Light-Adaptation of Photosystem II Is Mediated by the Plastoquinone Pool†

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ABSTRACT: During the first few enzymatic turnovers after dark-adaptation of photosystem II (PSII), the relaxation rate of the EPR signals from the Mn cluster and $Y_D^\bullet$ are significantly enhanced. This light-adaptation process has been suggested to involve the appearance of a new paramagnet on the PSII donor side [Peterson, S., Åhrling, K., Högblom, J., and Styring, S. (2003) *Biochemistry 42*, 2748−2758]. In the present study, a correlation is established between the observed relaxation enhancement and the redox state of the quinone pool. It is shown that the addition of quinol to dark-adapted PSII membrane fragments induces relaxation enhancement already after a single oxidation of the Mn, comparable to that observed after five oxidations in samples with quinones (PPBQ or DQ) added. The saturation behavior of $Y_D^\bullet$ revealed that with quinol added in the dark, a single flash was necessary for the relaxation enhancement to occur. The quinol-induced relaxation enhancement of PSII was also activated by illumination at 200 K. Whole thylakoids, with no artificial electron acceptor present but with an intact plastoquinone pool, displayed the same relaxation enhancement on the fifth flash as membrane fragments with exogenous quinones present. We conclude that (i) reduction of the quinone pool induces the relaxation enhancement of the PSII donor-side paramagnets, (ii) light is required for the quinol to effect the relaxation enhancement, and (iii) light-adaptation occurs in the intact thylakoid system, when the endogenous plastoquinone pool is gradually reduced by PSII turnover. It seems clear that a species on the PSII donor side is reduced by the quinol, to become a potent paramagnetic relaxer. On the basis of XANES reports, we suggest that this species may be the Mn ions not involved in the cyclic redox changes of the oxygen-evolving complex.

Photosystem II (PSII)[1] in plants, algae, and prokaryotic organisms catalyses the light-dependent electron transfer from water to plastoquinone. During this process, water is oxidized to molecular oxygen. When a quantum of light reaches the primary electron donor, P680, an electron is ejected, reducing Q_A, the first quinone acceptor. $P680^+$ is then quickly rereduced by the closely situated redox active tyrosine (Y_Z), thereby ensuring stable charge separation. The charge on Q_A^- is passed to another plastoquinone, Q_B. After two turnovers, reduced plastoquinone is released from its special site in the protein, and an oxidized plastoquinone takes its place. The electrons and protons are ultimately used to reduce $NADP^+$ to NADPH. Y_Z^{ox} is reduced by the oxygen-evolving complex (OEC) that consists of four manganese ions, a calcium, and a chloride ion. The OEC is capable of storing, in a cyclic manner, four oxidizing equivalents before it oxidizes water to molecular oxygen in a concerted process and the cycle starts over. The intermediate oxidation states of the OEC are denoted $S_0 \cdots S_4$, where the suffix refers to the number of electron holes stored (*1*). For recent reviews, see ref *2*.

The plastoquinone bound to the Q_B site accepts two electrons from Q_A and is doubly protonated before it leaves the Q_B site in favor of a nonreduced plastoquinone. The plastoquinone pool present in the thylakoid membrane thus accepts the electrons from PSII. Quinols from this pool supply electrons to the Cyt b_6f complex but at a slower rate than the PSII-mediated quinone reduction (*3*). In PSII membrane fragments, the plastoquinone pool is depleted, and it becomes necessary to add an exogenous quinone as electron acceptor to ensure efficient advancement of the reaction center beyond the first few flashes. For a review of the chemistry of the iron−quinone complex of photosystem II, see ref *4*.

The S-states of the OEC can be studied by electron paramagnetic resonance spectroscopy (EPR). For a review of the EPR signals arising from fully active oscillating centers, see ref *5*. Both the S_0 and the S_2 state have complex manganese multiline signals arising from $S = 1/2$ ground states, with minimum motifs consisting of antiferromagnetically coupled $Mn^{II}-Mn^{III}$ and $Mn^{III}-Mn^{IV}$ di-μ-oxo bridged dimers, respectively. The other two manganese ions are

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[1] Abbreviations: Cyt, cytochrome; DMSO, dimethyl sulfoxide; DQ, duroquinone; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance spectroscopy; H_2Q, hydroquinone; MeOH, methanol; MES, 2-(*N*-morpholino)ethanesulphonic acid; NADP, nicotinamide adenine dinucleotide phosphate; OEC, oxygen-evolving complex; $P_{1/2}$, power of half-saturation; P680, primary electron donor; PPBQ, phenyl-*p*-benzoquinone; PSI, photosystem I; PSII, photosystem II; RT, room temperature (22 ± 1 °C); Y_D, tyrosine residue D2-Y161; Y_Z, tyrosine residue D1-Y161.

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thought to have the oxidation states $Mn^{III}-Mn^{III}$ or $Mn^{IV}-Mn^{IV}$ through the oxidation cycle, with $Mn^{IV}-Mn^{IV}$ currently the most favored motif (see, e.g., ref *6*). The S_1 and S_3 states appear to have diamagnetic ground states, with parallel-polarization EPR signals arising out of excited $S = 1$ or $S = 2$ spin states (*7*−*10*).

