THERMODYNAMIC LIMITATIONS OF PHOTOSYNTHETIC WATER OXIDATION AT HIGH PROTON CONCENTRATIONS*
Ivelina Zaharieva, Jörg M. Wichmann, and Holger Dau
Freie Universität Berlin, FB Physik, Arnimallee 14, 14195 Berlin, Germany
Address correspondence to: Prof. Holger Dau, Freie Universität Berlin, FB Physik, Arnimallee 14, 14195 Berlin, Germany, Tel: +49 30 838 53581; Fax: +49 30 838 56299; E-mail: holger.dau@fu-berlin.de

In oxygenic photosynthesis, solar energy drives the oxidation of water catalyzed by a Mn4Ca complex bound to the proteins of Photosystem II (PSII). Four protons are released during one turnover of the water-oxidation cycle (S-state cycle) implying thermodynamic limitations at low pH. For proton concentrations ranging from 1 nM (pH 9) to 1 mM (pH 3), we have characterized the low-pH limits using a new experimental approach: a specific pH-jump protocol combined with time-resolved measurement of the delayed chlorophyll fluorescence (DF) after ns-flash excitation. Effective pH values are determined for low-pH inhibition of the light-induced S-state transitions: $pK_1 = 3.3 \pm 0.3$, $pK_2 = 3.5 \pm 0.2$, $pK_3 \approx pK_4 = 4.6 \pm 0.2$. Alkaline inhibition is not observed. An extension of the classical Kok model facilitates assignment of these 4 pH-values to specific deprotonation steps in the reaction cycle. Our results provide important support to the extended S-state cycle model and criteria needed for assessment of quantum chemical calculations on the mechanism of water oxidation. They also imply that in intact organisms, the pH in the lumen compartment hardly can drop below 5, thereby limiting the $\Delta$pH contribution to the driving force of ATP synthesis.

Light-driven water oxidation by plants, algae and cyanobacteria is a pivotal process in biological solar-energy conversion (1). Its evolutionary development, roughly 3.5 Billion years ago, has boosted life on Earth by facilitating the efficient use of water as a source of electrons and protons for the synthesis of energy-storing carbohydrates and biomass in general. The long-standing scientific interest in photosynthetic water oxidation recently has been invigorated by the vision of future technological systems which, akin to plants and cyanobacteria, use water as a substrate for light-driven formation of energy-rich and storable compounds (H2 or other fuel materials) (2-5). We believe that insights into energetics and reaction mechanisms of the natural paragon could provide inspiration and guidelines for the development of new technologies (6-8). However the biological process is understood only insufficiently (9).

In oxygenic photosynthesis, solar energy drives the oxidation of water and the accumulation of 'energized' electrons by reduction of quinones (Fig. 1). In this process, 4 electrons and 4 protons are removed from 2 molecules of water (10-11). The reaction is denoted as water oxidation because the electrons are removed from the oxides ($O^2-\text{)}$ of two 'substrate' water molecules, followed by O-O bond formation and dioxygen ($O_2$) release. In photosynthesis, the process is catalyzed by a Mn4Ca complex bound to the proteins of Photosystem II (PSII) (12-14). The light-driven oxidation of one specific tyrosine residue ($Y_Z$) in PSII provides the oxidant ($Y_Z^{1+}$) and thus the driving force. $Y_Z^{1+}$ is formed and reduced 4 times per released $O_2$ molecule; 4 protons are released into the aqueous phase. Accordingly the following net equation describes the process of photosynthetic water oxidation:

$$2 \text{H}_2\text{O} + 4 \text{Y}_Z^{1+} \rightarrow 4 \text{H}^+ + 4 \text{Y}_Z^0 + \text{O}_2 \quad (1)$$

Equation 1 implies that the driving force (which corresponds to the overpotential in electrochemical water oxidation) is diminished by the presence of the reaction products, namely dioxygen and protons, and approaches zero at high product concentrations (high $pO_2$, low pH). Equation 1 thus implies a principal thermodynamic limitation, but leaves open paths and mode of product backpressure (or product inhibition). Experimental studies are required to determine how energetics, efficiency and reaction kinetics of photosynthetic water oxidation relate to the diminished driving force at increased product content.
levels. The influence of elevated O2 concentrations has been investigated previously (15-16). In time-resolved X-ray experiments, no inhibition of the O2-evolution transition \( S_3 \rightarrow S_0 + O_2 \) was detected, up to \( pO_2 \) of 16 bar, suggesting that the Gibbs free-energy of any reaction intermediate is by more than 130 meV higher than for the product state \( (S_0 + O_2) \) (16), as recently confirmed by mass spectroscopy (17) and for intact cells (18). (Here and in the following we use, for simplicity, the term free energy or \( \Delta G \) instead of standard free energy or \( \Delta G^0 \).)

