

Extent and Rate of Proton Release by Photosynthetic Water Oxidation in Thylakoids: Electrostatic Relaxation Versus Chemical Production[†]

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ABSTRACT: The detailed chemical mechanism of the four steps of photosynthetic oxidation of two molecules of water to yield molecular oxygen plus four protons is under contention. The observed release of protons is a composite of the chemical production and more indirect reactions such as electrostatically induced shifts of acid/base equilibria of peripheral amino acids. In thylakoids we studied the extent and the rate (at microsecond time resolution) of proton release and uptake by each of the four oxidation steps. The *pattern of net proton release* in thylakoids varied drastically (between 0.3 and 2 H⁺/e⁻) as a function of pH. It differed substantially from the pH-dependent patterns of PSII-enriched membrane fragments and core particles, but the stepped progression toward release of dioxygen (the Kok parameter triple) was about the same. This implied an electrostatic origin of this variation and, within the observed limits, a lack of (inhibitory) feedback of the uncompensated charge on the electron transfer from the catalytic Mn cluster to Tyr_Z⁺. The *rate of rapid proton transfer* to the amphiphilic, surface-adsorbed indicator neutral red was proportional to its concentration. The shortest half-transfer time was 12 μs, substantially shorter than the time for electron transfer from Mn to Tyr_Z⁺ at any oxidation step. Rapid deprotonation thus occurred at the level of Tyr_Z⁺. By rapid deprotonation acts the four light-driven oxidation steps S₀ ⇒ S₁ ⇒ S₂ ⇒ S₃ ⇒ S₄ created between 3.4 (at pH 7.4) and 4.5 (pH 6.3) bases per photosystem II. On the last step there was a compensating slow release of 0.6 proton (pH 7.4) and slow uptake of 0.5 proton (pH 6.3) in milliseconds, respectively. The slow event was attributed to the oxygen-evolving step, S₄ → S₀. Our results are in favor of a concerted electron–proton transfer mechanism from water to a manganese–base cluster and are also in favor of one reaction step with water (S₄ → S₀) as opposed to two two-electron reactions with a peroxide intermediate formed upon S₁ ⇒ S₂.

Photosynthetic water oxidation by photosystem II (PSII)¹ of higher plants is catalyzed by a tetranuclear manganese cluster which is located at the luminal side in the thylakoid membrane [for recent reviews, see Andersson and Styring (1991) and Debus (1992)]. The catalytic center is stepped through five increasingly oxidized states, named S₀ to S₄ with the highest one reacting spontaneously to the lowest, S₄ → S₀, under release of dioxygen. Each of the four steps from S₀ to S₄ is driven by one quantum of light. During one cycle two water molecules are oxidized to yield dioxygen plus four protons.

In dark-adapted material (with the catalytic centers predominantly in state S₁) excitation with a series of short light flashes (single turnover of PSII) produces an O₂ burst after the third flash which mainly causes the transition S₃ ⇒ S₄ → S₀. The oxygen burst is not paralleled by the outburst of four protons. Instead, proton release into the lumen is distributed over all transitions. The pattern of proton release as a function of flash number has been under contention for

many years (Junge et al., 1977; Fowler, 1977; Saphon & Crofts, 1977; Förster & Junge, 1985; Renger et al., 1987; Wille & Lavergne, 1982). Recent studies on photosystem II-enriched membrane fragments (BBY) and thylakoids have shown that it can be noninteger (Lavergne & Rappaport, 1990; Jahns et al., 1991). In BBY membranes it depends strongly on pH (Rappaport & Lavergne, 1991). On the basis of studies with modified thylakoids (Jahns & Junge, 1992a,b) and photosystem II core particles (Wacker et al., 1990; Lübbers & Junge, 1990; Lübbers et al., 1993) it has been suggested that it may also depend on the protein periphery of the catalytic center. This has strengthened the notion that proton release does not primarily reflect the reactions with bound water, but may be transiently overlaid with protolytic reactions of other groups, ligands to the catalytic center or even more remote amino acid residues which electrostatically react to the deposition of a positive charge in the center (Renger, 1988; Rappaport & Lavergne, 1991; Jahns & Junge, 1992b).

There is evidence that some protolytic reactions occur already at the level of the intermediate electron carrier Tyr_Z⁺. Proton release has been time resolved by the neutral red technique. For the third flash, which causes mainly the transition S₃ ⇒ S₄ → S₀, one portion of proton release occurs with a half-rise time of 200 μs (Förster & Junge, 1985), thus faster than electron transfer from the manganese cluster to the intermediate carrier Tyr_Z⁺ (about 1 ms; Babcock et al., 1976). A biphasic deprotonation on S₃ ⇒ S₄ → S₀ has also been indirectly inferred (Lavergne et al., 1992), on the basis of measurements of certain absorption transients in the blue, which are interpreted as local electrochromism in response to the net charge of the catalytic Mn center (Lavergne, 1987; Velthuys, 1988; Saygin & Witt, 1985).

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¹ Abbreviations: BBY, photosystem II-enriched membrane fragments; BCP, bromocresol purple; BSA, bovine serum albumin; core particles, oxygen-evolving reaction center core preparations of PSII; Chl, chlorophyll; CR, cresol red; Cyt_b₆f, cytochrome *b*₆f protein complex; D1, protein subunit of photosystem II; DCBQ, dichlorobenzoquinone; DNP-INT, dinitrophenyl ether of iodonitrothymol; fwhm, full width at half-maximum; LHC, light-harvesting complex; NR, neutral red; PSII, photosystem II; Tyr_Z, tyrosine 161 on D1.

It still remained to be shown to what extent and in which redox transition some of the four chemically produced protons are detectable. The production of protons upon electron abstraction from water does not depend on pH. The strong pH dependence of the overall pattern of proton release then should offer a clue for variation of the relative contributions of chemistry and electrostatics. Different rates of these two types of reactions may be used as kinetic labels to the respective sources.

Following previous work with neutral red (Förster & Junge, 1985), we attempted a kinetic resolution of the protolytic reactions of the water oxidase, avoiding, however, certain technical complications with this dye which have only recently been recognized (Lavergne & Rappaport, 1990). These complications are avoided when unstacked thylakoids are used (Jahns et al., 1991), as in this work.

We studied flash-induced proton release from water oxidation in unstacked thylakoids as a function of the pH and the buffer concentration. The Kok parameters (S_1 , α , β) of dark-adapted samples, the initial population of state S_1 , and the photochemical double hits and misses were determined from measurements of the oxygen yield and cross-checked against stable absorption changes in the near-UV which have been attributed to manganese oxidation (Dekker et al., 1984; Lavergne, 1987). The Kok parameters varied only slightly with pH. The stoichiometric pattern of proton release, on the other hand, was strongly pH dependent. The pH dependence (in thylakoids) differed from the one reported for BBY membranes (Rappaport & Lavergne, 1991; Renger et al., 1992) and PSII core particles (Wacker et al., 1990; Lübbers & Junge, 1990; Jahns et al., 1992; Lübbers et al., 1993).

The time course of proton release was mainly uniphasic and fast in the first three oxidation steps. It was biphasic in the fourth, $S_3 \Rightarrow S_4 \rightarrow S_0$. In any redox transition the rate of the fast component, as detected by neutral red, was proportional to the dye concentration. At high concentration the half-rise time of rapid proton transfer ($\sim 12 \mu\text{s}$) was shorter than the rise time of electron transfer from the Mn cluster to Tyr_Z in any of the four transitions. The slow component of proton release, during $S_3 \Rightarrow S_4 \rightarrow S_0$, on the other hand, was independent of the indicator concentration. Its rate seemed to be clamped to the rate of electron transfer between the manganese cluster and Tyr_Z⁺. This component indicated proton release at alkaline pH (7.4), and it turned into proton uptake at acidic pH (6.3).

