Charge Recombination and Proton Transfer in Manganese-Depleted Photosystem II[†]

Fabrice Rappaport*[,‡] and Jérôme Lavergne[§]

Institut de Biologie Physico-Chimique, CNRS UPR 9072, 13 Rue Pierre et Marie Curie, 75005 Paris, France, and CEA-Cadarache, DEVM-LBC, 13108 Saint Paul-lez-Durance, France

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ABSTRACT: The proton transfer reactions induced by the oxidation and reduction of the secondary donor, tyrosine Y_Z, have been studied in photosystem II after inactivation (Mn-depletion) of the oxygen-evolving complex. The rate of the recombination reaction of Y_Z^{ox} with the reduced primary acceptor Q_A^- appears modulated by a protonatable group with p$K \approx 6$ in the presence of Y_Z^{ox}. The finding of monophasic recombination kinetics requires that the proton equilibration of this group is faster than the recombination rate. The same group modulates the extent of proton release, from 0 below pH 5 to 1 per center above pH 7. The kinetics of proton appearance and disappearance in the bulk medium are markedly dependent on the material used. In PSII core particles, the release is observed in the 100 μs range and the uptake accompanies the recombination reaction. In PSII membranes, both of these reactions are markedly delayed, so that the uptake considerably lags behind the completion of the recombination reaction. An electrochromic shift of a chlorophyll is present during the whole lifetime of Y_Z^{ox}, suggesting a charged character of this species. A fast decreasing phase of this signal was observed in particles in the same time range as proton release. These results are discussed in the framework of a model where the proton originating from the formation of the neutral oxidized tyrosine radical ($Y_Z^{\bullet}$) remains locally trapped. In turn, this proton shifts the pK of a nearby group from a value ≥ 9 to a value of 6.

It has been broadly recognized in recent investigations of the photosynthetic oxygen-evolving complex that a key role in the catalytic function rests on the way in which the system handles the protolytic reactions involved during oxidant accumulation and water oxidation (Krishtalik, 1986; Rappaport & Lavergne, 1991; Baldwin et al., 1993; Lavergne & Junge, 1993; Gilchrist et al., 1995; Hoganson et al., 1995). Although substantial progress has been gained in this domain, the present picture is still unclear in several respects. The stoichiometry and release rate of protons during the four successive steps of the Kok cycle (Joliot & Kok, 1975) appear to vary depending on the biological material and techniques. In thylakoids or PSII[1] membranes, the release is modulated by the S states, with, around neutral pH, a minimum on the $S_1 \rightarrow S_2$ transition and a maximum on the $S_3 \rightarrow (S_4 \rightarrow) S_0 + O_2$ transition (Saphon & Crofts, 1977; Förster & Junge, 1985; Rappaport & Lavergne, 1991). The pH dependence of this pattern was, however, found to be different in these two types of materials (Lavergne & Junge, 1993; Haumann & Junge, 1996). In purified oxygen-evolving PSII core particles, a uniform release of one proton was observed on each transition (Wacker et al., 1990; Lübbers et al., 1993). Whereas it is clear that such variations must be due to the interference of protonatable groups responding to the electrostatic influence of the Mn-containing catalytic center, it remains debated which material reflects best the intrinsic pattern. Some authors tend to favor the uniform release observed in particles (Gilchrist et al., 1995; Hoganson et al., 1995; Haumann & Junge, 1994), whereas others argue in favor of the oscillating pattern (Rappaport & Lavergne, 1991; Witt, 1991; Haumann & Junge, 1996). To some extent, similar discrepancies concern the question of the proton release rate. It is again broadly accepted that the appearance of protons in the aqueous lumen may be considerably delayed with respect to the release from the catalytic site by a diffusion barrier consisting of protein or membrane-bound buffering groups. Nevertheless, there is only partial agreement between results based on the use of dyes or on the electrochromic absorbance change reflecting the local electrostatic balance on the donor side of PSII. The results of Haumann and Junge (1994), using the amphiphilic dye neutral red in thylakoids, or hydrophilic dyes in particles (Lübbers et al., 1993), support very fast (≤ 10 μs) release occurring on each of the S-transition before the intermediate carrier, tyrosine Y_Z, is reoxidized. Rappaport et al. (1994), analyzing the kinetics of the electrochromic change, found evidence for such fast phases only for two transitions, namely, the oxygen-evolving step $S_3 \rightarrow (S_4 \rightarrow) S_0 + O_2$ and, at low pH, the $S_0 \rightarrow S_1$ step. The views of Junge's group that imply a rapid and stoichiometric proton release triggered by the oxidation of Y_Z irrespective of the S state have been incorporated as a key feature in a recent hypothesis (Gilchrist et al., 1995; Hoganson et al., 1995) in which the oxidized tyrosine Y_Z would dump a proton to the bulk and then abstract, during its reduction, both an electron and a proton (a H atom) from the oxygen-evolving complex.

In this context, we undertook an investigation of the simplified system in which the Mn cluster has been destroyed, e.g. by Tris-washing (Blankenship et al., 1975). This leaves us with only two carriers on the donor side, the

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* Corresponding author. Tel.: +33 1 43 25 26 09. FAX: +33 1 40 46 83 31. E-mail: rappaport@ibpc.fr.

[‡] CNRS.

[§] CEA-Cadarache.

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[1] Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonate); MES, 2-[*N*-morpholino]ethanesulfonic acid; PSII, photosystem II; OEE, oxygen-evolving enhancer; Y_Z, Y_D, tyrosine D_1161, tyrosine D_2160.

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primary donor P_{680} and the secondary donor Y_Z [Tyr D1−161, see Diner and Babcock (1996)]. In this system, the oxidation rate of Y_Z is almost 1000-fold slower than in oxygen-evolving material and displays a marked pH dependence (Conjeaud & Mathis, 1980). Due to the absence of the native reductant, the lifetime of Y_Z^{ox} is also considerably increased. The oxidation of Y_Z in Tris-washed material was reported to trigger a proton release (Renger & Voelker, 1982; Förster & Junge, 1984). When electron transfer toward the secondary quinone acceptor Q_B is inhibited, the re-reduction of Y_Z^{ox} proceeds mainly through recombination with the primary quinone Q_A^-, allowing a study of the proton re-uptake process. In this paper, we describe the pH dependence of the electron and proton transfer reactions occurring under such conditions. We also investigate the coupling between proton release and the local electrochromic absorption change reflecting electrostatic events on the donor side.

MATERIALS AND METHODS

PSII-enriched membrane fragments were prepared as described by Ghanotakis and Babcock (1983), omitting the second Triton incubation. The samples were frozen and kept at a concentration of 4 mg of chlorophyll/mL in a medium containing 0.3 M sucrose, 5 mM $MgCl_2$, 10 mm NaCl, and 2 mM Mes pH 6.5. They were diluted for Tris treatment at 0.2 mg of chlorophyll/mL in 0.8 M Tris at pH 8.0, and then incubated under room light for 5 min and centrifuged for 30 min at 35000*g*. The pellet was resuspended in a medium containing 0.3 M sucrose, 10 mM NaCl, and 10 mM of the appropriate buffer (Mes for pH lower than 6.5 and Hepes for higher pH). The centrifugation and resuspension steps were performed twice for washing. The inactivation of the water oxidase was checked from the absence of oscillating absorption changes in the UV, and SDS−PAGE showed that the extrinsic polypeptides (OEE) were lost.

