,4-naphthoquinone ($E_m = +58$ mV, pH 7), anthraquinone-2-sulfonate ($E_m = -195$ mV, pH 6.5), 2-hydroxy-1,4-naphthoquinone ($E_m = -100$ mV, pH 6.5) and N,N,N',N'-tetramethyl- p-phenylenediamine (TMPD, $E_m = +300$ mV, pH 6.5), nitrotetrazolium blue chloride (NBT), 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red), 4-hydroxy-3,5-diiodobenzonitrile (ioxynil), sodium oxalate, sodium glycolate, potassium ferricyanide and additional chemicals were purchased from Sigma Aldrich, as well as the enzymes horse heart cytochrome c, horseradish peroxidase, superoxide dismutase from human erythrocytes (2,500 units/mg protein) and catalase from bovine liver (2,000-5,000 units/mg protein). Nanostructured TiO₂ -electrodes on conductive ITO glass were obtained from Solaronix S. A., Aubonne, Switzerland (20 nm particle size, 0.15 μ m thickness and 80% 20 nm and 20% >100 nm particle size, 0.6 - 1 μ m thickness). Water was purified with a reverse osmosis (RO) filter (Neptune model L993162, Pure Limited, UK) to a specific resistivity to >18 m Ω cm.

If not indicated otherwise, all redox potentials refer to those vs SHE.

2.1.1 Synthesis and preparation of nano-crystalline WO₃ films

Nano-crystalline WO₃ films on conductive FTO glass were prepared according to Lui et al. (2015): 2 g of tungsten powder was dissolved in 20 mL of 30 % hydrogen peroxide and stirred for 5 h at room temperature. 2 mg of platinum black was added to decompose the excess hydrogen peroxide and the platinum was removed by filtration.

20 mL of acetone was then added to the solution to stabilize the resulting tungsten oxide hydrate from condensation and precipitation. 10 % by weight of the polymer polyethylene glycol (20,000) was added and stirred into the solution to increase the viscosity of the light yellow paste. Films of the paste were deposited directly after the synthesis on FTO coated glass slides. The FTO glass was cleaned beforehand with detergent and water and then rinsed with deionized water, acetone and isopropanol followed by heating at 450°C for 30 min. The films were deposited by spin-coating the sample 1) for 1.30 min at 1000 rpm to obtain a 650 nm film or 2) for 1.30 min at 3000 rpm to obtain a 150 nm film. After the film deposition, the coated FTO glass was heated at 450°C for 30 min.

2.1.2 Synthesis and preparation of TiO₂ electrodes with an amorphous TiO₂ blocking layer

Nanostructured TiO₂ electrodes on conductive FTO glass with a non-conductive layer of TiO₂ underneath (100 nm thickness) were prepared by spray pyrolysis according to Oja et al. (2014).

2.1.3 Electrochemical Impedance Spectroscopy (EIS) of TiO₂ and WO₃ films on a TCO coated glass

When an n-type semiconductor is placed into an electrolyte, its Fermi Level is adjusted with respect to the redox potential of the solution, aiming for an equilibrium situation. This causes band bending of the conduction band. Changing the voltage of the semiconductor artificially through the use of a potentiostat causes the semiconductor and redox couple Fermi levels to separate, and hence the level of band bending owing to electron depletion in the semiconductor will change depending on the applied voltage. If the applied voltage is such that there is no band bending or charge depletion, then the semiconductor is at its flatband potential, V_b (Gelderman et al. 2007).

To obtain experimental evidence of the flatband potential of TiO₂ and the WO₃ film and the electron density of the materials at the experimental conditions applied here, a Mott-Schottky plot of the metal oxides on ITO/FTO was determined using Electrical Impedance Spectroscopy (EIS). A small amplitude sinusoidal ac voltage $V(t)$ was applied

at the sample and the amplitude and phase angle (relative to the applied voltage) of the resulting current $I(t)$ was measured. Ohm's law was used to determine further on the impedance $Z(\omega)$:

$$V(t) = V_0 + V_m \sin(\omega t). \quad (2.1)$$

$$I(t) = I_0 + I_m \sin(\omega t + \theta). \quad (2.2)$$

$$Z(\omega) = \frac{V(t)}{I(t)}. \quad (2.3)$$

Here, V_0 and I_0 are the bias potential and the steady-state current flowing through the electrode, respectively, V_m and I_m are the maximum voltage and current at the sinusoidal signal and θ is the phase angle of the resulting current.

In quantitative terms, impedance is the complex ratio of the voltage to the current in an alternating circuit (AC). In practice, this means that a small sinusoidal AC voltage, $V(t)$, is applied to an electrode and the amplitude and phase angle of the current, $I(t)$, is measured at different frequencies. Impedance extends the concept of resistance to AC circuits, and possesses both magnitude and phase, unlike resistance, which has only magnitude. With the help of a Nyquist plot, which is recorded during the EIS, the impedance can be determined in this case using the equation for a constant phase

element, CPE (**Figure 2.1**). A constant phase element is an equivalent electrical circuit component that models the behaviour of a double layer; also called an imperfect capacitor (due to e.g., non-homogeneity of the surface such as roughness; Gelderman et al. 2007). Using the Boltzmann distribution to describe the distribution of electrons in the space charge region and Gauss' law relating the electric field through the interface to the charge contained within that region, the Mott-Schottky equation is obtained:

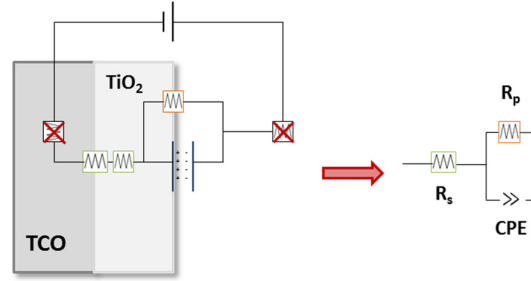


Figure 2.1 Modified Randles circuit used to model the TiO_2 electrolyte-electrode interface for calculations of the capacitance of a constant phase element (CPE). R_s is a series resistant, simulating the internal resistance at the TCO/ TiO_2 interface and inside TiO_2 , R_p is transient resistance reproducing the transient resistive effects of TiO_2 during charging and discharging.

$$\frac{1}{C^2} = \frac{2}{\varepsilon \cdot \varepsilon_0 \cdot A^2 e N_D} \left(V - V_{fb} - \frac{k_b \cdot T}{e} \right). \quad (2.4)$$

Where

C = interfacial capacitance, ε = dielectric constant, ε_0 = permittivity of free space, A = area, e = electronic charge, N_D = number of donors, V = applied potential, V_{fb} = flatband potential, k_b = Boltzmann's constant, T = absolute temperature.

Here, impedance spectra were recorded using the Autolab PG12 instrument including a Frequency Response Analyzer (FRA) and coupled with Nova electrochemical software. EIS data were gathered using a 10 mV amplitude perturbation between 10,000 Hz and 0.1 Hz in the dark. The EIS data were simultaneously fit to the equivalent circuit described with the model in **Figure 2.1** using the Zview software (Scribner Associates).

2.2 Equipment

A Thin Layer Quartz Glass Spectroelectrochemical cell Kit (OTTLE cell) was obtained from ALS Co., Ltd, Japan, with an optical path length of 0.5 mm and 1 mm, a gold mesh working electrode (100 mesh, gold gauze) and a platinum electrode (80 mesh, platinum gauze).

2.3 Culture conditions for His₆-tagged *Thermosynechococcus elongatus*

A strain of *T. elongatus* with a histidine tag on the CP47 protein of PSII was obtained as a liquid culture from Dr Karim Maghlaoui (Imperial College London). The cultures were grown in a rotary shaker (120 rpm) at 45 °C under continuous illumination using fluorescent white lamps of intensity of about 50 $\mu\text{E m}^{-2} \text{s}^{-1}$ in DTN-medium (Muhlenhoff and Chauvat 1996) supplemented with 10 mM sodium bicarbonate. For maintenance, the cells were grown in the presence of kanamycin (40 $\mu\text{g ml}^{-1}$) in closed flasks. For PSII preparations, the cells were grown in 5 L Erlenmeyer flasks to a maximum volume of 2.5 L. In total 15 L were cultured for PSII preparations.

2.4 Isolation, purification and characterization of PSII core complexes from *Thermosynechococcus elongatus*

Photosystem II particles were isolated from a CP47 His-tagged mutant from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 by Ni²⁺ affinity chromatography, based on a protocol described by Suigiura and Inoue (1999) with modifications by Boussac et al. (2004): cells were harvested when the optical density at 730 nm reached ~1.0 by using a cell concentrator pump (Watson-Marlow Pumps Group), followed by centrifugation and washing in Buffer 1, containing 40 mM MES (pH 6.5), 15 mM MgCl₂, 15 mM CaCl₂, 10 % (v/v) glycerol, 1.0 M betaine. Cells were resuspended in the same buffer to a concentration of chlorophyll a above 1 mg mL⁻¹. 0.2 % (w/v) bovine serum albumin, 1 mM benzamidine, 1 M aminocaproic acid and 50 µg mL⁻¹ DNase I were added and the suspension was passed twice through a chilled Cell Disruptor (Constant Systems, Model T5) at 25 kpsi. From this moment onward, the samples were kept in near darkness and at 4 °C. The crude extract was then spun down at 5000 x g for 5 minutes to pellet cell debris.

Thylakoids were collected by ultracentrifugation (180 000 x g, 4 °C), then washed twice in Buffer 1 and resuspended at a final chlorophyll a concentration of 1 mg mL⁻¹ with the addition of 1 % (w/v) *n*-dodecyl-β-maltoside (β-DDM; Biomol, Germany) and 100 mM NaCl. The suspension was mixed gently for a few minutes followed by 15 min ultracentrifugation (180 000 x g, 4 °C) to remove insoluble material. The supernatant was gently mixed with a Ni²⁺ chelating resin (ProBond, Life technologies) to a 1 : 1 volume ratio for about 30 min, before pouring into the chromatographic column to allow for binding. The resin was allowed to settle in the column, the excess liquid was removed and the supernatant was left to rapidly flow through.

The resin was washed overnight with Buffer 2 containing 40 mM MES (pH 6.5), 15 mM MgCl₂, 15 mM CaCl₂, 100 mM NaCl, 15 mM imidazole, 0.03 % (w/v) β-DDM, 10 % (v/v) glycerol, and 1.0 M betaine. When the wash was colourless (optical density at 673 nm < 0.05), the bound PSII was eluted using Buffer 3, containing 40 mM MES (pH 6.5), 15 mM MgCl₂, 15 mM CaCl₂, 200 mM NaCl, 300 mM imidazole, 0.06 % (w/v) β-DDM, 10 % (v/v) glycerol, and 1.0 mM betaine. The eluted PSII was concentrated using centrifugal filter tubes (Amicon Ultra) with a molecular weight cut-off of 100 000 NMWL by spinning at 4000 x g until most of the Buffer 3 had passed through and washed three times with Buffer 1.

The PSII was finally concentrated to a chlorophyll a concentration of $\sim 3 \text{ mg} \cdot \text{mL}^{-1}$ and stored in Buffer 1 (storage buffer) in liquid nitrogen.

2.4.1 Preparation of Mn-depleted PSII

- (1) Some Photosystem II samples were depleted of the Mn_4CaO_5 complex according to M. Kato et al. (2012) by incubating Photosystem II ($[\text{Chl a}] = 0.5 \text{ mg/mL}$) for one hour in the dark at room temperature in a buffer, containing 15 mM CaCl_2 , 15 mM MgCl_2 , 0.03% β -DM, 10 % (v/v) glycerol, 1.0 M betaine, 40 mM MES-NaOH (pH 6.5), 10 mM hydroxylamine (NH_2OH) and 0.5 mM EDTA. Hydroxylamine and EDTA were removed by washing the samples five times using Millipore Ultrafree-15 centrifugal filters with a 100 kDa cut-off by centrifugation at $10,000 \times g$ for 30 min, using the same buffer without NH_2OH and EDTA.
- (2) Another protocol which was applied to remove the Mn_4CaO_5 cluster involved a treatment of the Photosystem II samples with Tris (Boussac et al. 2004): the samples were incubated in room light at 4°C at $[\text{Chl}] = 0.5 \text{ mg/mL}$ in a solution containing 1.2 M Tris-NaOH (pH 9.2) and centrifuged for 3 h at $170,000 \times g$. The pellet was resuspended in a solution containing 1 M CaCl_2 for 30 min in the dark at 4°C . The sample was furthermore centrifuged again for 3 h at $170,000 \times g$ and the Mn-depleted PSII samples were resuspended again in buffer 1.

2.4.2 Oxygen evolution measurements

Oxygen evolution activity was assayed with a Clark-type oxygen electrode (Oxylab, Hansatech) at 25°C in the presence of 0.5 mM DCBQ and 1.0 mM potassium ferricyanide, using saturating red light (590 nm cut-off filter; $13,000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). The activity in the various preparations were about $3500\text{-}5000 \mu\text{mol O}_2 \cdot \text{mg Chl a}^{-1} \cdot \text{h}^{-1}$ under these conditions.

2.4.3 Substitution of the bicarbonate ligand with oxalate or glycolate

The redox potential of the non-heme iron was modified by substituting the bicarbonate ligand with either oxalate or glycolate according to Petrouleas et al. (1994). Isolated PSII core particles were incubated for 2.5 h at a chlorophyll concentration of 0.5 mg/mL in buffer 1 containing either 30 mM sodium oxalate or sodium glycolate and washed twice in the same buffer without any further additions using Millipore Ultrafree-15 centrifugal filters with a 100 kDa cut-off by centrifugation at 4,000 x g for 30 min.

2.4.4 PsbE-His23Ala PSII core complexes

Isolated PSII core complexes from *T. elongatus* mutants lacking the Cyt *b*₅₅₉ heme iron were obtained from Dr Alain Boussac (iBiTec-S, CNRS UMR 8221, CEA Saclay, France). The PsbE mutant was constructed in *T. elongatus* WT*3 cells, i.e. in a strain in which the psbA1 and psbA2 subunits were deleted (Sugiura et al. 2015). The axial histidine ligand of the heme iron on the PsbE subunit, PsbE-His23, was substituted for an alanine, which cannot operate as a ligand to the heme iron, leading to the assembly of the apo-Cyt *b*₅₅₉ in the cells.

2.5 Isolation and characterization of PSII enriched membrane fragments from *Spinacia oleracea*

Photosystem II enriched membrane fragments from spinach (BBY membranes) were prepared in the dark at 4°C using only green light according to a protocol, combining established procedures (Berthold et al. 1981; Volker et al. 1984; Johnson et al. 1994). 500 g fresh market spinach were carefully washed and stored overnight at 4°C in the dark to minimize the starch content in the leaves. On the following day, the spinach leaves were homogenized in a pre-cooled blender in buffer 1, containing 20 mM tricine-NaOH (pH 8.1), 0.4 M NaCl, 2 mM MgCl₂, and 0.2 % BSA, freshly added at the day of the preparation. 300 mL buffer 1 were used per 100 g of spinach. The homogenate was carefully filtered through a cheese cloth into large centrifuge tubes which were kept on ice; foaming was avoided. The filtrate was centrifuged for 10 min at 6,000 x g and the supernatant was discarded. The pellet was resuspended in 80 mL buffer 1 with a

watercolour brush and the suspension was carefully vortexed to homogenize the solution. The solution was furthermore centrifuged at 35,000 x g for 7 min, the supernatant was discarded and the precipitate was suspended in 40 mL buffer 2, containing 20 mM MES-NaOH (pH 6.5), 15 mM NaCl, 5 mM MgCl₂, 1 mM sodium bicarbonate and 0.2 % BSA, freshly added at the day of the preparation. The suspension was inverted about 5-6 times without foaming and centrifuged at 35,000 g for 7 min. The supernatant was discarded and the pellet was gently resuspended with a watercolour brush in 7 mL buffer 3, containing 25 mM MES-NaOH (pH 6.5), 0.4 M sucrose, 15 mM NaCl, 5 mM MgCl₂ and 1 mM bicarbonate. The sample volume was measured in a pre-cooled graduated cylinder and the chlorophyll concentration was determined while incubating the suspension for 30 min on ice: 10 µL of the sample was added to 40 µL buffer 3. 10 µL of the diluted sample was added to 990 µL acetone (80 %). The sample was centrifuged for 2 min at 14,500 rpm in an Eppendorf centrifuge and the chlorophyll concentration was determined measuring the absorption of the sample at 663 nm (chlorophyll a) and 645 nm (chlorophyll b). The total chlorophyll concentration was calculated according to Arnon (1949):

$$[\text{Chl}] = (A_{663} \cdot 8.02 + A_{645} \cdot 20.2)/1000 \quad (2.6)$$

The chlorophyll concentration was adjusted to 3 mg/mL with buffer 3 and buffer 4 (buffer 3 with 33 w/v Triton X-100), keeping a ratio buffer 3/buffer 4 of 2/11 of the total volume and a chlorophyll to Triton X-100 ratio of 1:20 with 2/11 of the total volume being buffer 4. Foaming was avoided and the sample was incubated without stirring for 15 min when the desired concentration was achieved. The sample was centrifuged again at 35,000 x g for 15 min. The supernatant was discarded and the pellet was suspended in 40 mL buffer 3 and centrifuged at 3,000 x g for 3 min. The supernatant was centrifuged once more at 35,000 x g for 15 min. If the resulting supernatant was still green, the pellet from the previous step was suspended again in buffer 3 and centrifuged at 35,000 x g for 20 min. This was repeated until the supernatant was clear. The precipitate was suspended in a small volume of buffer 5, containing 20 mM MES-NaOH (pH 6.5), 0.4 M sucrose, 15 mM NaCl, 5 mM MgCl₂, 20 % (v/v) glycerol and 1 mM bicarbonate and the chlorophyll concentration of the sample was determined as described above. The PSII enriched membranes were divided into aliquots, frozen with liquid nitrogen and stored at -80°C.

2.5.1 Preparation of Mn-depleted PSII enriched membranes

- (1) Some PSII enriched membranes from spinach were depleted of the Mn_4CaO_5 cluster by treating them with hydroxylamine (Johnson, Krieger & Rutherford, 1995), similarly to the procedure described above for Photosystem II core complexes isolated from *T. elongatus* (section 2.4.1): the PSII enriched membranes were incubated for 1h on ice in the dark with $[\text{Chl}] = 0.5 \text{ mg/mL}$ in a buffer containing 5 mM NH_2OH , 400 mM NaCl and 50 mM MES-NaOH (pH 6.5). To remove the hydroxylamine, the sample was washed at least twice in the same buffer without NH_2OH using Millipore Ultrafree-15 centrifugal filters with a 100 kDa cut-off by centrifugation at $4,000 \times g$ for 30 min, using the same buffer without NH_2OH and EDTA.
- (2) In some samples, the Mn_4CaO_5 cluster was removed as well by incubating the PSII enriched membranes in a buffer containing Tris, which was carried out as described above (section 2.4.1).

2.5.2 Oxygen evolution measurements

Oxygen evolution activity was assayed with a Clark-type oxygen electrode (Oxylab, Hansatech) at 25 °C in the presence of 0.5 mM DCBQ and 1.0 mM potassium ferricyanide, using saturating red light (590 nm cut-off filter; $5,000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). The activity in the various preparations were about 300 - 500 $\mu\text{mol O}_2 \cdot \text{mg Chl a}^{-1} \cdot \text{h}^{-1}$ under these conditions.

2.5.3 Removal of the bicarbonate ligand

The depletion of the bicarbonate ligand at the PSII acceptor side of PSII enriched membranes was carried out as described in Shevela et al. (2007) with some modifications: $\text{CO}_2/\text{HCO}_3^-$ was removed from the buffer (400 mM sucrose, 20 mM CaCl_2 , 10 mM MgCl_2 and 50 mM MES-NaOH, pH 6.5, BC^+ buffer) by flushing with argon for 60 min (BC^- buffer). The PSII enriched membranes were depleted of $\text{CO}_2/\text{HCO}_3^-$ by 50-fold dilutions with the BC^- buffer and subsequent dark incubation on ice for 2 h under argon atmosphere. The PSII enriched membranes were collected by centrifugation at

4,000 x g for 30 min and were washed at least twice in the BC^- medium. They were stored in the dark and on ice under argon atmosphere until they were used. If not indicated otherwise, the samples used for the spectroelectrochemical titrations were prepared freshly at the day of the experiment.

2.5.4 Treatment with sodium formate

The treatment of the PSII enriched membranes with sodium formate to replace the bicarbonate ligand was carried out according to the method described by Shevela et al. (2007) with minor modifications: samples were treated with the BC^+ buffer containing 50 mM NaHCO_2 at 20 °C and pH 5.0 for 15 min. The treated samples were diluted 50-fold in the BC^- medium (pH 6.5) containing 50 mM NaHCO_2 . The PSII enriched membranes were collected by centrifugation at 4,000 x g for 30 min, washed once in the same buffer and finally resuspended to the desired concentration.

2.6 Spectroscopic measurements

2.6.1 Fluorescence spectroscopy

To prove whether the substitution of the bicarbonate ligand with glycolate and oxalate was successful, the relaxation of the chlorophyll fluorescence yield after excitation with a saturating actinic flash was measured. Chlorophyll fluorescence induction and decay were monitored with a Photon Systems Instruments (Brno, Czech Republic) FL3000 dual modulation kinetic fluorometer. Both measuring and saturating flashes were provided by computer-controlled photodiode arrays. A single saturating flash (625 nm) was provided at the onset of the experiment for 50 μs and the fluorescence decay was monitored for 60 s with a flash duration of the measuring light (455 nm) of 9 μs and a delay time between the flashes of 8 μs . The kinetics of the electron transfer between Q_A^- and Q_B were examined in the unmodified PSII and compared to PSII with the bicarbonate ligand substituted by either oxalate or glycolate. Data were analyzed using proprietary software provided by Photon Systems Instruments (FluorWin).

2.7 Spectroelectrochemical redox titrations in an OTTLE-type cell

2.7.1 Assembly of the OTTLE-cell and electrode modifications

Spectroelectrochemical redox titrations of potassium ferricyanide, horse heart cytochrome c and *T. elongatus* in solution were performed at 25°C (PSII enriched membranes from spinach at 15°C) in an optically transparent thin-layer (spectro)electrochemical (OTTLE) cell (optical pathlength 0.5 mm, Als Co., Ltd.). The working electrode (100 mesh, gold gauze, Als Co., Ltd.) was cleaned prior to each measurement by exposing it to an oxygen plasma (Emitech K1050X Plasma Asher, ashing power 70 W, ref. power 2 W, ashing time 30 s, bleed delay time 12 s) and modified afterwards in the case of cytochrome c and Photosystem II complexes by incubation under Argon flushing in a 1 mM aqueous solution of 4,4'-dithiodipyridine (Sigma Aldrich) according to Taniguchi et al. (Taniguchi et al. 1982). The electrode was carefully rinsed with water before usage. The reference and counter electrode were a Ag/AgCl (3 M KCl_(aq), diameter 6 mm) electrode and a platinum electrode (80 mesh, platinum gauze, Als Co., Ltd.), respectively. The Ag/AgCl reference electrode was calibrated using a saturated calomel reference electrode. The electrode potential was controlled by a potentiostat (Metrohm / Eco Chemie Autolab potentiostat/ galvanostat, electrochemical analyser (PGSTAT12)).

2.7.2 Redox titrations of potassium ferricyanide

Redox titrations of 1 mM potassium ferricyanide were carried out in MES buffer, pH 7.0. The reduction was followed by the increase of the absorption bands at 303 nm and 418 nm using a UV-visible spectrophotometer (HP 8452 diode array spectrophotometer). The fraction of reduced potassium ferricyanide, proportional to the spectral intensity, was plotted against the applied potential, and the data were fitted according to the converted Nernst equation (1.6).

