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Substrate water exchange in photosystem II core complexes of the extremophilic red alga *Cyanidioschyzon merolae*☆

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ABSTRACT

The binding affinity of the two substrate–water molecules to the water-oxidizing Mn_4CaO_5 catalyst in photosystem II core complexes of the extremophilic red alga *Cyanidioschyzon merolae* was studied in the S_2 and S_3 states by the exchange of bound ^{16}O-substrate against ^{18}O-labeled water. The rate of this exchange was detected *via* the membrane-inlet mass spectrometric analysis of flash-induced oxygen evolution. For both redox states a fast and slow phase of water-exchange was resolved at the mixed labeled m/z 34 mass peak: $k_f = 52 \pm 8\ s^{-1}$ and $k_s = 1.9 \pm 0.3\ s^{-1}$ in the S_2 state, and $k_f = 42 \pm 2\ s^{-1}$ and $k_{slow} = 1.2 \pm 0.3\ s^{-1}$ in S_3, respectively. Overall these exchange rates are similar to those observed previously with preparations of other organisms. The most remarkable finding is a significantly slower exchange at the fast substrate–water site in the S_2 state, which confirms beyond doubt that both substrate–water molecules are already bound in the S_2 state. This leads to a very small change of the affinity for both the fast and the slowly exchanging substrates during the $S_2 \rightarrow S_3$ transition. Implications for recent models for water-oxidation are briefly discussed.

1. Introduction

The molecular oxygen we breathe is produced by cyanobacteria, algae and higher plants *via* light-induced water oxidation in photosystem II (PSII). This reaction occurs within the oxygen evolving complex (OEC) that catalyzes the oxidation of two water molecules into molecular oxygen, four protons and four electrons. This complex is comprised of four manganese atoms and one calcium atom, which are connected by five μ-oxo bridges. These atoms are arranged in a chair-like structure with a distorted cubane base (Mn_4CaO_5 cluster) [1–6]. Four consecutive flashes lead *via* sequential charge separations in the chlorophyll-containing reaction center of PSII to the accumulation of four oxidizing equivalents in the OEC, which subsequently liberates O_2. To describe this reaction cycle, which was deduced from flash-induced oxygen evolution patterns [7], Kok and coworkers introduced the S state model, also known as the Kok cycle, with five different oxidation states of the OEC [8]. The S state model denotes the number of stored oxidizing equivalents in a particular S state by the index i (S_i: S_0–S_4). The molecular mechanism captured by this elegant kinetic scheme is presently not fully understood and different mechanisms have been proposed [3,9–16].

Cyanidioschyzon merolae is an extremophilic red microalga that naturally grows at extremely low pH (pH 0.2–4) and moderately high temperatures (40–56 °C) [17,18]. The composition of the extrinsic lumenal proteins of PSII stabilizing the OEC differs somewhat from that of higher plants, green algae and prokaryotic cyanobacteria [19]. Higher plants and green algae both contain PsbO, PsbP, and PsbQ (and possibly PsbR), whereas red algae contain PsbO, PsbP, PsbQ' (CyanoQ homologue), PsbU and PsbV. Cyanobacteria contain PsbO, PsbU, PsbV and the cyanobacterial homologues of PsbP and PsbQ: CyanoP and CyanoQ. Notable is the intermediate composition of extrinsic proteins in red algae compared to prokaryotes and eukaryotes. A 17 Å electron density map derived from electron microscopy and single particle analysis of the *C. merolae* dimeric PSII particles has recently been published together with a functional study of this complex under extreme conditions [20]. In the same study it was found that *C. merolae* PSII retained significant photosynthetic activity in an unusually broad range of pH, temperatures and light intensities. Since the mechanism of water-oxidation is known to be highly dependent on the prompt release of protons produced during water-oxidation [21], it is highly interesting to study if this greater pH stability of PSII is related only to the above-described differences in the protein composition, or if the kinetic

