Analyses of pH-Induced Modifications of the Period Four Oscillation of Flash-Induced Oxygen Evolution Reveal Distinct Structural Changes of the Photosystem II Donor Side at Characteristic pH Values†

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ABSTRACT: This study presents a thorough analysis of the reaction pattern of flash-induced oxygen evolution in spinach thylakoids as a function of pH ($4.5 \leq pH \leq 9$) and the redox state of tyrosine Y_D in polypeptide D2. Evaluation of the experimental data within the conventional Kok model [Kok, B., Forbush, B., & McGloin, M. (1970) *Photochem. Photobiol. 11*, 457–475] led to the following results: (1) the probability of the miss factor is strongly pH dependent (with a pronounced minimum near neutral pH) while the double hit factor is less affected; (2) a marked increase of the apparent S_0 population arises at alkaline pH in dark-adapted samples where most of the Y_D is reduced, but this effect is absent if the percentage of PS II containing the oxidized form Y_D^{ox} is high; and (3) the lifetimes of S_2 and S_3 exhibit a characteristic pH dependence that is indicative of conformational changes of functional relevance within the water-oxidizing complex and its environment; (4) the kinetic interaction of redox states S_2 and S_3 with Y_D is characterized by a change of its behavior at a threshold pH of 6.5–7.0; and (5) at acidic pH values the extent of S_2 and S_3 reduction by Y_D decreases concomitant with the occurrence of a very fast decay kinetics. On the basis of a detailed discussion of these results and data from the literature, the water oxidase is inferred to undergo structural changes at pH values of 5–5.5 and 6.5–7.0. These transitions are almost independent of the redox state S_i and modify the reaction coordiates of the water oxidase toward endogenous reductants.

Photosynthetic oxygen evolution takes place within a manganese-containing functional unit, referred to as water-oxidizing complex (WOC),[1] of the photosystem II (PS II) which is anisotropically incorporated into the thylakoid membrane. The overall reaction comprises a sequence of four univalent oxidation steps (Kok et al., 1970) energetically driven by the cation radical $P680^+$ which is formed as the result of the primary charge separation within the PS II reaction center [for a recent review, see Renger (1992)]. A redox-active tyrosine residue of polypeptide D1 [symbolized by Y_Z and identified by Debus et al. (1988a) and Metz et al. (1989)] acts as intermediary carrier for the stepwise electron abstraction from the WOC by $P680^+$. In addition to the main oxidative pathway via Y_Z, the WOC in its different redox states S_i (i indicates the number of oxidizing redox equivalents stored in the WOC) can also interact with another redox-active tyrosine Y_D located in polypeptide D2 (Debus et al., 1988b; Vermaas et al., 1988). These comparatively slow reactions probably comprise the redox couples $P680/P680^+$ and Y_Z/Y_Z^{ox} [for a recent discussion, see Messinger et al. (1993)]. Although the kinetic pattern of the above-mentioned reactions is well resolved, key mechanistic questions of photosynthetic O_2 formation are still far form being understood [for reviews, see Renger (1987, 1993), Hansson and Wydrzynski (1990), Debus (1992) and Rutherford et al. (1992)]. Apart from special questions like the entry of substrate water into the redox cycle (Bader et al., 1993) and the formation of the oxygen–oxygen bond, the problem of structure–function relationships is of general interest for all enzymatic reactions. In this respect protons are of central relevance. Not only can they directly participate in several redox reactions but they are also important determinants of the protein structure either by forming hydrogen bonds or by affecting the electrostatic interaction within a protein or between different subunits.

The oxidation of two water molecules to O_2 is necessarily coupled with the concomitant formation of four protons which are released into the lumen of the thylakoids. Originally, the proton release pattern coupled with the sequence of the univalent oxidation steps in WOC was thought to provide information on the state of the substrate water molecules bound to the catalytic site. This interpretation was questioned on the basis of theoretical consideration (Renger, 1978; Wydrzynski et al., 1985), and it was predicted that the univalent redox steps $S_i \rightarrow S_{i+1}$ can exhibit noninteger pH-dependent stoichiometries because the reaction sequence takes place within a protein matrix (Renger, 1987). This idea is confirmed by recent studies indicating that the proton release pattern depends on pH [for a recent review, see Lavergne and Junge (1993)] and on the nature of the protein matrix surrounding the WOC (Wacker et al., 1990).

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[1] Abbreviations: D1 and D2, polypeptides of the photosystem II core; FWHM, full width at half-maximum; DCIP, 2,6-dichlorophenolindophenol; Hepes, *N*-(2-hydroxymethyl)piperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morphholino)ethanesulfonic acid; pH_c, critical pH value for a conformational change; PS I, photosystem I; PS II, photosystem II; P680, primary electron donor in PS II; Q_A and Q_B, primary and secondary plastoquinone acceptors of PS II; S_i, redox state i of the water oxidase; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; WOC, water-oxidizing complex; Y_D and Y_D^{ox}, redox-active tyrosine of polypeptide D2 in its reduced and oxidized form, respectively; Y_Z, redox-active tyrosine of polypeptide D1 that acts as the electron donor to $P680^+$; Y_D thylakoids and Y_D^{ox} thylakoids, thylakoids with a high or low amount of the reduced form of tyrosine D, respectively; Y_i, normalized oxygen yield due to the ith flash of a flash train.

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Apart from the protolytic reactions due to the redox steps leading to water oxidation, additional pH effects have to be considered. It was found that the oxygen evolution capacity strongly depends on pH with a flat maximum between 6.0 < pH < 8.0 and steep declines in the acidic and alkaline range beyond the plateau region (Renger et al., 1977; Homann et al., 1983; Schlodder & Meyer, 1987). Preincubation at pH = 3.0 was found to cause Ca^{2+} release concomitant with an increase of the stability of S_2 (Ono & Inoue, 1988; Shen et al., 1992) and blockage of the oxidation steps beyond the modified $Y_Z^{ox}S'_3$ state, thereby leading to the inhibition of O_2 evolution. This effect can be reversed by Ca^{2+} addition [for reviews see Debus (1992) and Rutherford et al. (1992)]. Likewise, the reversible inhibition of oxygen evolution at somewhat higher pH values (4–4.5) was shown to be coupled with Ca^{2+} release (Krieger & Weiss, 1992). At alkaline pH, on the other hand, an unusually high S_0 population was reported [Plijter et al., 1986; Lockett et al., 1990; see however Renger and Hanssum (1988)]. With respect to the possibility of pH-induced structural changes it should be mentioned that at alkaline pH the inactivation of the WOC activity leads to drastic effects such as the release of part of the extrinsic proteins and of the functional manganese (Pulles et al., 1976; Briantais et al., 1977; Maison-Peteri et al., 1981; Kuwabara & Murata, 1982, 1983; Sandusky et al., 1983; Wacker and Renger, in preparation).

