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Redox interaction of Tyrosine-D with the S-states of the water-oxidizing complex in intact and chloride-depleted Photosystem II

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Abstract

The light-induced oxidation of Tyrosine-D in Photosystem II has been studied by time-resolved measurements of the EPR Signal II_{slow} at room temperature. When induced with single turnover flashes, the oxidation of Tyrosine-D undergoes a period-four oscillation as a function of flash number, showing Tyrosine-D^+ formation in the S_2 and S_3 oxidation states of the water-oxidizing complex. The kinetics of Tyrosine-D oxidation by the S_2 and S_3 states are almost identical in the pH range of 4.5 to 7.8, and show the same pH dependence for the S_3 state as has previously been observed for the S_2 state (Vass and Styring (1991) Biochemistry 30, 830–839). It is concluded from the pH-dependent oxidation kinetics that a proton binding with a pK around 7.0–7.2 retards electron transfer from Tyrosine-D to the water-oxidizing complex both in the S_2 and in the S_3 states. In addition, our results imply that the S_2/S_1 and S_3/S_2 redox couples have about the same redox potential relative to that of the Tyrosine-D^+/Tyrosine-D couple. Removal of chloride from Photosystem II induced an approximately 10-times slowdown in the Tyrosine-D oxidation kinetics by the S_2 state. This result indicates that Tyrosine-D can interact with the S_2 state in the absence of chloride. The retarded oxidation kinetics observed under these conditions are consistent with the previously demonstrated stabilization of the chloride-free S_2 state. We also observed the flash-induced oxidation of Tyrosine-Z in a large fraction of the chloride depleted Photosystem II centers. In this system Tyr-Z^+ was abnormally stable and decayed biphasically with 500 ms and 12–15 s half-times.

Key words: Photosystem II; Oxygen evolution; Water-oxidizing complex; Tyrosine-D; Chloride ion depletion; Manganese cluster

1. Introduction

Photosystem II (PS II) couples the oxidation of water with the reduction of plastoquinone (for recent reviews see [1–3]). Even though PS II contains at least 20 subunits, the primary photochemistry takes place in the relatively small reaction center complex, which is constituted by a heterodimer of two hydrophobic proteins, D1 and D2. The D1/D2 heterodimer binds the primary electron donor P680, the primary electron acceptor pheophytin, and the first (Q_A) and second (Q_B) quinone electron acceptors [4]. Both the D1 and the D2 proteins contain a redox active tyrosine residue, denoted as Tyr-Z and Tyr-D, respectively, which serve as electron donors to P680. Tyr-Z is tyrosine-161 on the D1 protein [5,6], and Tyr-D is tyrosine-160 on the D2 protein [7,8]. The catalytic manganese cluster involved in the oxidation of water is also thought to be bound by the D1/D2 heterodimer [9–12].

Water oxidation requires the accumulation of four oxidizing equivalents. This is accomplished by cycling the water-oxidizing complex through five oxidation states during illumination. These are termed S-states and designated S_n, where n stands for the number of oxidizing equivalents stored [13,14]. Molecular oxygen

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Abbreviations: Chl, chlorophyll; DAD, diaminodurene; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholine-ethane-sulfonic acid; P680, primary electron donor of PS II; PS II, Photosystem II; Q_A, primary electron acceptor of PS II; Q_B, secondary quinone acceptor of PS II; Tyr-Z, tyrosine-161 on the D1 protein, electron carrier between P680 and the water-oxidizing complex; Tyr-D, tyrosine-160 on the D2 protein, accessory electron donor in PS II; PpBQ, phenyl-*p*-benzoquinone; S_0-S_4, charge storage states of the water-oxidizing complex.

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is released in the S_3-S_4-S_0 transition, in which S_4 is a short-lived intermediate.

During the light induced redox cycling of the water-oxidizing complex, four electrons are transferred sequentially from the catalytic manganese cluster to $P680^+$ via Tyr-Z, which is an intermediary electron carrier. The oxidation of Tyr-Z by $P680^+$ occurs in the 20–200-ns time scale [15], whereas the reduction of Tyr-Z^+ by the water oxidizing complex takes place in the microsecond to millisecond time scale, depending on the oxidation state of the manganese cluster [5,16].

The other redox active tyrosine, Tyr-D, also becomes oxidized in the light but, unlike Tyr-Z^+, Tyr-D^+ is stable in the dark for tens of minutes to hours [17–19]. Although Tyr-D does not participate in the steady-state electron transfer from water to $P680^+$, it is in a complex charge equilibrium with the water-oxidizing complex: in the S_0 state the water-oxidizing complex slowly reduces Tyr-D^+ in tens of minutes, concomitant with the formation of the S_1 state [19–22]. In the S_2 and S_3 states, however, the water-oxidizing complex oxidizes Tyr-D in a few seconds [18–23].

Oxidation of the redox-active tyrosines gives rise to characteristic EPR signals called Signal $II_{very\ fast}$ from Tyr-Z^+ [24] and Signal II_{slow} from Tyr-D^+ [18]. These signals have similar spectral characteristics but decay with very different kinetics (hence the denotations 'very fast' and 'slow') [5,25]. The spectra have recently been assigned to the neutral, deprotonated form of the tyrosine radicals [25].

Previously we have characterized in details the redox interaction of Tyr-D with the S_0 and S_2 states of the water-oxidizing complex [19]. That study made possible the estimation of the midpoint redox potential of the Tyr-D^+/Tyr-D couple relative to those of the S_1/S_0 and S_2/S_1 couples: $E_m(S_1/S_0) - E_m(\text{Tyr-D}^+/\text{Tyr-D}) >= 40$ mV, $E_m(\text{Tyr-D}^+/\text{Tyr-D}) - E_m(S_2/S_1) >= 170$ mV [19]. These redox potential differences could well explain both the oxidation of Tyr-D by the S_2 state and the reduction of Tyr-D^+ by the S_0 state.

We also showed that two protonation events influence the charge equilibrium between Tyr-D and the water-oxidizing complex. A proton binding (pK 7.5) in the vicinity of Tyr-D retards its oxidation by the S_2 state. This pK has been assigned to His190, in the D2 protein, which is most likely hydrogen bonded to Tyr-D [10,19]. In addition, the binding of a proton near the water-oxidizing complex (pK 5.6) accelerates the oxidation of Tyr-D by the S_2 state and retards the reduction of Tyr-D^+ by the S_0 state [19].