2.7.3 Redox titrations of horse heart cytochrome c

Redox titrations of 1 mM horse heart cytochrome c were performed in 50 mM HEPES buffer with 2 mM CaCl_2 , pH 7, under nitrogen atmosphere in the absence and presence of the redox mediators (100 μM each) 2,6-dichloro-1,4-benzoquinone ($E_m = +310$ mV, pH 7), p-benzoquinone ($E_m = +293$ mV, pH 7), *N,N,N',N'*-tetramethyl-p-phenylenediamine ($E_m = +260$ mV, pH 7), 2,5-dimethyl-p-benzoquinone ($E_m = +176$ mV, pH 7) and 1,4-naphthoquinone ($E_m = +58$ mV, pH 7) with a final concentration of cytochrome c of 2 μM in the electrolyte. The reduction of the heme in cytochrome c was followed by the increase in the α -band absorption at 550 nm (Myer et al. 1979) using a UV-visible spectrophotometer (HP 8452 diode array spectrophotometer), where the absorption of the redox mediators was negligible compared to that of the protein. The fraction of reduced heme, proportional to the spectral intensity, was plotted against the applied potential, and the data were fit according to the converted Nernst equation (1.6).

2.7.4 Redox titrations of Photosystem II

Redox titrations of Photosystem II in the OTTLE cell were carried out under conditions reported earlier by Shibamoto et al. (2009): Photosystem II was dissolved with $[\text{Chl}] = 150$ $\mu\text{g/mL}$ in a buffer containing 50 mM MES-NaOH (pH 6.5), 0.2 M KCl, 0.1 % dodecyl- β -D-maltoside, 1 M glycine-betaine and 1% taurine. A combination of redox mediators was added: 100 μM anthraquinone-2-sulfonate ($E_m = -195$ mV, pH 6.5), 100 μM 2-hydroxy-1,4-naphthoquinone ($E_m = -100$ mV, pH 6.5) and 200 μM *N,N,N',N'*-tetramethyl-p-phenylenediamine (TMPD, $E_m = +300$ mV, pH 6.5) if not stated otherwise. The sample was kept in the OTTLE cell in complete darkness and chlorophyll a fluorescence was excited at each potential step approximately every 12 min for about 5 s to monitor the change in fluorescence, indicating the state of the electrochemical equilibrium. The excitation was carried out with a DUAL-PAM-100 P700 & Chlorophyll Fluorescence Measuring System (Heinz WALZ GmbH), using the pulse amplitude modulation (PAM) method and a very weak monochromatic beam (460 nm, $3 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). The emission (> 625 nm, RG665 filter) was detected in line with the measuring beam at the back of the OTTLE cell with a Photomultiplier-Detector Unit (DUAL-DPM). The

fraction of reduced Q_A , which is proportional to the fluorescence intensity, was plotted against the applied potential, and the data were fit according to the converted Nernst equation (1.6) (with $T = 288.15$ K for PSII enriched membranes). If not indicated otherwise, the redox titrations were carried out in the glove box under nitrogen atmosphere and an oxygen concentration of 20 ppm.

2.7.4.1 Excitation energy transfer efficiency in PSII dimer complexes and the non-linearity of chlorophyll fluorescence

Although the Nernstian behavior of the fluorescence yield change against the electrode potentials ensures a linearity between the fluorescence yield and the redox state of Q_A , Comayras et al. (2005) showed that the Nernstian behavior does not necessarily correspond to a linearity: they demonstrated that the fluorescence yield of Photosystem II dimer complexes is not linearly related to the redox state of Q_A , since excitation energy transfer between the two monomers is possible and therefore, the excitons yielded by a photosystem unit with a reduced Q_A becomes available for a neighboring unit with an oxidized Q_A for the induction of charge separation (Comayras et al. 2005). This leads to a decrease in the fluorescence yield. Comayras et al. (2005) derived the following model equation for the relationship between the fluorescence yield, $F(x)$, and a fraction of closed photosystem units x , which represent the fraction of Q_A^- :

$$F(x) = x^2 + x(1 - x) \frac{1+\rho}{1+\rho R}. \quad (2.7)$$

Here, R corresponds to the ratio of maximum fluorescence to basal fluorescence, F_m/F_0 , and the parameter ρ to the transfer efficiency between the two units of the dimer. The case $\rho = 1$ describes a situation, where the intra-dimer transfer with respect to exciton decay is very fast, resulting the following equation:

$$F(x) = x^2 + \frac{2x(1-x)}{1+R}. \quad (2.8)$$

For $R = 1$, equation (2.8) becomes $F(x) = x$, and the mole fraction of reduced Q_A depends linearly on the measured fluorescence yield, which would be a true value for the redox state of Q_A .

Strictly speaking, equation (2.8) is only valid for PSII dimers, such as the ones isolated and titrated from *T. elongatus*. The isolated and used PSII enriched membranes from spinach are abundantly surrounded by the light harvesting complexes II (LHC II). In this case and in thylakoids and cells, the excitation energy diffusion among PSII dimers through LHC II should be considered in the fluorescence yield. Traditionally, a homogeneous model, often called “lake model” has been applied to describe the relationship between $F(x)$ and x (Paillotin 1976; Lavergne and Trissl 1995):

$$F(x) = \frac{x}{1+J-Jx}. \quad (2.9)$$

Here, J describes the connectivity between the LHC II and the PSII core complex and is calculated according to $J = R-1$.

The experimentally obtained data for the mole fraction of Q_A^- were corrected for PSII enriched membranes from spinach similarly to Shibamoto et. al. (2010) (see section 3.4.2). To determine the F_m/F_0 ratio, a weak measuring pulse was used to measure the fluorescence minimum level, F_0 , and the fluorescence maximum level, F_m , was determined by providing a single saturating flash (625 nm) at the onset of the experiment for 50 μ s and monitoring the fluorescence for 200 s with a flash duration of the measuring light (625 nm) of 9 μ s and a delay time between the flashes of 8 μ s, using a FL3000 dual modulation kinetic fluorometer (Photon Systems Instruments, Brno, Czech Republic). To test, whether the flash was saturating and the F_m level was reached, a small amount of $Na_2S_2O_4$ was added to the sample after the flash. For PSII enriched membranes, a value of $R = 2.39$ ($J = 1.39$) was obtained. This value is in a good agreement with the ones obtained by Lavergne et al. (1995).

For the calculation of the corrected Q_A/Q_A^- midpoint potential (see section 3.4), accounting for the non-linear behavior of chlorophyll fluorescence in PSII enriched membranes from spinach, equation (2.9) was applied. The experimentally obtained mole fraction of Q_A^- was considered to be $F(x)$ and the corresponding, corrected values for the mole fraction of Q_A^- , x , were calculated.

2.8 Protein electrochemistry of PSII in metal oxide hybrid systems

2.8.1 Electrode preparation and immobilization of PSII

The electrodes were treated at 450°C for 10 min before usage and cooled to room temperature. The method developed was based on experience from previous studies involving protein immobilisation on surfaces (Fantuzzi et al. 2004; Astuti et al. 2004; Panicco et al. 2008). 40 μL of PSII solution in the storage buffer containing 0.03 % β -DDM at $[\text{Chl a}] = 4 \mu\text{g/mL}$ (for obtaining a monolayer of PSII on the electrode surface) or $[\text{Chl a}] = 400 \mu\text{g/mL}$ (for studies on multilayers) were used to cover the electrode surface. It was found empirically that the lowest amount of PSII needed to give a measurable direct photocurrent was approximately 2 $\mu\text{g/mL}$, thus double that concentration was chosen as this concentration gave a reasonable signal to noise ratio. The higher concentration for multilayers was chosen to be 100 times greater. The PSII-electrode was incubated in a humidified atmosphere in the dark at 4°C overnight. Before the measurements, the electrode was rinsed with ultrapure water (Neptune model L993162, Purite, UK) to remove non-immobilised PSII and placed in a vessel containing the electrolyte buffer used for the electrochemical measurements. The vessel was kept in the dark and on ice prior to the measurement.

The final amount of PSII on the TiO_2 surface was determined by concentration measurements of chlorophyll a using a NanoDrop Spectrophotometer (Thermo Scientific, NanoDrop 1000). PSII was rinsed off the surface prior to a measurement with 40 μL methanol (99.9%) and solubilized. The amount of chlorophyll a was determined by measuring its absorption in methanol at 665 nm using an extinction coefficient of $79.95 \text{ mg} \cdot \text{mL}^{-1} \cdot \text{cm}^{-1}$ (Boussac et al. 2004) and correcting for volume changes occurring during extraction. The amount of PSII was deduced based on 35 chlorophylls/ PSII (Umena et al. 2011). Taking into account the size of the PSII monomers (approximately 10^{-12} cm^2 based on the crystal structure; Zouni et al. 2001) and the roughness of the TiO_2 surface, the accessible area was estimated to be about 4-5 times that of the geometrical area of the TiO_2 layer. Based on this estimate the amount of PSII on the surface was found to be $1.2 \text{ pmol} \cdot \text{cm}^{-2}$ when the low chlorophyll concentration (4 $\mu\text{g/mL}$) was used, corresponding to the formation of a monolayer/sub-monolayer on the electrode surface.

2.8.2 Electrochemistry of PSII adsorbed onto metal oxide electrodes

Electrochemical measurements were carried out using a PGSTAT12 electrochemical analyser controlled by GPES software (Eco Chemie Utrecht).

For photocurrent studies involving the investigation of electron transfer pathways from the protein to the metal oxide (chapter 4), an open glass cell was used with a platinum wire as a counter electrode and a saturated calomel electrode (SCE) as a reference. An external bias of +644 mV vs SHE (if not indicated otherwise) was applied before each measurement for 250 s to let the system equilibrate in the dark. The first measurement of each series always consisted of five illumination intervals with a duration of 10 s followed by a 60s dark interval. This initial illumination time provided a sufficient duration for the photocurrent to equilibrate and plateau. Therefore, only the photocurrent after the fifth illumination interval was considered for data analysis. Additives such as DCBQ, DCMU and quercetin to the electrolyte caused an extension of the equilibration time during illumination, but the photocurrent plateaued already after the third illumination interval. Therefore, all measurements with DCMU, DCBQ, quercetin and ioxynil in the electrolyte buffer were recorded with three illumination intervals of 20 s each followed by a 100s dark interval. For the data analysis, only the photocurrent corresponding to the third illumination interval was considered. All measurements were carried out at 25°C. An electrolyte buffer solution containing 20 mM CaCl_2 , 40 mM MES and 5% glycerol at pH 6.5 (if not indicated otherwise) was used. 10 mM stock solutions of DCBQ and quercetin were prepared in ethanol (99.8 %) and 10 mM DCMU stock solutions were prepared in DMSO (99.9 %).

Continuous illumination was provided by a Xenon lamp and the light was filtered through a 590 nm cut-off filter producing red light with an intensity of $800 \mu\text{E m}^{-2}\text{s}^{-1}$ in the cell. The applied light intensity did not induce any detectable photocurrent from the TiO_2/ITO or the WO_3/FTO surface in the absence of PSII. 10 mM stock solutions of DCBQ, ioxynil and quercetin were prepared in ethanol (99.8 %) and 10 mM DCMU stock solutions were prepared in DMSO (99.9 %).

For investigations of the origin of cathodic photocurrents (chapter 5), the same set-up and the same electrolyte buffer were used as described above. The bias dependent studies of the photocurrent generated by PSII were carried out starting from positive potentials and were consequently decreased in either 50 mV or 100 mV steps, whereas

each potential was applied for 150 s. In order to minimize photodegradation of the immobilized PSII, the photocurrents were recorded during this time using short illumination intervals. The length of each illumination period was chosen according to the time needed to obtain a stable photocurrent, which was reached within 20 s. Each illumination interval was followed by a period of 15 s in the dark. The photocurrent was considered to have reached equilibrium when two subsequent illumination intervals showed the same current values within the standard deviation.

Chapter 3.

Spectroelectrochemical titrations of Photosystem II in an OTTLE cell

3.1 Evaluation of the OTTLE cell with a redox titration of potassium ferricyanide

Firstly, to correctly determine the redox potential of the Q_A/Q_A^- -couple in PSII, the function and accuracy of the OTTLE cell was tested. A redox titration of an aqueous solution of 1 mM $Fe^{III}(CN)_6^{3-}$ in MES buffer, pH 7.0, was carried out by monitoring the absorption at 303 nm and 418 nm.

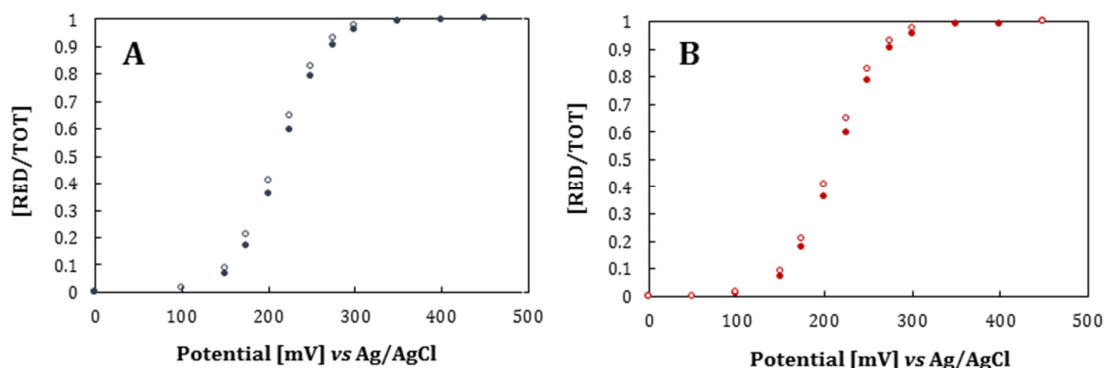


Figure 3.1 (A) Redox titration of 1 mM $Fe^{III}(CN)_6^{3-}$ in MES buffer, pH 7.0, 25°C: Nernst plot of the fraction of reduced species at 418 nm. Closed circles correspond to the reductive titration, open circles to the oxidative titration. (B) Redox titration of 1 mM $Fe^{III}(CN)_6^{3-}$ in MES buffer, pH 7.0, 25°C: Nernst plot of the fraction of reduced species at 303 nm. Closed circles correspond to the reductive titration, open circles to the oxidative titration.

Reductive and oxidative titrations were in a very good agreement (**Figure 3.1 (A)** and **(B)**) and led to a reproducible redox potential close to the literature value of +420 mV (Njus et al. 1983):

Absorption	418 nm, reductive	418 nm, oxidative	Absorption	303 nm, reductive	303 nm, oxidative
E_m [mV]	+409.69 ± 0.67	+407.45 ± 0.15	E_m [mV]	+410.17 ± 0.63	+406.72 ± 0.15

3.2 Evaluation of the OTTLE cell with a redox titration of horse heart cytochrome c in the presence and absence of redox mediators

The cell was tested for its application for redox titrations of biological systems. Horse heart cytochrome c was titrated in a 50 mM HEPES buffer with 2 mM CaCl_2 at pH 7 in the absence (**Figure 3.2 (A)**) and presence of 2,6-dichloro-1,4-benzoquinone ($E_m = +310\text{ mV}$ pH 7), p-benzoquinone ($E_m = +293\text{ mV}$, pH 7), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine ($E_m = +260\text{ mV}$, pH 7), 2,5-dimethyl-*p*-benzoquinone ($E_m = +176\text{ mV}$, pH 7) and 1,4-naphthoquinone ($E_m = +58\text{ mV}$, pH 7) as redox mediators (**Figure 3.2 (B)**). Given a literature value of horse heart cytochrome c of $E_m = +260\text{ mV}$ at pH 7 (Margalit and Schejter 1973), the redox potential determined in the OTTLE cell in the absence of redox mediators is satisfying, although, the reductive and oxidative titration differ quite significantly from each other and the fitting error in the reductive titration is unexpectedly high.

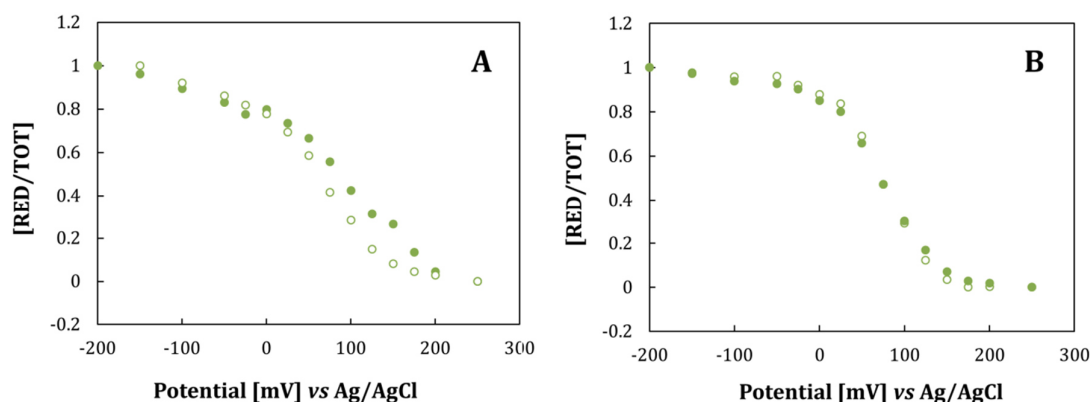


Figure 3.2 (A) Redox titration of $20\text{ }\mu\text{M}$ horse heart cytochrome c in HEPES buffer, pH 7.0, 25°C : Nernst plot of the fraction of reduced species at 550 nm in the absence of redox mediators. Closed circles, reductive titration; open circles, oxidative titration. **(B)** Redox titration of $20\text{ }\mu\text{M}$ horse heart cytochrome c in HEPES buffer, pH 7.0, 25°C : Nernst plot of the fraction of reduced species at 550 nm in the presence of the redox mediators 2,6-dichloro-1,4-benzoquinone, p-benzoquinone, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, 2,5-dimethyl-*p*-benzoquinone and 1,4-naphthoquinone. Closed circles, reductive titration; open circles, oxidative titration.

This problem was eliminated by titrating the protein in the presence of suitable redox mediators, which covered a potential range from 0 mV to +350 mV. The reproducibility of the reductive and oxidative titration and the good agreement with the literature value

demonstrates the importance of redox mediators for a spectroelectrochemical titration: the lack of suitable mediators may result in an insufficient interaction of the protein with the working electrode and therefore, in an incorrectly estimated redox potential.

- Redox mediators	550 nm, reductive	550 nm, oxidative	+ Redox mediators	550 nm, reductive	550 nm, oxidative
E_m [mV]	+233.28 $\pm$ 7.08	+248.71 $\pm$ 2.48	E_m [mV]	+272.82 $\pm$ 3.21	+273.15 $\pm$ 1.54

3.3 Spectroelectrochemical redox titrations of Photosystem II isolated from *T. elongatus*

The redox titration of horse heart cytochrome c shows that the redox potential of protein cofactors can be determined spectroelectrochemically in the OTTLE cell with a high accuracy. In a next step, the cell was used to carry out redox titrations of Photosystem II isolated from *T. elongatus*, aiming for the determination of the midpoint potential of the Q_A/Q_A^- couple. Here, the reduction state of the electron acceptor Q_A modulates the fluorescence yield of chlorophyll a in PSII as described above and it can therefore be used to monitor the concentration of Q_A and thus determine the redox potential of the Q_A/Q_A^- -couple.

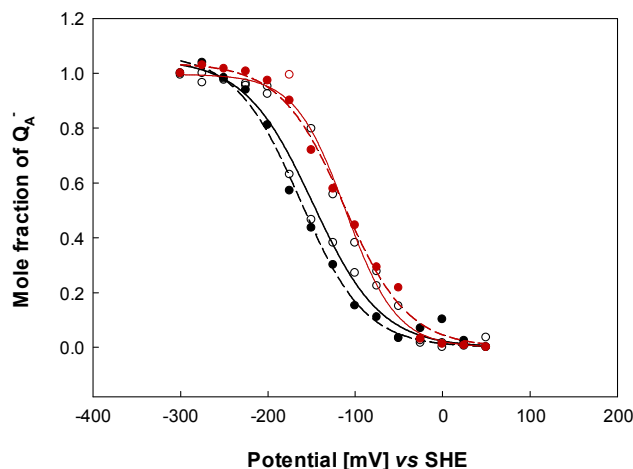


Figure 3.3 Two independent redox titration curves of Q_A in intact PSII core complexes from *T. elongatus* measured as fluorescence yield. Measurements were carried out in the presence of oxygen. Closed circles reductive titration; open circles oxidative titration. The curves were fitted according to equation (1.6); the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potential of the black Nernst curves is $-155 \text{ mV} \pm 5 \text{ mV}$ and the red ones correspond to $-110 \text{ mV} \pm 5 \text{ mV}$.

In a first attempt, the conditions of Shibamoto et al. were reproduced: chlorophyll a fluorescence was recorded as a function of an applied potential in the presence of the redox mediators anthraquinone-2-sulfonate ($E_{m,7} = -195 \text{ mV}$), 2-hydroxy-1,4-naphtho-

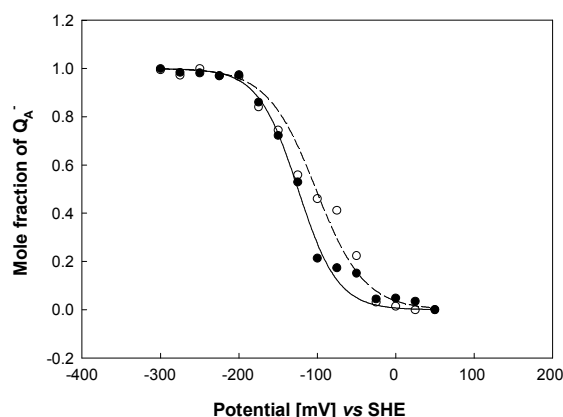


Figure 3.4 Redox titration curve of Q_A in intact PSII core complexes from *T. elongatus* measured as fluorescence yield. Measurements were carried out in the presence of oxygen and after cleaning the working electrode before the measurement. Closed circles reductive titration; open circles oxidative titration. The curves were fitted according to equation (1.6); the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potential of the Nernst curves is $-115 \text{ mV} \pm 5 \text{ mV}$.

gold mesh priorly to each measurement by exposing it for 2 min to an oxygen plasma and renewing the 2,2-dithiodipyridine layer on the electrode, resulted in reproducible reductive and oxidative titrations obtained in single measurements (**Figure 3.4**) and the fittings of the Nernst plots resulted in a midpoint potential of $E_m(Q_A/Q_A^-) = 115 \text{ mV} \pm 5 \text{ mV}$, although the potential region from -50 to -100 mV indicates the electrochemical interference of oxygen with the measurement. Due to the presence of the detergent n-dodecyl- β -D-maltoside in the buffer, oxygen could not be removed sufficien-

quinoxaline ($E_{m,7} = -100 \text{ mV}$) and

N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD, $E_{m,7} = +300 \text{ mV}$) in the presence of oxygen. The results of two independent redox titrations shown in **Figure 3.3**: multiple titrations resulted in significant differences of the midpoint potential, ranging from -155 mV to -110 mV, which were determined by fitting their oxidative and reductive titrations to a one-electron Nernst curve according to equation (1.6).