The dark-stable state of the OEC is the S_1 state. Thus, three flashes of light are required to forward the dark-adapted OEC into the S_0 state. This in turn means that the first observation of the S_0 state is upon completion of the first S cycle after a period of dark-adaptation. In studies of the S_0 signal we found that the power saturation behavior of the S_0 state was remarkably different to that of the S_2 state (*11*). This naturally led to a further study of the S cycle on the second turnover of the enzyme (*12*). In that study, we found a difference in the power saturation behavior of the donor side paramagnets between samples in the first and in subsequent turnovers of the enzyme. We studied the S_2 state multiline signal, after one flash of light and five or more flashes of light, and found that the relaxation behavior of the OEC changed after the first turnover of the enzyme (i.e., after the first molecule of oxygen was released). Other groups have studied light-induced changes in the relaxation behavior of the multiline and $Y_D^{\bullet}$. The first observation of such an effect was by Beck et al. (*13*, *14*). They showed that the relaxation behavior of the multiline signal varied with the length of dark-adaptation prior to 200 K illumination, identifying a resting and an active state of the enzyme. Koulougliotis et al. (*15*) showed that the resting S_1 state was diamagnetic. $Y_D^{\bullet}$ studies reveal that the phenomenon is not exclusive to the S_2 state but an effect experienced by the OEC and Y_D irrespective of the S state (*12*, *16*, *17*). Lorigan and Britt (*18*) have shown that the active state S_2 multiline signal has biphasic relaxation behavior. In our recent publication (*12*), we showed that the OEC undergoes an adaptation to light after a period of dark, which commences after two flashes and is complete after 10 flashes, corresponding to about two turnovers the OEC. The relaxation behavior is best explained in terms of the appearance of a paramagnet close to the multiline center (*12*) on the second turnover of the enzyme.

The appearance of a paramagnet on the donor side must involve a redox reaction, separate from the P680-driven electron transport chain. As we detected no additional $S = 1/2$ EPR signals during the light-adaptation process, we concluded that a two-electron transfer was taking place and that an even-spin system was responsible for the relaxation enhancement. Preliminarily, we proposed that the change in relaxation behavior was brought about by the artificial quinone used as an electron acceptor. As the change appears after several electrons have reduced the quinone, it must be that a quinol brings about the change. Thus, the relaxation enhancement is likely the result of a double reduction of a species near the multiline signal spin center. In this study, we probe the relationship between the relaxation rate of the S_2 multiline signal and $Y_D^{\bullet}$ signal and the redox state of the quinone pool by the addition of different quinones and quinols to PSII samples, followed by illumination. We present data that clearly links the relaxation properties of the Mn giving rise to the multiline signal to the state of the quinone pool.

MATERIALS AND METHODS

PSII membrane fragments were prepared according to ref *19*, frozen in liquid N_2, and stored at −80 °C until needed, in a buffer containing 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) at pH 6.0, 15 mM NaCl, 10 mM $MgCl_2$, and 400 mM sucrose. Oxygen activity was measured with a Clarke oxygen electrode. Typical O_2 activities for these membrane fragments were 800 (μmol of O_2)(mg of Chl)$^{-1}$ h^{-1}. For EPR experiments, samples were diluted to ∼1.8 (mg of Chl) mL^{-1} in the same buffer, with or without 3% (v/v) methanol (MeOH). Pre-illumination and flash−freeze procedures were carried out as described previously (*20*). Prior to the train of flashes, the samples were dark-adapted for 18 min at room temperature. The exogenous quinones, phenyl-*p*-benzoquinone (PPBQ) or duroquinone (DQ) or their respective quinol (see below) were added in the dark to a final concentration of 0.5 mM, 1 min before the flashes were given. Illumination was carried out using a Nd:YAG laser, giving 350 mJ per 7 ns flash at 532 nm. The samples were frozen immediately at 200 K and then transferred to liquid N_2 in readiness for EPR. Samples given long-term dark-adaptation, overnight on ice, were kept as dark as possible and only given one preflash prior to the final flashes. PSII membrane fragments for continuous illumination were prepared in the dark at ∼10 (mg of Chl) mL^{-1}, given no preflash, frozen to 200 K in a liquid N_2 flow cryostat, and illuminated for 5 min with white light (2400 μmol of photons m^{-2} s^{-1}).

Thylakoids from spinach were prepared according to ref *21* and prepared in the same manner as above for flash illumination. $P_{1/2}$ values were measured in samples with and without 2 mM EDTA. No electron acceptor was added to the thylakoids. The uncoupled O_2 activity was 380 (μmol of O_2)(mg of Chl)$^{-1}$ h^{-1}.

Exogenous quinones PPBQ and DQ were reduced by treating the quinone dissolved in dimethyl sulfoxide (DMSO) with $NaBH_4$ for 5 min while stirring and then acidifying with HCl. The resulting quinols were stored in small aliquots and kept at −80 °C until needed.