Joliot and Joliot discovered, for excitation with a sequence of saturating flashes of light, a periodic flash-number dependence of the oxygen evolution yield (19). To explain this periodicity, in 1970 the paradigmatic Kok cycle was proposed (Fig. 2A), which describes the sequential accumulation of 4 oxidizing equivalents (by the Mn complex) before O2 formation (20). It was found that the rate constants of electron transfer (ET) are only moderately pH dependent (21-22) so that suppression of water oxidation at low pH has remained unexplained. In EPR experiments, Styring and coworkers found a pH-dependent efficiency (or yield) of the individual S-state transitions (23), later (partially) confirmed in FTIR experiments (24). These studies represent major progress, but could not provide a complete and clear-cut characterization of the thermodynamic limitations at high proton concentration.

A crucial experimental difficulty lies in discrimination between the various influences on PSII which relate directly or indirectly to the proton concentration, namely: (i) influence on the efficiency (or yield) of individual S-state transitions, (ii) acceleration of S-state decay in the dark-interval between saturating flashes, (iii) transient or (iv) irreversible deactivation of PSII (damage). Using a new experimental approach, we will discriminate between (i) to (iv) and explore the thermodynamic limits of PSII water oxidation in a quantitative way. The results support an extension of Kok’s classical reaction cycle (25-26) and bear further mechanistic implications.

**EXPERIMENTAL PROCEDURES**

PSII membrane particles (27) were suspended in Buffer I (500 mM glycine betaine, 15 mM NaCl, 5 mM MgCl₂, 5 mM NaCl₂, 1.25 mM MES, 1.25 mM MOPS and 1.25 mM HEPPS, pH 6.4). Addition of Buffer II changed the pH from 6.4 to a desired value in the range between 3.0 and 8.9; it differed from Buffer I in the concentration of the 3 buffering substances (200 mM instead of 1.25 mM). Buffer III shifted back the pH to 6.4 (600 mM). The individual pH-jump protocols are schematically shown in Fig. S1 A-H.

The DF transients were recorded after ns-flash excitation (~5 ns, 532 nm, ≥2 mJ cm⁻², (28-29)). To obtain a low-noise signal which exhibits the typical flash-number dependence of dioxygen formation (DFₘₛ), the DF amplitude was averaged from 1 and 3 ms after the respective flash (28-30). For estimation of the efficiency of S-state transition at different pH, the DFₘₛ pattern for the flashes applied after the pH jump were simulated on basis of an adapted Kok model. To determine the \( pK \) values of the individual S-state transitions, the pH dependence of the resulting transition efficiencies were simulated assuming a titration-curve behavior (Eq. S5). For further details, see SD Materials and Methods.

**RESULTS**

In our experiment, we exploit a unique feature of water oxidation in PSII: Saturating ns-flashes of green Laser light (5 ns, 532 nm) can be used to drive the catalysts synchronously through its reaction cycle. The flashes 1 to 4 induce the transitions \( S_1 \rightarrow S_2 \) (1st flash), \( S_2 \rightarrow S_3 \) (2nd), \( S_3 \rightarrow S_0 \) (3rd), and \( S_0 \rightarrow S_1 \) (4th flash); subsequent flashes drive further rounds of the reaction cycle (Fig. 2A) (20,31). O₂ is formed only in the course of the \( S_3 \rightarrow S_0 \) transition. Therefore a period-of-four oscillations in the flash-number dependence of the O₂ yield is observed, ideally with maxima at flash 3, 7, 11, .. (20) (Fig. 2B). The damping and (phase) shift of maxima at higher flash-numbers reflect partial de-synchronization explainable by the less-than-unity quantum efficiency of the individual transitions, which has been described in terms of a miss probability (20). In Fig. 2B, two alternative methods for detection of the flash-number dependence of the O₂-formation yield are compared. The filled circles relate to detection by an oxygen electrode whereas the open circles describe the oscillations in the amplitude of the delayed fluorescence in the millisecond range after flash excitation (DFₘₛ) (28-30,32-33). We find that
the flash-number dependencies of the two signals are identical. Since detection by an oxygen electrode is incompatible with our pH-jump protocol, we have chosen the DF$_{ms}$ signal for detection of the O$_2$ flash pattern.

