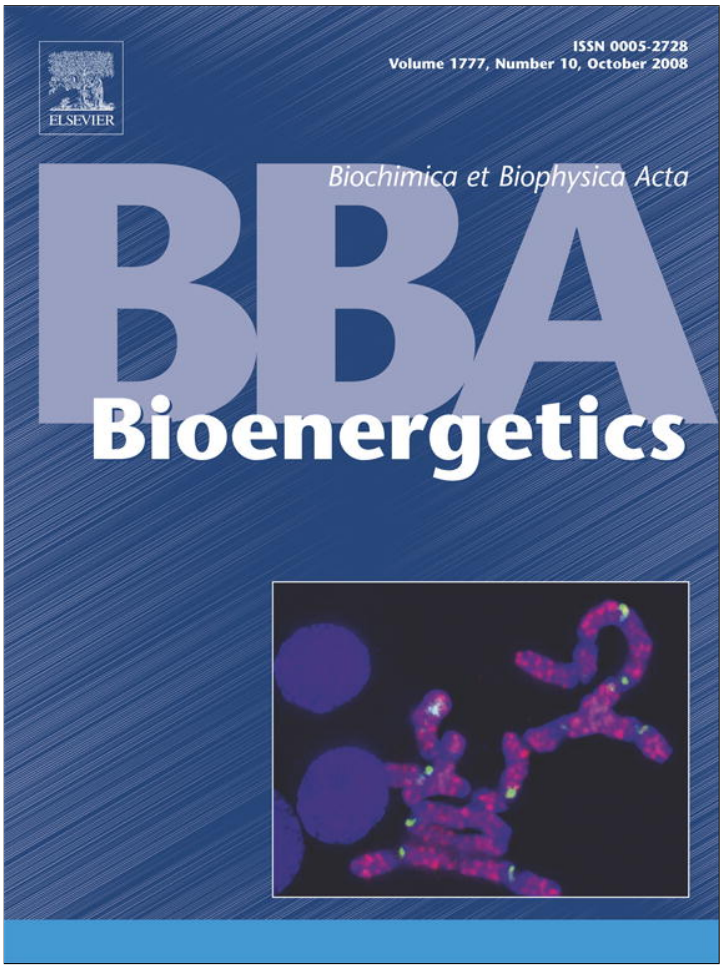

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The terminal reaction cascade of water oxidation: Proton and oxygen release

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ABSTRACT

In cyanobacteria, algae and plants Photosystem II produces the oxygen we breathe. Driven and clocked by light quanta, the catalytic Mn_4Ca-tyrosine centre accumulates four oxidising equivalents before it abstracts four electrons from water, liberating dioxygen and protons. Aiming at intermediates of the terminal four-electron cascade, we previously have suppressed this reaction by elevating the oxygen pressure, thereby stabilising one redox intermediate. Here, we established a similar suppression by increasing the proton concentration. Data were analysed in terms of only one (peroxy) redox intermediate between the fourfold oxidised Mn_4Ca-tyrosine centre and oxygen release. The surprising result was that the release into the bulk of one proton per dioxygen is linked to the first and rate-limiting electron transfer in the cascade rather than to the second which produces free oxygen. The penultimate intermediate might thus be conceived as a fully deprotonated peroxy-moiety.

1. Introduction

Photosynthetic water oxidation takes place in Photosystem II (PSII), a multiprotein-complex of about 30 subunits [1]. Absorption of light initially oxidises a chlorophyll *a* entity (P_{680}) (for a recent survey see [2]) to yield $P_{680}^{+\cdot}$. $P_{680}^{+\cdot}$ oxidises a redox-active tyrosine (Y_Z) within nanoseconds, and further on, the catalytic Mn_4CaTyr-centre. Excitation with single-turnover flashes of light stepwise oxidises the Mn_4CaTyr-centre in a cyclical process (for recent reviews, see [3–7]). In the dark, the centre is reset into its first oxidation state S_1, so that it reaches the fourth oxidation state, S_4, after excitation with the third in a series of short flashes of light. Only thereafter it reacts with bound water (or water derivatives) to release dioxygen and protons in a still ill characterised reaction cascade. It necessitates the transfer of four electrons into the Mn_4CaTyr-centre, resetting S_4 into S_0. The rate-limiting step of this cascade relaxes typically in 1.3 ms, and it dominates both, the first reactions (i.a. the electron transfer to the oxidised tyrosine [8–13] and the last (the appearance of oxygen in the medium [14–19]). Intermediates are apparently short-lived, and this is why they have been difficult to detect.