EPR spectroscopy was carried out on a Bruker ESP300e X-band spectrometer equipped with an Oxford He cryostat and temperature control system. Spectra were taken within 48 h of the samples receiving the final flashes.

Theoretical Calculations. Determination of power of half saturation, $P_{1/2}$, was carried out by first estimating the signal intensity of the multiline signal at various applied powers of microwave radiation, detected at 7 K. The signal intensity was estimated by adding the peak height of three characteristic multiline peaks downfield of $g = 2$. To check that this gave an accurate measurement of $P_{1/2}$, the signal intensity was also estimated from the sum of three peak heights upfield of $g = 2$ (see Figure 1A), for each type of sample. We found that the variation between the two methods of measurement was small (≤8%). The intensity of the $Y_D^{\bullet}$ signal was obtained from the double integration over the whole signal using WinEPR software.

The power of half saturation was then found from fitting the expression

$$I = I_0 \sqrt{P}/\sqrt{(1 + P/P_{1/2})^b} \qquad (1)$$

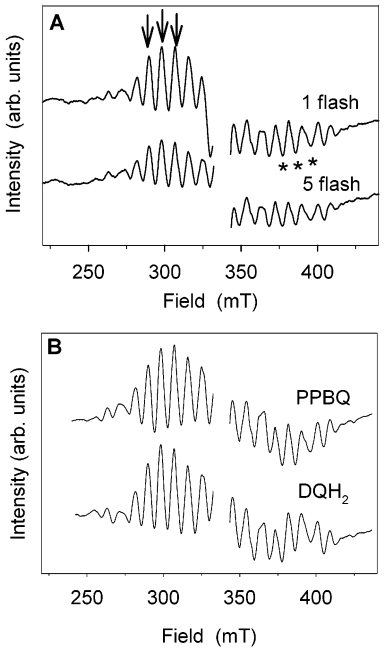

FIGURE 1: S_2 state multiline signal recorded in PSII membrane fragments given (A) one and five flashes and (B) continuous illumination at 200 K. Marked on the one-flash spectrum in (A) are the peaks used routinely for amplitude analysis (arrows) and those used for controls (asterisks). In panel B, either PPBQ or DQH_2 has been added to the sample. EPR conditions: microwave frequency 9.42 GHz, power 6.32 mW; modulation frequency 100 kHz; modulation amplitude 2 mT; and temperature 7 K.

(*22*) to the data. Here I is the measured signal intensity, I_0 is the unsaturated signal intensity, P is the applied microwave power, $P_{1/2}$ is the power of half saturation, and b is the inhomogeneity parameter ($b = 1$ for an inhomogeneously broadened line). For a more detailed description, see ref *12*.

RESULTS

In PSII membrane fragments, the plastoquinone pool is depleted. To maintain Q_A oxidized through a multiflash protocol, it is necessary to use an artificial electron acceptor. The artificial electron acceptor also stabilizes each S state, so that the back reaction to the S_1 state does not occur to any significant extent even after months of storage in liquid N_2. We most commonly use PPBQ for these purposes. We also routinely use 3% MeOH in our samples as small monoalcohols have been shown to maximize the S_2 state multiline signal, and MeOH specifically allows the observation of the S_0 state multiline signal (*20*, *23*).

In the presence of an artificial electron acceptor such as PPBQ, we see a dramatic change in the relaxation behavior of the S_2 state multiline signal when the spectrum is collected after one flash as compared with when it is collected after the system having been given five flashes (i.e., on the second turnover of the enzyme after a period of dark-adaptation) (ref *12* and Table 1). The relaxation of the five-flash multiline signal is significantly enhanced as compared to the one-flash signal. Figure 1A shows typical one- and five-flash spectra from which $P_{1/2}$ measurements were taken. We observe no significant change in the overall shape of the signal between the one- and five-flash samples. The five-flash multiline signal has lower intensity because of dephasing of the PSII centers leading to a lower S_2 state population after five flashes than after one flash. We estimate, using Kok parameters (*1*), that the S_2 state population of the five-flash sample is about 65% of that of the one-flash sample.

Table 1: Comparison of $P_{1/2}$ Values[a]

sample	dark-adapt. (min)	no. of flashes	Multiline Signal: typical $P_{1/2}$ (mW)	Multiline Signal: range $P_{1/2}$ (mW)	$Y_D^\bullet$: typical $P_{1/2}$ (mW)
PSII with PPBQ	o/n [b]	0			0.025
	o/n	1	16		0.069
	o/n	5	>300		0.170
PSII with PPBQ	20	0			0.040
	20	1	22	16−88	0.070
	20	4			0.133
	20	5	130	54−318	0.187
PSII with DQ	20	0			0.061
	20	1	20	20−92	0.088
	20	5	∼210	78−400	0.177
PSII with PPBQ, no MeOH	20	1	47	16−47	
	20	5	153	40−153	
PSII with DQH_2	20	0			0.037
	20	1	∼150	135−275	0.175
PSII with $PPBQH_2$	20	1	∼200	40−220	0.169
thylakoids	20	1	48	16−84	
	20	5	110	50−177	
PSII with PPBQ[c]	o/n		24		
PSII with DQH_2[c]	o/n		68		

[a] Typical $P_{1/2}$ values of the S_2 multiline signal and the $Y_D^\bullet$ signal for PSII membrane fragments given flash illuminations at RT or continuous illumination at 200 K as indicated, in the presence of exogenous quinone or quinol and thylakoid membranes given 1 and 5 flashes. Unless otherwise stated, samples have 3% MeOH. Experimental temperature: 7 K. [b] Overnight on ice. [c] Samples exposed to 5 min of white light at 200 K.

