Function of Tyrosine Z in Water Oxidation by Photosystem II: Electrostatical Promotor Instead of Hydrogen Abstractor†

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ABSTRACT: Photosynthetic water oxidation by photosystem II is mediated by a Mn4 cluster, a cofactor X still chemically ill-defined, and a tyrosine, YZ (D1-Tyr161). Before the final reaction with water proceeds to yield O2 (transition S4 S0), two oxidizing equivalents are stored on Mn4 (S0 S1 S2), a third on X (S2 S3), and a forth on YZ (S3 S4). It has been proposed that YZ functions as a pure electron transmitter between Mn4X and P680, or, more recently, that it acts as an abstractor of hydrogen from bound water. We scrutinized the coupling of electron and proton transfer during the oxidation of YZ in PSII core particles with intact or impaired oxygen-evolving capacity. The rates of electron transfer to P680+, of electromechanism, and of pH transients were determined as a function of the pH, the temperature, and the H/D ratio. In oxygen-evolving material, we found only evidence for electrostatically induced proton release from peripheral amino acid residues but not from YZox itself. The positive charge stayed near YZox, and the rate of electron transfer was nearly independent of the pH. In core particles with an impaired Mn4 cluster, on the other hand, the rate of the electron transfer became strictly dependent on the protonation state of a single base (pK ≈ 7). At pH <7, the rate of electron transfer revealed the same slow rate (tt/2 ≈ 35 μs) as that of proton release into the bulk. The deposition of a positive charge around YZox was no longer detected. A large H/D isotope effect (≈2.5) on these rates was also indicative of a steering of electron abstraction by proton transfer. That YZox was deprotonated into the bulk in inactive but not in oxygen-evolving material argues against the proposed role of YZox as an acceptor of hydrogen from water. Instead, the positive charge in its vicinity may shift the equilibrium from bound water to bound peroxide upon S3 S4 as a prerequisite for the formation of oxygen upon S4 S0.

Photosystem II of higher plants and cyanobacteria is a multisubunit protein—pigment complex which oxidizes two molecules of water and produces dioxygen at the expense of four quanta of light (7–3). After photo-oxidation of the primary electron donor P680 (which is presumably a specialized chlorophyll a dimer that is located close to the lumenal side of the thylakoid membrane), the electron vacancy on

P680+ is filled in nanoseconds (4, 5) by an electron from a redox-active tyrosine, YZ [D1-Tyr161 (6–8)]. The latter is in turn reduced in micro- to milliseconds by the oxygen-evolving complex (OEC) that catalyzes the oxidation of water (9). It contains four manganese atoms [Mn4 (10, 11)], probably a further redox cofactor, X, whose chemical nature is still ill-defined (12–14), and Ca2+ and Cl– ions (2, 15). Clocked by four quanta of light, the OEC cycles through the increasingly oxidized states S0 S1 S2 S3 S4 S5. The release of dioxygen is associated with the last transition S4 S0, which spontaneously advances in the dark (16).

YZ and its counterpart YD (D2-Tyr161) have been proposed to be located at a distance of 10–15 Å from P680 (17–19), in positions that correspond to residues L-Arg135 and M-Arg162 on the L and M subunits of the bacterial reaction center (BRC) (20–22). The electrogenicity of the electron transfer from YZ to P680+ (23–26) and data on local electrochromic band shifts in PSII (27) are compatible with this notion.

The distance between the Mn cluster and YZ is under debate. On the basis of EPR, some authors have claimed

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that YZ is relatively far away from Mn, at a distance of 8–20 Å (17, 28–30). Correspondingly, YZ has not been said to be directly involved in a reaction with water but just as an electron transfer component in the consecutive sequence of redox reactions water → manganese → YZ → P880. Contrastingly, a much smaller distance between YZ and Mn of only 4.5 Å has recently been inferred on the basis of ENDOR data (31, 32), and these and other data (see below) have been interpreted to show a more direct involvement of YZ in the water chemistry. On the basis of the observation that the hydrogen bonds to YZox were found to be less ordered than those of YDox (31, 33–36), and that the rotational mobility of YZox was slightly larger (34, 37) in calcium-depleted PSII, it has been hypothesized that the oxidized YZ rapidly releases its hydroxyl proton into bulk water to serve as an abstractor of hydrogen from bound water. We have previously argued in favor of its hydroxyl proton into bulk water to serve as an abstractor of hydrogen from bound water (32, 38–40). These data were, however, obtained on material with impaired oxygen evolution. It is thus an open question of whether YZ serves as (a) an electron acceptor or (b) an abstractor of hydrogen from bound water. We have previously argued in favor of an electron acceptor function of YZ because of the detectable charge around it and the lack of detectable proton release into the bulk from YZox (41, 42). The fact that we have reported rapid proton release [at rise times down to 10 µs (43, 44)] has been misinterpreted by some authors as a deprotonation of YZ into the bulk. This has prompted us to carry out this detailed study.

We analyzed the reactions involving YZ in a systematic way, both in fully functional and in inactive (Mn-depleted) core particles. We measured the rate of electron transfer from YZ to P880+, the release of protons and electrochromic band shifts of intrinsic pigments as a function of the pH, of the isotopic ratio of H2O/D2O, and of the temperature. The results give clues for solving the seeming incompatibility of two observations from the literature, namely the status of YZox as an electroneutral radical (45) and the lack of detectable proton release together with the large electrochromism (41) that is caused by YZox. We consider two equally possible solutions. (1) YZox is a YZ...H+–B− pair wherein B− denotes a base in close vicinity to YZ. (2) YZ is in the anionic form (YZ−) under physiological conditions, and it is oxidized to the neutral YZ2+. In both cases the oxidized YZ2+ is hardly appropriate as an acceptor of hydrogen from water.