The membranes were used at 10 μg of chlorophyll per mL in the presence of 10 μM DCMU and 50 μM of the oxidant octocyanotungstate (this substance was a kind gift of Professor A. J. Stemler). PSII particles were prepared according to Diner and Wollman (1980) from a mutant strain of *Chlamydomonas reinhardtii* lacking PSI and ATPase (Fud50.F15). They were diluted for experimental use at 2 μg of chlorophyll per mL in a medium containing 10 mM NaCl, 0.003% Triton X-100, 10 mM buffer, and 5 μM octocyanotungstate.

For proton release measurements no buffer was added to the medium. The dye concentration was in the 20−40 μM range depending on the pH region investigated. The dyes used were bromocresol green ($4.0 \leq pH \leq 6.0$), bromocresol purple ($5.5 \leq pH \leq 7.0$), and phenol red ($6.5 \leq pH \leq 8.0$). The absorption changes of the dyes were measured at 570 nm. At this wavelength, no significant absorption change is detected in the absence of the dye. For each of these dyes, the absorption changes were suppressed in the presence of a buffer, showing that the observed signals were entirely due to pH changes.

Absorption changes were measured with the Joliot-type spectrophotometer (Joliot & Joliot, 1984; Joliot et al., 1980) where the absorption is sampled at discrete times with short monochromatic flashes. Saturating xenon flashes of 2 μs half-width duration, filtered with a broad-band red filter, were used for actinic illumination.

The amount of proton released per reaction center was calibrated by adding a known volume of a HCl solution at pH 4.0 to the medium and by measuring the absorption changes induced by this addition. The concentration of PSII reaction centers was estimated from the flash-induced absorption change at 325 nm, which gives the amount of Q_A^- formed, taking an extinction coefficient of 13 mM^{-1} cm^{-1} (van Gorkom, 1974).

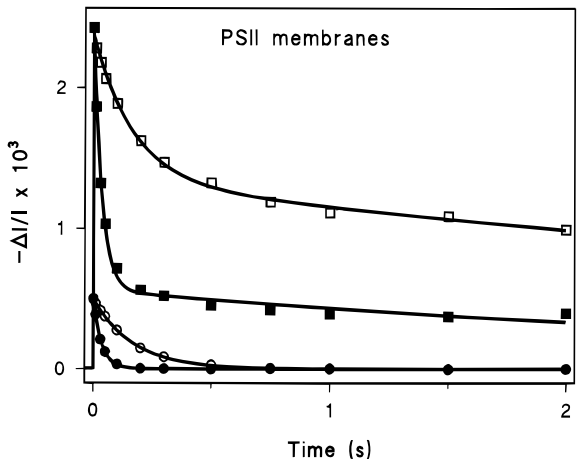

FIGURE 1: Flash-induced absorption changes in PSII membranes at 315 nm (squares) and at 292 nm (circles) at pH 5.5 (solid symbols) and 7.5 (open symbols). The lines are best fits with a sum of two exponentials. The amplitude of the second exponential was found to vanish for the 292 nm changes. The same rate constant was found for the fast phase at all wavelengths.

RESULTS

1. Transient Absorption Changes in the UV

We studied the time course of flash-induced absorption changes at 315 and 292 nm, using either Tris-washed PSII-enriched membranes in the presence of DCMU, or PSII core particles from *Chlamydomonas*. In the latter case Tris-washing and inhibitor addition are not because since the Mn cluster has been lost during the preparation and no secondary quinone acceptor is present. Under these conditions the charge-separated state $Y_Z^{ox}Q_A^-$ is formed after a few microseconds and is expected to decay through charge recombination. At 315 nm the absorption changes reflect predominantly, if not only, the redox changes of Q_A, whereas at 292 nm, an isosbestic wavelength of the (Q_A^-−Q_A) spectrum, only the changes due to (Y_Z^{ox}−Y_Z) are observed (van Gorkom, 1974; Diner & De Vitry, 1984 ; Dekker et al., 1984). Figure 1 shows the flash-induced absorption changes at these wavelengths, recorded at pH 5.5 (open symbols) and 7.5 (closed symbols). The decay of Q_A^- (squares) is biphasic, whereas that of Y_Z^{ox} (circles) is monophasic. The curves drawn in Figure 1 show the result of fitting the Q_A^- decay with a sum of two exponentials and that of Y_Z^{ox} with a single exponential. The time constant of the latter was found identical with that of the fast phase of the Q_A^- decay. While the rate of the fast phase depends on pH, that of the slow phase is almost pH independent. The same features were observed with PSII particles (not shown).

In agreement with previous reports (Babcock & Sauer, 1975; Yerkes et al., 1983; Dekker et al., 1984), this result shows that a fraction of Q_A^- does not decay through recombination with Y_Z^{ox}, presumably because of another reduction pathway for Y_Z^{ox} competing with the recombination reaction. When multiple flashes are used (e.g. with a time spacing corresponding to completion of the fast phase), the

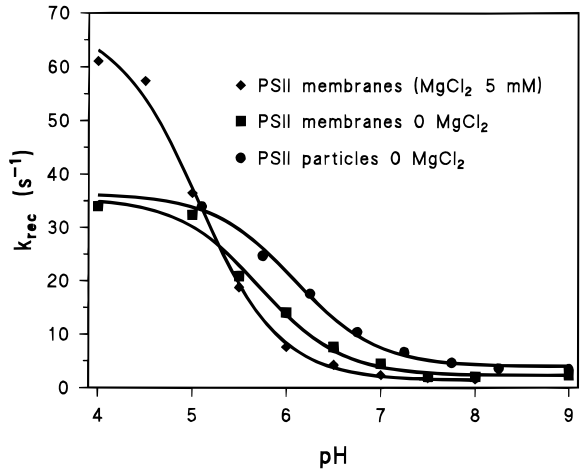

FIGURE 2: Rate constant of the recombination reaction $Y_Z^{ox}Q_A^- \rightarrow Y_ZQ_A$ (computed as described in the text) as a function of pH. The solid curves are best fits using the equation: $k_{rec} = [k_{rec}^p + k_{rec}^u 10^{(pH-pK)}]/[1 + 10^{(pH-pK)}]$, as explained in the Discussion.

fraction of slowly recovering centers is progressively increased, in agreement with the competition model (not shown). Concerning the nature of the competing donor to Y_Z^{ox}, some possibilities can be ruled out. It cannot be the reduced form of the exogenous acceptor (i.e. octocyanotungstate) since omission of this acceptor did not affect the extent of the slow phase of Q_A^- reoxidation (but did slow down its rate). No absorption changes associated with the oxidation of cytochrome b_{559} [see Buser et al., *(*1990)] could be detected. On the other hand, in PSII membranes, the decay kinetics of Y_Z^{ox} were accompanied with the formation of a trough around 430 nm (not shown), which may be ascribed to the oxidation of a chlorophyll, such as "Chl-z" (Thompson & Brudvig, 1988). Another, nonexclusive, candidate for the auxiliary donor is a Mn fraction remaining bound to the PSII donor side. It was reported that among the 4 Mn atoms present in the intact cluster, 0.5−1 atom is resistant to Tris-washing (Kuwabar & Murata, 1983). We found that repetitive washing and/or use of EDTA only slightly diminished the amplitude of the slow phase. As shown by Ananyev et al. (1996), however, EDTA is a poor chelator for Mn.