This work aims at characterising short-lived intermediates of the terminal reaction cascade by a thermodynamic approach. Aside from water, being both educt and solvent, only the products, dioxygen and protons, are diluted in the bulk, whereas the other reagents are solid. With this in mind, we have previously shifted the internal equilibrium of the reaction cascade by elevating the oxygen partial pressure. The suppression of oxygen evolution has been tentatively attributed to the stabilisation of a peroxy-intermediate [20,21]. Half-suppression occurred by an only tenfold increase (2.1 bar) over the ambient oxygen pressure (0.21 bar). Here it is attempted to elicit a similar effect by increasing the proton concentration. A minimal construct capable of photosynthetic oxygen production from water was used, namely Photosystem II core particles from the cyanobacterium *Synechocystis* sp. PCC 6803 containing a total of 35 chlorophyll molecules [22]. UV-absorption transients at 360 nm were monitored. They are partially attributable to redox transitions of the Mn cluster [11–13]. The distinct millisecond component detected after the third light flash in a series was taken as diagnostic for oxygen evolution [20,23]. And this signal, coined the “millisecond phase” in the following, was investigated under variation of both, the pH and the oxygen pressure.

2. Materials and methods

Cells of the modified “wild-type” (WT*) [24] of *Synechocystis* sp. PCC 6803 were cultivated as described in [25] but in an atmosphere enriched with 4% CO_2 under illumination with (cool-white) fluorescent light at 0.8–1.4 mW cm^{-2}. The medium was supplemented with 15 mM glucose. Oxygen evolving PSII-core particles were prepared as described previously [26]. They contained a total of 35 chlorophyll molecules [22]. Nine batch preparations of core particles were pooled and stored at −80 °C. Aliquots were thawed and suspended at a chlorophyll concentration of 9 μM in a standard medium containing 0.2 mM 2,5-Dichloro-*p*-benzoquinone (DCBQ) as an electron acceptor, and the pH was adjusted by adding either of two buffers. For the pH

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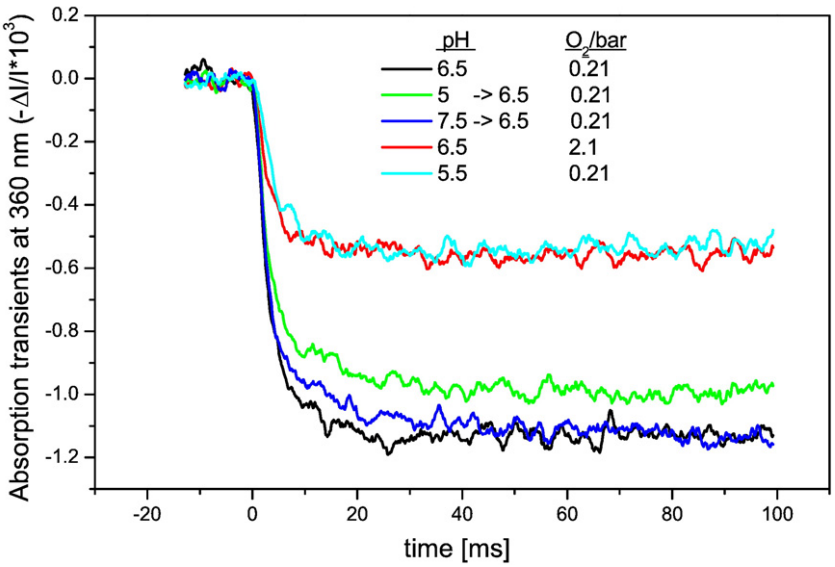

Fig. 1. Absorption transients at 360 nm representing the millisecond phase of the electron transfer into the catalytic Mn_4CaTyr-centre. Original transients upon the third flash in a row were corrected for events at the reducing side of PSII, as described elsewhere [20]. Black: the ms-phase under standard conditions (1 bar air [=0.21 bar O_2], pH 6.5). Red: partial inhibition by a tenfold increase of the oxygen pressure (2.1 bar, pH 6.5). Light blue: inhibition by acidification at 0.21 bar O_2. Green and blue: reversibility of the inhibition by high and low pH at 0.21 bar oxygen: samples were incubated at pH 5 (green) or pH 7.5 (blue) for 25 min and retitrated to pH 6.5 within 5 min. Four transients averaged.

range between 5 and 6.7, the buffer was 50 mM 2-(*N*-Morpholino) ethanesulphonic acid), and for pH 7 to 7.5 it was 50 mM *N*-(2-Hydroxyethy)piperazine-*N*′-(2-ethanesulphonic acid). At pH 6.7, the particular choice of buffer had no influence on oxygen evolution under continuous illumination. The sample was pre-equilibrated for 30 min with the given gas atmosphere in the dark before measurements.

Dark adapted samples (9 μM Chl) were filled into a square cuvette with 1 cm path-length and excited by a series of six laser flashes of a Nd: YAG laser (532 nm, FWHM ~9 ns, 100 ms between flashes). Absorption transients at a wavelength of 360 nm were recorded. Photometric transients were digitised on a Nicolet Pro92 transient recorder. The original time resolution was 100 μs/address. For presentation in the figures, the original data were smoothed over 20 points.