These shifts in the single titrations were partially overcome by the development of a cleaning protocol for the working electrode: cleaning the

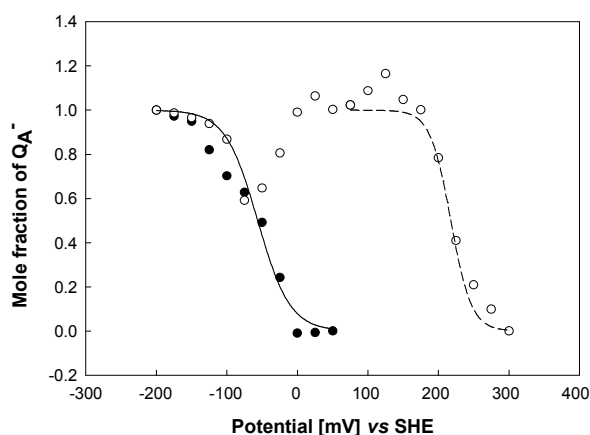


Figure 3.5 Redox titration curve of Q_A in intact PSII core complexes from *T. elongatus* measured as fluorescence yield. Measurements were carried out in the absence of oxygen in the glove box. Closed circles reductive titration; open circles oxidative titration. Both curves were fitted according to equation (1.6). The midpoint potential of the Nernst curves are $-60 \text{ mV} \pm 5 \text{ mV}$ and $+224 \text{ mV} \pm 7 \text{ mV}$, respectively.

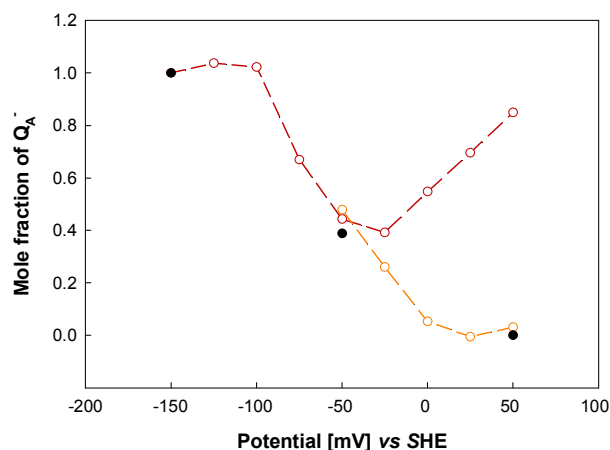


Figure 3.6 Three-step reductive titration curve (black dots) of Q_A in intact PSII core complexes from *T. elongatus* measured as fluorescence yield and two independent oxidative titrations. Measurements were carried out in the absence of oxygen. Red curve: full-step oxidative titration from -150 mV to +50 mV. Orange curve: oxidative titration from -150 mV to -50 mV (one step) and from -50 mV to 50 mV (step-wise).

overlapped with the reductive titration only in the range from -200 mV to -75 mV. At more positive potentials, the fluorescence of the sample increases again to the same magnitude as obtained at potentials < -150 mV and began to decrease at +150 mV following a Nernst behaviour to the initial level. This up-shift of the midpoint potential obtained during the oxidative titration could be explained by the fact that PSII was kept at negative potentials for a significant amount of time, which possibly could have reduced the Mn_4CaO_5 -cluster, resulting in its release or at least in the release of the Ca^{2+} -ion. Similar phenomena were observed by Krieger et al. (1995), who attributed this to the reductive loss of the Mn_4CaO_5 cluster. This work showed that the presence of the manganese determined whether the Ca^{2+} -ion was bound and the reduction of the manganese was the trigger for the loss of the Ca^{2+} -ion and hence for the upshift of the E_m of the Q_A/Q_A^- -couple. The sensitivity of intact PSII towards the application of low potentials was also demonstrated by Ido et al. (2011, see also Fufezan et al. 2007). This work was a further proof for the existence of the high and low potential form of Q_A .

tly prior the measurement by purging the solution with argon. Several attempts to further reduce the oxygen concentration in the cell were unsuccessful and the set-up was assembled in the glove box. From then on, measurements were carried out under nitrogen atmosphere with an oxygen concentration of 20 ppm.

Surprisingly, the titrations in the absence of oxygen in the glove box showed a completely different behaviour (**Figure 3.5**): the reductive titration was shifted significantly towards higher potentials, resulting in a midpoint potential of $E_m(Q_A/Q_A^-) = -60 \text{ mV} \pm 5 \text{ mV}$. The oxidative titration

Further on, it was tested whether PSII remained intact when it was kept only for a limited amount of time at potentials < -100 mV. The potential steps in the reductive titration were set to three (+50 mV, -50 mV and -150 mV) and the total amount of time spent at -150 mV was reduced to 1 h, whereas the fluorescence intensity was measured every 15 min to ensure the complete reduction of Q_A during this time. **Figure 3.6** shows the result of two independent oxidative titrations and their corresponding reductive titration: the first oxidative titration (from -150 mV to +50 mV in potential steps of 25 mV) agreed well with the reductive titration in up to a potential of -50 mV, where the fluorescence intensity started to increase again. In a second oxidative titration, going in one step from -150 mV to -50 mV in 30 min, the oxidative titration could be completed and the fluorescence intensity decreased to the initial level following the reductive titration. **Figure 3.7** shows one the reductive titrations (black closed circles), the two combined oxidative titrations (red and orange open circles) and their respective fittings according to equation (1.6). Reductive and oxidative titration were in a good agreement and resulted in a midpoint potential of $E_m(Q_A/Q_A^-) = -60 \text{ mV} \pm 5 \text{ mV}$.

This value is in a good agreement with the one previously obtained by Krieger et al. ($E_m = -81 \text{ mV} \pm 26 \text{ mV}$) and Ido et al. ($E_m = -60 \text{ mV} \pm 48 \text{ mV}$). Interestingly, the conditions of the redox titration (glove box, nitrogen atmosphere) seem to play a role for the stability of PSII in a strong reductive environment. One possible explanation for the increase of the midpoint potential of Q_A during the oxidative titration could be the loss of the bicarbonate anion ligated to the non-heme iron before or during the titration. In fact, this was observed first in EPR based redox titrations of *T. elongates* (A. W. Rutherford, J. Douglass, S. De

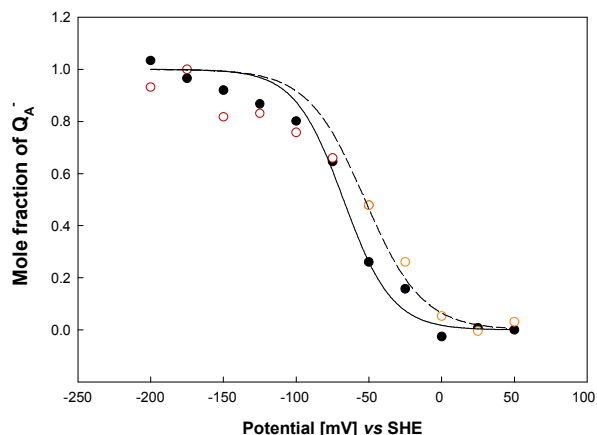


Figure 3.7 Combined redox titration curves of Q_A in intact PSII core complexes from *T. elongatus* measured as fluorescence yield. Measurements were carried out in the absence of oxygen in the glove box. Black closed circles, reductive titration; red open circles, first oxidative titration from -150 mV to +50 mV in potential steps of 25 mV; orange open circles, second oxidative titration, in which the first potential step was from -150 mV to -50 mV and then the potential was decreased further in 25 mV steps to +50 mV. The resulting midpoint potential of the Nernst curve is $-60 \text{ mV} \pm 5 \text{ mV}$.

Causmaeker and W. Nitschke, personal communication). The bicarbonate is known to play a significant role at the electron acceptor site (van Rensen 2002; Shevela et al. 2012), where it is thought to stabilize the Q_A -Fe- Q_B structure and thus, allow efficient electron transport and the protonation of Q_B . Additionally, it has a significant effect on the stability of PSII and in initiating and/or facilitating the assembly of the inorganic core of the OEC from OEC-depleted PSII reaction centres. There are also indications for the functioning of HCO_3^- in the assembled OEC (Shevela et al. 2012).

Bicarbonate can be removed from PSII

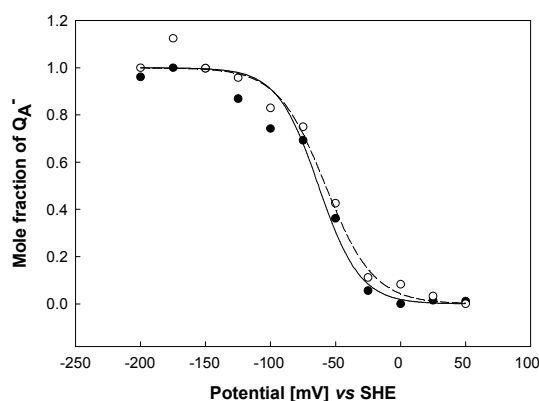


Figure 3.9 Redox titration curves of Q_A in intact PSII core complexes from *T. elongatus* from non-frozen samples, measured as fluorescence yield. Measurements were carried out in the absence of oxygen in the glove box. Closed circles reductive titration; open circles oxidative titration. The curves were fitted according to equation (1.6); the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potential of the Nernst curves is $-75 \text{ mV} \pm 5 \text{ mV}$.

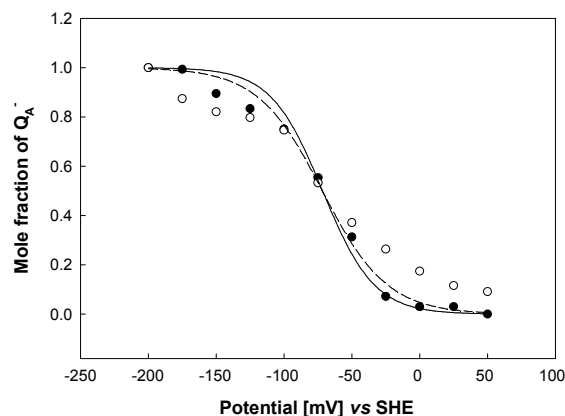


Figure 3.8 Redox titration curves of Q_A in intact PSII core complexes from *T. elongatus* measured as fluorescence yield. Measurements were carried out in the presence of 5 mM sodium bicarbonate in the buffer and in the absence of oxygen in the glove box. Closed circles reductive titration; open circles oxidative titration. The curves were fitted according to equation (1.6); the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potential of the Nernst curves is $-65 \text{ mV} \pm 5 \text{ mV}$.

by 1) diluting the samples into buffers that were depleted of bicarbonate by bubbling with CO_2 -free air, nitrogen or argon; 2) boiling the buffer; 3) incubating the samples at pH 5.0, which is below the pK_a of bicarbonate ($pK_a = 6.3$) and which induces therefore its protonation and results in the subsequent dissociation of H_2CO_3 into CO_2 and water and 4) adding chemical analogues of bicarbonate such as formate or acetate to the sample, which replace bicarbonate at its binding site (Shevela et al. 2007). Here, the combination of pH 6.5, which is slightly above the pK_a of bicarbonate, the nitrogen atmosphere in the

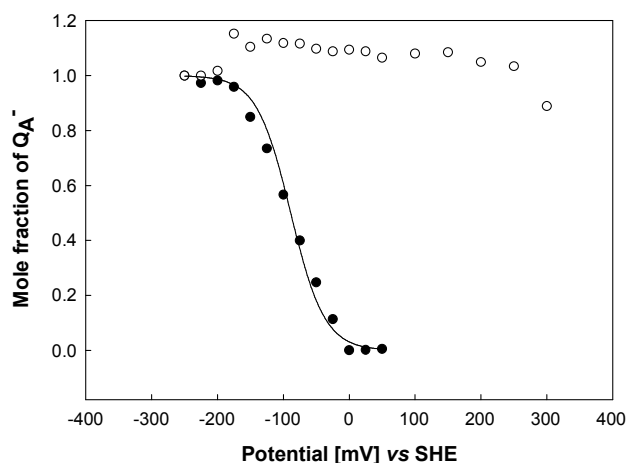


Figure 3.10 Redox titration curves of Q_A in intact PSII core complexes from *T. elongatus* measured as fluorescence yield. Measurements were carried out as previously, but in the absence of the redox mediator TMPD. Closed circles reductive titration; open circles oxidative titration. The curves were fitted according to equation (1.6); the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potential of the Nernst curves is $-90 \text{ mV} \pm 2 \text{ mV}$.

3.8) and resulted in a Q_A midpoint potential of $E_m(Q_A/Q_A^-) = -65 \text{ mV} \pm 5 \text{ mV}$. This value is in a good agreement with the one previously obtained during the reductive titration in the absence of bicarbonate (**Figure 3.5**). Therefore, the presence and/ or absence of the bicarbonate ligand seems to play a major role in maintaining the stability of intact PSII and the OEC. To test, whether the binding of the bicarbonate ligand is influenced by freezing isolated PSII directly after the preparation, the Q_A/Q_A^- midpoint potential was determined in a non-frozen sample. Interestingly, both, the reductive and oxidative titration were possible and the Nernst curves resulted in a midpoint potential of $E_m(Q_A/Q_A^-) = -75 \text{ mV} \pm 5 \text{ mV}$ (**Figure 3.9**).

Furthermore, the influence of the redox mediators on the Q_A/Q_A^- midpoint potential was investigated. Ido et al. (2011) observed already a down-shift of the Q_A/Q_A^- midpoint potential in Mn-depleted PSII in the presence of the redox mediator mix used by Shibamoto et al. (2009, 2010) to $-20 \text{ mV} \pm 30 \text{ mV}$. Especially TMPD has been suggested to interact with the Q_B site and the Mn_4CaO_5 cluster (Gauthier et al. 2006). Upon the removal of TMPD from the redox mediator mix, the midpoint potential was down-shifted

glove box and the long duration of a single titration (up to more than 8 h) could result in the removal of the bicarbonate ligand and therefore, in a structural change of the acceptor site of PSII and additionally, in the destabilization of the OEC. This would cause a more positive midpoint potential of Q_A and would explain the up-shift of the potential towards a significantly higher midpoint potential during the oxidative titration (**Figure 3.5**). The hypothesis was tested by carrying out a titration in the presence of 5 mM sodium bicarbonate, which enabled indeed a full oxidative titration in the glove box (**Figure**

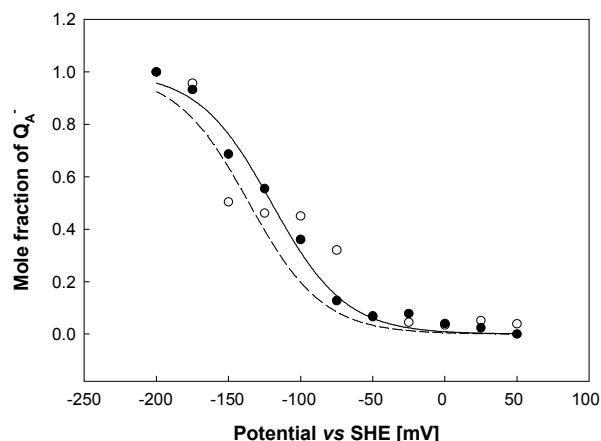


Figure 3.11 Redox titration curves of Q_A in intact PSII core complexes from *T. elongatus* measured as fluorescence yield. Measurements were carried out in the presence of 5 mM bicarbonate in the buffer and in the absence of oxygen in the glove box. TMPD was replaced in the redox mediator mix used by Shibamoto et al. (2009, 2010) by 100 μ M duroquinone and 100 μ M phenazine-methosulfate. Closed circles reductive titration; open circles oxidative titration. The curves were fitted according to equation (1.6); the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potential of the curves is estimated to be $-120 \text{ mV} \pm 5 \text{ mV}$.

to $E_m(Q_A/Q_A^-) = -90 \text{ mV} \pm 2 \text{ mV}$ (**Figure 3.10**) and in comparison to previous titrations, an oxidative titration was not possible at all in the absence of sodium bicarbonate in the buffer. When TMPD was exchanged by 100 μ M duroquinone ($E_m = +35 \text{ mV}$, pH 6.5 (Denke et al. 1998)) and 100 μ M phenazine methosulfate ($E_m = +110 \text{ mV}$, pH 6.5 (Zhu and Davidson 1998)), a down-shift of both, the reductive and oxidative titration was observed (**Figure 3.11**) and a midpoint potential of $E_m(Q_A/Q_A^-) = -120 \text{ mV} \pm 5 \text{ mV}$ was determined. But care should be taken with this midpoint potential, since both, oxidative and reductive titration cannot be fitted precisely to a one-electron Nernst curve. In fact, the titration curves indicate a poor

communication of the protein with the redox mediators and strengthen the importance of the choice of the correct redox mediator set for the redox titration of a protein cofactor.

Furthermore, given the indication of the instability the OEC during the titration and the loss of the bicarbonate anion at the same time, the Q_A/Q_A^- redox potential was determined in PSII samples lacking the Mn_4CaO_5 -cluster (see experimental section 2.4.1 (2)). This undertaking turned out to be difficult: **Figure 3.12** shows that despite complete loss of oxygen evolution activity and therefore, removal of the Mn-cluster, the redox potential of the Q_A/Q_A^- -couple did not change significantly with respect to the intact sample ($E_m(Q_A/Q_A^-) = -100 \text{ mV} \pm 5 \text{ mV}$) and the fittings of the reproducible reductive and oxidative titrations indicate that the data do not correspond to a one-electron Nernst curve. Interestingly, the oxidative titration is in a good agreement with the reductive one and it was possible to carry it out even in the absence of additional sodium bicarbonate in the buffer. The midpoint potential remained unchanged when 5 mM sodium bicarbonate

was added. Also the titration of a freshly-prepared, non-frozen sample lacking the Mn_4CaO_5 -cluster showed a similar behaviour: the midpoint potential was estimated according to fittings to be $E_m(\text{Q}_\text{A}/\text{Q}_\text{A}^-) = -100 \text{ mV} \pm 5 \text{ mV}$. Given the observations independently made by Krieger et al. (1993, 1995), Johnson et al. (1995), Ido et al. (2011) and Shibamoto et al. (2009, 2010) that removal of the Ca^{2+} -ion

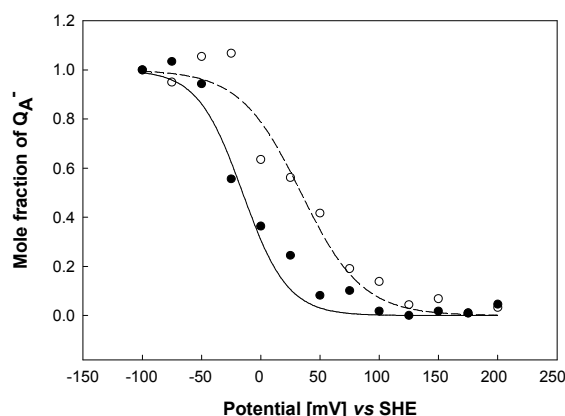


Figure 3.13 Redox titration curves of Q_A in PSII core complexes from *T. elongatus* lacking the Mn_4CaO_5 -cluster measured as fluorescence yield. Measurements were carried out in the presence of the redox mediators (each had a concentration of $100 \mu\text{M}$) phenazine ethosulfate ($+85 \text{ mV}$, pH 6.5), 1,2-naphthoquinone, ($+175 \text{ mV}$, pH 6.5) duroquinone ($+35 \text{ mV}$, pH 6.5) and 2,3,5,6-tetramethyl-phenylene-1,4-diamine (DAD, $+270 \text{ mV}$; Fultz and Durst 1982). Closed circles reductive titration; open circles oxidative titration. The curves are fitted according to equation (1.6); the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potential of the reductive titration curves is estimated to be $-15 \text{ mV} \pm 5 \text{ mV}$ and the one of the oxidative titration $+33 \text{ mV} \pm 5 \text{ mV}$.

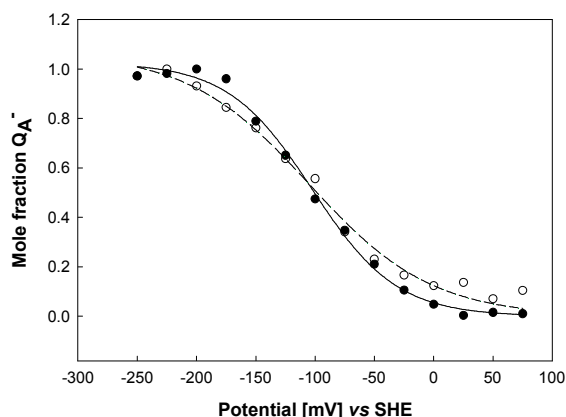


Figure 3.12 Redox titration curves of Q_A in PSII core complexes from *T. elongatus* lacking the Mn_4CaO_5 -cluster measured as fluorescence yield. Measurements were carried out with the redox mediator mix used by Shibamoto et al. (2009, 2010). Closed circles reductive titration; open circles oxidative titration. The curves are fitted according to equation (1.6); the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potential of the curves is estimated to be $-100 \text{ mV} \pm 5 \text{ mV}$.

(and the Mn-cluster) results in a shift of the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ midpoint potential of about $\Delta 150 \text{ mV}$ to higher potentials, the obtained results are difficult to explain. The stability of PSII with respect to the binding of the bicarbonate ligand does not seem to be affected by the cluster removal; the curve fittings rather seem to indicate that the interaction of the redox mediators with the protein was quite poor during the measurement. Different redox mediator combinations were further on tested to investigate their effect on the potential of the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ -couple in PSII samples lacking the Mn-clu-

ster. Combinations of different redox mediators were able to shift the redox potential significantly to higher values, but their interaction with PSII remained rather poor, as **Figure 3.13** shows one example; e.g., reductive and oxidative titrations often resulted in quite different midpoint potentials and the curve fittings indicated a weak interaction of the protein with the redox mediators. Since the experimental set-up and the conditions of the redox titrations were exactly the same as the ones used by Shibamoto et al. (2009, 2010), the question arises based on the described observations, whether the PSII core complexes used in the titration were actually the same. Given the difficulty in determining the potential of the Q_A/Q_A^- couple in PSII samples lacking the Mn-cluster and the poor communication of various redox mediators with the protein - despite the fact that at least one of the used redox mediator set worked well for Shibamoto et al. (2009, 2010) - it could well be the case that e.g., the used PSII samples were actually mutations: the *T. elongatus* cell cultures, which have been used for PSII isolation and for the redox titrations, grew in the same incubator as *T. elongatus* mutants, expressing a different PsbA gene encoding for the D1 protein.

Cyanobacterial species have several PsbA variants, which encode for the D1 protein (Clarke et al. 1993; Sugiura et al. 2010b; Kiss et al. 2012; Sugiura and Boussac 2014). The expression of the different genes depends on environmental conditions, in particular, specific up and down regulations of one of these genes under high-light conditions is e.g., indicative of important photo-protection mechanisms. The thermophilic cyanobacterium *T. elongatus* has three psbA genes in its genome (Nakamura et al. 2002) and from the translated sequences and among the 344 residues of the PsbA proteins, 31 differ between PsbA1 and PsbA2, 21 between PsbA1 and PsbA3 and 27 between PsbA2 and PsbA3 (Sugiura and Boussac 2014). The PsbA1 gene is expressed under “normal” conditions, whereas the PsbA3 expression occurs under high-light or UV-light conditions and the transcription of the PsbA2 gene has been reported to take place under microaerobic conditions (Sicora et al. 2009). Although, Q_A is bound to PsbD (D2), measurements of its redox potential in the PsbA3 mutant resulted in a value of $E_m(Q_A/Q_A^-) = \sim -102$ mV (Kato et al. 2012). This midpoint potential fits surprisingly well to the one obtained during the first redox titrations, which were carried out outside the glove box ($E_m(Q_A/Q_A^-) = -115 \text{ mV} \pm 5 \text{ mV}$, **Figure 3.4**).

Q_A is located in close proximity (14 Å) to the Q_B binding site, which is located on the D1 protein and linked to Q_A via the Q_A -D2-H214-Fe-D1-H215- Q_B bridge (see also section

1.2.3). Switching from PsbA1 to PsbA3 leads to the substitution of an amino acid at the position 270 at the electron acceptor site (Ser in PsbA1 and Ala in PsbA3 and PsbA2), which has been proposed to modify the structure of the Q_B site by changing the D1-H215 hydrogen bond strength (Sugiura et al. 2010). Docking calculations and FTIR studies also suggests that the hydrogen bond strength between D2-H214 and Q_A is affected by the hydrogen bond strength between Q_B and D1-H215 (Ishikita et al. 2011). Therefore, a change in the Q_B site is likely to be propagated to Q_A via this hydrogen bond network, leading to a change in the E_m of the Q_A/Q_A^- couple.