The competition model predicts the same rate for the decay of Y_Z^{ox} and Q_A^-, as was observed. This rate should be (k_{rec} + k_d), where the first term denotes the recombination rate constant and the second one that of the competing reduction pathway. On the other hand, the relative amplitude at 315 nm of this phase is $k_{rec}/(k_{rec} + k_d)$, so that one can readily calculate both rate constants from the fit parameters. As shown in Figure 2, k_{rec} is markedly accelerated at low pH, by a factor of 15 between pH 8 and 4. The rate k_d becomes also faster when lowering the pH (not shown), although to a smaller extent, so that the relative amplitude of the slow phase of the Q_A^- decay is diminished (compare the 315 nm traces at pH 7.5 and 5.5 in Figure 1).

It has been reported by Conjeaud and Mathis (1980) that the rate of reduction of P_{680}+ by tyrosine Y_Z in Tris-treated PSII membranes is pH dependent. This dependence appeared to be modulated by the ionic strength of the buffer. To address the influence of this parameter on the rate of recombination between Y_Z^{ox} and Q_A^-, we repeated the above experiments in the presence of 5 mM $MgCl_2$. As may be seen in Figure 2 (diamonds) a marked effect was indeed

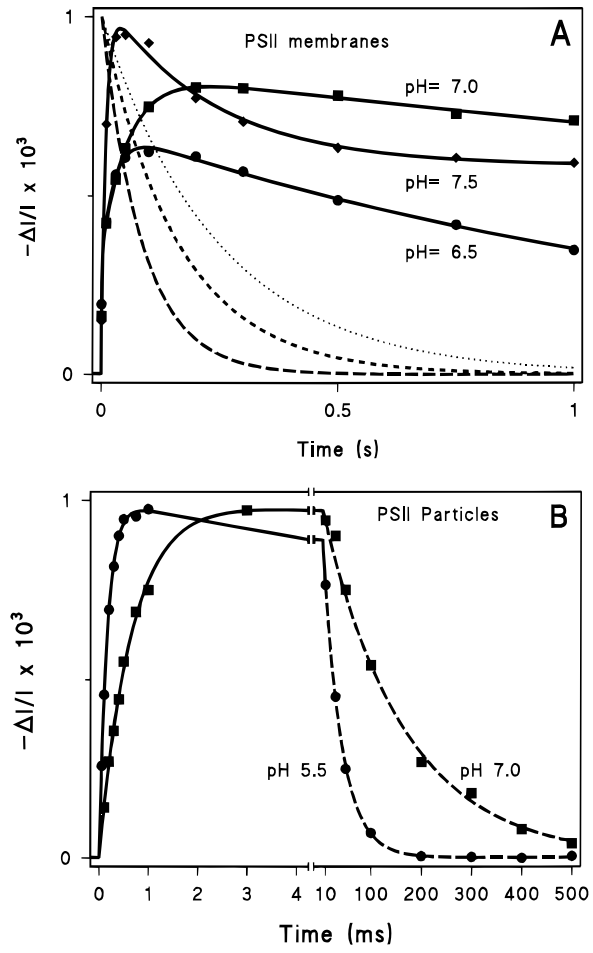

FIGURE 3: Transient absorption changes (570 nm) of pH-indicating dyes in PSII membranes (A) and PSII particles (B) at various pH's. The nonsolid curves show the decay kinetics of Y_Z^{ox} for comparison. In panel A, the pH values are 6.5, 7.0, and 7.5 for respectively, the dashed, short-dashed, and dotted curves.

observed, consisting of a shift of the curve by about 1 unit toward lower pH and an almost doubled rate at low pH.

2. Proton Release

We studied the kinetics of proton release and rebinding induced by the oxidation and reduction of Y_Z during the charge separation−recombination cycle, using hydrophilic dyes. As previously reported for Tris-washed inside out thylakoids (Renger & Voelker, 1982) or thylakoids in the presence of neutral red (Förster & Junge, 1984), we observed such a proton release, both in PSII membranes and particles. The kinetics of proton release and rebinding, however, turned out to be markedly dependent on the material used. In the following, we assume that no or negligible protonation occurs on the acceptor side, in agreement with previous work in thylakoids (Polle & Junge, 1986; Jahns et al., 1991) or PSII membranes (Rappaport & Lavergne, 1991). As discussed below, some correction may be required in the case of core particles, where Bögershausen and Junge (1995) detected an uptake of about 0.3 H^+/center at pH 5.5.

PSII Membranes. The time-course of the absorption changes which reflect the appearance and disappearance of protons as sensed by the hydrophilic dye is shown in Figure 3A at various pHs. Comparing with Figure 1, the proton kinetics appear to diverge markedly from those of electron transfer. In PSII membranes the rates of proton release (tens of ms) and re-uptake (hundreds of ms to s) are much slower

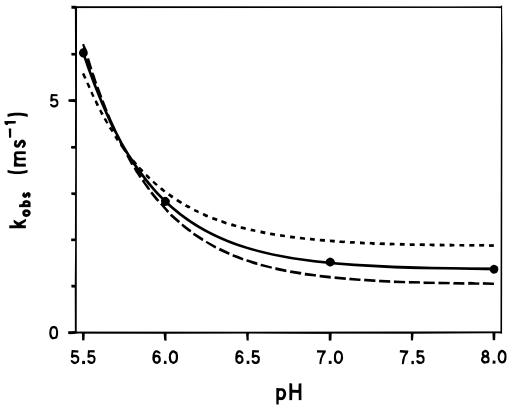

FIGURE 4: Rate of the observed proton release as a function of pH in PSII particles. The data were fitted according to $k_{obs} = k_{prot}(10^{-pH} + 10^{-pK})$ (see text). The solid line is the best fit, obtained for $k_{prot} = 1.5 \times 10^9$ M^{-1} s^{-1} and pK = 6.05. For comparison, the dashed and short-dashed curves correspond to the fit of the data with a single free parameter (k_{prot}), imposing a pK of 6.2 and 5.8, respectively.