In the present paper the effect of pH on the WOC is analyzed by measurements of the period four oscillations of the O_2 yield in dark-adapted isolated spinach thylakoids induced by a train of short flashes. By this means, the effect of pH on the interaction between the WOC in the redox states of S_2 and S_3 with Y_D (and other endogenous redox components) is studied in detail, and the question of a high S_0 population at alkaline pH is addressed.

On the basis of the data of this study and data taken from the literature, the WOC is inferred to undergo structural changes of functional relevance near neutral pH. Additional changes are inferred to take place at pH values of 5–5.5.

MATERIALS AND METHODS

Thylakoids were prepared from market spinach according to Winget et al. (1965) with modifications. After the final isolation step, the samples were resuspended in weakly buffered freezing medium at pH 6.5 (400 mM sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 10 mM Mes/NaOH) to a chlorophyll concentration of 6 mg/mL, which was determined according to Welburn and Lichtenthaler (1984). For storage the samples were frozen as small aliquots in liquid nitrogen and kept at −80 °C. Long-term storage of thylakoids under these conditions leads to the reduction of Y_D^{ox} in up to approximately 75% of the PS II centers [referred to as Y_D thylakoids, see Messinger and Renger (1990a, 1993); see also Vass et al. (1990)].

In order to obtain samples with the same amount of $S_1Y_D^{ox}$ as starting material for measurements at all pH values, the following procedure was used (all sample handlings were performed in the dark or in dim green light): after thawing on ice the thylakoids were diluted with buffer A [20 mM NaCl, 5 mM $MgCl_2$, 50 mM Tricine (pH 7.6)] to a chlorophyll concentration of 1 mg/mL. The samples (100 μL) were then illuminated with a saturating xenon flash (FWHM = 15 μs) and subsequently incubated in the dark for 15 min at room temperature (23 °C) to allow complete reduction of S_2 to S_1, thereby giving rise to thylakoids with a high population of state $S_1Y_D^{ox}$. Finally, the samples were centrifuged at 4 °C, and the pellets were stored on ice. To ensure comparable O_2-flash yield pattern of both sample types, S_1Y_D thylakoids were carried through the same procedure omitting only the preflash. Three minutes before starting the O_2-flash yield measurements, the samples were resuspended with buffer A, B, C, or D to a chlorophyll concentration of 1 mg/mL in order to obtain the desired pH. Buffers B, C, and D were identical to buffer A with the exception that 50 mM Hepes (buffer B), 50 mM Mes (buffer C), or 50 mM succinate (buffer D) was used instead of Tricine. Buffers A–D were also used as flow buffers for the Joliot-type electrode. The pH was adjusted at 9 ± 1 °C with a pH-meter that was calibrated at the same temperature with pH standard solutions purchased from Merck.

The flash-induced O_2 oscillation patterns were measured at 10 °C with a modified Joliot-type electrode (Joliot, 1972) that keeps the temperature of the electrode constant within ±0.2 °C. Two minutes before the measurement was started, a 10-μL aliquot of these samples was transferred rapidly into the Joliot-type electrode, which allows complete temperature adaptation under our conditions. The polarization voltage of −600 mV was switched on 30 s before excitation with a flash train (2 Hz) of short (FWHM = 3 μs) saturating xenon flashes (General Radio, Stroboslave 1539A). The amperometric signals were separated from the constant background signal with an isolation transformer and amplified by a laboratory-built polarograph. The amplified signal was recorded with an externally triggered Nicolet 1072. For better resolution, the signals were recorded with a sampling rate of 3 ms per point starting 50 ms before and ending 120 ms after each flash. The peak heights of the signals were taken to be proportional to the amount of oxygen evolved in the corresponding flash.

The probabilities of misses, double hits, and the apparent S_0 population were determined by a least-squares fit method comparing the relative O_2 yields of the first 12 flashes of the train with calculated sequences [on the basis of the conventional Kok model (Kok et al., 1970)] as outlined in Messinger et al. (1991).

For practical reasons, in the case of the S_2 and S_3 lifetime measurements, a slightly different procedure was used to obtain $S_1Y_D^{ox}$ thylakoids and the desired pH values: small aliquots (17 μL) of the S_1Y_D thylakoids were illuminated prior to dilution with one flash (as above) and then kept on ice for more than 1 h as described previously (Messinger & Renger, 1990a). Both sample types (S_1Y_D and $S_1Y_D^{ox}$) were then either washed once with a buffer of the desired pH (buffer A, B, C, or D) and resuspended in the same medium to 1 mg of chlorophyll/mL, or alternatively buffer solutions with a slightly higher or lower pH were directly added to these thylakoids. The diluted sample was then kept in the dark on ice while the S_2 or S_3 lifetime measurement was performed with aliquots from it.

The S_2 and S_3 lifetimes were measured in the conventional way [for a review see Joliot and Kok (1975)] by exciting dark-adapted samples with one (S_2 formation) or two (S_3 formation) preflashes and monitoring the O_2 yield pattern induced by a flash train (2 Hz) given at various dark times, t_d, after the preflash(es). These patterns were deconvoluted into normalized S_i-state populations within the framework of the conventional Kok model by the use of a least-squares fit method, taking the misses and double hits of the normal sequence (2 Hz) as fixed values. The rate constants were derived from a semilogarithmic plot of the normalized S_2 or S_3 populations as a function of t_d.

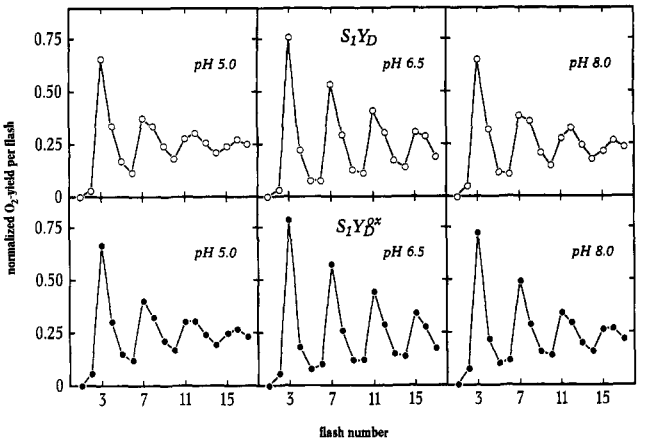

FIGURE 1: Normalized O_2 yield as a function of the flash number in a train of saturating single-turnover flashes (2-Hz flash frequency) in dark-adapted spinach thylakoids with a high (○, S_1Y_D thylakoids) or a low (●, $S_1Y_D^{ox}$ thylakoids) population probability of the reduced form of tyrosine D, measured at 10 °C and pH = 5.0 (left), 6.5 (middle), and 8.0 (right). The O_2 yields of each sequence are normalized to one-third of the sum of the corresponding oxygen yields induced by the first 12 flashes. For further details see Materials and Methods.

