Biochimica et Biophysica Acta 1817 (2012) 1208–1212

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Probing the turnover efficiency of photosystem II membrane fragments with different electron acceptors ☆

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ARTICLE INFO

Article history:
Received 31 January 2012
Received in revised form 21 March 2012
Accepted 29 March 2012
Available online 6 April 2012

Keywords:
Photosystem II
Electron acceptor
Oxygen evolution
Membrane-inlet mass spectrometry
Turnover frequency
Artificial photosynthesis

ABSTRACT

In this study we employ isotope ratio membrane-inlet mass spectrometry to probe the turnover efficiency of photosystem II (PSII) membrane fragments isolated from spinach at flash frequencies between 1 Hz and 50 Hz in the presence of the commonly used exogenous electron acceptors potassium ferricyanide(III) (FeCy), 2,5-dichloro-*p*-benzoquinone (DCBQ), and 2-phenyl-*p*-benzoquinone (PPBQ). The data obtained clearly indicate that among the tested acceptors PPBQ is the best at high flash frequencies. If present at high enough concentration, the PSII turnover efficiency is unaffected by flash frequency of up to 30 Hz, and at 40 Hz and 50 Hz only a slight decrease by about 5–7% is observed. In contrast, drastic reductions of the O_2 yields by about 40% and 65% were found at 50 Hz for DCBQ and FeCy, respectively. Comparison with literature data reveals that PPBQ accepts electrons from Q_A^- in PSII membrane fragments with similar efficiency as plastoquinone in intact cells. Our data also confirm that at high flashing rates O_2 evolution is limited by the reactions on the electron-acceptor side of PSII. The relevance of these data to the evolutionary development of the water-splitting complex in PSII and with regard to the potential of artificial water-splitting catalysts is discussed. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: from Natural to Artificial.

1. Introduction

Photosystem II (PSII) is the multi-subunit pigment–protein enzyme that uses solar energy to drive water-splitting in plants, algae, and cyanobacteria [1–4]. This unique reaction begins with the absorption of light and the generation of a stable and directed charge separation in the photochemical reaction center (RC) of PSII, followed by the formation of the radical pair $P680^{\bullet+}Q_A^{\bullet-}$ (for reviews, see [5,6]). The cation radical $P680^{\bullet+}$, which has a midpoint potential of about +1.3 V, energetically drives the sequential oxidation of two water molecules to dioxygen and four protons that is catalyzed within the oxygen-evolving complex (OEC) located at the *electron-donor side* of PSII [7,8]. The heart of the OEC is a Mn_4CaO_5 cluster [9,10], which acts as a storage device, accumulating redox equivalents during repeated one-electron oxidations by $P680^{\bullet+}$ via the redox-active tyrosine (Y_Z). Thus, the Mn_4CaO_5 cluster links the one-electron photochemistry with the four-electron water oxidation chemistry. According to the model developed by Kok and co-authors [11], the Mn_4CaO_5 cluster attains five different oxidation states (called S_i states; with $i=0$ to 4 indicating the number of oxidizing equivalents stored within the cluster) during water oxidation, and dioxygen is evolved when the highly reactive S_4 state is reached and spontaneously decays into the S_0 state. Meanwhile, on the *electron-acceptor side* of PSII, the $Q_A^{\bullet-}$ formed as a results of the primary charge separation (see above) reduces plastoquinone (PQ) (Fig. 1A) at the Q_B site to plastoquinol (PQH_2) in two one-electron reduction steps that are coupled with the uptake of two protons from stroma/cytoplasm (recently reviewed in [12]). PQH_2 is then replaced by a PQ from the thylakoid membrane that might be pre-bound at a Q_C site [13].