MATERIALS AND METHODS

Oxygen-evolving PSII core particles were prepared from 12-day-old pea seedlings according to van Leeuwen et al. (46) with modifications as in ref 44. Concentrated core particles were stored at −80 °C in 20 mM Bis-Tris/HCl, 400 mM sucrose, 20 mM MgCl2, 5 mM CaCl2, 10 mM MgSO4, and 0.03% (w/v) β-DM at pH 6.5 until use. For one experiment, we used PSII core particles prepared according to ref 47. They were stored in 20 mM MES, 400 mM sucrose, and 10 mM CaCl2 at pH 6.5. After thawing, core particles were suspended in 5 µM chlorophyll (except for measurements at 827 nm, see below), 5 mM CaCl2, 5 mM MgCl2, and 20 mM buffer (pH 4–7, MES; pH 6–8, Bis-Tris; pH 7–8, Tricine; and pH 8–11, Tris). The buffers were omitted in experiments on pH transients.

Inactivation of core particles was achieved by pH 9 treatment. A suspension with 5 µM chlorophyll was titrated to pH 9 for 5 min, and then the pH was readjusted to the desired value directly before the measurements. Oxygen evolution was below 10% of a control after this treatment. It should be noted that the behavior of samples treated at pH 9 in flash-spectroscopic measurements was indistinguishable from that of samples that were depleted of manganese by the classical Tris-wash treatment (48, 49). The pH 9 treatment thus probably also resulted in the release of Mn from its binding site (50).

The substitution of D2O for H2O was performed as follows. After thawing, the PSII-containing material was suspended in a medium with D2O (99.7% pure), at pH 6.5 (L = lyonium ion, H or D) and at the final chlorophyll concentrations for the measurements. Samples were then incubated in the light (2 mW cm−2) for 5 min at room temperature and, after readjusting the pH to the desired value, dark-adapted for 15 min prior to the first flash train. It should be noted that the maximum H/D isotope effects were already achieved after a much shorter incubation time, e.g. less than 1 min, as is apparent from measurements of absorption changes at 827 nm as a function of the incubation interval (data not documented).

The reduction of P880+ was measured as flash-induced absorption changes at 827 nm (12). The beam of a stabilized cw laser diode was focused through a 5 cm long cuvette and a pinhole at a distance of about 1 m on a fast avalanche photodiode. This setup suppressed the fluorescence of the sample and scattered light. A Q-switched, frequency-doubled Nd:YAG laser served as the excitation source (flash duration of 6 ns, 532 nm). The maximal electrical bandwidth of the system was dc to 150 MHz (4 ns per address). Transients were digitized and averaged for signal-to-noise improvement on a Tektronix DSA602 recorder. Samples contained 50 µM chlorophyll and 1 mM hexacyanoferrate(III) plus 0.5 mM DCBQ as electron acceptors. Samples of PSII core particles with impaired oxygen evolution also contained 0.5 mM hexacyanoferrate(II) as electron donor. Under these conditions, QA− was stable for at least 5 ms at pH 8.

Flash spectrophotometry at wavelengths other than 827 nm was performed with the setup described in ref 51. A xenon flash lamp (flash duration of 10 µs) was used for excitation. The optical path length was 1 cm. The maximal electrical bandwidth of the system was 100 kHz (5 µs per address). Transients were digitized and averaged on a Nicolet Pro30 recorder. For the electron acceptor conditions of the samples, see the legends to the figures.

Proton release was measured with various pH-indicating dyes at 30 µM (pH 4.5–6, methyl red at 548 nm; pH 5–7, bromocresol purple at 575 nm; and pH 6.5–8.5, phenol red at 559 nm) (44). From transients in the presence of the dyes, background transients obtained under the same conditions but in the further presence of 20 mM buffer were subtracted.

Local electrochromism due to YZox was recorded at 443 nm as previously described (52).

Oxygen evolution was measured under continuous white light illumination with a Clark-type electrode with 4 µM chlorophyll at 20 °C with 500 µM DCBQ as an electron acceptor.

The time course of absorption transients was analyzed in terms of exponentials using commercial routines.
RESULTS

Rate of Oxidation of YZ in Photosystem II with Impaired Oxygen Evolution

Influence of pH. Oxygen evolution by PSII core particles was inactivated by incubation at pH 9 (see Materials and Methods). Electron transfer from YZ to P680\(^+\) was monitored at 827 nm. Absorption transients at this wavelength can be attributed to the radical cation P680\(^+\) (53–56). Figure 1 shows raw transients due to the oxidation/reduction of P680 in the time interval from 100 ns to 100 \(\mu\)s after the first exciting flash at six pH values ranging from pH 4 to 10.5 (note the logarithmic time scale). The overall decay time of P680\(^+\) was strongly pH-dependent; it decreased from about 200 \(\mu\)s at pH 4 to 1 \(\mu\)s at pH > 8. The major portion of the pH-dependent decay could be attributed to electron transfer to P680\(^+\) from YZ. Our data are in line with other data obtained on Tris-washed PSII (49, 57, 58). The approximation of the decay of P680\(^+\) in Figure 1 by a single exponential was rather poor. Three exponentials were more appropriate, according to

\[
\Delta A(t) = a_{f}e^{-kt_{f}} + a_{s}e^{-kt_{s}} + a_{v}e^{-kt_{v}}
\]  

wherein \(a_{f}\), \(a_{s}\), and \(a_{v}\), and \(k_{f}\), \(k_{s}\), and \(k_{v}\) correspond to the extents and rates of the fast, slow, and very slow components, respectively. Figure 2A (top) shows the pH dependence of the respective rate constants of the fast \((k_{f}\), squares\) and the slow component \((k_{s}\), circles\) over a wide pH range from 4 to 10. Figure 2B (middle) shows the respective extents, and Figure 2C (bottom) shows the extent of the very slow component \((k_{v}\), open triangles\).

\(k_{f}\) was little dependent on the pH, and between 6 and 10.5, it averaged to 7 \(\times 10^{5}\) s\(^{-1}\) which is equivalent to a half-decay time of about 1 \(\mu\)s. \(k_{s}\), on the other hand, decreased by a factor of 5 from 10\(^5\) s\(^{-1}\) \((t_{1/2} = 7 \mu s)\) at pH 8 to about 2 \(\times 10^{4}\) s\(^{-1}\) \((t_{1/2} = 35 \mu s)\) at pH 4.

