$P680^{+\bullet}$ Reduction Kinetics and Redox Transition Probability of the Water Oxidizing Complex as a Function of pH and H/D Isotope Exchange in Spinach Thylakoids†

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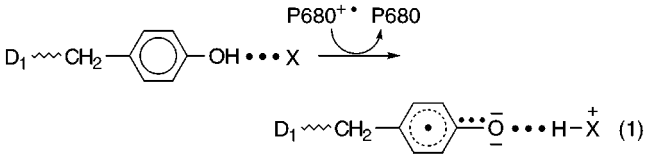
Received November 19, 1998; Revised Manuscript Received January 26, 1999

ABSTRACT: The rise of fluorescence as an indicator for $P680^{+\bullet}$ reduction by Y_Z and the period-four oscillation of oxygen yield induced by a train of saturating flashes were measured in dark-adapted thylakoids as a function of pH in the absence of exogenous electron acceptors. The results reveal that: (i) the average amplitude of the nanosecond kinetics and the average of the maximum fluorescence attained at 100 μs after the flash in the acidic range decrease with decreasing pH; (ii) the oxygen yield exhibits a pronounced period-four oscillation at pH 6.5 and higher damping at both pH 5.0 and pH 8.0; (iii) the probability of misses in the S_i-state transitions of the water oxidizing complex is affected characteristically when exchangeable protons are replaced by deuterons [at pH $<$6.5, the ratio $\alpha(D)/\alpha(H)$ is larger than 1 whereas at pH $>$7.0 values of $<$1 are observed]. The results are discussed within the framework of a combined mechanism for $P680^{+\bullet}$ reduction where the nanosecond kinetics reflect an electron transfer coupled with a "rocket-type" proton shift within a hydrogen bridge from Y_Z to a nearby basic group, X [Eckert, H.-J., and Renger, G. (1988) *FEBS Lett. 236*, 425−431], and subsequent relaxations within a network of hydrogen bonds. It is concluded that in the acidic region the hydrogen bond between Y_Z and X (most likely His 190 of polypeptide D1) is interrupted either by direct protonation of X or by conformational changes due to acid-induced Ca^{2+} release. This gives rise to a decreased $P680^{+\bullet}$ reduction by nanosecond kinetics and an increase of dissipative $P680^{+\bullet}Q_A^{-\bullet}$ recombination at low pH. A different mechanism is responsible for the almost invariant amplitude of nanosecond kinetics and increase of α in the alkaline region.

Photosynthetic water oxidation to molecular oxygen and four protons takes place at a manganese-containing operational unit referred to as water oxidizing complex (WOC)[1] (for reviews, see references *1*−*3*). This process is energetically driven by the strongly oxidizing chlorophyll cation radical $P680^{+\bullet}$ which is formed as a result of the primary charge separation in photosystem II (PS II) (for a review, see ref *4*). $P680^{+\bullet}$ extracts electrons stepwise from the WOC with tyrosine Y_Z [Tyr 161 of polypeptide D1 (*5*, *6*)] acting as intermediary electron carrier. The kinetics of both $P680^{+\bullet}$ reduction by Y_Z and WOC oxidation by Y_Z^{OX} depend on the redox state, S_i, of the WOC (for compilation of data, see ref *7*). The former reaction exhibits multiphasic kinetics which are dominated by electron transfer from Y_Z to $P680^{+\bullet}$ in the nanosecond time domain provided that the WOC is functionally competent (*8*, *9*). The extent of the nanosecond kinetics depends on S_i. On the other hand, the single electron-transfer steps in the WOC can be described by monoexponential kinetics with increasing half-life times and activation energies when the redox state S_i increases from S_0 to S_3 (see ref *10* and references cited therein).

The oxidized form Y_Z^{OX} appears as a neutral radical in EPR studies (*11*). The proton from the phenolic group is either localized in the neighborhood as originally discussed in (*9*) and supported by later studies (*12*, *13*) or released into the lumen as an essential step of the recently proposed hydrogen abstractor model of water oxidation (*14*). Based on the weak temperature dependence of the nanosecond kinetics, a mechanism was proposed (9) where electron transfer from Y_Z to $P680^{+\bullet}$ is coupled with the shift of the hydrogen-bonded proton of the OH group of Y_Z to a nearby base X (within a double well potential):



(1)

This proton shift is expected to be very fast, and therefore this reaction will be referred to as "rocket-type" mechanism. Based on several studies with mutants of *Synechocystis* PCC6803 where His 190 has been replaced by other residues (*15*−*23*) and on the results of structure modeling (*24*−*26*), the basic group X is now identified as His 190 of polypeptide D1. To explain the existence of multiphasic kinetics and the dependence of their normalized amplitudes on redox state S_i, it was proposed that the nanosecond kinetics reflect relaxation processes which lead to a shift of the redox equilibrium $P680^{+\bullet}Y_Z \rightleftharpoons P680Y_Z^{OX}$ toward the right side

† The financial support by the Deutsche Forschungsgemeinschaft (Re 354/10-3 and Re 354/17-4) is gratefully acknowledged.

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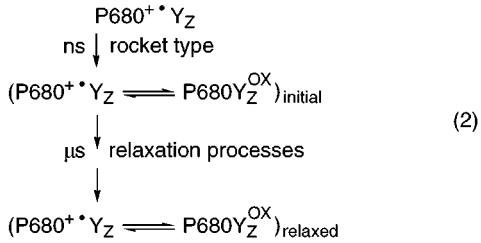
[1] Abbreviations: ^{3}Car, carotenoid triplet; Chl, chlorophyll; HEPES, *N*-2-(hydroxyethyl)piperazine-*N*′-(hydroxypropane)sulfonic acid; LHCII, light harvesting complex II; MES, morpholinoethanesulfonic acid; P680, photoactive chlorophyll of PS II; PS II, photosystem II; WOC, water oxidizing complex; Y_Z, redox active tyrosine between WOC and P680.

10.1021/bi9827520 CCC: $18.00
Published on Web 04/27/1999

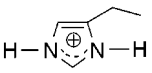
(*12*, *27*). The nanosecond kinetics of $P680^{+\bullet}$ reduction by Y_Z^{OX} remain invariant to replacement of exchangeable protons by deuterons (*28*) while the microsecond kinetics are markedly affected. Based on these findings, it was concluded that the relaxation processes in the microsecond time domain which give rise to higher population of state $P680Y_Z^{OX}$ are coupled with proton movement in the environment of Y_Z^{OX} (*12*, *29*, *30*). The above-mentioned results on $P680^{+\bullet}$ reduction kinetics can be summarized by the following simplified model:



(2)

where the population probability of $P680^{+\bullet}$ is higher in the initial than in the relaxed state(s) (*30*).