The relaxation behavior is measured as a change in intensity of the signal with increasing power (Figure 2A). The curve is then fit to eq 1, and the data is plotted with the unsaturated signal intensity (i.e., the slope of the linear region in Figure 2) for each experiment normalized to compensate for different S_2 state populations. The intensity for the multiline signal at any one power is approximated from the sum of three peaks on the low-field side of $g = 2$ (see Figure 1A and *Theoretical Calculations*). These peaks avoid potential contributions from $Fe^{2+}Q_A^-$ and oxidized Cyt b_{559} and areas where the S_0 multiline signal may contribute in the five-flash samples.

Table 1 and Figure 2 display the results of a number of $P_{1/2}$ experiments, aimed at elucidating the relationship between the quinone acceptor and the relaxation enhancement of the S_2 multiline signal observed after multiple flashes. For each experiment, multiline signal $P_{1/2}$ values from a representative data set are displayed. As there is a variability associated with this measurement, we have also tabulated the range of values observed for a certain type of sample. Even though a range of values is obtained, a consistent trend is observed: in each set of samples, the multiline signal from the five-flash sample relaxes faster than the multiline signal from the one-flash sample. Typically, $P_{1/2}$ values are 20 mW for a one-flash sample and ∼200 mW for a five-flash sample, depending on sample treatment and electron acceptor used.

A PPBQ-containing sample that had been dark-adapted overnight gave a one-flash multiline signal $P_{1/2}$ of 16 mW

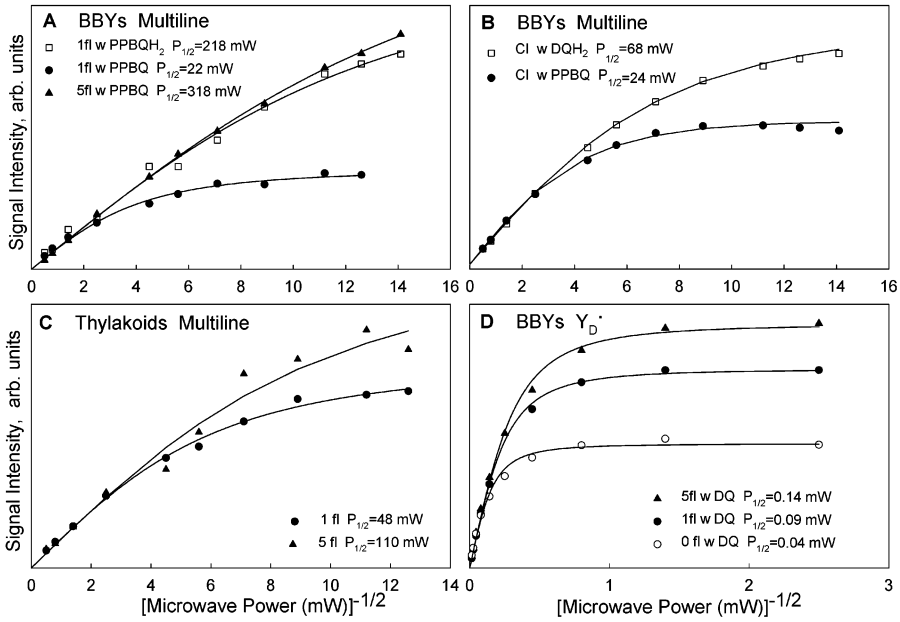

FIGURE 2: Normalized microwave power saturation curves and estimates of $P_{1/2}$ for various samples as indicated at 7 K. Unless otherwise stated, PSII membrane fragments (BBYs) were used. $P_{1/2}$ in all cases was estimated using eq 1. 3% MeOH was present in the sample buffer. (A) S_2 state multiline signal for samples given one and five flashes in the presence of PPBQ and $PPBQH_2$ as indicated. (B) S_2 state multiline signal for continuously illuminated samples with PPBQ and DQH_2 as indicated. (C) S_2 state multiline signal for thylakoid preparations without exogenous electron acceptors. (D) $Y_D^\bullet$ signal for samples after zero, one, and five flashes in the presence of DQ. EPR settings for $Y_D^\bullet$ signal: microwave frequency 9.42 GHz, modulation frequency 3.13 kHz, and modulation amplitude 0.2 mT.

and a five-flash multiline signal $P_{1/2}$ of >300 mW (Figure 2A and Table 1). Equivalent samples that had been dark-adapted for only 20 min on ice prior to flash treatment showed a similar tendency, although the one-flash $P_{1/2}$ values were slightly higher than in the long-term dark-adapted material. Using DQ instead of PPBQ as a quinone acceptor again gave similar results. Short-term dark-adapted samples containing PPBQ but with no MeOH were also prepared, and these displayed the same $P_{1/2}$ enhancement upon advancing the system from one to five flashes as those containing MeOH.