The oxidation of Tyr-D by the S_3 state is also well documented by using EPR [18] or flash-induced oxygen evolution measurements [20,22]. However, the kinetics of this process are not yet known to the same extent as those of Tyr-D oxidation by the S_2 state. The available results indicate similar kinetics for the oxidation of Tyr-D by the S_2 and S_3 states at neutral pH [18,20,22], but the effects of protonation on the oxidation of Tyr-D by the S_3 state have not been studied.

Since the charge exchange between Tyr-D and the S-states is governed by the respective redox potential differences, changes in the stability of the S_2 (or S_3) state are expected to influence the Tyr-D oxidation kinetics. Removal of Cl^- from the water-oxidizing complex is known to induce an approx. 10-times increase in the stability of the S_2 state, and to block the formation of higher S-states [27–29]. Thus, Cl^- depletion will probably influence the Tyr-D oxidation kinetics, which might provide further insight into the nature of the modifications brought about by Cl^- removal in the water-oxidizing complex.

Here we have characterized the effects of protonation on the oxidation of Tyr-D by the S_3 state, and studied also Tyr-D oxidation by the S_2 state in Cl^--depleted PS II. The results show that oxidation of Tyr-D in the S_3 state has practically the same kinetic characteristics as found in the S_2 state. In contrast, Cl^- depletion induced a substantial retardation of Tyr-D oxidation, in agreement with the known stability increase of the S_2 state in the absence of Cl^-. In Cl^--depleted samples we also observed a fraction of centers with slowed down reduction of Tyr-Z^+.

2. Materials and methods

Sample preparation

PS II-enriched membranes (BBY particles [26]) were prepared from spinach thylakoids and were stored at −80°C in 0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 20 mM MES (pH 6.3), at 3–4 mg Chl/ml.

Before the flash experiments, BBY particles were kept in darkness at room temperature for 30 min to allow centers in the S_0Tyr-D^+ state to decay to the S_1Tyr-D state [20,21]. This treatment serves to synchronize the centers in the S_1 state. The dark incubation was followed by chemical reduction of Tyr-D^+ with 1 mM DAD and 5 mM sodium ascorbate at 1 mg Chl/ml, in the dark for 30 min at room temperature [21]. The reducing chemicals were removed by repeated dilution and centrifugation. The membranes were thereafter resuspended in either 50 mM L-glutamic acid (pH 4.5–5.0), MES (pH 5.0–7.0) or Hepes (7.0–8.0) buffers with the same additions as in the BBY storage buffer.

Cl^- depletion was achieved by replacement of Cl^- with SO_4^{2-} according to [28]. BBY particles were washed twice in 0.4 M sucrose, 1 mM NaCl, and 4 mM MES (pH 6.5), and then diluted (10-fold) with 0.4 M sucrose, 50 mM Na_2SO_4, and 40 mM MES (pH 7.5) and incubated for 15 min in darkness. The membranes were collected by centrifugation and suspended in 0.4

M sucrose, 5 mM $MgSO_4$, and 40 mM Hepes (pH 7.0). After this treatment, the remaining oxygen evolution of the PS II membranes was about 10% of the nontreated control, which could be restored to about 90% of the original activity by the addition of 40 mM NaCl.

During the Cl^- depletion treatment, which was performed in the dark, a large fraction (about 40%) of Tyr-D^+ was reduced. Further reduction of the Cl^--depleted PS II membranes by DAD/ascorbate led to complete loss of Tyr-D^+. In these samples, however, only a very limited light-induced oxidation of Tyr-D could be observed (not shown), which is probably due to the inactivation of the water-oxidizing complex in the Cl^--depleted and reduced PS II membranes. Thus, we restricted our kinetic studies of Tyr-D oxidation to the fraction of Tyr-D that was reduced during the Cl^--depletion procedure.

EPR measurements

Room temperature (21°C) EPR measurements were performed at 9.799 GHz, in a 330 μl quartz flat cell at 1.5–2.0 mg Chl/ml in the presence of 1 mM EDTA. After the sample reduction, P*p*BQ dissolved in dimethyl sulfoxide was added to a final concentration of 0.2 mM as an electron acceptor to avoid acceptor side limitations of S-state turnovers. Oxidation of Tyr-D was induced by single-turnover flashes of saturating intensity from a Nd-YAG laser (Quanta-Ray DCR-3G, 8 ns, 300 mJ, 532 nm) directed into the EPR cavity. The flash frequency was 3 Hz. The formation of Tyr-D^+ as well as the rapid formation and subsequent slow rereduction of Tyr-Z^+ was followed by measuring the rise and decay kinetics (in the seconds time scale) of Signal II at its low-field peak (347 mT) with a Bruker ESP 300 EPR spectrometer, equipped with a Bruker 8102 standard cavity. Data acquisition was performed with the ESP 300 program of the EPR spectrometer. Exponential analysis of the kinetic traces was done with an IBM AT computer using a least-squares curve fitting program. Tyr-D oxidation kinetics were analyzed in terms of mono- or biphasic exponential rise, above and below pH 7.0, respectively. For the analysis of kinetic traces after two or three flashes, the zero point of the exponential fitting was set to the time of the second or third flash, respectively. In addition, a baseline constant was included to correct for the increase of the signal between the first and second or third flashes.

3. Results

S-state dependence of Tyr-D oxidation

The redox interaction of Tyr-D with the water-oxidizing complex has been demonstrated in both the S_2 and S_3 states, either by directly monitoring Tyr-D oxidation with EPR measurements [18,19] or by following the reduction of the S_2 and S_3 states by Tyr-D using flash-oxygen measurements [20,22].

