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EPR kinetic studies of the S_{-1} state in spinach thylakoids

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Abstract

The $Y_Z^{\bullet}$ decay kinetics in a formal S_{-1} state, regarded as a reduced state of the oxygen evolving complex, was determined using time-resolved EPR spectroscopy. This S_{-1} state was generated by biochemical treatment of thylakoid membranes with hydrazine. The steady-state oxygen evolution of the sample was used to optimize the biochemical procedure for performing EPR experiments. A high yield of the S_{-1} state was generated as judged by the two-flash delay in the first maximum of oxygen evolution in Joliot flash-type experiments. We have shown that the $Y_Z^{\bullet}$ re-reduction rate by the S_{-1} state is much slower than that of any other S-state transition in hydrazine-treated samples. This slow reduction rate in the S_{-1} to S_0 transition, which is in the order of the S_3 to S_0 transition rate, suggests that this transition is accompanied by some structural rearrangements. Possible explanations of this unique, slow reduction rate in the S_{-1} to S_0 transition are considered, in light of earlier observations by others on hydrazine/hydroxylamine reduced PS II samples.

Keywords: Photosystem II; Oxygen evolving complex; Tyrosine Z reduction kinetics; Super-reduced state; Hydrazine

Photosystem II (PSII) catalyzes the oxidation of water to molecular oxygen in cyanobacteria and plants. The oxidation takes place in the catalytic site of the oxygen evolving complex (OEC) which is believed to be a tetranuclear manganese cluster (Mn cluster) with its associated inorganic and proteinaceous co-factors. The release of O_2 is a four photo-driven electron process, demonstrated by the periodicity of four observed in the O_2 release pattern by saturating single flashes [1,2]. To describe the O_2 production mechanism, a charge-accumulating intermediate with five oxidation states for the OEC was proposed by Kok et al. The oxidation states are called S_i states, where i denotes the number of oxidizing equivalents stored in the active site under normal cycling. The oxidizing equivalents are created by the photo-oxidation the primary donor (P_{680}) and transferred to the Mn cluster via a redox active tyrosine known as Y_Z [3,4]. The strongly oxidizing P_{680}^{+} oxidizes Y_Z to neutral tyrosine radical ($Y_Z^{\bullet}$). $Y_Z^{\bullet}$ is then reduced by the OEC, which advances from S_i to S_{i+1}. There is another spectroscopically similar tyrosine (Y_D) on polypeptide D2 which can be photooxidized but is not a part of the main electron transfer pathway in PSII [5].

The lower S state transitions are generally agreed to correspond to Mn oxidations [6–8]. The S_0 to S_1 transition corresponds to oxidation of a Mn^{II} to Mn^{III} while it is still unclear whether Mn oxidation occurs in the S_2 to S_3 transition (see [9]). EPR spectroscopy has been applied to trace the Mn valences in the S-cycle. The S_2 state, which is the most studied state by EPR gives rise to a multiline signal with about 20 hyperfine lines at liquid helium temperatures [10]. The S_2 multiline signal is attributed to a ground state with spin $S = 1/2$ of the Mn cluster. This Kramers system corresponds either to a $Mn^{IV}(Mn^{III})_3$ or $Mn^{III}(Mn^{IV})_3$ formal oxidation state of the Mn cluster. It has been recently shown

Abbreviations: Chl, chlorophyll; EPR, electron paramagnetic resonance; HEPES, *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid; OEC, oxygen evolving complex; PPBQ, phenyl-*p*-benzoquinone; PSII, photosystem II; MSP, Manganese stabilizing protein; Y_Z, redox-active tyrosine of D1 polypeptide; Y_D, redox-active tyrosine of D2 polypeptide

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that a multiline signal can also be detected from the S_0 state, which is also expected to be paramagnetic with a half-integer spin based on these Mn oxidation state assignments [11,12].