Important information on the maximal rate of water-splitting by PSII can be gathered from the experiments monitoring the dependence of the PSII turnover efficiency on the frequency of single turn over flashes. Such an approach may indicate at what flash rate one of the above described steps becomes rate-limiting. Such information is valuable for future experiments, where both effective S state cycling of the OEC and high repetition rates are required for data collection. One of the methods that has been previously applied to reflect the dependence of the OEC turnover on high flash frequencies was the polarographic detection of O_2 evolution by PSII [14–16]. With one exception [16], all these studies have been *in vivo* studies performed either with leaf disks [14] or whole cells of cyanobacteria [15]. However, the estimation of net O_2 production by PSII is quite

Abbreviations: MIMS, membrane-inlet mass spectrometry; PSII, photosystem II; OEC, oxygen-evolving complex; PPBQ, 2-phenyl-*p*-benzoquinone; DCBQ, 2,5-Dichloro-*p*-benzoquinone; FeCy, $K_3[Fe(CN)_6]$; PQ, plastoquinone

☆ This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: from Natural to Artificial.

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doi:10.1016/j.bbabio.2012.03.038

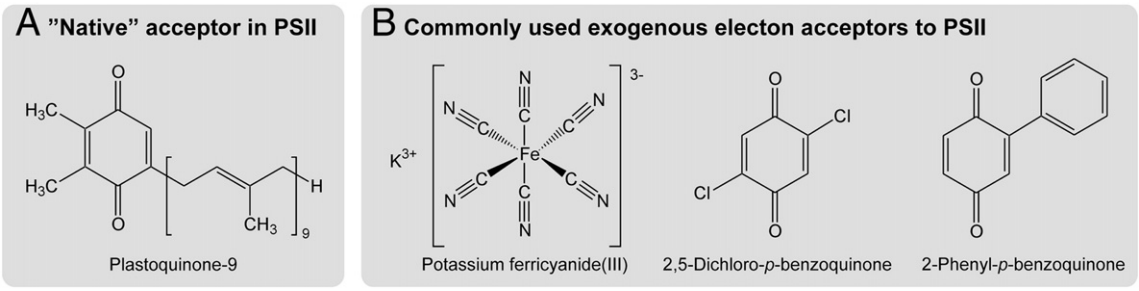

Fig. 1. Structures of the electron acceptors to PSII membrane fragments used in this study.

complicated in whole cells due to overlapping reactions of O_2 production by PSII and its concomitant partial consumption by mitochondria (respiratory activity). Similarly, in thylakoids photoreduction of O_2 can occur at PSI (Mehler reaction) [17] and at the PQ pool [18]. In addition, there are also some other drawbacks in the various polarographic approaches (for recent review, see [19]). Among them are the following: (*i*) low sensitivity and high background (membrane covered Clark-type electrode); (*ii*) the sensitivity of bare Pt-cathodes towards added redox compounds such as artificial electron acceptors (Joliot-type electrodes); (*iii*) the fast consumption of O_2 by bare Pt-electrodes that creates anaerobic conditions, which in chloroplasts and whole cells can lead to a blockage of light-induced electron transport due to the over-reduction of the PQ pool (Joliot-type electrodes); and (*iv*) the formation of harmful H_2O_2 at the bare Pt-cathodes that may affect the photosynthetic sample (Joliot-type electrodes). Recently, a fast repetition rate measurement of chlorophyll (Chl) *a* fluorescence has been used to study the S state cycling at high flash rates in intact cells of algae, cyanobacteria and in leaves of higher plants [20]. This novel and elegant approach successfully coped with the above-mentioned limitations of the polarographic methods and revealed a large increase in both misses and double hits at high flash frequencies. However, in this study, the increased miss parameter was attributed to the decreased turnover efficiency of the intrinsic OEC reactions, and not to the "acceptor-side" reactions of PSII [20]. This conclusion is in conflict with earlier results obtained by O_2 evolution measurements that where interpreted to demonstrate that the reactions at the PSII acceptor side are rate limiting at high flash frequencies [16].