The timing diagram in Fig. 2C illustrates our experimental approach (see also Fig. S1). In Phase I (S-state preparation at pH 6.4), the OEC was advanced to the desired S-state by either zero (S$_1$), one (S$_2$), two (S$_3$), or three (S$_0$) saturating flashes spaced by 700 ms, a period ensuring a maximal transition probability (32) (that is, a minimal value of Kok's miss parameter). In Phase II (changed pH), the pH of the medium was changed by addition and rapid mixing of Buffer II, facilitating a jump towards the chosen pH-value (here in the pH-range from 3 to 9). At the changed pH, a single laser pulse initiated the next S-state transition. Subsequently the pH was brought back to pH 6.4 (by addition of Buffer III). In Phase III (probe flashes at pH 6.4), a train of laser pulses was applied (probe flashes, see Fig. 2C).

In Fig. 2D typical results are shown: the O$_2$ flash-patterns in the probe phase (at pH 6.4) for preceding pH jumps down to 4.2. Any shift (delay) of the maximum of the oscillations indicates a lowered efficiency of the transition that was initiated by a single flash during the pH-jump phase. For the S$_1$→S$_0$ transition (3rd flash) at changed pH, the flash pattern is delayed by one flash indicating an S$_3$→S$_0$ efficiency that is close to zero at pH 4.2. A smaller, but still sizeable delay is observed for a pH-jump in the S$_2$→S$_1$ transition, whereas the S$_1$→S$_2$ transition is only marginally affected and no delay is observed for S$_0$→S$_1$. For application of the 3rd (S$_3$→S$_0$) and especially the 4th flash (S$_0$→S$_1$), a decreased amplitude of the oscillations in the probe phase is observed, explainable by deactivation of a sizeable PSII fraction by the preceding low-pH jump.

Visual inspection reveals the order in the low-pH sensitivity of the individual transitions (S$_3$→S$_0$ > S$_2$→S$_1$ > S$_1$→S$_2$ > S$_0$→S$_1$) and partial deactivation (for 3rd and 4th flash at low pH). Curve-fitting of the probe pattern on the basis of the classical Kok-model facilitated quantification of (i) the relative transition probability (= efficiency) at the changed pH (Fig. 3) and (ii) the active fraction of PSII (Fig. 4).

The transition probabilities for the four S-state transitions at pH values ranging from 3 to 9 are shown in Fig. 3. Simulations based on the Henderson-Hasselbach equation (Eq. S5) result in effective pK values for the efficiency drop at low pH of 3.3 (1st flash, S$_1$→S$_2$), 3.9 (2nd, S$_2$→S$_3$), and 4.5 (3rd, S$_3$→S$_0$), whereas the efficiency of the S$_0$→S$_1$ transition ($\phi_{S0\rightarrow S1}$, 4th flash) is pH-independent. In the alkaline range, the efficiency is found to be maximal and pH-independent for all S-state transitions. (In Fig. 3, the numerical values of the transition probabilities of the 4th-flash transition at low pH are exactly equal to 100% and slightly lower at higher pH. This results merely from inadequacies of the used Kok-model to describe S-state deactivation and transient inactivation of PSII.)

In the above experiments, the pH differed from the standard pH (6.4) not only during the flash-induced transition itself but also for 1.5 s before and after the flash. A discrimination between accelerated S-state decay at low pH (34) and the pH-sensitivity of the light-induced transition itself requires additional experiments. To assess and quantify the influence of pH exposure in the dark, we used the protocol shown in Fig. 2C but without flash-application in the pH-jump phase. Specifically for PSII poised in the S$_3$ state, strong acceleration of the S-state decay is observed, a process describable by an effective pK of 3.7 (Fig. S2).

