Biochimica et Biophysica Acta 1837 (2014) 1411–1416

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Electrochemically produced hydrogen peroxide affects Joliot-type oxygen-evolution measurements of photosystem II☆

Long Vo Pham, Johannes Messinger *

Department of Chemistry, Chemistry Biology Center (KBC), Umeå University, Linnaeus väg 6, SE-901 87 Umeå, Sweden

ARTICLE INFO

Article history:
Received 22 November 2013
Received in revised form 18 January 2014
Accepted 22 January 2014
Available online 29 January 2014

Keywords:
Photosystem II (PSII)
Oxygen evolving complex (OEC)
Water oxidation
Manganese
Hydrogen peroxide (H_2O_2)

ABSTRACT

The main technique employed to characterize the efficiency of water-splitting in photosynthetic preparations in terms of miss and double hit parameters and for the determination of S_i ($i = 2,3,0$) state lifetimes is the measurement of flash-induced oxygen oscillation pattern on bare platinum (Joliot-type) electrodes. We demonstrate here that this technique is not innocent. Polarization of the electrode against an Ag/AgCl electrode leads to a time-dependent formation of hydrogen peroxide by two-electron reduction of dissolved oxygen continuously supplied by the flow buffer. While the miss and double hit parameters are almost unaffected by H_2O_2, a time dependent reduction of S_1 to S_{-1} occurs over a time period of 20 min. The S_1 reduction can be largely prevented by adding catalase or by removing O_2 from the flow buffer with N_2. Importantly, we demonstrate that even at the shortest possible polarization times (40 s in our set up) the S_2 and S_0 decays are significantly accelerated by the side reaction with H_2O_2. The removal of hydrogen peroxide leads to unperturbed S_2 state data that reveal three instead of the traditionally reported two phases of decay. In addition, even under the best conditions (catalase + N_2; 40 s polarization) about 4% of S_{-1} state is observed in well dark-adapted samples, likely indicating limitations of the equal fit approach. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: Keys to Produce Clean Energy.

1. Introduction

Molecular oxygen is important for life on Earth owing to its role in the glycolysis and citric acid pathways that yield in aerobic organisms the energy-rich ATP molecules. The generation of the present day oxygen-rich atmosphere started about 2–3 billion years ago [1]. At that time, prokaryotic cyanobacteria developed the ability to split water into molecular oxygen and metabolically bound hydrogen, using sunlight as energy source [2,3]. Within the molecular structure of a plant cell, photosynthesis is performed by a specific intracellular organelle — the chloroplast. Chloroplasts contain a membrane system, the thylakoid membrane, that comprises lipids, quinones and a very high content of protein complexes: light harvesting complexes (LHC's), photosystem II (PSII), cytochrome b_6f, photosystem I (PSI) and the ATP synthase. The unique photosynthetic oxidation of water into molecular oxygen, protons and electrons is performed by the Mn_4CaO_5 cluster in PSII [4–9]. In the late 1960's, Pierre Joliot and his co-workers built an electrode, now referred to as Joliot-type electrode, that allowed measuring the oxygen evolution yields per flash induced in algae by a series of single-turnover light flashes. In this way, he made the fundamental discovery that photosynthetic oxygen evolution occurs with a periodicity of four, in which the first maximum is observed after the third flash (Fig. 1A) [10,11]. This periodicity disappeared after several cycles, and a small oxygen yield was already observed after the second flash. This complex pattern of photosynthetic oxygen evolution was explained by Kok and co-workers within an elegant model [12]. The revolutionary idea of Kok's model was that it postulated that each PSII reaction center, which generates in response to light a positive and a negative charge, is connected to only one unit, the oxygen-evolving complex (OEC), that first stores four oxidizing equivalents before it reacts with two water molecules to generate oxygen [13]. This basic reaction scheme was expressed by five oxidation states, labeled S_0, S_1, S_2, S_3 and S_4, that the OEC can attain [12]. Of these states only the singly oxidized S_1 state is dark-stable, while S_2 and S_3 return to S_1 via charge recombination with $Q_B^{-/2-}$ or via reduction by tyrosine D (Y_D; D2-Tyr160). The S_0 state is oxidized to S_1 by the long lived radical form of Y_D, Y_D^{ox}. Oxygen evolution (and substrate water-binding) occurs spontaneously within 1–2 ms after the highly reactive S_4 intermediate is reached, which sets the OEC back into the S_0 state. The damping of the period four oscillation and the small oxygen yield after the 2nd flash was explained by Kok et al. by introducing two parameters that affect the probabilities for the light-induced transitions between these S_i states. The miss parameter (α) gives the probability that an

Abbreviations: ATP, adenosine triphosphate; BSA, bovine serum albumin; FIOP, flash-induced oxygen evolution pattern; LHC, light harvesting complex; PSII, photosystem II; MOPS, 3-(N-morpholino)propanesulfonic acid; OEC, oxygen-evolving complex; PSI, photosystem I; S_i-state, oxidation state of the Mn_4CaO_5 cluster; Y_D, tyrosine D (D2-Tyr160), reduced form; Y_D^{ox}, oxidized form of tyrosine D

☆ This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: Keys to Produce Clean Energy.

* Corresponding author. Tel.: +46 907865933.
E-mail address: Johannes.Messinger@umu.se (J. Messinger).