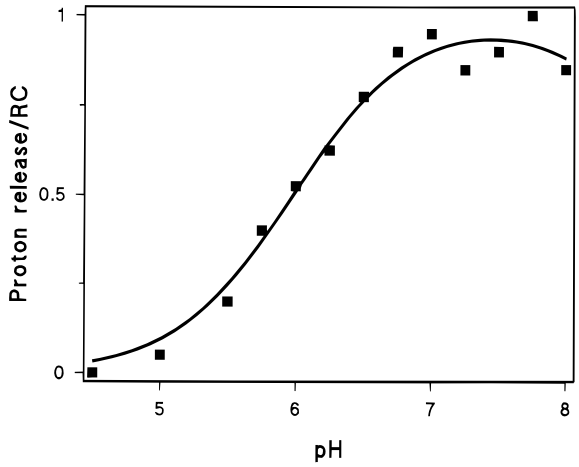

FIGURE 5: Proton release per reaction center as a function of pH in PSII particles.

than the oxidation (tens of μs, Conjeaud & Mathis, 1980) and reduction of Y_Z (tens of ms). Renger and Voelker (1982), using Tris-washed inside-out thylakoids also noted the slowness of proton release in their system. The delay between the electron and proton kinetics is especially striking during the reprotonation process since the lifetime of the flash-induced bulk pH change is much longer than the lifetime of Y_Z^{ox} which caused this change. As proposed by Renger and Voelker (1982), the pH changes in the aqueous medium appear kinetically limited by a diffusion barrier since we observed (data not shown) that addition of imidazole accelerates the rate of proton transfer to the dye (i.e. a 5-fold acceleration in the presence of 20 μM imidazole). This mobile buffer is known to accelerate surface to bulk proton movement by collisional transfer [see the review by Gutman and Nachliel (1990)]. The extent of the acceleration is dependent on the concentration of added imidazole as expected for a collisional mechanism. The process of proton re-binding induced by the reduction of Y_Z^{ox} appears more complicated: (i) we observed no effect on the rate of absorption changes upon addition of imidazole (not shown); (ii) increasing the pH leads to an overall slowing down of this process and induces a biphasic behavior at pH > 7.0 (see Figure 3A).

PSII Particles. In PSII particles (Figure 3B), the proton release is markedly faster than in PSII membranes (hundreds of μs), although still slower than Y_Z oxidation. On the other hand, the re-uptake phase is now concomitant with the reduction of Y_Z^{ox} and follows the same pH dependence. As may be seen in Figure 3B, the rate of the observed release is also accelerated when decreasing the pH. Figure 4 shows the variation of this rate as a function of pH. Because the detection of the flash-induced pH changes in this material is much faster than the reduction of Y_Z^{ox}, we could measure the full extent of the proton release. Figure 5 shows the amount of proton release per PSII reaction center as a function of pH. The data are well fitted by assuming a single protonatable species undergoing a pK shift upon oxidation of the tyrosine Y_Z (see Discussion).

It may be noted that both at pH 7.0 or 5.5 the proton-indicating absorption changes decay to the base line at long times. This supports our assumption of a negligible uptake on the acceptor side since a substantial fraction of the centers remained blocked in the Q_A^- state (e.g. 25% at pH 5.5 and 58% at pH 7.0). We found no indication of a rapid uptake transient, at variance with Bögershausen and Junge (1995) using a different core particles preparation. We also observed that the proton release in PSII particles was insensitive to the presence of DCMU, which is known from previous work in PSII membranes or thylakoids to preclude proton uptake (Polle & Junge, 1986; Jahns et al., 1991; Rappaport & Lavergne, 1991). Nevertheless, below pH 5.5 we did observe a slightly negative asymptote of the dye absorption changes, consistent with the occurrence of a small uptake at low pH. This effect, however, is of little quantitative consequence on the proton release titration of Figure 5.

3. Electrochromic Changes in the Blue Region

The oxidation of Y_Z is known to induce absorption changes in the region of the absorption peaks of chlorophyll *a* (Diner & de Vitry, 1984; Dekker et al., 1984; Lavergne, 1984; Saygin &Witt, 1985; Diner & Tang, 1995; Mulkidjanian et al., 1996). These changes have the shape of the first derivative of an absorption band, suggesting an electrochromic shift of a chlorophyll. It has been recently observed [B. A. Diner and J. Lavergne, unpublished; see Diner and Tang (1995)] that, in a mutant from *Synechocystis* in which the difference spectrum of ($P_{680}^+ - P_{680}$) is blue shifted, the spectrum of ($Y_Z^{ox} - Y_Z$) is also shifted to the same extent. This result supports the electrochromic interpretation and identifies P_{680} as the field-sensitive pigment. As the electrochromic signal is an indicator of the local electrostatic field, one may expect that its amplitude is sensitive to proton release from the protein to the bulk. We thus measured the absorption changes observed upon oxidation of Y_Z in intact and Mn-depleted PSII. The results are shown in Figure 6 (open squares, intact material; solid circles, Mn-depleted material). The ($Q_A^- - Q_A$) spectrum, shown for comparison, was obtained in the presence of DCMU and hydroxylamine. Under these conditions, Y_Z^{ox} is reduced by hydroxylamine in a few tens of milliseconds and Q_A^- is stable for several tens of seconds (Bennoun, 1970). The spectrum was thus obtained from the extent of the absorption changes measured 1 s after a series of flashes. The spectrum of ($Y_Z^{ox} - Y_Z$) in Tris-washed PSII membranes was obtained as the difference between the spectrum of ($Q_A^- - Q_A$) and the absorption changes measured 2 ms after a flash in the presence of

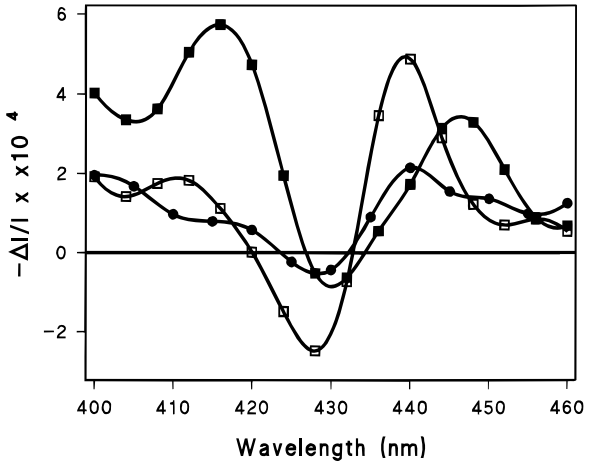

FIGURE 6: Spectra of (Q_A^-−Q_A) (solid squares) and (Y_Z^{ox}−Y_Z) in intact and Tris-washed PSII membranes (open squares and solid circles, respectively) at pH 7.0. Using the extinction coefficient for the (Q_A^-−Q_A) spectrum reported by Dekker et al. (1984) one $\Delta I/I$ unit corresponds to a variation of extinction coefficient of 1.3 mM^{-1} cm^{-1}. Thus, $\Delta\epsilon$(415 nm) = 7.4, $\Delta\epsilon$(445 nm) = 4.25 for the (Q_A^-−Q_A) spectrum; $\Delta\epsilon$(428 nm) = −0.55, $\Delta\epsilon$(440 nm) = 2.75 for the (Y_Z^{ox}−Y_Z) spectrum in Tris-washed samples and $\Delta\epsilon$(428 nm) = −3.2, $\Delta\epsilon$(440 nm) = 6.3 for the (Y_Z^{ox}−Y_Z) in oxygen evolving PSII.