A reduction in the amplitude of the probe-phase oscillations is observed only for low-pH exposure of PSII poised in the S$_0$ state, indicating formation of an inactive S$_0$ state (S$_{0\#}$). The extent of S$_{0\#}$ formation upon dark-exposure to low pH for 3 s is shown in Fig. 4, suggesting an apparent pK of 4.6. Notably, S$_{0\#}$ formation is slowly reversed at the control pH indicating that formation of this state does not occur by irreversible 'damage' to PSII (Fig. S4). For all other S-state, the short time period of exposure to low and high pH (3 s) precludes transient or permanent inactivation of PSII, as confirmed by an O$_2$-activity assay (Fig. S7).

Our finding of optimal efficiency of the S$_0$→S$_1$ transition also at low pH (Fig. 4) is in conflict with previous reports (23-24) (detailed quantitative comparison to the previously obtained pK values in Supplemental Data). Previously a clear
separation between reduced transition efficiency and PSII inactivation in the S₀ state had not been achieved. Therefore PSII inactivation by S₀ transition could mimic a reduced efficiency of the S₀→S₁ transition in these earlier studies.

On the basis of our results on the pH-dependence of S-state deactivation (pH-jump without flash), refined simulations were approached which facilitated a correction for S-state decay of the data shown in Fig. 3 (see SD for details). The thereby obtained pH dependence of the efficiency of the light-induced S-state transition itself (Fig. 3, red lines) represents a central result of our study. The half-inhibition pH of the S₁→S₂ transition is determined to be 3.3±0.3; for the S₂→S₁ transition the value is 3.5±0.2. The low-pH efficiency drop of the S₁→S₀ transition is characterized by an effective pK of 4.6±0.2. We note that for the S₃→S₀ transition a Hill coefficient of 2 (n=2 in Eq. S5, steeper red line in Fig. 3) results in a better description than a Hill coefficient of 1. (Also the 1ˢᵗ flash data might be simulated better assuming that n equals 2. However at these extremely low pH values, the data is less reliable because other pH-dependent process might start to interfere. Therefore we used a conservative simulation approach for this transition, that is, a n-value of unity.)

Up to this point, the analysis has been based exclusively on the oscillation pattern of the ms-fluorescence in the probe phase. Now we turn to the DF decays detected within 10 µs to 60 ms after the Laser flash applied at the changed pH. These DF decays carry unique mechanistic information on the efficiency loss at low pH. The competition between forward reaction (i.e. the S-state transition) and losses by charge recombination is a major determinant of the transition efficiency (or transition yield, \( \Phi_s = 1 - m_r \)). The DF decays reflect the competing charge recombination losses. Therefore the time integral of the DF fluorescence provides a relative measure of the probability for miss events by charge recombination (32).

In Fig. 5, the integrated delayed fluorescence is compared to the transition efficiency (from red lines in Fig. 3, corrected for the S-state dark decay). For the S₁→S₂ and S₂→S₁ transitions, a good correlation is observed implying that the efficiency drop at low-pH indeed is explainable by competition between pH-dependent forward reactions and recombination processes (Fig. 5, upper panel). For S₁→S₀, however, this correlation breaks down at low pH (Fig. 5, lower panel) suggesting that a pH-limitation comes into play which does not result in charge recombination within the 60 ms time window of the DF measurement. Therefore we propose that there are two deprotonation-limited processes in the S₁→S₀ transition: the first one competing with fast charge recombination (pK slightly higher than 4.6) and a second one unrelated to rapid recombination losses (pK slightly below 4.6). The existence of two pH-dependent process of similar pK also explains the steep pH dependence of the efficiency drop at low pH (Hill coefficient of 2, see Fig. 3).

In summary, we assessed the influence of proton concentrations ranging from 1 nM (pH 9) to 1 mM (pH 3) on: (a) the efficiency of distinct S-state transitions, (b) the rate of S-state decay, and (c) deactivation by short-term exposure to extreme pH values. The used experimental approach facilitated full discrimination between the three above mentioned types of influences. Accelerated S-state decay is observed only in the S₀-state (Fig. S2, pK ~3.7). We did not detect any low-pH inhibition of the S₀→S₁ transition itself, however discovered that low-pH exposure of PSII in the S₀-state results in transient formation of an inactive state (S₀̅). Detailed characterization of this interesting state requires further investigation. In all other S-states, neither low-pH nor high-pH exposure (for 3 s) causes transient deactivation or lasting damage.