0005-2728/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbabio.2014.01.013

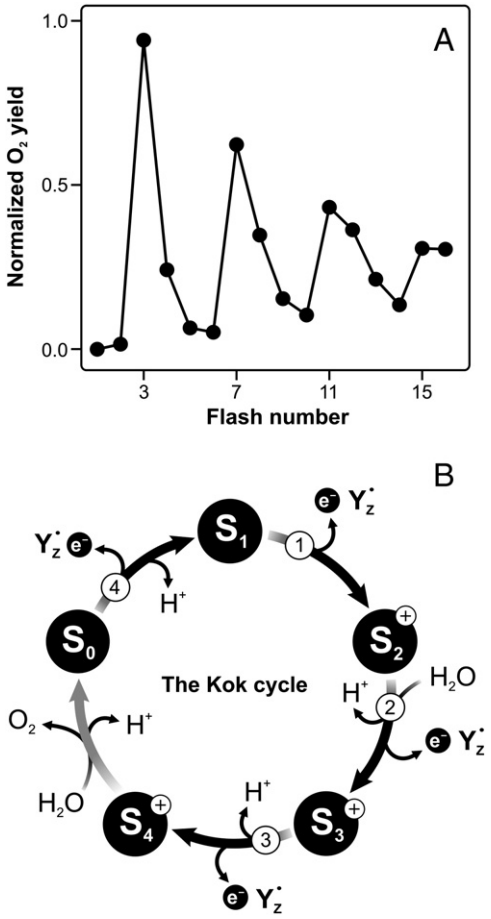

Fig. 1. The oxygen evolution pattern of dark-adapted thylakoid sample as a function of flash at the frequency of 2 Hz (A) and a modern version of the Kok cycle that includes proton release and substrate water binding [12,15,16,21] (B).

OEC does not advance to the next higher S_i state after a saturating flash excitation, while the double hit parameter (β) gives the percentage of centers that undergo two transitions during one flash ($S_i \rightarrow S_{i+2}$). A modern version of the Kok cycle is displayed in Fig. 1B. It includes the strictly alternating removal of electrons and protons [6,14,15] and two separate water binding events [16]. The Kok model allows fitting flash-induced oxygen evolution patterns (FIOPs) with the simplifying assumption that the miss and double hit parameters are S_i state independent. Several attempts have been made to derive their expected S_i state dependence, but no consensus has been reached as yet [17–20].

The Joliot-type electrode consists of a bare platinum cathode, onto which the PSII sample is placed directly. The Pt-cathode is polarized by about −750 mV against a Ag/AgCl anode so that it reduces the O_2 formed after flash excitation [11]. The induced fast transient is uncoupled electronically from the constant background signal caused by O_2 dissolved in the flow buffer and recorded on a personal computer. The membrane-less cathode not only provides the fast response and high sensitivity required for obtaining FIOPs, but also exposes PSII directly to this potential and to possible reaction intermediates generated during O_2 reduction at the platinum surface [11]. One such likely intermediate is hydrogen peroxide [22–24]. This is of concern since it is known that the Mn_4CaO_5 cluster of the OEC can be reduced by small water-like reductants such as NH_2OH, NH_2NH_2, H_2S and H_2O_2 to states below the natural S_0 state [25–30]. For example, it was shown that the addition of 0.03% H_2O_2 to PSII causes at alkaline pH (pH 8.8) a two-electron reduction of the S_1 state into the S_{-1} state [30]. When chloroplasts were given a single flash of light to form the S_2 state prior to the addition of H_2O_2, the FIOP (measured 5 min later when all hydrogen peroxide is gone) displays a high oxygen yield after the fourth flash, indicating that most of the S_2 state was converted in a fast reaction into the S_0 state [30–32]. In addition, PSII may also be able to use H_2O_2 as substrate for flash-induced molecular oxygen formation [33–37].

In this report, we investigate the effects of in situ (on the bare Pt-cathode) produced hydrogen peroxide on FIOPs and S_i state lifetimes.

2. Materials and methods

2.1. Sample preparation

Spinach thylakoid samples were isolated from spinach leaves as described previously [38,39]. After the final isolation step, the thylakoid membranes were frozen as small beads in liquid nitrogen and then stored at −80 °C. Before the FIOP experiments were performed, the frozen stock solution of thylakoid sample was defrosted in the dark on ice and diluted to [Chl] = 0.5 mg/ml with measuring buffer (5 mM $MgCl_2$, 20 mM NaCl, 50 mM MOPS/NaOH at pH = 7.0). The chlorophyll concentration was calculated according to Porra [40].

2.2. S_1Y_D and $S_1Y_D^{ox}$ thylakoids

FIOP experiments were performed using either preflashed or non-preflash thylakoid samples, respectively. S_1Y_D thylakoid samples contained a high percentage (>80%) of the reduced form of tyrosine D (Y_D) due to long-term (several months) dark-storage at −80 °C [39,41]. $S_1Y_D^{ox}$ thylakoids with approximately 90% oxidized tyrosine D, were prepared by giving one saturating flash to an aliquot of the S_1Y_D sample (pH 7.0, 20 °C), followed by a 5 minute dark-incubation at room temperature [41].

2.3. Preparation of inactive catalase

Catalase from bovine liver (CAS: 9001-05-2, product number C9322-5G, 3809 U/mg) was purchased from Sigma-Aldrich and was either used directly, or when indicated, after inactivation with the inhibitor NaN_3 in the presence of H_2O_2 [42]. The catalase powder was dissolved in measuring buffer (50 mM MOPS/NaOH, 20 mM NaCl, 5 mM $MgCl_2$, pH = 7.0) to a final concentration of 40.000 U/ml (10.5 mg/ml). Then, 1 M NaN_3 (final concentration) was added and the mixture was incubated for 10 min. Finally, 1 M H_2O_2 (final concentration) was added to the solution, which was incubated for 4 h at room temperature. The inactivated catalase was collected and separated from the free inhibitors by centrifugation in a molecular weight cut-off tube (500 μl, pore size 100.000 kDa; 30–40 min centrifugation at 10.000 ×g). The retained solution (50 μl) was further purified by repeated washing steps (4–5 times) with measuring buffer. After the last centrifugation, the protein concentration was determined using the Bradford assay [43,44]. The residual enzymatic activity was probed by Beers assay [45,46]. The solution was then frozen and stored at −20 °C until used.

