Unequal misses during the flash-induced advancement of photosystem II: effects of the S state and acceptor side cycles

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Abstract
Photosynthetic water oxidation is catalyzed by the oxygen-evolving complex (OEC) in photosystem II (PSII). This process is energetically driven by light-induced charge separation in the reaction center of PSII, which leads to a stepwise accumulation of oxidizing equivalents in the OEC (Si states, \( i = 0–4 \)) resulting in \( \text{O}_2 \) evolution after each fourth flash, and to the reduction of plastoquinone to plastoquinol on the acceptor side of PSII. However, the \( S_i \)-state advancement is not perfect, which according to the Kok model is described by miss-hits (misses). These may be caused by redox equilibria or kinetic limitations on the donor (OEC) or the acceptor side. In this study, we investigate the effects of individual \( S \) state transitions and of the quinone acceptor side on the miss parameter by analyzing the flash-induced oxygen evolution patterns and the \( S_2 \), \( S_3 \) and \( S_0 \) state lifetimes in thylakoid samples of the extremophilic red alga \( \text{Cyanidioschyzon merolae} \). The data are analyzed employing a global fit analysis and the results are compared to the data obtained previously for spinach thylakoids. These two organisms were selected, because the redox potential of \( Q_A/Q_A^- \) in PSII is significantly less negative in \( \text{C. merolae} \) \( (E_m = -104 \text{ mV}) \) than in spinach \( (E_m = -163 \text{ mV}) \). This significant difference in redox potential was expected to allow the disentanglement of acceptor and donor side effects on the miss parameter. Our data indicate that, at slightly acidic and neutral pH values, the \( E_m \) of \( Q_A^-/Q_A \) plays only a minor role for the miss parameter. By contrast, the increased energy gap for the backward electron transfer from \( Q_A^- \) to Pheo slows down the charge recombination reaction with the \( S_3 \) and \( S_2 \) states considerably. In addition, our data support the concept that the \( S_2 \rightarrow S_3 \) transition is the least efficient step during the oxidation of water to molecular oxygen in the Kok cycle of PSII.

Keywords Photosynthesis · Photosystem II · Mechanism of water oxidation · Flash-induced oxygen oscillation pattern (FIOP) · Unequal miss parameter · \( \text{Cyanidioschyzon merolae} \)

Introduction
Cyanobacteria, algae, and higher plants capture sunlight to store its energy in the chemical bonds of carbohydrates, proteins, and lipids. The electrons and protons used for atmospheric \( \text{CO}_2 \) fixation are extracted from water. The electrons are then energized to the reduction potential required for \( \text{CO}_2 \) reduction in two light-driven charge separation reactions performed in the reaction centers of photosystems II (PSII) and photosystem I (PSI), and stabilized by multiple electron transfer events to allow the slow chemistry of water oxidation and \( \text{CO}_2 \) fixation to occur. The side product of solar-driven water oxidation is molecular oxygen, which sustains all aerobic life on Earth (Govindjee et al. 2005; Renger 2008; Barber 2009; Shevela et al. 2012; Blankenship 2014; Cardona et al. 2015).
The reaction sequence in the PSII complex, which catalyzes water oxidation to dioxygen, can be divided into three main events that occur at different time scales. Photon capture in the light-harvesting antenna of PSII and excitation energy transfer to the reaction center lead to the formation of the excited state of the primary donor, P680\(^*\), which is a chlorophyll (Chl) dimer. P680\(^*\) transfers its excited electron within picoseconds to the primary acceptor pheophytin, Pheo (Fig. 1a) (Mamedov et al. 2015; Mirkovic et al. 2017; Romero et al. 2017). The subsequent electron transfer steps, which increase the spatial separation and decrease the potential difference between the photo-generated charge pair, minimize the chance for charge recombination reactions. These processes occur over a wide time span (10 ns–100 µs) and include on the acceptor side electron transfer from Pheo\(^{•−}\) to the firmly bound plastoquinone \(Q_A\), and further to the second, exchangeable plastoquinone, \(Q_B\). On the donor side, P680\(^{•+}\) is reduced by tyrosine Z. Y\(_Z\) (D1-Tyr161), which in turn oxidizes the oxygen-evolving complex (OEC) of PSII. Repetition of these steps allows for multi-electron and multi-proton chemistry to occur at the 100 µs–1 ms time scales. These reactions form plastohydroquinone on the acceptor side and molecular oxygen in the OEC on the donor side of PSII (Renger and Renger 2008; Blankenship 2014).

At the heart of the OEC is the Mn\(_4\)CaO\(_5\) cluster (Fig. 1b, center). This inorganic core unfolds its full catalytic potential within the special protein and water environment of its binding pocket. The structure of this complex catalytic site has been revealed by years of research employing EPR, EXAFS, and X-ray crystallography measurements, as well as theoretical calculations for recent contributions (e.g., Messinger et al. 1997; Peloquin et al. 2000; Zouni et al. 2001; Cinco et al. 2002; Robblee et al. 2002; Ferreira et al. 2004; Yano et al. 2006; Kulik et al. 2007; Dau and Haumann 2008; Siegbahn 2014; Romero et al. 2017; Suga et al. 2015; Young et al. 2016). This pocket positions a network of hydrogen-bonded water molecules around the inorganic unit (Umena et al. 2011), thereby orchestrating the proton-coupled electron transfer events required for the accumulation of four oxidizing equivalents and the subsequent water oxidation chemistry (Lavergne and Junge 1993; Rappaport and Lavergne 1997; Dau and Haumann 2008; Service et al. 2010; Rappaport et al. 2011; Nakamura et al. 2016; Nilsson 2016).