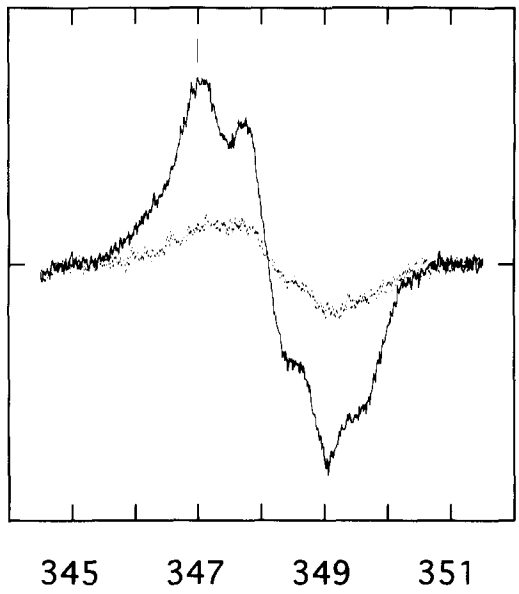

Fig. 1. EPR spectra of Signal II_{slow} in isolated PS II membranes. BBY particles were washed with 1 mM DAD and 5 mM sodium ascorbate to reduce Tyr-D^+. Signal II_{slow} was measured in the presence of P*p*BQ as electron acceptor either in the dark (dotted spectrum) or after illumination with one saturating laser flash (solid spectrum). The bar indicates the field position used in the kinetic measurements. EPR conditions: $T = 294$ K; microwave power, 20 mW; modulation amplitude, 0.5 mT.

Here we aim at a detailed comparison of the Tyr-D oxidation kinetics in the S_2 and S_3 states. In our previous work, where the kinetics of Tyr-D oxidation in the S_2 state were studied [19], only a limited formation of the S_3 state could be achieved, most likely due to acceptor side limitations of electron transfer in our PS II membrane preparation. To improve this situation, P*p*BQ was added as an artificial electron acceptor to the PS II membranes in which Tyr-D^+ had been reduced by DAD/ascorbate. In the reduced samples Signal II_{slow} was completely lost in the absence of P*p*BQ (spectra not shown). Subsequent addition of P*p*BQ in the dark induced a small EPR radical signal in the $g = 2$ region (Fig. 1), which possibly arises from reduced P*p*BQ or from Tyr-D^+ accidentally oxidized in the presence of P*p*BQ. Illumination of these samples with a single saturating flash at room temperature restored the majority of Signal II_{slow} (Fig. 1) due to the reaction S_2Tyr-D → S_1Tyr-D^+. The amplitude of the radical observed in the dark is about 12–15% of the amplitude of the total light inducible Signal II_{slow} at the position of the low field peak.

To allow studies of Tyr-D oxidation in the S_3 state with only minor influence from centers in the S_2 state,

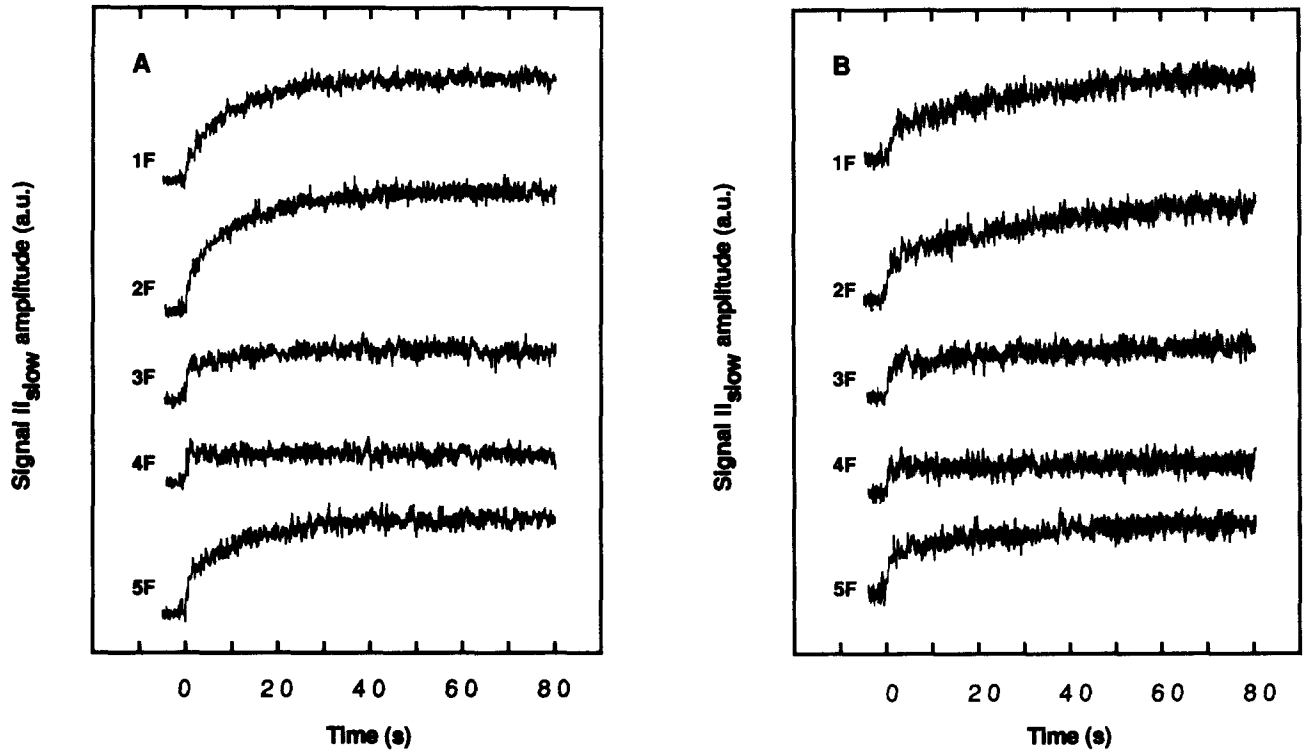

Fig. 2. Signal II_{slow} formation after flash illumination at pH 6.1 (A) and pH 5.1 (B). BBY particles were treated with DAD/sodium ascorbate to reduce Tyr-D^+. The samples were illuminated with a series of saturating laser flashes with a frequency of 3 Hz. The oxidation of Tyr-D was followed by measuring the rise kinetics of Signal II_{slow} at its low field peak (347 mT, Fig. 1) with the same EPR conditions as in Fig. 1. (conversion time, 80 ms/point; time constant, 40 ms).