The UV-transients after the third laser flash were normalised to yield the same extent of the rapid rise (representing the turnover of Photosystem II in each given sample) and they were corrected to eliminate the contributions of the preceding *S*-state transition and of the artificial electron acceptor DCBQ by the same procedure as previously described (see [20,22]).

3. Results

3.1. Effects of oxygen and pH on the dark equilibrium between the oxidation states, S_i

The extent of the UV-absorption jump upon laser flash 2 was independent of the oxygen pressure [20,22], and also of the pH in the range from 5.0 to 7.5 (data not shown). The normalised transients of absorptions at 360 nm thus suggested that the dark equilibrium of S_0/S_1 was unchanged. The latter is in line with previous results under variation of the pH obtained on spinach thylakoids [27]. EPR-experiments in collaboration with J.H. Su and J. Messinger revealed a fully expressed S_2-multiline signal after the first flash of light (unpublished data) in PSII-core particles equilibrated with high O_2 pressure. It corroborated an oxygen- and pH-independent dark equilibrium between S_0 and S_1.

3.2. On the reversibility of the pH effects on the millisecond phase

In PSII-core particles the effects of high oxygen pressure on the millisecond phase are fully reversible in PSII-core particles at neutral pH [20]. Reversibility of the pH-induced effects was not *a priori* granted. In spinach thylakoids suspended at pH 5 and 8, for instance, there is a time-dependent decrease of the oxygen evolving activity. It has been attributed to the degradation of Photosystem II [27]. We checked the reversibility of pH effects in PSII-core-particles.

Previously we found that lowering the pH from 6.7 to 5.7 (under air) suppressed the millisecond phase from 100% to 64.4% [22]. This was here corroborated, we observed 65% at pH 5.5. In both sets of experiments, the time lapse between the start of exposure to acid pH and firing of flashes was short, namely 1 min. Longer incubation at the given pH, say for 30 min, caused additional effects that are documented in Fig. 1 and Table 1.

Fig. 1 shows typical differences of UV-absorption transients at 360 nm. As a standard, the black and red transients demonstrate the known suppressive effect of a tenfold increase of the oxygen pressure from 0.21 bar (black) to 2.1 bar (red) at the same pH (6.5). When keeping the oxygen pressure constant at 0.21 bar preincubation for 25 min at pH 5 reduced the extent of the millisecond phase further, in

Table 1
Inhibition of the millisecond phase in dependence on the pH and the oxygen pressure after 30 min of incubation

pH	bar O_2	UV-jump $-\Delta I/I*10^3$ (ms-phase)	Relative extent (%)
7.5	0.21	0.61	54
7	0.21	0.86	76
6.5	0.21	1.13	100
6	0.21	0.85	75
5.5	0.21	0.54	48
5	0.21	0.41	36
7.5	2.1	0.15	13
7	2.1	0.44	38
6.5	2.1	0.56	50
6	2.1	0.35	31
5.5	2.1	0.16	14
5	2.1	0.08	7
5	5	0.01	1
5→6.5	0.21	0.99	88
7.5→6.5	0.21	1.11	98

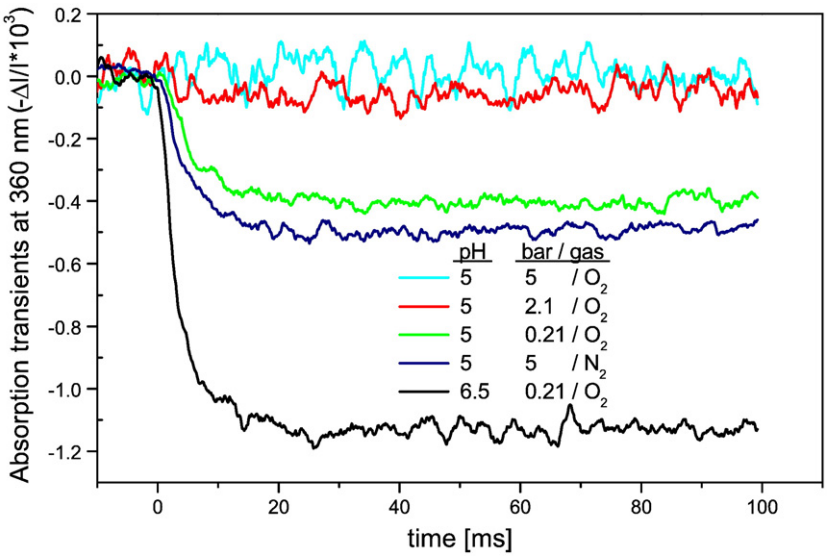

Fig. 2. Absorption transients at 360 nm representing the millisecond phase of the electron transfer into the catalytic Mn_4CaTyr-centre. Original transients upon the third flash in a row were corrected for events at the reducing side of PSII, as described elsewhere [20]. Black: 0.21 bar O_2, pH 6.5, Red: 2.1 bar O_2, pH 5, Light blue: 5 bar O_2, pH 5. Green: At pH 5, ambient oxygen pressure (0.21 bar) was already slightly inhibitory, and its replacement by 5 bar N_2 (no oxygen present) was stimulating (dark blue). Four transients averaged.