Given the difficulty in measuring the midpoint potential of the Q_A/Q_A^- -couple from *T. elongatus* and the ambiguous resulting values of about -70 mV in intact systems in the presence of sodium bicarbonate and about -100 mV in PSII lacking the Mn-cluster, it could well be the case that the *T. elongatus* cells used for PSII isolation were actually a mixture between the “normal” cells, expressing the PsbA1 gene and mutated cells, expressing the PsbA3 gene, which were grown in the same incubator. This hypothesis could be tested e.g., by measuring the thermoluminescence of the $S_2Q_A^+$ -charge recombination (Kato et al. 2012). The peak intensity in the PsbA1 and PsbA3 mutant is different in the presence of DCMU and a peak shift occurs in the case of the PsbA3 mutant in the presence of bromoxynil. Since the differences were marginal in first thermoluminescence experiments, a mixture of the two species could hardly be identified with this method.

Another possibility could be that the binding constant of the bicarbonate ligand is lower in the PsbA3 mutant due to changes in the Q_B pocket and the binding of the ligand was already destabilized prior to the measurement by transferring the sample into the glove box. The addition of sodium bicarbonate enabled a full titration, but the question still remains why the titration of the Mn-depleted samples showed a very similar behaviour to the intact system, especially, since it seems to be the case that with the loss of the bicarbonate ligand during the titration and a shift of the Q_A/Q_A^- potential occurs to much higher potentials, indicating the formation of the so called “high potential” form of PSII, accompanied by the loss of the Mn-cluster (e.g., Ido et. al. 2011). Interestingly, a similar behaviour was observed in the PsbA3 mutant: no potential shift for the E_m of the Q_A/Q_A^- -couple was observed in redox titrations of Mn-depleted PSII expressing the PsbA3 gene (A. W. Rutherford and A. Boussac, personal communication), which is another indication that the titrated samples likely contained the PsbA3 mutant.

To avoid further difficulties resulting from working with a potential mixture of PSII complexes containing different D1 proteins, the species was changed and further redox

titrations to determine the Q_A/Q_{A^-} midpoint potential were carried out with PSII enriched membranes isolated from spinach.

3.4 Spectroelectrochemical titration of Photosystem II isolated from *Spinacia oleracea*

Since the various attempts to determine the redox potential of the Q_A/Q_A^- couple in *T. elongatus* did not lead to a clear result as described above, the spectroelectrochemical titrations were carried out with PSII enriched membranes from spinach. The same set-up was used with the same chlorophyll concentration and redox mediators, but the titrations were carried out at 15°C instead of 25°C to ensure the stability of the PSII enriched membranes during the duration of the measurement. Interestingly, a clear result was obtained for measurements at pH 6.5 and pH 7.0: **Figure 3.14 (A)** shows an average of three independent redox titrations at pH 6.5, resulting in a Q_A/Q_A^- midpoint potential of $E_m = -144 \text{ mV} \pm 11 \text{ mV}$.

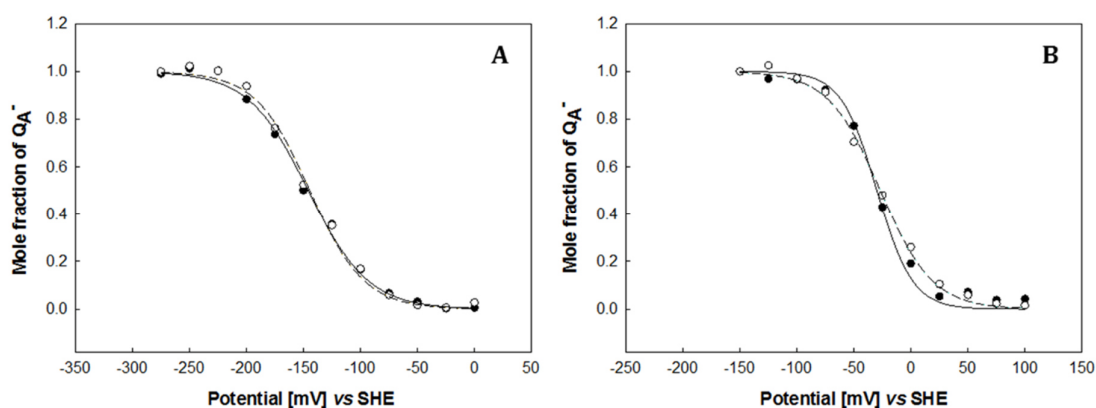


Figure 3.14 Average of three redox titrations of Q_A in (A) intact and (B) Mn-depleted PSII enriched membranes from spinach measured as fluorescence yield. Closed circles, reductive titration; open circles, oxidative titration. The curves were fitted according to equation (1.6) with $T = 288.15 \text{ K}$; the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potential of the Nernst curve in (A) is $-144 \text{ mV} \pm 11 \text{ mV}$ and in (B) $-28 \text{ mV} \pm 6 \text{ mV}$.

The depletion of PSII enriched membranes of the Mn_4CaO_5 cluster by treating them with hydroxylamine according to section 2.5.1 resulted in a midpoint potential for the Q_A/Q_A^- couple of $E_m = -28 \text{ mV} \pm 6 \text{ mV}$ (**Figure 3.14 (B)**). Identical results were obtained when freshly prepared intact and Mn-depleted membranes were used. These results are in a good agreement with the ones previously reported by Shibamoto et al. (2009, 2010) and Allakhverdiev et al. (2011): $E_m = -140 \text{ mV} \pm 2 \text{ mV}$ (intact PSII) and $E_m = +20 \text{ mV}$ (Mn-depleted PSII) and $E_m = -146 \text{ mV} \pm 1 \text{ mV}$ (intact PSII) and $E_m = -14 \text{ mV} \pm 1 \text{ mV}$

(Mn-depleted PSII), respectively, although Allakhverdiev et al. (2011) carried out the redox titrations at pH 7 using a potentiometric set-up.

The experimental set-up used here, successfully reproduces the conditions used by Shibamoto et al. (2009, 2010) and leads to almost identical values, but the question remains, how the contradictory values by Krieger et al. (1995) and Ido et al. (2011) of $E_m = -84 \text{ mV} \pm 16 \text{ mV}$ (intact PSII) and $E_m = +65 \text{ mV} \pm 25 \text{ mV}$ (Mn-depleted PSII) and $E_m = -60 \text{ mV} \pm 48 \text{ mV}$ (intact PSII) and $E_m = +60 \pm 30 \text{ mV}$ (Mn-depleted PSII), respectively, were obtained.

3.4.1 The bicarbonate ligand and the influence on the Q_A/Q_A^- midpoint potential

Herbicide binding to the Q_B site has been shown to have a significant effect on the redox potential of the Q_A/Q_A^- couple (see section 1.4.2), which, in the case of phenolic herbicides, is proposed to be caused by a change in the hydrogen bond network around the non-heme iron at the acceptor side. An important role in stabilizing this hydrogen bond network is attributed to the bicarbonate ligand, as described above and an indication of its influence on the Q_A redox potential was already observed in PSII complexes isolated from *T. elongatus* (see section 3.3).

To investigate, whether the bicarbonate anion influences with its presence at the acceptor site the redox potential of the Q_A/Q_A^- couple, intact and Mn-depleted PSII enriched membranes were depleted of the bicarbonate ligand according to section 2.5.3. This depletion resulted in a significant change of the Q_A/Q_A^- potential (**Figure 3.15**): for the intact PSII enriched membranes, a value of $E_m = -65 \text{ mV} \pm 5 \text{ mV}$ was obtained and the Mn-depleted samples resulted in a potential of $\pm 57 \text{ mV} \pm 2 \text{ mV}$. These values are remarkably close to the ones obtained by Krieger et al. (1995) and Ido et al. (2011). When the titration was carried out in intact and Mn-depleted PSII enriched membranes with the bicarbonate ligand being present and additional 10 mM sodium bicarbonate in the solution, the Nernst curves resulted in Q_A midpoint potentials of $E_m = -130 \text{ mV} \pm 5 \text{ mV}$ (intact) and $E_m = -29 \text{ mV} \pm 3 \text{ mV}$ (Mn-depleted), respectively (see **Figure 3.16 (A) and (B)**). These obtained redox potentials are in a very good agreement with the ones previously obtained in titrations of the fresh and frozen PSII enriched membranes and the question arises, whether the two redox potentials with their respective low and high

potential forms for the intact and Mn-depleted PSII reported in the literature originate from the absence and/or presence of the bicarbonate anion ligated to the non-heme iron.

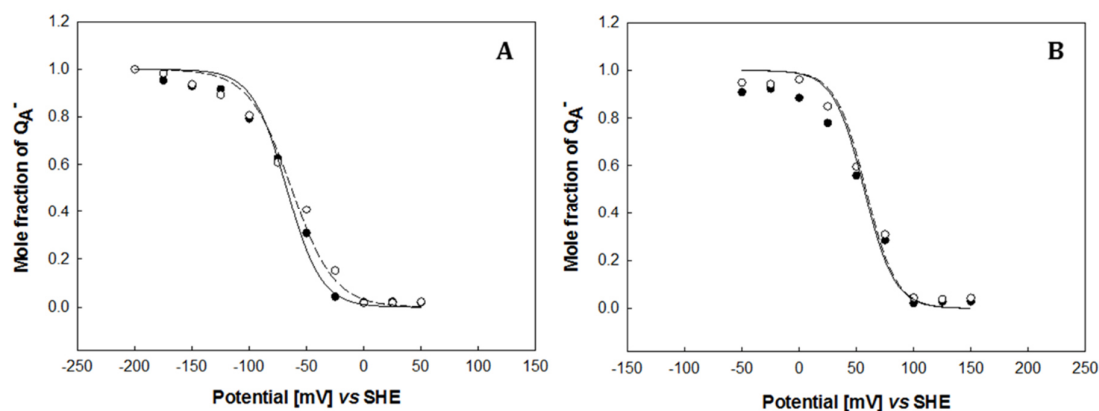


Figure 3.15 Average of two redox titrations of Q_A in (A) intact and (B) Mn-depleted PSII enriched membranes from spinach depleted of the bicarbonate ligand at the electron acceptor site, measured as fluorescence yield. Closed circles, reductive titration; open circles, oxidative titration. The curves were fitted according to equation (1.6) with $T = 288.15$ K; the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potential of the Nernst curve in (A) is $-65 \text{ mV} \pm 5 \text{ mV}$ and in (B) $+55 \text{ mV} \pm 6 \text{ mV}$.

To test, whether bicarbonate depletion in intact and samples lacking the Mn-cluster can be reversed again by addition of sodium bicarbonate to the electrolyte prior to the measurement, the samples were firstly depleted of the bicarbonate anion according to the procedure (section 2.5.3) and then 10 mM sodium bicarbonate was added to the buffer prior to the measurement. Indeed, a potential shift occurred again with respect to the midpoint potential observed for the Q_A/Q_A^- couple in the bicarbonate-depleted samples in **Figure 3.15 (A)** and **(B)**: the resulting midpoint potential was shifted to $-133 \text{ mV} \pm 3 \text{ mV}$ for the intact and $-19 \text{ mV} \pm 3 \text{ mV}$ for the Mn-depleted sample (**Figure 3.17**).

This observation leads to the question, whether the binding constant of the bicarbonate ligand can be affected by reducing environments and its removal can therefore be triggered during the course of the redox titration by e.g., exposing the samples to low potentials.

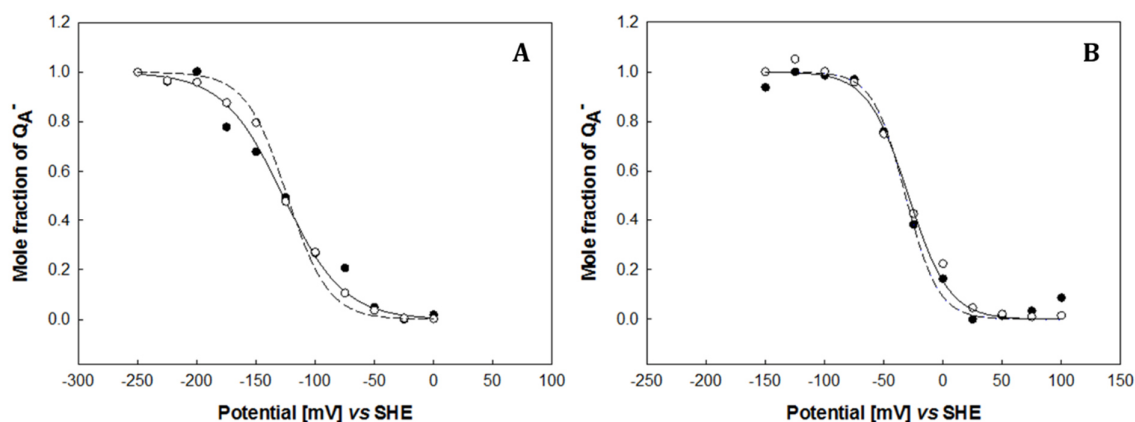


Figure 3.16 Average of two redox titrations of Q_A in (A) intact and (B) Mn-depleted PSII enriched membranes from spinach with 10 mM sodium bicarbonate present in the buffer solution, measured as fluorescence yield. Closed circles, reductive titration; open circles, oxidative titration. The curves were fitted according to equation (1.6) with $T = 288.15$ K; the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potential of the Nernst curve in (A) is $-130 \text{ mV} \pm 5 \text{ mV}$ and in (B) $-29 \text{ mV} \pm 3 \text{ mV}$.

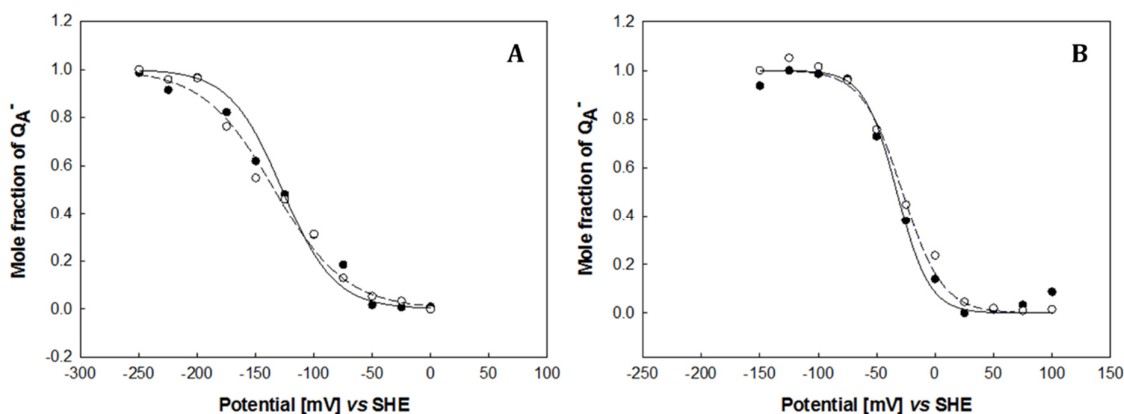


Figure 3.17 Average of two redox titrations of Q_A in (A) intact and (B) Mn-depleted PSII enriched membranes from spinach, which were firstly depleted of the bicarbonate ligand and secondly, prior to the measurement, 10 mM sodium bicarbonate were added again to the buffer solution. They were measured as fluorescence yield. Closed circles, reductive titration; open circles, oxidative titration. The curves were fitted according to equation (1.6) with $T = 288.15$ K; the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potential of the Nernst curve in (A) is $-133 \text{ mV} \pm 3 \text{ mV}$ and in (B) $-19 \text{ mV} \pm 3 \text{ mV}$.

3.4.1.1 Discussion

Krieger et al. (1995) observed already that when active PSII samples were incubated for 15 min or longer under very reducing conditions (e.g., with an excess of sodium dithionite in the buffer) at about -420 mV, a shift in the E_m of Q_A occurred to the high-potential value, indicating the transformation of PSII into the inactive form. A similar effect is hypothesized to occur here as well and account for the potential-shift. The isolation and preparation of the PSII enriched membranes were carried out in contrary to the preparation of Krieger et al. (1995) and Ido et al. (2011) in the presence of 1 mM sodium bicarbonate (see section 2.5). This variation of the protocol taken together with the titration results presented in **Figure 3.15 (A) and (B)** and **Figure 3.16 (A) and (B)**, suggests that the bicarbonate ligand was present after the preparation and maintained its position as well during the freezing process. The depletion of the bicarbonate ligand caused a change in the hydrogen bond network around Q_A and the non-heme iron, which extended as discussed before to the axial ligands D1-H198 and D2-H197 of P_{D1} and P_{D2} , respectively, and further in the PsbA subunit down to the direct metal ligands of the OEC, such as D1-E189 and D1-D170 (see section 1.2.3). This change in the hydrogen bond network is accompanied as well by a missing negative charge from the HCO_3^- -ion, which is likely to cause an effect on the direct redox environment and on the energetic properties of on Q_A .

The question remains, whether it is possible that a redox event causes a K_d change of the bicarbonate ligand, so that its removal is facilitated. In a first test, the intact PSII enriched membranes were washed once before the titration in the bicarbonate free buffer to lower the binding and furthermore, by carrying out the reductive titration to a potential of -250 mV. Then, the sample was left in the titration set-up for 2 h at -250 mV before the oxidative titration was carried out. **Figure 3.18** shows the result: indeed, the redox poisoning of the sample at -250 mV resulted in the change of the midpoint potential of the Q_A/Q_A^- couple from $-135 \text{ mV} \pm 3 \text{ mV}$ (reductive titration) to $+29 \text{ mV} \pm 2 \text{ mV}$ (oxidative titration). This result is in a good agreement with the once obtained by Krieger et al. (1995), who associated this phenomenon with the loss of the Mn-cluster due to redox poisoning. Additionally, it is demonstrated here that not only the stability of the Mn-cluster is affected by the application of a highly reducing potential: the midpoint potential of the Q_A/Q_A^- couple changes to the one of the Mn-depleted sample with the

bicarbonate anion being removed (**Figure 3.15 (B)**). This time-dependent experiment demonstrates as well again that four potentials forms of the Q_A/Q_A^- couple exist, which agree well with the ones reported in the literature.

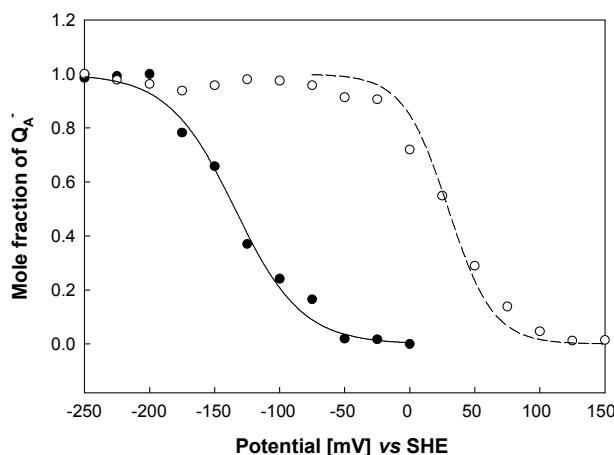


Figure 3.18 Average of two redox titrations of Q_A measured as fluorescence yield in intact PSII enriched membranes from spinach, which were washed once in bicarbonate-depleted buffer prior to reductive titration. The oxidative titration was further on carried out after leaving the sample in the set-up for 2 h at an applied potential of -250 mV. Closed circles, reductive titration; open circles, oxidative titration. The curves were fitted according to equation (1.6) with $T = 288.15$ K; the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potentials of the Nernst curves are $-135 \text{ mV} \pm 3 \text{ mV}$ and $+29 \text{ mV} \pm 2 \text{ mV}$, respectively.

Furthermore, it was investigated whether the binding of other anions than bicarbonate affects the Q_A redox potential, since the replacement of bicarbonate with formate or acetate was not only shown to inhibit the overall oxygen evolution activity in thylakoids isolated from spinach by 50 % and 10 %, respectively (Shevela et al. 2007) and to influence the electron transfer rate from Q_A to Q_B (e.g., Sedoud et al. 2011 for investigations of formate binding), but acetate-binding to the non-heme iron was also proposed to change the environment of Q_A and Q_B through the histidine ligand network of the NHI and to affect charge recombination events and photoinhibition in PSII (Roach et al. 2013).

In first experiments, bicarbonate was replaced by formate according to section 2.5.4. This replacement caused indeed a small change in the midpoint potential: the E_m increases to $-99 \text{ mV} \pm 3 \text{ mV}$ (**Figure 3.19**). This upshift increases the probability of direct charge recombination of P_{D1}^+ and Q_A^- to the ground state, leading to a lower yield of the

generation of $^3\text{P}_{\text{D1}}$ and thus highly toxic $^1\text{O}_2$. Given this effect of formate on the redox potential, it would be of interest to investigate the effect of acetate binding and other anions such as oxalate and glycolate, which change as well the redox potential of the NHI (see chapter 5).

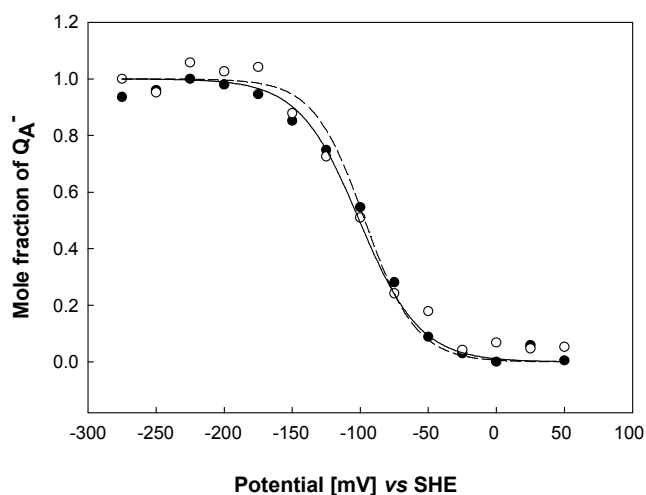


Figure 3.19 Average of two redox titrations of Q_A measured as fluorescence yield in intact PSII enriched membranes from spinach with the bicarbonate anion replaced by formate. Closed circles, reductive titration; open circles, oxidative titration. The curves were fitted according to equation (1.6) with $T = 288.15 \text{ K}$; the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potential of the Nernst curves is $-99 \text{ mV} \pm 3 \text{ mV}$.

3.4.2 Excitation energy transfer in PSII enriched membranes and the influence on the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ midpoint potential

Due to the possibility of excitation energy transfer between the two photosystem units as described earlier (section 2.7.4.1), the calculated midpoint potentials were corrected according to equation (2.9), using the 'lake' model for PSII enriched membranes from spinach. These corrections have been carried out throughout this section where indicated and their significance should be exemplified here.

Figure 3.20 (A) and **(B)** show the experimental data (black curves) for intact PSII (**Figure 3.20 (A)**) and Mn-depleted PSII (**Figure 3.20 (B)**) of the mole fraction of Q_A^- , calculated based on the fluorescence, against the electrode potential, which follows a Nernst behaviour. The red and green Nernst plots were computed from equation (2.9)

with the experimentally obtained value of $R = 2.39$ and represent the situation, where chlorophyll fluorescence is linear to the amount of centres with Q_A being reduced (i.e. $R = 1$).