On the basis of the period four oscillation during flash-induced oxygen evolution, first measured by Joliot et al. (1969), and additional experiments, five oxidation states of the OEC were identified and named by Kok and coworkers as the \(S_0\), \(S_1\), \(S_2\), \(S_3\), and \(S_4\) states (Fig. 1b) (Kok et al. 1970; Joliot and Kok 1975). For different, earlier proposals, see Joliot et al. (1969) and Mar and Govindjee (1972). Proton release occurs on all transitions, except the \(S_1\)→\(S_2\) transition.

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**Fig. 1** The electron transfer cofactors of the PSII reaction center (left, a) and the Kok cycle of solar-driven water oxidation and the structure of the Mn\(_4\)CaO\(_5\) cluster (right, b). \(S_i\)-state-dependent miss parameters, proton release, and substrate water binding are indicated. Numbers on the arrows correspond to flash numbers driving the first cycle, which starts from the dark-stable \(S_1\) state. The oxidation states of Mn are indicated in the order Mn1, Mn2, Mn3, Mn4 of the Mn\(_4\)CaO\(_5\) cluster, which is depicted in the center of the Kok cycle (see Kok et al. 1970). \(\alpha_i\) signifies the miss parameter connected to the oxidation of the respective \(S_i\) state \((i=0–4)\). Color code: O red, Mn purple, Ca yellow. W1–W4 are terminal water ligands (W2 may be a hydroxo) of Mn4 and Ca. The structures shown are based on pdb 5TIS (Young et al. 2016).
(Rappaport and Lavergne 1991; Schlodder and Witt 1999). This is important, to allow $Y_{Z^\bullet}$, which has a near constant oxidation potential throughout the reaction cycle, to stepwise oxidize the Mn$_4$CaO$_5$ cluster. Thus, in the positively charged $S_{2}^{+}$ state, initially a proton needs to be released to allow oxidation of the neutral $S_{i}^{0}$ state to the $S_{i}^{+}$ state (Rappaport and Lavergne 1991; Klauss et al. 2012). This step is also likely coupled with the binding of one water molecule to the Mn$_4$CaO$_5$ cluster (Haumann et al. 2005; Suzuki et al. 2008; Siegbahn 2009; Suga et al. 2015, 2017; Capone et al. 2016; Retegan et al. 2016; Ugur et al. 2016; Kim and Debus 2017). Similarly, $S_{3}^{n}$ state needs to be formed by a proton release from $S_{3}^{+}$ before the OEC can be further oxidized and in turn form $O_2$ from the two substrate ‘water’ molecules (Rappaport et al. 1994; Rappaport and Lavergne 2001; Dau and Haumann 2007; Klauss et al. 2015).

The most stable oxidation state of the OEC is the $S_1$ state, in which the four Mn ions have the oxidation states III, IV, III, IV, III, in the order of numbering in Fig. 1b (Krewald et al. 2015). The $S_2$ (III, IV, IV, IV) and $S_3$ (IV, IV, IV, IV) states decay to the $S_1$ state within seconds to minutes via redox reduction by the reduced form of tyrosine $Y_D^{\text{RED}}$ (D2-Tyr160) or recombination with $Q_0^-$ (Diner 1977; Rutherford et al. 1982; Robinson and Crofts 1983; Rutherford and Inoue 1984; Vermaas et al. 1984, 1988; Nugent et al. 1987). In addition, a very slow decay was reported, but the electron donor for this phase was not yet identified (Styring and Rutherford 1987). By contrast, the $S_0$ state is slowly oxidized to the $S_1$ state by the long-lived $Y_D^{\text{OX}}$ radical, but is stable if $Y_D$ is reduced (Styring and Rutherford 1987; Vass and Styring 1991; Messinger and Renger 1993). The highly reactive $S_4$ state oxidizes two water molecules into molecular oxygen. Through this process, four electrons are injected into the OEC, effectively resetting the system for the next cycle into the $S_0$ state (III, IV, IV, III, III).

The damping of the period four oscillation in flash-induced oxygen evolution shows that the $S_i$ state transitions do not occur with 100% efficiency. This inefficiency is described in the Kok model mostly by the miss parameter, which gives the average probability that an $S_i$ state does not advance, upon saturating flash illumination, into the next higher $S_{i+1}$ state (Forbush et al. 1971; Delrieu 1974; Messinger and Renger 2008). Typical values for the $S_i$-state-independent miss parameter range between 10 and 15% (Messinger and Renger 1994; Isgandarova et al. 2003). Depending on the width of the exciting flash, typically also 0–5% of double advancements ($S_i$ to $S_{i+2}$ transitions) contribute to the $S_0$ state mixing with increasing flash number. Under saturating illumination, the miss parameter has been proposed to be governed by either the redox equilibria between the redox cofactors at the donor or acceptor sides, or by kinetic limitations (Renger and Hanssum 1988; Meunier and Popovic 1990; Shinkarev and Wraight 1993; Christen et al. 1999; de Wijn and van Gorkom 2002; Han et al. 2012). To minimize the possibility of kinetic limitations, we employed in this study a flash frequency of 2 Hz. In addition, we explicitly account for back reactions of the $S_i$ states by our global fit analysis (Vass et al. 1990; Isgandarova et al. 2003; Pham and Messinger 2016). Thus, the efficiency of the $S_i$ state turnovers is expected to be governed by redox equilibria between all the electron transfer cofactors of PSII. These equilibria prevent, in a random fashion, a certain percentage of centers (miss parameter $\alpha$) from advancing to the next higher oxidation state $S_i$ ($i = 0, 1, 2, 3, 4$) by preventing a stable charge separation. In this regard, especially the presence of $Q_A^-$, Pheo$^-$, P680$^-$, and/or $Y_{Z^\bullet}$ at the time of flash excitation is of note, which may vary with a period two on the acceptor side and a period four on the donor side (Naber et al. 1993; Shinkarev and Wraight 1993). In addition, inefficiencies of the $S_i$ state transitions caused by deprotonation events (Forbush et al. 1971) or structural equilibria, as that in the $S_3$ state between the EPR multiline and the $g = 4.1$ state (Retegan et al. 2016), may lead to a $S_i$ state dependence of the miss parameter. Recent work by several groups provided experimental support for the $S_i$ state dependence of the miss parameter. While fluorescence experiments by de Wijn and van Gorkom (2002) and FTIR experiments by Takumi Noguchi’s group (Suzuki et al. 2012) suggest that the miss parameters $\alpha_i$ increase from $\alpha_0$ to $\alpha_3$, Stenbjörn Styring and coworkers concluded on the basis of EPR experiments that the highest miss factor is associated with the $S_2 \rightarrow S_3$ transition (Han et al. 2012). We have recently developed a new kinetic Kok model that allows fitting a regular 2 Hz flash-induced oxygen evolution pattern (FIOP) simultaneously with 44 FIOPs obtained during the $S_2^\bullet$, $S_3^\bullet$, and $S_0^\bullet$-state lifetime measurements (Isgandarova et al. 2003; Pham and Messinger 2016). This global analysis, which works akin to decay-associated spectral analysis, indicated that the majority of misses occurs in spinach thylakoids at neutral pH in either the $S_2 \rightarrow S_3$ or the $S_3 \rightarrow S_0$ transition. In addition to misses, a small percentage of double hits ($\beta$; $S_i \rightarrow S_{i+2}$) is observed if light pulses of ms duration are employed.