This implied that the detectable protolytic reactions were predominantly a response to the oxidation of Tyr_Z. Proton production during the transition $S_4 \rightarrow S_0$ was largely compensated by proton binding through bases which had been created during the four rapid oxidation steps. Apparently the chemical production of protons during $S_4 \rightarrow S_0$ was synchronized with the resetting (by electron transfer from water to the manganese cluster) of the pKs of these bases. This might explain why only uncompensated protons were detected with the characteristic slow rate of this electron transfer.

MATERIALS AND METHODS

Unstacked thylakoids were prepared from 13-day-old pea seedlings as described elsewhere (Polle & Junge, 1989) and stored at -80°C until use. Thylakoid membranes were unstacked by mild EDTA treatment (1 mM/mM Chl) (Jahns et al., 1991). Unstacking was assayed by monitoring the apparent half-rise time of proton uptake from the suspending medium by PSII (Polle & Junge, 1986b). It was less than 5 ms in completely unstacked thylakoids.

Flash-spectrophotometric measurements were performed with the setup described in Junge (1976). The sample was

held in a cuvette with a 1- or 2-cm optical path length. The saturating exciting flash was provided by a xenon flashlamp ($\sim 10 \mu\text{s}$ fwhm, 2 mJ cm^{-2} , Schott RG 610) or a Q-switched ruby laser ($\sim 50 \text{ ns}$ fwhm, energy at 694 nm adjusted with gray filters to $\sim 2 \text{ mJ cm}^{-2}$). Absorption transients in dark-adapted material were recorded with fresh samples dispensed automatically into the cuvette from a light-shielded storage vessel. To improve the signal-to-noise ratio, up to 750 transients were averaged. Samples were normally excited by trains of four or nine flashes spaced 250 ms apart.

Proton release from water oxidation was recorded in two alternative ways: (a) in the thylakoid lumen by using the amphiphilic dye neutral red (NR) and (b) in the suspending medium by using hydrophilic dyes such as cresol red after permeabilization of the membrane for protons with nigericin. *Neutral red* is adsorbed at the membrane and monitors pH transients at the surfaces (Ausländer & Junge, 1975). From the traces obtained in the presence of the indicator, curves recorded in the absence of the dye were subtracted ($\pm \text{NR}$). With unstacked thylakoids the buffering of pH transients in the suspending medium by the nonpermeant buffer bovine serum albumin (BSA) is not only selective for the suspending medium but also fast enough to obtain a kinetically competent response to pH transients in the lumen (Jahns et al., 1991). (Time resolution of proton release has not been achieved in previous studies with BBY membranes and core particles because of stacking of the former and aggregation of the latter.)

After thawing, thylakoids were resuspended at $20 \mu\text{M}$ chlorophyll in 10 mM NaCl, 2 mM hexacyanoferrate(III), 2.6 g/L BSA, and $7 \mu\text{M}$ DNP-INT. For some measurements this medium contained additionally 10 mM MgCl_2 . DNP-INT was added in order to inhibit plastoquinone reoxidation by the Cyt_b₆f complex and to abolish proton release from this process into the thylakoid lumen (Trebst et al., 1978). Hexacyanoferrate served to preoxidize the plastoquinone pool during dark adaptation to allow at least nine turnovers of PSII. Samples were prepared under dim light and dark adapted at room temperature for 20 min before measurement. Prior to dark adaptation, the medium pH was adjusted to the values given in the figure captions. During the measuring interval it was stable within ~ 0.1 pH unit in the presence of BSA and ~ 0.3 pH unit in its absence.

Results obtained with neutral red were cross-checked by using *hydrophilic dyes* in the suspending medium. Nigericin was used to accelerate the passage of protons from the lumen into the medium. pH transients were then detected by using bromocresol purple (BCP, pH range < 7.3) and cresol red (CR, pH range > 7.3), both indicators at a concentration of $15 \mu\text{M}$. The pH transients attributable to water oxidation were obtained as follows: (i) Proton uptake at the PSII acceptor side was measured in the absence of nigericin as the difference of the results of two experiments $\pm \text{dye}$. (ii) The sum of proton uptake and proton release was obtained in the presence of $4 \mu\text{M}$ nigericin ($\pm \text{dye}$). The difference of the transients, ii - i ($\pm \text{nigericin}$), represented proton release from water oxidation. In these experiments the flash distance was chosen longer than in the measurements with neutral red, namely, 500 ms, for completion of the accelerated equilibration of the transmembrane pH difference.

Oxygen evolution as a function of flash number was measured with a modification of a standard commercial Clark-type electrode. The surface of the platinum electrode was enlarged to increase the sensitivity. To prevent interaction of the redox cofactors (e.g., hexacyanoferrate) with the electrode, the platinum surface was covered with a thin Teflon membrane

(thickness, 12 μm). The advantage of the Teflon cover was that the same concentrations of redox additives could be used in the polarographic and spectrophotometric experiments. Only the chlorophyll concentration was 10-fold larger in the former. The concentration of membrane adsorbed chemicals (neutral red, DNP-INT) was scaled up accordingly.

For oxygen measurements 5 mL of stock containing thylakoids equivalent to 200 μM chlorophyll, 10 mM NaCl, 2 mM hexacyanoferrate, 70 μM DNP-INT, 2.3 g/L BSA, and 150 μM neutral red was prepared; the pH was adjusted to the desired value, and 200 μL of the sample was put on the electrode for a dark-adaptation time of 30 min (optical path length of the sample, ~ 1.5 mm). Saturating flashes spaced 2 s apart were provided by the xenon flashlamp through a light guide and a long-pass RG 610 filter. A flash interval longer than that used in the spectrophotometric measurements was chosen to account for the limited velocity of O_2 diffusion through the Teflon membrane. The electrode was interfaced to the amplifier and signal averager (Tracor Northern transient recorder; DC coupling; electrical bandwidth, 10 Hz). Patterns of oxygen yield as a function of flash number were analyzed by using a least squares fit algorithm of our own design. They were interpreted in terms of the three Kok parameters (Kok et al., 1970), namely, the dark distribution of centers over the states S_0 and S_1 and the probabilities of misses (α) and double hits (β). The latter two were assumed not to vary from transition to transition [but see Meunier and Popovic (1990) and Shinkarev and Wraight (1993)].

Absorption transients in the near-UV were recorded flash-spectrophotometrically at a wavelength of 320 nm. The binary oscillations due to semiquinone anion formation at the PSII acceptor side on odd flashes were eliminated by using DCBQ (200 μM) instead of hexacyanoferrate as electron acceptor (Lavergne, 1991). The nonoscillating absorption transients due to the reduction of DCBQ were determined in a parallel experiment under repetitive excitation (ten groups of nine flashes per fresh sample) and then subtracted from the traces that were obtained with dark-adapted material. The resulting step-shaped traces showed quaternary oscillations similar to those described for BBY membranes (Lavergne, 1991; Renger & Hanssum, 1992).

The patterns of proton release as a function of flash number were analyzed under the assumption that the first four flashes of a series given to dark-adapted thylakoids released four protons. They were deconvoluted by using the Kok parameters (as determined from the oxygen pattern) to yield the amount of protons liberated on every S transition. The same routine was applied for the simulations of the UV pattern.