The extents of the fast and the slow kinetic components were pH-dependent. They are shown in Figure 2B. At pH > 7, the fast component \((\text{squares})\) was dominant with an amplitude of about 80% at pH 10. Its relative extent decreased to about 10% at pH 5.5 with an apparent pK of 7 (line). The slow component \((\text{Figure 2B, circles})\) prevailed at pH < 7. Its amplitude increased from about 10% at pH 8 to 70% at pH 5.5, and again decreased below pH 5.5. The increase of the amplitude of the slow component thus occurred at the expense of the extent of the fast component, again with an apparent pK of 7.

At pH < 6, an additional very slow component \((k_{v}\) rose up. Its total extent increased to about 60% at pH 4 \((\text{Figure 2C, open triangles})\). Its average rate constant \((k_{v}\) was about 1500 s\(^{-1}\) \((\text{half-decay time of 450 } \mu \text{s})\). We suspected that the very slow component could be attributed to the recombination of the charge pair P680\(^+\)/QA\(^–\). To test this possibility, we directly monitored the reoxidation of QA\(^–\) at 320 nm where the quinone anion strongly absorbs \((59–61)\). The upper part of Figure 3 shows UV transients at pH values ranging between 4 and 9 on the first flash given to inactive centers. At pH 9, the decay was rather slow and due to oxidation of QA\(^–\) by external acceptors. It speeded up at more acidic pH. The bottom trace in Figure 3 shows the difference between transients obtained at pH 9 minus 4. The overall half-decay time of the difference signal was 450 s. It was coincident \((\text{Figure 2C, solid triangles})\) with the one determined for the very slow component of the reduction of P680\(^+\) \((\text{Figure 2C, open triangles}; \text{see also ref 62})\). The coincidence supports the notion that the latter could be attributed to the charge pair recombination between P680\(^+\) and QA\(^–\). A fit of the difference transient in Figure 3 by two exponentials \((\text{see the solid line})\) revealed two compo-
ponents of equal extent with half-rise times of 135 and 870 ms (see Table 1). Taken together, the results which are documented in Figures 2 and 3 and Table 1 showed that the contribution of the charge recombination to the reduction of P680+ was negligible between pH 6 and 10, whereas it rose up to about 60-70% at pH 4. Forward electron transfer from YZ to P680+ was, however, still well separated from the charge recombination reaction because the former was at least 4 times faster than the latter even at the lowest pH values.

We summarized the rates of electron transfer between QA, P680+, and YZ in Table 1.

### Effect of Temperature

We measured the influence of temperature on the rate of electron transfer from YZ to P680+ at pH 5 and 9. At pH 5 mainly the slow kinetic component (ks) prevailed (relative extent of 65%) and at pH 9 the fast one (kf, relative extent of 80%, compare Figure 2B). Figure 4 shows Arrhenius-type plots of kf (squares) and ks (circles) as a function of the temperature. The fast component revealed a small activation energy of 0.15 eV (pH 9, Table 1). The slow component (ks) revealed a greater one, 0.30 eV (at pH 5, Table 1). A biphasic deconvolution of the kinetic traces at pH 6.3 yielded similar activation energies for the slow and fast components, namely 0.28 and 0.16 eV, respectively (dotted lines in Figure 4). At pH 4.0, the very slow components (kv) that are due to charge pair recombination (see Figure 2C) revealed an even weaker temperature dependence (data not shown) which corresponded to an activation energy of 0.1 eV.

### H/D Isotopic Substitution

We studied the effect of protium/deuterium substitution on the rate of YZ oxidation. This parameter was again studied at pH 5 and 9 where the slow and fast components prevailed. Figure 5 shows raw transients at 827 nm at these pH values as measured in H2O and D2O. We found that kf was essentially independent on the substitution of D2O against H2O. The H/D isotope effect was less then 1.1 (Table 1). The slow component ks, on the
other hand, was substantially retarded in D_2O. Its H/D isotope effect was 2.5 at pH values between 7 and 5 (Table 1; see also ref 63). Charge recombination as observed at pL < 5 (prevailing k_w) was apparently insensitive for isotopic substitution (data not shown).

Rate of Oxidation of YZ in Oxygen-Evolving Centers

Figure 6 shows transients at 827 nm due to the oxidation of P_680^- on the first and third flash given to dark-adapted active centers at pH 6.5 in either H_2O or D_2O. The reduction of P_680^- was multiphasic, and it occurred mainly in the nanosecond time range. Similar rates were observed on transitions S_0 → S_1 and S_1 → S_2 on one hand and on S_2 → S_1 and S_1 → S_3 on the other (Table 1). The former rates were greater than the latter (Table 1), in agreement with previous reports (5, 64–66). This behavior has been attributed to one extra positive charge on the Mn cluster which was brought in on transition S_1 → S_2 (see refs 5 and 67 and below). It was observed between pH 5.5 and 7. In this pH region, the oxygen-evolving capacity of the centers was about constant (compare Figure 10). Below pH 5, the nanosecond components were largely replaced by microseconds components which was accompanied by the reversible inactivation of oxygen evolution (see Figure 10), in line with previous reports (68, 69). An irreversible inactivation was also observed above pH 7.5, presumably due to the release of Mn (50). The nanosecond components were practically independent of H/D substitution; their isotope effect was less than 1.1 (Figure 6 and Table 1). That they were only weakly dependent on temperature between 35 and 0 °C (see Table 1) was in line with a previous report (70). Table 1 summarizes relevant data from the literature together with our results on the rate of YZ oxidation in intact PSII core particles.