a time-dependent way down to 36% (Table 1). When the sample was titrated back to pH 6.5 the signal was largely restored to 88% (green transient in Fig. 1). Incubation at pH 7.5 for 25 min lowered the extent even further, to 13% (Table 1), but back titration to pH 6.5 within 5 min (blue transient) restored the signal amplitude to 98% (Fig. 1). Thus, the pH-induced suppression of the $S_4 \rightarrow S_0$ transition was largely reversible at pH 5 and almost fully reversible at pH 7.5. The pH-dependent degradation of PSII-core particles was small and it was neglected in the following.

3.3. The millisecond phase of UV-transients at 360 nm as a function of the pH and the oxygen pressure

The millisecond phase of UV-transients at 360 nm as a function of the pH and the oxygen pressure is presented in Figs. 1 and 2 by original transients. Fig. 3 shows its extent as a function of the pH at two different oxygen pressures, 0.21 bar (red) and 2.1 bar (black). Data are summarised in Table 1. The two points at pH 6.5 in Fig. 3 reproduced the previously observed half inhibition of the millisecond phase by about 2.1 bar oxygen. On top of this inhibition by oxygen, there was a pH-dependent one at both sides of pH 6.5, acid and alkaline. In contrast to its behaviour at pH 6.5, where 2.3 bar O_2 half suppressed the millisecond phase, at pH 5.0 it was more strongly suppressed by the same pressure (7% remaining, see Table 1, Fig. 2), and even more so at 5 bar O_2 (1%; Table 1 and Fig. 2). At about neutral pH, the suppression by high oxygen went never below 20% even if the pressure was elevated up to 28 bar i.e. more than 100-times above the ambient level [20,21]. The strong synergistic action of the proton and the oxygen concentration, both being only tenfold augmented over standard (pH ~ 7, 0.21 bar), suggested that, there may exist different sites of attack by oxygen and protons from the bulk (see Discussion). Fig. 4 shows a phenomenological fit of the inhibition as function of the

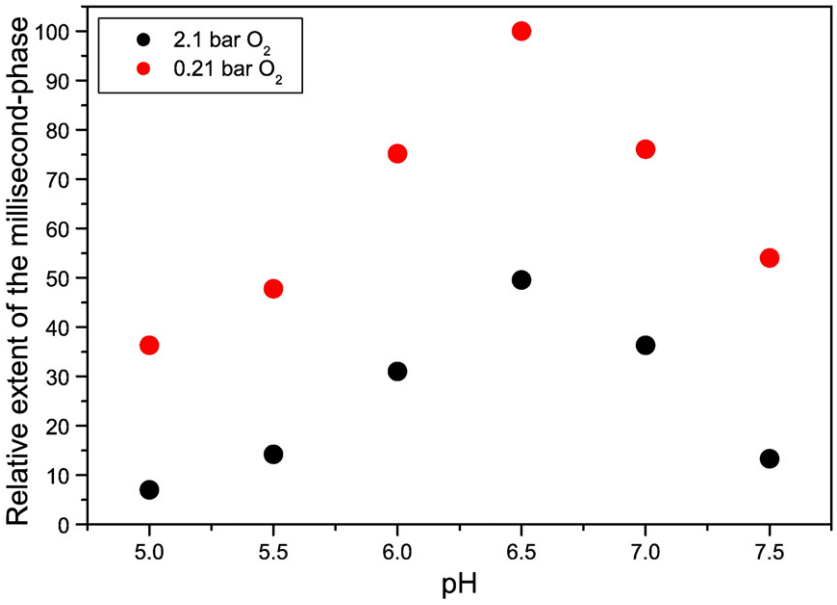

Fig. 3. Extent of the millisecond phase in dependence of the oxygen pressure (Red: 0.21 bar; Black: 2.1 bar) and pH. At pH 6.5, half inhibition was reached by 2.1 bar oxygen, and at pH 5.5 already by 0.21 bar oxygen.