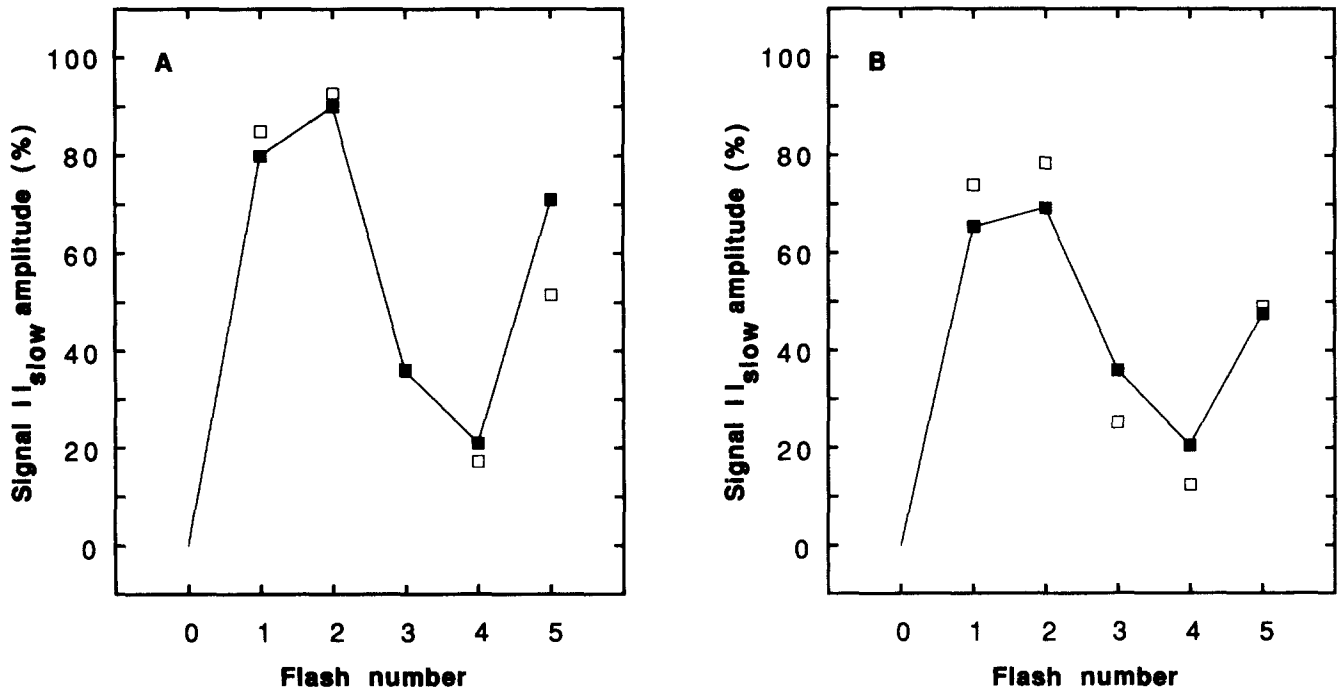

Fig. 3. Flash-induced oscillation of the amplitude of Signal II_{slow}. The experimental amplitudes (filled squares) were measured at pH 6.1 (A) and pH 5.1 (B) 80 s after the flashes. These amplitudes are shown as a percentage of the maximal signal size that could be induced by continuous illumination of the same sample. The simulated values (open squares) were calculated by assuming 0:100:0:0 distribution of the $S_0:S_1:S_2:S_3$ states before the flashes. The miss and double hit parameters were 0.15 and 0.03 at pH 6.1, and 0.12 and 0.03 at pH 5.1. An additional assumption to simulate the oscillation at pH 5.1 was that Tyr-D oxidation after few flashes occurred at this pH in only 80% of the centers, which were able to oxidize Tyr-D in continuous light. Measuring conditions as in Fig. 2.

long dark incubation (30 min) was applied before Tyr-D$^+$ was reduced by the DAD/ascorbate. The long dark adaptation is known to synchronize the centers in the S_1 state before the flashes [20,21]. It thus minimizes the amount of centers which remain in the S_2 state after two flashes (see below).

The flash-induced oxidation of Tyr-D was followed by monitoring the rise of Signal II_{slow} at the position of the low field peak. Such kinetic traces, recorded after 1 to 5 flashes at pH 6.1 and pH 5.1, are shown in Fig. 2A and B. The total amplitude of the light-induced increase in Signal II_{slow} shows a clear period-four oscillation as a function of flash number. The oxidation of Tyr-D is large after one and two flashes but small after three and four flashes. The oscillation pattern can be well simulated using an extension of the Kok model of S-state turnovers [13,14], which takes into account the oxidation of Tyr-D in both the S_2 and the S_3 states [22](Fig. 3A and B). It is of note that at pH 5.1 only about 70% of the total light-inducible signal was observed during the first few flashes, while the rest could only be induced by continuous illumination.

From the simulation of the oscillatory pattern, about 96% of the reacting centers are expected to be in the S_2 state after the first flash. Thus, the kinetic trace after one flash clearly arises from the oxidation of Tyr-D by the S_2 state. The simulation also shows that immediately after two flashes, about 26% and 74% of the centers which are interacting with Tyr-D are expected to be in the S_2 and S_3 states, respectively. Thus, after the second flash the kinetic trace of Tyr-D oxidation is dominated by the S_3 state, with only one-quarter of the signal still arising from oxidation by the S_2 state.

pH effects on the oxidation of Tyr-D by the S_2 and S_3 states

The analysis of Tyr-D oxidation by the S_2 state, measured after one flash, gave essentially the same results as obtained earlier in DCPIP/ascorbate treated samples in the absence of artificial electron acceptors [19]. The oxidation kinetics were monophasic at or above pH 7.0 (not shown) and become clearly biphasic at lower pH levels (Fig. 2A, B). The half-time for the slow phase increased from about 1 s at pH 7.5 to more than 30 s at pH 4.6 (Fig. 4A). The pH dependent lifetime, and rate constant, of Tyr-D oxidation by the S_2 state could be well fitted by assuming a single proton binding with pK about 7.0 (Fig. 4A) in agreement with our previous results [19]. The relative amplitude of the fast phase (0.5–1 s) showed a similar increase with lowering the pH as found earlier [19] (not shown).

Analysis of the Tyr-D oxidation kinetics after two flashes is complicated by the fraction of centers which remain in the S_2 state (see above). The effect of this residual S_2 population on the Tyr-D oxidation kinetics was taken into account by including additional exponential components (one above pH 7.0 and two below pH 7.0) into the fitting process. The lifetime of these components, which represent the S_2 state, were fixed at the values obtained from the analysis of the one-flash curves, while their amplitudes were fixed according to the S_2/S_3 ratio after two flashes obtained from the simulation of the oscillatory patterns (Fig. 3).