By analyzing the equilibrium portion of P_680^+ (e.g., after nanosecond electron transfer was completed; see Table 1), we calculated the difference ΔE_i between the redox potentials E_{m}(P_680^+/P_680) and E_{m}(YZ_{ox}/YZ) in the states S_0 and S_1 and S_2 and S_3, respectively, thus in the presence of zero (i = 0) and one (i = 1) positive net charge on the Mn cluster.

$$ΔE_i = \frac{2.3RT}{F} \log \frac{[P_{680}]_{\text{red}}}{[P_{680}]_{\text{ox}}}$$

On the first flash, the equilibrium proportion of P_680^+ was only 5–10% (see ref 71 and this work). The calculated midpoint potential of P_680 was by 80 mV more positive than that of YZ. Upon the second (and third) flash, about 20–25% of P_680^+ remained oxidized. This implied an initial energy gap of only 30 mV. Thus, the positive charge stored on the Mn cluster upon S_1 → S_2 increased the redox potential of YZ relative to P_680 by a surprisingly low figure of only 50 mV.

Proton Release upon the Oxidation of YZ

In oxygen-evolving PSII the oxidation of YZ is accompanied by fast proton release in both thylakoids (43, 72) and core particles (44). We have previously proposed that the variability of the extent of proton release as a function of the pH (43, 73–75) and of the preparation (47, 52, 74, 76) argues against the notion that it originates from the phenolic moiety of YZ itself ([pK of YZ_{ox} < −2 (77)]. This argument was corroborated by the pH dependence of the rate of proton release (44), showing its origin from electrostatically induced pK shifts of peripheral amino acids (for reviews, see refs 41 and 78).

We compared the rates of proton release as monitored by pH-indicating dyes in inactive core particles with those in active core particles. The upper traces in Figure 7 represent pH-indicating transients of bromocresol purple at pH 5.3 upon a first flash given to dark-adapted centers. In active centers, the transient due to proton release upon the oxidation of YZ (Figure 7, rise) persisted for several hundred milliseconds. In inactive centers, on the other hand, the proton was released (rise) and rebound (decay) with a half-time of about 40 ms. This half-time was shortened when hexacyanoferrate(II) was added as an external donor for YZ_{ox} (43) and of the preparation (75) arguments. We interpreted these findings to be in line with our previous results (42). (1) In oxygen-evolving centers, the oxidation of YZ creates a positive net charge in the catalytic center that causes a pK shift of peripheral amino acids. The pK shift persists independent of the position of this net charge on YZ_{ox} or on the Mn cluster. This interpretation is backed up by extensive studies on the kinetics of proton release (42–
proton release in both materials at pH indicates that the same peripheral groups contributed to proton release from active centers fairly well. In inactive centers and invariant, about 2 with $pK$ values of the various groups. The smooth line in Figure 8 was calculated for the contributions of three peripheral acid groups to proton release with different $pK$ values (see the text). The dashed line represents the extents of proton release per PSII center (right coordinate scale) in inactive core particles.

44), and by studies on local (52, 79) and transmembrane (26) electrochromism. (2) In inactive centers, the proton which is released upon the formation of $Y_Z^{ox}$ is rebound when the latter is re-reduced (for a description of this push-and-pull mechanism of proton release/uptake, see refs 80–82). The lower traces in Figure 7 show the upper pH transients on an expanded time scale. In oxygen-evolving material, proton release occurred much faster at pH 5.3, with a half-time of about 15 μs, than in inactive centers where the half-time was about 40 μs (Table 1).

In Figure 8, we compared the rates of proton release in oxygen-evolving material (solid triangles, data replotted from ref 44) and in inactive centers (open triangles) as a function of the pH. The rate of the slow kinetic component of electron transfer from $Y_Z$ to $P_{680}^{-}$ in inactive material was replotted from Figure 2 for comparison ($k_s$, dotted line). In active centers, the rate of proton release decreased steeply between pH 5 and 7 and less steeply up to pH 8. This behavior can be rationalized by the participation of various peripheral amino acids with different $pK$s depending on the chosen pH in the medium (44). The rate of proton release from each group depends linearly on its $pK$ (44), and the overall rate of the total release comprises the individual contributions of the various groups. The smooth line in Figure 8 was calculated for the simultaneous contributions of three groups with $pK$ values of 7.5, 6, and 4.5. It describes the rates of proton release from active centers fairly well. In inactive centers and between pH 8 and 6, the rate of proton release was similar to that in active material. This result likely indicates that the same peripheral groups contributed to proton release in both materials at pH >6. At pH <6, the rate of proton release in inactive centers was lower than that in active centers and invariant, about 2 × 10$^{-6}$ s$^{-1}$ (35 μs). This rate was about the same as that of the main component ($k_s$) of the electron transfer from $Y_Z$ to $P_{680}^{-}$. There are two alternative interpretations for the lower rates of proton release in inactive centers at acidic pH (Figure 8).

(a) Inactive centers may have lost some peripheral groups whose acid $pK$s give rise to the fastest proton release at acidic pH. (b) The release of protons in inactive centers may be limited by the rate of electron transfer or vice versa. Possibility (a) implied that the ratio of protons over electrons decreased below pH 6 in inactive centers. Such a decrease was, however, not observed (Figure 8, dashed line, right coordinate scale). Instead, the extent decreased only below pH 5, concomitant with the increase of charge pair recombination. Under these conditions, no protons were apparently released in the presence of $P_{680}^{-}$. These results indicated that possibility (b) was more likely.

We determined the rate of proton release in inactive centers in both H$_2$O and D$_2$O as a function of the pH (raw data not shown). At pH 8, the H/D isotope effect was 1.6. At pH 5, it was much greater, namely 4 (Table 1). Similar H/D effects were, however, observed in active centers. The variability of the H/D isotope effect as a function of the pH was compatible with the contributions of various peripheral acid groups. If the $pK$ difference between the acid and water ($pK = -2$) decreases by 3 units, one expects an increase of the isotope effect by a factor of about 2 (83).