Apart from this multistep "forward" reaction $P680^{+\bullet}$ can also be reduced by $Q_A^{-\bullet}$ with a kinetics of 100−200 μs (*31*, *32*). Accordingly, retardation of the relaxation processes will lead to an increase of the relative extent of the dissipative recombination reaction between $P680^{+\bullet}$ and $Q_A^{-\bullet}$. As a consequence, the probability of misses in the S_i-state transitions increases and the characteristic period-four oscillation of flash-induced oxygen evolution exhibits a higher damping due to an enhanced probability of misses (*29*, *30*). A pronounced effect is expected to arise when the rocket-type reaction is blocked by protonation of His 190, thus breaking the hydrogen bond between the OH group of Y_Z and X (see eq 1). The p*K*-value for the formation of the imidazolium state:



of histidines is about 6 in solution (see textbooks of biochemistry), and therefore this type of kinetic modulation might be of relevance under in vivo conditions because the pH of the lumen can drop down to values of around 5 under continuous illumination (*33*). To address this problem, in samples that are as close as possible to in vivo conditions, comparative experiments of the $P680^{+\bullet}$ reduction kinetics and the period-four oscillation of oxygen evolution were performed with isolated spinach thylakoids. The results obtained highly support the idea that in the acidic range of $5.0 < pH < 7.0$ the extent of the very fast rocket-type Y_Z-oxidation by $P680^{+\bullet}$ becomes progressively diminished with decreasing pH while it remains virtually unaffected in the alkaline region. On the other hand, the probability of misses (α) in the period-four oscillation of oxygen yield increases at both pH 5.0 and 8.0 compared with the α-value at pH 6.5. Likewise, the isotope H/D exchange effect on the α-values changes its direction at around pH 7.0.

MATERIALS AND METHODS

Experimental Part. Thylakoids were isolated from spinach as described by Winget et al. (*34*). For H/D exchange experiments, concentrated samples of 4−8 mg of Chl/mL were suspended in a large excess of D_2O (99.9% D, Eurisotop) buffer solution.

Flash-induced changes of the fluorescence quantum yield with a time resolution of about 500 ns were monitored with home-built equipment (*35*) as outlined in (*27*). For the sake of a clear separation of the unresolved nanosecond kinetics from the slower components, the first point of the rise was monitored at 1 μs after the actinic laser flash. All experiments were performed at 4 °C. The suspension contained isolated thylakoids at a concentration of 20 μg of Chl/mL, 10 mM NaCl, and 50 mM each of the following buffers: succinic acid (pL = 4.0−5.0), MES (pL = 5.5−6.5), and HEPES (pL 7.0−8.0), where L = H or D.

The flash-induced O_2 oscillation patterns as a function of pH were measured at 4 °C with a modified Joliot-type electrode (*36*) as described in (*37*). The same buffer solutions were used as for the fluorescence measurements. In the "D_2O samples", the buffer solutions were adjusted by using a glass electrode with corrections according to Glasoe et al. (*38*).

Data Evaluation. For analysis of fluorescence data, two general phenomena have to be taken into account: (I) the transient flash-induced changes of the fluorescence yield reflect the superposition of time-dependent populations of different quenchers; and (II) the fluorescence yield is a nonlinear function of quencher concentration.

Two types of quenchers are relevant for point (I): (a) redox components in PS II and (b) quenching states in the antenna. With respect to type (a), the fluorescence yield is determined by the redox states of P680, Pheo, and Q_A (other redox components can be ignored for the sample type and experimental conditions used in this study). The radical ion states $P680^{+\bullet}$ and $Pheo^{-\bullet}$ are efficient nonphotochemical quenchers (*39*−*41*) while Q_A acts as photochemical quencher (*42*). A deconvolution into effects due to individual quenchers can be achieved on the basis of kinetic differences. The flash-induced primary charge separation leads to the radical pair $P680^{+\bullet}Pheo^{-\bullet}$ which becomes stabilized by electron transfer from $Pheo^{-\bullet}$ to Q_A. The latter process is characterized by lifetimes of about 300 ps (*43*−*45*). Accordingly, at about 1 ns after the flash the state $P680^{+\bullet}PheoQ_A^{-\bullet}$ is attained and quencher $Pheo^{-\bullet}$ disappeared. Therefore, at a time resolution of about 1 μs, only the reduction of $P680^{+\bullet}$ and reoxidation of $Q_A^{-\bullet}$ contribute as type (a) quenchers to the fluorescence transients. Three different reactions determine the signals: (i) $P680^{+\bullet}$ reduction by Y_Z; (ii) recombination reaction between $Q_A^{-\bullet}$ and $P680^{+\bullet}$, and (iii) $Q_A^{-\bullet}$ reoxidation by Q_B ($Q_B^{-\bullet}$). The first reaction exhibits multiphasic kinetics in the nanosecond and microsecond time domains (*8*, *9*, *12*, *30*) and causes a fluorescence rise owing to disappearance of quencher $P680^{+\bullet}$. In marked contrast, the second reaction, with typical 100−200 μs kinetics (*31*, *32*), is not coupled with a net change of the fluorescence yield because quencher $P680^{+\bullet}$ is eliminated at the expense of the synchronous formation of quencher Q_A. The third reaction takes place with kinetics of a few hundred microseconds at room temperature (*46*, *47*). It has been shown that a kinetic separation of reactions (i) and (iii) can be achieved when the flash-induced fluorescence transients were measured at 4 °C and monitored with a sweep time of 100 μs as outlined in ref *27* and *30*.

With respect to type (b) quenchers, only carotenoid triplets (^{3}Car) need to be considered. Recent studies with isolated LHC II revealed that in the time domain of 1−100 μs the fluorescence yield only depends on formation of ^{3}Car and its decay with a characteristic 3 μs kinetics under aerobic conditions (*27*, *48*, *49*). Accordingly, after correction for these 3 μs kinetics, the fluorescence rise reflects the kinetics of $P680^{+\bullet}$ reduction by Y_Z whereas the $P680^{+\bullet}Q_A^{-\bullet}$ recombination is "invisible".

The nonlinearity between fluorescence yield and quencher concentration [point (II)] originates from excitation energy transfer between different photosynthetic units of PS II (50). There exist two different types of PS II (α- and β-centers) (*51*), and only α-centers are connected by excitation energy transfer. It has been shown that under conditions where only redox changes of quencher Q_A take place in PS II the normalized variable fluorescence $F_v/F_{v,max}$ as a function of the normalized quencher concentration [Q] is given by the equation (*52*):

$$\frac{F_v}{F_{v,max}} = a\frac{(1-p)(1-[Q])}{1-p(1-[Q])} + (1-a)(1-[Q]) \quad (3)$$

where $F_v = F - F_o$ and $F_{v,max} = F_{max} - F_o$, a = the fraction of α-centers, and p = the probability of excitation energy transfer between them.

In the present study, the fluorescence rise is determined by quencher $P680^{+\bullet}$, and therefore F_v and [Q] in eq 3 have to be replaced by $F_v(t)$ and $[P680^{+\bullet}(t)]$, respectively. In this case, however, a problem arises for the normalization of the $P680^{+\bullet}(t)$ concentration. If one accepts that a saturating laser flash generates state $P680^{+\bullet}Q_A^{-\bullet}$ in all PS II centers, the "true" maximum value of $F_{v,max}$ can only be achieved if a complete transformation into state $P680Q_A^{-\bullet}$ takes place. This, however, does not occur due to competing $P680^{+\bullet}Q_A^{-\bullet}$ recombination. Therefore, the maximum value of the difference $F(100\ \mu s) - F_o$ is used as a suitable approximation of $F_{v,max}$. The values measured after the first and fifth flashes at pH 6.5 are used because in this case the contribution of $P680^{+\bullet}Q_A^{-\bullet}$ recombination to the total $P680^{+\bullet}$ reduction is estimated to be about 5% or less (vide infra). In this way, the time course of the normalized fluorescence rise $[F(t) - F_o]/[F_{max}\ (100\ \mu s) - F_o]$ is transformed into $[P680^+(t)]$ by using eq 3 and values of $a = 0.7$ and $p = 0.5$. These values provide the best description of experimental data in thylakoids when Q_A is the only quencher (*52*, *53*). It is reasonable to assume that replacement of quencher Q_A by quencher $P680^{+\bullet}$ does not affect the values of a and p.