In summary, the first four experiments of Table 1 show that the light-adaptation process takes place in PSII membrane fragments with either PPBQ or DQ as electron acceptor and irrespective of the presence of MeOH. Minor differences between the experiments are evident: a sample that has been dark-adapted overnight has the lowest possible $P_{1/2}$ value for the one-flash multiline signal, about 15−20 mW. Samples given only a short dark-adaptation (∼20 min) have more elevated one-flash $P_{1/2}$ values, 20−90 mW, with the lower end of the range more common. The five-flash samples, however, took a range of values, regardless of the initial dark-adaptation time but consistently higher than the corresponding one-flash samples. Samples with no MeOH in the final buffer appeared to favor elevated $P_{1/2}$ values in the one-flash samples. The most consistent results were obtained with PPBQ, indicating that it is the better electron acceptor in these samples.

The power saturation behavior of $Y_D^\bullet$ was also studied, at the same temperature as for the multiline signal (7 K). The $P_{1/2}$ of the EPR signal arising from the dark-stable tyrosine $Y_D^\bullet$ has been shown to vary with flash number (*16*). As reported earlier (*12*), the relaxation behavior of the $Y_D^\bullet$ signal followed that of the multiline signal through the light-adaptation process. Here, we found a range of $Y_D^\bullet$ results but with a clear trend. A typical value for the $Y_D^\bullet$ signal in the S_2 state was 0.07 mW for the one-flash sample and 0.18 mW for the five-flash sample (Table 1 and Figure 2D). In other words, there is an increase of $P_{1/2}$ between one and five flashes for both the multiline and the $Y_D^\bullet$ signal. The zero-flash sample (S_1 state) had a lower $Y_D^\bullet$ $P_{1/2}$ still, 0.045 mW. A zero-flash sample dark-adapted overnight had a $P_{1/2}$ value of ∼0.024 mW. This mirrors the one-flash multiline signal result, showing a low first-turnover $P_{1/2}$ that is reduced further by long-term dark-adaptation. We also studied the S_1 state on the second turnover, obtained after four flashes. $Y_D^\bullet$ in this S_1 sample had an elevated $P_{1/2}$ of 0.133 mW. This confirms that the light-adaptation effect is related to the second turnover of the enzyme and is not a specific S_2 state effect.

If the multiline signal and $Y_D^\bullet$ relaxation enhancement is induced by quinones reduced by Q_A, as postulated, then the addition of exogenous quinol to the sample should give the same effect. We therefore prepared the quinols $PPBQH_2$ and DQH_2. Samples were treated with quinol in the same manner as quinone—1 min in the dark before the final train of flashes was given. As seen in Table 1 and Figure 2A, this resulted in a fast-relaxing S_2 multiline signal already on the first flash after dark-adaptation. (These samples were only studied after one flash, as there were insignificant amounts of oxidized quinone available to allow further S state advancement.) The result was similar for DQH_2 and $PPBQH_2$, but DQH_2 was more consistently able to donate electrons.

The $Y_D^\bullet$ signal behaved in a fashion similar to the multiline signal in the one-flash samples containing quinol, displaying an elevated $P_{1/2}$ value (0.17 mW). The zero-flash sample, however, had a low $P_{1/2}$ (∼0.04 mW). This shows that the addition of quinol in the dark is not sufficient to obtain the

fast-relaxing state; light is also required. In other words, quinol induces the change in relaxation behavior of the donor side paramagnets, and the event is light-driven.

The same effect was seen using continuously illuminated samples (Figure 2B and Table 1). In samples (here dark-adapted overnight on ice) illuminated with white light at 200 K in the presence of MeOH and PPBQ, a typical $P_{1/2}$ of 24 mW was obtained. A similar sample treated with DQH_2 showed a typical $P_{1/2}$ of 64 mW. It is therefore possible to instill second-turnover behavior in samples illuminated at 200 K by treating them with reduced quinone.

The nonsaturated spectra from the two types of continuously illuminated S_2 samples are displayed in Figure 1B. As these samples are very dense ($\sim$10 (mg of Chl) mL^{-1}), they are the best suited for spectral comparison. It is clear that there are no significant differences in the multiline signal itself, nor in its intensity, accompanying the change in relaxation rate reported above.

We have in our earlier report (*12*) excluded oxygen as the source of the relaxation enhancement. It could, however, be possible that a paramagnetic species could be formed if the quinol reduces oxygen. We found that oxygen is consumed in the oxygen electrode upon addition of duroquinol to a PSII buffer solution. This effect was enhanced by light. Addition of catalase showed that the major species formed was hydrogen peroxide. Insignificant amounts of superoxide were formed, as assayed by the addition of superoxide dismutase. Hydrogen peroxide is diamagnetic, but superoxide has a distinct EPR spectrum (*24*) not observed in our samples. This control suggests that any reduction of O_2 by quinol in our samples does not result in the paramagnetic superoxide.