The analysis of the Signal II_{slow} induction traces after two flashes revealed monophasic kinetics for the oxidation of Tyr-D by the S_3 state above pH 7.0 and

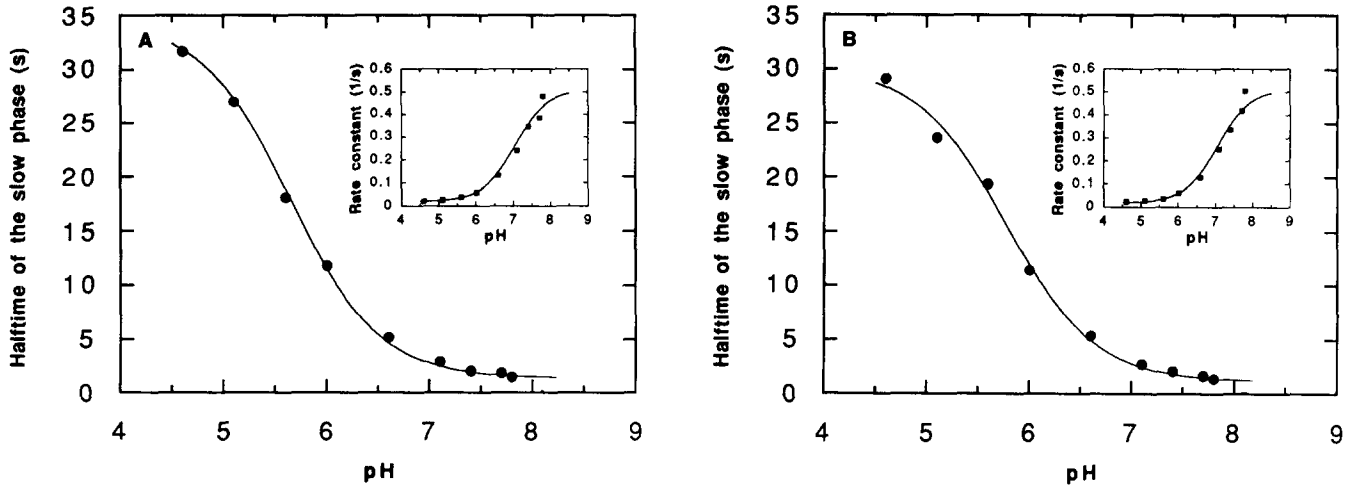

Fig. 4. pH dependence of the kinetics for Tyr-D oxidation in the S_2 (A) and the S_3 state (B). The half-time of the slow phase for the S_2Tyr-D → S_1Tyr-D$^+$ electron transfer was obtained from the analysis of one-flash traces similar to those in Fig. 2. The half-time of the slow phase for the S_3Tyr-D → S_2Tyr-D$^+$ electron transfer was obtained from the analysis of the two-flash traces as described in the text. The solid curves represent one-proton titration curves with pK 7.0 and 7.2 in (A) and (B), respectively. The insets show the same data expressed as rate constants.

biphasic kinetics below pH 7.0. This is very similar to the situation after one flash, i.e., in the S_2 state. The pH dependent lifetimes and amplitudes obtained for the slow phase (1–28 s) were well reproducible. However, the amplitudes of the fast phase (0.5–1 s) were less reliable. This is due to uncertainties in determining the extent of Tyr-D oxidation between the first and second flashes. Thus, the present analysis is concentrated on the comparison of the slow phases of Tyr-D oxidation by the S_2 and S_3 states.

The rate of the slow phase of Tyr-D oxidation by the S_3 state, calculated after two flashes, is strongly pH dependent, and increases from about 1 s at pH 7.5 to about 28 s at pH 4.6 (Fig. 4B). Above pH 6.0, the oxidation rates are almost identical to those obtained in the S_2 state, while they are somewhat faster below pH 6.0 (compare Fig. 4A and B).

The pH dependence for the oxidation of Tyr-D by the S_3 state (Fig. 4B) can be well explained by binding of a proton with pK 7.2, in fast equilibrium with the bulk solution, that retards electron transfer from Tyr-D to the water splitting enzyme. This pK is similar to that obtained for the slowdown of electron transfer from Tyr-D in the S_2 state, and it is highly likely that the same titrable group is observed in both reactions.

We have also analyzed the kinetic traces obtained after three flashes without correction for any residual S_2, which is expected to be less than 15% on the basis of the simulation shown in Fig. 3. Despite the substantially smaller signal-to-noise ratio, the lifetimes of the slow phase obtained from the analysis of the three-flash traces were almost identical to those after the second flash (not shown).

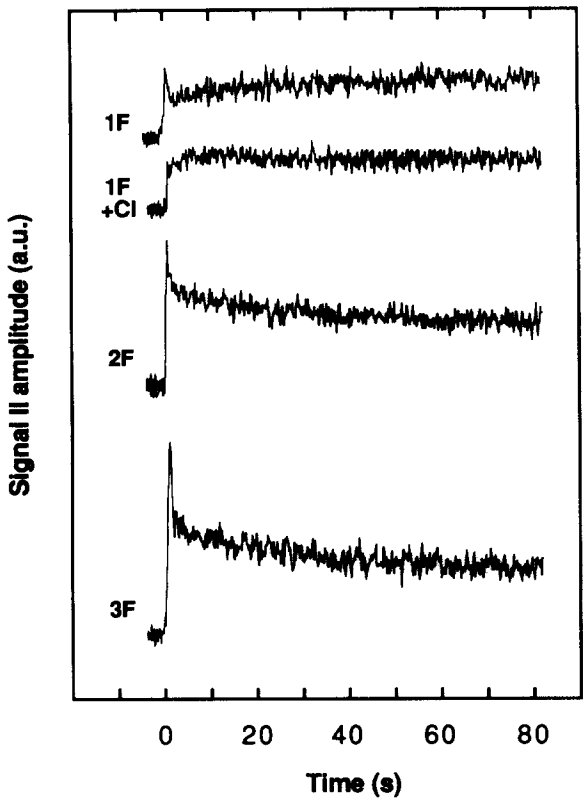

Fig. 5. Signal II formation and decay after flash illumination in Cl^- depleted BBY particles. Cl^--depleted BBY membranes, in which Tyr-D^+ was spontaneously reduced in about 30% of the centers, were illuminated with a series of saturating laser flashes. The oxidation of Tyr-D and Tyr-Z was followed by measuring the rise kinetics of Signal II under the same conditions as in Fig. 2, both in the absence of Cl^- from the suspension medium (1, 2 and 3 flashes) or in the presence of 40 mM NaCl (shown after the first flash).