RESULTS

The Kok Parameters Resulting from Oxygen Yield and UV-transients as a Function of pH. Figure 1 shows original traces (left panels) of oxygen evolution (A) and absorption transients at 320 nm (B) following flash excitation of dark-adapted, unstacked thylakoids at pH 6. Both traces exhibited the standard oscillatory behavior with a period of 4 and damping. Similar measurements were performed from pH 5.5 to 8.5. The relative amount of oxygen produced on every flash was determined from the original traces as indicated and plotted as a function of flash number in the right panel of Figure 1A (solid circles). It was interpreted in standard terms by using an approach with three parameters to yield the initial distribution of the catalytic centers over states S_0 and S_1 (with $S_0 = 1 - S_1$) and the proportion of misses (α) and double hits (β). The calculated oxygen yield per flash is shown as a line in Figure 1A. The results of this analysis are given

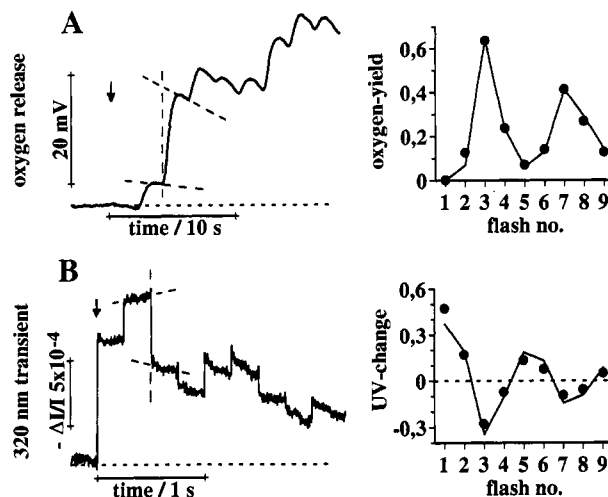


FIGURE 1: Oxygen evolution (A) and absorption transients at 320 nm (B) induced by a train of nine flashes in dark-adapted unstacked thylakoids at pH 6. (A) Left: Oxygen evolution measured with a Teflon-covered Clark-type electrode in the presence of 130 μM neutral red (for other additions, see Materials and Methods); flash spacing, 2 s; electrical bandwidth, 10 Hz; no averaging. The first flash is marked by an arrow. The signal extent by each flash was determined as indicated for the third flash. Right: Normalized extents of oxygen evolution (closed circles) taken from the original transient in the left panel (the sum of the first four flashes = 1) as a function of flash number. The line resulted from a fit with the following Kok parameters (%): misses (α) = 10.3, double hits (β) = 4.9, $S_1 = 94.9$ ($S_2 = S_3 = 0$). (B) Left: Absorption transients at 320 nm. The trace represents the difference of transient signals from dark- and light-adapted material: xenon flash spacing, 0.25 s; electrical bandwidth, 100 Hz; 30 traces averaged; standard medium, except 200 μM DCBQ used instead of hexacyanoferrate. The extents of the jumps were determined as indicated for the third flash. Right: Normalized extent as a function of flash number (closed circles; the sum of the first four flashes = 0). The line was calculated with the above set of Kok parameters plus the relative extinction coefficients of the four S transitions given by Lavergne (1991).

Table 1: Kok Parameter Triple and Total Extent of Oxygen Evolution in Dark-Adapted Unstacked Thylakoids as a Function of pH^a

pH	misses (α)	double hits (β)	S_1 (%)	extent
5.5	13.1	4.1	100.0	0.95
6.0	10.3	4.9	94.9	1.00
6.5	11.1	5.4	92.8	0.94
7.0	12.8	4.6	87.0	0.98
7.5	13.0	5.8	88.8	0.85
8.0	12.6	5.3	80.0	0.74
8.5	12.0	5.5	75.1	0.50

^a The total extent of oxygen yield was determined from traces like those in Figure 1A averaged over the first eight flashes. The total extent was normalized to 1 at pH 6.

in Table 1. Only minor variations of misses, α , and double hits, β , as a function of pH and a slight and monotonous decrease of the dark proportion of S_1 from 100% at pH 5.5 to 75% at pH 8.5 were observed. The total extent of the polarographic signal was close to 100% at pH 5.5 and decreased to 50% at pH 8.5 (Table 1), similar to data reported in the literature (Renger et al., 1977). This reflected the irreversible deactivation of centers at alkaline pH, which was also obvious from measurements of oxygen evolution under continuous light (not shown). The same pH dependence of the fit parameters S_1 , α , and β as documented in Table 1 was observed in samples without added neutral red. This excluded a direct interference of this indicator with the redox reactions.

At pH 6 the absorption jumps at 320 nm (Figure 1B) were also well described by the Kok parameters which resulted

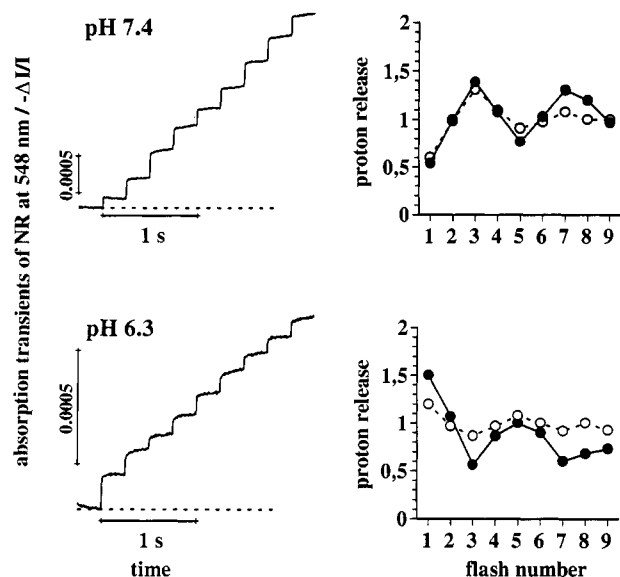


FIGURE 2: Pattern of proton release from water oxidation in dark-adapted unstacked thylakoids. Left: Original transients obtained with neutral red (\pm NR, 13 μ M; wavelength, 548 nm); upper trace at pH 7.4, lower trace at pH 6.3; xenon flash spacing, 0.25 s; electrical bandwidth, 100 Hz; 100 signals averaged. Note the different ordinate scales at the two pH values. Right: Extent of proton release as a function of flash number. Solid circles: Extents resulting from the left-hand traces. The average extent over the first four flashes was set equal to one. Open circles: Extents resulting from pH transients in the suspending medium as measured with hydrophilic dyes (\pm nigericin; see Materials and Methods) determined at 575 nm, with cresol red at pH 7.4 and bromocresol purple at pH 6.3.

from oxygen yield, namely, $\alpha = 10.3\%$, $\beta = 4.9\%$, and $S_1 = 94.9\%$. We calculated the expected UV transients by using the relative extinction coefficients of the successive S transitions as determined by Lavergne for PSII membrane fragments (Lavergne, 1991), namely, $-0.016:0.324:0.176:-0.484$ (when starting from S_0 and normalizing the sum to 1). The UV pattern changed only slightly as a function of pH. This was fully understood in terms of changes of the S-state distribution in the dark. Additionally, it proved that the centers were well synchronized. Furthermore, we showed that the stable UV changes in thylakoids were comparable with the ones occurring in PSII membrane fragments (Lavergne, 1991) and reaction center core particles (van Leeuwen et al., 1992).

The results of the oxygen and UV measurements can be summarized as follows: In thylakoids the S-state distribution is only slightly dependent on pH, as is the number of misses and double hits determined via oxygen release. Especially at acidic pH, where the most drastic changes of the pattern of proton release were observed (see following section), the S-state distribution in the dark was almost ideal, with 100% population of S_1 .

Extent of Proton Release as a Function of Flash Number, pH, and Mg^{2+} . Figure 2 shows original absorption transients of neutral red (left panels) which indicate proton release by water oxidation into the thylakoid lumen. The extents of the acidification steps, determined about 200 ms after each flash, were plotted as a function of flash number (right panels, solid circles). The average extent over the first four flashes was arbitrarily set as one proton per active photosystem II per flash. Different pH values produced different patterns of proton release as a function of flash number. The yield ratio of the first over the third flash was 1.5:0.5 at pH 6.3 and 0.5:1.5 at pH 7.4. The yields of the second and the fourth flash, on the other hand, remained constant, close to 1. A superimposed decrease of the flash-induced jumps at pH 6.3 and a slight increase at pH 7.4 were apparent upon proceeding

