Photosynthesis Research **38**: 279–296, 1993.

Minireview

Proton release during the redox cycle of the water oxidase

Jérôme Lavergne[1] & Wolfgang Junge[2]
[1]*Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France;*
[2]*Biophysik, FB Biologie/Chemie, Universität Osnabrück, 49069 Osnabrück, Germany; Correspondence to either author*

Received 10 June 1993; accepted in revised form 1 October 1993

Key words: electrochromic changes, oxygen-evolving complex, Photosystem II, proton release, protolytic reactions, Tyrosine Z

Abstract

Old and very recent experiments on the extent and the rate of proton release during the four reaction steps of photosynthetic water oxidation are reviewed. Proton release is discussed in terms of three main sources, namely the chemical production upon electron abstraction from water, protolytic reactions of Mn-ligands (e.g. oxo-bridges), and electrostatic response of neighboring amino acids. The extent of proton release differs between the four oxidation steps and greatly varies as a function of pH both, but differently, in thylakoids and PS II-membranes. Contrastingly, it is about constant in PS II-core particles. In any preparation, and on most if not all reaction steps, a large portion of proton transfer can occur very rapidly (< 20 μs) and before the oxidation of the Mn-cluster by Y_z^+ is completed. By these electrostatically driven reactions the catalytic center accumulates bases. An additional slow phase is observed during the oxygen evolving step, $S_3 \Rightarrow S_4 \rightarrow S_0$. Depending on pH, this phase consists of a release or an uptake of protons which accounts for the balance between the number of preformed bases and the four chemically produced protons. These data are compatible with the hypothesis of concerted electron/proton-transfer to overcome the kinetic and energetic constraints of water oxidation.

Abbreviations: BBY-membranes – Photosystem II-enriched membrane fragments prepared after Berthold, Babcock and Yocum (1981); BSA – bovine serum albumin; Chl – chlorophyll; CAB-protein – chlorophyll a/b - binding protein; core particles – oxygen evolving reaction center core particles of Photosystem II; Cyt – cytochrome; DCBQ – 2,5-dichloro-p-benzoquinone; IML – intermittent light; P-680 – primary electron donor of Photosystem II; PS II – Photosystem II; Y_z – tyrosine residue on the D1 polypeptide, electron carrier between manganese and P-680; $\Rightarrow$ – photochemical reaction

1. Introduction

Four protons must be released during the tetravalent oxidation of two molecules of water to molecular oxygen. Their release into the thylakoid lumen, concomitant with the uptake of protons during quinone reduction on the opposite side of Photosystem II, is one of the sources of transmembrane protonmotive force which drives the synthesis of ATP.

The complete reaction cycle (the Kok-cycle) of water oxidation (see Joliot and Kok 1975) is made up of four univalent steps each powered by one quantum of light. One concern of this review is to evaluate whether the pattern of proton release over these four steps is diagnostic for the catalytic mechanism, involving manganese, other cofactors and (bound) water. On some given transition, proton release may not necessarily arise from water oxidation, but from oxidized intermediates (such as, for example, a protonated μ-oxo bridge between manganese atoms upon the oxidation of one of its manganeses). In addition to chemically produced protons there may also be electrostatically expelled

protons originating from neighboring amino acid side chains responding to the deposition of a positive charge in the catalytic center. The electrostatic response may already be initiated at the level of the secondary electron donor of Photosystem II. Independent of whether the generated bases are manganese ligands or remote amino acid side chains, it is worth asking whether their reprotonation during, say the oxygen evolving transition $S_3 \Rightarrow S_4 \rightarrow S_0$ is a prerequisite for the completion of the reaction cycle because it lowers the energetic barrier of an otherwise too endergonic reaction step.

The experimental side of this topic is not any less complicated than the interpretational one. From the pioneering work of Fowler (Fowler 1977) until the present, the results on proton release have shown marked oscillations, not only as a function of flash number but also as a function of authors and date. Although the authors of this review do believe that things are now getting clearer in several respects, they do not claim to have reached the ultimate truth and, in fact, they have not even reached total consensus amongst them. The message here is simply: work in progress, with end in sight.

The basic experimental design has been to detect the amount of protons released upon each of a series of so-called single turnover flashes. Then by applying the Kok model (Kok et al. 1970) the contribution of each of the successive oxidation step (S-transitions) can be estimated. Although this strategy is readily described, its implementation has presented difficulties; it has been a field of controversies, rectifications and oscillations, even from the same laboratories. It is obvious that a rigorous assignment of the stoichiometric and kinetic pattern of proton release can only be expected under two prerequisites: 1) The Kok-parameters, namely the population of the more stable states (S_0 and S_1) in the dark, and the proportion of double hits and misses are determined in parallel measurements of oxygen evolution and/or of certain transients of UV-absorption, attributed to the different 'S' states. 2) Proton release/uptake attributable to water oxidation is clearly discriminated from protolytic reactions due to other sources. We start with a short description of the techniques used to measure protolytic reactions, as applied to different types of biological material. Some general considerations are then exposed on the detection and, in particular, on kinetic aspects of proton release and on the various possible mechanisms of proton release during an oxidative transition. This is followed by a review of recent results on stoichiometries and kinetics obtained in different materials by our research groups. The complex picture that results is finally discussed in the light of the current concepts on the mechanism of water oxidation.

2. Materials and techniques

Various biological materials have been used for research on this topic, each presenting specific advantages and disadvantages. As the signal-to-noise ratio is about reciprocal to the number of chlorophyll (Chl) molecules per Photosystem II (PS II), it increases from thylakoids (500–700 Chl) to BBY-membranes (250 Chl) to oxygen evolving reaction center complexes (50–100 Chl). Thus, the latter should be the best system. Several recipes for preparing detergent-solubilized PS II particles with preserved O_2-evolving capacity have been reported (Ghanotakis et al. 1984, 1987, Rögner et al. 1987, Haag et al. 1990, Van Leeuwen et al. 1991, Kirilovsky et al. 1992). Compared with the 'D1-D2-Cyt*b*-559' particles (e.g., Nanba and Satoh 1987)) that are the closest analog to the bacterial reaction center preparations, the O_2-evolving reaction center core particles retain core antenna polypeptides and some extrinsic 'Oxygen Evolution Enhancing' polypeptides. These preparations, however, show significant modifications of the oxygen-evolving system, compared with native material, such as in having slower O_2-evolving reaction, decreased stability of the higher S-states and, as will be seen, different proton release stoichiometry. It is worth asking whether this 'degradation' of the finely-tuned machine, that exists in vivo, gives insight into the intrinsic catalytic mechanism.

The so called 'BBY' preparation of granal membranes (Berthold et al. 1981), based on a mild Triton X-100 solubilization of stroma lamellae, is expected to preserve better the integrity of the oxygen-evolving complex. This should not be taken for granted, however, since our current results suggest different pH dependences of the proton release pattern in thylakoids and BBYs. The significance of this discrepancy is not clear to the

authors, but it cannot be ruled out that the oxygen-evolving complex is affected by the preparation procedure.

Studies on oxygen evolving reaction center core particles (core particles) and BBY-membranes, unlike those in thylakoids, do not suffer from the superimposition of the protolytic events in Photosystem II with those resulting from Photosystem I and the Cyt b_6f complex. But the protolytic events at both the oxidizing and the reducing end of Photosystem II contribute to the net pH-transient in the medium. It is advantageous in thylakoids, on the other hand, that the membrane spatially separates proton release by water oxidation and proton uptake by plastoquinol.

Two types of approaches have been chosen to detect the release of protons by water oxidation in thylakoids: (a) The difference of pH transients in the suspending medium is recorded with and without a protonophore added. The protonophore (uncoupler) accelerates the relaxation of the pH-difference across the thylakoid membrane (typical half-decay time in the absence of a protonophore is about 10 s (Junge et al. 1986)). Either glass electrodes (Fowler 1977) or hydrophilic pH-indicating dyes (Saphon and Crofts 1977) have been used for detection of pH-transients in the medium. The latter are practically selective for pH-transients in the medium because of the very large volume ratio (> 1000 : 1) of the medium over the lumen in thylakoids (Polle and Junge 1986a,b). Both the glass electrode (intrinsic rise time >10 ms) and the indicator dyes, which detect protons from the lumen only after their passage across the membrane, have limited time resolution. (b) A particular amphiphilic dye, neutral red, has been used as an indicator for pH-transients in the lumen of thylakoids (Ausländer and Junge 1975). Compared to hydrophilic indicators, it has a much greater sensitivity for pH-transients in the lumen. This is due to the adsorption at the membrane-water interfaces which causes an effective 'upconcentration' in the lumen (Junge et al. 1979, Hong and Junge 1983). The dye originally responds to events at both membrane-water interfaces. It is, however, made selective by the addition of a non-permeant buffer, like bovine serum albumin, in order to 'quench' the pH transients in the medium. With this dye the different time windows of proton release by water oxidation (≤ 1 ms) and by plastoquinol oxidation (about 10 ms) have been detected (Ausländer and Junge 1975). The kinetic competence of this indicator dye is high in unstacked thylakoids, but is flawed in stacked ones, as discussed further down.

3. Kinetics of protolytic events

It is worthwhile recalling some kinetic properties of protolytic reactions. According to the fundamental work of Eigen (1963), protolytic reactions in aqueous environment are among the most rapid reactions outside of photochemistry. The protonation of a base in water is diffusion controlled with a rate constant ranging from 10^{10} to 10^{11} M^{-1} s^{-1}. The rate constant of the off-reaction, k_-, on the other hand, is related to the constant of the on-reaction, k_+, by the dissociation constant K_o of the acid: $k_- = K_o \cdot k_+$. The validity, in principle, of Eigen's concepts for acid/base groups at the surface of proteins and membranes has been experimentally and theoretically established by Gutman and Nachliel (1990). The response rate of pH-indicating dye, B, to a sudden injection of protons into water (in the limit of a small perturbation) is very high. The effective rate constant is $k_{eff} = k_+ \cdot [B^-]$. With a conservative estimate for k_+, namely 10^{10} M^{-1} s^{-1}, and for the concentration of the base, $[B^-] = 1$ mM, the effective rate constant, k_{eff}, is 10^7 s^{-1}. The dye responds with a relaxation time of 100 ns. If there are further buffering molecules around, the relaxation time will be even shorter.