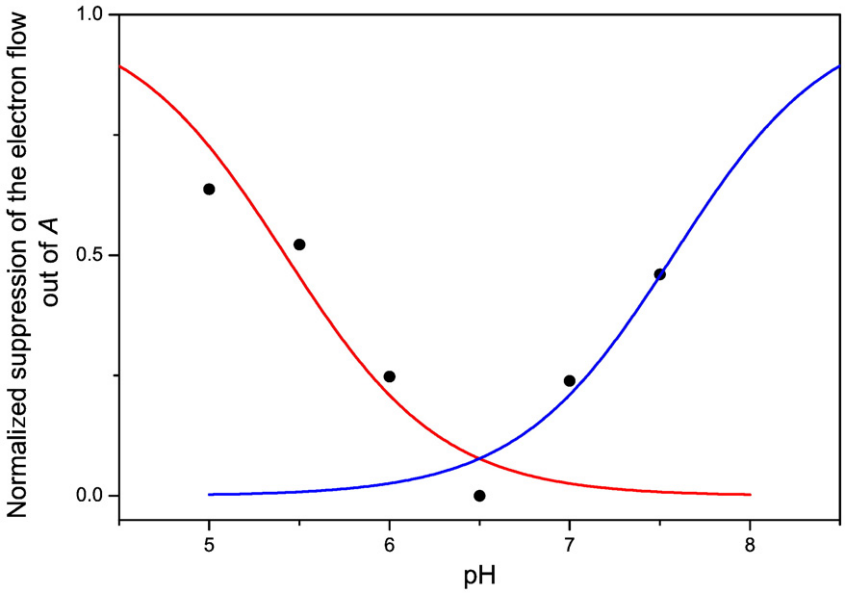

Fig. 4. pH-titration of the relative inhibition of the millisecond phase at 0.21 bar oxygen. Two inhibitory components for the reduction of S_4 were found, one in the acid pH region titrating with p*K* 5.4 (red line) and the other one with p*K* 7.6 in the alkaline pH range (blue line). The Hill coefficient was set to 1 in both cases.

pH in terms of two titration curves, with p*K*s of 5.4 (red line) and 7.6 (blue line), both with a Hill coefficient of 1.

In order to rule out any effect by hydrostatic pressure alone and to prove the specific suppressive effect of oxygen, the measurements were repeated at pH 6.7, 6 and 5 under increased nitrogen pressure. At pH 6.7 there was no difference between transients at 19 bar nitrogen (ultra-pure N_2, virtually no oxygen present) and at ambient oxygen pressure (0.21 bar) [20]. At pH 6 and pH 5, however, ambient oxygen pressure (0.21 bar) was already slightly inhibitory, as evident from the stimulating effect of its replacement by nitrogen, namely +4% at pH 6 and +20% at pH 5 (see Fig. 2). In other words, at acid pH the millisecond-reaction was more sensitive to oxygen than it was at about neutral pH.

4. Discussion

Photosynthetic oxygen production from water is pivotal for aerobic life on earth, and its, in essence, enigmatic mechanism is of paramount interest for attempts to technically produce solar fuels.

Although great progress has been made in development of systems to mimic the catalytic centre of water oxidation [28–30] and in unravelling the crystal structure of PSII [31–36] current structural concepts of the metal cluster with ligands including bound water (derivatives) suffered from three shortcomings, (i) the still limited spatial resolution of 3.0 Å [34], (ii) the reduction of manganese during the collection of X-ray scattering data [37], and (iii) the lack of mutual influence between certain structurally assigned ligands of the metal cluster and the cluster itself [26,38–40]. This is why the exact position of the metals is still under debate, and the position in the metal cluster of the educt, water, or its intermediates (e.g. peroxide) is simply unknown. A comprehensive understanding requires both, the spatial arrangement of reaction partners and their detailed reaction scheme. The latter was addressed in this work.

We studied the effect of oxygen and of the pH on the terminal reaction cascade in PSII-core-particles and found it sensitive to only tenfold increase over standard of the concentration of both, oxygen and protons.

In algal and cyanobacterial cells various inhibitory effects of oxygen on photosynthetic-reactions have been previously reported (for a review see [41]), inter alia lower rates of oxygen evolution, reduced fluorescence signature (F_v/F_m ratios) and electron transport rates [42–44]. However, the target for this action of oxygen within the complex reaction pattern in the cell has remained obscure. By studying these effects in PSII-core particles, the minimal unit capable of oxygen evolution, they could be attributed to the site of oxygen evolution, proper. The same holds true for the effects of pH. We found that both acid and alkaline pH reversibly suppressed the terminal reaction cascade leading from the highest oxidation state of the Mn_4CaTyr-centre, S_4, to the lowest, S_0.

4.1. The inhibition by slightly alkaline pH

The inhibition by slightly alkaline pH titrated with an apparent p*K* of 7.6 and a Hill coefficient of one (Fig. 4, blue line). It was fully reversible (Fig. 1). It can only be speculated about the identity of the group responsible for this inhibition. Previously, EPR measurements have revealed the inhibition of the $S_3 \Rightarrow S_4 \rightarrow S_0$ transition at alkaline pH [45]. The slightly higher p*K* of 8 in this work on PSII-membrane fragments from spinach might have been due to different material. Bernat et al. [45] have attributed this inhibition to an alkaline-induced decrease of the redox-potential of the Y_Z^{ox}/Y_Z couple, such that Y_Z^{ox} is unable to oxidise the S_3 state fast enough to avoid dissipative side reactions. Other authors have invoked structural changes [27,46]. The here observed suppression, say at pH 7.5 is, however, neither attributable to Mn-depletion, which occurs only at pH around 9–10 [47–51], nor to the release of the extrinsic proteins occurring at pH values greater than 8 [52–54] because these effects are irreversible, whereas the here observed inhibition was reversible.