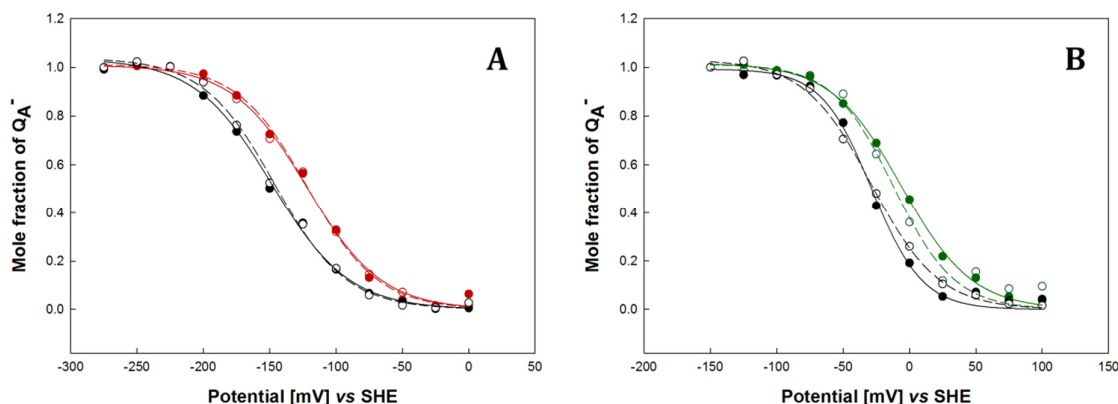


Figure 3.20 Nernstian plots of the mole fraction of Q_A^- against the electrode potential measured as fluorescence yield (black plots, see **Figure 3.14** (A) and (B)) and calculated according to equation (2.9) using the 'lake' model for excitation energy transfer in PSII enriched membranes from spinach and $R = 2.39$. (A) intact PSII; (B) Mn-depleted PSII. Closed circles, reductive titration; open circles, oxidative titration. The curves were fitted according to equation (1.6) with $T = 288.15$ K; the solid line corresponds to the reductive titration, the dashed line to the oxidative titration. The midpoint potential of the Nernstian plot in (A) is $E_m = -122$ mV in (B) -9 mV.

The midpoint potential for the Q_A/Q_A^- -couple obtained from the calculated Nernst plots is $E_m = -119$ mV for intact PSII and $E_m = -9$ mV for Mn-depleted PSII. **Figure 3.21** demonstrates the significance of this correction by showing plots of the theoretical mole fraction of Q_A^- from 0 to 1 and the corresponding calculated mole fraction, $F(x)$, by using equation (2.9) and different values for R . Choosing the cases $R = 1$ (for ideal connectivity between the PSII monomers), $R = 2.39$ (experimentally obtained here) and $R = 6$ (example of an extreme value), the graphs illustrate the deviation from the ideal situation $R = 1$, where the measured fluorescence yield is linearly related to the fraction of reduced centres of Q_A (see **Figure 3.21** and the dashed line for $R = 6$ and the dotted line for $R = 2.39$).

Table 3.1 lists the determined midpoint potentials and the corresponding values corrected for the non-linear behavior of chlorophyll fluorescence based on the excitation

energy transfer in PSII dimer complexes (see section 2.7.4.1 and Shibamoto et al. (2010)).

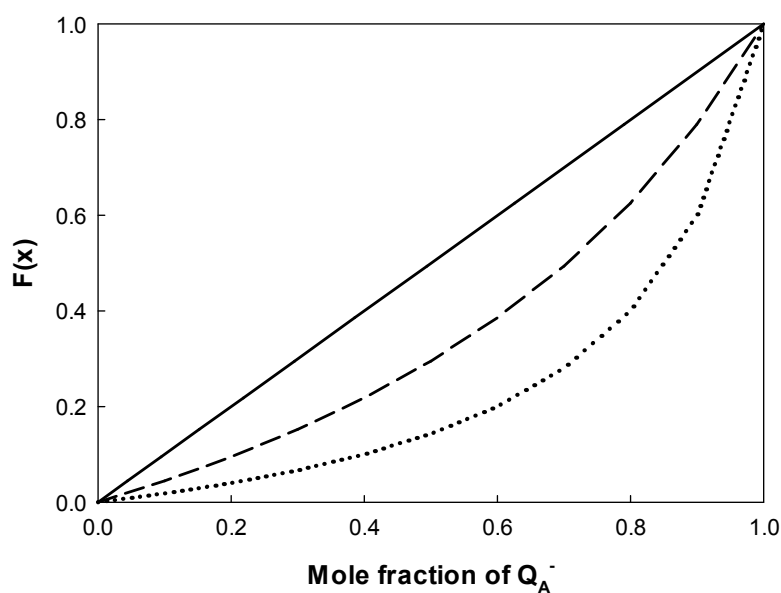


Figure 3.21 Plots of the experimentally obtained mole fraction of Q_A^- based on the fluorescence yield and the corresponding calculated mole fraction of Q_A^- using equation (2.9) and $R = 1$ (solid line), $R = 2.39$ (dashed line) and $R = 6$ (dotted line).

Sample	$E_m (Q_A/Q_A^-)$	Corrected $E_m (Q_A/Q_A^-)$
PSII +bicarbonate ligand	-144 mV	-122 mV
PSII -Mn-cluster, + bicarbonate ligand	-28 mV	-9 mV
PSII -bicarbonate ligand	-65 mV	-46 mV
PSII -Mn-cluster, -bicarbonate ligand	+55 mV	+73 mV

Table 3.1 Midpoint potentials of the Q_A/Q_A^- couple in intact and Mn-depleted BBY membranes at 15°C as a result of various redox titrations with the bicarbonate ligand being present and/or absent and the corrected E_m values considering the non-linear behaviour of fluorescence (section 2.7.4.1) with $R = 2.39$.

It seems to be the case that Shibamoto et al. (2009, 2010) and Allakhverdiev et al. (2011) worked under conditions, under which they maintained the bicarbonate anion ligand in place, which is the reason why they obtained in their measurements the lower potential forms, whereas Krieger et al (1995) and Shibamoto et al. (2011) titrated Q_A in samples lacking the bicarbonate ligand.

The redox titrations with PSII enriched membranes isolated from spinach provided clear results for the Q_A/Q_A^- midpoint potential and demonstrated as well the influence of the bicarbonate ligand on the potential.

Furthermore, it would be interesting to investigate this phenomenon in other species. First measurements on PSII isolated from *T. elongatus* (section 3.1.1) indicated already that bicarbonate plays as well a crucial role in cyanobacteria, although, its exact influence needs to be investigated further in samples, which are clearly expressing either the PsbA1 or PsbA3 gene. Furthermore, it would be of interest to determine the midpoint potential as well in different organisms, e.g., which contain chlorophyll d and f as major light absorbing pigments, since Shibamoto et al. (2010) showed already that the Q_A midpoint potential is species dependent. One example would be *Leptolyngbya* sp. strain JSC-1, which exhibits an extensive photoacclimative response to growth in far-red light including the synthesis of chlorophylls d and f (Gan et al. 2014) and which is currently grown in our laboratory.

Chapter 4.

Electrochemical investigations of Photosystem II in metal oxide hybrid systems

4.1 Introduction

Knowledge of Photosystem II has inspired the field of artificial photosynthesis, in which robust and cheap catalysts are being developed for photochemical and electrochemical generation of fuels using solar energy (Barber and Tran 2013; Sherman et al. 2014; Young et al. 2012).

The enzyme itself is often considered to have applications in a range of photoelectrochemical devices as described already above (Kato et al. 2014; Badura et al. 2011; Yehezkeli et al. 2014). However, the use of isolated PSII for energy generation appears unrealistic, not only because of the energy, time and effort required for isolating it from the living cell, but also because PSII undergoes photodamage. Indeed the D1 protein, which is the location of the damage, is the most rapidly turned-over protein in the thylakoid membrane (Edelman and Mattoo 2008). Its degradation strongly depends on the incident light intensity and it can have a half-life of only 30 min (Kanervo et al. 1993). Nevertheless, applications of isolated PSII that do not require the scale or longevity needed for energy production are conceivable (e.g. sensors for pollutants and herbicides (Giardi et al. 2001; Maly et al. 2005). Additionally, the utilization of PSII in devices could become advantageous if a new form of PSII with enhanced photostability is either engineered or isolated from an organism living in extreme conditions (Treves et al. 2013).

PSII is a particularly interesting system for studying electron transfer from protein-bound cofactors to electrode materials since it is the only reaction center capable of taking electrons from water and thus it does not need electron donors that could react with the electrode directly. A well understood electronic coupling between PSII and an electrode surface could allow an additional avenue of research on the enzyme itself.

4.2 Photocurrent generated by Photosystem II adsorbed on a nanostructured titanium dioxide/ indium tin oxide electrode: electron transfer pathways

Photocurrents from PSII immobilized onto electrode surfaces have been studied for decades (for example Agostiano et al. 1984; Lemieux and Carpentier 1988; Yehezkeli et al. 2012; Kato et al. 2012, 2013; Mersch et al. 2015).

Anomalous results with the photocurrents generated by PSII in these metal oxide hybrid systems indicate that the present understanding of the reactions occurring is incomplete, as described already in section 1.4.3.1. In particular, on metal oxides, the addition of the herbicide DCMU, which is expected to shut down the mediated electron transfer by competing with exogenous quinone acceptors at the Q_B site, resulted in significant residual photocurrents which could not be accounted for by direct electron transfer from $Q_A^{\bullet-}$ in the contact layer (Rao et al. 1990; Kato et al. 2012). Until now, efforts have been focused mainly on the phenomenon of the photocurrent itself and its maximization. However, the characterization of the electron pathway from the protein to the electrode and the understanding the involvement of mobile mediators are both important for developing this methodology, both for studying the enzyme and for any potential applications.

Little if any work has been done on characterizing the electron pathway from the protein to the electrode and the role of the mobile mediators (see section 1.4.3.1). The focus on obtaining maximum photocurrents has led to the use of protein multilayers (in the presence of mediators), but this gives rise to heterogeneity, with direct and mediated electron transfer to the electrode potentially occurring at the same time.

In a first part of the project, the PSII layer thickness on the electrode surface was controlled by working with a monolayer/sub-monolayer and with multilayers of PSII. Work with the monolayer inevitably resulted in much smaller photocurrents, but it allowed much less ambiguous results than working with multilayers. Furthermore, the effect of the herbicide DCMU and redox mediators on the photocurrents generated with electrodes using monolayers and multilayers of PSII was investigated and the electrode structure and morphology to control access to the ITO was changed. The results allow the proposal of a new model for the electron transfer in this kind of PSII/metal oxide semiconductor system.

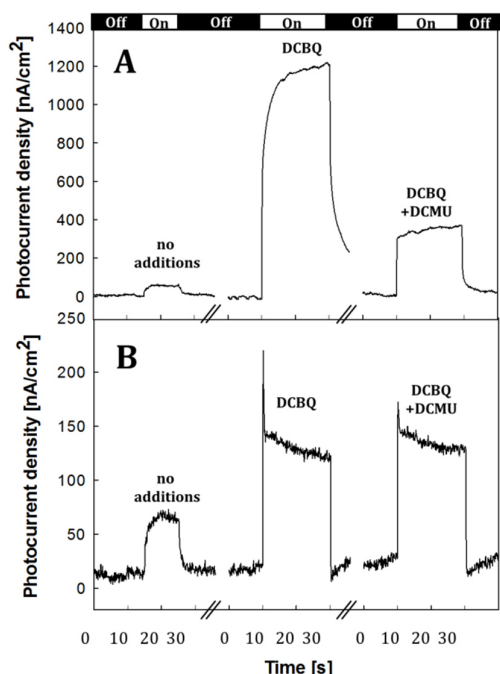


Figure 4.1 Photocurrent response from PSII multilayers (A) and monolayers (B) adsorbed onto a nanostructured TiO₂ film in the absence and presence of the mediator DCBQ and the herbicide DCMU. (A) The photocurrent recorded from PSII multilayers (first trace), the presence of 100 μ M DCBQ (second trace) and 10 μ M DCMU (third trace). (B) The photocurrent recorded from a PSII monolayer (first trace) in the presence of 100 μ M DCBQ (second trace) and in the presence of 100 μ M DCBQ and 10 μ M DCMU (third trace). Note: The bar at the top shows the length of the illumination periods, 10 s for the trace on the left and 20 s for the others (see experimental section).

Kinetics of the photocurrent generation as an indication of the formation of different PSII layers on the electrode. Since the first immobilization experiments of PSII on TiO₂ films, the observation has been made that the addition of an artificial electron acceptor such as DCBQ gave rise to a sharp increase in photocurrent, although the overall magnitude of photocurrent differed substantially (Rao et al. 1990; Lemieux and Carpentier 1988; Kato et al. 2012). The results were interpreted as an interaction of the electron acceptor with the Q_B binding side and therefore, accelerating the electron transfer from PSII to the electrode (Kato et al. 2012). Nevertheless, immobilization procedures of PSII onto electrodes indicate that the actual concentration of photoactive PSII and its electronic connection to the electrode is unknown. The addition of DCBQ as an electron mediator enables the possibility of connecting all PSII complexes electronically to the electrode, even those ones, which are not in close proximity to the electrode surface.

To study the origin of photocurrent and the function of DCBQ further in detail, two extreme PSII concentrations were chosen in this study for an immobilization onto TiO₂/ITO electrodes:

[Chl] = 4 μ g/mL and [Chl] = 400 μ g/mL. The two concentrations resulted in a significant difference in the magnitude of the overall photocurrent in the presence of DCBQ and gave rise to a substantial difference in the induction and relaxation photocurrent kinetics (**Figure 4.1 (A) and (B)**). This observation was associated with different protein layer thicknesses on the electrode surface and the ability of controlling the electron transfer rate from PSII to the electrode with DCBQ by overcoming

diffusion-based limitations.

The effect of the herbicide DCMU and the electron mediator DCBQ. In various systems, where mediated photocurrent generation by PSII was recorded, a remaining amount of photocurrent was observed, when DCMU was added to the electrolyte (Lemieux and Carpentier 1988; Rao et al. 1990; Rao et al. 1990; Amako et al. 1993; Ulas and Brudvig 2011; Kato et al. 2012; Lemieux and Carpentier 1988), which could be observed as well in our studies (**Figure 4.1 (A)**). Interestingly, DCMU did not lead to a decrease of the photocurrent, when PSII was immobilized in form of a monolayer (**Figure 4.1 (B)**). This observation led to the conclusion that the recorded photocurrent originated from Q_A as a primary electron donor, since the Q_B acceptor site was blocked by the herbicide for electron transfer to the electrode. However, this explanation was not compatible with the enhancement of the photocurrent observed in the presence of electron mediators such as DCBQ: the enhancing effect of DCBQ could only be explained by mediating the electron transfer

between the Q_B site and the electrode. To address this question and to determine the effect of DCBQ and DCMU, PSII was immobilized in two concentrations on the electrode, leading to the formation of two different protein layer thicknesses on the electrode and the formation of mono- and multilayers. Firstly, several possible explanations for the lack of a DCMU effect on the DCBQ enhanced photocurrent were tested when PSII was immobilized as a monolayer:

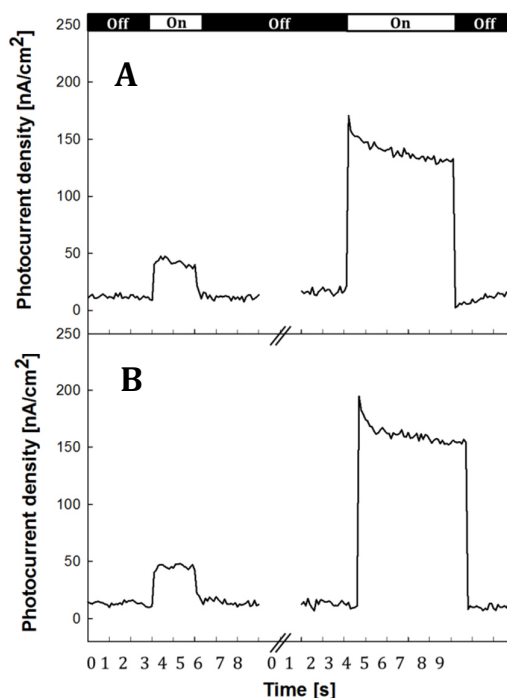


Figure 4.2 (A) Photocurrent response from PSII immobilized onto TiO_2 as a monolayer with $10 \mu M$ DCMU added to PSII prior to its immobilization (first trace) and after the addition of $100 \mu M$ DCBQ to the measuring buffer (second trace). (B) Photocurrent response from PSII immobilized onto TiO_2 as a monolayer in the presence of $10 \mu M$ DCMU in the measuring buffer (first trace) and after the addition of $100 \mu M$ DCBQ (second trace). Note: The bar on top of the charts shows the length of the illumination periods.

- i) The possibility that DCMU had restricted access to the Q_B site in the immobilized PSII. DCMU was added to the immobilization buffer containing PSII prior to its immobilization on the electrode. DCBQ addition still induced a comparable enhancement of the photocurrent occurring from PSII (**Figure 4.2 (A) and (B)**) indicating that the lack of a DCMU effect was not simply due to restricted access of DCMU to the Q_B site.
- ii) The possibility that DCMU binding affinity was decreased in the immobilized PSII was tested by increasing the concentration of DCMU in the measuring buffer. The DCBQ-induced enhancement of photocurrent in the PSII monolayer was unaffected by DCMU up to concentrations of 100 μM (**Figure 4.3**). An immobilization induced shift in the binding affinity (nanomolar; Zimmermann et al. 2006) by several orders of magnitude seems unlikely. Further control experiments showed that a gradual slight decrease of the photocurrent with time was due to photodamage of the protein and was unrelated to the effect of DCMU.

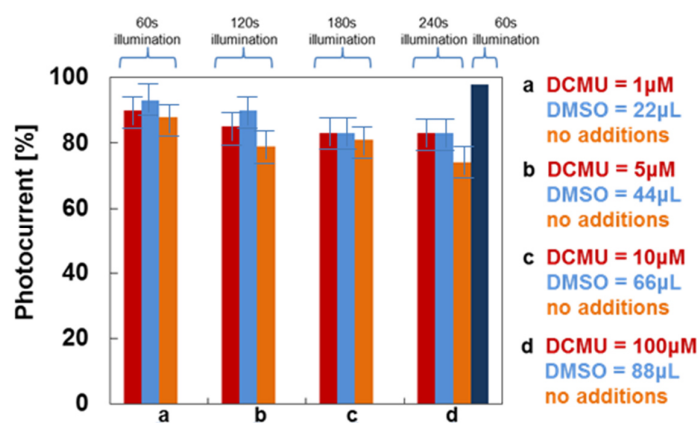


Figure 4.3 Influence of increasing concentrations of DCMU and sequential illuminations on the photocurrent. PSII was immobilized onto TiO_2 as a monolayer and photocurrent measurements were recorded in the presence of 100 μM DCBQ. The photocurrent obtained on the first illumination in the presence of DCBQ was taken as 100%. Control experiments in the absence of DCMU are also shown (with and without DMSO, the solvent for DCMU). The results indicate that the PSII monolayer is unaffected by the DCMU treatment and that the insensitivity of the photocurrent is not due to the PSII immobilization since it is unlikely that this affects the binding constant by several orders of magnitude.

The experiments were performed sequentially with increasing concentration of DCMU with the consequence that the PSII was exposed to the light for increasing periods of time as indicated above the bar chart. When compared with a control sample (dark blue bar), which was kept in the dark for 240 seconds and then illuminated for 60 seconds, a small decrease in photocurrent was measured. This small decrease, which was not significantly affected by the presence of DCMU, was attributed PSII photo-degradation.

iii) The possibility that immobilization generates a situation in which DCBQ becomes fixed or trapped between the protein and the TiO_2 surface was discounted since the enhancement of the photocurrent by DCBQ was reversed when the DCBQ was removed by replacing the buffer (**Figure 4.4**).

Based on the experiments described above, it seems likely that DCBQ and DCMU function as an electron mediator and as a Q_B site inhibitor respectively, as

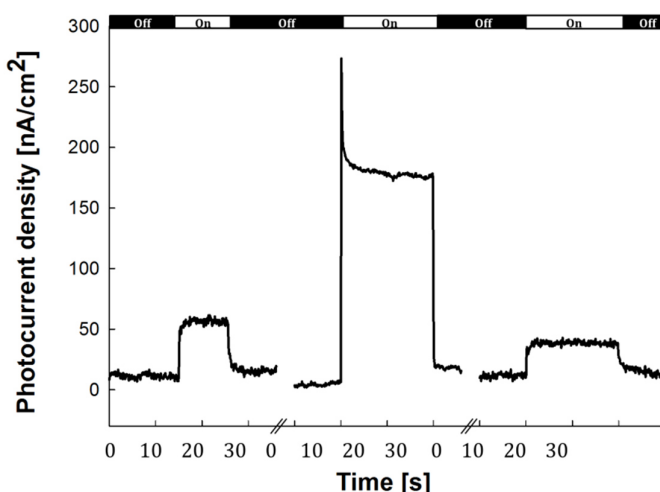


Figure 4.4 Photocurrent density from PSII immobilized as a monolayer onto TiO_2 without mediator (first trace), in the presence of $100\ \mu\text{M}$ DCBQ in measuring buffer (second trace) and after the replacement of the measuring buffer with a fresh solution without DCBQ (third trace). The loss of the photocurrent in the third trace indicates that DCBQ is neither bound to PSII nor to TiO_2 . Note: The bar at the top shows the length of the illumination periods.

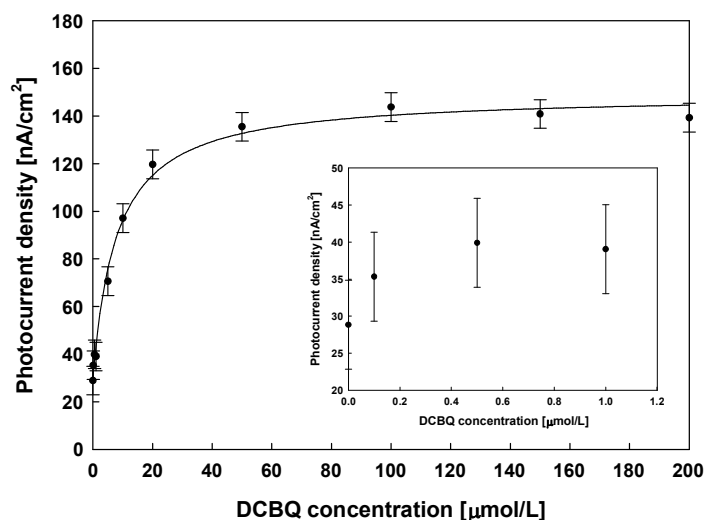


Figure 4.5 Photocurrent density from PSII immobilized as a monolayer onto TiO_2 as a function of increasing DCBQ concentrations in the measuring buffer with error bars. The maximum photocurrent was recorded in the presence of $100\ \mu\text{M}$ DCBQ. Inset shows the photocurrent recorded for DCBQ concentrations up to $1\ \mu\text{M}$.

expected. The results in **Figure 4.1 (A) and (B)** thus indicate that $\text{Q}_\text{A}^\bullet$ is able to donate electrons directly to the nanostructured TiO_2 . Two potential mechanisms can be suggested to explain why DCBQ enhances the photocurrent from the PSII monolayer and why DCMU had no effect on the photocurrent under these conditions: i) DCBQ takes electrons directly from Q_A and delivers electrons to the TiO_2 or the ITO; ii) DCBQ takes electrons from the TiO_2 surface

and delivers them to the ITO.

Both mechanisms require the diffusion of DCBQ in solution. This is expected for the mediator, but was confirmed by i) the loss of photocurrent when DCBQ is removed from the buffer as mentioned above, and ii) the observation that increasing concentration of DCBQ resulted in a hyperbolic increase in photocurrent with a K_m of 8 μM (**Figure 4.5**). This value is however more than 10 times smaller than that measured in oxygen evolution measurements in solution (Sato et al. 1992), suggesting that the interaction of DCBQ with PSII does not involve the Q_B site.