Flash-induced oxidation of Tyr-D in Cl^--depleted PS II

Oxidation of Tyr-D by the water-oxidizing complex in the S_2 and S_3 states and the reduction of Tyr-D^+ in the S_0 state is explained by the different redox potentials of these redox species, i.e., $E_m(S_1/S_0) < E_m$(Tyr-D^+/Tyr-D) $< E_m(S_2/S_1)$, $E_m(S_3/S_2)$ [19,21]. The stability, and in turn the redox potential, of different S-states is often changed accompanying modifications to the water-oxidizing complex. This might also influence the oxidation rate of Tyr-D. One example of a modification that influences the Mn cluster is the removal of Cl^- by substitution with SO_4^{2-}. This treatment is denoted Cl^--depletion, and is known to induce a substantial stabilization of the S_2 state. This effect is manifested in the approx. 10-times longer lifetime of the S_2 state at room temperature [27–29], corresponding to an approximately 50–70 mV decrease in the $E_m(S_2/S_1)$ relative to that in the presence of Cl^- [29].

In order to explore the effects of Cl^- on the charge equilibrium between Tyr-D and the water-oxidizing complex, the flash-induced oxidation kinetics of Tyr-D were studied in Cl^--depleted PS II membranes.

During the Cl^--depletion treatment, about 30–40% of Tyr-D^+ was reversibly reduced. In such samples a single flash induced a very fast rise of Signal II, which was faster than the resolution of our EPR spectrometer (Fig. 5). The initial jump of Signal II decayed in about 1 s. This decay was superimposed on a much slower rise of the signal which is evident in the one-flash trace in Fig. 5. After 60–80 s the level of the signal was saturated, and spectral measurements revealed that a substantial fraction of Tyr-D had been oxidized by the flash (not shown). Following two or more flashes, the size of the initial jump largely increased relative to that after one flash (Fig. 5). This initial increase was followed by a biphasic decay to a level which was substantially higher than before the flashes (Fig. 5).

Our interpretation of this complex behavior is that the flashes result in the formation of Signal II_{slow}, i.e., the oxidation of Tyr-D, in the Cl^--free PS II in a slow reaction that is completed in about 80 s (Fig. 5). Superimposed on this, there is a rapid increase and a relatively slow decay of Signal II_{fast} that originates from Tyr-Z^+. Readdition of Cl^- suppressed most of

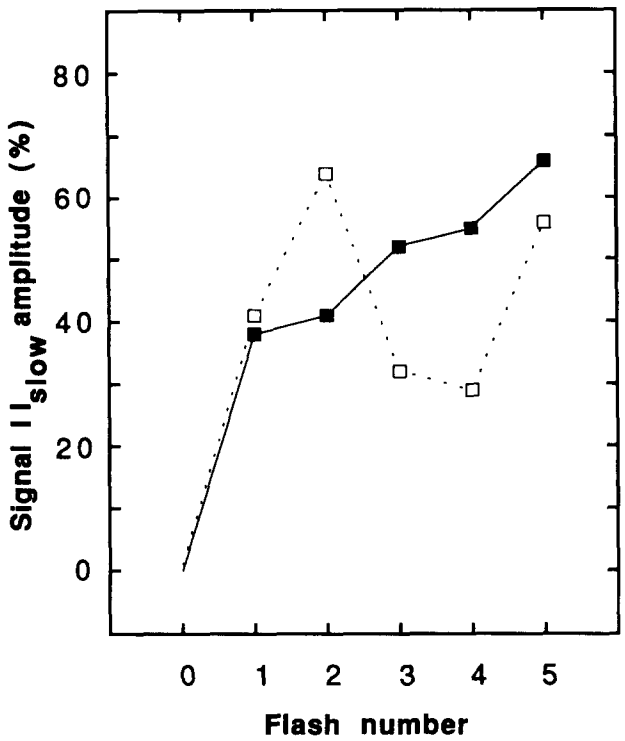

Fig. 6. Flash-induced oscillation of Signal II_{slow} amplitude in Cl^--depleted and Cl^--repleted BBY particles. The amplitudes were obtained 80 s after the flashes. They are shown as a percentage of the maximal signal increase that could be induced by continuous illumination in Cl^--depleted (closed squares) and Cl^--repleted (open squares) BBY particles.

the rapid initial increase and the subsequent decay of Signal II (Fig. 5). At the same time, the very slow rise kinetics of Signal II_{slow} were changed to faster ones, similar to those observed in the intact samples at the same pH (pH 7.0) (Fig. 5). This clearly demonstrates that Tyr-Z^+ formation occurs in centers that are reversibly modified by Cl^--depletion.

In contrast to the nontreated control, Tyr-D oxidation in the Cl^--depleted samples did not show the normal period-four oscillation. Instead, the total amount of oxidized Tyr-D, observed after the equilibrium between the rise and decay kinetics, was substantially increased after the first flash. Illumination with subsequent flashes resulted only in small further additions to the amplitude of Signal II_{slow} (Fig. 6). Cl^- repletion partially restored the flash-induced oscillation of Tyr-D oxidation (Fig. 6), even though with a higher damping than observed in the intact samples (compare with Fig. 3).

The slowly rising component of Signal II after one flash in the Cl^--depleted samples has a half-time of about 16 s at pH 7.0. This is much slower than the 1.7–2 s obtained in the Cl^--repleted or non-Cl^--depleted samples at the same pH (Fig. 4A). Since the slow rise kinetics result in the formation of Tyr-D^+, and they are transformed into faster kinetics by Cl^- readdition, we suggest that this slow (16 s) phase arises from the oxidation of Tyr-D by the modified S_2 state of the Cl^--depleted PS II.

Since there was a net increase of Signal II after 80 s, the kinetic traces after two or three flashes were analyzed on the assumption that the initial fast induction of Signal II is followed by a decay that is superimposed on a monophasic rising component. From the flash traces it is obvious that the decay of Signal II after the flashes is biphasic (best seen in the 3-flash trace in Fig. 5). With these assumptions 300–500 ms and 12–15 s decay phases and a 12–16 s rising phase were obtained. The total amplitude of the transient signal corresponds to a radical species in about 30% of PS II centers when compared to the size of Signal II_{slow} obtained after the complete oxidation of Tyr-D.