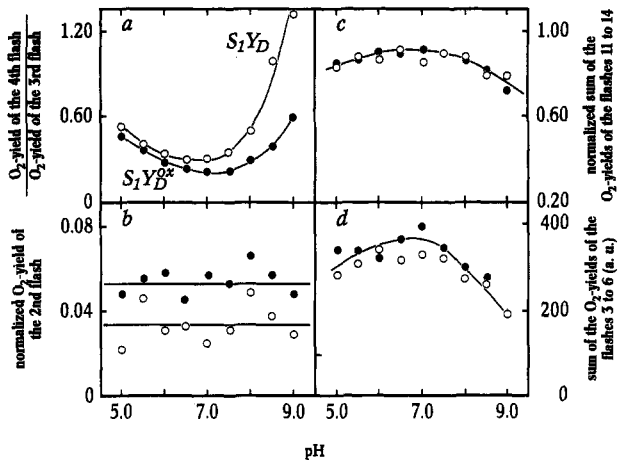

FIGURE 2: Characteristic parameters of O_2 oscillation patterns as a function of the medium pH in S_1Y_D (○) and $S_1Y_D^{ox}$ thylakoids (●): (a) ratio of the O_2 yield due to the 4th and the 3rd flash, respectively; (b) O_2 yield of the 2nd flash normalized to the sum of the O_2 yields induced by flashes 3–6; (c) sum of the O_2 yields due to flashes 11–14 normalized to that of flashes 3–6; (d) sum of the O_2 yields induced by flashes 3–6. For further details see text and Materials and Methods.

For comparison, the flash-induced O_2 oscillation pattern and the S_2 and S_3 lifetimes were also measured after incubation of the thylakoids (1 mg of chlorophyll/mL) with 10 μM gramicidin D.

RESULTS

pH Dependence of the Period Four Oscillation Pattern of O_2 Evolution. The O_2 oscillation pattern was recently shown to be virtually invariant to temperature between 0 and 20 °C, whereas, the decay reactions of S_2 and S_3 exhibit a strong thermal activation in this range (Messinger & Renger, 1990b; Messinger et al., 1993). Accordingly, the measurements of this study were performed at 10 °C, because under these conditions especially the fast kinetics of S_2 and S_3 relaxation can be better resolved.

Typical O_2 oscillation patterns obtained with S_1Y_D thylakoids (top, 75% Y_D) and $S_1Y_D^{ox}$ thylakoids (bottom, 90% Y_D^{ox}) are depicted in Figure 1 for three physiological pH values. At first glance, these data reveal several striking phenomena: (1) the O_2 oscillation patterns of $S_1Y_D^{ox}$ thylakoids are less damped than those of S_1Y_D thylakoids over the whole pH range, even though the reduction of S_2 and S_3 by Y_D is relatively slow at 10 °C [see Messinger et al. (1993)]; (2) the differences between $S_1Y_D^{ox}$ and S_1Y_D thylakoids are much more pronounced at pH 8.0 than at neutral and acidic pH values; and (3) regardless of the redox state of Y_D, the damping of the oscillations reaches a minimum at neutral pH.

In an attempt to characterize these features in more detail, the following qualitative parameters were used: (a) the ratio of the O_2 yields due to the 4th (Y_4) and the 3rd (Y_3) flash, Y_4/Y_3, reflecting the probability of misses (α) and the apparent S_0/S_1 ratio in dark-adapted samples; (b) the O_2 yield of the second flash (Y_2) normalized to the sum of the oxygen yields induced by flashes 3–6, $Y_2/\sum_{i=3}^{6} Y_i$, indicating the probability of double hits (β); (c) the ratio $\sum_{i=11}^{14} Y_i/\sum_{i=3}^{6} Y_i$ as a measure of the effective acceptor pool size; and (d) the sum $\sum_{i=3}^{6} Y_i$ as a measure of the initial oxygen-evolving capacity of the thylakoids. These parameters are shown in Figure 2 as a function of pH in the range between pH 5.0 and 9.0.

As expected from Figure 1, the Y_4/Y_3 ratio of the S_1Y_D and the $S_1Y_D^{ox}$ samples is minimal around neutral pH values [see also Wraight et al. (1972)] and always attains somewhat larger values in S_1Y_D thylakoids than in $S_1Y_D^{ox}$ samples over the whole pH range analyzed in this study (see Figure 2a). A closer inspection shows that the Y_4/Y_3 minimum is located between pH 7.0 and 7.5 in $S_1Y_D^{ox}$ thylakoids, while that of S_1Y_D samples is slightly shifted to pH 6.5–7.0. The most pronounced differences between both sample types, however, are observed at pH values above pH 7.0. In this region the difference of the Y_4/Y_3 ratio between both sample types increases significantly and reaches rather high values for S_1Y_D thylakoids; i.e., at pH 8.5, the O_2 yields induced by the 4th and 3rd flash are almost identical in S_1Y_D thylakoids, and at pH 9.0 the O_2 yield of the 4th flash markedly exceeds that of the third flash. The differences between the pH dependencies of Y_DS_1 and $Y_D^{ox}S_1$ thylakoids show that the increase of the Y_4/Y_3 ratio at alkaline pH comprises at least two effects: one that is independent of the redox state of Y_D and another one that depends on the presence of Y_D in its reduced state.

In contrast to the strong pH dependence of the Y_4/Y_3 ratio, the normalized O_2 yield of the second flash (Figure 2b) is almost pH independent within the error range of this parameter. In general, slightly lower Y_2 values are found in S_1Y_D thylakoids than in samples enriched in $S_1Y_D^{ox}$. The latter effect might have an interesting implication. As the value of β correlates almost linearly with the rate constant of Q_A^- reoxidation under our experimental conditions (Messinger et al., 1993), the rate of this reaction is estimated to be slower by about 20% in Y_D compared with Y_D^{ox} thylakoids. This result could be an indication for a long-range electrostatic acceleration of Q_A^- reoxidation by Q_B (Q_B^-) due to the positively charged microenvironment of Y_D^{ox}. An effect of this type could be rationalized by differences in the distances between Y_D and the binding sites of Q_A and Q_B, respectively. It has to be emphasized that these conclusions based on β values are rather indirect. Experiments are in progress to analyze a possible long-range modulation of the kinetics of acceptor side reactions by the redox state of Y_D.

The parameter $\sum_{i=11}^{14} Y_i/\sum_{i=3}^{6} Y_i$ as a measure of the effective acceptor pool size has a value close to 1 for thylakoid samples at neutral pH and is virtually temperature independent between 0 and 40 °C (Messinger et al., 1993). Figure 2c shows that

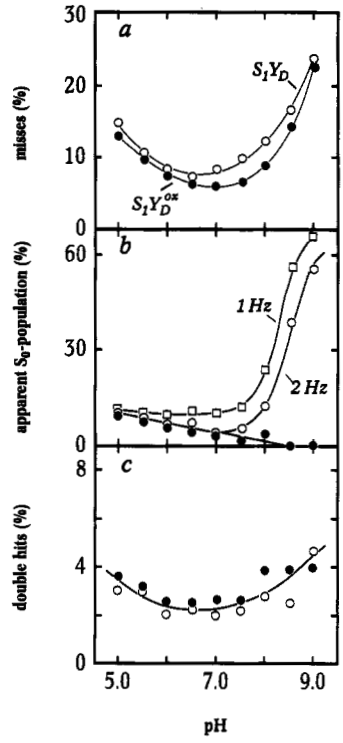

FIGURE 3: Probability of misses (a, top), double hits (c, bottom), and apparent normalized dark population of S_0 (b, middle) as a function of pH in S_1Y_D (○, □) and $S_1Y_D^{ox}$ thylakoids (●) at 10 °C. The flash frequency was 2 Hz (○, ●) and 1 Hz (□), respectively.

this parameter is not affected by the redox state of Y_D. It decreases slightly at acidic and alkaline pH. This small decline is probably the result of two effects: a retarded kinetics of the plastohydroquinone reoxidation at low pH values (Siggel, 1975) and a light-induced deactivation of a small part of the PS II centers during the flash train at alkaline pH mainly due to the susceptibility of S_2 (Briantais et al., 1977).