2.4. Experiments of flash-induced oxygen evolution patterns (FIOPs)

The FIOP experiments of S_1Y_D or $S_1Y_D^{ox}$ thylakoids were performed with an unmodulated home-built Joliot-type electrode in measuring buffer (see above) at 20 °C and pH 7.0 [39,47] in the absence of artificial electron acceptors. 10 μl aliquots of the thylakoid suspension were transferred to the surface of bare Pt-cathode in very dim green light, and the polarization voltage of −750 mV was switched on 40 s (or longer, if specified) before exposing the sample to a series of 16 Xe-flashes (2 Hz; Perkin Elmer, LS-1130-4). A personal computer was used to trigger the flash lamp and to record the data at a sampling rate of 3600 points/s.

2.5. S_i-state lifetime measurements

The S_2, S_3 and S_0 state lifetimes of both non-pre-flashed (S_iY_D) and pre-flashed ($S_iY_D^{ox}$) thylakoid samples were measured with the Joliot-type oxygen electrode by exciting dark adapted samples with one (S_2), two (S_3) or three (S_0) pre-flash(es) while they were resting on the electrode surface. After the desired dark times (t_d), a flash train of 16 flashes (2 Hz) was given to PSII samples and the resulting FIOPs were recorded and deconvoluted into S_i-state population as described below [19,48,49]. The polarization was switched on at the times indicated (40 s to 20 min) prior to giving the train of 16 flashes. About 16–18 time points were collected per S_i state decay.

2.6. Analysis of FIOPs

The oxygen yields of the first 16 flashes of each FIOP were analyzed within the framework of an extended Kok model that was programmed within an Excel spreadsheet. This model included the reduced S_{-1} state of Mn_4CaO_5 cluster and a damping parameter (d) that accounts for the loss of active PSII centers during the flash train [19,49,50]. The program is based on the formulas:

$$\begin{bmatrix} [S_{-1}]_n \\ [S_0]_n \\ [S_1]_n \\ [S_2]_n \\ [S_3]_n \end{bmatrix} = \begin{bmatrix} \alpha_{-1} & 0 & 0 & 0 & 0 \\ \gamma_{-1,n} & \alpha_0 & 0 & \beta_n & \gamma_{30} \\ \beta_n & \gamma_{0,n} & \alpha_1 & 0 & \beta_n \\ 0 & \beta_n & \gamma_{1,n} & \alpha_2 & 0 \\ 0 & 0 & \beta_n & \gamma_{2,n} & \alpha_3 \end{bmatrix} * \begin{bmatrix} [S_{-1}]_{n-1} \\ [S_0]_{n-1} \\ [S_1]_{n-1} \\ [S_2]_{n-1} \\ [S_3]_{n-1} \end{bmatrix} * d \quad (1)$$

and

$$Y_n^{fit} = (1-\alpha_3) * [S_3]_{n-1} + \beta_n * [S_2]_{n-1}. \quad (2)$$

Where $[S_i]_{n-1}$ and $[S_i]_n$ are the S_i state populations before and after the n^{th} flash, α_i is the S_i state dependent miss probability, β_n is the flash number dependent double hit probability (β can be higher on the first flash under certain circumstances), γ is the single hit probability (e.g. $\gamma_{1,n} = 1-\alpha_1-\beta_n$), d is the damping parameter (see above) and Y_n^{fit} is the oxygen yield generated after the n^{th} flash.

All fits presented in this report are based on S_i state independent (equal) miss and double hit parameters, since these are most commonly used and we found our conclusions to be invariant towards various possible S_i state dependent approaches. The fits were performed within a spreadsheet program (Microsoft Excel) using the 'GRG nonlinear' method of the 'Solver' subroutine of Excel to minimize the deviation dy_n^2 between the experimental and calculated oxygen yields by varying a specified set of parameters:

$$dy_n^2 = \sum_{n=1}^{F} \left[Y_n^{exp} - Y_n^{fit} * \left(\sum_{n=1}^{F} Y_n^{exp} / \sum_{n=1}^{F} Y_n^{fit} \right) \right]^2. \quad (4)$$

Here, Y_n^{exp} is the experimental oxygen yield of the n^{th} flash and F stands for the number of analyzed flashes (or independent data points). The S_i-state populations were normalized according to Eq. (5), and the fit quality was determined by Eq. (6).

$$\sum_{i=-1}^{3} [S_i] = 1 \quad (5)$$

and

$$FQ = \frac{dy_n^2}{F-P} \quad (6)$$

where, P is the number of free parameter used in the fit procedure. A systematic fit approach was used in which the miss and double hit parameters were determined first from the 2 Hz FIOP of $S_1Y_D^{ox}$ thylakoids and then kept constant for the subsequent S_i-state lifetime analysis.

2.7. Rate constant and half-time calculations

The rates of S_2 and S_3 state decay were determined by fitting the time dependents of the S_2 or S_3 state population by the sum of two first order decay reactions, where the amplitude of the fast phase reflects the amount of Y_D (Eq. (7)). In some cases, also fits with three phases were tested.

$$S_i(t_d) = A_{i,fast} * \exp^{-k_{i,fast}*t_d} + A_{i,slow} * \exp^{-k_{i,slow}*t_d} \quad (7)$$

where, $k_{i,fast}$ and $k_{i,slow}$ are the rate constants, and $A_{i,fast}$ and $A_{i,slow}$ are the amplitudes of the fast and slow phase of decay ($A_{i,fast} + A_{i,slow} = 1$).