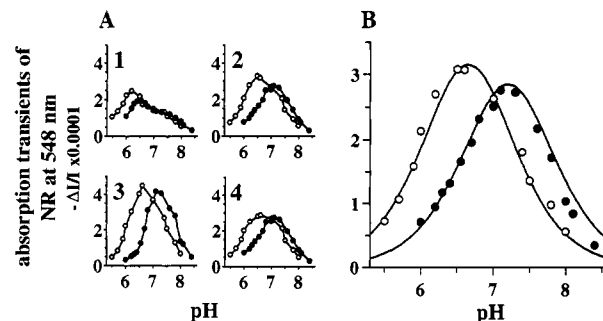


FIGURE 3: Absorption transients of neutral red induced by the first four flashes in dark-adapted unstacked thylakoids as a function of pH. (A) Extent of the first four flashes derived from traces similar to those in the left-hand panels of Figure 2 in the absence (solid circles) and presence (open circles) of 10 mM $MgCl_2$. (B) Sum of extents over the first four flashes, normalized at an average release of 1 proton per flash. The lines represent best fits of the data by the curve of the differential sensitivity of an indicator dye in response to very small pH transients (Junge et al., 1979). The assumed pK was 7.20 (solid circles) and 6.65 (open circles).

further to flash number 9. This was understood to result from the increasing acidification of the thylakoid lumen during the series of flashes. When the stepwise acidification was started at pH 6.3, it caused an upward run on the differential response curve of neutral red (apparent pK of 7.2) and accordingly a downward run at pH 7.4. This effect has been previously used to calibrate the flash-induced pH jumps (about 0.03 pH unit per single turnover of PSII; Junge et al., 1979). The patterns of proton release did not markedly change when only 100 μ M instead of 2 mM hexacyanoferrate was added to the samples [not shown; but see Lübbers et al. (1993)].

The right panel in Figure 2 also shows the patterns of proton release recorded by using two hydrophilic indicator dyes (open circles), bromocresol purple (pH 6.3) and cresol red (pH 7.4). They were obtained by difference measurements \pm nigericin. This protonophore was added to accelerate the equilibration of the luminal acidity with that of the suspending medium (see Materials and Methods). Qualitatively these patterns reproduced the inversion of the yield ratios for the first and the third flash resulting from the experiments with neutral red. The deviations from the latter are attributed to the less stable pH and the diminished signal-to-noise ratio due to the procedure of taking differences (\pm nigericin) of differences (\pm indicator) of small signals.

The pattern of absorption transients of neutral red as a function of flash number was measured at a medium pH ranging from pH 6.0 to 8.4 with or without $MgCl_2$ added. Addition of Mg^{2+} diminishes the surface potential, and this was used to vary the relation between the medium pH and the surface pH at the luminal side of the membrane. Addition of $MgCl_2$ also caused partial restacking of the membranes, which did not matter in these experiments, because all signal extents were determined about 200 ms after each flash (e.g., after complete buffering of pH transients in the partitions). The respective extents of the first four flashes are plotted in Figure 3A (solid circles, without $MgCl_2$; open circles, with $MgCl_2$). These extents depend, of course, on the sensitivity of the indicator, which is a function of the luminal surface pH. This pH, on the other hand, is modified by the surface potential caused by negatively charged lipid heads and amino acid side chains (Barber, 1980). Addition of salt diminishes the surface potential, thereby increasing the surface pH in relation to the pH in the suspending medium. The sum of the absorption changes over the first four flashes is plotted in Figure 3B (circles). The bell-shaped curves fitted to the data points represent the calculated differential sensitivity of a

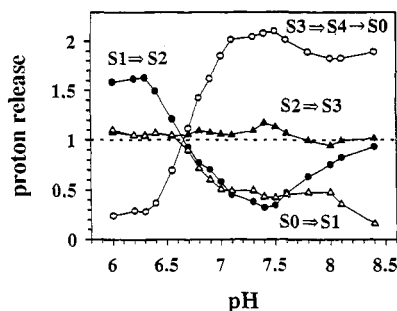


FIGURE 4: Extent of proton release from water oxidation in dark-adapted unstacked thylakoids as a function of pH: result of a deconvolution of data as in Figure 3 (no MgCl_2) with Kok parameters resulting from experiments as in Figure 1 (Table 1) to yield the contributions of the four transitions $S_0 \Rightarrow S_1$ (open triangles), $S_1 \Rightarrow S_2$ (solid circles), $S_2 \Rightarrow S_3$ (solid triangles), and $S_3 \Rightarrow S_4 \Rightarrow S_0$ (open circles).

univalent pH indicator according to $\Delta A \sim 10^{-(\text{pH} + \text{pK})} (10^{-\text{pH}} + 10^{-\text{pK}})^{-2} \Delta [\text{H}^+]_t$, wherein pH denotes the medium pH; pK, the apparent pK of the indicator at the given surface potential; and $\Delta [\text{H}^+]_t$, the total concentration jump of protons in the thylakoid lumen induced by one flash (including all protons, bound and free). The good fit was obtained if the apparent pK was assumed to be 7.2 in the absence of MgCl_2 and 6.65 in its presence. This is in reasonable agreement with the published apparent pKs of adsorbed neutral red under these two salt conditions (Hong & Junge, 1983). The same deviation of approximately 0.6 pH units of the courses of signal extents over the pH in the presence or absence of MgCl_2 was observed in all four flashes (Figure 3A). Except for the above pH shift, the pH-dependent variations of the extents of proton release between the first and the third flash were not affected by the salt conditions. This meant that the proton-delivering groups were probably exposed to about the same pH value at the membrane-bulk interface as was neutral red.

Extent of Proton Release as a Function of S Transition.

The raw data resulting from experiments with neutral red documented in Figure 3A were normalized and deconvoluted with the Kok parameter triple (Table 1; missing pH values were interpolated) to yield the extent of proton release per active reaction center as a function of the four transitions, $S_i \Rightarrow S_{i+1}$. The results are depicted in Figure 4. The proton pattern now appears much sharper at both ends of the pH scale. The extent of proton release on transition $S_0 \Rightarrow S_1$ (open triangles) declined from 1.1 to 0.2 when going from pH 6 to 8.4. The release on $S_1 \Rightarrow S_2$ was minimal at pH 7.4 and increased at both ends of the pH scale. The increase at acidic pH was accompanied by a sharp decrease of proton release on transition $S_3 \Rightarrow S_4 \Rightarrow S_0$. We give three examples of patterns (starting from $S_0 \Rightarrow S_1$): 1.1:1.6:1.1:0.2 at pH 6, 0.5:0.4:1.1:2 at pH 7.4, and 0.2:0.9:1.1:1.9 at pH 8.4. The only transition whose proton release was independent of pH was $S_2 \Rightarrow S_3$. It was the only transition where the pH dependence (namely none!) was the same for thylakoids (this work), BBY membranes (Rappaport & Lavergne, 1991), and core particles (Lübbbers & Junge, 1990; Wacker et al., 1990; Jahns et al., 1992). For the other redox transitions the respective pH dependences differed between these systems.

Time Resolution of Proton Release. It has been demonstrated that neutral red is a kinetically competent indicator of pH transients in the lumen of *unstacked* thylakoids (at low salt; Jahns et al., 1991). This material was used for the following studies.

We measured proton release under *repetitive flash excitation* (0.1 Hz) using either a xenon flashlamp or a Q-switched ruby laser as light source. Figure 5A shows the response of

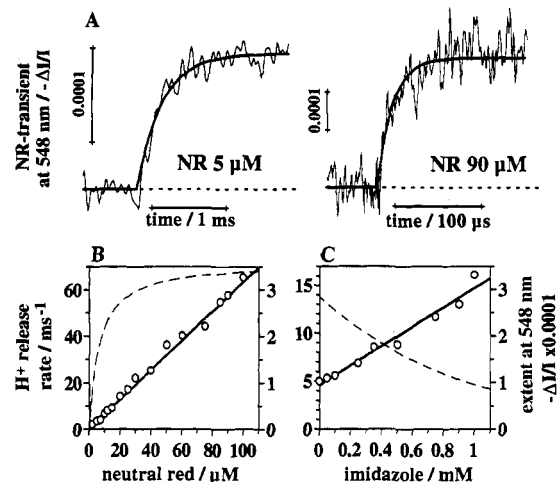


FIGURE 5: Time-resolved proton release under repetitive flash excitation. (A) Time-resolved transients of neutral red ($\pm \text{NR}$), pH 7.2. Left: 5 μM NR; time resolution, 17 μs per address; electrical bandwidth, 30 KHz; 150 signals averaged; optical path length, 2 cm; xenon flashes at 0.1 Hz. The smooth curve represents the single-exponential best fit with $t_{1/2} = 235 \mu\text{s}$. Right: 90 μM NR; time resolution, 800 ns per address; electrical bandwidth, 1 MHz; 750 signals averaged; optical path length, 1 cm, ruby laser flashes at 0.1 Hz; fitted half-rise time, 12 μs (smooth curve). Note the 10-fold-expanded time scale in the left-hand trace. (B) Rate of proton release as a function of the concentration of neutral red in the medium (left-hand ordinate scale) at pH 7.2. The straight line represents a linear regression with a slope of $0.65 \mu\text{M}^{-1}\text{ms}^{-1}$. The broken line shows the signal extent as a function of the concentration of neutral red (right-hand ordinate scale). (C) Rate of proton release as a function of the concentration of imidazole at constant neutral red (10 μM), pH 7.2. Left-hand scale, rate; right-hand scale, signal extent (broken line).