To understand the mechanism of water oxidation, it becomes crucial to define the Mn valences of S intermediate state. One approach has been to study the 'functional limits' of formal reduction of the redox accumulator within the OEC. It is known that 'super-reduced' states of the water splitting enzyme can be created by the incubation of samples with exogenous reductants, such as hydrazine [13,14], hydroxylamine [15–17] and nitric oxide [18]. The S_{-1} super-reduced state was first introduced to describe the observation of a two-flash delay in the first maximum of O_2 evolution when the samples were incubated with hydroxylamine [16]. More reduced states of the OEC, i.e., S_{-2} and S_{-3} are also proposed to explain further delays in the first oxygen burst, which result from increased reductant concentration and incubation time [13]. By analysis of the oxygen evolution patterns, the existence of the S_{-4} and S_{-5} states in hydrazine-treated thylakoids were also reported but these intermediates are relatively unstable. Although super-reduced states are generally proposed as intermediates in the process of photo-activation, there are also situations in which these states occur in vivo. For example, the S_{-1} and S_{-2} states were observed in mutants lacking the manganese stabilizing protein [19] or a part of the lumenal E loop of the CP47 protein [20,21]. The occurrence of S_{-1} state in cyanobacteria and other microalgae is proposed to result from a highly reducing cellular environment [22,23]. The S_{-3} state was recently detected in vivo in cucumber leaves under dark chilling conditions [24].

Even though the delay in the O_2 evolution is explained by introducing super-reduced states, the nature of the interaction of reductants with the OEC remains ambiguous. An examination of the literature [14] suggests that while hydroxylamine and hydrazine behave similarly under some conditions, hydroxylamine is the kinetically stronger reductant. In addition, hydrazine, under mild conditions of exposure, acts essentially to produce a two-step retardation [14], while hydroxylamine may act this way, or induce a one-electron reduction. In the latter case, this is seen both by O_2 yield flash pattern analysis [13,14] and by the appearance of the S_0 multiline signal [25]. Even though a formal reduction of the Mn in the OEC seems clear under these circumstances, there are still some controversies in the literature in this regard. Two separate studies, using Mn K edge XANES to monitor the mean Mn oxidation states in the OEC as a function of hydroxylamine treatment yielded different results. Guiles et al. showed that the absorption edges for dark-adapted PSII preparations with or without hydroxylamine incubation were virtually identical [26] for treatment regimes involving hydroxylamine/PS II mole ratios of ~3. A clear shift to lower energy was observed after a single turnover in the treated samples, corresponding to full reduction to S_0 (a net two electron process). This pattern was retained at higher hydroxylamine treatment ratios, although some lowering of the mean edge position was observed, corresponding to formation of Mn^{2+}. By contrast, Riggs-Gelasco et al. [17] showed that, under comparable treatment conditions, hydroxylamine induced a reductive edge shift in the dark, which was reversible on illumination. However, the latter studies employed solubilized PS II core complex material, lacking the 16 and 23 kDa extrinsic proteins, while the former study involved intact PS II membrane particles. Access of reductant to the OEC site may be qualitatively, as well as quantitatively, different in these two cases [27].

In this work, we have used time-resolved EPR to determine the rate for the S_{-1} to S_0 transition to further understand the nature of super-reduced states. For technical reasons, we are restricted to the use of the milder reductant, hydrazine, under conditions where it operates as a two-electron retardant. By applying fast, time-resolved EPR techniques, we were previously successful in obtaining the complete kinetic parameters for the S transitions in thylakoids and PSII membranes [28,29]. The technique is based on measuring the reduction rate of the $Y_Z^{\bullet}$ radical which allows determination of the S-transition rates. The $Y_Z^{\bullet}$ species is paramagnetic giving rise to a transient EPR signal which is conventionally called Signal II_{vf} in intact systems [30]. By deconvoluting the signals for individual S-state contributions, we have shown that $Y_Z^{\bullet}$ reduction is relatively fast (~100 μs) for transitions up to S_3, while the last transition, $S_3 \rightarrow S_0$, which is coupled to the release of O_2, is in the millisecond time regime.