What can be said, on the other hand, of the proton release rate from the catalytic center or its environment? Let us assume that a protonated group (thus with, initially, $pK_{red} > pH$) acquires a new, lower pK ($pK_{ox} < pH$) as a consequence of the oxidation step. The rate constant of deprotonation is given by $k_{eff} \cong k_+ \cdot K_{ox}$. With $k_+ = 10^{10}$ M^{-1} s^{-1} and $pK_{ox} = 4$, the effective rate constant is 10^6 s^{-1}, equivalent to a relaxation time of 1 μs (or 10 μs if $pK_{ox} = 5$). An acid group in direct contact with water can only experience a pK-shift by a 'chemical' mechanism (see below), whereas a pK-shift which is induced by electrostatic interaction with the newly created positive charge is only expected for groups which are somehow buried in the protein. The assumption of a diffusion-controlled protonation is then no longer valid, and the electric field affects the rate

constants, both of deprotonation and protonation. Anyway, it is obvious that pH-indicators, if only present at sufficiently high concentration, should not be limiting for the time-resolution of deprotonation events. The theoretically expected high time resolution of indicator dyes is not met, however, if the indicator is kept far apart from the primary groups. In the latter case, proton transfer to the indicator dye involves intermediate diffusion steps often together with transient buffering reactions. This can greatly delay the response of the dye as observed for proton uptake by PS II in stacked thylakoid membranes (Polle and Junge 1986b; Junge and Polle 1986) and with aggregated core particles (Lübbers and Junge 1990). Exceptions to the rules of rapid protolytic reactions may also occur for particular mechanistic reasons. Carroll and Norton (1992) give an example of a protonation of a μ-oxo-bridge in a synthetic Mn-cluster, which is attributed to slow geometric and structural rearrangements following the primary redox transition.

4. Possible mechanisms for proton release

In general, a deprotonation which is coupled to the oxidation of a center X proceeds as follows. The center X has different pKs in its two redox forms. When $pK_{red} >> pH >> pK_{ox}$, the reduced center X is fully protonated, and its oxidation will cause the release of one full proton. However, if $pK_{red} \approx pH$ (X is only partially protonated when reduced), or if $pK_{ox} \approx pH$ (X partly deprotonated when oxidized), the oxidation step will be accompanied by non-integer, pH dependent proton release. A non-integer pattern was first proposed as a possibility for water oxidation (Renger 1988) and established for the acid/base reactions in bacterial reaction centers (McPherson et al. 1988, Maróti and Wraight 1988). The linkage between the pK shift and redox transition can lie anywhere between the two extremes, from purely chemical to purely electrostatic. At the chemical limit the redox group is directly involved in proton binding, so that the magnitude of the pK shift is determined by the distortion and the occupation of molecular electronic orbitals. The electrostatic case corresponds to chemically distinct redox and protonatable groups so that the pK shift may be approximated by through-space coulombic interaction between point charges. An intermediate case, that we may call ligand deprotonation, involves both features: the redox and proton binding centers are some distance apart, but share common electronic orbitals. These various possibilities are illustrated in Fig. 1.

Obviously, some deprotonation steps in the photosynthetic water oxidation cycle *must be* chemical deprotonations, precisely those involving oxidation of the water substrate. Energetic considerations (Krishtalik 1986, 1990) make it very unlikely that each of the S-transitions consists of the abstraction of one electron from the water substrate. The current view is that most, if not all, of the oxidations from S_0 to S_4 are carried out on charge storing intermediates such as the Mn atoms of the cluster (see Debus 1992 for a comprehensive review). Then, if one assumes the plausible (but still unwarranted) idea that *all* oxidation steps up to S_4 involve Mn atoms and that electron abstraction from water only occurs on the $S_4 \rightarrow S_0$, O_2-evolving reaction, one may envisage the following mechanisms for proton release on each of the intermediate transitions: (i) Electrostatic deprotonation of protonatable group(s) (such as amino acid side chains); and (ii) Mn-ligand deprotonation. One should keep in mind that 'deprotonation' may also mean 'OH^- binding' and that the latter process could correspond to binding of the water substrate. This is not a casual remark, since, under all likelihood, binding of OH^- rather than H_2O should be part of the catalytic strategy for lowering the activation barrier (Brudvig and De Paula 1987, Krishtalik 1990). In this framework, complex protolytic events should accompany the $S_4 \rightarrow S_0$ step: Chemical deprotonation of the substrate, together with reprotonation of the bases that lost protons during the oxidations from S_0 to S_4.

From the foregoing, special interest should be given to the available data concerning the pKs of Mn ligands. Chemical modeling (Pecoraro 1988, Bossek et al. 1990, Philouze et al. 1992) as well as EXAFS results on the Mn–Mn distances in the photosynthetic Mn-cluster (Yachandra et al. 1986, George et al. 1989, Sauer et al. 1992, Penner-Hahn et al. 1990) suggest the involvement of μ-oxo bridges for ligating the Mn atoms. Information on the protonation state and shifts of the bridging oxygens in synthetic systems is now available. Various di-μ-oxo (MnIII,MnIII) complexes were found to have pKs in aqueous medium ranging from 8.3 to 11 for

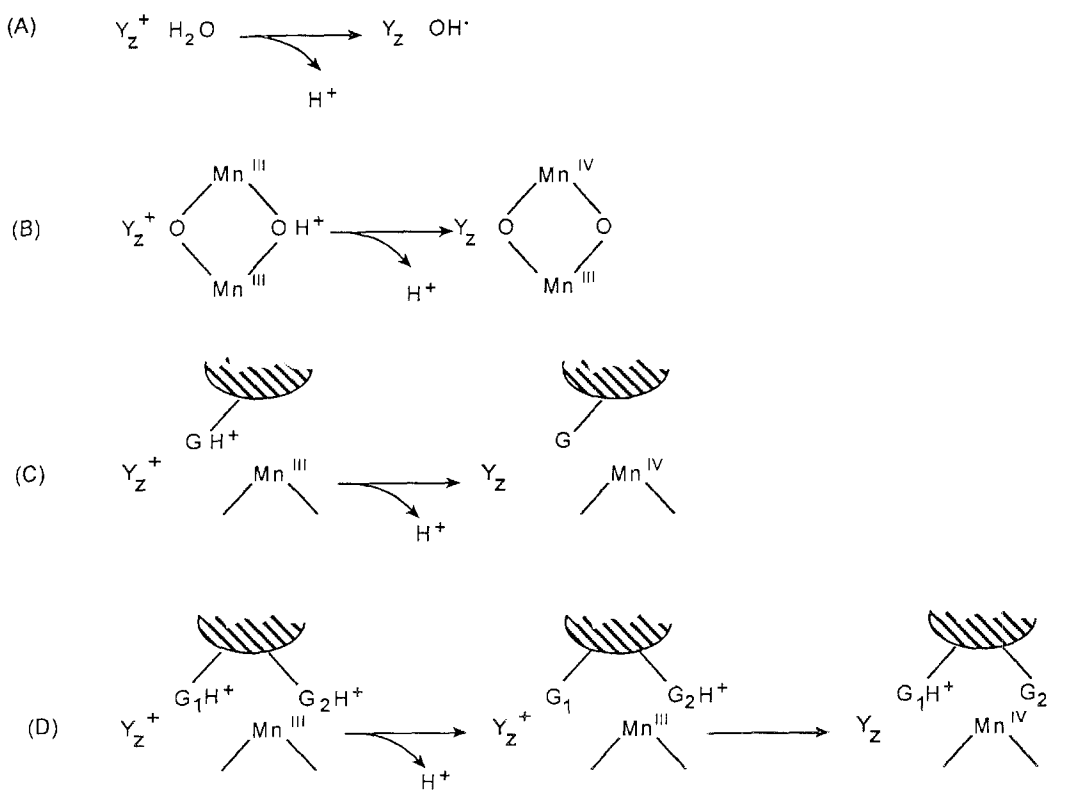

Fig. 1. A scheme illustrating various possibilities for proton release on the donor side of Photosystem II. (A) Chemical deprotonation. The particular example shown (formation of an $OH^\cdot$ radical) was chosen for its simplicity rather than likelihood, since the redox potential of the $OH^\cdot/H_2O$ couple (in aquo) ($E_0 = 2.85$ V) is much higher than that of Y_Z^+/Y_Z. (B) Deprotonation of a Mn ligand. (C) Electrostatic deprotonation of a group close (but not liganded) to a Mn atom. Depending on geometry and rate factors, the deprotonation may be initiated before the electron transfer from Mn to Y_Z^+. (D) Push-pull process involving two groups. The deprotonation is initiated by Y_Z^+ acting on G_1 and stabilized, after the electron transfer step, by proton transfer from G_2 to G_1.

protonation of one of the bridging oxygens, the other one remaining unprotonated (Thorp et al. 1989, Machanda et al. 1991). A Mn-complex with pK = 11 had also been reported (Cooper and Calvin 1977) to have a pK $\approx$ 2.3 in the (MnIII,MnIV) state, thus a ΔpK $\approx$ 8.7. Machanda et al. (1992) described three successive redox states of another dimer: The (MnIII,MnII) form has both of its bridges protonated, the (MnIII,MnIII) has one bridge fully deprotonated and the second one protonated with pK = 9.15, but the (MnIV,MnIII) state is fully deprotonated. Thus, the release pattern for the two successive oxidation steps would be 1:1 below the pK, 1.5:0.5 around the pK, and 2:0 above the pK.

Concerning kinetic aspects, it should be made clear that transient electrostatic or ligand deprotonation may accompany the transfer of the positive charge from P-680 to Mn. The lifetime of $P\text{-}680^+$ (sub μs range) seems a bit too short to induce electrostatic deprotonation, but such is not the case for Y_Z^+ (with lifetimes from 100 micro- to some milliseconds depending on the S-transitions). If this happens (and, as will be shown, there is consistent evidence that it does), a complex process may be expected, with initial deprotonation caused by Y_Z^+, followed by rearrangement when the positive charge reaches its stable location (Mn).

5. The stoichiometric pattern

Patterns of proton release are discussed in terms of the fractional production of protons attributable to the transitions $S_0 \Rightarrow S_1 : S_1 \Rightarrow S_2 : S_2 \Rightarrow S_3 : S_3 \Rightarrow S_4 \rightarrow S_0$. The absolute ratio of protons released over electrons transferred through Photosystem II has not been determined in the cited studies. Instead, the average proton release per flash over the complete

cycle was assumed to represent four protons per active Photosystem II.