neutral red at low (left) and high (right) dye concentration at pH 7.2. The acceleration caused by the higher concentration is apparent (note the 10-fold-expanded time scale at 90 μM neutral red). A series of measurements was carried out with variation of the concentration of neutral red, and the data were approximated by a single exponential, illustrated by the lines in Figure 5A. The resulting rate constants are plotted as a function of the neutral red concentration in Figure 5B (open circles). The rate was proportional to the neutral red concentration above 10 μM . At 100 μM neutral red a rate of $62\,000 \text{ s}^{-1}$ was observed, corresponding to a signal half-rise time of 12 μs . No saturation of the rates occurred up to this concentration. The use of higher concentrations was not practical because the optical density of the sample was too high. Below 10 μM the rate deviated from the linear behavior. When the measured rates were extrapolated to zero neutral red, an intrinsic rate of approximately 2000 s^{-1} (half-rise time of 350 μs) was determined. These results pointed to a bimolecular collision between the fixed proton donors and the mobile neutral red.

We asked whether the response of neutral red to an acidification of the lumen was also accelerated by addition of another mobile and amphiphilic buffer. Proton release was recorded at a constant concentration of neutral red (10 μM) but now with increasing amounts of the membrane-permeable, amphiphilic buffer imidazole ($\text{pK} \sim 7$). The rate constants as a function of imidazole concentration are depicted in Figure 5C (open circles). Starting at $\sim 5000 \text{ s}^{-1}$, the addition of 1 mM imidazole led to a rate constant of $16\,000 \text{ s}^{-1}$. The rate constants were approximately proportional to the imidazole concentration with no saturation occurring up to 1 mM. The use of higher concentrations was again impractical because the added buffer quenched the extent of the neutral red signal in the lumen (Figure 5C, broken line, left ordinate scale). A similar acceleration of proton detection by added buffers has

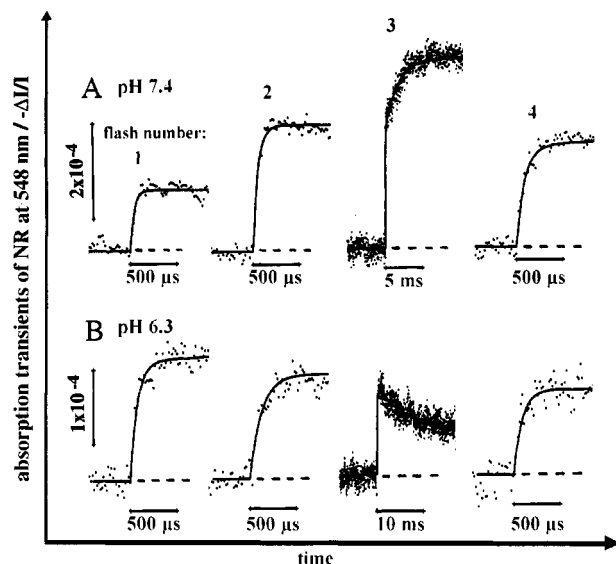


FIGURE 6: Time-resolved proton release after the first four flashes in dark-adapted unstacked thylakoids: time resolution, 17 μ s per address; electrical bandwidth, 30 KHz; optical path length, 2 cm; \pm NR, 45 μ M; 200 transients averaged per ruby laser flash. (A) pH 7.4, flashes 1–4. (B) pH 6.3, flashes 1–4. Note the different time scale for flash no. 3 at both pH values. Solid lines represent best fits of the data with the half-rise times and extents given in Table 2.

Table 2: Half-Rise Time and Relative Extent (in Parentheses) of Proton Release as a Function of Flash Number^a

	pH	flash no.			
		1	2	3	4
H ⁺	7.4	30 (0.5)	40 (1.0)	40 (0.9) 1200 (0.6)	50 (0.8) 1500 (0.2)
H ⁺	6.3	50 (1.2) 7000 (0.3)	80 (1.1)	70 (1.0) 5000 (–0.5)	60 (0.9)
e [–]	6.0	110 ^b	350 ^b	1300 ^b	30 ^b
	6.5	60 ^c	310 ^c	1200 ^c	330 ^c

^a Conditions: 45 μ M neutral red, dark-adapted unstacked thylakoids, pH 7.4 and 6.3. Half-rise times (μ s) resulted from fits by two exponentials. Extents were normalized to the average over the first four flashes. ^{b,c} The third row gives the half times of electron transfer (μ s) from the manganese center to TyrZ⁺ for PSII membrane fragments as determined by Dekker et al. (1984) (^b) and Lavergne et al. (1992) (^c) for comparison.

been previously described for proton release in the bacteriorhodopsin photocycle (Heberle & Dencher, 1992; Dencher et al., 1991).

The rate of proton release under repetitive flashes was measured over a temperature range from 1 to 27 °C (13 μ M neutral red, pH 7.2). The overall rates resulting from a fit by a single exponential led to an Arrhenius activation energy of 35 kJ mol^{–1} (data not shown), which is larger than is characteristic for diffusion-controlled reactions.

Figure 6 shows proton release in *dark-adapted unstacked thylakoids* at a high concentration of neutral red (45 μ M) at two pH values (7.4 and 6.3). Two hundred traces were averaged per flash; each trace was measured with a separate sample. For records of the second to the fourth flash, 1–3 conditioning xenon flashes were fired and the measuring light was only applied to monitor the response to the final laser flash under investigation. The transients were analyzed in the same way as the ones recorded under repetitive excitation by fitting them by one or two exponentials (lines in Figure 6).

At pH 7.4 (Figure 6A, upper traces) there was a monophasic rise on the first and second flashes. The extents and half-rise times were 0.5 proton at 30 μ s and 1 proton at 40 μ s (see Table 2). On the third and fourth flashes the rise was biphasic with fast phases of 0.9 and 0.8 proton at half-rise times of 40 and

50 μ s. On the third flash a slow phase of 0.6 proton at 1.2 ms was apparent. The slow phase on flash number 4 was smaller, 0.2 proton at 1.5 ms. At pH 7.4 and the given neutral red concentration 3.2 protons were released with half times shorter than electron transfer (Table 2) from the manganese cluster to TyrZ⁺ (1st, 2nd, and 3rd flash) and in the range of this transfer time (4th flash), respectively. Only 0.8 proton was released slowly, mainly on the third flash.

In summary, the data recorded at pH 7.4 (Figure 6A) showed that the series of four flashes rapidly created 3.2 base equivalents in the vicinity of manganese-TyrZ⁺. A millisecond reaction step was mainly observed on transition S₄ → S₀ which finally liberated dioxygen. Out of the four protons which were supposedly produced during this transition, only 0.8 was discernable by its slow appearance. Its rate was independent of the concentration of the indicator dye (see below). The larger portion, 3.2 protons, was obviously trapped by previously created bases, and thereby escaped detection.