According to earlier measurements of the average O_2 yield per flash and of the rates of O_2 evolution, the number of functionally competent WOC's significantly declines at acidic and alkaline pH values [see, e.g., Renger et al. (1977), Homann et al. (1983), and Schlodder and Meyer (1987)]. On the other hand, Figure 2d reveals that the sum of the oxygen yields induced by flashes 3–6 exhibits a much less pronounced pH dependence, with a broad maximum around pH 7.0. The shape of this curve was found to be markedly dependent on the incubation time. If the thylakoids were incubated at the measuring pH for about 1 h instead of the 3 min as used in Figure 2d, the value of the sum $\sum_{i=3}^{6} Y_i$ was reduced to about 25% at pH 5.0 and to about 30% at pH 8.0 compared with that measured at pH 7.0 (data not shown). This result clearly indicates that thylakoids in redox state S_1 slowly lose in the dark the oxygen evolution capacity at acidic and alkaline pH. Therefore, the measured pH dependencies of the functional integrity of WOC represent a superposition of (i) the "true" effect of the protonation state and (ii) a time-dependent activity loss. If one assumes that a 3-min incubation time is sufficient to attain the equilibrium protonation state of the WOC, then our results show that the oxygen evolution capacity is rather weakly dependent on this state in the range of $5 \leq$ pH ≤ 8.5 but susceptible to time-dependent degradation at acidic and alkaline pH. The situation seems to be somehow different in PS II membrane fragment where the extent of $\sum_{i=3}^{6} Y_i$ exhibits a more pronounced pH dependence, especially in the alkaline region (U. Wacker, J. Messinger, and G. Renger, in preparation).

The data described so far were obtained with intact thylakoid preparations. Therefore, the question arises as to what extent the results in the alkaline region might be influenced by an acidification of the thylakoid lumen due to the protons released during the S_i-state transitions induced by the single-turnover flash excitations. To check for this possibility, the same experiments were performed after incubation of the thylakoids with 10 μM gramicidin D. Almost identical results were obtained (data not shown). This finding indicates that no significant "acidification effect" arises. Obviously, the slight uncoupling of thylakoids that is known to occur during a freeze/thaw procedure [see Santorius (1990) and references therein] is sufficient to prevent the formation of a significant proton concentration difference between lumen and stroma under our excitation conditions (flash frequency of 2 Hz).

The most striking feature of the results described so far is the huge difference in the Y_4/Y_3 ratio between Y_D and Y_D^{ox} thylakoids at alkaline pH values. In order to investigate whether this difference is due to an increase of the miss parameter or of the apparent S_0 population, the O_2 oscillation patterns were deconvoluted into the usual Kok parameters (α, β, and initial S_i-state population). The results depicted in Figure 3 clearly show that there are only minor differences in the miss and double hit parameters between Y_D and Y_D^{ox} samples, while a huge difference arises for the apparent S_0 population at alkaline pH values. The apparent S_0 population increases up to 50% (at pH 9.0) in Y_D thylakoids while it drops close to zero in Y_D^{ox} thylakoids. The dramatic increase in the apparent S_0 population after short time incubation of Y_D thylakoids at alkaline pH could originate from two different effects: (i) a reaction of the type $S_1Y_D \rightarrow S_0Y_D^{ox}$ taking place during the dark incubation (3 min) at alkaline pH before the measurement, or (ii) a fast back-reaction of S_2 and S_3 with Y_D during the dark time (500 ms) between the flashes of the train, giving rise to the increase of the apparent S_0 population. The significance of the latter effect can be simply checked by measurements of the O_2 oscillation patterns at different flash frequencies. The results obtained at 1 Hz (instead of the normally used flash frequency of 2 Hz) show that in Y_D^{ox} samples practically no changes are observed (data not shown), while in Y_D thylakoids a significant increase of the apparent S_0 population results when the dark time between the flashes is increased (see Figure 3b, open squares).

pH Dependence of the S_2 and S_3 Lifetimes. The above findings suggest that the extent and/or the rate constant for the interaction of Y_D with the S_2 and/or S_3 state of WOC are higher under alkaline pH conditions than at neutral and acid pH values. In order to test this idea, the S_2 and S_3 lifetimes were measured at different pH values between 4.5 and 8.75 by varying the dark time between the 1st and 2nd flash (S_2 decay) or the 2nd and 3rd flash (S_3 decay) of the flash train [for review see Joliot and Kok (1975)]. Usually the decay kinetics are biphasic: a fast phase due to the reduction by Y_D, and a slow phase which represents mainly a charge recombination with electrons from the acceptor site of PS II (other endogenous electron donors cannot be excluded from a contribution to the slow reduction).

Typical time courses of the S_2 and S_3 decay in Y_D thylakoids at 10 °C are presented as semilogarithmic plots in Figure 4A for pH 5.5 (left) and pH 7.5 (right). Figure 4B shows at a different time scale the data of the fast phases, which were obtained by subtraction of the corresponding slow phases. In general, the following conclusions can be gathered from these data: (1) the rate constant of the slow S_2 decay seems to be almost unaffected by pH, while that of the slow S_3 decay is