1. Materials and methods

Thylakoid membranes were isolated from market spinach leaves in dim light. About 1 kg of leaves were deveined and homogenized in 50 mM HEPES–NaOH (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$ and 0.4 M sucrose. The homogenate was filtered through eight layers of cheesecloth and one layer of nylon mesh before centrifugation at $1000 \times g$ for 10 min. The pellet was resuspended in 1 L of 50 mM HEPES–NaOH (pH 7.5), 50 mM NaCl and 5 mM $MgCl_2$ and then centrifuged at 1000 g for 10 min. The pellet of thylakoid membranes was finally resuspended in homogenizing buffer at 1.5 mg Chl/mL. The O_2 evolution of thylakoids was between 220 and 250 μmol O_2 (mg of Chl)$^{-1}$ h^{-1} in the presence of 2 mM NH_4Cl as uncoupler and artificial electron acceptors (0.25 mM PBQ and 0.25 mM $K_3Fe(CN)_6$). Chl concentration was determined in 80% acetone according to Porra et al. [31].

For hydrazine treatment, 100 mL of thylakoids at 1.5 mg/mL was first incubated with 0.2 mM hydrazine on ice in the dark for 10 min prior to the addition of 1 mM PBQ and 1 mM $K_3Fe(CN_6)$. This procedure essentially removes excess reductant by oxidation [15,25] and with the sample volumes

employed in these studies is the most efficient means of achieving this removal. The hydrazine was added from a buffered 1 M stock solution. The EPR experiment was started immediately after the 10 min incubation to avoid long exposure of the sample to the reaction mixture. The hydrazine-treated sample was kept in the dark on ice and introduced into the EPR cell by a Gilson minipulse 3 pump using a tube from a long measuring cylinder. The tube allows a displacement of an illuminated aliquot in the EPR cell with a fresh dark-adapted aliquot. Saturating 10 μs xenon flashes from an EG and G electro-optic flash lamp were used to excite the sample. A non-magnetic optical fiber was used to illuminate the sample residing in the EPR cavity.

All EPR measurements were performed on a Bruker ESP 300E spectrometer equipped with a TM011 cavity. The ESP 300E spectrometer computer controlled the EPR data acquisition and triggering the flash lamp and the pump. The flash lamp was triggered within a fixed delay time after the data acquisition was started. The $Y_Z^{\bullet}$ decay kinetic measurements were performed at the low-field peak (g = 2.010) of the $Y_D^{\bullet}$ EPR signal, previously described [28,29], using instrumental parameters of 100 kHz modulation frequency, 100 mW microwave power, 4 G modulation amplitude and 20 μs time constant. From all the $Y_Z^{\bullet}$ decay kinetic signals, a field independent flash artifact signal measured at g = 1.99 was subtracted. The $Y_Z^{\bullet}$ decay kinetic of each flash was stored in a separate file in the EPR computer. About 3 L of thylakoid membranes at 1.5 mg/mL was used to obtain the $Y_Z^{\bullet}$ decay kinetics of the S_{-1} to S_0 transition presented here.

The O_2 flash patterns were obtained using a home-built unmodulated Joliot type electrode at 25 °C. A train of 16 flashes, generated by a computer at a rate of 4 Hz, were digitized and recorded on the computer to determine the S-state composition of the sample.

2. Results

To generate the formal S_{-1} state, the sample was incubated with hydrazine. Fig. 1 shows the steady-state O_2 activity of thylakoid samples versus incubation time with 0.2 mM hydrazine. Steady-state O_2 activity directly correlates with the intactness of OEC. In comparison with hydroxylamine, hydrazine treatment is known to be less damaging, however, a long exposure of the sample with hydrazine also causes irreversible damage, destroying the O_2 activity [32]. As it is shown in Fig. 1, under the experimental conditions that were used here, about 30% of O_2 activity is lost after 2 h incubation following hydrazine treatment. To minimize the deactivation effect, the exposure time was therefore kept under 30 min for flash experiments described below. Since under these conditions the O_2 activity remains almost unaffected, it is very likely that most centers function normally resetting back to the S_0 state after illumination. At longer incubation time, further reduction causes irreversible changes by generating Mn^{+2} ions that are expected to be labile and released from the OEC site.