In contrast, the S_0 oxidation to S_1 was described well by a mono exponential reaction: $S_0Y_D^{ox} \rightarrow S_1Y_D$ (Eq. (8)).

$$S_0(t_d) = S_0Y_{D\ (Initial)}^{ox} * \exp^{-K_{01}*t_d} + C \quad (8)$$

where, C is a constant that reflects the S_0Y_D fraction.

3. Results and discussion

3.1. Flash-induced oxygen evolution pattern (FIOPs)

Fig. 2A shows a flash-induced oscillation pattern (FIOP) of spinach thylakoids obtained after 40 s polarization of the electrode, which in our set up is the minimum time required for achieving a stable polarization at −750 mV against the Ag/AgCl anode. Under this condition, a deep oscillation was observed with maxima at the 3rd, 7th and 11th flash, and low oxygen yields after the 5th and 6th flashes. Extension of the

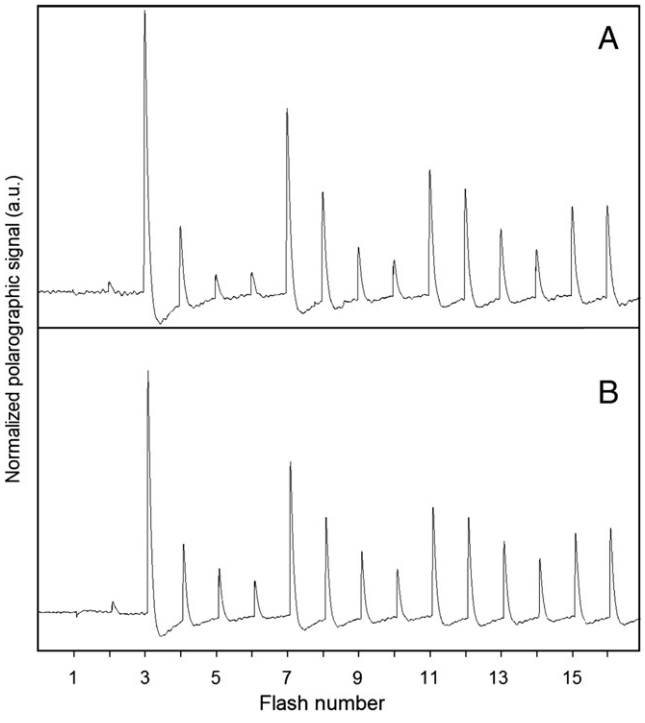

Fig. 2. Original FIOPs obtained after 40 s (A) and 20 min (B) polarization of −750 mV in the absence of catalase. The data are normalized to the sum of the oxygen yields obtained by flashes 3–6.

Table 1
Fit parameter for FIOPs of spinach thylakoids obtained after various polarization times and experimental conditions.

V_{polar}, mV	−750					−600	
t_{polar}	40 s	20 min	20 min	20 min	20 min	40 s	20 min
Protein addition	No	No	Catalase	No	Catalase	No	No
Buffer saturated with	Air	Air	Air	N_2	N_2	Air	Air
Miss (α)	9.5	9.2	10.9	9.7	9.3	9.0	9.8
Double hit (β)	1.1	1.9	1.0	1.2	1.2	1.2	2.3
Damping (d)	98.0	98.8	98.1	97.7	97.8	98.6	98.9
S_1-state	94.0	81.0	91.1	88.8	91.4	95.7	85.1
S_0-state	2.1	1.9	2.5	2.8	2.2	0.0	0.0
S_{-1}-state	3.9	17.2	6.4	8.5	6.5	4.3	14.9
FQ (*10^6)	7.3	3.6	9.4	5.5	4.7	4.2	8.1
Oxygen yield (%)	100	57	86	84	92	100	63

polarization time decreased the maximum at the 3rd flash and led to a corresponding increase of the yields after the 5th and 6th flashes. This is seen well in Fig. 1B, which displays a pattern obtained from an aliquot of the same thylakoid preparation after 20 min polarization at −750 mV. Analysis of these two patterns within the Kok model yields very similar values for the miss and double hit parameters for both data sets, but indicates a significant increase in the S_{-1} population at the expense of the S_1 state population (Table 1). We note that this effect is largely independent of the polarization voltage in the typical range of −600 mV to −750 mV (Table 1). Only a small and constant S_0 population is found. This suggests that the S_1 state is reduced to the S_{-1} state during the polarization time by a two electron reductant. Alternatively, a two-electron reductant could accumulate that then reduces the S_2 state into S_0 during the dark-time between the first and second flashes. On the basis of previous data in the literature, and since no reductants were added, H_2O_2, formed at the electrode surface by partial reduction of O_2, appears to be the most likely two-electron reductant that can account for our observation.

To further characterize the S_{-1} state formation and for identifying the two electron reductant we repeated the experiment at various polarization times with and without the addition of catalase, inactivated catalase or BSA. Fig. 3 shows that the increase of the S_{-1} population in thylakoids without additions is nearly linear with the time of polarization, demonstrating that the reductant is indeed formed during the polarization time. The addition of inactivated catalase or BSA had neither an effect on the extent nor on the time course and of S_{-1} state formation. In contrast, the addition of active catalase almost completely suppressed the reduction of S_1 to S_{-1}. The fit results in Table 1 show that purging the flow buffer of the Joliot-electrode with N_2, which strongly reduces the dissolved O_2 concentration, had a similar effect as adding catalase. Therefore, our data conclusively identify H_2O_2, formed by the reduction of dissolved oxygen from the flow buffer, as reductant that reduces the S_1 state to the S_{-1} state during prolonged polarization times. Table 1 also shows that at long polarization times, especially in the absence of catalase and in the presence of O_2, a significant decrease in the total oxygen yield was observed.