Abbreviations: *C. merolae*, *Cyanidioschyzon merolae*; PSII, photosystem II; PSIIcc, photosystem II core complexes; OEC, oxygen evolving complex; S states (S_i), oxidation states of the OEC; W_f, fast exchanging substrate–water; W_s, slowly exchanging substrate–water; k_s, rate constant of slow water exchange; k_f, rate constant of fast water exchange; TR-MIMS, time resolved membrane inlet mass spectrometry; FIOPs, flash induced oxygen evolution pattern

☆ This paper is dedicated to the memory of Warwick Hillier (18.10.1967–10.01.2014). Using membrane-inlet mass spectrometry and FTIR spectroscopy Warwick made many important discoveries regarding substrate–water binding to the OEC and the mechanism of water-oxidation. He was a very good scientist and friend who was highly appreciated throughout the photosynthesis community. In 2007 he was awarded the Robin-Hill Award of the International Society for Photosynthesis Research (ISPR).

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parameters of the OEC may also be changed, such as the binding affinity of the Mn_4CaO_5 catalyst for the two substrate–water molecules.

The time resolved membrane inlet mass spectrometry (TR-MIMS) approach used in this study gives information on how the affinity of the manganese catalyst for water substrate changes during the S state transitions [22]. The affinity can be directly probed by exchanging already bound $H_n^{16}O$ substrates (n = 0, 1, 2) with bulk $H_2^{18}O$. This exchange can be then probed by photo-generating oxygen release after various incubation times followed by the analysis of the isotopic composition of thus produced O_2 by isotope ratio mass spectrometry [13, 22–30]. Here, we present for the first time substrate–water exchange rates in the highly stable dimeric PSII core complexes isolated from the extremophilic red alga *C. merolae* [17]. We discuss the mechanistic implications of these data in the context of the substrate–water exchange rates obtained previously for the purified spinach and cyanobacterial PSII core complexes [23,25].

2. Materials and methods

2.1. Purification of C. merolae PSII dimers

Cell culturing, isolation of thylakoids and purification of dimeric PSII particles were performed essentially as described in [20]. Following solubilization of thylakoids (1 mg/mL Chl*a*) with 1% (w/v) dodecyl-β-D-maltoside, dimeric PSII core complexes were purified by anion exchange chromatography on DEAE Toyopearl 650M and DEAE Toyopearl 650S media, using a continuous NaCl gradient (0.05–0.15 M NaCl), as described in detail in [20]. The purity of dimeric PSII core complexes (PSIIcc) was assessed by SDS-PAGE, size exclusion chromatography (SEC) and spectrophotometrically, according to the procedures described in [20].

2.2. PSII activity measurement

The oxygen evolving activity of purified dimeric PSIIcc was measured using a Clark-type oxygen electrode (Hansatech). Measurements were performed at 30 °C in a buffer composed of 40 mM MES-KOH pH 6.1, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 25% (w/v) glycerol in the presence of 0.125 mM 2,6-dichloro-*p*-benzoquinone (Sigma, Germany) and 2.5 mM potassium ferricyanide (POCH, Poland) as the exogenous electron acceptors. Samples (1 μg/mL Chl) were illuminated with a white light intensity of 5000 μE/m^2/s, using a KL 2500 LCD white light source (Schott, Germany). Activities were calculated from initial rates of oxygen evolution curves. Each measurement was repeated 3 times. The average activity of dimeric PSII was 4500 μmol O_2/mg Chl/h, and was consistent across multiple preparations.