From these results, we draw the following conclusions. (1) In active centers and over the whole studied pH range, a proton which is liberated from $Y_Z^{ox}$ itself remains inside the protein for at least 300 μs, i.e. until an electron is transferred to $Y_Z^{ox}$ from Mn$\times$X. The observed rapid protons [which may appear slower (at alkaline pH) or faster (at acidic pH) than the reduction of $Y_Z^{ox}$] result from peripheral groups. (2) Only in inactive material and at pH <6 are the rates of proton release and of electron transfer from $Y_Z$ to $P_{680}^{-}$ similar. Only under these conditions may the proton which appears in the medium originate from the phenolic moiety of $Y_Z^{ox}$.

Local Electrochromism in the Presence of $Y_Z^{ox}$

The positive charge on or near $Y_Z^{ox}$ causes local electrochromic bandshifts of the inner pigments in both active and inactive PSII (27, 79, 84–87). This effect has been used to estimate the mutual orientations of pigments and cofactors (27). Figure 9 shows time-resolved transients of local electrochromism at 443 nm upon the oxidoreduction of $Y_Z$. In dark-adapted, active centers upon the first flash (left trace, pH 7), the amplitude of the initial fast rise is due to the electrochromic effect of the formation of $Y_Z^{ox}$ in nanoseconds plus a contribution of a chemical transient from Q$_A^-$ (60). The latter decays with a half-time of about 10 ms under the used electron acceptor conditions (see the legend to Figure 9 and ref 88). We determined the relative amplitude that could be attributed to Q$_A^-$ by adding 1 mM hexacyanoferrate(III) and monitoring the extent of the accelerated decay (half-time about 0.5 ms) due to the reoxidation of Q$_A^-$ (data not shown). The resulting relative extent of Q$_A^-$ was drawn as a dotted line in Figure 9. The remaining amplitude could be attributed to the local electrochromic effect of $Y_Z^{ox}$. In active centers (left), the latter extent decayed to about half its initial value (Figure 9, left) upon electron transfer from Mn to $Y_Z^{ox}$ on transition $S_1 \rightarrow S_2$ with a half-time of 80 μs (see also ref 42). In inactive centers this situation was completely changed. At pH 8 (Figure 9, inactive pH 8), the charge pair $Y_Z^{ox}$/Q$_A^-$ was formed in about 1 μs (compare Figure 2A). Whereas the relative extent due to Q$_A^-$ was as in active centers (dotted line), the extent that could be attributed to the electrochromism in response to $Y_Z^{ox}$ was about halved. At pH 6.5 (Figure 9, inactive pH 6.5), the latter extent was close to zero. At both pH values, no decay in microseconds of the electrochromic effect due to $Y_Z^{ox}$ was
material at pH dropped to the level which was observed in the impaired in the presence of YZ ox (compare Figure 7).

transients due to YZ ox in active and inactive centers as a electrochromism of YZ ox in active centers (open circles) probably due to the release of Mn. Consequently, the active centers were converted into inactive ones at pH (shown as a dashed line in Figure 10). At alkaline pH, the ultimately with the inactivation of the oxygen-evolving capacity pH 5.5 and 7.5. At more acidic pH, it decreased concomi-
tantly with the inactivation of the oxygen-evolving capacity (shown as a dashed line in Figure 10). At alkaline pH, the active centers were converted into inactive ones at pH >7.5, probably due to the release of Mn. Consequently, the electrochromism of YZ ox in active centers (open circles) dropped to the level which was observed in the impaired material at pH >7.5.

In inactive material, the electrochromism due to YZ ox (determined about 150 µs after the flash, open squares) was about constant between pH 9.5 and 8. This amplitude decreased to about zero at pH 6 with an apparent pK of 7.1 (Table 1). This pK was similar to that observed for the decrease of the amplitude of the fast component (k_f) of the oxidation of YZ (compare Figure 2B). Below pH 6, the signal extent became negative due to the superimposition of a transient from the equilibrium portion of P_{680}^+ (see Figure 2C and refs 71 and 87).

In the literature, several electrochromic difference spectra of YZ−YZ ox can be found that have been obtained with inactive centers from different starting materials at pH ≤7 (13, 27, 60, 84, 89). In this pH region, the electrochromism of YZ ox was negligible in core particles of the van Leeuwen/Bögershausen type (Figure 10, open squares). We performed the same experiments as with the latter material with a different type of core particles prepared according to Ghanotakis and Lübbers (see Materials and Methods). With oxygen-evolving material, the electrochromism due to YZ ox was similar in both core preparations (Figure 10, solid and open circles). In inactive centers, the same pH-dependent decrease of the electrochromic effect of YZ ox as in the van Leeuwen/Bögershausen-type cores was observed. Its apparent pK in the Ghanotakis/Lübbers-type core, however, was much lower, 6.1 (Figure 10, solid squares). These differences between the two types of core particles may reflect the different content of extrinsic polypeptides and detergent, possibly causing alterations of the dielectric permittivity at the luminal side of PSII proteins as previously proposed (26, 76).

**DISCUSSION**

**Comparison of the Rates of Electron Transfer from YZ to P_{680}^+ in Inactive and Active PSII**

The fast component (k_f) of YZ oxidation with a half-time of about 1 µs was dominant between pH 7.5 and 10.5 in inactive PSII core particles. Its features were similar to those of the nanosecond components of YZ oxidation in oxygen-evolving material (see Table 1, and refs 5 and 70). The activation energies of these components were low, 0.15 eV (this work) in inactive and ≤0.1 eV in active centers (90).

The slowing of the fastest portion of the oxidation of YZ from nanoseconds in active centers to about 1 µs in inactive material can likely be explained by the higher activation energy of the electron transfer in the latter case. An increase of the activation energy by 0.1 eV slows the reaction by a
factor of 50. The higher activation energy of electron transfer $Y_Z \rightarrow P_{680}^{+}$ in inactive centers is in line with a higher polarizability of the more hydrophilic binding pocket of $Y_Z$ (91). The latter was also apparent from the about halved amplitudes of the electrochromic shift of the absorption band of $P_{680}$ due to $Y_Z^{ox}$ (see below), and from the halved apparent electrogenericity of electron transfer from $Y_Z$ to $P_{680}^{+}$ (26).