We have shown here that the donor side relaxation behavior is linked to the change in the redox state of the exogenous quinone. To test if the same behavior can be attributed to the naturally present soluble plastoquinone, the experiment was carried out in thylakoid membranes. In this experiment, no exogenous electron acceptor was added, but we relied on the endogenous plastoquinone pool to keep Q_A oxidized. Again, we found similar behavior (Figure 2C). A one-flash sample had a typical $P_{1/2}$ of 48 mW with the range of 16−84 mW. A five-flash sample had a typical value of 110 mW with the range of 50−177 mW. As can be seen from Figure 2C, the data is more scattered, and the difference is smaller but still significant. It is clear that the light-driven reduction of the thylakoid plastoquinone pool also brings about relaxation enhancement of the PSII donor-side paramagnets. We conclude that the quinol-mediated light-adaptation process of PSII occurs in the natural system.

DISCUSSION

Here, we have provided direct evidence that the redox state of the quinone pool, whether exogenous or endogenous, controls the relaxation behavior of the S_2 multiline and $Y_D^\bullet$ signal. We have previously reported a significant relaxation enhancement of these signals in PPBQ-containing PSII membrane fragments commencing at the third flash after dark-adaptation. We explained this enhancement with the appearance of a new paramagnet on the PSII donor side and proposed this paramagnet to arise from a redox reaction with reduced members of the quinone pool in those samples (*12*). In the present study, the addition of reduced quinones to PSII membrane fragments induced the observed relaxation enhancement already on the first flash. This clearly establishes a correlation between the multiline and $Y_D^\bullet$ signal relaxation enhancement and the redox state of the quinone pool.

By studying the relaxation rate of the $Y_D^\bullet$ signal, we obtain access to the S states that do not give rise to readily detectable Mn-centered EPR signals. We found that when the relaxation enhancement was induced by adding quinol to a dark-adapted sample, $Y_D^\bullet$ was not affected in the zero-flash sample: a flash of light was required for $Y_D^\bullet$ $P_{1/2}$ to reach its second-turnover level. This light requirement may be an effect of quinol chemistry, as it was found that light enhanced the consumption of O_2 by DQ in the oxygen electrode (see above). Alternatively, a P680 turnover event may be necessary for the reduction to take place. This would imply transient conformational changes associated with P680 or OEC turnover.

We also found that the S_2 multiline signal relaxation rate in whole thylakoids, which perform multiple PSII turnovers utilizing the natural plastoquinone pool, experiences the same second-turnover relaxation enhancement as the PPBQ-treated membrane fragments. This demonstrates that the quinol-mediated light-adaptation process is a naturally occurring phenomenon, with quinols from the thylakoid plastquinone pool reducing a site in PSII.

Crane et al. (*25*) have reported that the endogenous plastoquinone pool of chloroplasts becomes up to 80% reduced within 5 min of light exposure. In the dark on the other hand, 50−96% (*26*) of the plastoquinone pool is oxidized. Kirchhoff et al. (*3*) showed that only about 50−60% of the plastoquinone pool is involved in the light-saturated linear electron transport from PSII to the Cyt b_6f complex. In stacked thylakoids, about 70% of the PSII centers are located in small domains where one to two PSII share a local pool of a few plastoquinone molecules. They find that the capacity of electron transfer from water to plastoquinone is expected to exceed that of the Cyt b_6f mediated transfer by a factor of 3−5. In other words, there is a build-up of PQH_2 in stacked membranes, which occurs during exposure to light.

The investigations discussed above show a build-up of reduced plastoquinone during the light part of the reaction as linear electron transport through the Cyt b_6f complex is slower than electron transport through PSII. There is therefore additional PQH_2 available to act as a reductant for an electron acceptor in PSII during light-adaptation.

In PSII membrane fragments treated with PPBQ or DQ, maximal relaxation enhancement of the S_2 multiline signal is reached after 10 flashes, at the third enzymatic turnover after dark-adaptation. The multiline signal $P_{1/2}$ obtained on the fifth flash is approximately half of the maximum (*12*). In this material, PSI is absent, and the linear electron transfer through the thylakoid enzymes thus broken. In whole thylakoids with no exogenous quinones added, we have a lower degree of control over the experiment. The range of values reported for the one-flash multiline signal (Table 1) are relatively high, indicating a higher quinol population in the natural plastoquinone pool than in corresponding membrane-fragment samples. The range of $P_{1/2}$ values from five-flash thylakoids are, on the other hand, lower than corresponding membrane-fragment experiment. This probably

reflects the natural competition between light-adaptation chemistry and linear electron transport from PSII to the Cyt b_6f complex. Despite this, the distinction between one- and five-flash data in the thylakoids (Figure 2C) is strong enough to show that light-adaptation does take place in this material.