5.1. Thylakoids

The 1:0:1:2 stoichiometric pattern for proton release during the above sequence of oxidation steps became widely accepted for almost ten years, expressing a consensus between several independent techniques and groups (an exception was Hope and Morland (1979), who maintained the 1:1:1:1 pattern). Indeed this 1:0:1:2 stoichiometric pattern was close to the result of Fowler (1977) using a pH electrode, then supported by A. R. Crofts' group from experiments with hydrophilic dyes (Saphon and Crofts 1977) and on delayed chlorophyll *a* fluorescence (Bowes and Crofts 1981), by Wille and Lavergne (1982) with a spin probe and by Förster and Junge (1985) using the neutral red technique.

There were, however, questionable aspects in the above reports (see below) and there was a common prejudice in the expectation of the proton stoichiometry to be a set of integers. Fowler had actually reported a non-integer pattern (0.75:0:1.25:2), but suggested this could reflect a heterogeneity between two types of centers, each with its own integer stoichiometry.

The following set of experiments has finally led to reject the 1:0:1:2 pattern in favor of, in general, non-integer stoichiometries in thylakoids. The absorption changes of neutral red caused by each flash of a series are multiphasic: a fast component in the ms or sub-ms range displays a marked flash number (S-state) dependence of its amplitude and rate, while a slower component (tens of ms) is about identical upon each flash. It thus seemed that only the fast phase with its clear S-signature and kinetic range matching the electron transfer reactions could be relevant (Förster and Junge 1985). Although no convincing interpretation could be proposed for the slow phase, it seemed reasonable, at that time, to exclude it from the analysis. The amplitude of the fast phase was close to zero on the first flash (corresponding predominantly to the S1 $\Rightarrow$ S2 transition), maximum on the third one ($S_3 \Rightarrow S_0$, O_2 evolving step) and in-between on the second flash, thus quite consistent with the 1,0,1,2 pattern. This interpretation of the neutral red data was questioned by Lavergne and Rappaport (1990) who showed that the entire amplitude of the response of neutral red (fast and slow components) had to be assigned to proton release from water oxidation. Their rationale to explain the slow component was based on studies by Polle and Junge (Junge and Polle 1986, Polle and Junge 1986b,c) on the delay between the intrinsically rapid proton uptake by PS II from the partitions between stacked thylakoids and its slow detection by hydrophilic indicator dyes in the suspending medium. The lateral relaxation of the alkalization jump along the narrow partition domain into the medium is rather slow (about 100 ms). Then, assuming that BSA has no access to the narrow partition domain, neutral red, which is adsorbed to *both surfaces* of the membrane, will initially respond to proton release into the lumen *and* uptake in the partitions. The uptake response will disappear along with the lateral equilibration of the partitions. The transient superimposition of the alkalization response masks a portion of the rapid lumenal acidification signal and accounts for the slow apparent 'acidification' phase which, in fact, reflects the relaxation of the interfering contribution. It also accounts for the observation by Hong et al. (1981) (then differently interpreted) that in the presence of DCMU, which blocks electron transfer from Q_A^- and proton uptake at the acceptor side of Photosystem II, a rapid lumenal acidification is monitored by neutral red. Lavergne and Rappaport (1990) pointed out that this phenomenon is accompanied by the disappearance of the slow phase, as expected from the suppression of the alkalization transient.

The slow lateral pH-equilibration between the partitions between stacked thylakoids and the external space had been shown by Polle and Junge (1989) to be drastically accelerated by small and mobile hydrophilic buffers, such as phosphate. It was also accelerated when thylakoids were totally unstacked by mild EDTA-treatment (Polle and Junge 1986b). Thus, in both these situations, one expects suppression of the slow component of the neutral red response with concomitant increase of the fast phase, so that the total amplitude is conserved. Both of the above situations were experimentally tested in collaborative work between our two groups and the results were in nice agreement with the prediction (Jahns et al. 1991).

The reinterpretation of the neutral red results had obvious consequences regarding the proton release stoichiometry. If one takes the full amplitude of the

response rather than only the rapid component (in stacked thylakoids), the whole oscillating sequence is shifted upwards: The signal on the first flash ($S_1 \Rightarrow S_2$), for instance, is not zero any more but close to half a proton. At first glance, the proton release pattern at pH 7–7.5 looks like 1:0.5:1:1.5 instead of 1:0:1:2 (Lavergne and Rappaport 1990). As explained earlier, a non-integer pattern should depend on the pH and information on the pKs of proton release groups can be derived therefrom. Thus both our research groups pursued this plan, but using different materials. Before describing the results of Rappaport and Lavergne (1991), obtained with BBYs, we give an account of recent results obtained in unstacked pea thylakoids (Haumann and Junge 1993). The stoichiometric pattern of proton release as a function of the redox transition, $Si \Rightarrow Si + 1$, was derived from the flash sequence data, using the Kok-parameters (S_1/S_0-distribution, double hits, misses) which were determined from parallel measurements of oxygen evolution. The result is illustrated in Fig. 2A. The most pronounced features are: 1) The only marginally pH-dependent proton yield of 1 for the transition $S_2 \Rightarrow S_3$, a feature which is shared by all other materials (see below). 2) A decrease from 1 to less than 0.5 starting at pH 6.7 and towards alkaline pH for the transition $S_0 \Rightarrow S_1$. 3) A mutually compensating behavior of the respective proton yields for $S_1 \Rightarrow S_2$ and $S_3 \Rightarrow S_4 \rightarrow S_0$, with the former dominating at pH 5.8–6.6 (up to 2 protons compared to 0.3 for $S_3 \Rightarrow S_4 \rightarrow S_0$) and the latter around pH 7.5 (1.5 vs. 0.5 protons). The crossover point where both released one proton was at pH 6.7. The above pH-dependence was corroborated by an alternative technique, with hydrophilic pH-indicating dyes (instead of neutral red) and with and without the added protonophore nigericin (Haumann and Junge 1993). These results strongly deviated from the pH-dependence of proton release published for BBY-membranes (Fig. 2B) and core particles (Fig. 2C).

5.2. *BBY-membrane fragments*

These preparations have lost the vesicular topology, but not the appressed structure of thylakoids. The global pH changes contributed both by the donor and acceptor sides of Photosystem II may be monitored by using hydrophilic dyes in the absence of ionophores (Rappaport and Lavergne 1991).

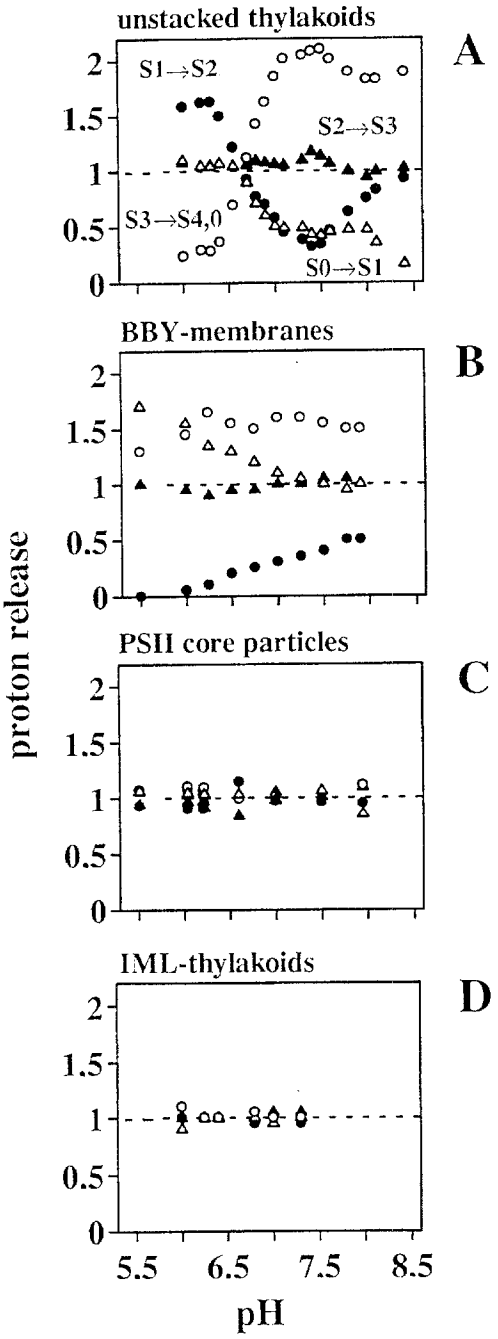

Fig. 2. Patterns of proton release by water oxidation as a function of pH. Original patterns as function of flash number were deconvoluted to yield the extent at any of the four transitions, namely $S_0 \Rightarrow S_1$ (open triangles), $S_1 \Rightarrow S_2$ (closed circles), $S_2 \Rightarrow S_3$ (closed triangles), and $S_3 \Rightarrow S_4 \rightarrow S_0$ (open circles). Four different types of material, all capable of oxygen evolution, were used; from top to bottom: unstacked pea thylakoids (Haumann and Junge 1993); BBY-membranes (Rappaport and Lavergne 1991); PS II core particles (Lübbers et al. 1993); and IML-thylakoids (Jahns and Junge 1992b).

These authors checked that under their experimental conditions (using DCBQ as an electron acceptor), the uptake on the acceptor side was constant upon each flash (no binary oscillation) and could be subtracted. The damping parameters (misses and double-hits) were estimated from the measurement under similar conditions of the UV absorption changes associated with the S-states (Lavergne 1991). The initial state was set close to 100% S_1 by preilluminating the sample with one flash and letting it fully deactivate before starting the flash sequence measurement.