The extremophilic red alga *Cyanidioschyzon merolae* grows in hot springs at a very low pH (0.2–4.0) and moderately high temperatures (40–56 °C) (Ciniglia et al. 2004; Ferris et al. 2005). The intracellular pH of *C. merolae* is tightly controlled metabolically and maintained in the pH range of 6.3–7.1 over the extracellular pH range of 1.5–7.5 (Zenvirth et al. 1985; Enami et al. 2010). The photosynthetic apparatus of this alga is an evolutionary intermediate of cyanobacteria and higher eukaryotic phototrophs, containing combined prokaryotic and eukaryotic structural and functional features. Both PSII and PSI complexes from this acido-thermophilic alga are characterized by unprecedented robustness across a wide range of external conditions and recently molecular mechanisms underlying such resilience.
to adverse conditions have been revealed including reaction center-based non-photochemical quenching in PSII, accumulation of photoprotective carotenoid zeaxanthin (in PSII and PSI), and remodeling of the external light-harvesting antenna in PSI (Krupnik et al. 2013; Haniewicz et al. 2018).

The PSII complex in C. merolae is reminiscent of its counterpart in cyanobacteria, but the OEC is protected by the additional fourth extrinsic subunit PsbQ’, over and above the three proteins present in cyanobacteria, PsbV, PsbU, and PsbO (Ohta et al. 2003). The PsbQ’ subunit has been localized by electron microscopy coupled to single particle analysis (Krupnik et al. 2013) as well as recently by X-ray crystallography (Ago et al. 2016) on the lumenal side of PSII in the vicinity of the CP43 and PsbV proteins, close to the membrane plane. The function of PsbQ’ has been recently elucidated by reconstitution of this protein with the cyanobacterial PSII followed by measurement of the $Q_A$ redox potential in thus modified complex. In these experiments, the redox potential of $Q_A$ was shown to be positively shifted when PsbQ’ was attached to the PSII complex, likely resulting in a decrease in the amount of destructive triplet Chl in the primary donor special pair (being Chl $a$ in Synechocystis and Chl $a$ in Acaryochloris) pointing towards high conservation of the electron transfer processes in oxygenic photosynthesis.

On the acceptor side, the potential of $Q_A^{-}/Q_A$ is significantly less negative in C. merolae ($E_m = -104$ mV) than in spinach ($E_m = -163$ mV) (Shibamoto et al. 2010). Given that the midpoint potentials of the other redox cofactors, including $Q_B^{-}/Q_B$, $Q_mBH_2/Q_mB^-$ and Pheo$^-$/Pheo are similar in these organisms (no comparable data available in the literature), the less negative $E_m$ of $Q_A^{-}/Q_A$ is expected to increase the $Q_A^-$ population and thereby the miss parameter in comparison to spinach PSII centers. In addition, the larger energy gap between Pheo$^-$/Pheo and $Q_A^{-}/Q_A$ is expected to increase the lifetime of the $S_0$ and $S_1$ states in C. merolae PSII centers (Fig. 2). Interestingly, Allakhverdiev and coworkers reported a shift of redox potentials of $Q_A$ and Pheo depending on the presence or absence of extrinsic proteins in the cyanobacterial PSII complexes of Synechocystis sp. and Acaryochloris marina (Allakhverdiev et al. 2010, 2011). Yet, the energetics of the water-splitting reaction in PSII RC was conserved, even though the potentials of $Q_A^-$ and Pheo$^-$ were relatively shifted depending on the identity of the primary donor special pair (being Chl $a$ in Synechocystis and Chl $d$ in Acaryochloris), pointing towards high conservation of the electron transfer processes in oxygenic photosynthesis.

To analyze the effects of the altered $Q_A^{-}/Q_A$ midpoint potential in detail and to separate it from $S_i$-state-dependent effects, we measured flash-induced oxygen yield patterns of C. merolae thylakoids at three different pH values and after H/D exchange, and analyzed the data using our global fit analysis approach. High pH is expected to simplify deprotonation reactions needed to advance the Mn$_4$CaO$_5$ cluster into the next $S_i$ state and to thereby reduce the donor side misses. It may also be expected that high pH makes it more difficult to protonate $Q_B^2^-/Q_B^-$. This, in turn, may increase their midpoint potentials, likely leading to increased acceptor side misses. The results obtained in the present study are compared to literature data for spinach thylakoids measured under similar conditions.