It is interesting to note that the linear extrapolation of all data sets in Fig. 3 to zero polarization time indicates that about 4% of the PSII centers attain the S_{-1} state in dark adapted spinach thylakoids. Future studies have to show, whether this is an artifact of the equal miss fit approach, as suggested in a previous study using thylakoids isolated from

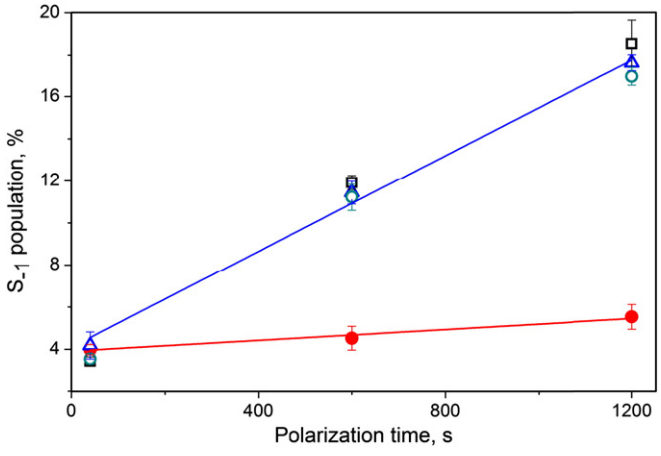

Fig. 3. Percentage of S_{-1} population as a function of polarization time (sec) in spinach $S_1Y_D^{ox}$ thylakoids with (red line and full circles) or without (blue line and open squares) the addition of catalase (10.000 U/ml = 2.63 mg/ml). Triangles and open circles indicate the S_{-1} population generated in the presence of BSA (2.63 mg/ml) or inactivated catalase (2.63 mg/ml), respectively. All experiments were repeated 3 times and fitted individually (error bars give the standard deviations). Other conditions: 20 °C, pH 7.0, [Chl] = 0.5 mg/ml, −750 mV (from 40 s to 20 min), t_{sed} = 3 min.

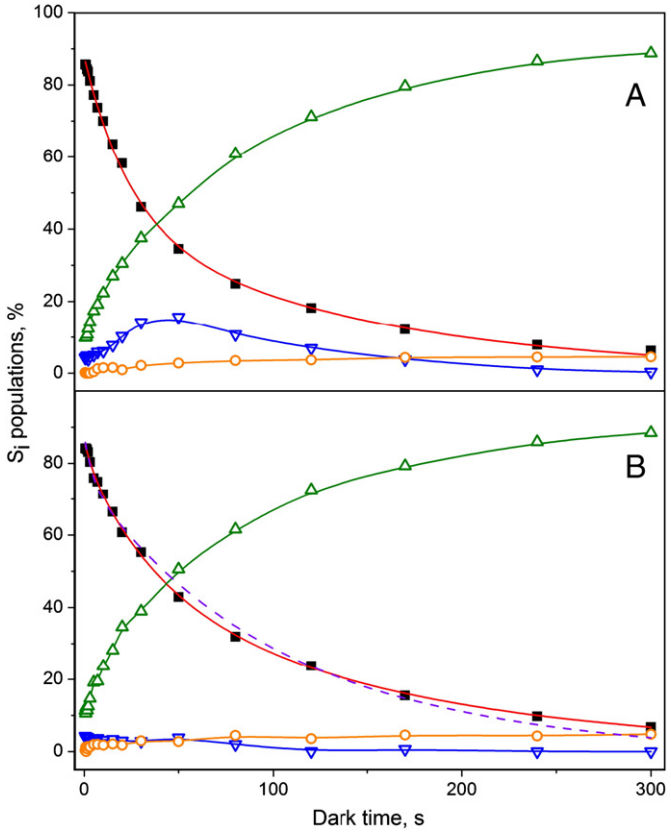

Fig. 4. S_2 state decay measurements in pre-flashed spinach thylakoids ($S_1Y_D^{ox}$). Both panels show the change in S_i state populations (square's, S_2; triangles, S_1; inverted triangles, S_0; circles, S_{-1}) as function of dark-time between the S_2 state generating flash and the subsequent flash train. A: no protein additions and air-saturated flow buffer, B: with added catalase ([catalase] = 10.000 U/ml) and nitrogen saturated flow buffer. A constant polarization time of 40 s prior to the flash train was used. Other conditions: [Chl] = 0.5 mg/ml, 20 °C, pH 7.0, −750 mV, t_{sed} = 3 min.

Table 2
Bi- and mono-exponential fits (Eqs. 7 and 8) of S_i state decays in pre-flashed ($S_1Y_D^{ox}$) spinach thylakoids in presence and absence of catalase in the sample and of O_2 in the flow buffer at 20 °C and pH 7.0. Normalized amplitude, rate constants (k) and half-life times ($t_{1/2}$) are specified.