The pH dependence of the release pattern computed for the successive transitions is shown in Fig. 2B. A constant release close to 1 is obtained for $S_2 \Rightarrow S_3$. For $S_0 \Rightarrow S_1$, the release becomes grater than 1 below pH 7. Upon the $S_1 \Rightarrow S_2$ transition, the stoichiometric coefficient remains below 1, rising from 0 below pH 6 to about 0.5 proton at pH 7.5. In order to fit these results by a formal model, at least four deprotonating groups are required, undergoing pK shifts in response to the oxidation steps of the charge-storing system (thus a set of four pKs for each S-state, meaning that 16 pK values are potentially involved). Among these pKs, only three lie within or close to the accessible pH range and could be estimated (the other ones correspond to groups that are fully protonated or unprotonated). Thus, a pK close to 6.0 in the presence of S_0, shifted to below 4.5 when S_1 is formed, accounts for the increase of proton release towards acidic pH observed for $S_0 \Rightarrow S_1$. This finding agrees with the finding by Vass and Styring (1991) of a pK $\approx$ 6.0 controlling the rate of the $Y_D^+S_0 \Rightarrow Y_DS_1$ reaction (Y_D is the tyrosine responsible for the EPR 'signal II-slow'). The bell-shaped curve (culminating at about 0.5 proton at pH 7.5) obtained for $S_0 \Rightarrow S_1$ involves a pK of about 8.2 in the presence of S_1 which is shifted to about 7.2 in the presence of S_2. Again, the latter pK of 7.2 agrees with the pH dependence for the back reaction of $S_2Q_A^-$ in DCMU-inhibited BBYs (Buser et al. 1992).

5.3. Oxygen evolving reaction center core particles

The first reports which suggested a featureless pattern of proton release in core particles (Wacker et al. 1990, Lübbers and Junge 1990) were obtained on samples prepared according to Ghanotakis et al. (1987) and Haag et al. (1990). Proton release was constant from the second flash on, whereas the net production after the first flash was much smaller (see Fig. 2 in Lübbers and Junge 1990 and Fig. 3 in Wacker et al. 1990). The diminution was caused by superimposition of proton uptake which was due to the photoreduction on the first flash of the oxidized non-heme iron at the acceptor side. The true pattern of proton release was therefore approximately 1:1:1:1.

This result was challenged for a time by Van Leeuwen et al. (1992b) on the grounds that the more rapid deactivation of the higher states in reaction centers might have damped out an oscillating pattern of proton release if the flashes in a series are too closely spaced. The rapid deactivation is, in fact, a property of their particular preparation (Van Leeuwen et al. 1991). It is not observed in the samples prepared according to Ghanotakis et al. (1987) as used by Lübbers and Junge (1990). Recent experiments with samples prepared after Van Leeuwen et al. (1991) in which the repetition period of light flashes was appropriately short (25 ms), confirmed an approximate 1:1:1:1 pattern (Jahns et al. 1992). The rapid deactivation of the S-states in this particular preparation is, in a way, fortunate, since it allows rapid dark-adaptation of the sample and repetitive excitation, thus saving material. It also minimizes the complication arising from the oxidation of the non-heme iron by 100 μM hexacyanoferrate (III).

One of our research groups (WJ) has scanned the pH-dependence of proton release in the range from 6 to 7.5, both for core particles prepared according to Ghanotakis et al. (1987) and Van Leeuwen et al. (1991). Oscillations of proton release were absent in the former (Lübbers et al. 1993) and much smaller than in thylakoids in the latter (O. Bögershausen, W. Drevenstedt and W. Junge, unpublished). As illustrated in Fig. 2C, the pH-dependence of the approximated 1:1:1:1 pattern of proton release was negligible (Lübbers et al. 1993). The oscillations of oxygen evolution and of the Mn-related UV-absorption transients were as clearly expressed in thylakoids as in BBY-membranes (Lübbers et al. 1993). There is presently no more lingering argument over this point with Hans van Gorkom's group.

5.4. IML-thylakoids

Another example of a structureless pattern release

has been observed in thylakoids prepared from pea plants grown under a regime of intermittent light (IML) with long dark periods (10 days of 2 min light followed by 2 h dark). These growth conditions severely impair the synthesis of chlorophyll *b* with the consequence that most of the light harvesting proteins around Photosystem II are lacking (Jahns and Junge 1992a). These thylakoids are intrinsically unstacked, so that the kinetic problem of the neutral red technique is absent. In this material, the pattern of oxygen release oscillates conventionally in the pH-range from 6 to 7.5. The pattern of proton release is approximately 1:1:1:1 and its pH-dependence, which is illustrated in Fig. 2D is much flatter than in control thylakoids (Jahns and Junge 1992b, 1993, Jahns et al. 1992). However, the case of IMLs is not fully clear, since in later experiments, Jahns and Lavergne (unpublished) found an almost normally oscillating pattern in this material.

6. Modification of proton release in thylakoids

6.1. The effect of DCCD

N,N'-Dicyclohexylcarbodiimide (DCCD) is a hydrophobic diimide which, in thylakoids, covalently binds to the proteolipid of the F-ATPsynthase and its β-subunit (see Jahns et al. 1988 for references). An inhibitory action on the acceptor side of Photosystem II has been later attributed to non-covalent action which is reversed by washing (Jahns and Junge 1990). A new type of covalent action has been discovered by Jahns et al. (1988). Incubation of stacked thylakoids with ≥10 DCCD to 1 chlorophyll diminishes the proton pumping action of Photosystem II under flashing light. Both the extent of proton release by water oxidation and the extent of proton uptake by quinone reduction decreased and a rapid electrogenic back reaction becomes apparent. The time lag (of about 3 ms) for the reduction of Photosystem I, which is attributed to the need for the protonation of the doubly reduced quinone as an intermediate electron carrier, is shortened. Oxygen evolution, on the other hand, is not affected (Jahns et al. 1988). Several lines of evidence have led to the conclusion that the covalent modification by DCCD closes the normal outlet of protons from water oxidation into the lumen. The need for electrostatic compensation apparently drives these protons through the protein across the membrane towards the site of plastoquinone reduction. There is evidence that all the reaction steps of the water oxidase are equally affected, which suggests a common outlet for protons into the lumen (Jahns and Junge 1989). Search for the target in Photosystem II of the covalent modification by DCCD has identified chlorophyll a/b-binding proteins (CAB-proteins or LHC II). Sites of ^{14}C-DCCD binding have been characterized by sequencing of cleavage fragments. According to the accepted folding model with three transmembrane helices, these sites are located at the luminal side of the thylakoid membrane (Jahns and Junge 1990). The DCCD-binding sites of the above studies are possibly also Ca^{2+}-binding sites on these proteins (Webber and Gray 1989). Further attempts to attribute these effects to any particular member of the CAB family have been unsuccessful (Jahns and Junge 1990, 1993). It is conceivable that certain members of the CAB-family, aside from their main function as antennae, serve another purpose, i.e. they shield the sites of proton production and channel protons into the thylakoid lumen.

6.2. Proton sequestering domains

The idea that protons from water oxidation and possibly also from plastoquinone oxidation are not always directly liberated into the lumen of thylakoids but rather into membrane sequestered domains has been promoted mainly by Richard Dilley and his coworkers (for a review, see Dilley 1991) based on experiments aimed at the use of protons for ATP synthesis and on the modification of membrane proteins by acetic anhydride. For Photosystem II, Peter Homann's research group has studied the influence of the metastable proton pool in the membrane on the release of functional Cl^- and on the stability against manganese extracting procedures (Theg and Homann 1982). Their protocol for the activation of these domains by low concentrations of uncouplers and by preincubation of thylakoids at low pH was adopted in flash spectrophotometric experiments to detect proton transfer from water oxidation to neutral red (Theg and Junge 1983). After this pretreatment, the release of protons from water oxidation was seemingly diminished until it reappeared after six flashes of light. It thus seems that the supposed domains become saturated after

transiently trapping the protons from the first six flashes. Titration of the domain capacity as a function of pH revealed a pK of about 7.5 and a Hill coefficient of 2 (Theg and Junge 1983). The pK of the domains was shifted towards acidity under oxidizing conditions (addition of hexacyanoferrate (III)). This has been interpreted by the location of the membrane sequestered proton releasing groups in the vicinity of a redox component (Cyt b_{559}?), the oxidation of which creates a positive electric field at these groups (Polle and Junge 1986a). This study also showed a doubled storage capacity of the domains per reactive Photosystem II when one half of the centers were blocked by DCMU. This implies delocalization of the domains over more than one Photosystem II.

7. Other indicators of the electrostatic balance in the catalytic center

If, on a given S-transition, one has abstraction of one electron and expulsion of p (a fractional number, in general) protons, the net charge variation is (1-p) elementary charges. Local electrostatic changes may actually be expected even when the transition is globally neutral (p = 1), if the redox center and deprotonating group are distinct (electrostatic or ligand deprotonation, as defined above): The dipole moment is modified and a close by molecule will sense an electrostatic potential change with sign and magnitude depending on its distances to the other charges. Various S-dependent responses are believed to have an electrostatic origin.

The *chlorophyll a fluorescence* yield of Photosystem II was found to oscillate under flashing light (Joliot et al. 1971, Delosme 1971). The *rate of reduction of P-680*$^+$ has been shown to oscillate depending on the S-state. Horst Witt and coworkers (Brettel et al. 1984, Meyer et al. 1989) used oxygen-evolving reaction centers from *Synechococcus* and found a half-rise time of 20 ns for reactions starting from states S_0 and S_1, and a biphasic rise with half times of 50 ns and 250 ns for centers starting from S_2 and S_3. The slow phases have been interpreted by coulombic retention of the electron traveling from Y_Z to P-680$^+$, in accordance with the then accepted pattern of proton release (which was not experimentally verified in their reaction center core preparation). A similar pattern of the rate, however, has been reported for BBY-membranes (Eckert and Renger 1988), where the pattern of proton release clearly differs from the then accepted one.

It was first suggested by Velthuys (1981) that the whole set of *S-dependent absorption changes* observed in the near UV and visible region could be interpreted as local electrochromic shifts reflecting the net electrostatic balance, in rough agreement with the then accepted 1:0:1:2 proton release pattern. Further investigations showed that this hypothesis was only partly correct: the UV changes are now largely attributed to oxidized minus reduced spectra of the redox centers (the Mn cluster and, possibly, an amino acid side group) (Dekker et al. 1984b, Lavergne 1987, 1991). On the other hand, the changes observed in the absorption bands of chlorophyll *a*, i.e. around 435 nm and 680 nm, are clearly electrochromic in nature (Dekker et al. 1984b, Saygin and Witt 1985, Lavergne 1987, 1991, Velthuys 1988). These *local* electrochromic changes should not be confused with the electrochromic change responding to the delocalized membrane potential (Junge and Witt 1968, Emrich et al. 1969).