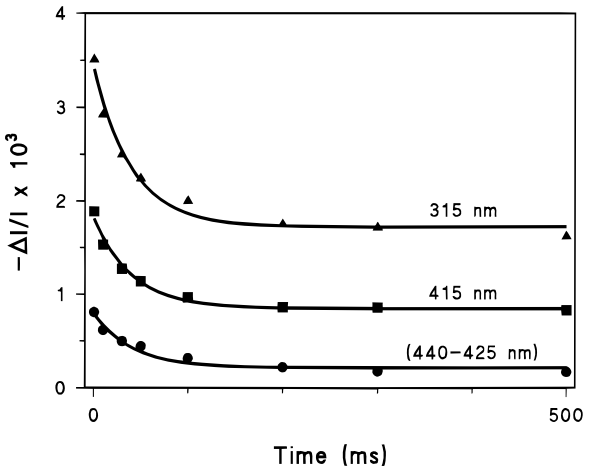

FIGURE 7: Flash-induced absorption changes in PSII particles at 315 nm (triangles), 415 nm (squares), and for the difference (440−425) nm (circles). The solid lines are the best fit of the data with a single exponential decay and constant offset. The same half-time was obtained at all wavelengths.

DCMU and in the absence of an exogenous electron donor. The spectrum of (Y_Z^{ox}−Y_Z) in intact PSII was obtained as the difference between the spectrum of (Q_A^-−Q_A) and the absorption changes measured 6 μs after a flash in the presence of DCMU [in oxygen-evolving PSII, the half-time of the reduction of Y_z by S_1 is in the 50−100 μs range; see Diner and Babcock (1996)]. The spectra were normalized to the same amplitude of the absorption change induced by the reduction of Q_A^- measured at 315 nm. The spectrum of (Y_Z^{ox}−Y_Z) displays similar features in both types of material, i.e. a trough around 428 nm and a peak at 440 nm. In the Tris-washed sample, however, the amplitude of the spectrum is markedly smaller than in intact PSII.

We also measured the transient flash-induced absorption changes in this region. Figure 7 shows the kinetics observed at various wavelengths in PSII particles. At 415 nm where the contribution of (Q_A^-−Q_A) is predominant, we obtained the same features as at 315 nm, i.e. a phase decaying in a few tens of milliseconds followed by a much slower one.

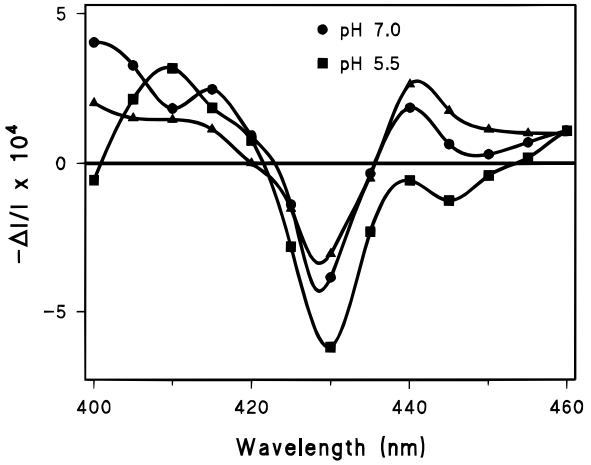

FIGURE 8: Spectra of the absorption changes between 100 μs and 1.5 ms after a flash at pH 7.0 (circles) and 5.5 (squares) in PSII particles. The spectrum of (Y_Z^{ox}−Y_Z) obtained by Diner and de Vitry (1984) with the same material is shown for comparison (triangles), normalized to the same amplitude as the solid circle spectrum for the difference (440−430 nm).

Also consistent with the results obtained in the UV region (Figure 1), the slow phase was hardly observed in the difference (440−425 nm) which cancels the contribution of (Q_A^-−Q_A) and reflects predominantly the electrochromic change associated with the oxidation of Y_Z (see Figure 6). The half-time of the transients were identical to those obtained in the UV. These results indicate that the positive charge which is at the origin of the electrochromic shift is retained during the whole lifetime of Y_Z^{ox}. The same conclusion was reached by Diner and Tang (1995) from similar experiments performed at −8 °C.

It may be expected (Rappaport et al., 1994) that the proton release event induced at short times by the oxidation of Y_Z is accompanied by a decrease of the electrochromic response. To address this issue, we measured the absorption changes occurring in the time range of the proton release observed in PSII particles. Figure 8 shows the difference between the changes measured at 100 μs and at 1.5 ms after a flash at pH 7.0 and 5.5, and for comparison, the difference spectrum of (Y_Z^{ox}−Y_Z) obtained by Diner and de Vitry (1984) with the same material. The concentration of Y_Z^{ox} does not vary significantly during this time interval which, on the other hand, covers most of the proton release kinetics. At pH 7.0, where the proton release is close to 1 per center (Figure 5), the (100 μs−1.5 ms) spectrum resembles the electrochromic spectrum associated with (Y_Z^{ox}−Y_Z) at longer times. Thus, as expected, a significant decrease (about half) of the electrochromic change accompanies the proton release event. According to this rationale, one expects a quite different picture at pH 5.5 where the proton release is small. The decrease of the electrochromic change in the sub-millisecond range should hence be smaller, and, conversely, the amplitude of the signal present at longer times should be larger than at pH 7, reflecting the higher net charge of the system. We found no clear confirmation of these predictions, however. As may be seen in Figure 8, a transient change is still observed in the (100 μs−1.5 ms) difference at pH 5.5. The large negative trough at 430 nm is likely due to a slow phase of P_{680}^+ reduction. The shoulder observed around 440 nm, however, is probably indicative of a contribution of the electrochromic spectrum, suggesting that some electrostatic relaxation is still occurring in the sub-millisecond time range, even when the proton release does

not take place. The spectrum observed at longer times (not shown) also displays no clear evidence of a larger electrochromic contribution. Tentatively, we suggest that at low pH in this material, another mode of electrostatic stabilization (possibly involving other ions and/or a conformation change) becomes a substitute for proton release. In PSII membranes, at variance with particles, we could not resolve at pH 7 an electrochromic decay in the 100 μs−1 ms range (the search for a possibly faster decay is precluded by the strong absorption changes of P_{680}^+ at shorter times).