**Materials and methods**

**Thylakoid preparation**

*Cyanidioschyzon merolae* cells were cultured at 42 °C and the thylakoids were isolated as described by (Krupnik et al. 2013), frozen in liquid nitrogen and stored at −80 °C until used. On the day of the FIOP experiments, the thylakoids were thawed in the dark on ice and washed three times with buffer containing 10 mM CaCl$_2$, 5 mM MgCl$_2$, 1 M betaine, and either 40 mM succinic acid/NaOH (pH 5.0), 40 mM MES/NaOH (pH 6.1), or 40 mM HEPES/NaOH (pH 8.0). In the case of D$_2$O experiments, the buffer was prepared in 99.9% D$_2$O at 40 mM MES/NaOD (pD 6.1). In the last step, the samples were diluted to [Chl] = 0.5 mg/ml. After this treatment, which required ~10 min, the relative flash-induced O$_2$ yields were 25% (pH 5.0), 100% (pH 6.1), and 45% (pH 8.0). No further decline in the activity was observed during the experiments. The *C. merolae* thylakoid...
sample had a small population of $Y_D^{RED}$ ($\approx 10\%$). Therefore, the dark-adapted thylakoid sample was used without preflash treatment.

**FIOPs and $S_i$-state lifetime measurements**

The FIOP measurements on *C. merolae* thylakoids were performed with an unmodulated home-built Joliot-type electrode at 20 °C without adding artificial electron acceptors (Joliot 1972; Messinger and Renger 1993). Stock solutions of catalase from bovine liver (3809 U/mg, Sigma-Aldrich) were prepared by dissolving the frozen catalase powder in the respective measuring buffer. The catalase concentrations were determined using the Bradford assay (Bradford 1976; Sedmak and Grossberg 1977) and the enzymatic activities were measured using Beers assay (Beers and Sizer 1952; Aebi 1984). Then, 10,000 U/ml catalase was added into the thylakoid samples and the mixture was incubated for 10 min. In addition, the measuring buffer in the reservoir of the Joliot-electrode was continuously bubbled with N₂ to remove O₂ and prevent electrochemical formation of H₂O₂ during measurements (Pham and Messinger 2014).

For each FIOP, a fresh 10-µl aliquot of the thylakoid sample was transferred to the surface of a bare Pt-cathode in dim green light and the sample was incubated for 3 min on the electrode to allow for settling and temperature adaptation. The polarization voltage of $-750$ mV was switched on 40 s before illuminating the sample with a series of 16 flashes (2 Hz; Perkin Elmer, LS-1130-4 flash lamp). A personal computer was employed to trigger the flash lamp and to record the data at a sampling rate of 3600 points/s. The $S_2$-, $S_3$-, and $S_0$-state lifetimes of thylakoid samples were measured in the same way by exciting dark-adapted samples with one ($S_2$ state formation), two ($S_3$ state formation), or three ($S_0$ state formation) pre-flash(es) while resting on the electrode surface. After the desired dark time ($t_d$), a series of 16 flashes (2 Hz) was applied. The polarization was always applied 40 s before the flash series.

**Data analysis of FIOPs**

For each FIOP, 16 oxygen yields were recorded. However, only the first 8 flash-induced oxygen yields were used for data analysis since thereafter, a strong decline of the O₂ yield was observed (Fig. 3). In the global fitting approach, 384 flash-induced oxygen yields comprising all time points of the $S_2$, $S_3$, and $S_0$ lifetime measurements were directly and simultaneously fitted by adjusting the rate constants of all $S_i$ state decays, the $S_i$-state-dependent miss parameters, the double-hit probability, the activity/damping parameter, and the initial percentages of $S_i$ states, $Y_D$ and $Q_B^{-}$ (Pham and Messinger 2016).

**Results and discussion**

The flash-induced oxygen evolution patterns (FIOPs) and the $S_i$-state lifetime measurements of *C. merolae* thylakoids were recorded at pH 5.0, 6.1, and 8.0 with a Joliot-type bare platinum electrode (Isgandarova et al. 2003). The results are presented in Figs. 3 and 4, as well as in Tables 1 and 2.

**pH dependence of the miss parameters**

Since the thylakoid preparation of *C. merolae* contained only about 10% of reduced $Y_P$, the FIOPs shown in Fig. 3 can be discussed qualitatively without considering the back reactions in the dark periods of 500 ms between the flashes. At pH 6.1, a clear period four oscillation with maxima after the 3rd, 7th, and 11th flash was seen, indicating a low average miss parameter (red spheres in Fig. 3). The overall amplitude of the 3rd oscillation (flashes 11 and onwards) was, however, untypically low. This FIOP was reminiscent to that of spinach BBY preparations, indicating a limited pool of plastoquinone bound on the acceptor side of *C. merolae* PSII complex embedded in thylakoid membrane fragments.

After transferring the thylakoid samples to pH 5, a FIOP with a clearly increased miss parameter was obtained (black squares in Fig. 3). This was visible by the decreased ratio of the O₂ yields induced by the 3rd flash ($Y_3$) to that of the 4th flash ($Y_4$). Similarly, the ratios $Y_7/Y_8$ and $Y_{11}/Y_{12}$ were diminished compared to those obtained at pH 6.1. Interestingly, the average O₂ yield per flash obtained at higher flash numbers did not decrease to the same extent as at pH 6.1. This can be explained by the finding that the total O₂ yield was only about 25% of that at neutral pH. The reduced number of active PSII complexes thus increased the number of plastoquinone molecules available for the active PSII
complexes. The strong decline in the number of active PSII centers in thylakoids of *C. merolae* was surprising, since a recent report showed that the O₂ rates of PSII core complexes isolated from the same organism are nearly invariant between pH 5.0 and 8.0 (Krupnik et al. 2013).