The kinetics are fitted by three exponentials plus a time-independent parameter. A target fit procedure with optimization by a special evolution strategy according to Ostermeier (*54*) was applied as outlined in (*27*, *30*).

The data evaluation of the oxygen yield measurements was performed within the framework of the conventional Kok model (*55*). For the sake of simplicity, a dependence of α on redox state S_i (*56*, *57*) has not been explicitly taken into consideration. The technical details of the fit procedure are described elsewhere (*58*).

RESULTS

Flash-Induced Changes of the Relative Fluorescence Quantum Yield in Dark-Adapted Thylakoids. Recent analyses revealed that the multiphasic $P680^{+\bullet}$ reduction by Y_Z in samples with an intact WOC can be monitored via flash-induced changes of the relative fluorescence quantum yield (*27*, *30*, *35*). Figure 1 shows traces obtained when dark-adapted isolated spinach thylakoids suspended in buffer solution of different pH are excited with a train of eight saturating laser flashes. The traces obtained at pH 6.5 correspond to published data (*27*, *30*). They exhibit a pronounced period-four oscillation of both the rise kinetics and the normalized fluorescence level attained 100 μs after the flashes [this parameter after flash number n of the train is symbolized by $F_n(100\ \mu s)$]. At this pH the oxygen evolution capacity attains its maximum (*59*, *60*), and, therefore, in the following, the "pH 6.5 samples" will be used as reference and referred to as control in this study (see also Materials and Methods). Marked deviations arise at lower pH (traces b and c) while an increase in the pH to 8.0 leads to comparatively moderate effects (trace d). A comparison of the traces measured at pH 5.0 (b) with those of the control (a) reveals that three striking features emerge at moderately acidic pH values: (i) the extent of the unresolved fast rise in the nanosecond time domain reflected by the data point at 1 μs after the flash markedly decreases in all flashes; (ii) the $F_n(100\ \mu s)/F_o$ level is diminished in all flashes; and (iii) the oscillation patterns of the extent of nanosecond kinetics and of $F_n(100\ \mu s)/F_o$ are less pronounced.

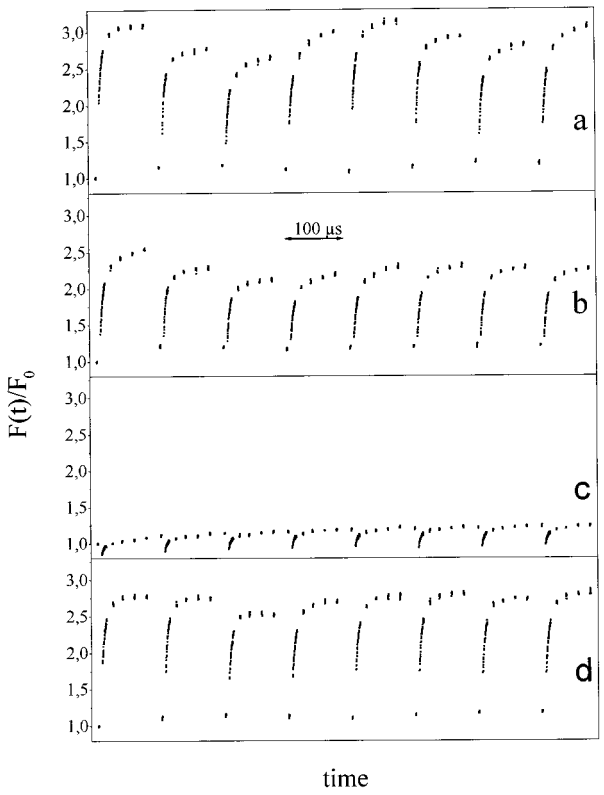


FIGURE 1: Normalized changes of the fluorescence quantum yield $F(t)/F_o$ induced by a train of saturating laser flashes (dark time 1 s) at 4 °C in dark-adapted spinach thylakoids suspended in buffer solutions of different pH values. The signals shown represent the average of two different thylakoid preparations and of eight different traces induced by the train of eight flashes in each sample. Sweep time: 100 μs. The first data point of the fluorescence rise is monitored 1 μs after the actinic laser flash. For other experimental details, see Materials and Methods. Panel a, control (pH 6.5); panel b, pH 5.0; panel c, pH 4.0, and panel d, pH 8.0.

A further lowering of the pH value down to 4.0 leads to a drastically altered pattern (trace c). In marked contrast to both the control (trace a) and the pH 5.0 sample (trace b), the flash-induced changes of the relative fluorescence yield are now of opposite direction; i.e., an "instantaneous" decrease is observed instead of the normal increase (compare panels b and c). The subsequent rise is dominated by a 3 μs kinetics, and its extent is mainly compensating the flash-induced decrease. This feature of the "pH 4 sample" resembles that observed for isolated LHC II which reflects exclusively formation (kinetically unresolved fluorescence decrease) and 3 μs decay of quencher ^{3}Car (*27*, *48*, *49*). In total, only a rather small increase above the F_o level is obtained in panel c. As a consequence, the $F(100\ \mu s)/F_o$ ratio is close to 1. Furthermore, any period-four oscillation completely disappeared. The results at pH 4.0 are readily explainable by a blockage of the WOC and a high degree of back-reaction between $P680^{+\bullet}$ and $Q_A^{-\bullet}$ (*59*). The latter reaction is not detectable by flash-induced fluorescence quantum yield changes [for details, see Materials and Methods and (*27*, *35*)].

In the following, the effects of pH will be analyzed only in a range where the WOC of isolated thylakoids is not significantly affected in its functional competence, i.e., in $5.0 \leq pH \leq 8.0$ [see data in (*59*, *60*)]. It has to be emphasized that any pH dependence of the normalized number of PS II complexes with a functionally fully competent WOC comprises at least two overlapping effects: (1) reversible protonation/deprotonation reactions of amino acid residues which are essential for the overall reaction; and (2) irreversible degradation of the protein matrix at unfavorable pH values. Both effects are often intermingled. However, as outlined in our previous studies (*60*), a reasonable separation of both phenomena can be achieved by selecting suitable incubation conditions. Based on these results, the experiments of this study were performed at a comparatively short incubation time of a few minutes.