The photocurrent measured as a function of an increasing DCBQ concentration deviated from hyperbolic behavior for concentration values below 1 μM , indicating a threshold below which DCBQ had no or little effect. This seems to suggest that DCBQ competes with another electron transfer route and its effect on the photocurrent can only be observed above a certain concentration in solution. At low DCBQ concentration the slow electron transfer through the metal oxide dominates, while at higher DCBQ concentrations (above 1 μM) the more favourable route provided by the mediator in solution will be preferred.

The results obtained using the PSII monolayer can be used to analyse the behaviour of the PSII multi-layers. For the multilayers of PSII, DCMU should drastically inhibit electron transfer (down to 10%) from PSII in all layers other than the contact layer. The data in **Figure 4.1 (A)** partially fit with this expectation, with the trace from the multilayers of PSII in the presence of DCBQ and DCMU showing a photocurrent amplitude that is twice that of the monolayer when DCMU is present (**Figure 4.1 (B)** right trace, note the scale difference between (A) and (B)).

The smaller amplitude of the photocurrent in the corresponding “monolayer” under these conditions is either due to the reduced DCBQ mediated electron flow from $Q_A^{\bullet-}$ or to the fact that the monolayer is incomplete, while the contact layer at the base of the multilayers is expected to be complete. Nevertheless, the slow rising kinetic, which is characteristic of the DCBQ-enhanced photocurrent in the multilayers of PSII is eliminated by DCMU (**Figure 4.1 (A)** right) leading to a photocurrent kinetic profile that is more similar to that of the monolayer. These changes in the kinetic profile suggest that the direct electron transfer from Q_A to DCBQ, which in solution is slow (see below), does not play a significant role in the electron transfer process to the electrode also in the multi-

layers. This explanation questions the role of electron acceptors such as DCBQ in case of PSII-monolayers on the electrode: if Q_A is the source of electrons, why is DCBQ still able to enhance the photocurrent?

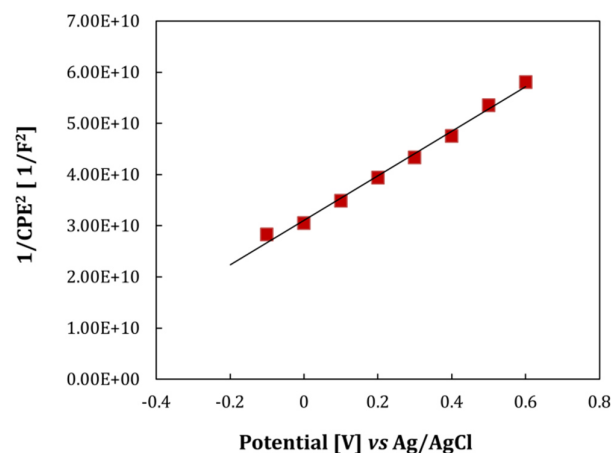


Figure 4.6 Mott-Schottky plot for TiO_2 in the electrolyte buffer solution containing 20 mM $CaCl_2$, 40 mM MES and 5% glycerol at pH 6.5. The resulting flat band potential was calculated to be -688 mV vs Ag/AgCl (-489 mV vs SHE) according to equation (2.4).

A model for electron transfer pathways. Literature reports on the position of the conduction band of TiO_2 indicate that at pH 6.5 the value is approximately -450 mV (Xu and Schoonen 2000). This value was confirmed in the here applied experimental conditions using electrochemical impedance spectroscopy: at the interface between the semiconducting electrode and the electrolyte occurs band bending, which is a result of the solid-

electrolyte interface. The Fermi level E_f in the semiconductor and electrochemical level E_{redox} in the electrolyte are equal at the equilibrium. A space charge region is formed at the interface, which provides a strong electric field. The band bending is also affected by an externally applied voltage. A unique potential exists for a given semiconductor and electrolyte at which the potential drop between the surface and the bulk is zero and there is no space charge layer: the flat band potential V_{fb} . This potential of a metal oxide can easily be determined as described in section 2.1.3 by determining a Mott-Schottky plot based on measuring the impedance of the material. **Figure 4.6** shows the Mott-Schottky plot for the TiO_2 used here, resulting in a V_{fb} of about -490 mV vs SHE.

Since the reduction potential of Q_A in intact PSII is -144 mV as verified in section 3.1.4 and photocurrents were recorded by applying a potential bias of +644 mV, even considering band bending, the concentration of traps and the electron mobility on the surface of the metal oxide is very small. Experimental evidence for poor electron mobility in similar conditions was shown in studies of interfacial electron transfer between proteins and TiO_2 where horse-heart cytochrome c with a reduction potential of +250 mV was immobilised onto mesoporous TiO_2 films (Topoglidis et al. 2008). Electron density of

the TiO_2 electrode was measured as a function of an applied bias and reported to show an insulating behaviour above an applied potential of -300 mV. This was considered to be caused by the position of the Fermi level, which lies deep in the band gap of the semiconductor at positive applied potentials. This caused a shift in the measured midpoint potential of the immobilised cytochrome c, indicating that a larger overpotential was needed to transfer electrons from the Fermi level of the semiconductor to the electrode.

Two models can therefore be outlined: in the first one, when no mediator (DCBQ) is present, electron transfer through the metal oxide is slow and the magnitude of the photocurrent is strongly dependent on recombination events (**Figure 4.7 (A)**); in the second one, in the presence of the mediator, the electrons transferred from the Q_A site and slowly moving on the TiO_2 surface would be rapidly extracted by the freely diffusing DCBQ to be directly delivered to the ITO. By bypassing the slow electron mobility through the mesoporous metal oxide, the DCBQ would provide an alternative and more favourable route for the electrons (**Figure 4.7 (B)**).

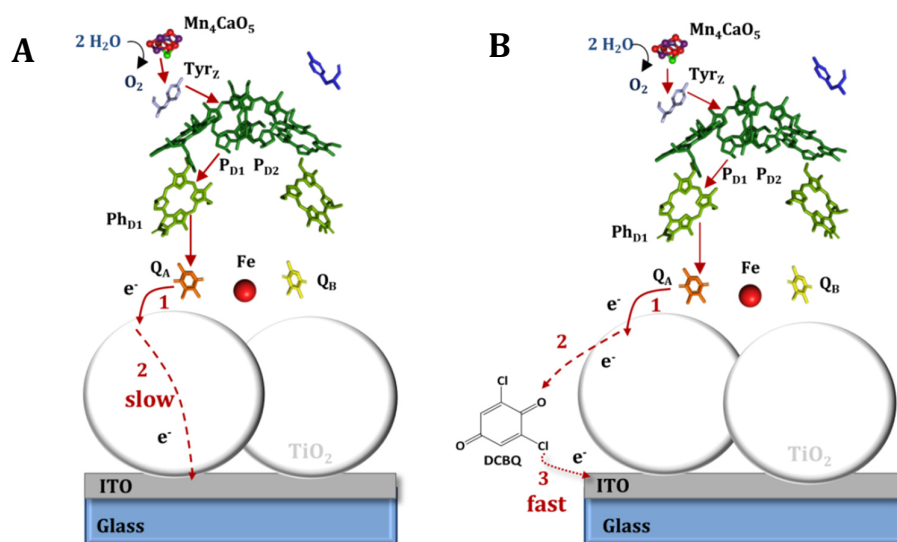


Figure 4.7 Model describing the electron transfer from PSII to ITO in the absence (A) and presence (B) of the electron mediator DCBQ. (A) Upon illumination electron transfer occurs from Q_A to the TiO_2 (1) and slow electron transfer occurs on the TiO_2 surface (2) due to the poor conducting properties of the material at an applied bias of +644 mV vs SHE. (B) The redox mediator DCBQ provides an alternative pathway for the electrons by picking up electrons from the TiO_2 surface and transferring them directly to ITO (3).

This would explain the observed enhancement of the photocurrent being completely insensitive to the presence of DCMU. To substantiate the theory further and to prove that electron mediators such as DCBQ do not interact with PSII, but rather mediate the electron transfer from the TiO₂ surface to the ITO, other electron mediators were tested.

Figure 4.8 (A) shows that with PSII in solution 10 % of the oxygen evolving activity remained when both DCBQ and DCMU were present, in line with previous observations (Boussac et al. 2004a). Assuming that this is due to DCBQ being able to accept electrons from Q_A[•] when the Q_B site is blocked, this indicates that the rate of electron transfer from Q_A[•] to DCBQ is ~10 times slower than the electron transfer rate to DCBQ when DCMU is absent. Consequentially the absence of an effect of DCMU on the DCBQ-enhanced photocurrent in the PSII monolayer (**Figure 4.1 (B)**) indicates that this photocurrent cannot be ascribed to DCBQ-mediated electron transfer between PSII and the electrode surface (see below).

Figure 4.8 (A) shows as well the oxygen evolution activity in solution with PpBQ, another commonly used electron acceptor with PSII (Sugiura and Inoue 1999). The activity was eliminated when DCMU was present (**Figure 4.8 (A)**, bar 11).

Clearly PpBQ is unable to accept electrons from Q_A[•] and yet PpBQ did enhance the photocurrent just as did DCBQ (**Figure 4.8 (B)**) and this enhancement was also unaffected by DCMU. We conclude that the PpBQ-enhanced photocurrent does not involve electron transfer from Q_A[•] to the mediator. Note, in **Figure 4.8 (B)** the differences in the magnitude of the photocurrents for the different mediators are likely to be due to differences in the reduction potentials and/or their different affinities for the TiO₂ surface.

The results presented in **Figure 4.8 (A)** and **(B)** and the ones described in the previous paragraphs argue strongly against a mechanism in which the mediator takes electrons directly from Q_A[•] and delivers them to the TiO₂ or the ITO. In the following, experiments are designed to test the alternative mechanism: the mediator taking electrons from the TiO₂ surface and delivering them to the ITO. **Figure 4.8 (B)** also shows an experiment using the redox mediator quercetin instead of DCBQ. Quercetin was chosen since it has a similar reduction potential to DCBQ (E_m (quercetin) = +331 mV; E_m (DCBQ) = +310 mV, both pH 7)), but does not act as an electron acceptor from PSII, as demonstrated in oxygen evolution experiments shown in **Figure 4.8 (A)**.

Figure 4.8 (B) shows that quercetin gives rise to an enhancement of the photocurrent, similar to DCBQ and PpBQ. This enhancement also occurs in the presence of DCMU.

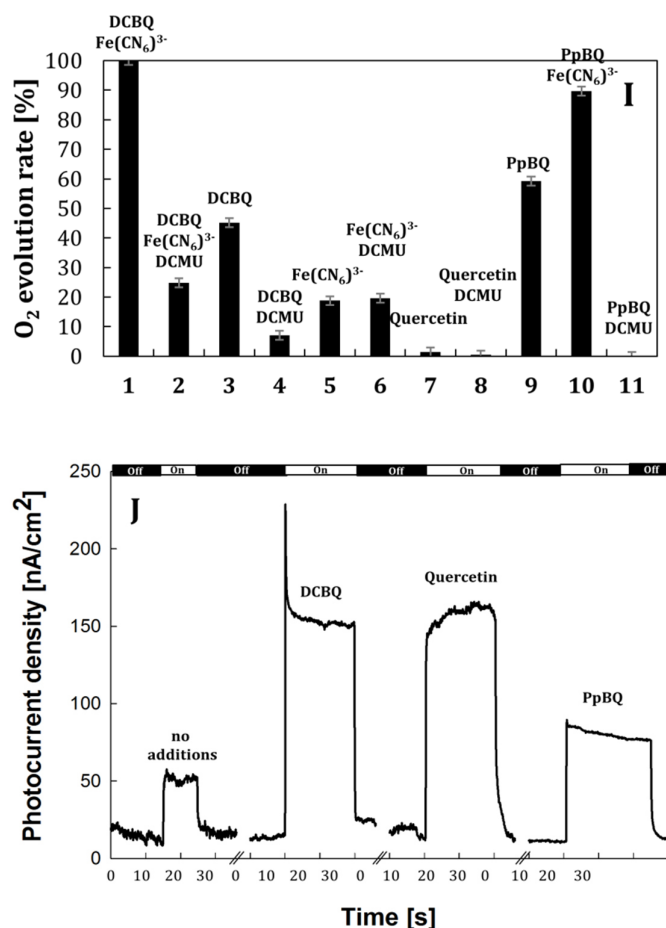


Figure 4.8 (A) Oxygen evolution measurements of PSII in the presence of 0.5 mM DCBQ, 1 mM ferricyanide (1); 0.5 mM DCBQ, 1 mM ferricyanide, 50 μ M DCMU (2); 0.5 mM DCBQ (3); 0.5 mM DCBQ, 50 μ M DCMU (4); 1 mM ferricyanide (5); 1 mM ferricyanide, 50 μ M DCMU (6); 0.5 mM quercetin (7); 0.5 mM quercetin, 50 μ M DCMU (8); 0.5 mM PpBQ (9); 0.5 mM PpBQ, 1 mM ferricyanide (10) and 0.5 mM PpBQ, 50 μ M DCMU (11). Error bars are indicated in grey. **(B)** Photocurrent response from PSII immobilized onto TiO₂/ITO electrode as a monolayer; unmediated, in the presence of 100 μ M DCBQ, 100 μ M quercetin or 100 μ M PpBQ in the measuring buffer. Note: The bar at the top shows the length of the illumination periods.

The results indicate that the mediator-induced enhancement of the photocurrent is due to electrons carried from the surface of the nanostructured TiO₂ to the exposed ITO by the mobile electron carrier. To confirm this model, photocurrents were studied using an electrode in which a layer of crystalline TiO₂, which is impermeable to any mobile electron carrier in solution (see scheme in **Figure 4.9**), was used to separate the nanostructured TiO₂ from the ITO conducting layer. Such layers have been shown to reduce the recombination of the injected electrons and block the interaction between the ITO surface and freely soluble redox active molecules (Burke et al. 2008). This separating layer eliminated any enhancement of the photocurrent by DCBQ (**Figure 4.10**). This is a good support for the mechanism that explains that the mediator-induced enhancement (which is only present without the

blocking layer) is due to DCBQ acting as an electron carrier, shuttling electrons from the TiO₂ to the ITO.

All results can therefore be explained by a model describing two situations: in the first case, when no mediator (DCBQ) is present, electron transfer through the metal oxide is slow and the magnitude of the photocurrent is strongly dependent on recombination events within PSII and on the electrode surface (**Figure 4.7 (A)**); in the second case, in the presence of the mediator, the electrons which are transferred from the Q_A site to the TiO_2

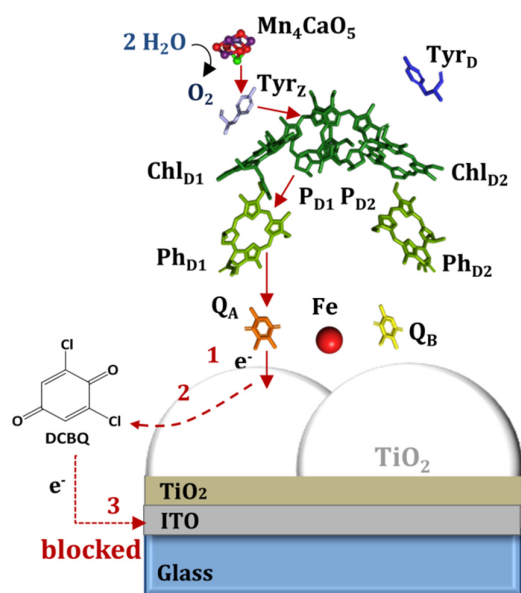


Figure 4.9 Model illustrating the DCBQ-mediated electron transfer from PSII to ITO when a blocking layer of crystalline TiO_2 was present between the nanoporous TiO_2 layer and the ITO surface. Upon illumination electron transfer occurs between Q_A and the mesoporous TiO_2 (1). The function of the blocking layer is to block the interaction of the mobile redox mediator DCBQ (2) with the ITO surface (3).

slowly move on the TiO_2 surface and then react rapidly with the freely diffusing DCBQ which then delivers them to the ITO (**Figure 4.7 (B)**). By bypassing the slow electron mobility through the nanoporous metal oxide, DCBQ provides an alternative and more rapid route for the electrons to reach the electrode.

By controlling the formation of the thickness of PSII layer immobilized onto TiO_2 /ITO electrodes and by studying the behaviour of the photocurrent in the presence and absence of external mediators and an Q_B -site inhibitor, it was shown that electrons are transferred to the TiO_2 directly from $Q_A^{\bullet}$. This is not unexpected since it is a relatively low potential electron carrier that is close to an exposed surface of the protein and electron transfer by the route has been

reported earlier. Unexpectedly, the rate-limiting step for photocurrent formation is electron transfer through the TiO_2 . Mobile electron carriers (DCBQ, PpBQ and quercetin) are able to take electrons from the TiO_2 to the ITO thereby enhancing the photocurrent.

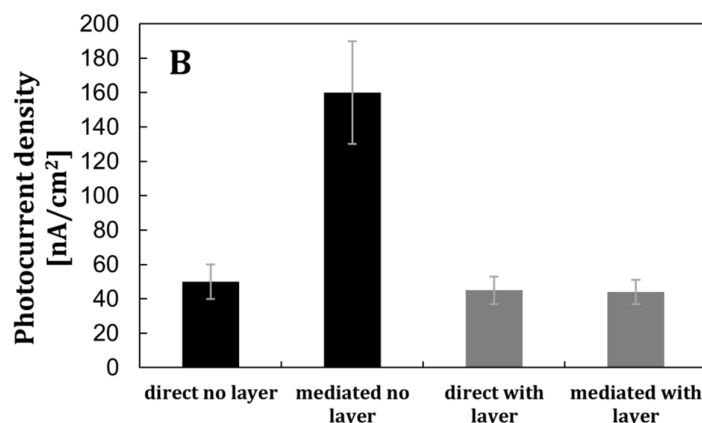


Figure 4.10 Bar chart showing the mediated and non-mediated photocurrent from the immobilised PSII in the absence and presence of a blocking TiO_2 layer on the ITO electrode. Photocurrent density recorded in the absence of the blocking layer, without (first bar) and with the mediator $100 \mu\text{M}$ DCBQ (second bar). Photocurrent density recorded in the presence of the blocking layer, without (third bar) and with $100 \mu\text{M}$ DCBQ (fourth bar). Error bars are indicated in grey.

4.2.1 Conclusion

The slow rate of electron transfer through the nanostructured TiO_2 is due to its conduction band being far above the reduction potential of Q_A in PSII and the applied bias (**Figure 4.11**) results in very low electron mobility. It has been suggested that a driving force of at least $\Delta G_{\text{inj}} = -0.2 \text{ eV}$ is required in order to obtain efficient electron injection from an excited dye-molecule into the conduction band of a semiconductor (Kalyanasundaram and Gratzel 1998). It seems likely that a similar requirement will apply to electrons injected from biological systems. A more appropriate material for work with PSII should have a conduction band edge at more positive values. Tin dioxide (SnO_2) would appear to be a better candidate given its conduction band edge at $\sim 250 \text{ mV}$ more positive than TiO_2 ($\sim -200 \text{ mV}$) and its better electron mobility. Even so with the Q_A potential being at -140 mV , electron transfer is still well away from what is required for efficient injection. Unfortunately, SnO_2 has shown a similar behaviour to TiO_2 in experiments with quercetin and DCBQ. Tungsten trioxide (WO_3) appears to be a promising candidate as well, given its conduction band edge at $\sim +50 \text{ mV}$, which is

approximately 200 mV more positive than the reduction potential of Q_A . The drawback with WO_3 is that it has been shown to have poor electron mobility compared to other metal oxides (Tacca et al. 2012). Even though there are different ways to prepare WO_3 films, resulting in different electronic properties, the films synthesized here did not lead to significantly higher photocurrents; electron mobility inside the material still seems to play a major role, which was supported by impedance spectroscopy. An alternative that has already been used is the meso-structured ITO, which is a degenerate semiconductor with metal-like conductivity and similar biological compatibility to other metal oxides. Even with this material, however, there are signs of anomalous photocurrent behavior (Kato et al. 2012, 2013), a reflection of lower than expected conductivity in mesoporous ITO electrodes due to dopant migration in the meso- structured material (Tiwana et al. 2011; von Graberg et al. 2011).

Given the advantages of using transparent mesoporous electrode materials (biocompatibility and that they afford the possibility of combining electrochemical and spectroscopic techniques) *meso*ITO is currently the subject of study. These studies could broaden the scope for electrochemically studies of the immobilized enzyme (both thermodynamically and kinetically) and for any potential applications.

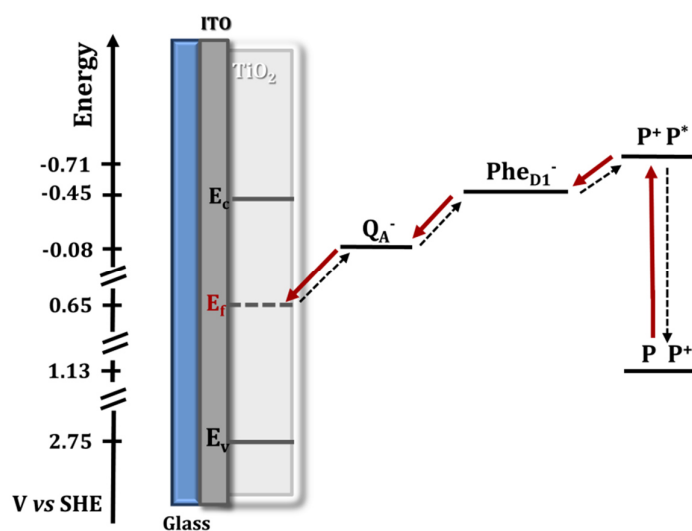


Figure 4.11 Schematic representation of the electron transfer steps from PSII to the TiO_2 /ITO electrode following the charge separation event and upon the application of a bias potential of +0.644 V vs SHE. The energetic levels of the PSII cofactors (Rappaport et al. 2002; Ido et al. 2011; Kato et al. 2009) involved in the electron transfer process are shown with respect to the position of the conduction band (E_c), Fermi level (E_f) and valence band (E_v) of TiO_2 (Gratzel 2001).

Chapter 5.

The origin of cathodic photocurrents generated by Photosystem II immobilized on electrode systems

5.1 Introduction

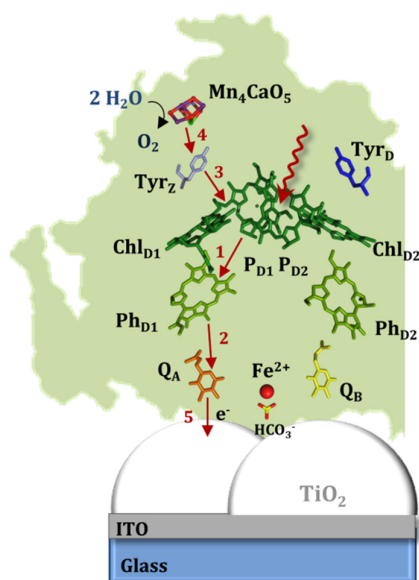


Figure 5.1 Schematic representation of the electron transfer steps occurring when Photosystem II is immobilized onto electrode surfaces, e.g., TiO₂/ITO electrodes. The arrangement of cofactors is based on the 1.9 Å crystal structure (pdb reference 3ARC). The numbers represent the order of electron transfer steps after charge separation.

The previously demonstrated existence of direct electron transfer from Q_A to the electrode surface in PSII/metal oxide hybrid systems (**Figure 5.1**) opens up new possibilities for electrochemical studies.

In further experiments, the origin of unexplained cathodic photocurrents (i.e. electron flow from the electrode) were investigated, which are described in literature reports where photocurrents generated by PSII or thylakoid membranes adsorbed onto various electrode materials are studied in dependence of an externally applied bias (e.g., Lemieux and Carpentier 1988; Agostiano et al. 1983; Terasaki et al. 2008): upon the application of potentials <+300 mV, anodic photocurrents, occurring from electron donation of PSII into the electrode, are replaced by cathodic photocurrents, indicating a light-dependent, catalytic electron flow from the

electrode to a substrate.