DISCUSSION

1. Kinetics of Proton Release and Uptake

PSII Particles. In this material, the proton release kinetics are fast (hundreds of μs) and the re-uptake is concomitant with the reduction of Y_Z^{ox}. As expected for a diffusion limited reaction, the rate of release k_{obs} may be adjusted by $k_{obs} = k_{prot}[H^+] + k_{deprot}$ (see Figure 4, solid line) where, k_{prot} and k_{deprot} are, respectively, the rate constants for proton binding and release, and their ratio k_{deprot}/k_{prot} is equal to the K_a of the group which releases the proton. The values thus obtained for k_{prot} and k_{deprot} are, respectively, 1.5×10^9 M^{-1} s^{-1} and 1.3×10^3 s^{-1}, corresponding to a p$K_a = 6.05$. This value of k_{prot} is consistent with a diffusion-limited reaction, since the rate of free diffusion of protons in water has been estimated to be $(1-10) \times 10^9$ M^{-1} s^{-1} (Gutman & Nachliel, 1990). It may not be excluded, however, that the detergent concentration used in this experiment is not quite sufficient to guarantee a monodisperse suspension of the particles so that the true diffusion-limited rate constant could be somewhat larger [see e.g. the 3-fold larger value reported by Bögershausen and Junge (1995)].

PSII Membranes. In this material, both the proton release and re-uptake, as monitored by the absorption changes of hydrophilic dyes, appear markedly delayed with respect to the electron transfer event that causes them, i.e. the oxidation and reduction of Y_z, respectively. As suggested by the acceleration of the proton release rate observed upon addition of imidazole, a "diffusion barrier" is probably involved. It presumably consists of an array of protein- or membrane-bound buffering groups among which the proton released by PSII hops many times before equilibration with the bulk phase occurs (Gutman & Nachliel, 1990). Förster and Junge (1984), using the dye neutral red, reported a much faster rate (10^4 s^{-1}) for the observed proton release in Tris-washed thylakoids than the one reported here or by Renger and Voelker (1982). In this case, the amphiphilic dye acts as the mobile intermediate interacting with the buffering barrier [see also Haumann and Junge (1994) for similar findings in oxygen-evolving thylakoids].

The very slow kinetics observed in this material for the re-uptake of the proton detected in the bulk medium raise an intriguing problem. If the appearance of the proton is limited by a diffusion barrier, the same rate limitation should control the reverse process. Nevertheless, the uptake rate (Figure 3A) is much slower than expected for a convolution of the recombination kinetics with the "barrier-limited" rate observed for the release phase. This paradox becomes still more obvious in the presence of imidazole, which accelerates the release but does not affect the uptake. It thus appears that, somehow, the communication between the buffering domain accessible to Y_Z and the aqueous medium is modified during the course of the experiment.

It should be stressed that in spite of the important delay for the exchange of protons with the bulk, the pH dependence of the recombination kinetics is similar with that observed for particles, implying, as further discussed below, a rapid proton equilibration of the group shifted to a pK close to 6 upon oxidation of Y_Z. This means that, locally, the proton release and uptake by this group occurs with similar fast rates as observed in particles. The picture that emerges is that of a pocket allowing rapid proton equilibration of the Y_Z region but equilibrating sluggishly with the bulk medium. The proton capacity of this pocket cannot be very small, as shown by experiments using multiple flashes. For instance, when a second flash is given right after the recombination phase, thus long before the complete uptake of the protons released to the bulk, the rate of P_{680}^+ reoxidation by Y_Z, or the recombination rate, remains the same as on the first flash. Some extra proton release to the bulk is also observed.

2. Dependence on pH of the Rate of the Recombination Reaction and of Proton Release

The rate of the recombination reaction $Y_Z^{ox}Q_A^- \rightarrow Y_ZQ_A$ shows a strong dependence on pH. As previously observed (Yerkes et al., 1983; Dekker et al., 1984) it is not increased by a factor of 10 per pH unit as would be expected for a stoichiometric proton uptake upon reduction of Y_Z^{ox}. To account for the dependence of the rate of the back-reaction on pH, we thus propose that a charge, present on a neighboring protonatable amino acid, modulates through Coulombic interaction the free energy gap between the $Y_Z^{ox}P_{680}$ and $Y_zP_{680}^+$ states. Within experimental accuracy, the recombination kinetics that we obtained are well fitted by a single-exponential decay, at any pH. This implies that the protonatable group which modulates the recombination rate is in rapid protolytic equilibrium with its environment, because otherwise, one would expect two kinetic phases, with relative amplitudes reflecting the fractions of the protonated and unprotonated states. Conversely, in the case of a fast equilibrium, the recombination process is influenced by the time-averaged protonation state of the interacting group, resulting in monophasic kinetics. One then expects the rate constant k_{rec} to depend on pH according to

$$k_{rec} = \frac{[k_{rec}^{\,p} + (k_{rec}^{\,u} \times 10^{(pH-pK)})]}{[1 + 10^{(pH-pK)}]}$$

where k_{rec}^{p} and k_{rec}^{u} are the rate constants in the presence of the fully protonated and unprotonated forms, respectively. The experimental data are satisfactorily fitted by such a function (curves in Figure 2), yielding a pK of 6.05 for the PSII particles (in agreement with the pK estimated above from the release rate) and a pK of 5.9 or 5.1 for PSII membranes in the absence or presence of Mg^{2+}, respectively.

If a protonatable group with pK close to 6 interacts electrostatically with Y_Z^{ox}, one expects that, conversely, the redox state of Y_Z will affect the pK of this group, leading to a pH dependent proton release. We observed (Figure 5) a release of about 1 proton per reaction center at pH ≥ 7 and a substoichiometric release at lower pH, decreasing to zero below pH 5. This is in qualitative agreement with results from Renger and Voelker (1982) who observed that the stoichiometry of proton release is close to one at pH ≥ 6 and decreases to zero at lower pH. To account for the release of about 1 proton per reaction center around pH 7.5, the pK

of the protonatable group must be ≥9 in the presence of reduced Y_Z (so that it is fully protonated at pH 7.5). This implies a p*K* shift of 3 units or more caused by the oxidation of Y_Z. As may be seen in Figure 5, the pH dependence of the release stoichiometry is satisfactorily fitted by this model.

In agreement with the results of Conjeaud and Mathis (1986) concerning the rate of P_{680}^+ reduction, we observed a marked effect of the ionic composition of the medium on the pH dependence of the recombination reaction. In the presence of 5 mM $MgCl_2$ (Figure 2), the modulating p*K* was shifted by almost one unit (5 instead of 6) and the rate constant k_{prot} was almost doubled. This p*K* shift underscores the effect of fixed charges which impose a negative potential at the surface of the membrane. As predicted by the Gouy−Chapman theory, divalent cations efficiently collapse this surface potential (Barber, 1980). The model proposed by Conjeaud and Mathis assumed that the surface potential was itself pH dependent due to the modified protonation state of the groups that create it. This complication was introduced because of the impossibility to account for the data in a particular model for the protolytic reactions of Y_Z. It turns out that our results and, as dicussed below, also theirs (P_{680}^+ reduction rate), are satisfactorily accounted for by the present model implying that deprotonation is carried out by one group fully protonated in the presence of reduced Y_Z and shifted to a p*K* of 5 to 6 (depending on the ionic composition of the medium) in the presence of Y_Z^+. We do not mean to exclude any variation of the surface potential as a function of the ambient pH, which is to some extent likely, but only point out that the observed pH dependencies are well fitted by neglecting such variations. We have no obvious explanation for the enhancement of k_{prot} by $MgCl_2$, which suggests that the field gradient between Y_Z^{ox} and P_{680} becomes steeper in the presence of divalent cations.