Average Amplitudes of the Fluorescence Rise Kinetics as a Function of pH. To test the idea of a suppression of the "rocket-type" $P680^{+\bullet}$ reduction by interruption of the hydrogen bridge between Y_Z and X (see eq 1) via protonation of group X, the extent of the nanosecond kinetics has to be determined as a function of pH. A possible modulation by individual S_i states will not be considered at this stage, and therefore Figure 2 shows the normalized average amplitudes of the three kinetic components (200 ns, 3 μs, and 20 μs) and the time-independent constant (see Materials and Methods) as a function of pH. An inspection of these data readily shows that compared with the control (pH 6.5), the normalized average amplitudes of the nanosecond kinetics decrease at lower pH but remain almost unaffected in the moderate alkaline region. The corresponding amplitudes of the 3 and 20 μs kinetics exhibit a rather weak dependence on pH while the time-independent parameter markedly increases at lower pH. The pH dependence of the normalized average amplitudes of the nanosecond kinetics of the flash-induced fluorescence rise in spinach thylakoids is in good qualitative agreement with previous measurements of 830 nm absorption changes in PS II preparations from thermophilic cyanobacteria (*61*). It indicates that the extent of fast $P680^{+\bullet}$ reduction by Y_Z becomes diminished at lower pH values. This finding

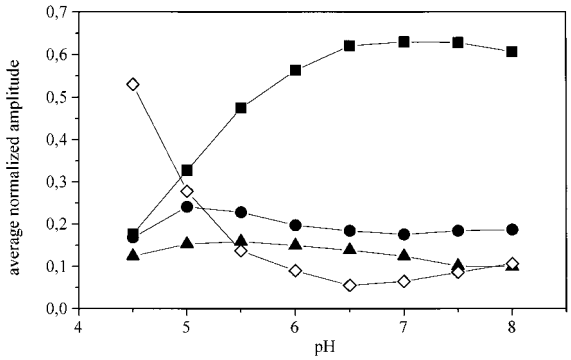


FIGURE 2: pH dependence of the normalized average amplitudes of the different kinetics and the time-independent parameter gathered from a target fit analysis of the data shown in Figure 1. The average amplitudes were determined according to $\bar{a}_i = \sum_{n=1}^{8} a_{n,i}$ where $a_{n,i}$ is the normalized amplitude of component i of the signal due to flash number n of the sequence. For details of data evaluation, see Materials and Methods. Filled symbols were used for the kinetics: 200 ns component (■), 3 μs component (●), and 20 μs component (▲), and an open symbol (◇) was used for the time-independent parameter.

is in line with the idea that the "rocket-type" electron/proton-transfer mechanism of Y_Z oxidation by $P680^{+\bullet}$ becomes progressively blocked upon acidification of the PS II donor side in both spinach thylakoids and thermophilic cyanobacteria.

Two effects are expected to arise if the pH dependence of the nanosecond kinetics would reflect a blockage of the "rocket-type" Y_Z oxidation by $P680^{+\bullet}$ concomitant with an increase of the dissipative back-reaction between $P680^{+\bullet}$ and $Q_A^{-\bullet}$: (a) the normalized average extent, $[F(100\ \mu s)/F_o]_{av}$, should decrease at low pH because the $P680^{+\bullet}Q_A^{-\bullet}$ recombination does not lead to fluorescence changes (*27*, *35*); and (b) the probability of misses in the oxidative S_i state transitions in the WOC has to increase when the extent of the $P680^{+\bullet}Q_A^{-\bullet}$ recombination reaction increases at the expense of $P680^{+\bullet}$ reduction by Y_Z. In the following, both expectations will be investigated.

Normalized Fluorescence Quantum Yield. The values of the fluorescence levels before $(F_o')_{av}$ and 100 μs after each flash, $[F(100\ \mu s)]_{av}$ (see Figure 1), averaged over the sequence of eight flashes and normalized to the dark level F_o before the first flash are depicted in Figure 3 as a function of pH. Two features emerge: (i) the $(F_o'/F_o)_{av}$ value of about 1.2 is virtually independent of pH at $4.5 \leq pH \leq 8.0$; and (ii) the $[F(100\ \mu s)/F_o]_{av}$ exhibit a striking decrease when the pH of the suspension is lowered from 6.5 to 4.5. The finding of $(F_o'/F_o)_{av} > 1$ originates from the slight oscillation of the initial fluorescence as a function of redox state S_i (see traces in Figure 1). This phenomenon is well established since its early discovery more than 25 years ago (*62*, *63*) and will not be analyzed in this study.

The most interesting feature with respect to a pH-dependent "rocket-type" mechanism of fast Y_Z oxidation by $P680^{+\bullet}$ is the marked decrease of $(F(100\ \mu s)/F_o)_{av}$ in the acidic region. This finding is expected when the normalized extent of the back-reaction between $P680^{+\bullet}$ and $Q_A^{-\bullet}$ increases with decreasing pH. It is therefore in line with the idea of a break of the hydrogen bond between the OH group of Y_Z and X (see eq 1) by the presumed protonation of His 190 to its imidazolium state. The population probability of

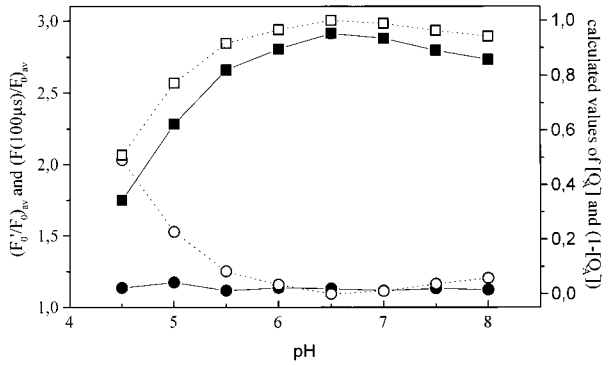


FIGURE 3: Average extent of the normalized fluoresence before, $(F_o'/F_o)_{av}$, and attained at 100 μs after each of the actinic flashes, $(F(100\ \mu s)/F_o)_{av}$, respectively, calculated population of state $P680^{+\bullet}Q_A^{\bullet}$ symbolized by [Q] and difference 1 − [Q] as a function of pH. Experimental data are taken from Figure 1; the calculation of [Q] was performed as described under Materials and Methods. The value of [Q] for the control is normalized to 1. The average values of $(F_o'/F_o)_{av}$ and $(F(100\ \mu s)/F_o)_{av}$ are obtained from the data of Figure 1 by the relation $(F/F_o)_{av} = (1/8)\sum_{n=1}^{8}(F_n/F_o)$ where F_n is either $(F_o')_n$ or $F_n(100\ \mu s)$ and n is the number of flashes in the train; F_o = the fluorescence before the first flash. The following symbols are used: (●) $(F_o'/F_o)_{av}$; (■) $(F(100\ \mu s)/F_o)_{av}$; (□) $[Q_A^{-\bullet}]$; (○) $1 - [Q_A^{-\bullet}]$. For further details, see text.