At pH 6.3 (Figure 6B, lower traces) the four flashes induced the fast release of 1.2, 1.1, 1, and 0.9 proton at half-rise times of 50, 80, 70, and 60 μ s, respectively (Table 2). In addition, the first flash exhibited a slow portion of 0.3 proton with a half-rise time of 7 ms. Thus proton release totaled 1.5 on this flash. The slow phase was probably caused by interference of a small transient proton uptake at the PSII acceptor side due to partial restacking of the membranes at acidic pH. At pH 6.3 the signal observed on the third flash differed completely from the one measured at pH 7.4. Instead of the slow release, a slow uptake of 0.5 proton with a half-rise time of 5 ms was observed. Therefore the net release was only 0.5 proton (fast release of 1 minus slow uptake of 0.5). In Figure 2 the uptake was only apparently absent because the time resolution was too low. With the previous notion that the sum over the first four flashes (taken 100 ms after each flash) equaled four protons, it turned out that at 1 ms after the third flash 4.5 protons were released up to state S₄ (including the fast release on the third flash). Whereas four bases had swallowed the chemically produced four protons during the slow oxygen-evolving transition, the excess of 0.5 base equivalent was reprotonated in a reaction with buffers in the lumen, including neutral red. The time course of this reprotonation, however, followed the time course of the steering redox reaction.

At pH 7.4 all flashes induced the release of approximately 1 proton with half times faster than electron transfer, except for the first flash. We asked whether the *net release* of only 0.5 proton in S₁ → S₂ (first flash, pH 7.4) was the result of rapid release of 1 proton, which was followed by the uptake of 0.5 proton with the rate of electron transfer from Mn to TyrZ⁺. With a half-rise time of only 100 μ s for the latter, the uptake might have escaped detection. This was addressed by measuring proton release on the first flash at pH 7.4 in the presence of 75 μ M neutral red plus 1 mM imidazole. Thirty micromolar DCMU was added to block the Q_B site and thereby proton uptake at the PSII acceptor side (Polle & Junge, 1986a), which might transiently interfere with proton release. The transient signal (data points and unexponential fit depicted in Figure 7) revealed a half-rise time of 13 μ s and an extent equivalent to 0.5 proton. For illustrative purposes we plotted simulated curves. They were composed of two exponentials, representing proton release (1 proton, 13 μ s) and proton uptake (0.5 proton, 100, 50, or 25 μ s). It was obvious that there was no indication for proton uptake upon S₁ → S₂ (first flash, pH 7.4).

We studied the dependence of the rates of the rapid (all transitions) and the slow components (S₃ → S₄ → S₀) of

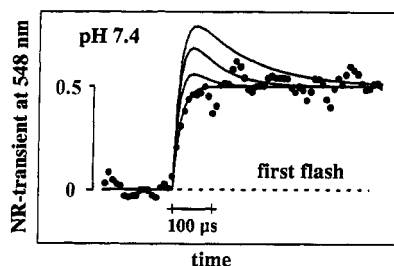


FIGURE 7: Time-resolved proton release on the first flash at pH 7.4: \pm NR, 75 μ M; imidazole, 1 mM; DCMU, 30 μ M; electrical bandwidth, 100 kHz; 10 μ s per address; 300 transients averaged; xenon flash. The upper three curves were calculated as differences between two exponentials, a rising one with a half time of 13 μ s and a relative extent of 1 and a decaying one with a half time of 100, 50, or 25 μ s (top to bottom) and an extent of 0.5. The lowest curve was calculated for one rising exponential alone with a half time of 13 μ s and an extent of 0.5.

protolytic reactions on the concentrations of neutral red and imidazole. Whereas the rates of rapid phases were proportional to the concentration, the slow components were not affected. Proton release was recorded at a neutral red concentration of 45 μ M plus 5 mM additional imidazole. The half-rise times of the fast releases on the first four flashes at pH 7.4 were approximately 2 times smaller than the values given in Table 2. The average figure was about 20 μ s (not documented). This corroborated the notion that all rapid phases represented an electrostatic response of amino acids to the abstraction of an electron from the manganese-TyrZ⁺ entity, whereas the slow phases were steered by the chemical reaction leading to the liberation of dioxygen.

DISCUSSION

There are at least three sources of proton release from the catalytic center of water oxidation (Lavergne & Junge, 1994): (1) chemical production of protons upon oxidation of water (or its intermediates), (2) deprotonation of ligands to the manganese cluster, and (3) deprotonation of more remote amino acid residues as an electrostatic response to the deposition of a positive charge in the center. The distribution of proton release over the four sequential oxidation steps of the water oxidase as a function of the medium pH differed between thylakoids (this article), PSII membrane fragments (Rappaport & Lavergne, 1991; Renger et al., 1992), and core particles (Lübbers & Junge, 1990; Wacker et al., 1990; Jahns et al., 1992). The patterns of oxygen release and of UV-absorption transients, on the other hand, were very much alike. The minimum structure which is capable of oxygen evolution, core particles, revealed an approximate 1:1:1:1 stoichiometry of proton release (Wacker et al., 1990; Lübbers & Junge, 1990; Jahns et al., 1992; Lübbers et al., 1994). There are two implications of the pH-dependent variation of the stoichiometric pattern in thylakoids: (1) *At least the pH-dependent excess/undershoot over 1 proton/electron is electrostatic in nature.* (2) *There is no evidence for any feedback of this variation on the normal four-step progress of water oxidation.*

The electrostatic response is expected to be sensitive to the properties of the protein periphery, namely, (1) the number and nature of polypeptides which surround the catalytic center, (2) the exposure of acid/base-residues to water, and (3) the dielectrically weighted distances between these residues and the locus of the respective positive charge and toward each other. There are only faint hints for a discrimination between these factors. The change of the stoichiometric pattern between normal thylakoids and thylakoids grown under intermittent light (Jahns & Junge, 1993; Jahns & Junge,

1992b), that lack most LHCII proteins, suggests a modifying role of antennae proteins.

Does the pronounced pH dependence of the pattern of proton release in thylakoids provide a clue to specific amino acids, namely, Asp-170, Glu-189, and His-190 on D1, which according to current structural models (Svensson et al., 1990; Ruffe et al., 1992; Nixon & Diner, 1992; Barry, 1993) form part of the catalytic center? Probably not, since there is hardly any detectable pH dependence of proton release in reaction center core particles, whose structure most closely resembles that of the center proper. Even if there is a rich structure of the pH dependence as in thylakoids and PSII membranes, ready attributions to single amino acids are precluded because of the following. We assume that the pH-dependent portion of the deprotonation is mainly based on electrostatics, and that the electric field is felt by several amino acids around any center. If one or several particular acids undergo deprotonation, the other groups are exposed not only to the field due to the positive charge (on TyrZ⁺ or the manganese cluster) but also to the one due to the negative charges (or the lack of positive charges) on the newly created bases. Thereby the center plus its environment of acid/base groups form an entity with collective pK values. Its deprotonation behavior is to be described by the Boltzmann-weighted sum over all protonation states of the ensemble. A rigorous simulation with the aim to yield the unperturbed pK values of individual groups can only be based on a structure at atomic resolution. Such an analysis has been exemplified for the related problem of proton uptake at the reducing end of bacterial reaction centers (Takahashi & Wraight, 1992; Beroza et al., 1991).

The origin of proton release, namely, chemical production due to stepwise oxidation of water, ligand deprotonation, or electrostatics, has remained open. At this point, it is worthwhile to invoke data on the *kinetics of proton release*. Let us first recall some basic properties of proton transfer from a donor to an acceptor molecule *in an aqueous environment* [see reviews by Eigen (1963) and Gutman and Nachliel (1990)]. There are three pathways for such proton transfer: spontaneous deprotonation (protolysis), direct collision between donor and acceptor (collision), and collision with water to yield OH⁻ (hydrolysis). If one assumes a diffusion-controlled on-rate of protonation (a conservative figure is $k_{on} = 10^{10} \text{ M}^{-1} \text{ s}^{-1}$), the rate of spontaneous deprotonation of a group that undergoes a pK shift, say, by 3 units from pK = 10 down to 7, is $k_{off} = Kk_{on} = 10^3 \text{ s}^{-1}$. The relaxation time is 1 ms. For a group whose pK is 5, the relaxation time is 100 times shorter, 10 μ s. Protons which are injected into a space containing 1 mM base have a lifetime as "free" protons of $[k_{on}(1 \text{ mM})]^{-1} = 100 \text{ ns}$. The rate constant for collisional transfer between a more acid donor to a more alkaline acceptor, on the other hand, is at least 1 if not 2 orders of magnitude less than k_{on} , $k_{col} = 10^8 \text{ M}^{-1} \text{ s}^{-1}$. If the base is mobile and present at 1 mM, the relaxation time is $[(k_{col}(1 \text{ mM}))^{-1}] = 10 \mu\text{s}$.