	Spinach thylakoid membrane											
	No additions, air-saturated buffer						Catalase, N_2-saturated buffer					
	Fast phase			Slow phase			Fast phase			Slow phase		
	$A_{i,f}$, %	$k_{i,f}$, s^{-1}	$t_{1/2}$, s	$A_{i,s}$, %	$k_{i,s}$, s^{-1}	$t_{1/2}$, s	$A_{i,f}$, %	$k_{i,f}$, s^{-1}	$t_{1/2}$(s)	$A_{i,s}$, %	$k_{i,s}$, s^{-1}	$t_{1/2}$(s)
$S_2Y_D^{ox}$	14	0.236	2.9	86	0.013	53	14	0.227	3.1	86	0.010	68
$S_3Y_D^{ox}$	10	0.311	2.2	90	0.012	58	0	–	–	100	0.012	58
$S_0Y_D^{ox}$				100	0.00102	680				100	0.00079	880

Table 3
Three component fits of S_2 state decays in S_1Y_D and $S_1Y_D^{ox}$ thylakoids in the presence and absence of catalase in the sample and of O_2 in the flow buffer at 20 °C and pH 7.0. Normalized amplitude, rate constants (k) and half-life times ($t_{1/2}$) are specified.

	Fast phase			Slow phase			Very slow phase		
	$A_{2,f}$, %	$k_{2,f}$, s^{-1}	$t_{1/2}$(s)	$A_{2,s}$, %	$k_{2,s}$, s^{-1}	$t_{1/2}$(s)	$A_{2,vs}$, %	$k_{2,vs}$, s^{-1}	$t_{1/2}$(s)
$S_2Y_D^{ox}$	6	0.187	3.7	38	0.0268	26	56	0.0066	105
S_2Y_D	45	0.208	3.3	25	0.0287	24	30	0.0066	105

Thermosynechococcus elongatus [19], or whether there exists a real S_{-1} state population under these conditions.

3.2. S_i-state decay

It is known that the S_2 state is especially reactive towards exogenous reductants [41,51]. It is therefore possible that S_2 lifetime measurements are affected by the formation of H_2O_2 on the electrode surface. In the S_2 state lifetime experiments one flash is given to advance the dark-adapted samples to the S_2 state. After various dark times then a train of 16 flashes was given to probe the S_i state populations. The polarization voltage of −750 mV was turned on always 40 s prior to the first flash of the detecting flash train (this maybe before or after the S_2 generating flash). The transient rise of the S_0 population in Fig. 4A shows that even at the shortest possible polarization time (40 s) a significant amount of two-electron reduction of S_2 to S_0 takes place. The addition of catalase abolishes this effect (Fig. 4B), confirming that this is not caused by a so far unknown PSII-intrinsic process, but by electrochemically produced H_2O_2.

Similar, but less pronounced effects were observed for the $S_3 \rightarrow S_2$ and $S_0 \rightarrow S_1$ state decays. The rate constants obtained by mono- (S_0) or bi- (S_2, S_3) exponential fits of these data are given in Table 2. The half-life times of the fast S_2 and S_3 decays (reduction by Y_D) and the slow S_3 decay (recombination with acceptor side electrons) are almost invariant to catalase addition. This is expected, since the Y_D reaction is fast as compared to the H_2O_2 reduction of S_2/S_3, and the S_3 state is known to react especially slowly with exogenous reductants such as NH_2OH or NH_2NH_2 [51]. In contrast, the half-life times of both the slow S_2 and the S_0 states are about 1.3 times longer in the absence of H_2O_2 (presence of catalase). This trend is consistent with our proposal, since catalase addition removes a competing pathway for S_i state decay. In the case of S_0, the formation of a small percentage (up to 5%) of both the S_{-2} and of S_2 state population was observed in experiments when catalase was absent. This confirms earlier suggestions that the S_0 state can be oxidized to S_2 by H_2O_2 [31] or be reduced by reductants to S_{-2} [51], i.e. that H_2O_2 can react with the Mn_4CaO_5 cluster as oxidant or reductant.

While the two phase fit describes the data of the S_2 state decay very well in the absence of catalase (red line in Fig. 4A), a systematic deviation is observed if H_2O_2 was removed by catalase (dashed line in Fig. 4B). Table 3 and Fig. 4B (red line) therefore present a three-phase fit. The presence of the three distinguishable phases was confirmed by S_2 lifetime measurements with S_1Y_D thylakoids. These data were fit independently and gave, despite the expectedly different amplitudes for the three phases of the decay, basically the same three rate constants as found above for the S_2 state decay in $S_1Y_D^{ox}$ thylakoids. In contrast, for the S_3 state in all cases one or two phases (fast, Y_D; and slow) were sufficient for describing its decay to S_2. Further studies will be required for a firm assign of the slow and very slow phases in the S_2 state decay to specific intrinsic electron donors; an attractive possibility would be Q_B^- and an electron donor of the protective branch (cytochrome b559, carotenoid, Chl_Z), respectively. Alternatively, Q_BH_2 may account for the very slow decay.

Similar, yet not identical, observations were reported by Tyystjarvi and coworkers using intact thylakoids isolated from pumpkin [52]. They observed two electron reduction pathways for the S_2Q_B and S_3Q_B decay in intact thylakoids, which were absent in BBY preparations of the same organism, or after the addition of exogenous electron acceptors. It was suggested that H_2O_2 produced by thylakoids in the dark may to some extent act as donor in the two-electron reduction of the S_2Q_B thylakoids, but not for S_3Q_B thylakoids.

4. Conclusions

For a reliable determination of (i) the S_i state distribution in dark-adapted PSII samples and (ii) the S_0 and S_2 life-times care must be taken to avoid electrochemical production of H_2O_2 at the surface of the Joliot type electrode. In the absence of such effects we observe three distinguishable phases in the S_2 state decay, and a residual S_{-1} state population in dark-adapted spinach thylakoids.