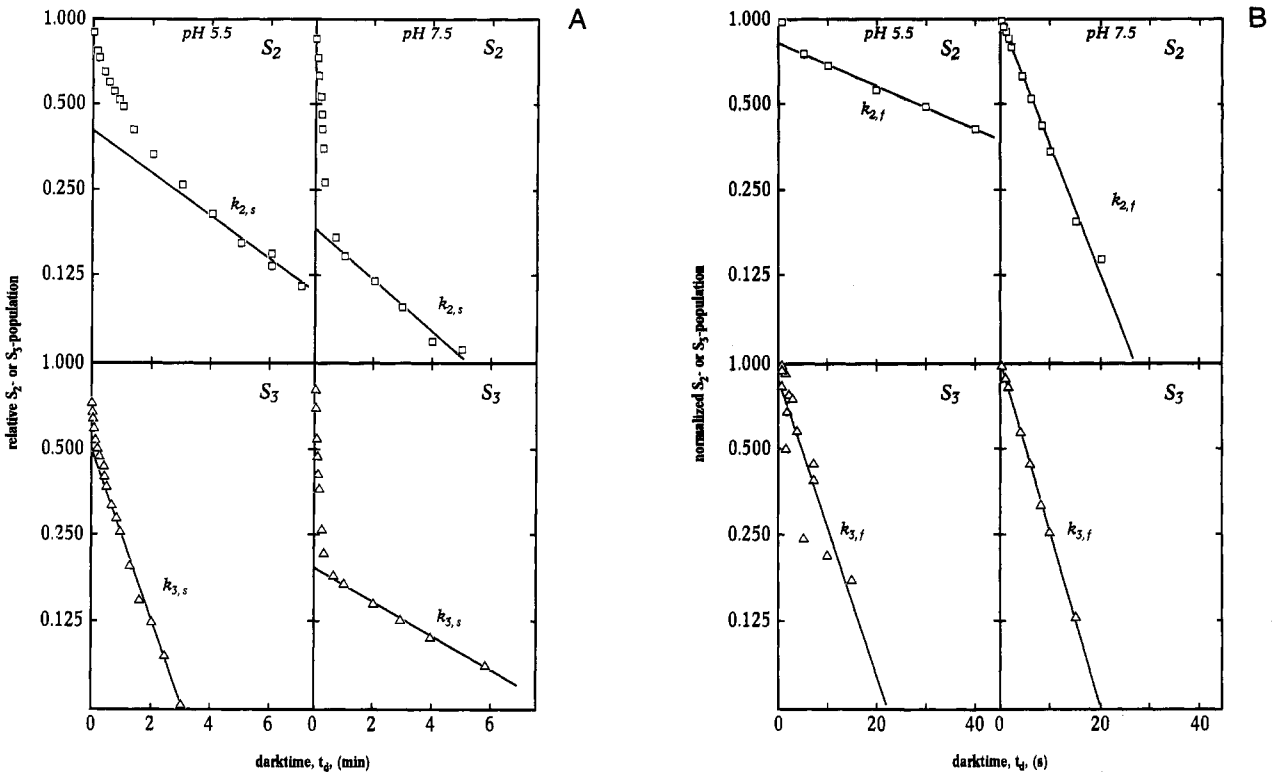

FIGURE 4: Relative S_2- (□) and S_3-state (△) population as a function of the dark time, t_d, between the 1st and 2nd (S_2) or the 2nd and 3rd (S_3) flashes of the train in S_1Y_D thylakoids at 10 °C and pH 5.5 (left) or pH 7.5 (right). The relative S_i-state populations were obtained by deconvolution of the O_2 yield pattern according to the normal Kok model [see Messinger et al. (1991)]. (A) Slow phase of the decay kinetics which were given in Tables 1 and 2. For further details see Materials and Methods. (B) Fast phase of the S_2 and S_3 decays, respectively, obtained from the measurements in (A) after subtraction of the slow phase. The time scales in panels (A) and (B) are different.

drastically changed (compare the slopes of the lines in Figure 4A); (2) in marked contrast to the behavior of the slow decay, the rate constant of the fast S_2 decay (see Figure 4B) is strongly affected by pH, while that of the fast S_3 decay is almost invariant; (3) surprisingly, the magnitude for the fast S_2 and S_3 decay is significantly smaller at pH 5.5 than at pH 7.5 (see Figure 4A); and (4) at pH 5.5 an additional very fast phase can be observed that is absent at pH 7.5 (see Figure 4B). With respect to the S_2 decay, the latter two points are in general agreement (see also Discussion) with the pH dependence found in EPR measurements for the extent of Y_D oxidation by S_2 formed via single-turnover excitation of ascorbate/DCPIP-treated PS II membrane fragments from spinach (Vass & Styring, 1991). Corresponding data for the S_3 decay were not reported in the literature.

In order to characterize these phenomena in more detail, the S_2 and S_3 lifetimes in Y_D and Y_D^{ox} thylakoids were measured at various pH values between pH 4.5 and 8.75. The data obtained are compiled in Tables 1–3, and the pH dependence of the half-lifetimes of the fast and slow S_2 and S_3 decay is shown in Figure 5. The data below pH 5.5 and above pH 8.0 will not be considered for a further analysis because in these pH regions the functional integrity of the WOC becomes severely diminished, especially at the longer incubation times which have been used during the S_2 and S_3 lifetime measurements (for the sake of clarity these data points are not connected by a curve in Figure 5). Two interesting phenomena emerge from the results in the pH range between 5.5 and 8.0 where the WOC remains highly active: (1) above a critical value of pH_c = 6.5–7.0 the S_2 and S_3 decays exhibit, for both the fast and the slow phases, a very similar pH dependency; and (2) compared with the pH dependence of the fast S_2 and S_3 decay, the corresponding slow phases exhibit an opposite behavior above pH_c, i.e., a stabilization of S_2 and S_3 toward the slow reduction by endogenous electron donors versus a destabilization toward the fast reduction by Y_D. Below pH_c, for both phases the same pH-dependent changes are observed. Figure 6 depicts the pH dependence of the extent

Table 1: Normalized Amplitudes (a_i) and Half-Life Times ($t^i_{1/2}$) of the Different Decay Kinetics of S_2 in Isolated Spinach Thylakoids as a Function of pH

	S_2 decay in Y_D thylakoids					
	very fast (vf)		fast (f)		slow (s)	
pH	a_{vf} (%)	$t^{vf}_{1/2}$ (s)	a_f (%)	$t^f_{1/2}$ (s)	a_s (%)	$t^s_{1/2}$ (s)
5.0	15	4.0	11	32	74	150
5.5	10	nd	46	39	44	250
6.0	6	2.8	57	23	37	270
6.5	4	1.7	59	13	37	110
7.0	0		73	8.8	27	150
7.3	0		76	7.2	24	180
7.5	0		80	7.2	20	200
7.7	0		67	3.9	33	260
8.5	58	0.34	19	5.6	23	(65)

Table 2: Normalized Amplitudes (a_i) and Half-Life Times ($t^i_{1/2}$) of the Different Decay Kinetics of S_3 in Isolated Spinach Thylakoids as a Function of pH

	S_3 decay in Y_D thylakoids					
	very fast (vf)		fast (f)		slow (s)	
pH	a_{vf} (%)	$t^{vf}_{1/2}$ (s)	a_f (%)	$t^f_{1/2}$ (s)	a_s (%)	$t^s_{1/2}$ (s)
5.0	11	0.5	11	3.5	88	32
5.5	4	0.5	26	6	70	56
6.0	6	0.5	31	11	63	69
6.5	10	0.5	52	13	38	130
7.0	0		70	7.4	30	150
7.5	0		77	4.7	23	310
8.0	0		75	4.5	25	480

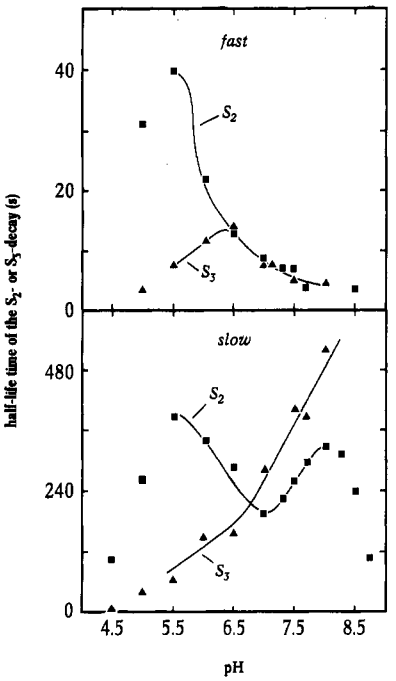

FIGURE 5: Half-life times of the fast (top) and the slow (bottom) phases of the S_2 (■) and S_3 decay (▲) as a function of pH in spinach thylakoids at 10 °C. The data for the fast phases were obtained with S_1Y_D thylakoids and the data of the slow phases with $S_1Y_D^{ox}$ thylakoids. For further details see Tables 1–3 and Materials and Methods.