state $P680Q_A^{-\bullet}$ at 100 μs after the flash symbolized by $[Q_A^{-\bullet}]$ will be diminished when the extent of the $P680^{+\bullet}Q_A^{-\bullet}$ recombination reaction increases [effects due to $Q_A^{-\bullet}$ reoxidation by $Q_B(Q_B^{-})$ are sufficiently small for measurements at 4 °C (see Materials and Methods)]. The extent of flash-induced formation of state $P680^{+\bullet}Q_A^{-\bullet}$ is almost independent of pH at 4.5 ≤ pH ≤ 8.0. Therefore, the difference between the maximum value of $[Q_A^{-\bullet}]$ at pH 6.5 and the corresponding value at a given pH can be interpreted as a pH-induced increase of the $P680^{+\bullet}Q_A^{-\bullet}$ recombination. This difference is depicted in Figure 3 as a function of pH (open circles). It exhibits a marked increase at decreasing pH. A normalization of $[Q_A^{-\bullet}]$ to 1 at pH 6.5 ignores any contribution of the $P680^{+\bullet}Q_A^{-\bullet}$ recombination reaction to the $(F(100\ \mu s)/F_o)_{av}$ value of the control. The probability of misses was independently determined from oxygen yield measurements (vide infra), and a value of 0.075 was determined for pH 6.5. If one assumes that this α-value originates entirely from the $P680^{+\bullet}Q_A^{-\bullet}$ recombination reaction, the "true" $[Q_A^{-\bullet}]$ value would be slightly higher, and the axis of the right side of Figure 3 is shifted by a few percent. This small shift, however, does not affect the general feature and therefore will not be discussed in detail. The increase of $1 - [Q_A^{-\bullet}]$ at decreasing pH is qualitatively in good agreement with the hypothesis of a blockage of the very fast "rocket-type" Y_Z oxidation by $P680^{+\bullet}$. A decrease of the extent of nanosecond kinetics implies an increased probability of $P680^{+\bullet}$ reduction via microsecond relaxation processes (see eq 2) and the competing recombination reaction with $Q_A^{-\bullet}$. An increase of the extent of the latter reaction, however, necessarily leads to an increase of the probability of misses in the S_i-state transitions of the WOC, and therefore this consequence can be experimentally checked by independent measurements, i.e., determination of the period-four oscillations of the oxygen yield in dark-adapted thylakoids that are excited with a train of short saturating flashes.

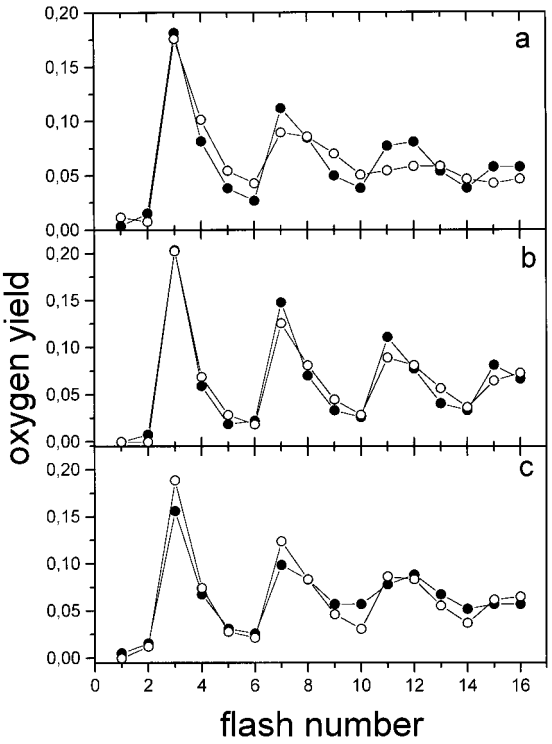


FIGURE 4: Normalized oxygen yield per flash as a function of the number of saturating single-turnover flashes in dark-adapted spinach thylakoids suspended in H_2O (filled circles) or D_2O (open circles) containing buffer of different pL (L = H or D). Panels a, b, and c describe results at pL = 5.0, 6.5, and 8.0, respectively. For experimental details, see Materials and Methods.

Period-Four Oscillation of Flash-Induced Oxygen Evolution. The filled circles in Figure 4 represent typical O_2 oscillation patterns which were measured with a Joliot-type electrode (*36*) in thylakoids suspended in H_2O-containing buffer solutions of different pH values. An inspection of these results shows that the oscillation is more pronounced at pH 6.5 (panel b) than at either pH 5.0 (panel a) or pH 8.0 (panel c), thus indicating an increased probability of misses in the acidic and alkaline range. The evaluation of these data, within the framework of the conventional Kok model, leads to the results depicted in Figure 5. The filled circles for the "H_2O samples" show that the probability of misses exhibits a minimum value of about 0.075 at pH 6.5 and increases both on the acidic and on the alkaline side. This feature is typical and nicely corresponds with our previously reported data (*60*). The increase of α at pH 5 and 8 fully accounts (within the limit of the experimental error) for the slight decrease of the average oxygen yield per flash at the corresponding pH values (*59, 60*) (data not shown). As a consequence, it can be concluded that after a short-time dark incubation of a few minutes, the number of functionally compentent WOC's is insensitive to pH in the range of 5−8 and the kinetic pattern of electron-transfer reactions is responsible for the pH dependence of the average oxygen yield per flash. At pH <5.0, the α-values steeply increase (data not shown) and the oxygen yield sharply drops down; i.e., it approaches zero at pH 4.0 depending on the incubation time. The marked increase of the α-values at decreasing pH is in contrast to a former study where α was reported to be virtually the same at pH 4.5 and pH 7.0 (*61*). It seems unlikely that this is due to different sample material. Limitations in the measurement

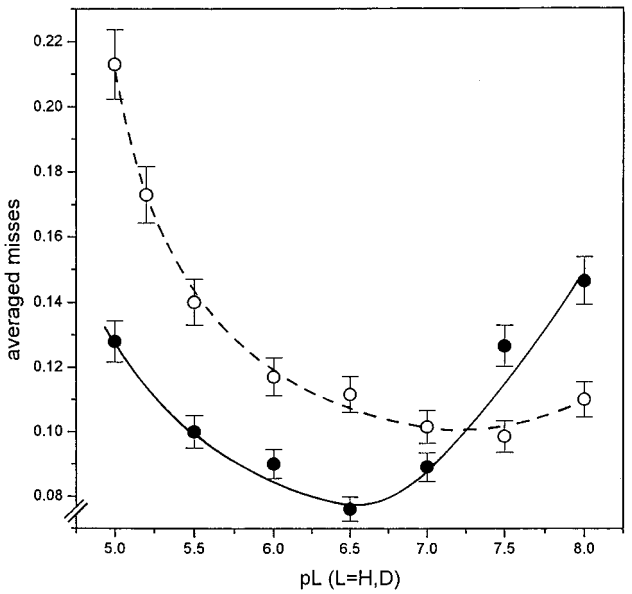


FIGURE 5: Probability of misses as a function of pH in "H_2O sample" (filled circles) and "D_2O sample" (open circles) as a function of pL. The determination of the α-values from oscillation patterns as shown in Figure 4 was performed within the framework of the conventional Kok model (see Materials and Methods). The data points represent the average of five measurements each performed with two different thylakoid preparations.

of the oxygen yield pattern in (*61*) seem to be a more plausible explanation for this discrepancy.

A comparison with the fluorescence data of Figure 3 reveals a very interesting phenomenon. At pH <6.5, qualitatively the same feature emerges from both methods, i.e., an increase of the α-values with progressing acidification. On the other hand, in the alkaline region the increase of the α-values gathered from the oxygen yield measurements has no corresponding counterpart in the extent of nanosecond kinetics of the fluorescence rise (see Figures 2 and 3). This difference is more clearly seen when the pH dependence of the normalized average amplitudes of the nanosecond kinetics is taken into account (see Figure 2). This disparity suggests that different mechanisms are responsible for the pH dependence of the α-values and the kinetics of $P680^{+\bullet}$ reduction by Y_Z in the acidic and alkaline regions.