Comparison of the FIOP obtained at pH 8.0 (blue triangles in Fig. 3) and pH 5.0 showed that while the first 6 normalized O₂ yields were nearly identical, clear deviations were seen thereafter. Specifically, the Y₇/Y₈ ratio was inverted. This was indicative of a significantly different flash number or Sᵢ state dependence of the miss parameters at these two pH values. The total O₂ yield was about 45% at pH 8.0 as compared to pH 6.1. This again resulted in a larger plastoquinone pool per active PSII complex.

The global fit program (GFP) analysis confirms these qualitative observations. Table 1 compares the best-fit parameters obtained at each pH by either enforcing equal misses or by allowing the miss parameter to be different for each Sᵢ state transition. At pH 6.1, an equal miss fit results in a miss parameter α of about 11%, while in the best Sᵢ-state-dependent miss fit, all misses (35%) occur in the S₂→S₃ transition. Importantly, the fit quality of the unequal miss fit is twice as good as that of the equal miss fit, strongly favoring the fit where α₂ parameter is largest. This is further supported by the finding that this fit result is systematically found once the equal miss constraint is released. However, when fits were started with a high value for α₁ or α₃ (all other αᵢ = 0), stable solutions were obtained with 35% miss in the respective Sᵢ state transition. The fit qualities (FQ) of these fits were in between the best fit and the equal miss fit (Table 2). Nevertheless, as soon as a few percent α₂ was included into the starting parameters.
conditions, the GFP finds the solution where $\alpha_2 = 35\%$. Forcing most misses into $\alpha_0$ resulted in unsatisfactory fits.

The finding that only one S state transition is responsible for all misses, whereas the other transitions occur at 100% efficiency, is counterintuitive and may be explained by shallow fit minima. Increasing manually the percentages of misses in other transitions demonstrates that the fit quality deteriorates by less than 15% if either $\alpha_1$ or $\alpha_3$ is increased up to 5% at the expense of $\alpha_2$. In contrast, redistributing 10% miss from $\alpha_2$ to $\alpha_1$ or $\alpha_3$, individually or combined, or increasing $\alpha_0$ by only 3% leads to a 40% worsening of the fit quality.

The fits for the pH 5.0 FIOP show the identical trends, except that the misses were higher: 18% for equal miss fits and 54% for the $S_2 \rightarrow S_3$ transition (Table 1). At this pH value, the unequal miss fit was nearly three times better compared to the equal miss fit. The fit where $\alpha_3$ is dominating has a nearly as good $F_Q$ as the one with $\alpha_2 = 54\%$; however, the value of $k_{21\text{,(fast)}}$ equals $k_{21\text{(slow)}}$, i.e., it was hitting the lower fit limit (Tables 1, 2). The fit where $\alpha_1$

![Table 1 Fit parameters obtained during a global fit of a 2 Hz FIOP and $S_3$, $S_2$, and $S_0$ lifetime measurements of C. merolae thylakoids at 20 °C and the indicated pH values](image-url)

| pH  | Equal miss | $S_i$-state-dependent miss (best fit) |
|-----|------------|---------------------------------------|
|     | $\alpha_0$, % | $\alpha_1$, % | $\alpha_2$, % | $\alpha_3$, % | $\beta$, % | $d$, % | $k_{32\text{,(fast)}}, s^{-1}$ | $k_{32\text{(slow)}}, s^{-1}$ | $k_{21\text{,(fast)}}, s^{-1}$ | $k_{21\text{(slow)}}, s^{-1}$ | $k_{01}, s^{-1}$ | Fit quality ($\times 10^{-8}$) |
| 5.0 | 18 | 11 | 22 | 0 | 0 | 40 |
| 6.1 | 18 | 11 | 22 | 0 | 0 | 0 |
| 8.0 | 18 | 11 | 22 | 54 | 0 | 0 |
| 48 |
| 35 |
| 6 |
| 1.6 | 3.8 | 3.8 | 1.6 | 2.5 | 2.7 |
| 98 | 96 | 96 | 97 | 95 | 98 |
| 2.0 | 1.5 | 0.028 | 3.2 | 0.32 | 4.4 |
| 0.0172 | 0.0030 | 0.0012 | 0.0165 | 0.0029 | 0.0012 |
| 1.4 | 2.1 | 0.07 | 0.8 | 1.4 | 2.4 |
| 0.0030 | 0.0015 | 0.0014 | 0.0022 | 0.0012 | 0.0012 |
| 2.3 | 0.0004 | 0.0007 | 0.00097 | 0.00015 | 0.0015 |
| 1625 | 970 | 600 | 615 | 495 | 362 |

Fits assuming either $S_i$-state-independent (equal) or $S_i$-state-dependent (unequal) miss probabilities are presented. Parameters: $\alpha_i$, miss parameter connected to the oxidation of the $S_i$ state ($i=0$–4); $\beta$ double-hit parameter; $d$, damping/activity parameter; $k_{32}$, $k_{21}$, and $k_{01}$, rate constants of $S_3$, $S_2$, and $S_0$ decay to the $S_1$ state. All fits are based on the following properties of the dark-adapted sample: 100% $S_1$ state population, $Y_D = 10\%$. A smaller value for the fit quality indicates a better fit. For further details see (Pham and Messinger 2016)