**DISCUSSION**

For three transitions between semi-stable states of the reaction cycle, effective pK values for the efficiency decrease of the light-induced transition at high proton concentrations have been determined: 3.3±0.3 (S₁→S₂), 3.5±0.2 (S₂→S₃), 4.6±0.2 (S₃→S₀, Hill coefficient ~2). Reduced transition efficiencies in the alkaline pH range are not observed, inter alia proving the insensitivity of our experiment to deceleration of the quinone chemistry at the acceptor side of PSII.

The efficiency decrease at low pH relates to the competition between forward reactions (S-state transitions) and recombination processes. At low pH, the forward reactions are blocked and the
yield for recombination reactions is increased. Comparison to the integrated recombination fluorescence indeed suggests that lacking deprotonation at low pH results in increased charge recombination thereby preventing the ET from the Mn complex to the Y2^{+}, for all three pH-dependent transitions (Fig. 5). Only in S3→S0, a process may be involved for that the observed low-pH block is not explainable by charge recombination within the experimentally accessed time range of 10 ms to 60 ms after flash excitation. This process likely involves the blockade of a later step in the reaction sequence that leads from the S3 to the S0 state. We propose that due to this low-pH blockade, a state is formed that decays either by slow charge recombination, within hundreds of milliseconds, or by a similarly slow one-electron side reaction.

Transitory inactivation by S0\textsuperscript{-} formation. In our study, an pK value of 4.6 is determined for the formation of the inactive S0\textsuperscript{-} state that is induced by low-pH exposure for about 3 s (Fig. 4). We note that the value of 4.6 reflects merely an apparent pK because, as indicated by the experiments of Fig. S4, low-pH exposure for 3 s is insufficient for reaching the equilibrium level of inactivation. The equilibrium pK rather may be close to 5.0 (Fig. S4). How does the protonation reaction associated with S0\textsuperscript{-} formation in the dark (within seconds) relates to the transitory S0\textsuperscript{-} formation in the flash-induced S3→S0 transition (see Fig. 6)? The formation of S0\textsuperscript{-} at low pH and its decay at the control pH, both take several seconds and thus are by about four orders of magnitude slower than formation and decay of S0\textsuperscript{+} (in the μs/ms domain). This observation strongly suggests that S0\textsuperscript{+} and S0\textsuperscript{-} are not identical states of the Mn complex. Nonetheless the same protonatable group or hydrogen-bond cluster may be involved in both S0\textsuperscript{+} and S0\textsuperscript{-} formation.

Extension of the Kok-cycle model and assignment of pK values. The here determined effective pK-values likely are related to deprotonation steps occurring at the catalytic site, that is the Mn complex and in its ligand environment. Any deprotonation at more distant sites would be unfavorable for the kinetics and energetics of the water-oxidation chemistry (10,35). Assignment to specific deprotonation steps in the light-driven reaction cycle of photosynthetic water oxidation is facilitated by an extended S-state model that describes not only the electron-transfer steps (from the Mn complex to Y2) but also essential deprotonation steps (proton transfer from the catalytic site to the aqueous phase; see (10,25-26) and Fig. 6).

Time-resolved X-ray (31,36) and other experiments (22,37) suggest that in the S3→S0 transition, a deprotonation step (S3\textsuperscript{+}→S3\textsuperscript{n}, pK2) precedes electron transfer, O-O bond formation and a second deprotonation step (S0\textsuperscript{n}→S0\textsuperscript{+}, pK4). Our results on the S3→S0 transition imply effective values for pK3 and pK4 close to 4.6. Also in the S2→S3 transition, proton removal from the Mn complex precedes the ET step (S3\textsuperscript{+}→S2\textsuperscript{+}) and predicts inhibition at low pH values; we find that pK3 likely is close to 3.5. The pH-independence of the S0→S1 transition is explainable by a rapid ET step (S0\textsuperscript{n}→S1\textsuperscript{n}) that precedes deprotonation (S1\textsuperscript{+}→S1\textsuperscript{n}). How does low-pH blockage of the latter deprotonation steps affect the efficiency of water oxidation?