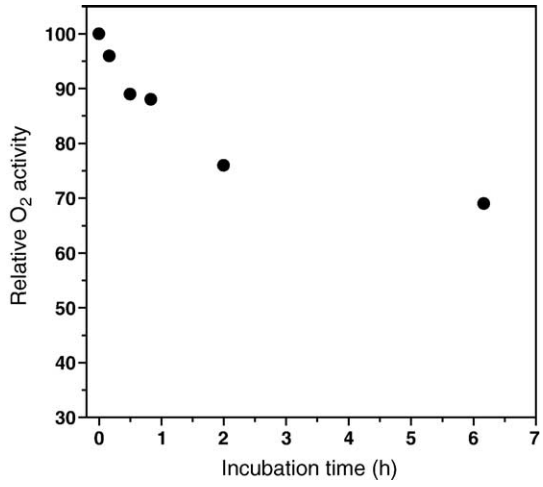

Fig. 1. Relationship between steady-state O_2 evolution activity and incubation time of thylakoids with 0.2 mM hydrazine. The thylakoid sample was incubated with hydrazine on ice and the oxygen activity of an aliquot was assayed in the presence of 1 mM PBQ and 1 mM $K_3Fe(CN_6)$ at each time point.

Having established that 10–30 min incubation post-hydrazine treatment does not cause appreciable loss in the O_2 activity, O_2 evolution flash patterns were obtained to determine the S-composition of the sample. Fig. 2 represents the flash-induced O_2 evolution pattern measured on an aliquot of a dark-adapted thylakoid sample in the absence and presence of 0.2 mM hydrazine. In untreated samples, which were dark-adapted for about 2 h, a pronounced first burst of O_2 evolution was observed after the third flash. This is because after sufficiently long dark-adaptation, all the centers including S_0 have decayed back to the dark-stable S_1 state, through slow oxidation by Y_D^{ox} in the case of S_0. Damping of the oscillations allows the miss probability (α) and double hit probability (β) to be determined. The α and β parameters are generally unchanged for the same sample concentration, flash lamp and experimental setup. Taking this into account, the most notable effect of hydrazine treatment on a dark-adapted sample is then the two-flash delay in O_2 formation (Fig. 2). This clearly indicates that 10 min incubation with hydrazine was long enough to reduce a substantial number of centers to the S_{-1} state, without causing significant irreversible damage, (see Fig. 1). This is because hydrazine is a two-electron donor which can efficiently convert an S_1-rich sample to an S_{-1}-rich sample. Unlike hydroxylamine, which may also act as a one-electron donor, the yield of S_{-1} state is then related to the initial ratio of S_0/S_1 [32]. The pattern in Fig. 2 indicates that about 80% of centers are in the formal S_{-1} state following hydrazine treatment.

The first step in measuring the $Y_Z^{\bullet}$ kinetics of the S_{-1} to S_0 transition was to ensure that each aliquot would be