Table 3: Half-Life Times $t^s_{1/2}$ of the Slow Decay Kinetics of S_2 and S_3 in Y_D^{ox} thylakoids as a Function of pH

pH	S_2 decay, $t^s_{1/2}$ (s)	S_3 decay, $t^s_{1/2}$ (s)	pH	S_2 decay, $t^s_{1/2}$ (s)	S_3 decay, $t^s_{1/2}$ (s)
4.50	100	6	7.50	260	400
5.00	270	40	7.70	300	390
5.50	370	60	8.00	330	520
6.00	340	150	8.25	310	
6.50	290	160	8.50	240	
7.00	190	290	8.75	110	
7.30	230				

of the fast (f) and very fast (vf) S_2 decay, normalized to the total S_2 decay, $(a_{vf} + a_f)/(a_{vf} + a_f + a_s)$, in Y_D thylakoids (closed squares in Figure 6). A strong pH dependence is observed with very small values at low pH (25% at pH 5.0) and high values above pH 7 (75–80%). In addition, the extent of the very fast phase normalized to the amplitude of both fast kinetics, $a_{vf}/(a_{vf} + a_f)$, in Y_D thylakoids (open squares in Figure 6) varies significantly with pH, showing an opposite dependence, with high levels at pH 5.0 and low levels at pH 7.0. Surprisingly, at pH 8.5 again a high extent of the very fast S_2 decay was observed (see also Table 1). A similar pH dependence was obtained for the fast and very fast S_3 decay (see Table 2). However, no exact determination of the very fast phase is possible due to limitations arising from the dark time of 200 ms between the first and second flash used for the S_3-state formation.

As in the case of the flash-induced O_2 oscillation patterns, practically no changes were observed in the rate constants or amplitudes of the different phases of the S_2 and S_3 decay if the measurements were performed after incubation with 10 μM gramicidin D (data not shown).

DISCUSSION

In this study two types of reactions in the WOC were analyzed in terms of their modulation by pH: (a) the interaction of the redox states S_2 and S_3 with tyrosine Y_D, reflected by the fast S_2 and S_3 decay, and (b) the reduction of S_2 and S_3 by the acceptor side (e.g., with Q_B^- as electron donor), monitored via the slow S_2 and S_3 decay.

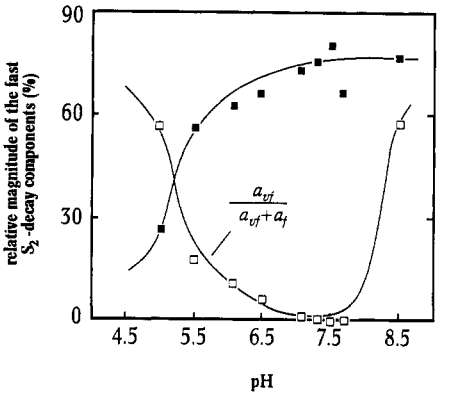

FIGURE 6: Amplitude of the sum of the fast and very fast phases (reaction of Y_D) normalized to the overall S_2 decay (■) and amplitude of the very fast phase normalized to the sum of the two fast phases (□) in Y_D thylakoids at 10 °C as a function of pH. For further details see Tables 1 and 2 and Materials and Methods.

The interpretation of pH-dependent rates of the electron transfer between functional groups incorporated into a protein matrix is a rather complex problem because several effects can contribute to the observed phenomena: (a) direct protolytic reactions at the redox-active species, (b) pK and/or redox potential shifts of functional group(s) due to the protonation or deprotonation of amino acids in the microenvironment, and (c) structural changes induced by the protonation state of the protein matrix, leading (e.g., via allosteric effects) to changes in the distance and/or mutual orientation and/or redox potential of functional groups that participate in the electron transport. In addition, for kinetic analyses it is also necessary to take into account the possible existence of transport barriers to protolytic reactions which may limit the rate of a particular electron transfer step. As a further complication, these barriers can be in turn a function of pH-dependent conformational states of the protein matrix.

These general considerations [see also Crofts and Wood (1978)] show that straightforward analyses of experimental data require not only detailed knowledge of the PS II structure but also of the protein dynamics which affects the proton transport properties. Both problems are far from being resolved for PS II. Therefore, only a qualitative discussion can be given of the results described in this study. Within this qualitative treatment special attention is paid to the role of (i) the protolytic reactions at the functional groups coupled with their redox transitions and (ii) pH-induced structural changes of the WOC and its neighborhood.

Protolytic Reactions within PS II. For the analysis of the first point, protolytic reactions need to be considered that are coupled with redox transitions of functional groups participating in the decay of S_2 and S_3. Although all of the protons released by water oxidation ultimately originate from the substrate, a particular H^+ release during the univalent oxidation steps within the WOC may originate from S_i state-dependent changes in the protonation state of the protein matrix. Consequently, a pH-dependent proton release into the aqueous bulk phase and a noninteger stoichiometry are expected for several redox transitions in the WOC (Renger, 1987). This idea has been fully confirmed by recent experimental findings [for review, see Lavergne and Junge (1993)].

With respect to the properties of Y_D, it is known that the OH group of the tyrosine residue becomes deprotonated upon oxidation of Y_D (Babcock et al., 1989) but the proton remains trapped at a nearby located residue of the protein matrix (Tang et al., 1993). This "trapped" proton probably does not exchange easily with the aqueous bulk phase. The latter idea is supported by the conclusion that Y_D is effectively shielded by a hydrophobic environment, based on structure models (Ruffle et al., 1992; Svensson et al., 1992) and on experimental data indicating that Y_D^{ox} is markedly less susceptible than the WOC to reduction by externally added hydrophilic electron donors like hydrazine and hydroxylamine (Sivaraja & Dismukes, 1988; Messinger et al., 1991; Messinger & Renger, 1993). Likewise, Y_D^{ox} becomes significantly unstable and equilibrates with the aqueous phase only in the presence of certain chaotropic substances (Messinger et al., 1993).