To obtain complementary information, the influence of a replacement of exchangeable protons by deuterons has been analyzed. This H/D exchange has recently been shown to exert different effects on the multiphasic kinetics of $P680^{+\bullet}$ reduction. The retardation of the microsecond kinetics in "D_2O samples" was ascribed to slower relaxation processes (*12*, *29*, *30*), thus giving rise to an enhanced extent of the competing $P680^{+\bullet}Q_A^{-\bullet}$ recombination. As a consequence, the probability of misses is expected to increase. The results obtained for the "D_2O sample" are also shown in Figure 4 (open circles). A comparison with the "H_2O sample" readily shows that the damping of the period-four oscillation pattern is more pronounced in the "D_2O sample" at pH 5.0 whereas the opposite is observed at pH 8.0. The data evaluation within the conventional Kok model leads to α-values which are shown in Figure 5 (open circles). At pL (L = H or D) <6.5, the expected ratio of α(D)/α(H) > 1 is observed. Values of about 1.6 are obtained. A drastically different feature, however, emerges at pH >7.0. In this case, the α(D)-values

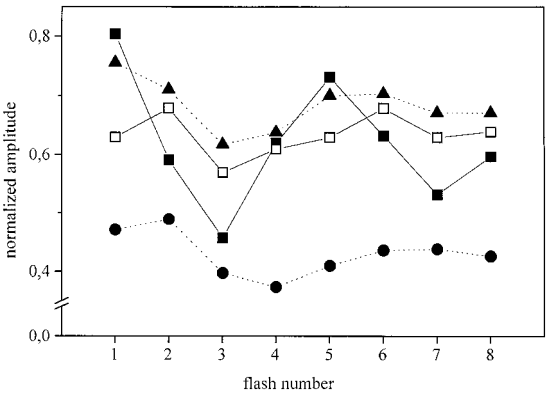


FIGURE 6: Normalized amplitudes of the nanosecond kinetics of fluorescence rise as a function of flash number in dark-adapted thylakoids suspended either in H_2O-containing buffer at pH 5 (filled circles), 6.5 (filled squares), or 8.0 (filled triangles) or in pD 6.5 D_2O-containing buffer (open squares).

at a certain pD are smaller than α(H) at the corresponding pH. This reversal of the α(D)/α(H) ratio is indicative for a different mechanism leading to an increase of α. A p*K* shift in the "D_2O sample" by about 1 unit could explain the data. The magnitude of this shift is significantly larger than data reported in the literature for the effect of D_2O on the p*K* of weak acids (*64*, *65*), and therefore it is difficult to ascribe the data of Figure 5 to the p*K* shift of a single protonizable residue. It appears more attractive to assume that several weak acidic groups give rise to this effect and these groups form the hydrogen bond network that determines the microsecond relaxation processes (see eq 2). A modulation of the relaxation processes by pH is plausible when taking into consideration that the number of bound protons drastically decreases with increasing pH. As a consequence, the hydrogen bond network and the proton rearrangement coupled with the relaxation processes are also expected to become modified.

Period-Four Oscillation of the Normalized Amplitudes of Nanosecond Fluorescence Rise Kinetics at Different pH Values and after H/D Exchange. To corroborate the idea of a pH-dependent change of mechanism, the oscillation patterns of the normalized amplitudes of the nanosecond kinetics were analyzed as a function of pH. The values measured for the fluorescence rise at different pH (see Figure 1) and normalized to $F_{v,max}$ (see Materials and Methods) were corrected for contributions by ^{3}Car quenching as outlined in (*27*, *35*). The results obtained at pH 5.0, 6.5, and 8.0 are depicted in Figure 6. As expected, a pronounced period-four oscillation with maxima after the first and fifth flashes is obtained at pH 6.5, in perfect agreement with recent findings (*27*). Likewise, the damping of this pattern largely increases at pH 5.0 and 8.0, in close correspondence with the pH dependence of the α-values shown in Figure 5. On the contrary, however, the amplitudes of the nanosecond kinetics are much smaller at pH 5.0 than at pH 8.0 as expected from the averaged data depicted in Figure 2.

Another interesting observation is the finding that both the oscillation pattern and normalized amplitudes of the nanosecond kinetics of the pH 8 sample are similar to those of the control after replacement of exchangeable protons by deuterons (compare filled triangles with open squares in Figure 6). The H/D exchange effect has been explained by a retardation of relaxation processes in the microsecond

domain (*30*). A closer inspection of the traces in Figure 1 suggests that a similar retardation is unlikely at pH 8 and therefore another mechanism has to be responsible for the modified oscillation pattern in the moderate alkaline region (see Discussion).

DISCUSSION

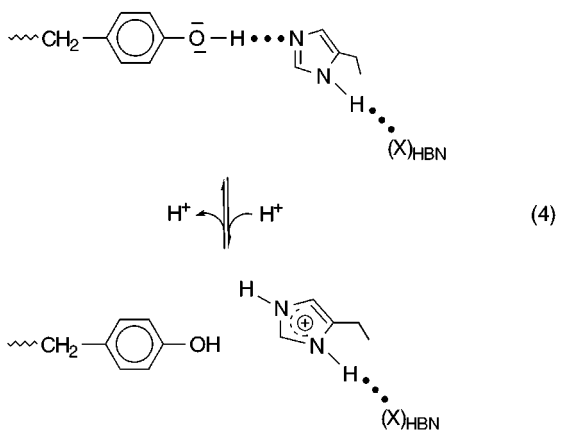
In the present study, isolated thylakoids were used as sample material because according to recent results the kinetics of PS II donor side reactions are slightly modified in detergent-treated samples; especially the S_3 oxidation by Y_Z is retarded by a factor of about 2 in PS II membrane fragments (*66*). Likewise, in the latter preparations, the probability of misses is markedly higher (*67*, *68*). Therefore, isolated thylakoids appear to be the most suitable sample type to analyze the reaction pattern of the PS II donor side in close relation to in vivo conditions. The results reported here reveal that in the range of $5.0 \leq pH \leq 8.0$, where the number of PS II complexes with a fully intact WOC is virtually not changed (see Results and refs *59*, *60*), three interesting features emerge: (i) the normalized amplitudes of the nanosecond kinetics of the flash-induced fluorescence rise remain virtually constant above pH 6.5 but decrease with progressing acidification; (ii) the extent of the $P680^{+\bullet}Q_A^{-\bullet}$ recombination reaction as reflected by a decrease of the normalized maximum fluorescence markedly increases when the pH is lowered from 6.5 to 5.0 whereas a shift into the moderate alkaline region (pH 8.0) leads to comparatively small effects; and (iii) the probability of misses, α, gathered from the period-four oscillation of flash-induced oxygen evolution exhibits a minimum value at pH 6.5 and almost symmetrically increases when the pH is changed either to 5.0 or to 8.0.

Based on these findings, it is evident that the origin of the enhanced α-values is different in the acidic and alkaline reagions. We have previously proposed that the $P680^{+\bullet}$ reduction by Y_Z with nanosecond kinetics occurs via a "rocket-type" mechanism where the electron transfer is coupled with a proton shift within a hydrogen bridge from the OH group of Y_Z to a nearby base X (*9*). The latter group is most likely the imidazole ring of His 190 in polypeptide D1 (15−26). The extent of $P680^{+\bullet}$ reduction by Y_Z further increases in the microsecond time domain by relaxation processes which give rise to a shift of the equilibrium $P680^{+\bullet}Y_Z \leftrightharpoons P680Y_Z^{OX}$ toward the right side (*12*, *30*). Based on H/D isotope exchange effects, the relaxation processes were inferred to be coupled with local proton movements (*12*, *29*, *30*).