The question whether the "acceptor" or "donor" side of PSII becomes rate limiting at high flash frequency was therefore revisited in the present study. Unlike to the previous reports, we employed isotope ratio membrane-inlet mass spectrometry (MIMS) combined with ^{18}O-labeling (reviewed in [21]) to monitor flash-induced $^{18}O_2$ evolution in PSII samples as a function of flash frequency. To exclude the competition of the O_2 evolution with O_2 uptake processes we used PSII membrane fragments (BBY preparations) isolated from spinach. Due to a special design of the MIMS cell (large sample volume and low consumption membrane; details described in [22]), the measurements were performed close to the air-saturated level of O_2. To minimize the influence of double hits, the PSII samples were excited with short (6 ns) laser flashes at frequencies between 1 and 50 Hz. The turnover efficiency of PSII particles was probed with three types of exogenous electron acceptors commonly used in photosynthesis research (for their structures see Fig. 1B): (*i*) potassium ferricyanide(III) ($K_3[Fe(CN)_6]$ or FeCy); (*ii*) 2,5-dichloro-*p*-benzoquinone (DCBQ); and (*iii*) 2-phenyl-*p*-benzoquinone (PPBQ).

2. Materials and methods

2.1. Chemicals and reagents

$H_2^{18}O$ (97% enrichment) was purchased from *Larodan Fine Chemicals AB*, Sweden. DCBQ (98%) and FeCy (99.99 + %) were obtained from *Aldrich*. PPBQ (95%) was purchased from *Sigma*. All reagents were used without further purification. DCBQ and PPBQ stock solutions (20 mM) were prepared in C_2H_5OH (95%) shortly before the experiments. The FeCy stock solution (150 mM) was freshly prepared in deionized and filtrated (*Millipore Quality*) water.

2.2. Sample preparation

PSII membrane fragments were prepared from fresh spinach leaves as in [23,24] with slight modifications described earlier [25]. Control rates of O_2 evolution of the studied samples were ~400–450 μmol O_2 mg(Chl)$^{-1}$ h^{-1} as measured by a Clark-type electrode at 25 °C in the presence of 200 μM PPBQ and 500 μM FeCy. Before the MIMS measurements PSII membranes were thawed in the dark on ice and diluted in the MS cell to a desired Chl concentration (30 μM) with SNM buffer (400 mM sucrose, 35 mM NaCl, and 40 mM MES/NaOH) at pH 6.0.

2.3. MIMS measurements and ^{18}O-labeling

The MIMS measurements (for recent review see [21]) were performed with an isotope ratio mass spectrometer (*ThermoFinnigan^{Plus}XP*) that was connected via a cooling trap (dry ice + C_2H_5OH; ~200 K) to a home built membrane-inlet cell with 600 μl-working volume (for more details, see [22]). The sample was separated from the high vacuum ($3 \cdot 10^{-8}$ mbar) by a ~150 μm-thick metallic mesh silicon membrane (*Franatech GmbH*, Germany) permeable only for gasses that was resting on a porous Teflon support (*Small Parts Inc.*, USA). Before the measurements, suspensions of the dark-adapted PSII samples in SNM buffer, as well as one of the electron acceptors (DCBQ, PPBQ, or FeCy) were loaded into the MS cell in the dark. For the $^{18}O_2$-labeling of the aqueous sample suspensions, $H_2^{18}O$ was added to the reaction mixture to a final enrichment of 20%. The Chl concentration of the PSII membranes in the MS cell after this addition was 30 μM, and those of the electron acceptors were the following: [DCBQ] = 250 μM; [PPBQ] = 250 μM; and [FeCy] = 5 mM. The sample suspension was kept at 10 °C and stirred constantly during measurements with a magnetic stir bar. PSII membranes were excited with a series of 10 short (6 ns) laser flashes provided by a frequency doubled Nd/YAG laser with flash frequency between 1 and 50 Hz (see below). Due to the enrichment of the sample suspension with $H_2^{18}O$, flash-induced O_2 evolution was detected with excellent S/N well above the low ^{36}Ar background as a doubly labeled ($^{18}O_2$) dioxygen at $m/z = 36$. In addition, non-labeled dioxygen ($m/z = 32$), ^{18}O-single-labeled dioxygen ($m/z = 34$), and argon ($m/z = 40$) were always simultaneously monitored (see Fig. S1 in Supplementary Data). While the rise of O_2 concentration at $m/z = 34$ ($^{16}O^{18}O$) gave discernible signals, at $m/z = 32$ the MIMS signal was indistinguishable from noise due to the large $^{16}O_2$ background signal. The amount of the $^{16}O^{18}O$ evolved was always consistent with the percentage of ^{18}O-labeling. The $m/z = 40$ signal (^{40}Ar) served as control (Fig. S1) in order to detect possible artifacts that can be caused by heating of the reaction suspension due to light illumination (no 'heating' artifacts were found in this study). For best reproducibility and to economize ^{18}O-water