After a few flashes almost all Tyr-D was oxidized. Nevertheless the rapid increase in the Signal II amplitude followed by the biphasic decay, now to the baseline, remained. The decay was dominated by a 300–500 ms component (approx. 80% relative amplitude) with a smaller contribution (approx. 20%) from a 12–15 s component similarly to that obtained from the analysis of the two- and three-flash traces. The decay kinetics and amplitudes of these phases were constant over a large number of flashes, indicating the existence of a compound, most likely Tyr-Z, which was transiently oxidized with high yield after two and more flashes. The assignment of the transient signal to Tyr-Z is supported by the spectral shape of the decay signal, which is very similar to that of the normal Signal II shape (Fig. 7).

Readdition of Cl^- largely suppressed the rapid increase in the amplitude of Signal II also after several flashes (not shown), and consequently the subsequent decay phases, indicating that these phenomena are also related to reversible modifications of the donor side in PS II in the absence of Cl^-.

4. Discussion

Even though Tyr-D does not mediate steady-state electron transport from water to $P680^+$, it has been shown to maintain a complex charge equilibrium with the water-oxidizing complex. This charge equilibrium leads to the oxidation of Tyr-D by the S_2 and S_3 states [18–23] and to the reduction of Tyr-D^+ by the S_0 state [19–22]. The detailed kinetic characterization of Tyr-D oxidation by the S_2 state, which we have performed in our previous paper [19], was extended in the present work to study the oxidation of Tyr-D by the S_3 state and by the modified S_2 state of Cl^--depleted PS II.

Oxidation of Tyr-D by the S_3 state

By using single turnover flashes to initiate the oxidation of chemically prereduced Tyr-D, a characteristic

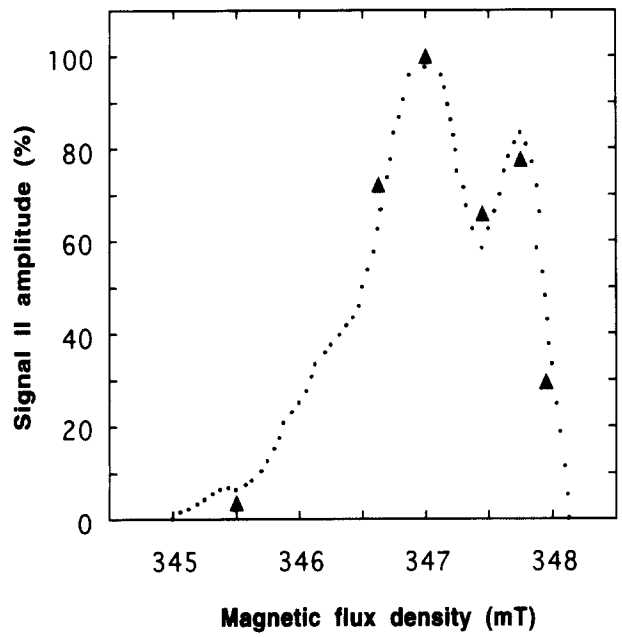

Fig. 7. Kinetic spectrum of the light-induced transient EPR signal in Cl^--depleted BBY particles. After complete oxidation of Tyr-D with 10 flashes, the samples were illuminated with a single laser flash and the induction of Signal II was measured at different field positions. The maximal amplitude of the induced signal (▲), measured immediately after the flash, is shown together with the shape of Signal II_{slow} (dotted line).

period-four oscillation was observed in the amount of Tyr-D^+ formed after the flashes. The oscillation was similar to that obtained earlier [18], and has been used here to estimate the amount of S_2 and S_3 centers which react with Tyr-D after one, two or three flashes. Our simulation of the oscillatory pattern indicate that after the first flash Tyr-D oxidation is totally dominated (in 96% of the reacting centers) by the S_2 state. After the second and third flashes the S_3 state was dominating the oxidation reaction (in about 74% and 85% of the reacting centers, respectively). Thus, our experimental protocol, which includes a long dark adaptation to synchronize the PS II centers in the S_1 state, and the addition of an artificial electron acceptor allows the formation of the S_3 state in the majority of the centers after two or three flashes.

The kinetic traces after one or two flashes were monophasic above pH 7.0, and become biphasic below pH 7.0. Since the lifetime of the faster phase (0.5–1 s) was close to the time delay between the exciting laser flashes (0.3 s), the relative amplitude of this phase after the second (and third) flashes could not be clearly resolved. Thus, our analysis is concentrated on the slow phase of the oxidation kinetics. In the pH range of 7.8 to 6.0 the half-rise time of the slow phase of Tyr-D oxidation was almost identical in the S_2 and S_3 states, and was increasing from about 1 s (at pH 7.8) to 12 s (at pH 6.0). Below pH 6.0, Tyr-D oxidation occurred somewhat faster in the S_3 (reaching 28 s at pH 4.6) than in the S_2 state (reaching 32 s at pH 4.6).

The pH dependence of the oxidation rates indicated a single proton binding with pK values of 7.0 and 7.2 in the S_2 and S_3 states, respectively. These pK values are very close to that obtained for the S_2 state (pK 7.3) in our previous study [19]. The pH dependent retardation of electron transfer towards the water-oxidizing complex with essentially the same pK both in the S_2 and S_3 states supports our previous conclusion that the proton binding occurs in the vicinity of Tyr-D. Based on computer graphics modelling of the protein structure of PS II, Tyr-D was suggested to form a hydrogen bond with His-190 of the D2 protein [10,30] and we predicted that protonation of His-190 is responsible for the pH-dependent kinetics of Tyr-D oxidation [19]. The existence of this hydrogen bonding interaction has also been supported by EPR studies on genetically engineered cyanobacterial mutants. The replacement of His-189 with Tyr or Leu in the D2 protein of *Synechocystis* 6803 resulted in the loss of normal Signal II_{slow}. Instead, a narrow light-inducible radical that could be assigned to Tyr-D^+ was observed [31,32].