* Rates for the fast $S_2$ and $S_3$ decay, as well as for $S_0$ oxidation are less reliable due to the small $Y_D$ population giving insufficient constraints for fitting the fast $S_2$ and $S_3$ decays; they are thus displayed in italics

![Table 2 Alternative fit parameters obtained during a global fit of a 2 Hz FIOP and $S_3$, $S_2$, and $S_0$ lifetime measurements of C. merolae thylakoids at 20 °C and indicated pH values](image-url)

| pH  | Equal miss | $S_i$-state-dependent miss (best fit) |
|-----|------------|---------------------------------------|
|     | $\alpha_0$, % | $\alpha_1$, % | $\alpha_2$, % | $\alpha_3$, % | $\beta$, % | $d$, % | $k_{32\text{,(fast)}}, s^{-1}$ | $k_{32\text{(slow)}}, s^{-1}$ | $k_{21\text{,(fast)}}, s^{-1}$ | $k_{21\text{(slow)}}, s^{-1}$ | $k_{01}, s^{-1}$ | Fit quality ($\times 10^{-8}$) |
| 5.0 | 0 | 0 | 0 | 0 | 0 | 0 | 43 |
| 6.1 | 0 | 54 | 0 | 35 | 0 | 0 | 0 |
| 8.0 | 0 | 0 | 0 | 57 | 47 | 0 |
| 0 |
| 54 |
| 1.6 | 1.6 | 2.9 | 2.9 | 1.4 | 2.1 | 3.1 |
| 97 | 98 | 95 | 95 | 95 | 95 | 98 |
| 2.5 | 1.8 | 0.15 | 0.33 | 0.017 | 0.023 | 6.9 |
| 0.016 | 0.011 | 0.0029 | 0.0027 | 0.0012 | 0.0011 | 0.0012 |
| 1.2 | 1.5 | 2.3 | 2.3 | 2.3 | 2.3 | 10* |
| 0.0024 | 0.0037 | 0.0013 | 0.0016 | 0.0011 | 0.0011 | 0.0015 |
| 2.0 | 2.0 | 0.00020 | 0.00016 | 0.00009 | 0.00090 | 0.01200 |
| 675 | 1330 | 536 | 645 | 890 | 600 | 610 |

The asterisk indicates that a parameter was constrained

* Rates for the fast $S_2$ and $S_3$ decay, as well as for $S_0$ oxidation are less reliable due to the small $Y_D$ population giving insufficient constraints for fitting the fast $S_2$ and $S_3$ decays; they are thus displayed in italics
is forced to be maximum, results in a fit that is nearly as poor as the equal miss fit. The large difference between the equal and \(S_i\)-state-dependent miss approaches comes from the—relative to \(Y_i/Y_{i+1}\)—large \(Y_i/Y_{i+1}\) ratio, which can only be modeled correctly with unequal misses, where \(\alpha_2\) or \(\alpha_3\) dominates.

In contrast to pH 5.0 and 6.1, the FIOPs obtained at pH 8.0 could not be fitted well when we assumed that all the misses occur in the \(S_2\rightarrow S_3\) transition or the \(S_3\rightarrow S_0\) transition (Tables 1, 2). For pH 8.0, an almost equally large percentage of misses occurred in the \(S_0\rightarrow S_1\) transition. Several good fits were also found where in addition to \(\alpha_0\) and \(\alpha_2\) also 5–20\% \(\alpha_1\) or \(\alpha_3\) were included.

Therefore, the quantitative GFP analysis of the \(C.\) merolae FIOPs obtained at three different pH values strongly supports unequal misses, whereby the majority of the misses occur in the \(S_2\rightarrow S_3\) or, less likely, in the \(S_3\rightarrow S_0\) transition at acidic and neutral pH, while at alkaline pH the \(S_0\rightarrow S_1\) transition becomes also inefficient. If proton release was the limiting factor for the efficiency of a \(S_i\) state transition, one would expect the miss probability to decrease with increasing pH. While this trend was observed for \(\alpha_2\) when comparing pH 5.0 and 6.1, this trend was not detected at pH 8.0.

Importantly, no binary oscillation of the miss parameter with the flash number (\(S_i\) state) was found when analyzing the pH 5.0 and 6.1 data. This supports the above analysis, which relied on the assumption that the contributions of the donor side dominate the miss parameter, while effects of the acceptor on the miss parameter are negligible.

In contrast, a pronounced binary oscillation of the miss parameter was observed at pH 8.0. This may be explained either by a high \(\alpha_0\) and \(\alpha_2\) or by high miss parameter connected to the presence of \((Q_A^-Q_B^-)\) on the acceptor side (Naber et al. 1993; Shinkarev and Wraight 1993). The pH jump experiments by Zaharieva et al. (2011) with spinach PSII membrane fragments, found effective pK values of 3.3, 3.5, and 4.6 for the inhibition of the \(S_1\rightarrow S_2\), \(S_2\rightarrow S_3\), and \(S_3\rightarrow S_0\) transitions at low pH, respectively. In contrast, none of the transitions showed decreased efficiencies at alkaline pH values. Furthermore, the \(S_0\rightarrow S_1\) transition was found to be pH independent between pH 3 and 9. On that basis, one would expect that the \(S_2\rightarrow S_3\) transition should be the only one affected by the pH under our experimental conditions. However, while the fits with large \(\alpha_3\) generally gave acceptable results, they were consistently inferior to those with large \(\alpha_2\). We thus suggest that the different time scales of incubation at the indicated pH, 1.5 s. vs > 10 min., are responsible for the deviating results. We also note that Zaharieva et al. (2011) observed relative changes in the efficiency of a specific \(S_i\) state transition caused by pH changes, not in the absolute \(\alpha_i\) values, and that pH effects on the acceptor side are minimized in that study by giving only one flash at the desired pH value.