2.3. Membrane-inlet mass spectrometry

An isotope ratio mass spectrometer (ThermoFinnigan Delta plus XP) connected to a membrane inlet sample chamber (165 μL) *via* a cooling trap (dry ice) was used for the time-resolved membrane inlet mass spectrometry measurements (TR-MIMS) described in this study. TR-MIMS measurements were performed at *m/z* 32, *m/z* 34 and *m/z* 36 to quantify the flash-induced oxygen production from PSII samples as described previously [22,31]. The samples were stored at −80 °C. Prior to measurements, the sample aliquots were thawed on ice and diluted in measuring buffer (pH 6.1, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 40 mM Mes, 1 M betaine) to a chlorophyll concentration of 0.3 mg/mL. Synchronization in the $S_1Y_D^{ox}$ state was achieved by pre-flashing each sample aliquot (300 μL, Chl [0.3 mg/mL]) once with a Xenon flash lamp (~5 μs FWHM), followed by dark adaptation for 1 h at 20 °C. After 40 min dark-adaptation the sample aliquot (165 μL) was loaded into the membrane inlet sample chamber. After 20 min of degasification under continuous stirring at 20 °C, the sample was advanced to the S_2 state with one flash (or two flashes at 2 Hz to attain the S_3 state) (~6 μs FWHM). Rapid injection ($t_{1/2}$ = 3 ms) of $H_2^{18}O$ (97.7%) with a gas tight syringe (Hamilton CR-700-50) into the cuvette enriched the sample with $H_2^{18}O$ (24% final). The dissolved oxygen in the $H_2^{18}O$ aliquot was removed prior to injection by adding glucose, glucose oxidase and catalase. The rapid $H_2^{18}O$ injection was initiated with a computer (LabView software), which triggered the release of 8 bars N_2 from a fast switching valve (FESTO MHE2-MS1H 3/2G-M7-K) that pushed a pneumatic piston (Festo AEVC-12-10-A-P) connected to the syringe plunger [22]. The same computer also controlled the timing of the subsequent flashes that initiate the O_2 release after defined incubation times. This time for substrate–water exchange it was varied between 0 s and 10 s by injecting $H_2^{18}O$ at various time points before giving two turnover flashes at 100 Hz (S_2 state exchange) or one turnover flash (S_3 state). The oxygen yields of the substrate–water exchange measurements were corrected for flash-induced double hits and injection artifacts as described in [22,23], and normalized by giving 4 flashes at 2 Hz 5 min after the third turnover flash. At very short incubation/exchange times corrections were also made to account for the isotopic enrichment and chlorophyll concentration present in the sample at the time of illumination [23].

2.4. Rate of $H_2^{18}O$ injection and mixing

The rate of $H_2^{18}O$ injection into the cuvette and the time for complete mixing with a sample was determined in separate experiments by measuring fluorescence as a function of time after fluorescein injection. The fluorescein solution (30 μM, 40 μL) was injected into the cuvette prefilled with a buffer solution (0.1 M Tris pH 8.0, 165 μL). The fluorescein was excited by a LED lamp (Luxeon V-Star blue, 1 W) *via* a band pass filter (Newport 470 nm–495 nm) and fiber optic tube. The resulting fluorescence (fluorescein — λex 490 nm; λem 525 nm) was guided *via* a bifurcated optical fiber and a second band pass filter (Newport 520 nm–547 nm). A photodiode (Hamamatsu S-2281/C9329) converted the fluorescence into an electrical signal that was measured with an oscilloscope.

2.5. Flash-induced oxygen-evolution pattern

The flash-induced oxygen-evolution pattern (FIOP) was obtained using TR-MIMS by giving 16 flashes separated by dark times of 25 s (Xenon flash lamp, ~5 μs FWHM). The experiments were performed at 20 °C and pH 6.1 ($CaCl_2$ 10 mM, $MgCl_2$ 5 mM, Mes 40 mM, betaine 1 M) at a chlorophyll concentration of 0.33 mg Chl/mL. Prior to loading into the MIMS cell, the sample was pre-flashed once and mixed with $H_2^{18}O$ (10% v/v), followed by dark adaptation for 1 h at 20 °C. An Excel spreadsheet program that was based on the extended Kok model was used to fit the FIOP.

3. Results

3.1. Flash-induced oxygen pattern (FIOP)

Fig. 1 shows the flash induced oxygen yield pattern (FIOP) of the *C. merolae* dimeric PSII core particles that was obtained at *m/z* 34 in the absence of artificial electron acceptors. The O_2 oscillation pattern shows the characteristic features of the Kok model, with a maximum of oxygen yield after the third flash. As typical for core preparations, only a very small second oscillation is observed due to the limited plastoquinone pool. The oscillation can be fit with a miss parameter $\alpha = 19$, a double hit parameter $\beta = 4$, and 100% S_1 state population in the dark-adapted sample [8,32]. The slightly high miss parameter is likely due to the long dark-times of 25 s that are required for resolving the oxygen yields after each flash individually.

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