For the interpretation of the pH effects observed in this study, different mechanisms are discussed for the moderate acidic and alkaline regions.

Interuption of the Hydrogen Bridge with Y_Z by Direct Protonation of His 190 in the Moderate Acidic Region. Within the framework of the combined "rocket-type"/relaxation model described by eq 2, the very fast Y_Z oxidation according to eq 1 could be rate-limited either by the proton or by the electron-transfer step. According to transition state theory, the rate of proton transfer in a hydrogen-bonded donor (D)−acceptor (A) pair is approximately $10^{12.8-\Delta pK_{DA}}\ s^{-1}$ (*69*) where ΔpK_{DA} is the difference of the p*K* values of D and A, respectively. Taking the p*K* values for the donor Tyr (p$K \approx 10$) and acceptor His (p$K \approx 6$) in solutions (see Textbooks of Biochemistry), a rate constant of about $(60\ ns)^{-1}$ is determined. This value is of the same order of magnitude as the experimentally monitored nanosecond kinetics of $P680^{+\bullet}$ reduction by Y_Z (*8*, *9*). However, it has to be emphasized that the rate constant of $(60\ ns)^{-1}$ estimated for proton transfer could deviate from reality by orders of magnitude because the p*K* values of amino acid residues in proteins are prone to substantial shifts due to mutual interactions of their protonizable groups (*70*). Therefore, ΔpK_{DA} could be significantly smaller than 4.0 and proton transfer within the bridge between Y_Z and His 190 much faster, i.e., a "rocket-type" reaction. As a consequence, the electron transfer from Y_Z to $P680^{+\bullet}$ would be the rate-limiting step of Y_Z oxidation. This idea is supported by the result of a recent analysis of this reaction (*71*) within the framework of the Marcus theory (*72*). It is clear that the strength of the hydrogen bond is of key relevance for the "rocket-type" mechanism. Recently the possibility has been controversially discussed that low-barrier hydrogen bonds (LBHB) might play a role in various enzymatic mechanisms (*73*−*75*). In model systems such as benzoylacetone, the energy barrier for the transition state in strong intramolecular hydrogen bonds has been calculated to be of the order of 10 kJ/mol (*76*). Although the LBHB model is an attractive proposal and the energy barrier exhibits a striking similarity with the experimentally determined activation energy for Y_Z oxidation by $P680^{+\bullet}$ (*9*), it seems more likely that this reaction is limited by the electron-transfer rate as outlined in ref *71* and that the hydrogen bond is significantly weaker than in a LBHB. The strength of the hydrogen bond between Y_Z and His 190 strongly depends on the distance between the O atom of Y_Z and the proximal nitrogen of His 190. It is clear that any structural change will affect this hydrogen bond. Therefore, the decrease of the extent of the nanosecond kinetics in the moderate acidic region can be readily explained by a protonation of the imidazole group of His 190. Based on the measured pH dependencies, the p*K* for the presumed imidazolium $\leftrightharpoons$ imidazole equilibrium can be estimated to be somewhat below 5.0. This value is in striking correspondence with our former conclusions on the p*K* of protonizable group(s) which was (were) inferred to control the average extent of microsecond kinetics. The latter proposal is based on a completely different assay, i.e., measurements of 690 nm absorption changes in thylakoids under repetitive flash excitation (*59*). A more precise experimental determination of the p*K* is prevented by the progressing irreversible destruction of the WOC when the pH is further decreased (*59*, *60*). A p*K* of 5.0 is markedly lower than that (p$K = 6.0$) known for the formation of the imidazolium state of His in solution (see Textbooks of Biochemistry). It is conceivable that the protonation of an imidazole ring in a hydrogen bond is more difficult and therefore the p*K* shifted toward lower values. Depending on the strength of the hydrogen bonding, also the p*K* value of Y_Z is expected to decrease. This idea is strongly supported by a recent study (which came to our knowledge after submission of this paper) where the p*K* value of hydrogen-bonded Y_Z was estimated to be 8.0−8.3 (*77*). The p*K* shift of Y_Z toward lower values is even larger than the change that is estimated for the protonation of the imidazole ring of His 190 from the

decrease of the extent of nanosecond kinetics. It should be mentioned that the samples used in (*77*) do not contain the manganese cluster. Therefore, the microenvironment of Y_Z is different as shown by measurements of the activation energy (*9*, *13*, *71*, *78*) and kinetic H/D isotope exchange effects of $P680^{+\bullet}$ reduction by Y_Z (*12*, *28*, *79*) and deduced from several studies using magnetic resonance spectroscopy (*80*). Accordingly, the p*K* values of Y_Z and His 190 in the native state of PS II could undergo alterations, but the general tendency of a shift toward lower values should not be changed. Furthermore, for a more detailed analysis, it has to be taken into account that the distal NH group of the imidazole ring of His 190 which is not part of the hydrogen bridge to Y_Z most likely acts as donor for another hydrogen bond with proton acceptor group(s) of the protein. In general, a network of hydrogen bonds is existing. Therefore, it appears to be oversimplifying and misleading when the mechanism of Y_Z oxidation by $P680^{+\bullet}$ is discussed exclusively on the basis of the p*K* values of individual amino acid residues. As a consequence of this idea, a more complex system with a global p*K* is a more appropriate description of the real situation. Accordingly, the blockage of the "rocket-type" reaction in the acidic region should be described by the following scheme:



(4)

where $(X)_{HBN}$ symbolizes all groups that participate in the presumed hydrogen bond network (HBN). This scheme of eq 4 also attempts to illustrate that the formation of the imidazolium state is probably coupled with some change of the mutual orientation between Y_Z and His 190. It has to be emphasized that a positive charge which is restricted to a localized imidazolium ring as shown on the right side of eq 4 reflects only approximately the charge distribution within a HBN. A partial delocalization throughout the whole HBN is probably more realistic.

For further detailed mechanistic analysis of this point, UV−resonance Raman spectroscopy in combination with H/D exchange might provide a promising tool. This technique was recently successfully applied for studies on the hydrogen bonding state of catalytically important distal histidine in horseradish peroxidase (*81*).