3. Mechanistic Implications

The problem we wish to discuss now is the nature of the protonatable group which controls the recombination kinetics and the proton release. Two models may be envisaged: (i) this group is Y_Z itself, or (ii) it is a distinct species reacting to the positive charge resulting from the oxidation of Y_Z. A first element to consider is that, under all likelihood, the oxidation of Y_Z results in a neutral, deprotonated radical ($Y_Z^\bullet$). Evidence for this rests on the comparison of the EPR spectra of Y_Z^{ox} and model tyrosines (Barry & Babcock, 1987; Evelo et al., 1989). This interpretation is supported by the p*K* of −2 of the tyrosil radical (Dixon & Murphy, 1976). The deprotonation of the oxidized tyrosine does not necessarily mean, however, that the proton is directly released to the bulk. A demonstration of this is found in the case of tyrosine Y_D, where magnetic resonance studies [reviewed by Diner and Babcock (1996)] have shown that the tyrosine proton becomes trapped by a nearby histidine ligand and remains hydrogen-bonded to the $Y_D^\bullet$ radical. Whether a similar mechanism applies to Y_Z is controversial. In the model proposed by Hoganson et al. (1995) and Gilchrist et al. (1995), the proton originating from $Y_Z^\bullet$ would be rapidly released into the bulk medium (*via* transient protonation of a neiboring group). In the native O_2-evolving system, the reduction of $Y_Z^\bullet$ would involve the concerted abstraction of an electron and a proton (a H-atom) from the water substrate or other intermediates. This model is mainly based on EPR data showing (in the Mn-depleted system) a significant mobility of the tyrosine ring in $Y_Z^\bullet$, at variance with $Y_D^\bullet$ (Tommos et al., 1995). It has been recently established, however, that $Y_Z^\bullet$ is hydrogen-bonded (Un et al., 1996; Tang et al., 1996), although in a more delocalized manner than in the case of $Y_D^\bullet$. It is thus quite possible that the proton released from $Y_Z^\bullet$ remains engaged in a H-bond with a nearby ligand.

We may now recast our discussion in the following terms: either $Y_Z^\bullet$ is the group with a p*K* close to 6 observed in the present work [direct deprotonation, as in the models of Hoganson et al. (1995) or Gilchrist et al. (1995)] or the proton released from the tyrosine remains trapped in its vicinity. In the latter case, a positive charge remains associated with Y_Z^{ox}, which can in turn interact electrostatically with a nearby group, that we denote "G", responsible for the protolytic reactions (indirect, or "domino" deprotonation). We believe that our results are best accommodated by the latter model. First, the observed p*K* is about 6, compared with that of −2 for the tyrosine radical *in vitro*, which would imply an unusually large shift due to the protein environment. Besides, if the p*K* of $Y_Z^\bullet$ was close to 6, its EPR or absorption spectra would be dependent on pH, which was not observed. A third evidence against direct deprotonation is the spectral shift of P_{680} associated with Y_Z^{ox}. This shift is most likely due to an electrochromic effect, implying a charged character of Y_Z^{ox}. The local trapping of the tyrosine proton accounts for this effect, as illustrated by the observation of a very similar shift associated with Y_D^{ox} (Diner & Tang, 1995). We thus assume that the proton given off by $Y_Z^\bullet$ is trapped by a group "A", possibly, but not necessarily, establishing a H-bond with $Y_Z^\bullet$. Possible candidates for A are His D1−190, whose mutation affects drastically the reduction rate of P_{680}^+ (Diner et al., 1991), and Glu D1−189. It should be noted that the release of a H-bonding proton is expected to be a slow process, even if its p*K* is low, because of the activation energy required for bond breaking (Hibbert & Emsley, 1990). This may account for the fact that the proton remains trapped during the lifetime of $Y_Z^\bullet$, and does not contradict the finding (Force et al., 1995) that, on a slower time-scale, the tyrosine proton is exchangeable. If the H-bonding partner (A) of $Y_Z^\bullet$ is a histidine nitrogen, an attractive possibility is that the deprotonating group (G) belongs to the same histidine. The H-bonding group is expected to be the pyridine nitrogen. The protonation of this nitrogen results in a markedly decreased p*K* for the pyrrole nitrogen (from its value of 14 in free imidazole at high pH) so that, for example, the two nitrogens of free imidazole at pH = 6 tend to become indistinguishable (Pullman & Pullman, 1963). In the present model, however, the structure remains dissymetric because of the proton trapped between $Y_Z^\bullet$ and the histidine and may keep a dipolar character, accounting for the electrochromic shift of P_{680}. The same mechanism may also be proposed for Y_D^{ox} (hydrogen-bonded to His D2−189), accounting for the finding by Vass and Styring (1991) that the protonation of a group with p*K* ≈ 7.4 accelerates the $Y_D^{ox}S_0 \rightarrow Y_DS_1$ reaction.

4. Thermodynamic Implications

According to the present results, the equilibrium between P_{680} and Y_Z^{ox} is modulated by the protonation state of group G, which has a p*K* of 6 in the presence of Y_Z^{ox} (in the following we adopt as standard conditions those of Figure 5: no Mg^{2+}, p*K* = 6). This group is fully protonated before the flash at pH ≤ 7.5, hence its p*K* before charge separation must be ≥9. The observed recombination rate is 35.5 or

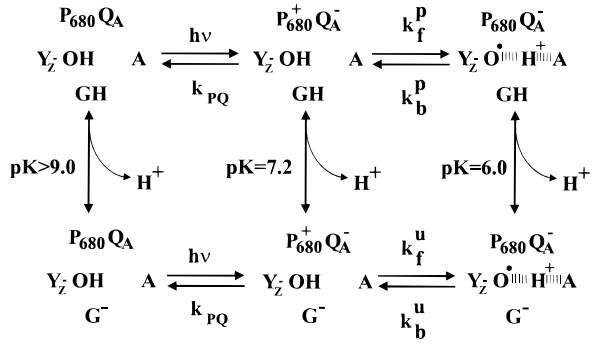

FIGURE 9: Scheme summarizing electron and proton transfer in the Mn-depleted system. k_f^p and k_f^u are the rate constants for the oxidation of Y_Z in the presence of protonated and unprotonated G ($k_f^p \approx 11.5 \times 10^3$ s^{-1}, $k_f^u \approx 350 \times 10^3$ s^{-1}), respectively. The backward rate constants k_b^p and k_b^u can be computed from the equilibrium constants derived from the present work: $k_f^p/k_b^p = 170$, $k_f^u/k_b^u = 2600$.