up to 7 groups of 10 flashes were recorded with 5 min brakes at various frequencies on one sample. Measurements were repeated in different orders 3–4 times. Test experiments at 2 and 50 Hz showed that no decline of O_2 yield occurs between the first and last flash group of such a series of experiments.

2.4. Laser illumination

The laser employed in this study was a factory modified Continuum Inlite II-20 that was specifically designed to operate up to 60 Hz flash frequency for limited periods of time ('Inlite II-60'). This frequency doubled Nd/YAG laser has a maximal output of 125 mJ/pulse at 532 nm. To obtain stable laser intensity at all frequencies, the laser was operated (with turned on Q-switch) until a stable average output energy of 25 mJ/pulse was reached (intensities of individual flashes may vary by 10–20%). If required, the lamp voltage was used to obtain this value. Only then a fast shutter (SH05 operated with SC10 Controller; both Thorlabs) was opened for the required period to let 10 flashes pass to illuminate the sample, which was at all other times well protected from light. The laser output energy was measured with a PE50-BB-DIF head connected to an Ophir Nova energy meter. In previous experiments [22] we established that 25 mJ/pulse is able to saturate samples up to chlorophyll concentrations of 50 μM (pathlength of the sample chamber is 8 mm). This was established by determining a number of ~250 Chl/RC for our BBY preparations (see Supplementary Material in [22]).

3. Results and discussion

In order to study the turnover efficiency of PSII at various flash frequency in the presence of the exogenous electron acceptors PPBQ, DCBQ or FeCy we used a MIMS set-up that was specially designed for the online detection of O_2 evolution under ambient and elevated O_2 concentrations (see Materials and methods). Fig. 2 displays oxygen evolution detected at $m/z = 36$ ($^{18}O_2$) that was induced by excitation of the PSII membranes in with 10 laser flashes at a frequency of 2, 20, and 50 Hz. Since the MIMS signals $m/z = 32$ ($^{16}O_2$), $m/z = 34$ ($^{16}O^{18}O$), and $m/z = 40$ (^{40}Ar) do not provide any additional information, for the sake of simplicity, these signals are not shown (but see Fig. S1). The MIMS experiments shown in Fig. 2 were performed either in the presence of 250 μM PPBQ (traces 1–3), 250 μM DCBQ (traces 4–6), or 5 mM FeCy (traces 7–9) as electron acceptors. A visual inspection of the MIMS data shows no significant differences in $^{18}O_2$ yields of the PSII samples in the presence of PPBQ or DCBQ upon flash illumination at 2 Hz. However, it is clearly seen that the $^{18}O_2$-MIMS signal obtained with FeCy at 2 Hz is about 25% smaller than those with PPBQ and DCBQ. This agrees well with earlier data by Fromme et al. that showed that with FeCy the maximum O_2-evolution rate is only achieved at frequencies of 0.3–0.5 Hz [16]. A further inspection of the spectra show that an increase of flash frequency (up to 20 and 50 Hz) visibly reduces $^{18}O_2$ yields in cases of DCBQ and FeCy, while $^{18}O_2$ evolution in the presence of PPBQ is almost unaffected by these flash rates.