Allosteric Interruption of the Hydrogen Bridge with Y_Z by Proton-Induced Ca^{2+} Release in the Moderate Acidic Region. Although the idea of His protonation provides a consistent picture, an alternative explanation can be offered. A detachment of Ca^{2+} from its binding site(s) due to protonation of ligands in the acidic region has been discussed recently and a p*K* value of about 4.7 deduced from measurements of pH-dependent Ca^{2+} release from PS II membrane fragments (*82*, *83*). This effect could lead to conformational changes which disturb the H-bonding geometry between Y_Z and His 190 and thereby block the "rocket-type" mechanism of $P680^{+\bullet}$ reduction according to eq 1. This idea is in line with our previous finding that the ratio of the normalized amplitudes of nanosecond and microsecond relaxation kinetics of 830 nm absorption changes becomes markedly shifted toward the latter when PS II membrane fragments are subjected to a very mild trypsin treatment which does not significantly affect the number of PS II complexes with a functionally competent WOC (*84*, *85*). The effect is stimulated by illumination with flashes (*86*) and can be largely reversed by Ca^{2+} in a specific manner including binding sites with two different affinity constants (*84*−*86*). The model of H^+-induced Ca^{2+} release also explains the blockage of the very fast electron transfer from Y_Z to $P680^{+\bullet}$ via the "rocket-type" mechanisms, but in this case a conformationally induced distortion of the hydrogen bond (strength and geometry) would be responsible for this effect. The latter idea is in line with previous conclusions on structural changes of the WOC in the range of pH 5.3−5.6 (*60*, *87*). Direct structural information on the distance between Ca and Y_Z is not available. However, some indirect conclusions can be gathered from data reported in the literature. Recent EXAFS studies revealed that the active site of the WOC is a Mn−Ca heteronuclear cluster where the distance between Mn and Ca is about 3.5 Å (*88*, *89*). The distance between Y_Z and the manganese cluster is a matter of current debate. Values in the wide range of from 3.5 to 15−20 Å have been reported (*71*, *90*−*93*). The very short distances (*90*) are now being shown to originate from incorrect data interpretation and therefore have to be enlarged to 8−12 Å (*94*−*96*). As outlined in ref *29*, the above-mentioned values can be reconciled with the idea of hydrogen bonding between ligands of Ca^{2+} and Y_Z either directly or via a chain of hydrogen bonds analogous to that recently reported for cytochrome $P450_{cam}$ (*97*). Accordingly, pH-induced Ca^{2+} release would lead to interruption of this chain and/or conformational changes which disturb the interaction between Y_Z and His 190.

A straightforward distinction between both models (direct His 190 protonation versus Ca^{2+} release triggered change of the hydrogen bond) cannot be achieved on the basis of the current stage of knowledge. However, regulation via protonation of His 190 appears to be more attractive because it provides a direct and simple steering mechanism. The pH-dependent regulation of $P680^{+\bullet}$ reduction in the moderately acidic range is probably of physiological relevance because the thylakoid lumen becomes acidified under strong light and attains pH values of about 5 (*33*) or even somewhat lower (*98*). Therefore, the pH region between 5 and 8 is the most relevant when considering in vivo conditions. Protonation of His 190 prevents fast reduction of $P680^{+\bullet}$ by Y_Z, thus favoring $P680^{+\bullet}Q_A^{-\bullet}$ recombination at the expense of the forward electron transfer. In this way a contribution to the dissipation of excess excitation energy by the reaction center would be achieved as originally proposed in ref *31*.

Increased Probability of Misses in the Moderate Alkaline Region. In the moderate alkaline region, a different mech-

anism has to be responsible for the increase of the α-values. The present data and previous measurements of 830 nm absorption changes (*61*) clearly show that the contribution of nanosecond kinetics to the overall $P680^{+\bullet}$ reduction remains almost insensitive to a pH shift from 6.5 to 8.0. Furthermore, the H/D kinetic exchange effect on the α-values becomes reversed at around 7.0 (see Figure 5).

A closer inspection of Figure 6 reveals that except for the marked differences in the normalized extent of the nanosecond kinetics (see also Figure 2), the period-four oscillation patterns of the amplitudes of nanosecond kinetics exhibit almost the same damping at pH 5.0 and 8.0 ("H_2O sample") and pD 6.5 ("D_2O sample"). The corresponding α-values gathered from the oxygen yield measurements are markedly larger than those of the control (see Figure 5). Therefore, it is clear that the general dependence of the $P680^{+\bullet}$ reduction by Y_Z on redox state S_i of the WOC as reflected by the period-four oscillation of the extent of nanosecond kinetics remains almost the same at different types of modulation induced either by pH shift (to 5.0 or 8.0) or by H/D exchange at pH 6.5. In marked contrast, however, the average extent of the very fast electron transfer from Y_Z to $P680^{+\bullet}$ via the "rocket-type" mechanism becomes markedly affected only in the acidic region (see Figure 2 and refs *29*, *61*). Therefore, in the near-alkaline region the increase of α can be caused either by a retardation of the slower relaxation processes in a similar way as recently shown for replacement of exchangeable protons by deuterons at pL 6.5 (*12*, *30*) or due to an alternative mechanism.

The rather small variation of $(F(100\ \mu s)/F_o)_{av}$ in the moderate alkaline region (see Figure 3) and a preliminary kinetic data evaluation are not in favor with a retardation of the relaxation reactions in the microsecond time domain. Therefore, it seems more likely that in the alkaline region structural changes occur which lead to a dissipation of oxidizing redox equivalents via a pathway other than $P680^{+\bullet}Q_A^{-\bullet}$ recombination.

There are different lines of evidence for pH-dependent structural changes of the WOC: (i) detailed analyses of the tryptic degradation pattern of inside-out vesicles and PS II membrane fragments revealed that the susceptibility of the WOC drastically increases above pH values of about 7.4 (99−101); (ii) a similar pH threshold was observed for the loss of Cl^- (*102*), the inhibition of CN^- in Cl^--deficient samples (*103*), and the drastic enhancement of S_1-state reduction by NH_2OH and NH_2NH_2 (*87*); (iii) the half-lifetimes of S_2 and S_3 reduction with Y_D exhibit characteristic changes at a pH value of about 6.5 (*60*, *104*); and (iv) the kinetic H/D exchange effect on the flash-induced proton/deuteron release pattern is markedly changed between pL 6.4 and 7.3 (*104*). Based on these findings, it appears plausible that also the increase of the α-values in the alkaline region is indicative of structural changes of the PS II donor side. These changes might be shifted toward higher pD values when exchangeable protons are replaced by deuterons. As a consequence, α(D)/α(H) ratios of <1 are observed above pH values of about 7.0.

Now questions arise on the nature of the dissipative reaction(s) that give(s) rise to an increase of α. The most simple explanation is an accelerated decay of the redox states S_2 and S_3 in the WOC. The idea is in agreement with our previous findings of a structurally induced faster reduction of S_2 and S_3 by the redox active tyrosine Y_D of polypeptide D2 (*60*, *104*). To corroborate this proposal, further studies are required which are in progress and will be presented in a forthcoming report.

CONCLUDING REMARKS

In conclusion, the results of the present study show that different types of mechanisms are responsible for the pH dependence of the reaction pattern that eventually leads to photosynthetic oxygen evolution. Below neutral pH, the kinetics of $P680^{+\bullet}$ by Y_Z become modulated via a progressive blockage of the "rocket-type" mechanism which is required for nanosecond kinetics. Concomitantly, the probability of misses in the S_i-state transitions of the WOC increases and becomes further enhanced when exchangeable protons are replaced by deuterons. The diminished amplitudes of nanosecond kinetics are ascribed to increasing protonation of the imidazole ring of His 190, but a proton-induced Ca^{2+} replacement in the WOC and/or its environment cannot be excluded as alternative mechanism. On the contrary, in the moderately alkaline region, the extent of nanosecond kinetics remains virtually unaffected accompanied by an increase of α-values which is not ascribed to an enhanced extent of $P680^{+\bullet}Q_A^{-\bullet}$ recombination but most likely to dissipative reaction(s) in the WOC. The different pH dependencies are likely to be of physiological relevance because pH shifts down to about pH 5 or even lower in the thylakoid lumen occur under natural illumination conditions (*33*, *98*).

ACKNOWLEDGMENT

We thank Dr. B. Diner for critical reading and valuable comments.

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BI9827520