Acknowledgements

The authors thank Sergey Koroidov for developing the LabView program that controls the flash lamp and records the data in our Joliot-type electrode. Dmitriy Shevela is acknowledged for his help with Fig. 1 and the helpful comments. This research project was supported by grants from the Swedish Research Council (VR), the Swedish Energy Agency, The K&A Wallenberg Foundation (The artificial Leaf Project) and the Umeå University (Solar Fuels Strong Research Environment).

References

[1] J.F. Kasting, J.L. Siefert, Life and the evolution of Earth's atmosphere, Science 296 (2002) 1066–1068.
[2] R. Buick, The antiquity of oxygenic photosynthesis: evidence from stromatolites in sulphate-deficient Archaean lakes, Science 255 (1992) 74–77.

[3] J. Xiong, C.E. Bauer, Complex evolution of photosynthesis, Annu. Rev. Plant Biol. 53 (2002) 503–521.
[4] V.K. Yachandra, K. Sauer, M.P. Klein, Manganese cluster in photosynthesis: where plants oxidize water to dioxygen, Chem. Rev. 96 (1996) 2927–2950.
[5] J. Messinger, Evaluation of different mechanistic proposals for water oxidation in photosynthesis on the basis of Mn_4O_xCa structures for the catalytic site and spectroscopic data, Phys. Chem. Chem. Phys. 6 (2004) 4764–4771.
[6] P.E.M. Siegbahn, Water oxidation mechanism in photosystem II, including oxidations, proton release pathways, O – O bond formation and O_2 release, Biochim. Biophys. Acta 1827 (2013) 1003–1019.
[7] E.M. Sproviero, J.A. Gascon, J.P. McEvoy, G.W. Brudvig, V.S. Batista, Quantum mechanics/molecular mechanics study of the catalytic cycle of water splitting in photosystem II, J. Am. Chem. Soc. 130 (2008) 3428–3442.
[8] H. Dau, C. Limberg, T. Reier, M. Risch, S. Roggan, P. Strasser, The mechanism of water oxidation: from electrolysis via homogeneous to biological catalysis, ChemCatChem 2 (2010) 724–761.
[9] W. Lubitz, E.J. Reijerse, J. Messinger, Solar water-splitting into H_2 and O_2: design principles of photosystem II and hydrogenases, Energy Environ. Sci. 1 (2008) 15–31.
[10] P. Joliot, A. Joliot, A polarographic method for detection of oxygen production and reduction of hill reagent by isolated chloroplasts, Biochim. Biophys. Acta 153 (1968) 625–634.
[11] G. Renger, B. Hanssum, Oxygen detection in biological systems, Photosynth. Res. 102 (2009) 487–498.
[12] B. Kok, B. Forbush, M. McGloin, Cooperation of charges in photosynthetic O_2 evolution — a linear four step mechanism, Photochem. Photobiol. 11 (1970) 457–475.
[13] J. Messinger, G. Renger, Photosynthetic water-splitting, in: G. Renger (Ed.), Primary Processes of Photosynthesis — Part 2: Basic Principles and Apparatus, The Royal Society of Chemistry, Cambridge, UK, 2008, pp. 291–349.
[14] H. Dau, M. Haumann, The manganese complex of photosystem II in its reaction cycle — basic framework and possible realization at the atomic level, Coord. Chem. Rev. 252 (2008) 273–295.
[15] A. Klauss, M. Haumann, H. Dau, Alternating electron and proton transfer steps in photosynthetic water oxidation, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 16035–16040.
[16] H. Suzuki, M. Sugiura, T. Noguchi, Monitoring water reactions during the S-state cycle of the photosynthetic water-oxidizing center: detection of the DOD bending vibrations by means of Fourier transform infrared spectroscopy, Biochemistry 47 (2008) 11024–11030.
[17] R. de Wijn, H.J. van Gorkom, S-state dependence of the miss probability in photosystem II, Photosynth. Res. 72 (2002) 217–222.
[18] G.Y. Han, F. Mamedov, S. Styring, Misses during water oxidation in photosystem II are S-state dependent, J. Biol. Chem. 287 (2012) 13422–13429.
[19] S. Isgandarova, G. Renger, J. Messinger, Functional differences of photosystem II from *Synechococcus elongatus* and spinach characterized by flash induced oxygen evolution patterns, Biochemistry 42 (2003) 8929–8938.
[20] H. Suzuki, M. Sugiura, T. Noguchi, Determination of the miss probabilities of individual S-state transitions during photosynthetic water oxidation by monitoring electron flow in photosystem II using FTIR spectroscopy, Biochemistry 51 (2012) 6776–6785.
[21] N. Cox, J. Messinger, Reflections on substrate water and dioxygen formation, Biochim. Biophys. Acta 1827 (2013) 1020–1030.
[22] A. Damjanov, M.A. Genshaw, J.O.M. Bockris, Role of hydrogen peroxide in reduction of oxygen at platinum electrodes, J. Phys. Chem. 70 (1966) 3761–3762.
[23] I. Katsounaros, W.B. Schneider, J.C. Meier, U. Benedikt, P.U. Biedermann, A.A. Auer, K.J.J. Mayrhofer, Hydrogen peroxide electrochemistry on platinum: towards understanding the oxygen reduction reaction mechanism, Phys. Chem. Chem. Phys. 14 (2012) 7384–7391.
[24] C.M. Sanchez-Sanchez, A.J. Bard, Hydrogen peroxide production in the oxygen reduction reaction at different electrocatalysts as quantified by scanning electrochemical microscopy, Anal. Chem. 81 (2009) 8094–8100.