2.3 s^{-1} in the presence of, respectively, GH or G$^-$. The recombination rate is $k_{rec} = k_{PQ}/(K + 1)$, where k_{PQ} is the rate constant for $P_{680}^+Q_A^-$ recombination and K the equilibrium constant $[Y_Z^{ox}P_{680}]/[Y_ZP_{680}^+]$. If $k_{PQ} = 6000$ s^{-1}, then $K = 170$ or 2600 in the presence of, respectively, GH or G$^-$. The value of 6000 s^{-1} was determined by Conjeaud and Mathis (1980) in the presence of Y_Z^{ox}. A slower rate (700 s^{-1}) for k_{PQ} was found by Diner et al. (1991) in a mutant lacking Y_Z. The difference between the two estimates may be due to an enhancement of the recombination rate due to the electrostatic influence of Y_Z^{ox}. Conjeaud and Mathis (1980), however, observed no dependence of k_{PQ} (in the presence of Y_Z^{ox}) on pH, which suggests that this rate constant is only weakly influenced by electrostatic effects on P_{680}. Hence, the value found by Conjeaud and Mathis may be more appropriate in the present discussion than the value reported by Diner et al. (1991), which could be affected by a structural change in the mutant.

The pK decrease of more than 3 units upon oxidation of Y_Z implies an electrostatic interaction of more than 180 mV between G and the (Y_Z−A) region. On the other hand, the equilibrium constant K (as observed from the recombination rate) is increased 15-fold (2600/170) upon deprotonation of G (or 30-fold in membranes with $MgCl_2$). This implies that the deprotonation increases the ΔG between P_{680} and Y_Z by only 70 meV. Thus, G also interacts electrostatically with P_{680}, and this interaction is 70 mV weaker than with Y_Z (i.e. ≥110 mV). Accordingly, the pK of G in the presence of P^+ should be about 7.2. The electrostatic interaction between G and P is consistent with the electrochromic data (see below), showing that ($Y_Z^{\bullet}$−H^+−A) interacts with P_{680} and that the amplitude of the shift is about halved when a proton is released from GH. The various equilibria involved are summarized in Figure 9.

Conjeaud and Mathis (1980, 1986) reported that the major part of P_{680}^+ reoxidation (60−80%) corresponds to an exponential decay, with a pH-dependent rate ($k_f = 11.5 \times 10^3$ s^{-1} at low pH, 350×10^3 s^{-1} at high pH). The model proposed by the authors assumed that reduced Y_Z (which had not been yet identified as a Tyr) had a pK of 7.5−8 (expressed for our standard conditions; their estimate was 6.3 after correction from the surface-potential). This pK was believed to be independent of the redox state of P_{680} and the oxidation of Y_Z would cause total deprotonation. Clearly, this model does not account for the present data, since it would imply a decrease of the proton release when increasing the pH, whereas the opposite was observed. Figure 9, on the other hand, provides a satisfactory explanation for the pH dependence of k_f. The protonation state of G modulates the ΔE_m between P_{680} and Y_Z, which will, in general, affect k_f. It is noteworthy, however, that the effect of pH on the equilibrium constant is essentially due to the effect on the forward rate constant k_f. Decreasing the pH from 8 to 4 causes a decrease of k_f by a factor of 30, compared with the effect of 15−30 (depending on the ionic composition of the medium) observed for the equilibrium constant. Together with the kinetic aspects discussed below, this suggests that Y_Z oxidation is rate limited by the concerted transfer of the proton to A and protolytic relaxation of G. The electrostatic influence of G controls this kinetic process by modulating the ΔpK between Y_Z and A. In our model (Figure 9), the pK of G is expected to be 7.2 in the presence of P_{680}+ and reduced Y_Z, in reasonable agreement with the pH dependence found for k_f. The view that the electron transfer from Y_Z to P_{680} in Mn-depleted PSII is rate limited by a proton transfer is supported by the finding (B. A. Diner, personal communication) that the reaction is slowed down in the presence of D_2O.

An important aspect of the observations of Conjeaud and Mathis (1980, 1986) is that the oxidation of Y_Z by P_{680}^+ appears essentially as a single exponential process with a pH dependent rate. As explained above for the recombination of $Y_Z^{ox}Q_A^-$, this implies that the protolytic equilibrium is faster than the electron transfer reaction, which has a half-time of 1−2 μs at high pH. For a pK of about 6 (corrected for surface potential; 7.2 when uncorrected), the relaxation rate of a diffusion-limited protolytic equilibrium should be less than 10^4 s^{-1}, i.e. slower by almost 2 orders of magnitude than the electron transfer rate. This suggests that in thylakoids, the local proton exchange on G is faster than diffusion-limited [see the review by Grunwald (1965) on "ultrafast" proton transfer reactions]. A similar conclusion has been attained by Maróti and Wraight (1989), about the rate of proton uptake on the acceptor side of the bacterial reaction center. Such a ultrafast transfer implies some kind of proton channel (e.g. bound water). The presence of a channel mediating proton transfer from G helps to rationalize the fact that in spite of its rapid proton equilibration properties, this group exerts marked electrostatic interactions with Y_Z and P_{680}, which would be severely screened if it were directly exposed to a liquid aqueous phase.

5. Electrochromic Changes

In a previous paper (Rappaport et al., 1994), we investigated the decay kinetics of the electrochromic shift associated with Y_Z^{ox} in oxygen-evolving membranes and interpreted the fast phase occurring on certain transitions (mainly during the $Y_Z^{ox}S_3 \rightarrow Y_ZS_0 + O_2$ reaction) in terms of proton release. A similar approach in Mn-depleted material (this work) has encountered only partial success. As shown in Figure 7, the electrochromic spectrum is markedly (about 2-fold) smaller in Tris-washed material (measured in the millisecond region, after proton release) than in oxygen-evolving material (measured in the μs region, before any fast phase of proton release). In line with our expectations, we observed (in PSII particles at pH 7) that when the electrochromic shift is measured at short times (100 μs), its amplitude is similar to that obtained in oxygen-evolving material. The signal then decreases to about half in the same time range as proton release (Figure 8). Nevertheless, we are confronted with two

difficulties. Firstly, we expected that at low pH (i.e. when no proton is released), this decrease would be suppressed, resulting in a "large" electrochromic change persisting in the millisecond range. At variance with this prediction, we still obtained evidence for an electrostatic relaxation in the sub-millisecond range, and, accordingly, the electrochromic spectrum in the millisecond range was of the "small" rather than "large" type. A second difficulty is the failure to observe in PSII membranes a transient decrease of the electrochromic shift in the 100 μs−1 ms region as obtained with particles at pH 7. This suggests that, in this material, the local proton equilibration is in fact already completed at 100 μs, i.e. during the lifetime of P_{680}^+. As discussed above, the results of Conjeaud and Mathis (1980, 1986) also support this view.

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