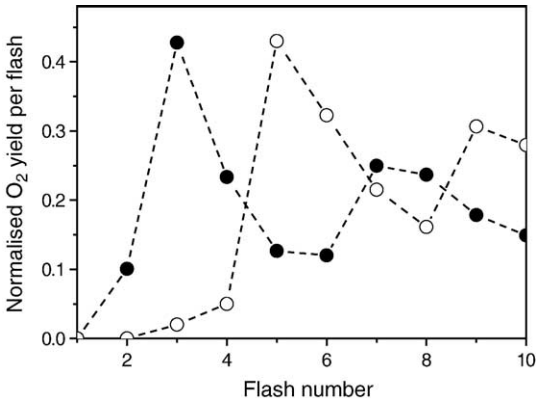

Fig. 2. Oxygen release patterns obtained on aliquots of the S_1-rich (filled circles) and S_{-1}-rich (open circles) samples using Joliot type electrode. For the S_1-rich sample, an aliquot was given a single flash and then dark-adapted for 10 min prior to receiving the measuring flashes. For the S_{-1}-rich state, an aliquot was incubated with hydrazine for 10 min prior to receiving the measuring flashes.

subjected to only one flash series. To acquire consistent $Y_Z^{\bullet}$ decay kinetics, an unexposed aliquot in the EPR cell was subjected to a train of flashes with 100 ms intervals and then replaced with a fresh S_{-1}-rich aliquot. After a complete run of the first 100 mL of sample, this was discarded and a fresh batch of treated sample was prepared. By preparing separate batches, the exposure time to the hydrazine was minimized to maintain the integrity of the sample. Fig. 3a shows the $Y_Z^{\bullet}$ decay kinetic traces of the 1st flash on a hydrazine-treated aliquot, summed over many aliquots. The signal is dominated by the $Y_Z^{\bullet}$ decay kinetics of the S_{-1} to S_0 transition due to the high initial S_{-1} population. The smooth curve represents the least-squares fit to the data of Eq. (1) for $t > 50$ μs.

$$I_i(t) = I_a e^{-t/\tau_a} + I_s e^{-t/\tau_s} \quad (1)$$

This assumes that the rise and fall kinetics are mono-exponential with the rise time governed by the spectrometer response time. I_a and τ_a are the signal amplitude and decay time constant for a given S state in O_2 evolving centers. I_s and τ_s represent the signal amplitude and decay time constant for a fixed fraction (10%) of inactive centers that turnover with a decay time constant of ~10 ms. The slow decaying component is that part of the signal which fails to recover during the 4 ms of data acquisition and appears as a base line offset.

The resulting halftime for the S_{-1} to S_0 transition is listed in Table 1 together with our previous results for the rate of S-state transitions in untreated thylakoids under similar conditions. The calculated rate for the S_{-1} to S_0 transition appears to be around 1.7 ms, which surprisingly is the most retarded transition and even slower than the S_3 to S_0 transition which is coupled to the oxygen chemistry. Fig. 3b and c shows the $Y_z^{\bullet}$ decay kinetics on the second and third turnover flashes, with 100 ms spacing between successive flashes. Although the signal to noise is insufficient for an individual quantitative analysis, it is apparent that a fast