We apply these considerations to the situation in the thylakoid lumen. Around neutral pH the intrinsic buffering capacity is equivalent to about 0.05 mol/mol of chlorophyll (Junge et al., 1979; Junge, 1982). At a specific internal volume in unstacked thylakoids (hypoosmolar solution) of 50 L/mol of chlorophyll the buffer concentration is about 1 mM. Neutral red is adsorbed at the membrane/water interface, which means that its effective concentration in relation to the "aqueous" volume of the lumen is more than 100-fold higher than in the medium (Hong & Junge, 1983). The buffering capacity of the lumen increases as a function of the concentration of added neutral red, starting from about 10 μ M in the medium.

Accordingly the lifetime of a *free proton* is expected to be 100 ns in the absence of this dye and far shorter in its presence. It is obvious that both the time range (10–350 μ s) and the dependence of the observed rate of proton transfer on the concentration of neutral red suggest a collisional mechanism of transfer. Still there seem to exist two alternatives to explain the observed behavior: (1) spontaneous protolysis (10 μ s) of the primary donor group and buffering by other water-exposed buffers (100 ns) which only more slowly react by collision with neutral red (say at 100 μ s) and (2) a pK shift of groups which are not in direct contact with water, which is why their spontaneous deprotonation into water is greatly delayed, but which can react by collision with neutral red and imidazole. The first possibility is unlikely. If there were groups undergoing rapid (10 μ s) spontaneous deprotonation, the direct, diffusion-controlled protonation should involve all groups in contact with water, intrinsic buffers, and neutral red, whereas the distribution of protons should depend on the relative abundance of each species. The relaxation time is expected to depend on the concentration of all bases [$\tau = (k_{on}\Sigma[B^-])^{-1}$] and thus to fall into the range of 100 ns or even less. It appeared, though, as if the electrostatic relaxation consecutive to or concomitant with the oxidation of the catalytic center involved groups which were *not in direct contact with water*. This, in turn, implied that the *full relaxation* by proton transfer to the intrinsic buffering groups took at least 350 μ s (in the absence of neutral red).

At high concentrations the major portion of *proton transfer to neutral red was faster than electron transfer from the manganese cluster to TyrZ⁺* (see Table 2). This holds true for any transition except, perhaps, $S_0 \Rightarrow S_1$ where the electron-transfer time is controversial between two groups (Dekker et al., 1984; Lavergne et al., 1992). We ignore here that the relaxation times of fast proton transfer seemed to differ slightly between the four redox transitions (Table 2), whether this was meaningful or not. Anyway, it followed that the primary response came from groups in the vicinity of TyrZ⁺. It is not yet clear whether electron transfer from the manganese cluster to TyrZ⁺ caused a compensating push-and-pull of protons, as previously proposed (Förster & Junge, 1985). The alkaline-directed resetting of the acid-shifted pK of the respective groups in the vicinity of TyrZ⁺ caused proton uptake at the same rate as the steering reaction, i.e., electron transfer from Mn to TyrZ⁺. If this was synchronous with proton release by other groups, neighbors to the now further oxidized manganese cluster proper, the mutually compensating events remained undetected. The alternative, indistinguishable at present, was that the primary acids were sensitive to the positive charge residing in the Mn–TyrZ⁺ system, irrespective of the specific charge location. This would be expected if the Mn–TyrZ⁺ core was embedded in a highly polarizable pocket within a more hydrophobic environment.

Was the fast phase of proton release, namely, the one attributable to TyrZ⁺, independent of pH and of the respective oxidation step? Clearly not. The most drastic deviation was observed for $S_1 \Rightarrow S_2$ at pH 7.4 where the extent of the rapid phase was only 0.5 without indication for rapid and experimentally unresolved compensating proton uptake (see Results and Figure 7). Considering the unproven possibility that rapid proton release was directly caused by the phenolic moiety of Tyr-161 (D1), our data showed at least that its pK was modified by the oxidation state of the water oxidase.

Mainly on transition $S_3 \Rightarrow S_4 \Rightarrow S_0$ there was an additional *slow phase* whose rise time coincided with the one of oxygen evolution during the dark reaction $S_4 \Rightarrow S_0$. At pH 7.4 there was an additional release of 0.6 proton and at pH 6.3 the

reuptake of 0.5 proton. The extent and the direction of this event compensated the pH-dependent variation of the total base production during the four light reactions, $S_0 \Rightarrow S_1 \Rightarrow S_2 \Rightarrow S_3 \Rightarrow S_4$. If the production up to $S_3 \Rightarrow S_4$ was less than 4, e.g., 3.4 at pH 7.4, there was a compensating slow release of 0.6 proton on $S_4 \Rightarrow S_0$. If the production was greater than 4 (a conservative estimate, without deconvolution, was 4.5 at pH 6.3), there was a slow uptake of 0.5 proton on the third flash. We attributed these slow phases to the net result of two counteracting events, namely, proton uptake due to a pK reset toward alkalinity of the bases which were produced during the four light-driven reactions and chemical production of four protons during the dark reaction, $S_4 \Rightarrow S_0$ (but see below). Both these events were caused by and probably synchronized with electron transfer from water to the oxidized manganese cluster.

It has been argued that thermodynamic and kinetic constraints of water oxidation can be relieved by (1) pooling electron-transfer steps from (bound) water to the manganese cluster into one four-electron or at least two-electron steps and (2) *coupling electron with proton transfer* to neighboring bases to overcome the electrostatic repulsion between electrons (Krishtalik, 1986, 1990). Broadly speaking, the latter is equivalent to hydrogen transfer to oxidant–base couples. A hypothetical 2×2 scheme, with peroxide formed as intermediate after transition $S_1 \Rightarrow S_2$, implied the chemical production of protons in this transition.

The pooling of four electron-transfer steps during $S_4 \Rightarrow S_0$ seems more plausible for two lines of circumstantial evidence: (a) Radmer and Ollinger (1986) found that $H_2^{18}O$, when added after state S_3 , still gave rise to the release of $^{18}O_2$ during $S_4 \Rightarrow S_0$. This has been taken as evidence for the entry of water only after state S_3 . The possibility of oxygen exchange between free and bound water (intermediates) cannot, of course, be excluded. (b) XANES experiments aiming at the X-ray absorption edge of manganese have demonstrated an upshift by about 1 eV during the light reactions (Yachandra et al., 1989; Ono et al., 1992; Guiles et al., 1987). The authors do not, however, unequivocally attribute this shift to manganese proper, but admit the possibility that a ligand to Mn is oxidized. On the basis of the foregoing, the alternative between 1×4 and 2×2 electron transfers was not settled. Our data on proton release, mainly those displayed in Figure 7, however, were clearly in favor of a 1×4 scheme. Whereas electron transfer between Mn and TyrZ⁺ during transition $S_1 \Rightarrow S_2$ was characterized by a half-rise time of 60–110 s (Renger & Hanssum, 1992; Dekker et al., 1984; Lavergne et al., 1992), only 0.5 proton was released within 13 μ s (pH 7.4), and there was no evidence for any further component of release/uptake from 25 s upward.