The very similar kinetics of Tyr-D oxidation indicate that the water-oxidizing complex behaves almost identically in the S_2 and S_3 states in respect to its reaction with Tyr-D. This also implies that the S_2/S_1 and S_3/S_2 redox couples have about the same redox potential relative to that of the Tyr-D^+/Tyr-D couple. The similar ability of the S_2 and S_3 states to oxidize Tyr-D is in agreement with their similar room temperature stabilities when compared to the short-lived S_4 state or to the much more stable S_0 and S_1 states. However, the stabilities of the S_2 and S_3 states are not completely identical, and some measurements of the S_2 and S_3 state lifetimes indicate a difference up to a factor of two to three [20,22]. The different stability of the S_2 and S_3 states reported in the literature might reflect different deactivation pathways for S_2 and S_3, i.e., S_2 reacting with electrons from the acceptor side, while S_3 is not [33]. In contrast, the reaction of the S_2 and S_3 states with Tyr-D is expected to proceed via the same pathway (see discussion in [19]), and thus to reflect more precisely the energetic difference between the S_2 and S_3 states. Thus, our results indicate that they have very similar midpoint redox potentials, which places the S_3/S_2 couple at about + 900–950 mV [19].

Oxidation of Tyr-D in Cl^--depleted PS II

The kinetics of oxidation of Tyr-D by the S_2 state in Cl^--depleted PS II membranes is about 10- times slower than in Cl^--sufficient PS II. This effect, which is largely reversible by readdition of Cl^-, is consistent with the increased stability of the Cl^--free S_2 state [27–29]. Based on the previous estimation of E_m(Tyr-D^+/Tyr-

D) – $E_m(S_2/S_1)$ > = 170 mV [19] and on the 50–70 mV decrease of $E_m(S_2/S_1)$ in Cl^--depleted BBYs [29], we estimate the redox potential difference between Tyr-D^+/Tyr-D and S_2/S_1 as > = 100–120 mV in Cl^--depleted PS II.

Superimposed on the slow oxidation of Tyr-D, a fast formation and a biphasic decay of Signal II was observed in the Cl^--depleted PS II membranes. The dominating fast phase (300–500 ms) of this decay corresponds to that of Signal II_{fast}, arising from the reduction of Tyr-Z^+ in the absence of fast electron donation from the water oxidizing complex [40]. Thus, it is straightforward to assign the fast phase of the observed signal, which corresponds to about 0.2 spins per reaction center, to the formation and decay of Tyr-Z^+. This assignment is supported by the kinetic spectrum of the total decay amplitude – that is dominated by the fast phase – which is very similar to that of normal Signal II.

The slower phase (12–15 s), in principle, can be assigned either to (i) a rather unstable population of Tyr-D^+, or (ii) to an unusually stable population of Tyr-Z^+. The loss of Tyr-D^+ in Cl^--depleted samples as observed here and noted earlier [41] is probably due to the elevated pH (7.5) during the preparation rather than a structural modification, and Tyr-D^+ still lives for tens of minutes to an hour in our Cl^--depleted PS II. Thus, the presence of a Tyr-D^+ population which would be stable only for 12–15 s can probably be excluded.

The second alternative, i.e., the presence of a stable Tyr-Z^+ population which decays biphasically (in the hundreds of milliseconds and in the seconds range) has already been suggested [35,36]. However, a recent study by Boussac and coworkers claims that no evidence for such a long lived Tyr-Z^+ population was found [37]. Instead, they suggested that not Tyr-Z^+ but an other oxidized species, a histidine, is formed during illumination which decays with about 900 ms and 30–40 s half-times (we obtained these values by analyzing the decay curve in Fig. 6 of [37]). Our results clearly show that Tyr-Z^+ is formed in Cl^--depleted PS II. However, at present we do not aim at clarifying this contradiction further. There might exist trivial explanations, like differences in the experimental material, that explain the variable results. However, it is of note that in Ref. 37 the flashes, which were used to generate Tyr-Z^+, were separated only by 4 s and traces from 128 flashes on the same sample were accumulated. It is likely that this protocol would leave the slowly decaying Tyr-Z^+ population (12–15 s half-time) unnoticed, since it would not decay between the flashes. Thus, the slow decaying phase we observed here most likely reflects the decay of stable Tyr-Z^+, which is formed after two or more flashes. It is also of note that stable Tyr-Z^+ population was observed after inhibition of water oxidation by other methods, too (e.g., by amine treatment [42] or Ca^{2+}-depletion [43].)

Depletion of Cl^- from PS II is known to inhibit the formation of higher S-states. The exact location of this block is still debated, and there are data favoring the interruption of either the S_2 to S_3 [28,29,34–36] or the S_3 to (S_4) S_0 [27,37,38] transition. These reports are, however, not totally conflicting since they all agree in that there are two positive charges stabilized at the donor side of Cl^--free PS II, and differ only in that the second positive charge is located on a modified S_3 state of the manganese cluster [27,38], on a stabilized Tyr-Z^+ [28,29,36] or on an oxidized histidine residue [37]. Our data (Fig. 6) are consistent with the water oxidizing complex reaching the S_2 state but not advancing beyond the S_3 state in the absence of Cl^-. This is shown by the large increase of Signal II_{slow} after the first flash (Figs. 5 and 6) indicating the oxidation of Tyr-D by the S_2 state, and by the lack of decline in the induced signal size after the third flash (Fig. 6) indicating that the S_0 state, which would have been unable to oxidize Tyr-D, is not formed. Unfortunately, it is not possible to decide from these data if the block is located after the S_2 or after the S_3 state, since the extent of Tyr-D oxidation is very similar after the first and second flash also in the nontreated control samples (Fig. 3). But it is possible that the quite large induction of Tyr-Z^+ after two flashes (in about 20–30% of the centers, Fig. 5) indicates that at least some centers are unable to advance from the S_2 state.

A last comment concerning a related report should be made. It was reported that Cl^--depletion modified the flash-induced oscillatory pattern of a fluorescence signal which is assigned to the S_2 state [39]. The measurement indicated a decreased amount of S_2 after the first flash, and was interpreted as showing the reduction of the S_1 state to the S_0 state or an unusually large miss on the first flash due to a structural reorganization of the water-oxidizing complex [39]. This finding can now be reinterpreted in the light of our data showing that the Cl^--depletion procedure decreases Signal II_{slow} leading to the reduction of Tyr-D^+, in our case, in 30–40% of the centers. Most of this Tyr-D can be reoxidized by a single flash via the S_2Tyr-D → S_1Tyr-D^+ process. Thus, Cl^- depletion does not convert the centers to the S_0 state in the dark, instead the decreased S_2-state formation after the first flash, observed in [39], is due to the S_2Tyr-D → S_1Tyr-D^+ charge transfer.

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