In addition further components have to be taken into account, because the reduction of S_3 and S_2 by endogenous electron donors very likely comprises for the fast (reduction by Y_D) as well as for the slow phase (mainly reduction by Q_B^-) the participation of the electron carriers Y_Z, P680 and Pheo, Q_A, respectively (Vass & Styring, 1991; Buser et al., 1992; Messinger et al., 1993). Therefore, possible protolytic reactions of these groups have to be considered, too.

In PS II complexes which are deprived of their oxygen evolution capacity, the oxidation of Y_Z by $P680^+$ is coupled with a proton release (Renger & Völker, 1982; Förster & Junge, 1984). In samples with intact WOC, however, the expected fast transient deprotonation during the short lifetime of Y_Z^{ox} could not be observed. Therefore, in intact systems a proton transport barrier is assumed to prevent the detection of this protolytic reaction (Renger et al., 1987; Lavergne & Junge, 1993), and it seems unlikely that the medium pH has a direct influence on this reaction. Analogously, the redox pairs $P680/P680^+$ and $Pheo/Pheo^-$ have very short lifetimes and are well shielded by the protein matrix. Therefore, if protolytic reactions could take place, they would be restricted to the microenvironment of these groups. This idea is supported by measurements of prompt and delayed fluorescence in broken chloroplasts showing no direct effect of pH in the range of 2.6–7.0 on the primary charge separation in PS II (van Gorkom et al., 1976).

On the PS II acceptor side a pH-independent working potential is assumed for Q_A/Q_A^-, whereas the $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ equilibrium is strongly pH dependent either due to a direct protonation of Q_B^- or due to a protonation of nearby located amino acid residue(s) [Robinson & Crofts, 1984; Vermass et al., 1984; see, however, Vass and Inoue (1986)].

pH Dependence of the S_2 and S_3 Lifetimes as Indicator of Conformational Changes in the WOC at pH 6.5–7.0. On the basis of the above-mentioned considerations, the pH dependence of the reactions $S_iY_D \rightarrow S_{i-1}Y_D^{ox}$ (i = 2, 3) should be dominated by the protolytic properties of S_2 and S_3. The oxidative forward reaction $Y_Z^{ox}S_2 \rightarrow Y_ZS_3$ was shown to be coupled with the release of one proton independent of the pH in the suspension [for review see Lavergne and Junge (1993)]. Therefore, the fast decay of S_3 can be described by

$$Y_DS_3 + H^+ \xrightarrow{k_{3,f}} Y_D^{ox}S_2 \quad (1)$$

and

$$d[Y_DS_3]/dt = -k_{3,f}[Y_DS_3][H^+] \quad (2)$$

Taking into account the high buffer capacity of the medium, the following pseudo-first-order kinetics should arise for the fast S_3 decay:

$$d[Y_DS_3]/dt = -k'_{3,f}[Y_DS_3] \quad (3)$$

with $k'_{3,f} = k_{3,f}[H^+]$. According to eq 3 the half-lifetime of the fast S_3 decay should increase with pH by a factor of 10 per pH unit.

Figure 5 (top) readily shows that the data cannot be fitted with eq 3: (i) in the range between pH 5.0 and 6.5 the half-lifetime of Y_DS_3 increases much less than expected; and (ii) above the critical pH value of pH_c = 6.5–7.0 the lifetime even decreases.

Therefore, it is concluded that the pH dependence of the fast S_3 decay cannot be explained by the assumption that the reactivity of S_3 is determined by the pH of the medium via a direct protolytic reaction occurring during the $S_2 \leftrightarrow S_3$ transition. In addition, the destabilization of the Y_DS_3 redox pair above pH_c cannot be caused by a deprotonation of a nearby located amino acid during this S_i-state transition, either.

An analogous description for the reaction $Y_DS_2 \rightarrow Y_D^{ox}S_1$ is further complicated by the fact that the stoichiometry of the proton release during the $S_1 \rightarrow S_2$ transition strongly depends on the type of preparation and on pH (Lavergne & Junge, 1993). In unstacked thylakoids, e.g., about 1.5 H^+ are released at pH = 6.0; then this value drops down to <0.5 at a pH of about 7.5 and rises again to values close to 1 above pH = 8.0 (Haumann & Junge, 1994).

Regardless of this complexity (which in principle implies the existence of at least two, on the basis of their protonation state, distinguishable S_2 states), it is clear that also the experimental data on the pH dependence of the fast S_2 decay (decrease of the lifetime with increasing pH) cannot be explained by the assumption that only the protolytic reactions coupled with the $S_1 \rightarrow S_2$ transition are responsible for the observed half-lifetimes.

The above analysis shows that beside the protolytic reactions coupled with the S_i-state transitions additional effects are responsible for the pH dependence of the interaction of Y_D with S_2 and S_3. The most striking feature of this pH dependence is the finding of a critical pH value at 6.5–7.0 where the properties of the decay kinetics of both S_2 and S_3 markedly change (see Figure 5). This pH value resembles that of pH-induced changes taking place within the WOC and/or its environment in the redox state S_1. It was found that above a pH of 7.2–7.5 the susceptibility to a proteolytic attack (by trypsin) of the donor side drastically increases in PS II membrane fragments (Völker et al., 1985). Likewise, analogous changes at characteristic pH values were observed for Cl^- binding (Lindberg et al., 1993) and for CN^- inhibition of O_2 evolution (Packham et al., 1982). Therefore, we conclude that all these effects are dominated by pH-induced conformational change(s) in the water oxidase via the (de)protonation of (an) amino acid(s). This (these) residue(s) is (are) inferred to be distant to the manganese-containing hole storage unit, because the pK value of this (these) group(s) seems to be only marginally modified by the electrostatic effects coupled with charge accumulation. In this respect it is also worth mentioning that additional buffer groups at the PS II donor side were found above pH 7.0 [Theg & Junge, 1983; for review see Dilley et al. (1987)]. The assumption of a conformational transition in PS II at pH_c implies that the pH-induced changes of the rate constants of the fast S_2 and S_3 decay are probably the result of a variation in the distance and/or the mutual orientation of the involved redox groups. A more detailed

analysis based on the pH dependence of S_1 reduction by NH_2-OH and NH_2NH_2 suggests the existence of two different structural changes at pH = 6.2–6.5 and around 7.4 (U. Kebekus, J. Messinger, and G. Renger, submitted for publication).

For the interpretation of the slow S_2 and S_3 decay components the protolytic reactions of the acceptor side have also to be taken into account. The pH dependencies of the $S_2Q_B^-$ and $S_2Q_A^-$ recombinations have been studied previously by fluorescence and thermoluminescence measurements (Robinson & Crofts, 1984; Rutherford et al., 1984; Vass & Inoue, 1986; Demeter & Salai, 1986). A comparison of these data with the results obtained in this study shows that the maximum of the half-lifetime for the slow S_2 decay at pH 5.5 seems to correspond with the previously reported decrease in the rate constant for the $S_2Q_B^-$ recombination between pH 7.0 and 5.3, while the second peak observed around pH 8.0 seems to be correlated to the stabilization of the $S_2Q_A^-$ redox couple in this pH region.