4.2. The inhibition of the millisecond phase at acid pH

The inhibition of the millisecond phase at acid pH is expected because water oxidation produces both, protons and oxygen, and both are released into the bulk. The acid inhibition we observed titrated with an apparent p*K* of 5.4 (Fig. 4, red line). Data were analysed in terms of a minimal reaction scheme with only two consecutive steps,

$$A \underset{b}{\overset{a}{\rightleftharpoons}} B + n\mathrm{H}^+ \underset{d}{\overset{c}{\rightleftharpoons}} C + \mathrm{O_2} + m\mathrm{H}^+$$

Scheme 1.

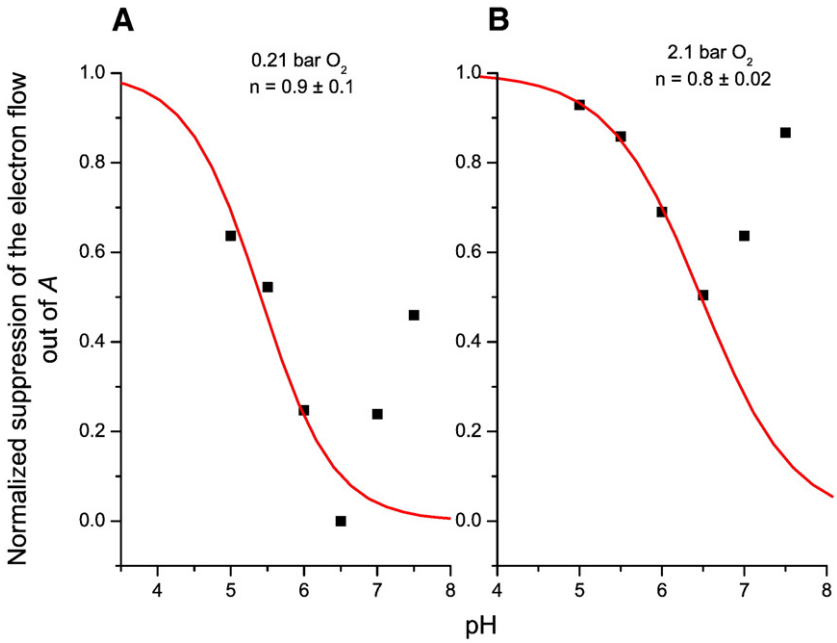

Fig. 5. The relative inhibition of the millisecond phase of electron flow during S_4 to S_0 in the acid pH range (5–6.5) (data points taken from Fig. 3). The fits according to Eq. (5) (red lines) resulted in about one proton, which is released in the first step of the consecutive reaction Scheme 2. A) 0.21 bar oxygen pressure, $n=0.9\pm0.1$. B) 2.1 bar oxygen pressure, $n=0.8\pm0.2$.

as previously [20,21,23] but now taking into account the release of n protons per oxygen in the first and m protons in the second step (see Scheme 1).

Herein A stands for the initial state S_4 of Mn_4CaTyr, C for S_0, and B for a redox intermediate. As previously, it was assumed that the first reaction in the cascade relaxed more slowly than the second, being rate-limiting. Starting from an initial concentration of $A=1$ the equilibrium concentration of $A=A_\infty$ is given by Eq. (1):

$$A_\infty = \frac{1}{1+\frac{a}{b\cdot H^n}+\frac{c}{d\cdot p\cdot H^m}\cdot\frac{a}{b\cdot H^n}} \tag{1}$$

Herein, a–d denote the forward and backward rate constants (see [20]), p the oxygen partial pressure, H the concentration of the proton in the bulk and m and n stoichiometric coefficients of H^+/O_2. The extent of the millisecond phase of UV-absorption transients, here taken for the extent of oxygen evolution, is then given by the difference: $1-A_\infty$. This holds, strictly speaking, only for a "non-absorbing" intermediate B, while it has been shown that an intermediate with half the extinction coefficient of A produces a slightly better fit to the previous data [23]. Here, we ignored this complication for simplicity by assuming that only one species, A, contributed to the UV-absorption. The pH-6.7-related rate constants, a/b' and c/d' have been previously determined to be 0.2 and 13.6 [23]. b/b' and d/d' are related to a/b and c/d (of Scheme 1) according to Eqs. (2) and (3) which explicitly invoke the pH-dependence:

$$\frac{a}{b\cdot H^n} = \frac{a}{b'\cdot 10^{-n^*(pH-6.7)}} \tag{2}$$

$$\frac{c}{d\cdot H^n} = \frac{c}{d'\cdot 10^{-n^*(pH-6.7)}} \tag{3}$$

The analysis of the pH and O_2 pressure dependence of the millisecond phase (Fig. 3) with Eq. (1) yielded a rather good fit both, at 0.21 and at 2.1 bar oxygen. Broadly speaking, the number of released protons was $n\approx1$ in the first step and $m\approx0$ in the second (see below).