A possible candidate for the chlorophyll pigment undergoing the shift is the primary donor P-680 (Schatz and Van Gorkom 1985): This phenomenon would be the equivalent on the donor side of the reaction center of the C-550 shift caused on its acceptor side by Q_A^- where the sensitive pigment, a pheophytin, is presumably the primary acceptor (Schatz and Van Gorkom 1985). In accordance with the hypothesis that P-680 is the electrochromic probe (as illustrated in Fig. 3), the oxidation of tyrosine Y_Z (the intermediate carrier between P-680 and the Mn cluster) is accompanied by a similar shift, with a larger amplitude, suggesting that Y_Z is closer to the probe than the Mn cluster.

The S-state dependence of the amplitude of the electrochromic change in the 435 nm region was studied in BBYs by Lavergne (1991). At variance with the null change expected on $S_0 \Rightarrow S_1$ in the framework of the 1:0:1:2 pattern of proton release (or, for that matter, 1:0.5:1:1.5), a signal was found on this transition (at pH 6.5), with inverted trough and peak compared to that observed on the $S_1 \Rightarrow S_2$ transition. This suggested a *negative charge change* on the first transition, which turned out to match the new release pattern later found in BBYs by Rappaport and Lavergne (1991), where more than one proton was released on $S_0 \Rightarrow S_1$ below pH 7. In

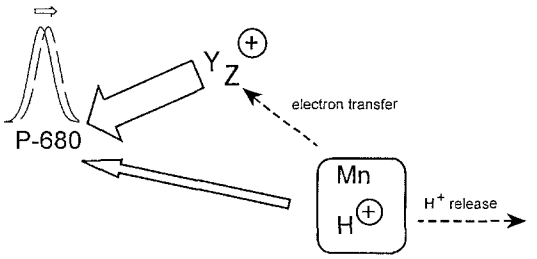

Fig. 3. A scheme rationalizing the electrochromic changes observed during the oxidation steps on the donor side of Photosystem II. The field-sensitive chlorophyll is assumed to be the primary donor P-680. Its spectral shift is larger when caused by the presence of a positive charge on Y_Z than on the, more remote, catalytic center ('Mn box'), as illustrated by the width of the open arrows. The contribution exerted by the Mn box depends on its net charge (balance of oxidation and deprotonation). Thus, a decrease in the shift may be caused by the electron transfer from the box to Y_Z^+ or by deprotonation of the box (see Fig. 5 for illustration of a case where deprotonation occurs first, followed by electron transfer).

the latter work, this agreement was extended by comparing the pH dependence of the electrochromic signal for each transition with the corresponding proton release stoichiometry. The two responses vary in a complementary way, suggesting that, as a first approximation, the electrochromic signal reflects the net electrostatic balance of charges in the catalytic center. Therefore, it was concluded (Rappaport and Lavergne 1991) that the chlorophyll probe is sufficiently remote from the catalytic center so that it is little sensitive to the geometrical details of charge distribution in this region. A second conclusion was that significant interference from other ion movements (e.g. Ca^{2+} release or Cl^- uptake) was unlikely.

The above remarks show that there is not much point in attempting to derive an 'intrinsic' proton release stoichiometry just from the electrochromic oscillations (Saygin and Witt 1985) rather than from direct proton measurements. The fact that the probe senses the global charge changes had to be experimentally demonstrated and could not be presumed a priori. Besides, it should be noticed that we have no way of calibrating the electrochromic response and that one can only deduce *relative* variations of the electrostatic potential. For instance, the electrochromic pattern cannot distinguish between the 1:0:1:2 model and the non-integer stoichiometry actually found around pH 7 (1:0.5:1:1.5).

Recent work by Van Leeuwen and Van Gorkom using core particles results in a puzzling picture. As mentioned above, these authors agree that the proton release pattern is approximately 1:1:1:1 in this material and different from the one in BBYs. However, the pattern obtained for the dependence of the blue electrochromic bandshift on the S-transitions and its pH titration (Van Leeuwen et al. 1992a) matched the results in BBY-membranes (Rappaport and Lavergne 1991). One thus faces the paradox that in core particles, this electrostatic response seems to become uncoupled from the proton release pattern, although it keeps the pH dependence obtained for this pattern in BBYs. A similar problem arises from Van Leeuwen's finding that the sub-μs electron transfer rate from Y_Z to $P\text{-}680^+$ has the same type of S-dependence as described by Brettel et al. (1984) for *Synechococcus* particles, suggesting an increase of the electrostatic potential sensed by Y_Z in the S_2 and S_3 states with respect to the S_0 and S_1 states, consistent with the deprotonation pattern of thylakoids or BBYs but inconsistent with the one in their material. Obviously, further work is needed to resolve this puzzle.

8. The kinetics of proton release

The rate of proton release is of interest for several reasons. It may allow us to decipher whether a protolytic step can be triggered by the positive charge on Y_Z^+ en route to the Mn cluster. The life-time of Y_Z^+, the product of the oxidation of Y_Z by $P\text{-}680^+$ in the sub-μs range, spans 50 μs to 1 ms, depending on the S-states (Dekker et al. 1984a, Lavergne et al. 1992, Renger and Hanssum 1992), which is in the time resolution range of pH indicating dyes. Kinetics of proton release might also provide information on intermediate steps during the $S_3Y_Z^+ \Rightarrow S_0Y_Z$ oxygen-evolving reaction. Finally, the pH dependence of these kinetics may help to characterize the groups involved, or to identify a possible binding of substrate OH^-.

8.1. Kinetic studies using the neutral red method

A first detailed study of the kinetics of proton release on the successive S-transitions was published by Förster and Junge (1985) (see also Förster et al. 1981), using neutral red. The main findings are as follows: There is a 1.2 ms phase accompanying the

$S_3 \Rightarrow S_4 \rightarrow S_0$ reaction, which is compatible with the rate of electron transfer in this transition. However, there is also a faster component (200 μs) which indicates that some proton transfer precedes the reduction of Y_Z^+. In the same line, the second flash, which promotes the step $S_2 \Rightarrow S_3$, causes proton release rising with a half time of 200 μs and therewith somewhat faster than electron transfer between the manganese center and Y_Z (300 μs, Dekker et al. 1984a). The rapid components of proton release in these two transitions have been attributed to the rapid dissociation and rebinding of protons during the transient oxidoreduction of Y_Z, caused by the prosthetic group proper, or by adjacent amino acid side chains. This interpretation is in line with observations in Tris-treated thylakoids that are unable to oxidize water but still carry out the reduction of P-680$^+$ by Y_Z. Proton release and proton uptake follows the oxidation and reduction of Y_Z (Renger and Voelker 1982, Förster and Junge 1984, Conjeaud and Mathis 1986).

Following the reappraisal of the responses of neutral red outlined earlier, new investigations were undertaken. Haumann and Junge (1993) observed the following: In unstacked thylakoids submitted to repetitive excitation (all S-states equally populated) more than 80% of the total extent of proton transfer to neutral red occurred as a fast phase with a rate proportional to the dye concentration. Indeed, the half-rise time ranged from 170 μs (5 μM neutral red) to 35 μs (27 μM) and further to 12 μs (100 μM). The latter situation is documented in Fig. 4. Such a concentration dependence is indicative of a bimolecular reaction between the indicator dye and the acid groups of the catalytic center. It implies, on the other hand, that the release of protons into the lumen is hindered by a rather high activation barrier, as if the acid groups were not in direct contact with water. It is noteworthy that the effective concentration of neutral red in the thylakoid lumen is 2 to 3 orders of magnitude larger than the average one in the suspension because of its adsorption at the membrane surface (Hong and Junge 1983). The result implied that under such conditions, the major portion of proton release can be triggered by Y_Z^+. Time resolution of proton transfer to neutral red in dark adapted thylakoids (S_1 initially populated) revealed fast proton transfer in all four transitions, $S_i \Rightarrow S_{i+1}$ (i = 0–3). On the oxygen evolving step, $S_3 \Rightarrow S_4 \rightarrow S_0$, there was an additional millisecond

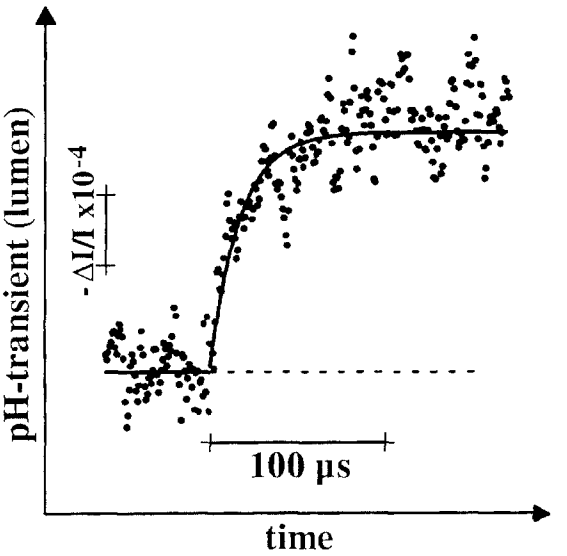

Fig. 4. Time course of proton transfer to neutral red from water oxidation. Repetitive excitation (0.1 Hz), unstacked pea thylakoids, 90 μM neutral red, 800 ns per address, analog electrical bandwidth 1 MHz. The curve is an exponential with 12 μs half rise time (Haumann and Junge 1993).

component, whose rate matched the one of $S_4 \rightarrow S_0$ and was independent of the concentration of neutral red. Most strikingly there was millisecond *release* of protons at pH 7.4 and millisecond *uptake* at pH 6.3. These observations were interpreted as follows: The primarily electrostatically driven deprotonation (rapid components) creates a set of 4–X (X < 1 at pH 7.4 and X > 1 at pH 6.3) bases during the transitions $S_0 \Rightarrow S_1 \Rightarrow S_2 \Rightarrow S_3 \Rightarrow S_4$. The effect of electron transfer from water to the manganese cluster during $S_4 \rightarrow S_0$ is then twofold: It liberates four protons and it resets the pKs of the previously formed bases into the alkaline direction. Only the excess of produced protons over bases at pH 7.4 is detectable as millisecond release and the excess of bases over produced protons at pH 6.3 as millisecond uptake of protons (Haumann and Junge 1993).