Our preferred assignment for the acidic to neutral pH (\(\alpha_2 >> \alpha_3, \alpha_1, \alpha_0\)) agrees well with the results of Han et al. (2012) who reported a similar trend by following the \(S_i\) state populations during a flash train via \(S_i\)-state-specific EPR signals. A high \(\alpha_2\) may be consistent with the proposed need of PSII samples in the \(S_2\) multiline (‘open cube’) configuration to first convert into the \(S_2\rightarrow S_1\) state (‘closed cube’) configuration, before they can reach the \(S_3\) state (Retegan et al. 2016). Other possible explanations for why the highest miss parameter is connected to the \(S_2\rightarrow S_1\) transition may include the need to bind one additional substrate (possibly in the form of hydroxide) and the small driving force for this transition (Suzuki et al. 2008; Cox et al. 2014; Kim and Debus 2017).

The increase of the miss probability of the \(S_0\rightarrow S_1\) and \(S_2\rightarrow S_3\) transitions in the alkaline pH cannot be easily explained by any of the above proposals, unless a second independent counter effect is brought into play. Since no artificial electron acceptors were used in this study, the period of two oscillations of the miss parameter may be taken as indication that the protonation of \(Q_B^-\) became a limiting factor between pH 7 and 8, or that the \(Q_A^-\) or \(Q_B^-\) midpoint potentials get more similar under these conditions, leading to a high \(Q_A^-\) population on every second flash. Further studies will be necessary to untangle the \(C.\) merolae PSII donor and acceptor side contributions at alkaline pH.

**pH dependence of \(S_i\)-state lifetimes**

For \(S_i\)-state lifetime experiments, the dark-adapted thylakoid sample (\(S_1\) state) was excited by 1, 2, or 3 flashes to enrich PSII in the \(S_2\), \(S_3\), or \(S_0\) state, respectively. Then, the dark time, \(t_0\), to the subsequent FIOP flash train (2 Hz) was varied in suitable steps to probe the fast and slow decay components (see insets in Fig. 4). The symbols in Fig. 4 show how the experimental flash-induced \(O_2\) yields varied as a function of \(t_0\) during the three lifetime measurements. Plotted are either the \(O_2\)-yields induced by flashes two to five (\(Y_2\)–\(Y_5\); \(S_2\) state decay) or \(Y_1\)–\(Y_4\) (\(S_1\) and \(S_0\) state decay). The \(S_2\) population can be qualitatively followed by the decay of \(Y_2\) (red dots), while \(Y_1\) tracks the \(S_1\) state population (black squares). The changes in the \(S_1\) and \(S_0\) populations can be estimated by amplitude changes of \(Y_3\) (green triangles) and \(Y_4\) (blue inverted triangles), respectively.

In the top row of Fig. 4, the pH dependence of the reaction \(S_2\rightarrow S_1\) was studied. The simultaneous decay of \(Y_2\) and the corresponding rise of \(Y_4\) demonstrated for all three pH values a clean one-electron reduction process. Due to misses and double hits, a small rise of \(Y_4\) and \(Y_5\) was also observed. Only a very small fast phase was discernible, which was followed by one slower kinetic phase. While there were not enough points for the fast phase to reliably analyze its pH dependence, it could be observed that the slow phase of \(S_2\)
decay was about 2–3-fold faster at pH 5.0 than at the two other pH values.

The pH dependence of the S₃ decay is best followed by the data presented in the central row. Due to the rapid S₃ decay at pH 5.0, the sequential decay of S₃ (Y₃) → S₂ (Y₂) → S₁ (Y₁) could be clearly discerned. Y₃ increased nearly in parallel to Y₂ due to misses. For the S₂ decay, a strong retardation of the slow decay was observed at the higher pH values. For the S₀ → S₁ reaction, a qualitative analysis was complicated, as the expected decrease of Y₄ was compensated by the contribution of O₂ to Y₄, which comes from centers in the S₁ state that due to α₁ are delayed by one flash in O₂ production. This time-dependent increase in the S₁ state population is caused by S₀ oxidation and by back reactions from the S₃ and S₂ states. In addition, the increasing Y₅ population further increases Y₄ by fast reduction of fractions of S₃ and S₂ between the flashes of the FIOP. Nevertheless, it appears that the S₀ oxidation exhibits a complicated pH dependence in the C. merolae PSII complex, with the slowest rate near neutral pH. To our knowledge, no previous data for the S₀ → S₁ reaction had been published at pH 5 or pH 8.

The lines in Fig. 4 connect the amplitudes calculated by the GFP using the parameters given in Table 1 for the best fits. Overall, despite some small deviations, a satisfactory agreement with the data were achieved. The fits confirm the trends discussed above for the slow S₂ and S₃ state decays at the three pH values studied. While the S₂ state decay is nearly pH independent (within a factor of 2), the rate of S₃ decay slowed by more than a factor of 10 between pH 5 and 8. This is consistent with the previous data obtained from spinach thylakoids (Messinger and Renger 1994).