The above mentioned qualitative observations are summarized with statistics (n = 3–4) in Fig. 3. The data presented here are normalized to the averaged $^{18}O_2$ yield induced by 10 laser flashes at 2 Hz in the presence of PPBQ (that is identical with that obtained in the presence of DCBQ; see traces 1 and 4 in Fig. 2). Fig. 3 reveals that in the presence of DCBQ or FeCy the PSII membranes lose at a flash rate of 50 Hz about 40–50% of their flash-induced O_2-evolving capacity compared to 2 Hz. The loss of O_2-evolving activity at flash frequencies between 2 and 10 Hz is somewhat more pronounced in the presence of FeCy (the activity decreased by about 25% at 10 Hz compared to that at 2 Hz) than in the presence of DCBQ (the activity decreased by about 10% at 10 Hz compared to that at 2 Hz). In contrast, the O_2-evolving capacity of PSII samples incubated with PPBQ is almost independent of the flash frequency. Only a very small drop (by about 5%) is seen at 40 and 50 Hz, and up to 30 Hz no significant decrease is measurable The observed phenomena cannot be explained by the different diffusion rate of acceptors through the membrane, since in PSII membrane fragments all acceptors can easily reach the acceptor side of PSII. They more likely reflect the different rates and/or efficiencies with which the acceptors bind into the Q_B pocket, receive protons (in case of PPBQ and DCBQ) and exchange against fresh acceptor molecules after reduction. The low yields of O_2 evolution with FeCy are most likely caused by the low binding efficiency of this inorganic complex to the Q_B-binding pocket. Interestingly, in a previous study by Haag et al. [26] the opposite result was found. This apparent contradiction can be straightforwardly explained by the different sample types involved: the present study describes the situation in PSII membrane fragments, where the electron transfer to artificial acceptors occurs within an almost native Q_B-site, while the previous study [26] describes the situation in spinach PSII core complexes, which are known to have a severe modification of the Q_B-binding site [27]. In case of a damaged Q_B-binding pocket no effective pre-binding of the quinones, stabilization of the semiquinone and protonation of the reduced quinones can occur and therefore the FeCy at its higher concentration outperforms the quinones by oxidizing $Q_A^{\bullet -}$. In principle, also a too tight binding

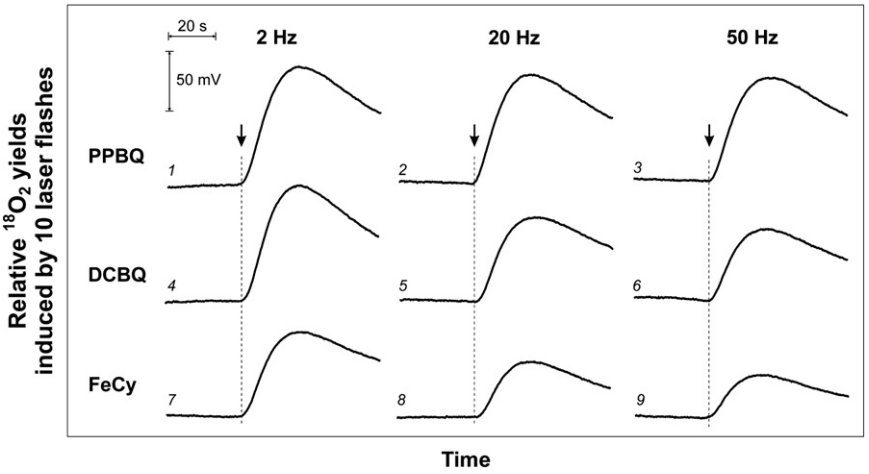

Fig. 2. MIMS measurements of oxygen evolution in spinach PSII membranes induced by a series of 10 saturating laser flashes (at 2, 20, and 50 Hz) and measured as $^{18}O_2$ ($m/z = 36$) at pH 6.0 and 10 °C. The measurements were performed in the presence of either 250 μM PPBQ (traces *1–3*), 250 μM DCBQ (traces *4–6*), or 5 mM FeCy (traces *7–9*) as artificial electron acceptors. The final $H_2^{18}O$ enrichment was ~20%. The *Chl* concentration of PSII membranes in the MIMS cell was 30 μg ml^{-1}.