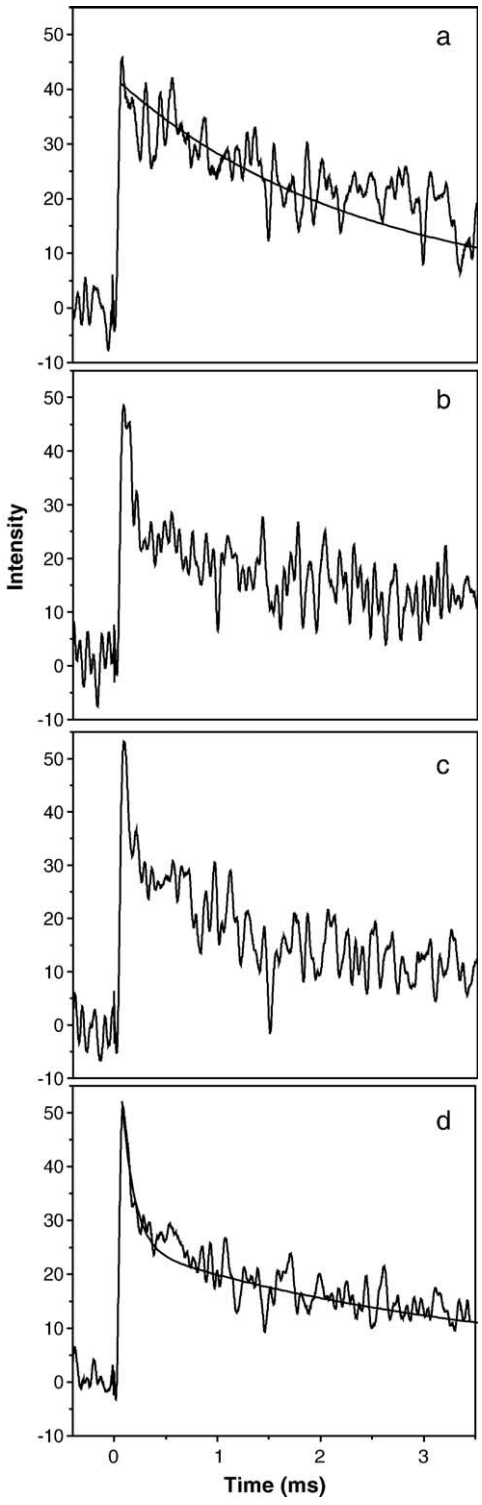

Fig. 3. $Y_Z^{\bullet}$ decay kinetics of the 1st (a), 2nd (b) and 3rd (c) flash on an S_{-1}-rich sample. The summation of kinetic traces (d) from 2nd, 3rd and 4th flash on the S_{-1}-rich sample. The smooth curves represent the fit to the data of Eq. (1). Fitting parameters for (a) were: $I_a = 0.9$ for the S_{-1} turnover and $I_s = 0.1$ for inactive centers. Fitting parameters for (d) included an additional term and were: $I_a = 0.75$ for the averaged contribution of S_0, S_1 and S_2 turnovers with a $t_{1/2} = 95$ μs; $I_b = 0.15$ for the fraction of S_{-1} ($t_{1/2} = 1.7$ ms) and S_3 ($t_{1/2} = 0.75$ ms) turnovers; $I_s = 0.1$ for the fraction of inactive centers. The signal amplitudes are divided by the number of averaged events: 4056 for (a–c) and 12168 for (d).

Table 1
Comparison of the halftime of the $S_{-1}\rightarrow S_0$ transition obtained here with the halftime of subsequent transitions from our previous work on untreated thylakoids [28]

$t_{1/2}$ (μs) $S_{-1}\rightarrow S_0$	$t_{1/2}$ (μs) $S_0\rightarrow S_1$	$t_{1/2}$ (μs) $S_1\rightarrow S_2$	$t_{1/2}$ (μs) $S_2\rightarrow S_3$	$t_{1/2}$ (μs) $S_3\rightarrow S_0$
1700	40–60	85	145	750

component dominates the $Y_Z^{\bullet}$ decay on the second and third turnovers. Moreover, the normalized amplitudes of the turnovers are similar in all cases. This is important, since the dark reduction by hydrazine also reduces $Y_D^{\bullet}$ to a low level. This re-establishes only slowly under repetitive flashing (requiring more than ~30 turnovers, data not shown). If the response in Fig. 3a were actually dominated by $Y_D^{\bullet}$ turnover in the presence of hydrazine, this would also be the case on the second and third flashes. The equivalence of the initial amplitudes also means that the slow decay seen in Fig. 3a is not the result merely of inactive centers, since these have largely reactivated to display normal turnover kinetics on the second and subsequent flashes.