In reaction center core particles prepared according to Ghanotakis et al. (1984), proton release by water oxidation has revealed an apparent rise time of 100 ms (Lübbers and Junge 1990). This long time has been attributed to particle aggregation. The preparation after Van Leeuwen et al. (1991), on the other hand, seemed to be monodisperse. When the detergent concentration was increased, the rise time shortened to reach a plateau. Proton release as

fast as 10 μs was observed (O. Bögershausen, W. Drevenstedt and W. Junge, unpublished). In contrast with the situation in thylakoids, the rate was nearly independent of the concentration and type (chemical nature and pK) of the added hydrophilic indicator. Instead, the overall rate increased proportional to the free proton concentration in the suspension with half rise times of 70 μs at pH 6 up to 7 μs at pH 5. In the pH-domain from 8 to 6 the rise was less steep but the lowest rate, which was observed at pH 8 was still 10^3 s^{-1}. This behavior may be expected for the spontaneous deprotonation of water exposed groups. The evaluation of the kinetic spectrum of the four transitions and its interpretation in terms of a minimum number of acid groups involved is in progress. At least at more acid pH, the deprotonation is surely initiated when the positive charge still resides on Y_Z.

Taken together, the kinetic results from thylakoids and core particles suggest the following: The dependence of the thylakoid response on the concentration of neutral red indicates proton transfer by a bimolecular collision between the dye and proton releasing groups. The unperturbed deposition into the lumenal space (ms range) may only be extrapolated at low dye concentration. In the case of core particles, the response of hydrophilic dyes indicates, on the contrary, protolysis of acids in contact with the aqueous medium. This suggests that a proteinaceous shield, which is responsible for the slow final expulsion of protons in the thylakoids, has been removed in the core particles and that this shield is somewhat permeable to neutral red. The problem is open whether neutral red directly accepts protons from the primary groups interacting with the redox carriers, or with secondary groups located in the shield. The group of WJ favors the second hypothesis, assuming that initial proton release which is triggered by Y_Z^+ occurs rapidly. In thylakoids it is followed by trapping on secondary basic groups. Examples for proton transfer via intermediate carriers have been reported for the quinone site of reaction centers from purple bacteria (Okamura and Feher 1992, Takahashi et al. 1992) and for bacteriorhodopsin (Gerwert et al. 1990). In core particles, the removal of the shield allows the primary groups to react directly with water. The less hydrophobic environment is expected to decrease their initial pK and also to decrease the pK induced by the oxidative transition. Possibly, such modifications may account for the different stoichiometric patterns (oscillating vs. non oscillating) in thylakoids and core particles. It may also account for the observed narrowing of the pH-optimum for oxygen evolution in the reaction center preparation.

8.2. Kinetic studies using the electrochromic response

A different approach was developed by Lavergne et al. (1992) for studies with BBY-membranes. In this material the response of hydrophilic dyes turned out to be slow (50 ms range), presumably because of diffusion barriers. On the other hand, the electrochromic signal described earlier provides an instantaneous probe of the electrostatic changes in the vicinity of the oxygen-evolving complex, which has been shown (Rappaport and Lavergne 1991) to correlate with the proton release pattern. As already mentioned (see Fig. 3), the chlorophyll band shift is not only modulated by the extent of proton release, it is also sensitive to the position of the positive charge during the electron transfer from the catalytic centre to Y_Z^+. The decreasing electrochromic response during this reaction reflects the removal of the positive charge some distance away from the probe. In order to gain information on the kinetics of proton release, the kinetics of the electrochromic response on each of the $Y_Z^+S_i \Rightarrow Y_ZS_{i+1}$ steps was compared with the one of electron transfer monitored through UV absorption changes (Lavergne et al. 1992).

Similar time courses were found for the electrochromic response and electron transfer only on the $Y_Z^+S_1 \Rightarrow Y_ZS_2$ transition, suggesting in this case that proton release is concomitant with Mn oxidation by Y_Z^+. On the other transitions (with a possible exception of $S_2 \Rightarrow S_3$ where the electron transfer kinetics is poorly resolved due to its small amplitude at the wavelength used) the electrochromic decay is globally faster than electron transfer, indicating that electrostatic relaxation precedes the oxidation of the catalytic center. It should be noted that Lavergne et al. (1992) argue that the electron transfer reaction $Y_Z^+S_0 \Rightarrow Y_ZS_1$ is much slower than previously thought ($t_{1/2} \approx 250$ μs instead of 30–50 μs (Dekker et al. 1984a) or sub-μs (Van Leeuwen et al. 1992a).

Analysis of the kinetics of the $Y_Z^+S_0 \Rightarrow Y_ZS_1$ and $Y_Z^+S_3 \Rightarrow Y_ZS_0$ transitions (see Fig. 5 illustrating the case of the latter reaction) revealed a qualitatively similar pattern, with a *lag phase* in the 25–50 μs

range for electron transfer, corresponding to a *fast decay phase* of the electrochromic response. The rest of the kinetics is accounted for by a slower phase ($t_{1/2} \approx$ 250 μs and 1.2 ms for, respectively, $Y_Z^+S_0 \Rightarrow Y_ZS_1$ and $Y_Z^+S_3 \Rightarrow Y_ZS_0$) for both signals. The fast decay phase of the electrochromic response suggests a proton release step triggered by Y_Z^+, in agreement with the neutral red data described above. The concomitant lag on the electron transfer reaction indicates that this proton release step is required to promote the Y_Z^+ reduction. The interpretation proposed by F. Rappaport, M. Blanchard-Desce and J. Lavergne (unpublished) is that the deprotonation occurs on a group closer to the Mn cluster than to Y_Z so that it increases the driving force towards Y_Z^+ reduction (as illustrated by the scheme shown in Fig. 4).

The amplitude of the fast phase of the electrochromic decay ($t_{1/2} \approx$ 30 μs) on the $Y_Z^+S_3 \Rightarrow Y_ZS_0$ (O_2-evolving) transition cannot account for the total amount (1.5) of protons released in the whole process, but rather for about one proton. Therefore,

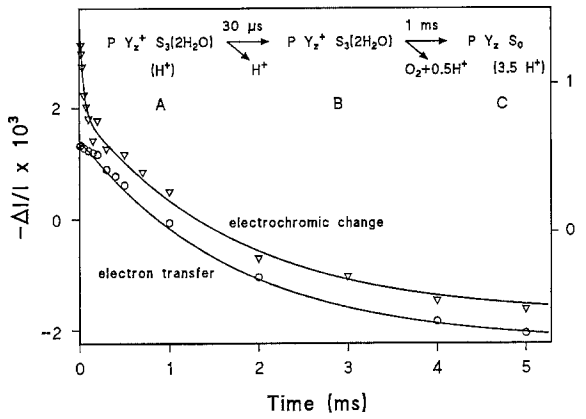

Fig. 5. Kinetics of the electron transfer reaction (bottom curve, open circles) and electrochromic response (top curve, open triangles) during the $Y_Z^+S_3 \Rightarrow Y_ZS_0$ transition in BBYs at pH 6.5. The electron transfer was monitored through absorption changes at 295 nm (left-hand ordinate scale) and the electrochromic signal as the difference of absorption changes at 440 nm and 424 nm (right-hand scale) (F. Rappaport, M. Blanchard-Desce and J. Lavergne, unpublished). The reaction scheme shown at the top has been proposed to account for these results. The release of one proton from the catalytic center is triggered by the electrostatic influence of Y_Z^+ (step A $\rightarrow$B, with $t_{1/2} \approx$ 30 μs). The lowered electrochemical potential of the catalytic center then allows the second (B $\rightarrow$C, $t_{1/2} \approx$ 1ms) step to take place, during which most (3.5) of the protons released by water oxidation are taken up by groups that went deprotonated during the preceding transitions.

the subsequent protolytic reactions involving the net release of 0.5 proton *and rebinding* of 3.5 protons to restore the Y_ZS_0 state occur concomitantly with the rate-limiting 1 ms phase accompanying O_2 release and S_0 reformation (see the scheme of Fig. 4). Thus, it turns out that some of the conclusions reached from the study of the electrochromic kinetics (Lavergne et al. 1992) are in nice agreement with those attained by WJ's research group from the neutral red experiments described above.

9. Conclusions

The pattern of proton release varies greatly depending on the type of preparation, although the progression of the catalytic center through the successive oxidation states driven by a series of flashes is similar. At the origin of this variability is presumably the different composition and/or conformation of the protein environment of the catalytic center. This periphery apparently modulates the electrostatic response to the deposition of a positive charge. Electrostatically driven deprotonation reactions are superimposed on chemically driven ones from bound water and, possibly, manganese ligands. The most rapid of the former reactions occur already when the positive charge still resides on Y_Z. In oxygen evolving reaction center core particles the rapid protolytic reactions appear to originate from groups at the water exposed surface, whereas in thylakoids they are shielded from the aqueous lumen by a proteinaceous cover. This cover may be involved in the phenomena related to proton sequestering domains and the short-circuiting of the proton pumping activity of PS II as induced by chemical modification of light-harvesting chlorophyll proteins. Deprotonation at the level of Y_Z is apparent for at least three, if not all four, S-state transitions (the question is still open for $S_1 \Rightarrow S_2$) both from time-resolved measurements of the pH transients and the electrochromic transients. It is an open question whether the deprotonation triggered by Y_Z^+ is only transient, being reversed and concomitantly replaced by a proton originating from a group closer to the manganese cluster. Evidence for kinetic components of proton release conforming with the rate of electron transfer from manganese to Y_Z is present to some extent in several transitions, but distinctly obvious on the oxygen-evolving step.

In this reaction, the fast phase (some tens of μs) reflects the release of one proton (or perhaps somewhat less) independent of pH, while the slow phase (ms range) accounts for the complement of the total release on this transition. Accordingly, at pH 7.4 (in thylakoids or BBYs), the ms-phase implies the additional release of about 0.5 protons, whereas at pH 6.3 (in thylakoids), it corresponds to an uptake. Clearly, most of the chemically produced protons from water oxidation are taken up by the bases that have been created on the foregoing oxidation steps and thus they escape kinetic resolution. There is a kinetic correspondence between a lag phase of electron transfer from manganese to Y_Z and a rapid phase of local electrochromism occurring in the time range of rapid deprotonation. This has been interpreted as a control of electron transfer by a deprotonation event, implying that the respective proton releasing group is closer to the manganese cluster than to Y_Z.