Both the nanosecond components in active centers and the 1 µs component in inactive ones of electron transfer from $Y_Z$ to $P_{680}^{+}$ were pH-independent (between 5.5 and 7.5 in active centers; see refs 64 and 69), nearly insensitive to H/D isotope substitution (for active material, see this work and refs 42 and 63), and revealed low activation energies. These features suggest that in both cases the rate of electron transfer was not limited by the transfer of a proton.

The slow component ($k_s$) of the oxidation of $Y_Z$ appeared in inactive centers at pH $<7.5$. The redistribution between the fast and slow modes of $Y_Z$ oxidation was described by a titration with an apparent single pK of 7.0. The features of the slow component were very different from those of the fast components. The rate decreased with decreasing pH; the H/D isotope effect was much larger, 2.5, and the activation energy was much higher, 0.3 eV. A counterpart of this slow component may occur in active centers only below pH 4.5 where electron transfer slows from nanoseconds to microseconds (69). We believe these features in inactive material indicate the limitation of electron transfer from $Y_Z$ to $P_{680}^{+}$ by a proton transfer reaction at pH $<7$. The pH-dependence of the slow component ($k_s$) was weak, and the rate decreased only by one order of magnitude when the pH was lowered from 8 to 4. This pH effect was likely explained by the electrostatic interactions between $Y_Z$ and the proton-transferring groups (see the next paragraph).

The extent of the very slow component ($k_v$) of $P_{680}^{+}$ reduction was increasing at acidic pH. This phase can be assigned to the recombination reaction $Y_ZP_{680}^{+}Q_0 \rightarrow Y_ZP_{680}Q_A$. The overall rate of this process matched at pH 4 the rate constant of the direct recombination that was observed in PSII where $Y_Z$ was mutated to phenylalanine (92) or destroyed (62). The observed effect is likely explained by a decrease of the equilibrium constant which reflects the ratio between $Y_Z^{ox}$ and $P_{680}^{+}$. We attributed this decrease to an increased midpoint potential of the couple $Y_Z^{ox}/Y_Z$ at acidic pH, which is likely caused by the increasing protonation of amino acid residues at the protein boundary. The latter effect likely also accounts for the slowing of the slower component ($k_v$) of forward electron transfer from $Y_Z$ to $P_{680}^{+}$ at acidic pH (see below). The fraction of $P_{680}^{+}$ that remained oxidized in equilibrium with $Y_Z^{ox}$ was found to be 50% at a pH of about 4.5; hence, the equilibrium constant at this pH was unity, and the difference between the midpoint potentials of $Y_Z$ and $P_{680}$ was zero. At pH 7.5, only about 10% of $P_{680}^{+}$ remained oxidized in equilibrium with $Y_Z^{ox}$. Thus, $\Delta E_m$ was $\approx0.1$ eV at neutral pH. We conclude that the midpoint potential of $Y_Z^{ox}/Y_Z$ increased by about 0.1 eV with respect to the potential of the couple $P_{680}^{+}/P_{680}$ when the pH was lowered from 7.5 to 4.5. These estimates agree well with previous reports on the differences of the redox potential of $P_{680}$ and $Y_Z$ in Mn-depleted PSII of about 0.1 eV at pH 6.5 (27, 92).

**Does $Y_Z^{ox}$ Release a Proton into the Bulk?**

It has been postulated by various authors (31, 32, 38–40) that $Y_Z$ abstracts a hydrogen atom from bound water on each of the four redox transitions of the catalytic cycle of water oxidation. This model implied that the hydroxyl proton of the phenolic moiety of $Y_Z$ was transferred each time to the bulk water of the lumen (40).

On one hand, this hypothesis seems to be hard to reconcile with the observations that in oxygen-evolving PSII the extents and rates of proton release vary as function of the S transition, of the pH, and of the preparation (for a review, see ref 41). Under certain conditions, proton release can be practically absent on transition $S_1 \rightarrow S_2$ in any PSII preparation (76). The variability of the extents has been unequivocally attributed to Bohr effects of peripheral acid groups (41, 78). Furthermore, electron transfer from Mn$_4X$ to $Y_Z^{ox}$ shows a negligible H/D isotope effect on some transitions (42, 63, 93, 94) which may not be expected if it is steered by the transfer of a hydrogen atom. These results have been discussed in detail elsewhere (42, 95).

The present work corroborates the above conclusions. In inactive centers and at alkaline pH, the rate of proton release was the same as in active centers and much lower than the rate of the oxidation of $Y_Z$. Thus, proton release greatly lagged behind electron transfer. The charge on or near $Y_Z^{ox}$ (see the next section) caused a large local electrochromic effect mainly on the absorption band of $P_{680}$ (27, 87, 96). If the proton from $Y_Z$ is released into bulk water, one would expect to observe the concomitant decay of this electrochromism due to the transfer of the positive charge from the low dielectric of the protein into the electrolyte. Such a coupling of the decay of electrochromism with the observable proton release was absent both in inactive centers at alkaline pH (this work) and in active ones (27, 52, 79). The electrochromism due to $Y_Z^{ox}$ thus only decayed when the latter was reduced [with half-times that vary as a function of the S transition in active centers (13, 87)]. We conclude that in oxygen-evolving centers (between pH 5 and 7.5) and in inactive material at alkaline pH the hydroxyl proton is not transferred into the bulk within the lifetime of $Y_Z^{ox}$ but is trapped inside the protein.