The S1→S2 transition itself is not associated with proton release and thus predicted to be pH-independent. The here found low-pH inhibition is, nonetheless, explainable within the framework model of Fig. 6: At low pH the S1\textsuperscript{n} state is formed thereby preventing induction of the S1\textsuperscript{+}→S2\textsuperscript{+} transition by a Laser flash. This conjecture is plausible and supported circumstantially by a several-fold increase in the initial level of the 1s\textsuperscript{+}-flash DF decay observable at pH values below 4 (Fig. S6).

Alkaline pH results in an increased driving force for water oxidation so that, from the viewpoint of thermodynamics, high-pH inhibition is not anticipated and indeed not observed in our investigation. The here reported findings are consistently explainable within the framework provided by reaction cycle of Fig. 6 (25-26), thus supporting the proposed basic sequence of ET and proton removal steps circumstantially.

Mechanistic implications. Mostly due to the non-equilibrium character of the light-induced processes, the here determined effective pK-values (pK1, 3.3; pK2, 3.5; pK3 and pK4 close to 4.6) might differ from the actual pK of the deprotonating group, but likely by less one pK-unit (estimate on basis of simple kinetic modeling). We tentatively assign pK1 to μ-hydroxo deprotonation and pK2 to deprotonation of a water species.
terminally ligated to Mn IV paralleled by µ-oxo bridge formation (10,38-39). For pK₃, assignment to CP43-Arg357 has been proposed (36,40); pK₄ might be related to deprotonation of a substrate water (after water ligation to Mn). However, also other protonatable groups could be involved, e.g. carboxylate sidechains like the Asp61 of the D1 protein. Proton shifts directly coupled to the ET step could complicate the assignment. Definitive identification of the 4 deprotonating groups represents an important task for future work; computational chemistry could contribute (41-42).

The pK value of the deprotonating group (pKᵢ) relates to the binding energy of the proton and facilitates calculation of the relative free-energy drop, $\Delta G^{H_i}$, associated with the respective deprotonation step (8,10):

$$\Delta G^{H_i} \approx 0.06 \text{ eV (pKᵢ - pH)} \approx 13.5 \text{ kcal/mol (pKᵢ - pH)} \ (2)$$

Computational approaches will become increasingly consequential in formulating and testing mechanistic models (41-42). For a specific reaction path (Fig. 14 in (43)), numerical calculation of the 4 $\Delta G^{H_i}$ resulted in values of -4.6, +8.1, +4.1, and -2.4 kcal mol⁻¹ (at pH 7), whereas the experimental pKᵢ-values suggest a clearly different set of energies (approximately -5, -5, -3, and -3 kcal/mol, according to Eq. 2). In another study the discrepancy between calculated and experimentally determined values was smaller, but still sizeable (44). This example illustrates that the 4 experimental pKᵢ-values, even though still imprecise, represent a criterion for evaluation of computed reaction paths.

**Water oxidation in intact organisms.** Product backpressure limits water oxidation by PSII already at surprisingly low proton concentrations. Between pH 4.5-5.5, reduced efficiency results from inhibition of two steps in the transition $S_3 \rightarrow S_0 + O_2$ (being $S_3^+ \rightarrow S_1^+$, pK₃, and $S_0^- \rightarrow S_0^0$, pK₄) as well as from transient deactivation by $S_{0\text{irr}}$-state formation. Only at clearly lower pH values, inhibition of the $S_2 \rightarrow S_3$ and $S_1 \rightarrow S_2$ transition as well as accelerated $S_3$-state decay would come into play, whereas the $S_0 \rightarrow S_1$ transition is fully pH-independent.

For intact plants illuminated at saturating light intensities, the pH in the lumen compartment and thus at the donor side of PSII has been estimate to be 5.7±0.5 (45). Accordingly, two steps in the transition $S_3 \rightarrow S_0 + O_2$ work close to their respective thermodynamic limits, whereas a clear free-energy surplus ($\Delta G^{H_i}$, Eq. 2) characterizes all other S-state transitions.

Acidification of the lumen compartment by the PSII donor side provides an essential contribution to the driving force of ATP synthesis, the so-called proton motive force (pmf) (1). The described thermodynamic limitations of the $S_3 \rightarrow S_0$ transition imply that the lumen pH can not drop much below pH 5, thereby limiting the driving force for ATP synthesis severely. (A high transthylakoid ∆pH might also come from alkalization of the stroma. However, the comparatively large volume and buffer capacity of the stroma space likely prevents a pronounced increase in the stroma pH.) To compensate, plants apparently control their transthylakoid ion fluxes such that a major fraction of the pmf stems from the membrane voltage formed upon illumination (45-46). Moreover, any mechanism of physiological relevance for the pH-dependent down-regulation of PSII (47-48) needs to have a threshold-pH higher than 5 (49), to be effective.