It has been convincingly argued that the electroneutral transfer of hydrogen atoms from water to electron/proton acceptor pairs (manganese plus base) is kinetically beneficial (Krishtalik 1990). As the number of bases created before the *oxygen-evolving step* varies between different sample preparations and as a function of pH, it will be interesting to study how this may affect the efficiency of this particular reaction step. More generally, it is somewhat difficult to reconcile the large variations observed for the stoichiometric pattern of proton release in different oxygen-evolving materials with the idea that the system must respect rather strict energetic constraints. A possibility to be explored in future research is that the local protolytic reactions and, hence, electrostatic conditions in the vicinity of the catalytic center remain more stable than suggested by the variability of the proton release pattern.

Acknowledgements

The authors are very grateful for the valuable experimental and intellectual input of their coworkers, especially of the present crew members: Fabrice Rappaport with JL, and Wolfgang Drevenstedt, Karin Lübbers, Michael Haumann and Oliver Bögershausen with WJ. J L wishes to thank Dr J-J Girerd for introducing him to the literature on the protonation of μ-oxo bridged manganese clusters. Financial support by the C.N.R.S. (to JL) and the Deutsche Forschungsgemeinschaft (TPB3-SFB171) and the Fonds der Chemischen Industrie (to WJ) is gratefully acknowledged.

References

Ausländer W and Junge W (1975) Neutral red, a rapid indicator for pH changes in the inner phase of thylakoids. FEBS Lett 59: 310–315

Berthold DA, Babcock GT and Yocum CF (1981) A highly resolved, oxygen-evolving Photosystem II preparation from spinach thylakoid membranes. FEBS Lett 134: 231–236

Bossek U, Weyhermueller T, Wieghardt K, Nuber B and Weiss J (1990) [L2Mn2(m-O)2(m-O2)](ClO4)2. The first binuclear (m-peroxo)dimanganese(IV) complex (L = 1,4,7-trimethyl-1,4,7-triazacyclononane). A model for the S_4–S_0 transformation in the oxygen-evolving complex in photosynthesis. J Am Chem Soc 112: 6387–6388

Bowes JM and Crofts AR (1981) The role of pH and membrane potential in the reactions of Photosystem II as measured by effects on delayed fluorescence. Biochim Biophys Acta 637: 464–472

Brettel K, Schlodder E and Witt HT (1984) Nanosecond reduction kinetics of photooxidized chlorophyll-a_{II} (P-680) in single flashes as a probe for the electron pathway, H^+-release and charge accumulation in the O_2-evolving complex. Biochim Biophys Acta 766: 403–415

Brudvig GW and De Paula JC (1987) On the mechanism of photosynthetic water oxidation. In: Biggins J (ed) Progress in Photosynthesis Research, pp 491–498. Martinus Nijhoff, Dordrecht

Buser CA, Diner BA and Brudvig GW (1992) Photooxidation of cytochrome b559 in oxygen evolving Photosystem II. Biochemistry 31: 11449–11459

Carroll JM and Norton JR (1992) Protonation of a bridging oxo-ligand is slow. J Am Chem Soc 114: 8744–8775

Conjeaud H and Mathis P (1986) Electron transfer in the photosynthesis membrane. Influence of pH and surface potential on the P 680 reduction kinetics. Biophys J 49: 1215–1221

Cooper SR and Calvin M (1977) Mixed valence interactions in di-μ-oxo bridged manganese clusters. J Am Chem Soc 99: 6623–6630

Debus RJ (1992) The manganese and calcium ions of photosynthetic oxygen evolution. Biochim Biophys Acta 1102: 269–352

Dekker JP, Plijter JJ, Ouwenand L and Van Gorkom HJ (1984a) Kinetics of manganese redox transitions in the oxygen evolving apparatus of photosynthesis. Biochim Biophys Acta 767: 176–179

Dekker JP, Van Gorkom HJ, Wensink J and Ouwehand L (1984b) Absorbance difference spectra of the successive redox states of the oxygen-evolving apparatus of photosynthesis. Biochim Biophys Acta 767: 1–9

Delosme R (1971) New results about chlorophyll fluorescence

in vivo. In: Forti G, Avron M and Melandri A (eds) Proceedings of the IInd International Congress on Photosynthesis, pp 187–195. Dr. W. Junk, The Hague

Dilley RA (1991) Energy coupling in chloroplasts: A calcium-gated switch controls proton fluxes between localized and delocalized proton gradients. Current Topics in Bioenergetics 16: 265–318

Eckert HJ and Renger G (1988) Temperature dependence of $P680^+$ reduction in oxygen-evolving PS II membrane fragments at different redox states S_i of the water oxidizing system. FEBS Lett 236: 425–431

Eigen M (1963) Protonenübertragung, Säure-Base-Katalyse und enzymatische Hydrolyse.Teil I: Elementarvorgänge. Angew Chem 12s: 489–588

Emrich HM, Junge W and Witt HT (1969) Further evidence for an optical response of chloroplast bulk pigments to a light-induced electrical field in photosynthesis. Z Naturforsch B 24: 1144–1146

Fowler CF (1977) Proton evolution from Photosystem II. Stoichiometry and mechanistic considerations. Biochim Biophys Acta 462: 414–421

Förster V, and Junge W (1984) Protolytic reactions at the donor side of PS II: Proton release in tris-washed chloroplasts with $t_{1/2} \times 100$ms. Implications for the interpretation of the proton release pattern in untreated, oxygen-evolving chloroplasts. In: Sybesma C (ed) Advances in Photosynthetic Research, pp 305–ff. Martinus Nijhoff/Dr W. Junk, The Hague/ Boston/Lancaster

Förster V and Junge W (1985) Stoichiometry and kinetics of proton release upon photosynthetic water oxidation. Photochem Photobiol 41: 183–190

Förster V, Hong YQ and Junge W (1981) Electron transfer and proton pumping under excitation of dark-adapted chloroplasts with flashes of light. Biochim Biophys Acta 638: 141–152

George GN, Prince RC and Cramer SP (1989) The manganese site of photosynthetic water-splitting enzyme. Science 234: 789–791

Gerwert K, Souvignier G and Hess B (1990) Simultaneous monitoring of light induced changes in protein side-group protonation, chromophore isomerization, and backbone motion of bacteriorhodopsin by time-resolved Fourier-transform infrared spectroscopy. Proc Natl Acad Sci USA 87: 9774–9778

Ghanotakis DF, Babcock GT and Yocum CF (1984) Structural and catalytic properties of the oxygen-evolving complex. Correlation of polypeptide and manganese release with the behavior of Z^+ in chloroplasts and a highly resolved preparation of the PS II complex. Biochim Biophys Acta 765: 388–398

Ghanotakis DF, Waggoner CM, Bowlby NR, Demetriou DM, Babcock GT and Yocum CF (1987) Comparative structural and catalytic properties of oxygen-evolving Photosystem II preparations. Photosynth Res 14: 191–199

Gutman M and Nachliel E (1990) The dynamic aspects of proton transfer processes. Biochim Biophys Acta 1015: 391–414

Haag E, Irrgang KD, Boekema EJ and Renger G (1990) Functional and structural analysis of Photosystem II core complexes from spinach with high oxygen evolution capacity. Eur J Biochem 189: 47–53

Haumann M and Junge W (1993) Extent and role of proton release by photosynthetic water oxidation in thylakoids: Electrostatic relaxation versus chemical production. Biochemistry (in press)

Hong YQ and Junge W (1983) Localized or delocalized protons in photophosphorylation? On the accessibility of the thylakoid lumen for ions and buffers. Biochim Biophys Acta 722: 197–208

Hong YQ, Förster V and Junge W (1981) A cyclic protolytic reaction around Photosystem II at the inside of the thylakoid membrane in DCMU-poisoned chloroplasts. FEBS Lett 132: 247–251

Hope AB and Morland A (1979) Proton translocation in isolated spinach chloroplasts after single-turnover actinic flashes. Aust J Plant Physiol 6: 1–16

Jahns P and Junge W (1989) The protonic shortcircuit by DCCD in Photosystem II. A common feature of all redox transitions of water oxidation. FEBS Lett 253: 33–37

Jahns P and Junge W (1990) Dicyclohexylcarbodiimide-binding proteins related to the short circuit of the proton-pumping activity of Photosystem II. Identified as light-harvesting chlorophyll *a/b*-binding proteins. Eur J Biochem 193: 731–736

Jahns P and Junge W (1992a) Thylakoids from pea seedlings grown under intermittent light: Biochemical and flash-spectrophotometric properties. Biochemistry 31: 7390–7397

Jahns P and Junge W (1992b) Proton release during the four steps of photosynthetic water oxidation: Induction of 1:1:1:1 pattern due to lack of chlorophyll *a/b* binding proteins. Biochemistry 31: 7398–7403

Jahns P and Junge W (1993) Another role of chlorophyll *a/b* binding proteins of higher plants: They modulate protolytic reactions associated with Photosystem II. Photochem Photobiol 57: 120–124

Jahns P, Polle A and Junge W (1988) The photosynthetic water oxidase: Its proton pumping activity is short-circuited within the protein by DCCD. EMBO J 7: 589–594

Jahns P, Lavergne J, Rappaport F and Junge W (1991) Stoichiometry of proton release during photosynthetic water oxidation: A reinterpretation of the response of neutral red leads to a non-integer pattern. Biochim Biophys Acta 1057: 313–319

Jahns P, Haumann M, Bögershausen O and Junge W (1992) Water oxidation: 1:1:1:1 proton-over-electron stoichiometry in CAB-protein depleted thylakoids and PS II core particles. In: Murata N (ed) Research in Photosynthesis II, pp 333–336. Kluwer Academic Publishers, Dordrecht

Joliot P and Kok B (1975) Oxygen evolution in photosynthesis. In: Govindjee (ed) Bioenergetics of Photosynthesis, pp 387–412. Academic Press, New York

Joliot P, Joliot A, Bouges B and Barbieri G (1971) Studies of System II photocenters by comparative measurements of luminescence, fluorescence and oxygen emission. Photochem Photobiol 14: 287–305

Junge W and Polle A (1986) Theory of proton flow along appressed thylakoid membranes under both non-stationary and stationary conditions. Biochim Biophys Acta 848: 265–273

Junge W and Witt HT (1968) On the ion transport system of photosynthesis: Investigation on a molecular level. Z Naturforsch 23b: 244–254

Junge W, Ausländer W, McGeer AJ and Runge T (1979) The buffering capacity of the internal phase of thylakoids and the magnitude of the pH changes inside under flashing light. Biochim Biophys Acta 546: 121–141