There is precedence for quinol-mediated reduction in PSII. In earlier studies (*27*, *28*) PSII membrane fragments, devoid of the 23 and 17 kDa extrinsic polypeptides, were treated with hydroxylamine (NH_2OH) and hydroquinone (H_2Q). They found that both compounds reduced the Mn of the OEC but that the region of attack was different. Hydroxylamine-treated samples (3 min, 100 μM) were EDTA sensitive (i.e., the reduced Mn could be removed from their binding site). The hydroquinone-treated samples (30 min, 200 μM), however, were not EDTA sensitive, suggesting that the Mn reduced by H_2Q were sequestered deeper in the protein and not the same as the Mn reduced by NH_2OH. Interestingly, the H_2Q-sensitive Mn were reactivated upon illumination as measured by oxygen evolution. The experiments described above strongly suggest that the Mn in the OEC exists as two separate populations, one nearer the protein surface than the other.

The quinol-dependent effect reported here requires light, as is evidenced by the low $P_{1/2}$ of $Y_D^\bullet$ in a zero-flash sample despite the presence of DQH_2. The identity of the actual species that provides the relaxation enhancement is not clear, but as a quinol is involved, it appears that a reduction of some species takes place. Our previous conclusion that light-adaptation is the result of dipolar interaction between the multiline signal and a new paramagnet (*12*) is thus strengthened. At this stage, we have been unable to identify an EPR signal from such a paramagnet. We have, however, excluded any $S = 1/2$ radical species as a source of the relaxation enhancement. Furthermore, any paramagnetism from an oxidized acceptor side iron is not involved in the light-adaptation process, as the redox state of this iron in the presence of artificial quionone is the same after one and five flashes (*29*). There is no evidence of $Fe^{2+}Q_A^-$ signals in these samples, as expected, except in samples flashed in the presence of quinol, in which the Q_B site is likely to be vacant (not shown). We observe no additional contribution from oxidized Cyt b_{559} in the five flash spectrum, thus excluding redox changes of this species as a cause of the enhancement. We have also excluded oxygen and byproducts of oxygen reduction (see above). However, the relative effect this paramagnet has on the multiline signal and $Y_D^\bullet$ was used to place it 7−15 Å away from the Mn giving rise to the multiline signal and also close to $Y_D^\bullet$ (23−46 Å) (i.e., on the donor side). Here, we postulate further the nature of this paramagnet.

Recent mechanistic modeling of the water-splitting reaction mechanism suggests that only two Mn ions and a Ca ion participate directly in charge accumulation and water oxidation (*30*). It is therefore tempting to explore the possibility that the other two Mn are capable of being reduced and through this reduction contribute the relaxation enhancement of $Y_D^\bullet$ and the S_2 multiline signal on the second turnover of the enzyme. A quinol-mediated two-electron reduction of the other two Mn seems clearly feasible in light of the investigation discussed above (*27*, *28*), where H_2Q was shown to selectively reduce a Mn population deeply sequestered in the protein.

The H_2Q reduction discussed above was undertaken in PSII reaction center complexes devoid of 17 and 23 kDa subunits. The incubation time to reach reduction of Mn to the 2+ oxidation state was 30 min in the dark and slower still if the preparation was stabilized with Ca^{2+}. The reduction observed in the present report is also induced by a quinol but in intact PSII membrane fragments and thylakoid membranes. In these more intact materials, the reduction takes place in less than 1 min and requires light, and no Mn^{II} is observed in EPR.

It is a commonly held view that two Mn undergo the redox changes required for water oxidation, and the other two Mn form an antiferromagnetically coupled even-spin system with $S = 0$ (see below). The currently favored interpretation is that the pair is in the $Mn^{IV}-Mn^{IV}$ state, but $Mn^{III}-Mn^{III}$ cannot be excluded. The exchange coupling for a $Mn^{III}-Mn^{III}$ dimer is small in magnitude as compared to that of a $Mn^{IV}-Mn^{IV}$ dimer (~ -10 cm^{-1} as compared to ~ -200 cm^{-1}) (*31*) so that a portion of the $Mn^{III}-Mn^{III}$ dimer population will be in the first excited state ($S = 1$). Through its paramagnetic excited state, such a species could act as an efficient relaxation enhancer of other paramagnets.

XANES studies provide evidence that the initial S_1 population may contain varying proportions of Mn^{III} and Mn^{IV}. Using K-edge energies either determined by inflection points or by energies at half-height (the most commonly used determination methods), reported data falls into two groups: those with S_1 edge energies of ~6551.5 eV (*32*, *33*) and those with edge energies of ~6553 eV (*34*, *35*). These values can be compared with edge energies for Mn^{III} in synthetic compounds of ~6551.3 eV and that of a mixed-valent $Mn^{III}-Mn^{IV}$ dimer of ~6553 eV (*36*). The differences of edge energies of PSII samples poised in the S_1 state persist through the S_2 and S_3 states, deconvoluted from one- and two-flash data. In the S_0 state, however, after the first turnover of the enzyme, the two groups of data converge, and the edge energies observed are 6550.5−6551 eV in all four reports. This edge energy is consistent with three Mn^{III} and one Mn^{II} in the S_0 state (*36*). These observations suggest that the oxidation state of some of the Mn ions can vary in the S_1 state without disrupting S state advancement upon flash excitation. Furthermore, it seems that irrespective of the original form of the S_1 state, three flashes produce a single form of the S_0 state, involving three Mn^{III} and one Mn^{II}. This does indeed correlate with the presently observed relaxation enhancement patterns of the multiline signal and $Y_D^\bullet$, where the first-turnover signals relax slowly if they have been thoroughly dark-adapted (less so if the dark period was shorter), and the second-turnover $P_{1/2}$ values are high irrespective of the initial state of the sample.