Product backpressure by dioxygen at elevated partial is physiologically irrelevant in oxygenic photosynthesis (16-18). In clear contrast, photosynthetic water oxidation is severely inhibited already at moderately increased proton concentrations. Our differentiated assessment of the thermodynamic low-pH limits has provided (i) insights into energetics and reaction cycle of the catalyst, i.e. the Mn₄Ca complex of PSII, and (ii) into the bioenergetics of oxygenic photosynthesis in general. Future progress could come from tracking the proton release in the time domain and from identification of the chemical identity of the deprotonating groups.
# REFERENCES


**FOOTNOTES**

*We thank Dr. M. Haumann for discussion and M. Fünning for preparing PSII particles. Financial support by the Deutsche Forschungsgemeinschaft (SFB 498, Berlin), the UniCat cluster of excellence (Unifying Concepts in Catalysis, Berlin), the German Ministry of Education and Research (BMBF; Bio-H₂, 03SF0318C and H₂ design cell, 03SF0355D), and the European Union (SOLAR-H₂, #516510) is gratefully acknowledged. IZ was generously supported by a fellowship of the Alexander-von-Humboldt foundation.

The abbreviations used are: DF, delayed fluorescence; ET, electron transfer; pmf, proton motive force; PSII, Photosystem II.
**FIGURE LEGENDS**

**Fig. 1.** Electron transfer and location of redox factors in Photosystem II (PSII) (13-14). Photon absorption is followed by formation of the primary radical pair, P680⁻Phe⁻. Subsequently at the (electron) acceptor side of PSII, Phe⁻ reduces the primary quinone acceptor, QA; at the donor side of PSII, P680⁺ is reduced by a redox-active tyrosine denoted as YZ. These processes are completed within less than 1 µs. Subsequently YZ ox oxidizes the catalytic center of water oxidation comprising an inorganic Mn₄Ca(µ-O)n core, coordinating residues and bound water molecules, whereas at the acceptor side an electron is transferred from QA to QB, the secondary quinone acceptor. Schematically indicated are the binding of two water molecules and the release of four protons. In intact organisms, this proton release results in acidification of the lumen compartment, which is separated from the stroma by the thylakoid membrane. At the acceptor side, the protonation of QB causes alkalization of the stroma. In plants and cyanobacteria, the thylakoid pH gradient resulting from lumen acidification and stroma alkalization contributes to the driving force of ATP synthesis.

**Fig. 2.** S-state cycle model and pH jump experiment. (A) Starting in the dark-stable S₁ state, each saturating flash of visible light initiates a sequence of electron transfer steps and eventually drives the S₁→S₁₊ transition. Each Sᵢ→Sᵢ₊ transition involves oxidation of the Mn complex, a protein-bound Mn₄Ca(µ-O)ₙ complex and its ligand environment. Water oxidation and O₂ formation follows S₄ formation induced by the third flash applied to dark-adapted PSII. Later we will extend Kok's S-state cycle model by taking into account explicitly also the removal of 4 protons from the Mn complex. (B) The flash-number dependence of the O₂-formation yield was detected for PSII particles on a Clark-type electrode (filled symbols). The amplitude of the delayed chlorophyll fluorescence (DF) at about 2 ms after a saturating Laser flash provides an alternative measure of the relative O₂-yield (open symbols). The maximal O₂-yield at the 3rd and 7th flash is explained by the S-state cycle in A. (C) Experimental pH jump protocol. (D) Representative data. The DF amplitude at 2 ms after Laser-flash application (DFₐₘ) is shown for the flashes of the 'probe phase' (at pH 6.4). The changed pH of 4.2 was present only during application of one specific flash of the sequence, namely during the 1st flash (curve labeled as S₁→S₂), 2nd (S₂→S₃), 3rd (S₃→S₀), and 4th (S₀→S₁). There was no pH change in the 'control' data. Inhibition of the S₃→S₀ transition at pH 4.2 results in a shift of the maximum from the 7th to the 8th flash (arrow in D).