Junge W, Schönknecht G and Förster V (1986) Neutral red as an indicator of pH transients in the lumen of thylakoids: Some answers to criticism. Biochim Biophys Acta 852: 93–99

Kirilovsky DL, Boussac A, Van Mieghem FJE, Ducruet JM, Sétif P, Yu J, Vermaas WFJ and Rutherford AW (1992) Oxygen-evolving Photosystem II preparation from wild type and Photosystem II mutants of *Synechocystis* sp. PCC 6803. Biochemistry 31: 2099–2107

Kok B, Forbush B and McGloin M (1970) Cooperation of charges in photosynthetic O_2 evolution: I. A linear four-step mechanism. Photochem Photobiol 11: 457–475

Krishtalik LI (1986) Energetics of multielectron reactions. Photosynthetic oxygen evolution. Biochim Biophys Acta 849: 162–171

Krishtalik LI (1990) Activation energy of photosynthetic oxygen evolution: an attempt at theoretical analysis. Bioelectrochem Bioenerg 23: 249–263

Lavergne J (1987) Optical-difference spectra of the S-state transitions in the photosynthetic oxygen-evolving complex. Biochim Biophys Acta 894: 91–107

Lavergne J (1991) Improved UV-visible spectra of the S-transitions in the photosynthetic oxygen-evolving system. Biochim Biophys Acta 1060: 175–188

Lavergne J and Rappaport F (1990) On the stoichiometry of proton release by the oxygen-evolving system. In: Baltscheffsky M (ed) Current Research in Photosynthesis, Vol I, pp 873–876. Kluwer Academic Publishers, Dordrecht

Lavergne J, Blanchard-Desce M and Rappaport F (1992) Oxidation and deprotonation reactions in the Kok cycle. In: Murata N (ed) Research in Photosynthesis, pp 273–280. Kluwer Academic Publishers, Dordrecht

Lübbers K and Junge W (1990) Is the proton release due to water oxidation directly coupled to events at the manganese centre?. In: Baltscheffsky M (ed) Current Research in Photosynthesis, Vol I, pp 877–880. Kluwer Academic Publishers, Dordrecht

Lübbers K, Haumann M and Junge W (1993) Photosynthetic water oxidation under flashing light: Oxygen release, proton release and absorption transients in the near UV. A comparison between thylakoids and a reaction center core preparation. Biochim Biophys Acta (in press)

Machanda R, Thorp HH, Brudvig GW and Crabtree RH (1991) Proton coupled electron transfer in high-valent oxomanganese dimers: Role of the ancillary ligands. Inorg Chem 30: 494–497

Machanda R, Thorp HH, Brudvig GW and Crabtree RH (1992) An unusual example of multiple proton-coupled electron transfer in a high-valent oxomanganese dimer [(phen)2 Mn(III) (O)2 Mn(IV) (phen)2](Clo4)3;(phen= 1,10-Phenathrolin). Inorg Chem 31: 4040–4041

Maróti P and Wraight CA (1988) Flash-induced H+-binding by bacterial photosynthesis reaction centers: Influences of the redox states of the acceptor quinones and primary donor. Biochim Biophys Acta 934: 329–347

McPherson PH, Okamura MY and Feher G (1988) Light induced proton uptake by photosynthetic reaction centers from *Rhodobacter sphaeroides* R-26. I. Protonation of the one-electron-states. Biochim Biophys Acta 934: 348–368

Meyer B, Schlodder E, Dekker JP and Witt HT (1989) O_2 evolution and Chl a_{II}^+ ($P\text{-}680^+$) nanosecond reduction kinetics in single flashes as a function of pH. Biochim Biophys Acta 974: 36–43

Nanba O and Satoh K (1987) Isolation of a Photosystem II reaction center consisting of D-1 and D-2 polypeptides and cytochrome *b*-559. Proc Natl Acad Sci USA 84: 109–112

Okamura MY and Feher G (1992) Proton transfer in reaction centers from photosynthetic bacteria. Annu Rev Biochem 61: 861–896

Pecoraro VL (1988) Structural proposals for the manganese centers of the oxygen evolving complex: An inorganic chemist's prospective. Photochem Photobiol 48: 249–264

Penner-Hahn JE, Fronko RM, Waldo GS, Yocum CF, Bowlby NR and Betts SD (1990) X-ray absorption spectroscopy of the photosynthetic oxygen evolving complex. In: Baltscheffsky M (ed) Current Research in Photosynthesis, pp 797–800. Kluwer Academic Publishers, Dordrecht

Philouze C, Blondin G, Menage S, Auger N, Girerd JJ, Vigner D, Lance M and Nierlich M (1992) A novel manganese-oxygen cluster type with a chain Mn4 unit: Relation to the reaction center of Photosystem II. Angew Chem 104: 1634–1636

Polle A and Junge W (1986a) Transient and intramembrane trapping of pumped protons in thylakoids. The domains are delocalized and redox-sensitive. FEBS Lett 198: 263–267

Polle A and Junge W (1986b) The slow rise of the flash-light-induced alkalization by Photosystem II of the suspending medium of thylakoids is reversibly related to thylakoid stacking. Biochim Biophys Acta 848: 257–264

Polle A and Junge W (1989) Proton diffusion along the membrane surface of thylakoids is not enhanced over that in bulk water. Biophys J 56: 27–31

Rappaport F and Lavergne J (1991) Proton release during successive oxidation steps of the photosynthetic water oxidation process: Stoichiometries and pH dependence. Biochemistry 30: 10004–10012

Renger G (1988) On the mechanism of photosynthetic water oxidation to dioxygen. In: Vänngard T (ed) Chemica Scripta 28A, pp 105–109. Cambridge University Press, Cambridge

Renger G and Hanssum B (1992) Studies on the reaction coordinates of the water oxidase in PS II membrane fragments from spinach. FEBS Lett 299: 28–32

Renger G and Voelker M (1982) Studies on the proton release pattern of the donor side of system II: Correlation between oxidation and deprotonization of donor D_1 in Tris-washed inside-out thylakoids. FEBS Lett 149: 203–207

Rögner M, Dekker JP, Boekema EJ and Witt HT (1987) Size, shape and mass of the oxygen-evolving Photosystem II complex from the thermophilic cyanobacterium *Synechococcus* sp. FEBS Lett 219: 207–211

Saphon S and Crofts AR (1977) Protolytic reactions in Photosystem II: A new model for the release of protons accompanying the photooxidation of water. Z Naturforsch, C: Biosci 32C: 617–626

Sauer K, Vittal K, Yachandra WK, Britt RD and Klein MP (1992) The photosynthetic water oxidation complex studied by EPR and X-ray absorption spectroscopy. In: Pecoraro VL

(ed) Manganese Redox Enzymes, pp 141–175. VCH, New York

Saygin Ö and Witt HT (1985) Evidence for the electrochromic identification of the change of charges in the four oxidation steps of the photoinduced water cleavage in photosynthesis. FEBS Lett 187: 224–226

Schatz G and Van Gorkom HJ (1985) Absorbance difference spectra upon charge transfer to secondary donors and acceptors in Photosystem II. Biochim Biophys Acta 810: 283–294

Takahashi E, Maróti P and Wraight CA (1992) Coupled proton and electron transfer pathways in the acceptor quinone complex of reaction centers from *Rhodobacter sphaeroides*. In: Müller A, Diemann E, Junge W and Ratajczak H (eds) Electron and Proton Transfer in Chemistry and Biology, pp 219–236. Elsevier, Amsterdam

Theg SM and Homann PH (1982) Light-, pH- and uncoupler-dependent association of chloride with chloroplast thylakoids. Biochim Biophys Acta 679: 221–234

Theg SM and Junge W (1983) The effect of low concentrations of uncouplers on the detectability of proton deposition in thylakoids. Evidence for subcompartmentation and preexisting pH differences in the dark. Biochim Biophys Acta 723: 294–307

Thorp H, Sasneskhi JE, Brudvig GW and Crabtree RH (1989) Proton coupled electrons transfer in [(bpy)2Mn(O) 2Mn(bpy)2]3+. J Am Chem Soc 3: 9249–9250

Van Leeuwen PJ, Nieveen MC, van de Meent EJ, Dekker JP and Van Gorkom HJ (1991) Rapid and simple isolation of pure Photosystem II core and reaction center particles from spinach. Photosynth Res 28: 149–153

Van Leeuwen PJ, Heimann C, Dekker JP, Gast JP and van Gorkom HJ (1992a) Redox changes of the oxygen evolving complex in Photosystem II core particles as studied by UV spectroscopy. In: Murata N (ed) Research in Photosynthesis, pp 325–326. Kluwer Academic Publishers, Dordrecht

Van Leeuwen PJ, Heimann C, Kleinherenbrink FAM and van Gorkom HJ (1992b) Kinetics of electron transport on the donor side of spinach photosystem core particles. In: Murata N (ed) Research in Photosynthesis, pp 341–344. Kluwer Academic Publishers, Dordrecht

Velthuys B (1981) Spectrophotometric studies on the S-state transition of Photosystem II and of the interactions of its charged donor chains with lipid soluble anions. In: Akoyunoglou G (ed) Photosynthesis II. Electron Transport and Photophosphorylation, pp 75–85. Balaban International Science Services, Philadelphia

Velthuys BR (1988) Spectroscopic characterization of the acceptor state Q_A^- and the donor state S_2 of Photosystem II of spinach in the blue, red and near-infrared. Biochim Biophys Acta 933: 249–257

Wacker U, Haag E and Renger G (1990) Investigation of pH-change-patterns of photosystem-II membrane fragments from spinach. In: Baltscheffsky M (ed) Current Research in Photosynthesis, pp 869–872. Kluwer Academic Publishers, Dordrecht

Webber AN and Gray JC (1989) Detection of calcium binding by Photosystem II polypeptides immobilized onto nitrocellulose membrane. FEBS Lett 249: 79–82

Wille B and Lavergne J (1982) Measurement of proton translocation in the thylakoids under flashing light using a spin-labeled amine. Photobiochem Photobiophys 4: 131–144

Yachandra VK, Guiles RD, McDermott AE, Britt RD, Dexheimer SL, Sauer K and Klein MP (1986) The state of manganese in the photosynthetic apparatus. 4. Structure of the manganese complex in Photosystem II studied using EXAFS spectroscopy. The S1 state of the oxygen-evolving Photosystem II complex from spinach. Biochim Biophys Acta 850: 324–332