In inactive centers and at acidic pH, the rates of the oxidation of $Y_Z$ and of the observed proton release were about equal. Furthermore, the generation of $Y_Z^{ox}$ did not cause an electrochromic effect. These observations can be understood as follows. Concomitantly with the oxidation of $Y_Z$ by $P_{680}^{+}$, the phenolic proton is at least transferred into the aqueous bulk phase. This removes the positive net charge as soon as it is formed. Electrochromic transients are therefore absent. The observed proton release is kinetically steered by the oxidation of $Y_Z$ or vice versa. The observed weak pH dependence of the slow component ($k_s$) may be rationalized by the participation of at least one intermediate group in the proton transfer between $Y_Z^{ox}$ and the boundary (97). The increasing protonation of the surface groups with decreasing pH will decrease the pK of this group and gradually slow the deprotonation of $Y_Z^{ox}$. Perhaps the most important aspect of these results is the experimental demonstration of a behavior where the phenolic proton is removed far away from $Y_Z^{ox}$. Only this situation would
Function of Tyrosine Z in Water Oxidation

Scheme 1: Two Hypothetical Models for the Functioning of Tyrosine YZ

(A) YZ is hydrogen bonded (dashed line) to a base B− with a pK of 7 in inactive PSII and with a pK of 4.5 in oxygen-evolving PSII. (Left) The transfer of the hydroxyl proton of YZox to B− is fast and not rate-limiting for electron transfer. (Right) Only when B is protonated the electron abstraction from YZ is limited by the transfer of the hydroxyl proton (to a second base A, as shown, or to the bulk water). (B) The pK of YZ itself is 7 in inactive centers and 4.5 in oxygen-evolving centers. (Left) These low pK values are in part achieved by modulating hydrogen bonds to the phenol oxygen of YZ− from bases A and B. (Right) Electron abstraction from YZ is limited by the transfer of the hydroxyl proton (possibly to bulk water) only where pH < pKYZ, where YZ is protonated.

prepare YZox for the postulated function of a hydrogen acceptor (31, 32, 38–40). Unfittingly, we found it only in core preparations with inactivated oxygen-evolving capacity and only at acidic pH. This corroborates our view that the phenolic proton of YZox remains in its immediate vicinity in active material. This view is hardly compatible with the postulated hydrogen acceptor function of YZox.

Mechanism of the Oxidation of YZ

Our results can be rationalized by two related models. Both models are based on the experimental evidence that YZox (31, 33–36, 98–101) and YZsin (102–105) are neutral radicals and hydrogen bonded to the CD loops of the D1 and D2 proteins.

Model 1 is shown in Scheme 1A (left). It assumes that YZ is neutral (protonated) in the ground state at physiological pH. Upon its oxidation by P680++, the pK of the tyrosyl side chain is shifted from presumably 10 to extremely low values (pK ≤ −2 in water; see ref 77) and the hydroxyl proton is removed. The low activation energy and the absence of a kinetic H/D isotope effect on the oxidation of YZ by P680++ implies that the phenolic proton is transferred to a very effective base B− with a higher rate than that of the electron transfer, i.e., in nanoseconds. The transition is then appropriately denoted as YZ-H⋯B− → YZ∗⋯H−B− (see also refs 42, 70, and 106). The positive charge of the proton gives rise to the observed local electrochromic transient. It is likely that the proton is rebound by YZox during its reduction, a proton rocking motion similar to the one that has been proposed to occur upon the oxidoreduction of YD (45). It should be noted that the rapid release of protons that we observed with pH-indicating dyes does not originate from YZox itself but from electrostatically induced pK shifts of at least three peripheral acid groups with different pK values.

In inactive material, the proton transfer starts to govern the rate of the oxidation of YZ if group B is protonated, e.g., below its pK of 7 (Scheme 1A, right). In this case, the proton of YZox is then transferred via a different pathway, e.g., to a different base A (as depicted in Scheme 1A, right) that is closer to the membrane/water interface or to bulk water. The main difference between active and inactive material according to model 1 is that the pK of B is 7 in inactive centers, but 4.5 in active ones. In active material, we expect that electron transfer from YZ to P680++ is rate-limited by proton transfer only below pH 4.5. Such a limitation has been observed. It is reversible (68, 69) unlike the one which is observed at alkaline pH and caused by the loss of Mn from the center.

Model 2 of YZ oxidation which is summarized in Scheme 1B differs from model 1 by the assumption that YZ is negative (YZ−, Scheme 1B, left) and electroneutral in its oxidized form, YZ'. The pK values of 7 in inactive PSII and of 4.5 in oxygen-evolving material are then attributed to YZ itself. According to model 2, the low activation energy of YZ oxidation and the absence of a H/D isotope effect in oxygen-evolving samples (at all studied pH values) and in inactive centers at pH > 7 are now simply explained by the absence of any proton transfer upon the oxidation of YZ−. The oxidation of the tyrosine anion to a neutral tyrosine radical is equivalent to the appearance of a positive charge at the place of YZ. This charge causes the observed local electrochromic effect when YZox is present. At pH values lower than the pK of YZ (pH < 4.5 in oxygen-evolving centers and pH < 7 in inactive ones), YZ is protonated (Scheme 1B, right). Then, the transfer of the hydroxyl proton (to water or to a nearby base) limits the rate of the electron transfer.

The difficulty of model 2 is the unusually low pK value of YZ. The pK of tyrosine is 10 in water, and one expects it to be shifted to even more alkaline values due to the decrease of the Born solvation energy when placed in the low dielectric of a protein (107). Still, functional pK values of tyrosine in the range of 6–4.5 have been reported for a family of native and artificial myoglobin mutants (ref 108 and references therein). In the latter case, the pK shift was due to the influence of a heme iron atom. In the case of YZ, the anionic state of tyrosine may be stabilized by Mn and Ca atoms in its vicinity. One positive charge spaced by 5 Å from YZ may already shift the pK by 3–5 units if we take ε in the range of 10–20 (27). Another example of a large pK shift is the Qb-binding site of bacterial reaction centers. The crucial L-Glu212 has an unusual pK of 10 (versus a pK of 4.5 in water) owing to the negative charge on L-Asp213 and to a possible hydrogen bond to the ubiquinone (109, 110). A large pK shift of YZ may result from the combined action of positive charges on manganese and calcium atoms, and from hydrogen bonds of neighboring amino acids to its phenolic oxygen. Model 2 assumes that the difference between active and inactive materials with respect to YZ oxidation is mainly a shift of its pK by about 2.5 units into the alkaline direction (from a pK of 4.5 to 7) due to the loss of the Mn cluster and an increased dielectric polarizability of the protein.