It was noteworthy that the data could not be fitted under the assumption that protons were exclusively released in the last oxygen evolving step, according to Eq. (4):

$$A_\infty = \frac{1}{1+\frac{a}{b}+\frac{c}{d\cdot p\cdot H^m}\cdot\frac{a}{b}} \tag{4}$$

This was because the maximum attainable inhibition is 0.83 with the determined value for $a/b'=0.2$ at pH 6.7 [23]. This value was the result of previous measurements at pH 6.5–6.7, where it has been shown that even very high oxygen pressure (28 bar) is incapable of inhibiting the reduction to S_0 by more than 75–80% [20,21,23]. A complete suppression could only be achieved by the interplay of both, acid pH and a high oxygen pressure (Table 1 and Fig. 2, light blue transient). This is only compatible with the two-step reaction Scheme 1, if proton release occurs during the first step or in both steps, but not exclusively in the last step of the reaction sequence.

The fit shown in Fig. 5, red lines (see also Table 2) resulted from the assumption that protons were exclusively released during the first reaction step according to Eq. (5):

$$A_\infty = \frac{1}{1+\frac{a}{bH^n}+\frac{c}{d\cdot p}\cdot\frac{a}{b\cdot H^n}} \tag{5}$$

Fig. 5 displays the amount of A_∞ (in equilibrium) in dependence of the pH and constant oxygen pressures (0.21 [air] and 2.1 bar). At both

Table 2
Fit parameters for the dependence of the millisecond phase as a function of the oxygen pressure

Condition	Proton release only in the first step (Eq. (5))	Proton release in both steps (Eq.(1))
0.21 bar O_2	$n=0.9$	$n=1.1$ $m^a=-0.3$
2.1 bar O_2	$n=0.8$	$n=0.6$ $m=0.2$

n and m are the H^+/O_2-stoichiometric coefficients under the assumption of a non-absorbing intermediate B.

[a] m denotes protons released concomitant with oxygen evolution.

$$X^3 \cdot M^3 \cdot Y^1 \cdot H_2O \cdot H_2O \xrightarrow[250\,\mu s]{30\,\mu s} X^2 \cdot M^3 \cdot Y^1 \cdot OH^- \cdot H_2O \xrightarrow{1ms} H^+ + X^0 \cdot M^2 \cdot Y^0 \cdot OO \xrightarrow{<<1ms} X^0 \cdot M^0 \cdot Y^0 + O_2$$
$$S_4^0 = A \qquad S_4^1 \qquad S_4^2 = B = S_2^* \bullet OO \qquad S_0 = C$$

Scheme 2.

oxygen pressures, 0.21 (Fig. 5A) and 2.1 bar (Fig. 5B), the proton stoichiometry was around 1 ($n = 0.9 \pm 0.1$ at 0.21 bar O_2; $n = 0.8 \pm 0.02$ at 2.1 bar O_2). Therefore, our data suggest that only one chemically produced proton is released into the bulk and that this occurs during the first of two consecutive reaction steps leading to the release of dioxygen.

In the data analysis only differences between steady state levels but not possible effects on the rates were considered. According to Scheme 1 the rate of *A*-decay should be lower at acid than at neutral pH. This appears to be the case when comparing the green (pH 5.0) and the black trace (pH 6.5) in Fig. 2. Because of limited signal-to-noise-ratio it was difficult to tell, with confidence whether or not the apparent kinetic effect was real.

How does the release of only one proton per electron during the first reaction in the cascade (see Scheme 1) relate to the pertinent literature on proton release associated with water oxidation? Here one has to discriminate between two different experimental approaches:

(i) Several groups, including our own, have studied the release of protons into the bulk phase using membrane bound [55–57] or water soluble pH-indicators [50,58–62] (reviewed in [63,64]). The published patterns of proton release as function of the flash number and the pH greatly varied between different materials, namely thylakoid membranes, PSII-enriched BBY membranes and PSII-core particles. The variance was ascribed to the overlay of the production of protons in the sequential oxidation of the Mn_4CaTyr-complex and electrostatically or conformationally induced p*K*-shifts of peripheral acid groups [64]. In PSII-core particles, suspended in detergent solution, as used in this work, the pattern of proton release in the series of redox transitions, namely $S_0 \Rightarrow S_1 \Rightarrow S_2 \Rightarrow S_3 \Rightarrow S_4 \rightarrow S_0$, was 1:1:1:1 [50,59,60,65,66]. It implied the release of one proton in the terminal reaction cascade $S_4 \rightarrow S_0$.
(ii) Two groups have previously detected a lag phase preceding the electron transfer in milliseconds into the Mn cluster. The respective duration was 30 μs (by UV-absorption [67]) and 250 μs (by time-resolved Röntgen-spectroscopy [68]) using PSII-enriched membranes. These lag phases were tentatively ascribed to internal proton transfer which precedes the rate-limiting step for electron transfer.