Three possible explanations can account for this phenomenon: i) a light-dependent electron transfer from the electrode surface to PSII, ii) electron transfer to a product produced by PSII upon illumination and iii) electron transfer to another species, which is present in the electrolyte and consumed in a light-dependent reaction by PSII.

The first explanation seems to be rather unlikely, although a few redox cofactors in PSII could potentially accept electrons from the electrode surface with respect to their redox potential (e.g., TyrD[•], TyrZ and the Mn₄CaO₅ cluster in the different S-states): i) only a limited number of electrons can be “stored” on PSII in this way (e.g., the reduction of the Mn-cluster would cause its release from the enzyme, leading to a fast decrease in photocurrent) and ii) the generation of cathodic currents is light-dependent and stable, indicating a constant electron flux from electrode to an acceptor upon illumination.

To investigate the other two possible explanations, PSII was immobilized as a monolayer onto nanostructured TiO₂/ITO electrodes as described in section 2.8.1. Photocurrents were recorded with the PSII-modified TiO₂/ITO electrode at different applied potentials under red light irradiation in an open glass cell at pH 6.5 and 25°C with a platinum wire as a counter electrode and a 3 M (KCl) Ag/AgCl electrode as a reference, as described in section 2.8.2.

5.2 Investigations of the cathodic photocurrent generation by PSII in metal oxide hybrid systems

Typical photocurrent responses from a bias dependent titration series of PSII from +750 mV to +50 mV vs SHE are shown in **Figure 5.2**. Upon the application of a potential <+300 mV, anodic photocurrents turn into cathodic photocurrents.

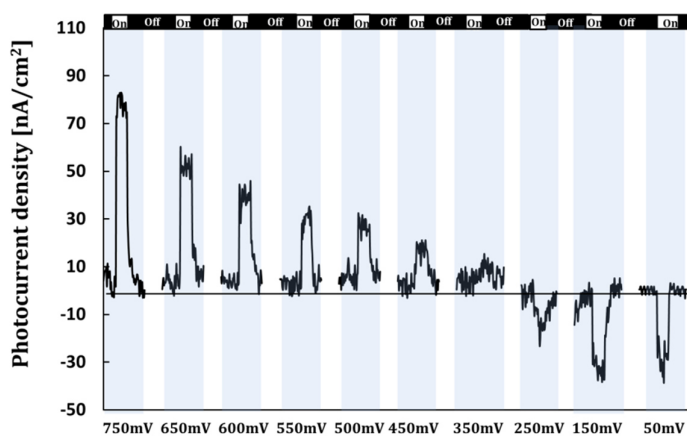


Figure 5.2 Photocurrent responses from a bias dependent titration series of PSII immobilized onto TiO₂/ITO electrodes ranging from +750 mV to +50 mV vs SHE. The bar on top of the charts shows the length of the illumination periods, which was set to 10 s at each potential step.

The question, which arises based on the discussed possible explanations above, is the one of the substrate, interacting with the electrode surface in the potential range of +300 mV to +50 mV.

One likely candidate for various reduction reactions in this potential range is oxygen (Katsounaros et al. 2014; Wood 1988). Oxygen is not only present in the electrolyte

during the measurement, but it is also a by-product of photosynthetic water oxidation (Cox and Messinger 2013; Dau et al. 2012).

Although the redox potential e.g., for the one-electron reduction reaction of oxygen to the superoxide anion radical is more negative ($E_m(\text{O}_2/\text{O}_2^{\bullet-}) = -160 \text{ mV}$, Wood (1988)), this process could still be possible due to i) the significant difference in concentration of superoxide and molecular oxygen in solution ($[\text{O}_2^{\bullet-}] \ll [\text{O}_2]$), leading to a shift of the redox potential towards more positive potentials and ii) observations made by Gurlo (2006), who reported on the physisorption of oxygen on n-type semiconducting materials even without the application of an external bias. The latter process leads to the trapping of an electron from the oxide's conduction band at the adsorbed oxygen molecule, which produces a superoxide anion radical ($\text{O}_2^{\bullet-}$) on the surface (Gurlo 2006). The flow of electrons from the semiconductor into the chemisorbed layer without the diffusion of

ionic species at the same time induces a space charge layer between the interior of the semiconductor and its surface, influencing the electrical conductivity and the work function of the semiconductor. The application of an external bias is found to favour or disfavour this process. Therefore, despite the observation of cathodic photocurrents at positive potentials ($< +300 \text{ mV}$) and the negative reduction potential for the one-electron reduction of oxygen to the superoxide anion radical, the

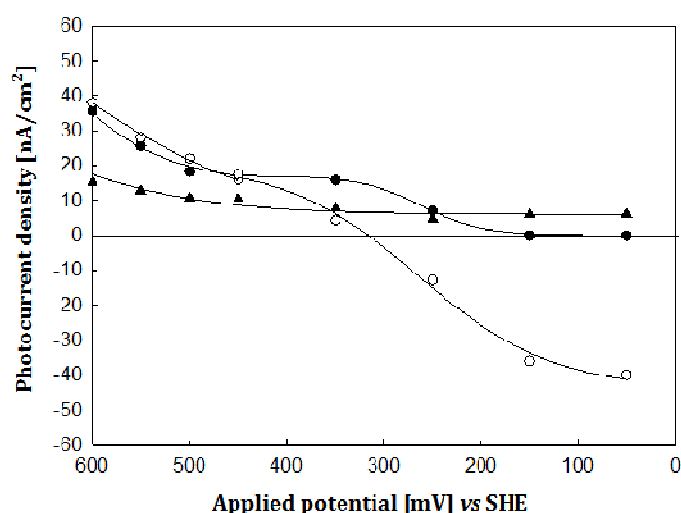


Figure 5.3 Photocurrent responses from a bias dependent titration series of PSII monolayers immobilized onto TiO_2/ITO electrodes in the presence of O_2 (○ trace), in the absence of O_2 (● trace) and in the presence of an insulating TiO_2 blocking layer (▲ trace). The illumination time was set to 20 s at each potential step (see section 2.8.2).

described process of O_2 interacting with TiO_2 and/or ITO might favor this reaction.

Another possible product of an oxygen reduction reaction (ORR) at the electrode surface is hydrogen peroxide: the two-electron reduction of oxygen to hydrogen peroxide has a positive reduction potential of $E_m(\text{O}_2/\text{H}_2\text{O}_2) = +310 \text{ mV}$ (pH 6.5, Wood 1988) and is therefore likely to occur as well.

Firstly, to investigate whether an oxygen reduction reaction at the electrode surface contributes to formation of cathodic photocurrents, photocurrents were studied using an electrode in which a layer of crystalline TiO_2 , impermeable to mobile electron carriers in solution, was used to separate the nanostructured TiO_2 from the ITO conducting layer. **Figure 5.3** shows that the introduction of this blocking-layer prevented indeed the formation of cathodic photocurrents at applied potentials $< +300\text{mV}$. This suggests the involvement of a freely diffusing molecule in the process. Furthermore, photocurrent measurements were carried out in the absence of oxygen in the measuring buffer, leading to a similar result: the photocurrent density at an applied bias $< +300\text{ mV}$ decreased to zero, but the observed currents beforehand remained anodic (**Figure 5.3**).

A cyclic voltammogram of the plain ITO electrode in the presence and absence of oxygen supported the hypothesis of potential oxygen reduction reactions occurring at the surface of the electrode (**Figure 5.4**): the CV recorded in the presence of oxygen deviated from the one recorded in the absence of oxygen around $+240\text{ mV}$, indicating that oxygen starts to electrochemically interact with the ITO surface at this potential.

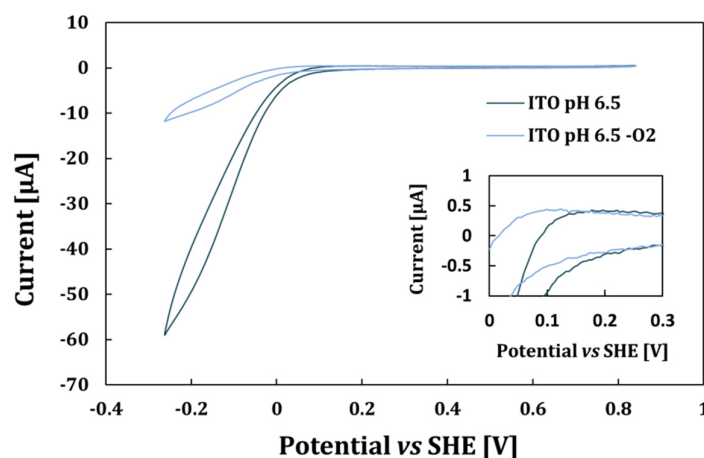


Figure 5.4 Cyclic voltammogram (scan rate: 50 mV/s) of the ITO electrode surface in the presence and absence of oxygen. The deviation from the baseline in the presence of O_2 at $\sim +240\text{ mV}$ (inset) indicates O_2 reduction reactions at the surface below this potential.

Furthermore, to address the question whether $O_2^{\bullet-}$ or H_2O_2 are produced in this oxygen reduction reaction, a cyclic voltammogram of the plain ITO electrode was recorded from +750 mV to +100 mV in the presence of 1 mM nitro blue tetrazolium (NBT^{2+}) in the measuring buffer. NBT^{2+} is a widely used specific reagent for the colorimetric detection of $O_2^{\bullet-}$ (Bielski et al. 1980) with a redox potential of $E_m = -50$ mV (pH 7; Fay and Kulasooriya 1972). Depending on the amount of $O_2^{\bullet-}$ present, NBT^{2+} undergoes either a two-electron reduction reaction to monoformazan (MF, **Figure 5.5**) or a four-electron reduction resulting in the formation of diformazan (DF).

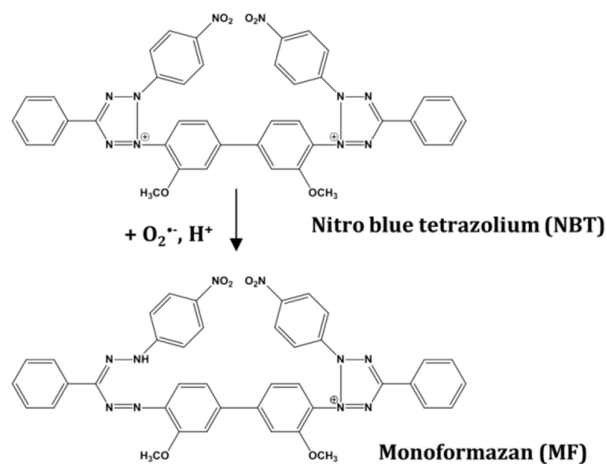


Figure 5.5 Reaction of nitro blue tetrazolium (NBT) with superoxide to monoformazan (MF).

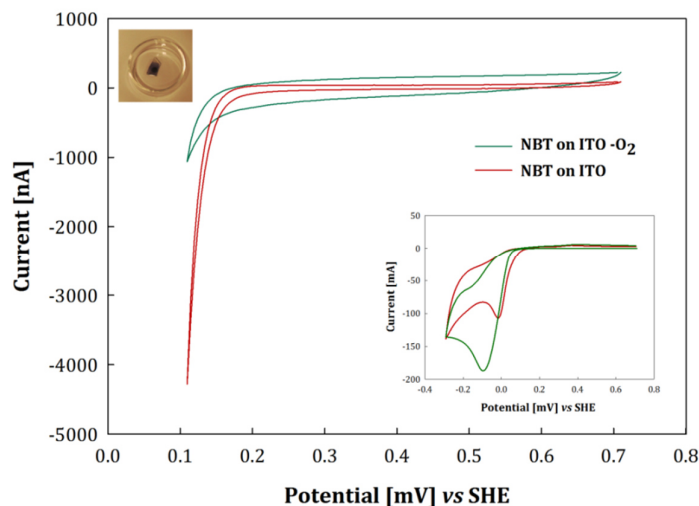


Figure 5.6 Cyclic voltammogram (scan rate: 50 mV/s) of nitro blue tetrazolium (NBT²⁺) in the measuring buffer in the presence and absence of O₂. The CV from +700 mV to +100 mV indicates the reduction of O₂ on the surface, which results in a coloured deposit on the electrode surface (see electrode picture). The cyclic voltammogram of NBT from +700 mV to -300 mV indicates its electrochemical reduction close to its redox potential $E_m = \sim -50$ mV (inset) under these conditions.

The cyclic voltammogram of the bare ITO electrode in the presence of the dye indicated that an oxygen reduction reaction occurred at the surface (**Figure 5.6**) and the absorption spectrum of the deposited film in DMSO confirmed the formation of MF during the scan (**Figure 5.7**).

Furthermore, a spectro-electrochemical measurement confirmed that H_2O_2 cannot be accounted for as the main product of the ORR observed at the ITO surface: when the applied potential at the plain ITO electrode was step-wise increased from +200 mV to +400 mV in the presence of Amplex Red and horseradish

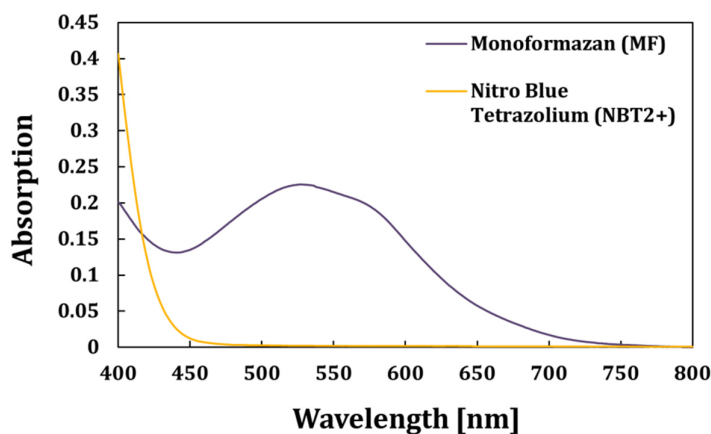


Figure 5.7 The absorption spectrum of the colored deposit of **Figure 5.5** in DMSO indicates the formation of the purple monoformazan (MF) on the ITO surface.

peroxidase in the measuring buffer, the recorded absorption spectrum of the buffer remained unchanged. If hydrogen peroxide had been produced in this potential range, it would have reduced Amplex Red to resorufin, which could have been observed by an increase in the absorption at 571 nm (Rodrigues and Gomes 2010). Nevertheless, colorimetric assays are not able to detect very small amounts of formed products and therefore, given the redox potential of the oxygen reduction reaction to H_2O_2 ($E_m(\text{O}_2/\text{H}_2\text{O}_2) = +310 \text{ mV (pH 6.5)}$), this process cannot be completely excluded at potentials around +300 mV, but the formation of the superoxide anion radical in the oxygen reduction reaction is the dominating product.

A further indication supporting this hypothesis was obtained in an initial bias-dependent titration series of PSII immobilized onto the TiO_2/ITO electrode in the presence of superoxide dismutase (SOD) and/or catalase in the electrolyte buffer. Experiments only with catalase (1300 U/mL) did not result in a change of the amount of anodic and cathodic photocurrent produced, suggesting that H_2O_2 cannot be the primary product of the oxygen reduction reaction occurring at the ITO surface. The presence of catalase (1000 U/mL) and SOD (100 U/mL) in the electrolyte buffer led to a decrease in the amount of cathodic photocurrents, which indicates that the SOD reacts with the product of the oxygen reduction reaction, $\text{O}_2^{\bullet-}$, in competition with PSII.

The question still remains how PSII reacts in a light-dependent, catalytic way with $O_2^{\bullet-}$ produced at the electrode to generate the cathodic photocurrent.

Given the extreme oxidizing and reducing chemistry occurring in the enzyme due to its ability to oxidize water and generate O_2 as a by-product, the redox potentials of the involved cofactors in PSII are spread over a wide scale, ranging from $E_m = -610$ mV (pH 7) for the Pheo/Pheo $^-$ redox couple at the acceptor side to the highly positive potentials for the chlorophylls forming the P_{680}^+/P_{680} couple ($E_m = 1.25$ V, pH 7) at the donor side (Rutherford et al. 2012). Thus, the formation of reactive oxygen species (ROS) likely occurs at the electron donor and acceptor side of PSII. One example is the one-electron reduction of molecular oxygen to superoxide by the reducing cofactors, which has been discussed for many years: light-induced production of $O_2^{\bullet-}$ by PSII has been demonstrated in several experiments and according to the redox potentials, Pheo $_{D1}^-$, the primary quinone electron acceptor Q_A^- ($E_m (Q_A/Q_A^-) = -144$ mV) and the Cyt b_{559} are proposed to primarily act as reductants for molecular oxygen in “leak” reactions (Pospisil et al. 2004; Bondarava et al. 2010). To prevent oxidative damage of proteins and lipids by these self-produced ROS, a complex defense system has been developed by the photosynthetic reaction centers and e.g., PSII has two cofactors, which are mainly discussed to act as reductants to $O_2^{\bullet-}$ (**Figure 5.8**; Pospisil et al. 2004; Pospisil 2014, 2012; Ananyev et al. 1994; Navari-Izzo et al. 1999; Nugent 2001):

- i) The non-heme iron at the acceptor side, which is a ferrous iron located between Q_A and Q_B and coordinated by four histidine residues at the D1 protein (D1H-215, D1H-272) and D2 protein (D2H-214, D2H-268) and the bidentate bicarbonate ligand (see **Figure 1.3 (A)**; Muh and Zouni 2013b). Evidence has been provided that the non-heme iron exhibits superoxide dismutase (SOD) activity, which catalyzes the reduction of $O_2^{\bullet-}$ to H_2O_2 (superoxide reductase, SOR) and the reaction of $O_2^{\bullet-}$ to molecular oxygen (superoxide oxidase, SOO; reviewed in Pospisil 2014). Both reactions are feasible, since the Fe^{3+}/Fe^{2+} redox couple has a pH-dependent redox potential of $E_m = +400$ mV (pH 7; reviewed in Pospisil 2014), which is between the standard potential of the $O_2^{\bullet-}/H_2O_2$ redox couple ($E_m = +890$ mV, pH 7; Pospisil 2012) and the standard potential of the $O_2^{\bullet-}/O_2$ couple ($E_m = -160$ mV).

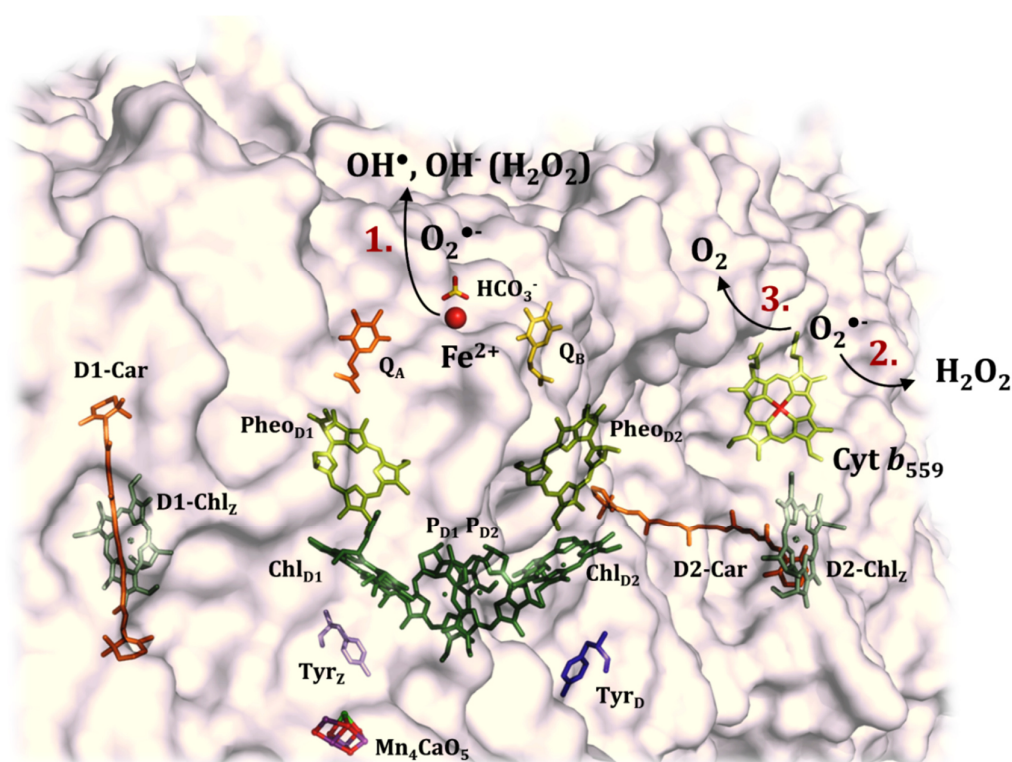


Figure 5.8 The arrangement of cofactors in PSII according to the 1.9 Å crystal structure (pdb reference ARC) with the metal centres potentially involved in the reduction of superoxide. (1) Reduction of O₂^{•-} by the ferrous non-heme iron to OH[•] and OH⁻ (or in a shortcut reaction to H₂O₂); (2) reduction of O₂^{•-} to O₂ by the ferrous iron of the LP form of Cyt b₅₅₉ and (3) of O₂^{•-} by the ferrous iron of the HP form of Cyt b₅₅₉ to H₂O₂.

- ii) The Cytochrome *b*₅₅₉ (Cyt *b*₅₅₉), which is a heme-bridged heterodimer located about 25 Å from Q_B (**Figure 5.8**) with a redox potential ranged from 400 mV to 0 mV (Pospisil 2014): The midpoint potential of the high potential form (HP) ranges from +310 mV and +400 mV (pH 7), the intermediate potential (IP) lies between +125 mV to +240 mV (pH 7) and the low potential form exhibits a redox potential, which is between -40 mV and +80 mV (pH 7). Experimental evidence has been provided that Cyt *b*₅₅₉ shows both, S₀O and S₀R activity (Pospisil 2012; Ananyev et al. 1994): The one-electron oxidation of O₂^{•-} is maintained by the ferric heme ion of the IP form of Cyt *b*₅₅₉, which is reduced in this reaction to ferrous heme iron. Since the standard potential of the O₂/O₂^{•-} couple is lower than the midpoint potential of the Fe³⁺/Fe²⁺ redox couple in the IP form, the oxidation of superoxide by Cyt *b*₅₅₉ to molecular oxygen is feasible. Another possibility to eliminate O₂^{•-} by Cyt *b*₅₅₉ is its one-electron reduction to

H_2O_2 , which is sustained by the ferrous heme iron of the HP form of Cyt b_{559} and which is oxidized in this reaction to the ferric heme iron (Pospisil 2012). Given the redox potential of the redox couple $\text{O}_2^{\bullet-}/\text{H}_2\text{O}_2$ of $E_m = +890$ mV (pH 7), this reaction is feasible, too.

To test which cofactor is involved in the light-dependent catalytic oxidation of $\text{O}_2^{\bullet-}$ produced at the ITO electrode, photocurrent measurements were carried out with PSII core complexes from a *T. elongatus* mutant lacking the heme iron in Cyt b_{559} (PsbE-His23Ala PSII, see section 2.4.4). **Figure 5.9** shows that no change in the formation of cathodic photocurrents can be observed during the measurement in the absence of the heme iron in Cyt b_{559} , excluding the involvement of Cyt b_{559} in the light-dependent reaction with $\text{O}_2^{\bullet-}$.