The significant differences in the pH dependence of the slow S_2 and S_3 decay are in agreement with previous conclusions drawn from measurements of the delayed luminescence and fluorescence in *Chlorella* as a function of ΔpH (Joliot & Joliot, 1980). In general, they are a clear indication for the different reactivities of the two S_i states toward endogenous electron donors (Y_D or $Q_AQ_B^-$). This corresponds well with the previously observed differences in the reactivity toward exogenous reductants like hydrazine and hydroxylamine (Messinger & Renger, 1990a; Messinger et al., 1991).

Extent of Fast Phase(s) of S_2 and S_3 Decay as Indicator of Conformational Changes at pH 5–5.5. A rather surprising result of this study is the finding that in thylakoids the extent of the two fast phases normalized to the overall decay, $(a_{vf} + a_f)/(a_{vf} + a_f + a_s)$, reflecting the percentage of PS II centers in the S_2 or S_3 state that react with Y_D, markedly decreases in the range of acidic pH (see Tables 1 and 2 and Figure 6). This phenomenon is interpreted to indicate that the reaction coordinates, i.e., the mutual orientation and/or distances of the redox groups involved, are a function of the pH in the aqueous bulk phase, so that at low pH values the WOC is able to react with Y_D only in a fraction of PS II centers. The alternative explanation by a pH-dependent redox state of tyrosine D, via a fast redox equilibrium between Y_D/Y_D^{ox} and an unknown electron acceptor with a redox potential that is sufficiently high to permit dark oxidation of Y_D at low pH, can be excluded on the basis of latest EPR measurements. It was found that in PS II particles the magnitude of the EPR Signal II_s (which reflects the amount of Y_D^{ox}) remains invariant to pH in the range of this study (Buser & Brudvig, 1992). These observations imply that the normalized extent of the fast S_2 (S_3) decay can be used as a measure of the relative amount of reduced Y_D only at pH values above 7.0.

A similar pH dependence was observed for the relative magnitude of EPR Signal II_s that can be generated at different pH values in dark-adapted PS II membrane fragments by excitation with a single-turnover flash (Vass & Styring, 1991; Buser & Brudvig, 1992; Buser et al., 1992). This finding was explained by the assumption of a pH-dependent competition of Q_A^- and Y_D for the oxidant S_2 formed after a single flash. The population of Q_A^- was assumed to be caused by either a limited PQ pool capacity in samples pretreated with DCIP/ascorbate (to achieve a high dark population of reduced Y_D), so that a significant fraction Q_A^- remains reduced after the first flash (Vass & Styring, 1991), or the existence of non-B-type centers (Buser & Brudvig, 1992). However, for several reasons both alternatives are not pertinent to rationalize the experimental data of this study: (i) the plastoquinone pool remains intact in thylakoids; (ii) an additional reductive pathway via Q_A^- should accelerate the decay of S_2 at acidic pH in contrast to the experimental observation (see Figure 5); and (iii) although non-B-type centers were shown to exist in thylakoids (Chylla & Whitmarsh, 1989), their Q_A^- reoxidation is so slow compared to the flash frequency that they do not evolve oxygen [for a review see Nedbal and Whitmash (1992)]. Therefore, for kinetic reasons, this type of non-B-type centers cannot contribute to the observed amperometric signals in the measurements of the O_2 oscillation patterns and of the S_2 and S_3 lifetimes gathered from these experiments.

In summary, the above-mentioned considerations lead to the conclusion that the diminished capacity of Y_D to reduce S_2 or S_3 at low pH relates to a phenomenon that reflects changes of the donor rather than the acceptor side of PS II. At present, the underlying mechanism for these changes in the reaction coordinates between Y_D and the S_2 and S_3 state is not clear. Interestingly, in the same pH region additional effects are reported to arise in PS II: (i) transition of cyt *b*559 from its high-potential form (pH 6.5) to the low-potential form (pH 4.5) that was correlated with a conformational change in its environment (Crofts & Horton, 1990); and (ii) a light-induced Ca^{2+} release from the PS II donor site, inferred to take place with a p*K* value of 4.7 (Krieger & Weiss, 1992). However, as Ca^{2+} depletion blocks the oxidation of the modified state S'_3 [for review see Debus (1992) and Rutherford et al. (1992)], it seems unlikely that this latter phenomenon directly correlates with the pH-dependent blockage of S_2 reduction by Y_D. Regardless of this particular problem, it appears reasonable to assume that structural changes induced by protonation of specific groups within the protein matrix are responsible for the observed "electronic decoupling" of Y_D from the water oxidase at low pH.

Apparent S_0 Population. The analyses of the O_2 oscillation patterns with the conventional Kok model reveals that the miss and double hit probabilities are similar in Y_D and Y_D^{ox} thylakoids, while, especially at higher pH values, the apparent S_0 population varies quite dramatically, depending on the redox state of tyrosine Y_D (see Figure 3). This result indicates that in spinach thylakoids the presence of Y_D is necessary for a high apparent S_0 population at alkaline pH. This observation could be explained either by a Y_D-induced reduction of S_1 into $S_0Y_D^{ox}$ or by the fast reduction of S_2 and S_3 by Y_D during the dark time between the flashes of the sequence. The significant dependence of the apparent S_0 population on the flash frequency in Y_D thylakoids together with the finding of a large extent of the very fast phase at pH = 8.5 shows that the latter mechanism is responsible for at least a large part of the effect. On the basis of the rate constant of the very fast phase and the differences between the O_2 oscillation patterns measured at 1 and 2 Hz, the true S_0 population in Y_D thylakoids is inferred to remain small, i.e., below an upper limit of about 15% at pH 8.5.

This result confirms a previous analysis of the O_2 oscillation patterns (Renger & Hanssum, 1988), but is in contrast to conclusions gathered from flash-induced UV absorption changes (Plijter et al., 1986) and EPR measurements (Locket et al., 1990). From both studies S_1 was inferred to convert into S_0 in the dark at high pH. Furthermore, these changes were shown not to be coupled with an oxidation of Y_D (Locket et al., 1990). Accordingly, it is hard to see how the reduced form of Y_D could drastically enhance the population of S_0. One possible explanation for the discrepancy of the values

estimated for the S_0 dark population could be the use of different sample material. PS II membrane fragments inactive in O_2 evolution at pH 8.3 were used in the latter two studies, while the thylakoids in this study and the samples used (thylakoids and PS II membrane fragments) in Renger and Hanssum (1988) still exhibited a considerable amount of flash-induced oxygen evolution (see Figure 2d). However, another point is much more important: in UV and EPR measurements, both O_2-inactive and -active PS II centers can contribute to the observed signals, whereas with the Joliot-type electrode only the behavior of the active centers is measured. Therefore, a further characterization of the apparent S_0 state in O_2-inactive samples is required in order to elucidate whether or not this state is identical with the normal S_0 state that can be populated either by three preflashes in dark-adapted thylakoids or by a suitable pretreatments with NH_2NH_2 (Messinger & Renger, 1993).

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