There are two ways in which such oxidation changes can effect the change in relaxation behavior of the ML signal. First, it could be due to a change in the intrinsic relaxation of the tetrameric Mn cluster, brought about by the reduction of two Mn^{IV} to two Mn^{III} in the second turnover of the enzyme. The shorter spin−lattice relaxation time of Mn^{III} ions then leads to an increase in $P_{1/2}$ (*37*). Second, if the Mn were organized as two separate pairs, the reduction of the second pair could lead to its paramagnetic excited state (see above) acting as an efficient relaxation enhancer of the dimer giving rise to the multiline signal.

The first option is the least favored by us. A change in the oxidation state of a pair of Mn from IV to III would lead to changes in the exchange couplings within a tetrameric cluster. Even small changes in exchange couplings have been shown to alter the hyperfine structure of multiline signals (*38*). Furthermore, the intrinsic hyperfine constants are typically smaller for a Mn^{III} ion than a Mn^{IV} ion (*39*), which would also lead to changes in the hyperfine structure. As shown in Figure 1, the multiline signal is nearly identical in the dark- and light-adapted states. This is a strong indication that no major modifications of the exchange couplings within the cluster have taken place (*38*, *40*). As the relaxation changes observed are strong, giving a 10-fold increase in the multiline $P_{1/2}$, minor modifications of these couplings are unlikely to bring about the full change. However, the complexity of the magnetic interactions within a tetrameric cluster are difficult to predict and give rise to unexpected effects.

The data discussed here is in our view more readily explained in terms of separate Mn dimers. The Mn^{III} dimer would enhance the relaxation of the multiline dimer through its paramagnetic excited state and would fit in with the estimate of the distance between the two paramagnets and Y_D. The recently emerging crystal structures (*41*, *42*) have so far identified only one Mn density, which makes the above interpretation more difficult. However, the crystal structures are both from strains of *Synechococcus*, and the data reported here is from plant material. The only available data on Mn stoichiometry in *Synechococcus* quotes one Mn per 18 Chl, 2.7 Mn per Q_A^- (*43*). The number of chlorophyll molecules identified in the crystal structures are 35 (*42*) and 36 (*41*). Similar analysis in highly active core preparations from spinach gives one Mn per seven Chl and 32 Chl in each reaction center (*44*). Of course, if cyanobacteria contain less Mn than plants, $P_{1/2}$ studies of cyanobacteria should reveal this difference. Such studies are currently being planned. We have so far not identified a $Mn^{III}-Mn^{III}$ dimer spectroscopically. However, if such a species exists in the light-adapted S_2 state (or S_2 generated in the presence of quinol) and not in a thoroughly dark-adapted state S_2 state, parallel polarization studies should be able to bring this out. We have commenced such studies.

A third option may be gradual changes of the protein lattice, facilitating energy release and thus enhancing the multiline and Y_D relaxation rates. This would be expected to result in different Raman relaxation behavior from the two types of samples, contrary to what was observed (*12*). It is unclear how the redox state of the quinone pool would affect the protein surroundings of the Mn and Y_D or how it would affect the couplings within the multiline spin center to bring about the changes observed.

We conclude that our data on flash-dependent changes of the Mn cluster and Y_D, and the correlation to the redox state of the quinone pool, are best accounted for by the appearance of a new paramagnet near the multiline spin center, induced by an increasing quinol population.

Although we have no direct evidence at this moment that the relaxation enhancement observed upon the second turnover of the enzyme is due to reduction of the second pair of Mn, we have presented compelling reasons why this may be so. We have shown experimentally that the light-adaptation effect is brought about by the reduced quinone pool, whether exogenous or endogenous. Other studies have shown that Mn can be reduced by quinols and still remain intact and bound in the protein. There is also XANES evidence that the initial oxidation levels in the S_1 state may vary. Our study indicates that the variation of that state could depend on the redox state of the quinone pool.

CONCLUSION

As the pool of quinones becomes increasingly reduced by PSII turnover, it is able to reduce a center near the multiline Mn. This center then becomes paramagnetic and enhances the relaxation rate of the S_2 multiline signal and the $Y_D^\bullet$ signal. The nature of this paramagnet is not known, but a suggested candidate is one of the Mn dimers of the OEC. We present the idea that double electron transfer may occur from quinol to the two Mn ions not involved in the redox cycling of water oxidation, changing from $Mn^{IV}-Mn^{IV}$ to $Mn^{III}-Mn^{III}$ in the light-adapted state. We have confirmed in this study that the relaxation enhancement is induced by the presence of quinols and that the reduction mediated by quinol is a light-driven event. Furthermore, we have shown that light-adaptation is part of the natural system, as the thylakoid plastoquinone pool itself gives rise to the enhancement without additional quinones.

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