In the present work, we detected effects attributable to the interchange of protons between the catalytic centre and the bulk. In other words, internal proton transfer was not directly monitored. Thus, the above statement that proton release is confined to the first of the two consecutive reaction steps and absent in the second one does not encompass internal proton transfers within the centre proper.

The finding in this work of a proton-to-O_2 stoichiometry of 1 during the terminal reaction cascade is compatible with the data given in (i). It is also evident from (i) that the first three transitions each generate one base in or around the catalytic centre. The data in (ii) invoke another (*non-redox*) intermediate as a precursor to the peroxy-intermediate [20,68–72]. If we take the rather rapid exchange of water at the centre during the lower oxidation states up to S_3 (for a review see [73]) as indicative of the binding of water in the form of H_2O (admittedly an arbitrary choice), we arrive at the following reaction Scheme 2 for the protolytic reactions in the reaction cascade leading from S_4^0 to S_0.

The lower line relates the upper to the previously defined three components (*A*, *B*, *C*) in the consecutive reaction, and the S_4^i denote sub-states of the state $S_4 = \langle S_4^0, S_4^1, S_4^2, \ldots \rangle$, where the catalytic centre, Mn_4CaY_Z-centre plus its ligands including bound water, holds four oxidising equivalents. *X* stands for the bases which are deprotonated during the foregoing oxidation steps and its superfix stands for their total proton binding capacity, *M* stands for the metal cluster Mn_4Ca and the superfix for the oxidation state, *Y* stands for the redox-active tyrosine and its superfix for the oxidation state, OO stands for the bound and deprotonated peroxy-moiety, and H^+ and O_2 stand for the liberated proton and oxygen in solution. The chemical identity of the three bases and their location around the metal cluster has still to be identified and so have the ligands to the deprotonated peroxide (for a recent model see [35]).

Reaction Scheme 2 is *tentative*. The step giving rise to the lag phase of 30 μs [67] and 250 μs [68] duration, respectively, precedes any electron transfer into manganese, as monitored directly by Röntgen absorption spectroscopy [68]. In Scheme 2, it has been assigned to an internal proton transfer between bound water and preformed bases. Because of its separation from electron transfer, this solid state reaction cannot be taken as straightforward evidence for the previously discussed "hydrogen abstraction" mechanism [74–78] but rather as evidence for proton transfer as a prerequisite of electron transfer [79]. On the other hand, the release into the bulk of one proton per dioxygen, as addressed in this work, occurs concomitant with the very first and rate-limiting electron transfer on the way from S_4 via S_2* to S_0. The rate-limiting step might therefore be conceived as proton-coupled electron transfer (PTET) (for evidence of PTET between Y_Z and P_{680}^{+P}, see [80,81]). The penultimate intermediate before the last electron transfer(s) which liberate dioxygen might be a fully deprotonated metal-*O*-*O*-metal moiety.

In the green algae *Chlamydomonas* (and so far only in this organism) a carboxy-anhydrase (CA) is specifically bound to PSII. Its removal slightly delays and readdition speeds the rate-limiting step of oxygen evolution by facilitating (through bicarbonate) the proton transfer out into the bulk [82]. This observation is in line with the above conclusion that the rate-limiting step involves proton release. The effect of CA, however, is specific to *Chlamydomonas*. In PSII-core complexes from *Synechocystis* bicarbonate does not affect UV-absorption transients at 360 nm, and it is neither reversibly nor irreversibly bound to PSII-enriched membranes from spinach [83,84].

Half-suppression of the reaction cascade by either product, oxygen and proton, occurred upon rising their concentration only tenfold over ambient level (neutral pH, atmospheric O_2). It implies a small driving force, only ≈5.6 kJ/mol or 59 mV, for both reaction steps [21,23].[1] Energetically, the performance of Photosystem II seems to be driven to the limits (see [85–87] for recent assessments), such that the remaining driving force for the terminal reaction cascade from water to dioxygen is rather small. Without further evolutionary engineering, nature does not have the option to raise the present atmospheric oxygen level very much above the present one.

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[1] Please note that the negative algebraic signs are missing for $\Delta G'_{B\rightarrow C}$ in Table 1 in [23].

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