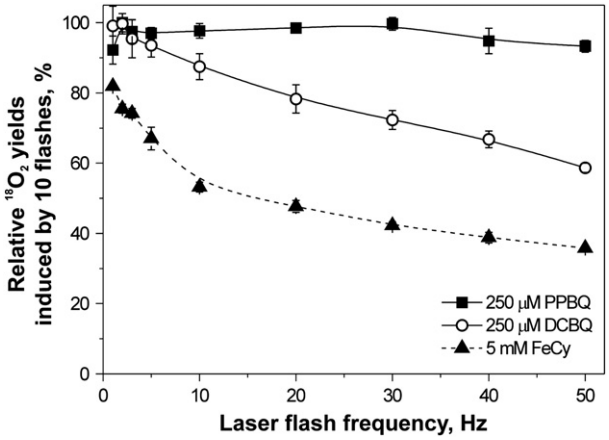

Fig. 3. Relative $^{18}O_2$ evolution ($m/z = 36$) of spinach PSII membrane fragments ([Chl] = 30 μg ml^{-1}) induced by a series of 10 laser flashes as a function of flash frequency measured by MIMS at pH 6.0 and 10 °C. The measurements were performed in the presence of either 250 μM PPBQ (closed squares), 250 μM DCBQ (open circles), or 5 mM FeCy (closed triangles) as artificial electron acceptors. The final $H_2^{18}O$ enrichment was ~20%. The results are an average of 3–4 experiments and normalized to the average $^{18}O_2$ yield induced by flashes at 2 Hz for DCBQ and PPBQ (which were found to be identical).

of hydroquinone could slow PSII turnover, but this seems to be a less likely explanation for this discrepancy.

Moreover, the protolytic properties of the non-protonizable FeCy and the protonizable PPBQ/DCBQ have to be considered. It has been shown previously that in the presence of FeCy continuous acidification of the PSII membrane fragments occurs due to the release of protons during water oxidation, while this phenomena is absent in the presence of PPBQ due to H^+-uptake caused by reduction of the acceptor [28]. However, the pH change is expected to be negligible in our experiments due to the small number of flashes (70), the low sample concentration, and the high buffer capacity (40 mM MES) of the employed reaction medium. This statement was confirmed by test experiments showing that the O_2 yield of flashes 1–10 is identical to that of the last flash group (flashes 61–70; data not shown). In principle, also inactivation of PSII or S-state destabilizing effects by reduced acceptor molecules or impurities need to be considered, but they are unlikely to be dominating, since the concentration of reduced acceptor is small due to the above mentioned small number of flashes, and impurities were minimized by purchasing relatively clean chemicals and preparation of acceptor solutions shortly before the experiments. While purification of the quinones prior use by recrystallization or sublimation is preferable, we point out that it is very unlikely that our data and conclusion are severely affected by impurities, since the least pure quinone (PPBQ; 95%) had the best overall performance.

The results presented here for PPBQ basically agree with the earlier data of Fromme et al. [16]. However, in the earlier data no difference between 1 Hz and 2 Hz was seen, and the total O_2 yield starts decreasing already at about 7 Hz (~150 ms dark times between flashes). This difference is likely due to the fact that Fromme et al. had a lower PPBQ/PSII ratio and also used 12-times more flashes (120 vs. 10) per flash group due to the lower sensitivity of Clark-electrodes as compared to mass spectrometers [16]. This likely leads to a decrease of available electron acceptor during the flash sequence, which in turn increases the times required for diffusion of fresh acceptor into the Q_B site.