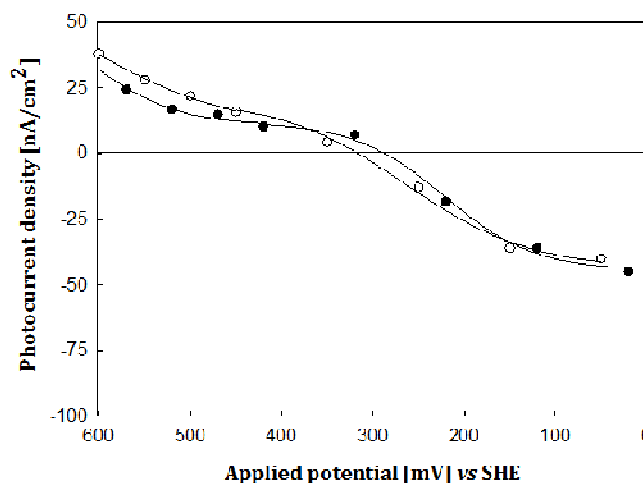


Figure 5.9 Photocurrent responses from a bias dependent titration series of a PSII monolayer in the presence of O_2 (○ trace) and of a PSII monolayer lacking the heme iron in Cyt b_{559} in the presence of O_2 (● trace) immobilized onto TiO_2/ITO electrodes. The illumination time was set to 20 s at each potential step (see section 2.8.2).

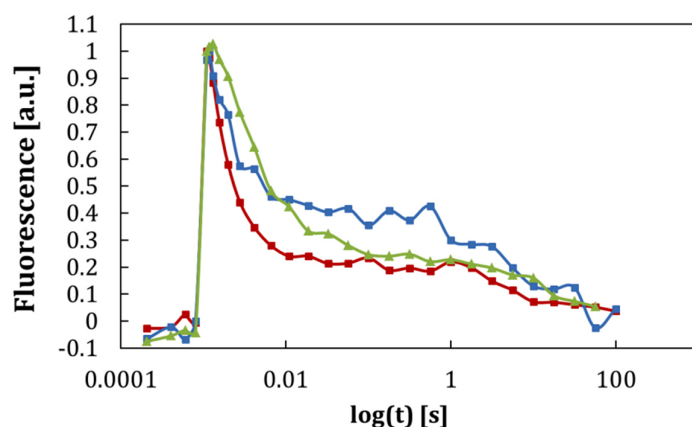


Figure 5.10 Fluorescence measurements reflecting the decay of Q_A^- after one actinic flash. The fluorescence decay was measured in a PSII control where the bicarbonate ligand was in place (red trace) and in samples treated with 30 mM sodium oxalate (blue trace) and 30 mM sodium glycolate (green trace).

To confirm the involvement of the non-heme iron in the formation of cathodic photocurrents, the reduction potential of the non-heme iron was changed by substituting the coordinating bicarbonate ligand with either oxalate or glycolate. The substitution of the bicarbonate ligand with oxalate raises the redox potential of the non-heme iron

to $E_m > +500$ mV in the pH range of 6 to 7.5 and glycolate lowers it to +340 mV, which is only weakly pH-dependent (Petrouleas et al. 1994). If the non-heme iron participates in the catalytic reaction with $O_2^{\bullet-}$ produced at the ITO surface, a change of its redox potential should affect the formation of cathodic photocurrents. Moreover, the incubation of PSII with either sodium oxalate or sodium glycolate affects the electron transfer rate from Q_A to Q_B , which is represented by a slower relaxation of the chlorophyll fluorescence yield after the excitation with a saturating actinic flash. This observation was used to confirm the successful substitution of the bicarbonate ligand with the respective carboxylate anion in fluorescence measurements (**Figure 5.10**).

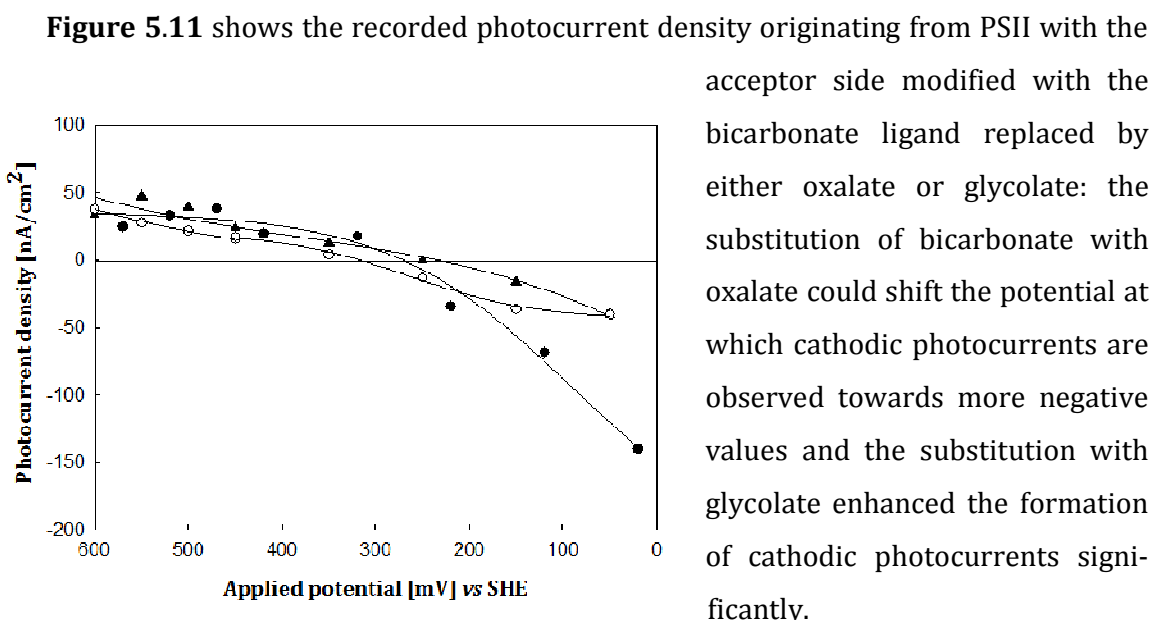


Figure 5.11 Photocurrent responses from a bias dependent titration series of PSII monolayers immobilized onto TiO_2/ITO electrodes when the HCO_3^- ligand was substituted by glycolate (● trace) or oxalate (▲ trace) and without any modifications of the acceptor site in the presence of O_2 (○ trace).

acceptor side modified with the bicarbonate ligand replaced by either oxalate or glycolate: the substitution of bicarbonate with oxalate could shift the potential at which cathodic photocurrents are observed towards more negative values and the substitution with glycolate enhanced the formation of cathodic photocurrents significantly.

This suggests that the non-heme iron plays a significant role in the reduction of $O_2^{\bullet-}$ produced at the ITO surface. A mechanistic model for this reduction reaction

has been suggested earlier, which involves the photocatalytic reduction of Q_A and explains as well the light-driven and catalytic nature of the measured cathodic photocurrent observed here (**Figure 5.12**; Pospisil et al. 2004; Pospisil 2012): the interaction of the non-heme iron with $O_2^{\bullet-}$ results in the formation of a ferric-peroxo species ($Fe^{3+}-OO^{\bullet-}$). Upon protonation, a ferric-peroxo species ($Fe^{3+}-OOH$) is formed, which can be reduced by an endogenous reductant such as the light-reduced primary quinone electron acceptor, Q_A . This leads to the abstraction of a hydroxylradical ($OH^{\bullet}$)

and the formation of a ferric-oxo species ($\text{Fe}^{3+}\text{-O}$). A second protonation reaction results in the abstraction of a hydroxyl anion (OH^-) and the ferric non-heme iron is reduced again to the ferrous iron by a second electron e.g., from Q_A . In general, a shortcut in this reaction cycle which is thermodynamically and kinetically favored is the cleavage of the Fe-O bond instead of the O-O bond (Attia et al. 2013). This cleavage would result in the

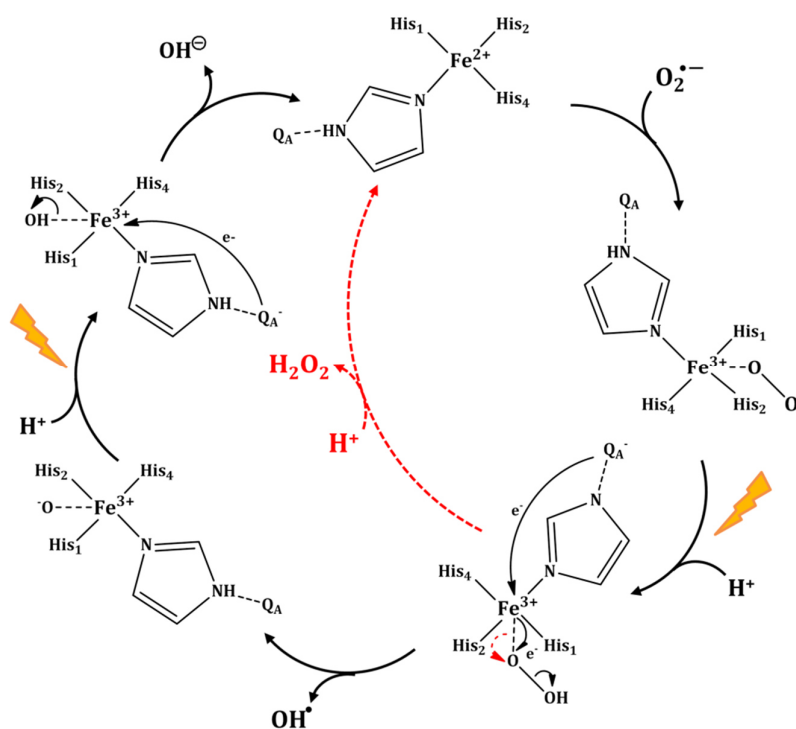


Figure 5.12 Proposed model for the catalytic, light-dependent reaction of $\text{O}_2^{\bullet-}$ with the non-heme iron at the PSII acceptor side. The route indicated in black involves the cleavage of the O-O bond in the iron-peroxo intermediate resulting in the formation of $\text{OH}^\bullet$ and OH^- . The route indicated in red represents a possible alternative pathway, which involves the cleavage of the Fe-O bond in the iron-peroxo intermediate leading to the formation of H_2O_2 . Redrawn with modifications from Pospisil et al. 2004.

formation of hydrogen peroxide upon protonation. The reaction is likely to occur as well, since the energetic cost of the O-O bond cleavage in ferric hydroperoxo complexes exceeds 47 kcal/mol, which is 30 kcal/mol higher than the energy required to elongate the Fe-O bond in Fe(III)-OOH . Nevertheless, Attia et al. (2013) identified only a small energy barrier (~ 15 and ~ 25 kcal/mol, respectively) for O-O bond cleavage in two of the

ferrous hydroperoxo spin states in SORs, the $S = 1$ and the ground-state $S = 2$. Even though these barriers are still higher than those reported for Fe-O bond cleavage, this reaction remains possible in SORs.

Therefore, according to the redox potential of the non-heme iron ($E_m(\text{Fe}^{3+}/\text{Fe}^{2+}) = +400 \text{ mV}$, pH 7), the reduction of bound peroxide according to the

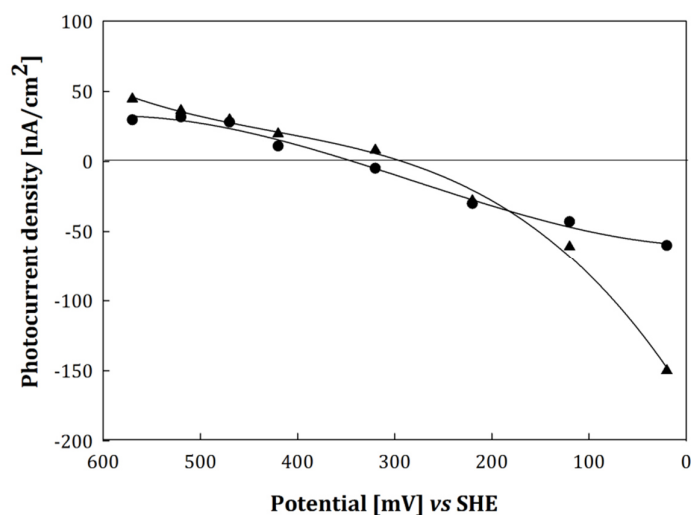


Figure 5.13 Photocurrent responses from a bias dependent titration series of PSII monolayers immobilized onto TiO_2/ITO electrodes in the presence of $10 \mu\text{M}$ DCMU in the electrolyte (● trace) and $100 \mu\text{M}$ ioxynil (▲ trace). The time of the illumination period was set to 20 s at each potential step (see section 2.8.2).

proposed mechanism is feasible. Thus, depending on the applied potential and the associated driving force for electron injection from Q_A into the electrode material, the electrons generated upon charge separation in PSII can be injected either into the electrode or be transferred to the non-heme iron to enable the reduction of $\text{O}_2^{\bullet-}$.

To confirm this hypothesis further, the bias dependency of the formation of cathodic

photocurrents was studied either in the presence of the urea type herbicide DCMU or the phenolic type herbicide ioxynil in the measuring buffer. Both herbicides cause a change in the redox potential of the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ redox couple as described already above: DCMU binds to the Q_B pocket and causes an upshift of the redox potential to $E_m(\text{Q}_\text{A}/\text{Q}_\text{A}^-) = -28 \text{ mV}$ (Krieger-Liszky and Rutherford 1998). Ioxynil downshifts the redox potential of the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ couple to $E_m(\text{Q}_\text{A}/\text{Q}_\text{A}^-) = -113 \text{ mV}$ by interacting with the D1-H215 residue, which coordinates the non-heme iron: Fourier transform infrared (FTIR) difference spectra indicate the formation of a strong hydrogen bond between the CO group of the phenolate form of the ioxynil and the NH group of the D1-H215 (Takano et al. 2008). The correlation of this hydrogen bond and the hydrogen bond between the CO group of Q_A^- and the NH group of the D2-H214, which is another ligand to the non-heme iron, is suggested to affect the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ redox potential. It is likely that the electron density is therefore shifted from the non-heme iron towards the hydrogen bonds, which would favor a nucleophilic attack from the superoxide anion to the Fe^{2+} . Although a more

negative redox potential of the Q_A/Q_A^- couple results as well in a higher driving force for electron injection into the TiO_2 conduction band, higher cathodic photocurrents should be recorded upon the treatment with ioxynil due to i) the higher driving force for Q_A^- to act as an electron donor for the non-heme iron in the $O_2^{\bullet-}$ reduction process and ii) the decrease in electron density at the non-heme iron, which favors the nucleophilic attack of $O_2^{\bullet-}$. This hypothesis is supported by the observation that in the presence of 100 μM ioxynil in the measuring buffer, the generated cathodic photocurrents were significantly increased in comparison to photocurrents recorded in the presence of 10 μM DCMU (**Figure 5.13**).

The results support the hypothesis that the light-dependent catalytic nature of cathodic photocurrents, which are observed when PSII is immobilized onto electrode materials and potentials $< +300$ mV are applied, originate from the formation of the superoxide anion radical in oxygen reduction reactions occurring at the electrode surface (**Figure 5.14**, step 1), which subsequently reacts with the non-heme iron at the PSII acceptor side light-dependent way (**Figure 5.14**, step 2), involving electron transfer from Q_A^- to the NHI. The observations made here explain not only the nature of the cathodic photocurrent, but are also of relevance for the stability of PSII on electrode systems and possibly under physiological conditions. In order to carry out bias dependent studies of PSII immobilized onto metal oxides, it is therefore of importance to avoid the interference with oxygen and move to anaerobic environments.

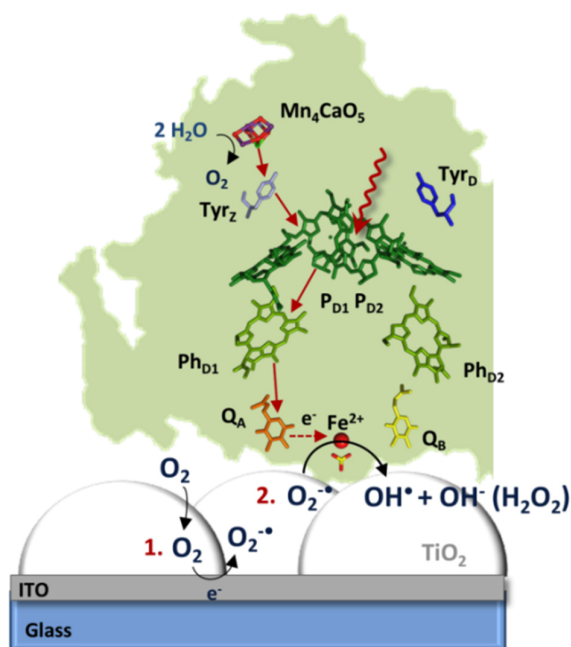


Figure 5.14 Model for the generation of cathodic photocurrents by Photosystem II immobilized on TiO₂/ITO electrodes upon the application of an external potential $< +300$ mV at the electrode. Step 1 represents the reduction of oxygen to the superoxide anion radical at the ITO surface, which subsequently reacts with the non-heme iron to OH[•] and OH⁻ (or H₂O₂, respectively, depending on the reaction route), step 2. The red arrows indicate the electron transfer pathway in PSII upon illumination. The dashed arrow from Q_A to the Fe²⁺-ion indicates the electron transfer from Q_A to the NHI when the driving force for electron injection into the metal oxide is too low, i.e. at potentials $< +300$ mV.

Chaper 6.

Conclusion and future work

In this work, the electron acceptor site of Photosystem II has been investigated using two main electrochemical methods: spectroelectrochemistry, to determine the redox potential of the cofactor Q_A , the primary quinone electron acceptor, and protein film voltammetry, to study the origin of anodic and cathodic photocurrents originating from Photosystem II in metal oxide hybrid systems.

6.1 Spectroelectrochemical redox titrations of Photosystem II

The midpoint potential of the Q_A/Q_A^- -couple was successfully determined in PSII enriched membranes from spinach in the presence and absence of the Mn_4CaO_5 -cluster

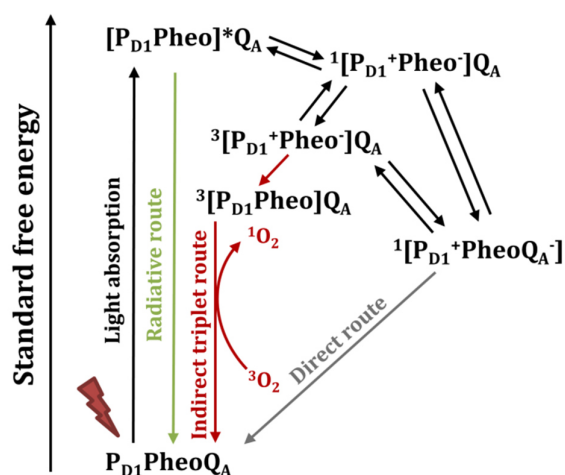


Figure 6.1 Scheme showing the energetics and recombination pathways in PSII. For simplicity only the charge recombination from the radical pair $P_{680}^{+}Q_A^{-}$ is shown, but this is formed from recombination of more stable charge pairs involving TyrZ, the S-states of the water splitting cycle and in the absence of Q_B . Note: the energy gaps are not to scale. According to: Sugiura et al. (2014).

by spectroelectrochemistry, reproducing literature values, which have previously been obtained in a similar experimental set-up. It was shown that the bicarbonate anion, a bidentate ligand to the non-heme iron at the electron acceptor site of PSII, has a significant influence on the potential: the values obtained for the intact and Mn-depleted PSII lacking bicarbonate, are about 80 mV higher than the ones containing bicarbonate. One possible explanation for the existence of the here determined different values for the Q_A/Q_A^- midpoint potential could be found in a protection mechanism against the formation of $^1O_2$: Photosystem II has two charge apart recombination

pathways for $P^{+}Q_A^{-}$ from the radiative route (**Figure 6.1**), and the size of the energy gap

between Q_A and $Pheo_{D1}$ determines the back-reaction rate and the recombination route (Sugiura et al. 2014): an indirect route via $P_{D1}^{++}Pheo_{D1}^{\bullet-}$, which then decays to the ^3P triplet state, lying about 1.3 eV above the ground state and which can easily promote the triplet to singlet oxygen formation, a highly reactive, damaging species; and a direct route, which is favored, when the energy gap between $Pheo_{D1}$ and Q_A is sufficiently large. The latter is likely to be the case during photoactivation, when the Mn-cluster is assembled and the midpoint potential of Q_A/Q_A^- is about 120 mV higher than with the Mn-cluster being in place (i.e. $E_m = -144$ mV and $E_m = -28$ mV). The absence and/or presence of the bicarbonate anion can favor this process further. Another function of the bicarbonate anion on the redox properties of Q_A could involve a protection mechanism in situations of elevated temperature and/or partial pressure, which affect the binding properties of the bicarbonate anion to the non-heme iron as well; here, the recombination reactions could protect PSII in rather extreme environmental conditions.

It would be of great interest as well to study the ‘bicarbonate effect’ and its influence on the Q_A/Q_A^- midpoint potential in cyanobacteria, e.g., in *T. elongatus*, since the here presented and discussed results suggest already an effect of the ligand on the energetic situation of PSII in cyanobacteria. Ideally, the same experiments are carried out with PSII core complexes isolated from *T. elongatus* samples, which encoded either the *PsbA1* or *PsbA3* gene to express the D1 protein in order to compare both mutants. Further comparative studies can be carried out as well with PSII complexes of different species as discussed, e.g., the thermo- and acidophilic primitive red algae *Cyanidioschyzon merolae* (*C. merolae*), which shows an unusual high photosynthetic activity. Moreover, the OTTLE cell set-up provides the opportunity to determine and re-measure the redox potential of other cofactors in PSII, e.g., the one of pheophytin a according to Kato et al. (2009).

The here obtained midpoint potentials question as well the previously calculated redox potentials of other cofactors, which were used to describe the energetic situation in Photosystem II (see section 1.2.3 and e.g., Rappaport et al. 2002, 2008). For instance, driving forces for the electron transfer from $Pheo_{D1}^{\bullet-}$ to Q_A and from $Q_A^{\bullet-}$ to Q_B need to be recalculated and calculations of the free-energy gap between $S_2Q_A^-$ and $S_1P^*Q_A$ considering the different midpoint potentials of Q_A/Q_A^- would allow the further quantification of charge recombination routes.

6.2 Investigations of Photosystem II in metal oxide hybrid systems

The clarification of the origin of photocurrent, which can be measured upon illumination of PSII in metal oxide hybrid systems, the identification of an alternative exit route for electrons to leave PSII via Q_A and the investigation of the effect of redox mediators such as DCBQ, the herbicide DCMU and oxygen on this system, leads to the next part of the project: the establishment of a set-up, where PSII can be studied electrochemically when it is immobilized onto metal oxide surfaces. Other electrode materials such as *meso*ITO and BiVO_4 are currently investigated, since their electronic properties are much more appropriate. A few literature reports demonstrate as well the successful application of modifications of tin oxide in these systems: Aksu et al. (2011) reported the application of porous tin-rich indium tin oxide for studies of electroactive biomolecules, since the material shows a high and durable electrical conductivity and is transparent (Aksu et al. 2011) and Frasca et al. (2013) described electrochemical studies of *human* sulfite oxidase on nanostructured antimony doped tin oxide (ATO) thin film electrodes (Frasca et al. 2013). As soon as a suitable material is found, it would be of great interest to study the electron transfer from PSII to the electrode in more detail, e.g., to analyse the kinetic profile in order to characterize the alternative electron transfer pathway from Q_A further. A combination of photocurrent measurement with the possibility of providing single flashes could allow as well a new kinetic analysis of the S-cycle, since the relatively slow electron transfer at the acceptor site from Q_A to Q_B (de Wijn and van Gorkom 2001) is not rate limiting any longer due to the existence of the identified alternative electron transfer pathway to the electrode directly from Q_A . Presumably, the fast electron transfer to the electrode could have an influence as well on the turnover rate of the Mn-cluster. The effect of surface modifications of the electrode material and different pH values would be of interest to investigate as well: their consequences for the electron transfer, but also for the binding properties of Photosystem II to the electrode.

Furthermore, the process of photoactivation could be potentially attainable with such a metal oxide hybrid system, by following the assembly of the Mn-cluster upon the provision of single turn over flashes and recording the increase of photocurrent at the same time.

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