Models 1 and 2 (Scheme 1) cannot be distinguished on the basis of our data alone. They predict, however, that the number and strength of hydrogen bonds to YZ may explain the higher midpoint potential and faster oxidation rate of the
latter compared to those of YZ. These properties likely change as a function of the material (active versus inactive), and of the pH in both the reduced and oxidized states. pH-dependent variations in the ENDOR spectra of YZox (111) suggested a protonation with a similar apparent pK of 6–7 in inactive material as reported above. Two hydrogen bonds to YZ (as tentatively depicted in Scheme 1) have been observed by the research group of D. Britt (121).

**Implications on the Structure of PSII and on the Mechanism of Water Oxidation**

There is good evidence (22, 26, 27, 112, 113) that tyrosines YZ and YD are placed symmetrically at ~14 Å from P680 and about 4 Å closer to the lumen. The position of Mn relative to YZ and P680 is controversial. The Mn cluster has been reported to be in close proximity to [less than 5 Å (31)] or at a greater distance from YZ [14–20 Å (27, 30, 114, 115)], or it may even consist of two Mn dimers (116) with a distance of about 10 Å between them (117).

The effect of the positive charge stored on Mn during the S1 → S2 transition on the redox equilibrium between YZ and P680 must be quite different for the above structural arrangements. If the whole tetraneuclear Mn cluster would be located at a distance of only 5 Å from YZ (as suggested in ref 31), one positive charge on Mn would raise the redox potential of YZ relative to P680 by about 180 mV (assuming an effective dielectric constant of 10; see ref 27 and references therein). This would shift the ΔG of the equilibrium between YZ and P680 for the S2 → S3 and S3 → S4 transitions relative to the S1 → S2 transition accordingly. Contrastingly, a rather moderate shift by only about 50 mV was observed (see Results). A distance between a compact tetra-Mn cluster and YZ of only 5 Å (as necessary for a hydrogen abstractor function of YZ) seems unlikely. It would furthermore be hard to reconcile with the observation of, at the same time, a small activation energy and a relatively low rate (t1/2 ≈ 60 μs) of electron transfer from Mn to YZox on transition S1 → S2 (42, 90). Alternatively, we suggest a triangular arrangement. Either (1) a tetraneuclear Mn cluster is located an only slightly smaller distance from YZ than from P680 (see also ref 27) or, alternatively, (2) one of the two Mn dimers (117), the one which (the auxiliary one) serves to store the positive charge on transition S1 → S2, is placed a similar distance from YZ and P680, the other (the catalytic one) is placed closer to YZ and participates in water oxidation. In both cases, a positive charge on Mn would increase the oxidizing potentials of both YZ and P680. If we take 10–13 and 15–20 Å as estimates of the distances between Mn and YZ and YZ and P680, respectively, E\text{m}(YZox/YZ) and E\text{m}(P680\textsuperscript{ox}/P680) are expected to rise by 125 and 80 mV, respectively, after the S1 → S2 transition (dielectric permittivity of 10). The energy gap between YZ and P680 then decreases by 45 mV, in line with the observations. The benefit is twofold. (1) The energy gap between these cofactors remains appropriate to assure the sequential electron transfer from Mn via YZ to P680\textsuperscript{ox}, and (2) the higher redox potential of P680 increases the driving force of the forward reaction (118).

Various models have been proposed for the mechanism of water oxidation. Some involve the stepwise oxidation of the substrate during at least some of the lower transitions of the catalytic cycle (10, 32, 38, 106, 119), whereas others pool the abstraction of four electrons from bound water on the final, oxygen-evolving transition S4 → S0 (42, 120). Currently discussed models of the first type involve the abstraction of hydrogen atoms from bound water during all four redox transitions of the catalytic cycle (38, 40). As argued above, our results in the current work as well as our previous investigations on electron transfer from Mn4X to YZox (42) make the functioning of YZ as a hydrogen abstractor on each step of the catalytic cycle unlikely. Our results are more compatible with a mechanism of water oxidation of the second type. We propose that two sequential two-electron transfer steps with a (bound) peroxide intermediate (see also ref 106) occur only on transition S1 → S0 and lead to the formation of dioxygen (42). In this concept, YZ serves two functions: (1) It is an electron transmitter between Mn4X and P680\textsuperscript{ox}. (2) The effective positive charge that is stored around YZox in the S4 state (as YZox-H\textcdots\textcdots\textcdots-B derived from YZox-H\textcdots\textcdots\textcdots-B in model 1, or as YZox derived from YZox\textcdots\textcdots\textcdots-B in model 2) may increase the oxidizing power of the catalytically active Mn by up to 100 mV depending on its distance from YZ (see above). During the S1 to S0 transition, this increase may be the prerequisite for a shift of the equilibrium between bound water and bound peroxide toward the latter (42, 106). YZox, as an electrostatical promotor, starts the reaction sequence that leads to the production of dioxygen.

**CONCLUSIONS**

Electron transfer, proton release, and local electrochromism upon the oxidoreduction of the tyrosine, YZ, were investigated in inactive and oxygen-evolving PSII core particles under variations of the pH, the temperature, and the H/D isotopic ratio. Only under special conditions (in inactive centers and at acidic pH) is electron transfer from YZ to P680\textsuperscript{ox} steered by the transfer of a proton to the protein/water boundary if not into the bulk. Under all other conditions (active centers in the physiological pH range, inactive centers at alkaline pH), the hydroxyl proton is either trapped by a nearby base inside the protein or intrinsically absent due to the low pK (about 7 in inactive and 4.5 in active centers) of YZ. These features make the functioning of YZox as a hydrogen abstractor from bound water unlikely. Instead, the positive charge on or near YZox may provide the final oxidizing potential that allows two two-electron abstractions from bound water at the Mn cluster during transition S1 → S4 → S0.

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