To show quantitatively this recovery of the centers after the first flash, a summation of the subsequent 2nd, 3rd and 4th flash data on the S_{-1}-rich sample is shown in Fig. 3d. These kinetic traces represent a mixture of S states with earlier transitions dominating the signal. According to the kinetic information given in Table 1, the fast phase corresponds to the $Y_Z^{\bullet}$ reduction by the S_0, S_1 and S_2 states (averaged $t_{1/2}$ = 95 μs) with the slow phase arising from the $Y_Z^{\bullet}$ reduction by S_{-1} and S_3. The contribution of S_{-1} and S_3 is due to the misses and double turnovers which are about 23% and 10%, respectively, based on our previous estimation for the same experimental setup [28,29]. The slowly relaxing component (~10%) is also apparent in these traces. The reappearance of the fast phase is a clear indication that the S-cycle runs normally after a single flash illumination of hydrazine-treated samples consistent with the high O_2 activity retained by the samples during the EPR experiments.

3. Discussion

The importance of 'water analog' reductants inducing super-reduced states in the OEC has long been recognized, as it can provide insight into the water oxidation chemistry. The two most extensively studied compounds, hydroxylamine and hydrazine, are generally believed to reduce Mn atoms in the OEC. Hydrazine is a mild reductant, which causes a clean two electron retardation in the Kok cycle under controlled conditions. In the experiments presented here, the samples were first synchronized to the S_{-1} state in the dark then unreacted reductant was removed by chemical oxidation to prevent the further reduction of the centers. The measurements were also performed at 100 ms flash intervals to minimize the reduction of the S_2 state with any unreacted hydrazine during the measurements. This is because hydrazine and hydroxylamine react in preference with the S_2 state even though the S_3 state is a stronger oxidant [14]. The first flash transient can therefore be assigned to the S_{-1} to S_0 transition with following transients corresponding to the normal Kok cycle transitions.

Regardless of the nature of S transitions, either a Mn or ligand oxidation, each transition is coupled to the $Y_Z^{\bullet}$ reduction. The reduction rates of $Y_Z^{\bullet}$ are dependent on the redox state of the Mn cluster in active samples. While most S state transitions appear to involve Mn oxidation, the change in rates cannot be simply explained by electrostatic effects. This is mainly because the S state transitions can be electroneutral, as an electron and a proton can be abstracted from the OEC. Interestingly, the $Y_Z^{\bullet}$ reduction kinetics in the S_{-1} to S_0 transition appeared to be much slower than in any of the subsequent S state transitions generally acknowledged to involve simple Mn oxidations. By extension, one would expect that if the S_{-1} to S_0 transition were to involve Mn(II) oxidation, it would be fast, as for the S_0 to S_1 transition. The S_{-1} to S_0 transition occurs with a half time of 1700 μs while the half time for the S_0 to S_1 transition is about 50 μs. This slow rate is only comparable to that of the S_3 to S_0 transition which is a unique transition accompanied by water oxidation and O–O bond formation. It is during this transition that the Mn-cluster is reduced through a concerted, multi-electron re-reduction process. As the O_2 release kinetics are closely coupled with the re-reduction of $Y_Z^{\bullet}$ in functional PS II turnover [33], some structural reorganization of the Mn cluster is expected in this process which presumably contributes to the slow rate of the S_3 to S_0 transition. Thus, it is also possible that the structure of the Mn-cluster in the S_{-1} state is significantly altered. As this transition is only a one-electron process leading to the S_0 state without any chemistry involved, the structural alternation must be as a direct result of the Mn reduction. This appears as a reversible process since the system exhibits normal turnover behavior within 100 ms on the second flash turnover (Fig. 3b–d). The contribution of changes at the extrinsic polypeptides to the slow rate should also be considered as it is shown that the susceptibility to proteolytic digestion of the 33-kDa MSP is increased on incubation of PS II membranes with hydroxylamine [34]. But such factors have less pronounced effects on the rate of early transitions demonstrated by measuring the $Y_Z^{\bullet}$ reduction kinetics of a cyanobacterial mutant lacking the 33-kDa MSP. We showed that the complete removal of the MSP leads to only a threefold slowing (averaged $t_{1/2}$ of 400 μs) in the early S state turnover kinetics and a fivefold slowing for the S_3 to S_0 transition [35]. It is worth noting that if the extrinsic polypeptides were released due to the hydrazine treatment, it would have had a global effect propagating to other transitions. Such effect was not seen on any other transition which is also supported by cross-linking experiments showing that hydrazine can readily reduce the Mn-

cluster even when the extrinsic polypeptides are cross-linked to the PSII complex [36].