[25] B. Bouges, Action de faibles concentrations d'hydroxylamine sur l'emission d'oxygene des algues chlorella et des chloroplastes d'epinards, Biochim. Biophys. Acta 936 (1971) 228–235.
[26] M. Sivaraja, D. Hunziker, G.C. Dismukes, The reaction of hydrogen sulfide with the photosynthetic water oxidizing complex and its lack of reaction with the primary electron acceptor in spinach, Biochim. Biophys. Acta 936 (1988) 228–235.
[27] B. Kok, B. Velthuys, in: A. Castellani (Ed.), Research in Photobiology, Plenum Press, New York, 1977, pp. 111–119.
[28] R.D. Guiles, V.K. Yachandra, A.E. Mcdermott, J.L. Cole, S.L. Dexheimer, R.D. Britt, K. Sauer, M.P. Klein, The S_0 state of photosystem-II induced by hydroxylamine — differences between the structure of the manganese complex in the S_0 and S_1 states determined by X-ray absorption-spectroscopy, Biochemistry 29 (1990) 486–496.
[29] J. Messinger, J.H. Nugent, M.C. Evans, Detection of an EPR multiline signal for the S_0^* state in photosystem II, Biochemistry 36 (1997) 11055–11060.
[30] B. Velthuys, B. Kok, Photosynthetic oxygen evolution from hydrogen peroxide, Biochim. Biophys. Acta 502 (1978) 211–221.
[31] W.D. Frasch, R. Mei, Kinetics of O_2 evolution from H_2O_2 catalyzed by the oxygen-evolving complex: investigation of the S_1-dependent reaction, Biochemistry 26 (1987) 7321–7325.
[32] W.D. Frasch, R. Mei, Hydrogen peroxide as an alternate substrate for the oxygen-evolving complex, Biochim. Biophys. Acta 891 (1987) 8–14.
[33] J. Mano, M. Takahashi, K. Asada, Oxygen evolution from hydrogen-peroxide in photosystem II — flash-induced catalytic activity of water-oxidizing photosystem II membranes, Biochemistry 26 (1987) 2495–2501.
[34] W.P. Schröder, H.E. Åkerlund, Hydrogen-peroxide production in photosystem II preparations, Curr. Res. Photosynth. 1–4 (1990) A901–A904.
[35] W.P. Schröder, H.E. Åkerlund, H_2O_2 accessibility to the photosystem II donor side in protein-depleted inside-out thylakoids measured as flash-induced oxygen production, Biochim. Biophys. Acta 848 (1986) 359–363.
[36] T. Wydrzynski, J. Ångström, T. Vänngard, H_2O_2 formation by photosystem II, Biochim. Biophys. Acta 973 (1989) 23–28.
[37] W. Hillier, T. Wydrzynski, Increases in Peroxide formation by the photosystem II oxygen-evolving reactions upon removal of the extrinsic 16, 22 and 33 kDa proteins are reversed by $CaCl_2$ addition, Photosynth. Res. 38 (1993) 417–423.
[38] G.D. Winget, S. Izawa, N.E. Good, Stoichiometry of photophosphorylation, Biochem. Biophys. Res. Commun. 21 (1965) 438–441.
[39] J. Messinger, G. Renger, Generation, oxidation by the oxidized form of the tyrosine of polypeptide D2, and possible electronic configuration of the redox states S_0, S_{-1}, and S_{-2} of the water oxidase in isolated spinach thylakoids, Biochemistry 32 (1993) 9379–9386.
[40] R.J. Porra, The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b, Photosynth. Res. 73 (2002) 149–156.
[41] J. Messinger, G. Renger, The reactivity of hydrazine with photosystem II strongly depends on the redox state of the water oxidizing system, FEBS lett. 277 (1990) 141–146.
[42] O.M. Lardinois, P.G. Rouxhet, Peroxidatic degradation of azide by catalase and irreversible enzyme inactivation, Biochim. Biophys. Acta 1298 (1996) 180–190.
[43] M.M. Bradford, Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[44] J.J. Sedmak, S.E. Grossberg, Rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250, Anal. Biochem. 79 (1977) 544–552.
[45] H. Aebi, Catalase invitro, Methods Enzymol. 105 (1984) 121–126.
[46] R.F. Beers, I.W. Sizer, A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase, J. Biol. Chem. 195 (1952) 133–140.
[47] P. Joliot, Modulated light source use with the oxygen electrode, Methods Enzymol. 24 (1972) 123–134.
[48] D. Shevela, V. Klimov, J. Messinger, Interactions of photosystem II with bicarbonate, formate and acetate, Photosynth. Res. 94 (2007) 247–264.
[49] D. Shevela, B. Nöring, H.J. Eckert, J. Messinger, G. Renger, Characterization of the water oxidizing complex of photosystem II of the Chl d-containing cyanobacterium *Acaryochloris marina* via its reactivity towards endogenous electron donors and acceptors, Phys. Chem. Chem. Phys. 8 (2006) 3460–3466.
[50] J. Messinger, G. Seaton, T. Wydrzynski, U. Wacker, G. Renger, S_{-3} state of the water oxidase in photosystem II, Biochemistry 36 (1997) 6862–6873.
[51] J. Messinger, U. Wacker, G. Renger, Unusual low reactivity of the water oxidase in redox state S_3 toward exogenous reductants — analysis of the NH_2-OH-induced and NH_2NH_2-induced modifications of flash-induced oxygen evolution in isolated spinach thylakoids, Biochemistry 30 (1991) 7852–7862.
[52] T.K. Antal, P. Sarvikas, E. Tyystjarvi, Two-electron reactions $S_2Q_B \rightarrow S_0Q_B$ and $S_3Q_B \rightarrow S_1Q_B$ are involved in deactivation of higher S states of the oxygen-evolving complex of photosystem II, Biophys. J. 96 (2009) 4672–4680.