In conclusion, microsecond time resolution of proton release in thylakoids has provided some discrimination between the chemical production of protons and their electrostatically driven release/uptake. The major portion of the electrostatic response occurred very fast at the level of TyrZ⁺. It was sustained upon further electron transfer from the manganese cluster to TyrZ⁺. Only in the last, oxygen-evolving transition, $S_4 \Rightarrow S_0$, were the created bases reprotonated. Proton binding, which was caused by electron transfer from water to the manganese cluster, was, at the same rate, largely compensated by the release of chemically produced protons. Only a small portion of uncompensated proton uptake/release was detectable with the characteristic millisecond pace of $S_4 \Rightarrow S_0$ and therefore only on this transition. Our results provide no evidence for a mechanism involving peroxide formation during $S_1 \Rightarrow S_2$, but they are compatible with a four-electron

mechanism under concerted transfer of protons during $S_4 \rightarrow S_0$.

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REFERENCES

- Andersson, B., & Styring, S. (1991) in *Current Topics in Bioenergetics*, Vol. 16 (Lee, C. P., Ed.) pp 2–81, Academic Press, London.
- Ausländer, W., & Junge, W. (1975) *FEBS Lett.* 59 (2), 310–315.
- Babcock, G. T., Blankenship, R. E., & Sauer, K. (1976) *FEBS Lett.* 61 (2), 286–292.
- Barber, J. (1980) *Biochim. Biophys. Acta* 594, 253–308.
- Barry, B. A. (1993) *Photochem. Photobiol.* 57, 179–188.
- Beroza, P., Fredkin, D. R., Okamura, M. Y., & Feher, G. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88 (13), 5804–5808.
- Debus, R. J. (1992) *Biochim. Biophys. Acta* 1102, 269–352.
- Dekker, J. P., Plijter, J. J., Ouwenand, L., & Van Gorkom, H. J. (1984) *Biochim. Biophys. Acta* 767, 176–179.
- Dencher, N. A., Heberle, J., Bark, C., Koch, M. H. J., Rapp, G., Oesterheld, D., Bartels, K., & Büldt, G. (1991) *Photochem. Photobiol.* 54, 881–887.
- Eigen, M. (1963) *Angew. Chem.* 12s, 489–588.
- Fowler, C. F. (1977) *Biochim. Biophys. Acta* 462, 414–421.
- Förster, V., & Junge, W. (1985) *Photochem. Photobiol.* 41, 183–190.
- Guiles, R. D., Yachandra, V. K., McDermott, A. E., Britt, R. D., & Dexheimer, M. P. (1987) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) pp 561–564, Martinus Nijhoff Publishers, Dordrecht.
- Gutman, M., & Nachliel, E. (1990) *Biochim. Biophys. Acta* 1015, 391–414.
- Heberle, J., & Dencher, N. A. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 5996–6000.
- Hong, Y. Q., & Junge, W. (1983) *Biochim. Biophys. Acta* 722, 197–208.
- Jahns, P., & Junge, W. (1992a) *Biochemistry* 31, 7398–7403.
- Jahns, P., & Junge, W. (1992b) *Biochemistry* 31, 7390–7397.
- Jahns, P., & Junge, W. (1993) *Photochem. Photobiol.* 57 (1), 120–124.
- Jahns, P., Lavergne, J., Rappaport, F., & Junge, W. (1991) *Biochim. Biophys. Acta* 1057, 313–319.
- Jahns, P., Haumann, M., Bögershausen, O., & Junge, W. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) pp 333–336, Kluwer Academic Publishers, Dordrecht.
- Junge, W. (1976) in *Chem. Biochem. Plant Pigm.* (Godwin, T. W., Ed.) 2nd Ed., Vol. 2, pp 233–333, Academic Press, London.
- Junge, W. (1982) in *Current Topics in Membranes and Transport*, p 431, Academic Press, London.
- Junge, W., Renger, G., & Ausländer, W. (1977) *FEBS Lett.* 79, 155–159.
- Junge, W., Ausländer, W., McGeer, A. J., & Runge, T. (1979) *Biochim. Biophys. Acta* 546, 121–141.
- Kok, B., Forbush, B., & McGloin, M. (1970) *Photochem. Photobiol.* 11, 457–475.
- Krishtalik, L. I. (1986) *Biochim. Biophys. Acta* 849, 162–171.
- Krishtalik, L. I. (1990) *Bioelectrochem. Bioenerg.* 23, 249–263.
- Lavergne, J. (1987) *Biochim. Biophys. Acta* 894, 91–107.
- Lavergne, J. (1991) *Biochim. Biophys. Acta* 1060, 175–188.
- Lavergne, J., & Rappaport, F. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) pp 873–876, Kluwer Academic Publishers, Dordrecht.
- Lavergne, J., & Junge, W. (1994) *Photosynth. Res.* (in press).
- Lavergne, J., Blanchard-Desce, M., & Rappaport, F. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) pp 273–280, Kluwer Academic Publishers, Dordrecht.
- Lübbers, K., & Junge, W. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) pp 877–880, Kluwer Academic Publishers, Dordrecht.
- Lübbers, K., Haumann, M., & Junge, W. (1994) *Biochim. Biophys. Acta* (in press).
- Meunier, P. C., & Popovic, R. (1990) *Photosynth. Res.* 23, 213–221.
- Nixon, P. J., & Diner, B. A. (1992) *Biochemistry* 31, 942–948.
- Ono, T., Nogushi, T., Inoue, Y., Kusunoki, M., Matsushita, T., & Oyanagi, H. (1992) *Science* 258, 1335–1337.
- Polle, A., & Junge, W. (1986a) *Biochim. Biophys. Acta* 848, 274–278.
- Polle, A., & Junge, W. (1986b) *Biochim. Biophys. Acta* 848, 257–264.
- Polle, A., & Junge, W. (1989) *Biophys. J.* 56, 27–31.
- Radmer, R., & Ollinger, O. (1986) *FEBS Lett.* 195 (1, 2), 285–289.
- Rappaport, F., & Lavergne, J. (1991) *Biochemistry* 30, 10004–10012.
- Renger, G. (1988) *Chem. Scr.* 28A, 105–109.
- Renger, G., & Hanssum, B. (1992) *FEBS Lett.* 299, 28–32.
- Renger, G., Gläser, M., & Buchwald, H. E. (1977) *Biochim. Biophys. Acta* 461, 392–402.
- Renger, G., Wacker, U., & Voelker, M. (1987) *Photosynth. Res.* 13, 167–184.
- Renger, G., Messinger, J., & Wacker, U. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) Vol. 2, pp 329–332, Kluwer Academic Publishers, Dordrecht.
- Ruffle, S. V., Donnelly, D., Blundell, T. L., & Nugent, J. H. A. (1992) *Photosynth. Res.* 34, 287–300.
- Saphon, S., & Crofts, A. R. (1977) *Z. Naturforsch., C: Biosci.* 32C, 617–626.
- Saygin, Ö., & Witt, H. T. (1985) *Photobiochem. Photobiophys.* 10, 71–82.
- Shinkarev, V. P., & Wraight, C. A. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90, 1834–1838.
- Svensson, B., Vass, I., Cedergren, E., & Styring, S. (1990) *EMBO. J.* 9 (7), 2051–2059.
- Takahashi, E., & Wraight, C. A. (1992) *Biochemistry* 31, 855–866.
- Trebst, A., Wietoska, H., Draber, W., & Knops, H. J. (1978) *Z. Naturforsch., C: Biosci.* 33C, 919–927.
- Van Leeuwen, P. J., Heimann, C., Dekker, J. P., Gast, P., & Van Gorkom, H. J. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) pp 325–326, Kluwer Academic Publishers, Dordrecht.
- Velthuis, B. R. (1988) *Biochim. Biophys. Acta* 933, 249–257.
- Wacker, U., Haag, E., & Renger, G. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) pp 869–872, Kluwer Academic Publishers, Dordrecht.
- Wille, B., & Lavergne, J. (1982) *Photobiochem. Photobiophys.* 4, 131–144.
- Yachandra, V. K., Guiles, R. D., McDermott, A. E., Cole, J. L., DeRose, V. J., Zimmermann, J. L., Sauer, K., & Klein, M. P. (1989) *Physica B (Amsterdam)* 158, 78–80.