**Fig. 3.** pH-dependence of the transition efficiency. The pH had been different from the standard value of 6.4 only for the indicated flash-induced transition (protocol of Fig. 2C). The resulting flash pattern (exemplary data in Fig. 2D) was simulated using an adaptation of Kok's model resulting in the shown transition probabilities, at the changed pH (open black circles). The black lines were obtained by calculation of standard pH-tritration curves for the indicated pK-values (the pK corresponds to the half-inhibition pH). The data also was corrected for S-state decay and deactivation (circles filled in red). Simulation of the corrected data by pH titration curves resulted in the shown pH values. For S₀→S₁, the transition efficiency is pH-independent.

**Fig. 4.** Inactivation by ΔpH-exposure of PSII residing in one specific S-state (filled symbols) or during Laser-flash application (open symbols). The filled symbols refer to the fraction of active PSII for an experiment in which the pH was changed for 3 s without application of any Laser flash during these 3 s. The indicated S-state had been populated before at pH 6.4 (protocols E-H in Fig. S1). The open symbols refer to the fraction of active PSII after application of a Laser flash at the changed pH inducing the indicated S₁→S₁₊ transition (A-D in Fig. S1). Inactivation of PSII was observed only if PSII in the S₀ state was exposed to high proton concentrations (low pH). The error bars represent standard errors (3-7 repetitions per pH value).
**Fig. 5.** Comparison of the integrated DF (closed symbols) and the transition efficiency (open symbols) of individual S-state transitions ($S_1\rightarrow S_2$, circles; $S_2\rightarrow S_3$, squares; $S_3\rightarrow S_0$, circles). We note that the open symbols represent the miss probability, $m_s$, that is related to the transition efficiency, $\Phi_s$, according to $m_s=1-\Phi_s$. The integrated DF represents a relative measure of the recombination losses occurring after the Laser flash (within the data acquisition period of 10 µs to 60 ms). For all shown data points, the DF integral was scaled by the same factor.

**Fig. 6.** Basic reaction cycle of photosynthetic water oxidation. The classical Kok model is extended to describe electron and proton removal from the Mn$_4$Ca complex and its ligand environment. The charge and oxidation states of the Mn complex are described by an S-state nomenclature in which the subscript indicates the number of accumulated oxidation equivalents and the superscript indicates the charge relative to the dark-stable $S_1$-state (+, positive; $n$, neutral). The states $S_1^-$ (dark-stable), $S_2^+$, $S_3^+$, and $S_0^-$ correspond to the states $S_1$, $S_2$, $S_3$, and $S_0$ of the classical Kok cycle; $S_3^-$ or $S_4^+$ may correspond to Kok’s $S_4$-state. Each proton is removed from the catalytic site; estimates of the corresponding pK values (pK$_1$ to pK$_4$) are obtained in the present investigation. At low pH, the $S_0$ state is reversibly inactivated by $S_{0\text{liq}}$ formation. The interrelation between the protonation reaction associated with pK$_4$ and pK$_{4\text{liq}}$ is still unclear.

We note that the ET from the Mn complex to $Y_Z^{\text{ox}}$ and the long-distance proton movement towards the aqueous phase cannot proceed in a concerted mode. However, direct coupling of the ET to local proton shifts is conceivable and not covered by the scheme.
Figure 2

A diagram illustrating the reaction cycle of P680 with oxygen yield and pH changes.

- Flash sequence: 4, 8, 12...
- Dark-stable state
- Flash sequence: 1, 5, 9...
- Flash sequence: 2, 6, 10...

Figure 3

Graph showing transition efficiency [%] with pH range from 3 to 9.

- pK 3.3: 1st flash
- pK 3.9: 2nd flash
- pK 4.5: 3rd flash
- pK 4.6: 4th flash

Graph of Oxygen Yield [r.u.] vs. Flash Number.

- Probe Flashes: 4, 8, 12...
- Probe-Phase O2 Pattern (pH 6.4)

Graph showing changes in pH with time.

- pH changes from 3 to 9
- Time [s]: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 26

Graph of O2 yield (DF_{ms}) vs. pH.

- Control
- S0 → S1
- S1 → S2
- S2 → S3
- S3 → S0
- S0 → S1
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*J. Biol. Chem.* published online April 4, 2011

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