The most surprising finding is the very strong pH dependence of the rates for the S₀ → S₁ transition. While the rate at pH 6.1 was well reproduced under all tested fit conditions and agreed within a factor of four with previous estimates in spinach thylakoids (Messinger and Renger 1994) and was practically identical with that determined with T. elongatus thylakoids (Messinger and Renger 1994; Isgandarova et al. 2003), the much faster oxidation rates at pH 5 and 8 were surprising. Since in the present experiments we were unable to obtain strong constraints for the fast decay kinetics, the values for k₀₀₁ varied at the two extreme pH values quite considerably depending on the fit scenario. At pH 5.0, values between 0.21 and 2.7 s⁻¹ were obtained, while k₀₀₁ was found to be in the range of 0.00007 and 0.035 s⁻¹ at pH 8.0. Thus, further studies are needed to determine the precise extent of the destabilization of S₀ at pH 5.0 and 8.0. Possible reasons for the faster S₀ oxidation could be related to the described two different positions of a water molecule near Y₁ that appear to influence the redox potential of Y₁D/Y₁OX (Umema et al. 2011; Saito et al. 2013; Sjöholm et al. 2017), and other pathways for S₀ to S₁ conversion may be possible under extreme pH conditions. At alkaline pH, also the easier removal of a proton may increase the rate of its conversion, since the S₀ → S₁ transition is coupled to a proton release (Rappaport and Lavergne 1991; Siegbahn 2013; Klauss et al. 2015).

### Effects of H/D exchange

To further understand the above pH effects, we performed a complete GFP analysis after H/D exchange at pD 6.1 (Table 3; Fig. 5). The data in Table 3 reveal that nearly all the fit parameters are identical under H₂O and D₂O. However, there is a twofold increase in the rate of S₀OX⁻ decay [k₃₂(slow)] and a sixfold increase of the rate in S₀ oxidation to S₁. In addition, a very small increase of α₂ from 35 to 37% was observed. The slightly higher value of α₂ in D₂O is in line with the idea that a proton release during the S₂ → S₃ transition is required, since a slowdown of that rate due to the greater O–D bond strength will increase the miss parameter by increasing recombination reactions between Qₓ⁻ and Y₁OX (Zaharieva et al. 2011). However, the small magnitude of this effect indicates that this can only be one of the several factors contributing to the low efficiency of this transition.

Similarly, the acceleration of the slow S₁ decay in D₂O and the much shorter S₃-state lifetimes at low pH (high proton concentration) are consistent with the need to take up a proton when returning to the S₂ state. In line with this idea, no clear H/D effect and a much weaker pH effect were observed on the rate of S₂ decay, which is not coupled to a proton uptake. This suggests that the pH has a strong effect on the redox potential of the S₁ state, while the effect on Qₓ⁻ is less important for the S₂ state lifetimes. The sixfold acceleration of the S₀ oxidation to S₁ by H/D exchange...
supports, indirectly, the surprising finding that low pH may induce the same effect (but even much stronger). The $S_0 \rightarrow S_1 Y_{\text{D}}$ reaction involves the following two half reactions:

$$S_0 \rightarrow S_1^+ + e^- \rightarrow S_1 + H^+ + e^-$$

$$Y_{\text{D}}^{\text{ox}} + H^+ + e^- \rightarrow Y_{\text{D}}.$$ 

While the formation of the $S_1$ state should be faster at high pH, the reduction of $Y_{\text{D}}^{\text{ox}}$ may occur faster at low pH. Indeed, a strong H/D effect was observed for the reactions $S_2 Y_{\text{D}} \rightarrow S_1 Y_{\text{D}}^{\text{ox}}$ and $S_3 Y_{\text{D}} \rightarrow S_2 Y_{\text{D}}^{\text{ox}}$ and was linked to the position of a water molecule near $Y_{\text{D}}$ (Isgandarova 2004; Sjöholm et al. 2017). Zaharieva et al. (2011) observed a rapid inactivation of centers in the $S_0$ state at low pH that occurs with a $pK_a$ of 4.6. We thus checked our data if a
specific slower phase of $S_0$ state inhibition could affect the $S_i$ state quantitation; no indications for that was found (data not shown).

**Comparison to spinach**

Overall, the miss parameters and $S_i$-state lifetimes found here for the PSII complex of the extremophilic red alga *C. merolae* were similar to those obtained earlier for spinach (Messinger and Renger 1994). This indicates that *C. merolae* PSII is not specifically adapted to acidic pH values, in agreement with earlier reports that the internal pH of *C. merolae* cells is regulated to values similar to those of higher plant chloroplasts. Consequently, the significantly different $Q_A^-/Q_A$ midpoint potentials are not an adaptation to pH, but more likely to growth temperature. This is supported by the nearly identical rates for the slow $S_2$ and $S_3$ state decays in *C. merolae* and spinach if measured at 20 and 10 °C, respectively (Table 4), which demonstrates the expected stabilization of the S states by the increased energy gap between Pheo$^-$/Pheo and $Q_A^-/Q_A$. In contrast, the average miss parameter is nearly identical between these two organisms at pH 5.0 and 6.1 if measured at the same temperature (20 °C; Table 5), supporting the idea that it is determined by the unaltered donor side reactions.

**Conclusions**

Global fits of the $S_3$, $S_2$, and $S_1$-state lifetime measurements provide a powerful tool for studying the efficiency of PSII complexes under various conditions, in different species or mutants. By comparing the data obtained here with *C. merolae* thylakoids to our previous spinach data, we have demonstrated that the redox potential of $Q_A^-/Q_A$ plays only a minor role for the miss parameter, but instead mostly affects the $S_i$-state lifetimes. This is likely due to the increased energy gap between the $Q_A^-/Q_A$ and Pheo$^-$/Pheo redox pairs, which increases the barrier for the acceptor side electrons for recombination with the holes on the donor side, which occurs via the pheophytin cofactor (see Fig. 2). In addition, our data support the notion that the $S_2 \rightarrow S_3$ transition is, at neutral pH, the least efficient step during the oxidation of water to molecular oxygen in the water-splitting enzyme, possibly due to the significant conformational changes occurring during the $S_2 \rightarrow S_3$ transition, which include the binding of one water molecule (Suzuki et al. 2008; Siegbahn 2009; Capone et al. 2016; Retegan et al. 2016; Ugur et al. 2016; Kim and Debus 2017; Suga et al. 2017).

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