As noted above, the reduction rates of $Y_Z^{\bullet}$ are only modestly dependent on the formal redox state of the Mn cluster in active samples up to S_3, within the normal S state cycle. The big change occurs on the S_3 to S_0 transition, which is characterized by several unique features:

(i) Slowing by about an order of magnitude in the $Y_Z^{\bullet}$ re-reduction kinetics.
(ii) The release of two protons in intact systems, as opposed to no more than one on any of the earlier S state transition steps [37].
(iii) The probable re-reduction of the Mn cluster in a concerted, multi-electron process. This accompanies water oxidation and O − O bond formation.

Förster and Junge showed that in hydroxylamine treated thylakoids which exhibited a two-step retardation, two protons were released on the first turnover, with a halftime of ~2 ms, and thereafter the release pattern and kinetics followed the expected course for normal PS II starting from the S_0 state [38]. Combined with the results presented here and the above discussion, the formal similarity between the S_3 to S_0 and 'S_{-1} to S_0' transitions is remarkable. A reasonable conclusion is then that two electron 'chemistry' occurs on the S_{-1} to S_0 transition, somewhat analogous to the four electron chemistry that occurs on the physiological S_3 to S_0 transition. This chemistry involves O═O bond formation between two bound substrate water molecules in the latter case, so presumably N − O bond formation between bound amine species and a water molecule in the former case. In effect, the OEC site may catalyze the reaction;

a) $[R\text{-}NH_2] + H_2O \rightarrow [R\text{-}NH\text{-}OH] + 2H^+ + 2\,e^-$

b) $[S_1 + Yz^{\bullet}] + 2\,e^- \rightarrow [S_0 + Y_Z]$

Scheme 1.

In (a), R is either NH_2 or OH. The reaction is essentially amine oxide formation, resulting in reactive, unstable species which would presumably undergo decomposition or other types of further, bi-molecular reaction (e.g., resulting in N_2). At neutral pH, the redox potentials of reaction (a) should be in the range ~0.6–0.8 V, based on the known values for NH_3 and primary aliphatic amines [39]. The reaction is indeed 'analogous' to the water oxidation reaction, in both redox potential and participation of one of the substrate species for which the OEC site is actually designed. We have recently shown that the O_2 release kinetics are closely coupled with the re-reduction of $Y_Z^{\bullet}$ in functional PS II turnover ($t_{1/2}$ = 0.75–0.85 ms, thylakoids [33]). The close match between the first turnover $Y_Z^{\bullet}$ re-reduction kinetics seen here ($t_{1/2}$ = 1.7 ms, hydrazine) and the corresponding proton release kinetics ($t_{1/2}$ ~2 ms, hydroxylamine [38]) in thylakoids, further supports the formal similarity between the S_3 to S_0 and 'S_{-1} to S_0' transitions, at least in intact systems. Because the XANES results for exogenous amine induced reduction of the OEC are somewhat ambiguous [17,26], the sequence of events summarized by Scheme 1 are as yet uncertain. However, if the reduction sequence observed in [26] obtains, then, the formal similarity between hydrazine reaction from S_1 and water oxidation turnover from S_3 is even stronger, for at least one model of the physiological process [40]. In both cases, the system is quasi-stably poised (in S_1 or S_3, respectively) with substrate bound, until Y_Z is oxidized. This then triggers a concerted, multi-electron reduction of the OEC followed by electron transfer to re-reduce $Y_Z^{\bullet}$, in a similar fashion in both cases. The extent to which these conclusions bear upon the existence of functional, true Mn reduced states below ~S_0 remains open.

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