By employing fluorescence techniques Ananyev et al. found in intact cells of *Chlorella pirenoidosa* also a slight increase of PSII turnover efficiency from 1 Hz to 2 Hz [20]. In addition, they observed with intact system an increase of the miss and double hit parameters at frequencies higher than 30 Hz, which corresponds to our observation of a very slight drop of O_2 yield above 30 Hz. Since an increase of the double hit parameter at high frequency is not feasible, this phenomenon was interpreted by Ananyev et al. as double misses instead [20]. Although we cannot provide a detailed analysis of the miss and double hits based on our data (only the total sum of O_2 produced by 10 flashes was recorded), the observed drop of O_2 yield from 100% at 30 Hz to 93% at 50 Hz may be assigned to an increase of the miss parameter from about 10% to about 20% (see Supplementary Data for simulations).

The strong dependence of the O_2 yield on the electron acceptor on one hand, and the generally good agreement of our PSII membrane fragments data in the presence of PPBQ with those of Ananyev et al. [20] obtained with *Chlorella* and *Euglena* cells on the other hand, suggest that the PSII turnover in plants and most algae PSII is limited by the reactions on the acceptor side, most likely by the exchange of reduced vs. fresh electron acceptor, that add up to a turnover time of O_2-evolution of about 20–30 ms. This is also in line with an overall rate limitation for O_2-formation of about 20 ms that can be estimated [29] on the bases of in vitro O_2 evolution rates of 5000–6000 μmol O_2 mg^{-1} Chl h^{-1} reported for PSII core complexes isolated from the thermophilic cyanobacterium *Thermosynechococcus elongatus* in the presence of PPBQ as artificial electron acceptor [30]. The above comparison to intact cells also shows that PPBQ is almost as efficient in oxidizing Q_A^- as the natural PQ. This may be due to the similar structure of the 'head' group (Fig. 1), allowing efficient binding in the Q_B-pocket. However, PPBQ is also known to lead to reductant-induced oxidation of the non-heme iron [31,32]. Therefore, it may take up always two electrons at a time and in this way may have twice much time for exchange of $PPBQH_2$ against PPBQ than the $DCBQH_2$/DCBQ redox couple.

This interpretation is in contrast to conclusions by Ananyev et al. [20], who stated that the reactions within the OEC are rate limiting. This assignment was based on the observed increase in misses and double hits (double misses), which was interpreted as showing that the OEC gets less efficient at flash frequencies above 30 Hz. However, the miss parameter reflects all equilibria and kinetics within PSII [33–35]. For example, an incomplete oxidation of Q_A^- within the time of two flashes will lead to a miss, because no stable charge separation is possible.

The rate limitation of O_2 production by the acceptor side has interesting consequences. In general this means that there was no evolutionary pressure to optimize the 1–2 ms turnover kinetics for O–O bond formation [36]. This is in contrast to the superb optimization of the energetics of S-state transitions and of the O–O bond formation via coupling of electron and proton transports within a delicate H-bonding network around the Mn_4CaO_5 cluster. Interestingly, Ananyev et al. [20] describe one organism (cyanobacterium *Spirulina maxima*) where the acceptor side appears to work at very similar speed to water-oxidation at the donor side.

Based on the absence of evolutionary pressure to optimize the turnover frequency of the OEC, we suggest that there is a chance that artificial catalysts for water-splitting can be developed that have higher turnover frequencies than PSII complexes.

4. Conclusions

Our presented experiments clearly show that for studies of the PSII water-splitting complex that require high flash frequencies PPBQ is the best electron acceptor. At high enough PPBQ/PSII ratios the turnover efficiency of PSII is not affected up to 30 Hz, and it only slightly drops when 40 and 50 Hz are employed. In contrast, at frequencies below 2 Hz DCBQ (and FeCy) is a better acceptor. The data obtained confirm that the water-oxidizing reactions on the donor side of PSII work generally faster than the slowest steps on the quinone-reducing side of PSII. Thus, the electron-acceptor side of PSII is concluded to be the rate-limiting for O_2-evolution.

Acknowledgements

This work was supported by the *Swedish Research Council (VR)*, the *Strong Research Environment Solar Fuels* (Umeå University) and the *Artificial Leaf Project (K&A Wallenberg Foundation)*.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2012.03.038.

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