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REGULAR PAPER

The inhibitory effects of acidification and augmented oxygen pressure on water oxidation

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Abstract Cyanobacteria, algae, and plants produce dioxygen from water. Driven and clocked by light quanta, the catalytic Mn_4Ca Tyrosine centre accumulates four oxidizing equivalents before it abstracts four electrons from water and liberates dioxygen and protons. Intermediates of this reaction cascade are short-lived (<100 μs) and difficult to detect. By application of high oxygen pressure to cyanobacterial PSII-core-complexes, we have previously suppressed the transition from the highest oxidation state of the centre to the lowest by stabilizing a (peroxy) intermediate. Here, we investigated the inhibitory interplay of acidification and augmented oxygen pressure. Starting from pH 6.5, acidification increasingly inhibited the reduction of the highest oxidized state and resulted in a lower oxygen partial pressure for half inhibition. Oxygen and proton interfere with different steps of the reaction cascade.

Keywords Photosynthesis · Water oxidation · Proton release · Photosystem II · Intermediate · Oxygen

Abbreviations

Chl	Chlorophyll
FWHM	Full width at half maximum
PSII	Photosystem II
S_i, $(i = 0 - 4)$	Redox states of the catalytic centre
Y_Z	Redox-active tyrosine-161 of PSII subunit D1

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Introduction

Almost all life on earth depends on biomass production provided by photosynthesis (known exceptions are the deep-sea ecological systems around the "black smokers" and "CO_2-lakes"). Photosynthetic organisms evolved perhaps as early as 3.6 billion years ago. The first photosynthetic organisms were anaerobic and used compounds like H_2S and organic acids as hydrogen source to reduce carbon dioxide. This kind of photosynthesis is called "anoxygenic" because no oxygen is released as a product of this reaction. More than 2 billion years ago, photosynthetic organisms evolved that used water as a hydrogen donor to reduce carbon dioxide according to the net equation:

$$6CO_2 + 12H_2O \xrightarrow{h\nu} C_6H_{12}O_6 + 6H_2O + 6O_2$$
$$\Delta G^\circ = 2872\text{kJ/mol}$$

This kind of photosynthesis is called oxygenic. Oxygenic photosynthesis changed the atmosphere on early earth from anaerobic to aerobic, and as a side effect, resulted in the formation of a protective ozone layer. It set the stage for the development of life outside of water owing to UV-shielding. Moreover, the availability of oxygen as a terminal electron acceptor during respiration in heterotrophic organisms and the resulting greater free energy gain compared to the one of fermentation was the prerequisite for the development of cell differentiation in multi-cellular organisms.

Oxygen is a highly reactive species capable of many side reactions. It interacts with triplets, radicals, and reduced compounds in numerous biochemical pathways inside the living cell, quite a few being harmful rather than useful, which has been evident when oxygen levels were

raised above ambient (0.21 bar O_2). As excellently reviewed by Raven and Larkum (2007), elevated oxygen pressure has been found to inhibit carbon dioxide fixation by ribulose-1,5-bisphosphate carboxylase/oxygenase, increase the competition between the Mehler-reaction and the photosynthetic carbon reduction cycle, inhibit carbon concentration in plants, elicit oxidative damage, and inhibit cell growth and respiration of photosynthetic organism. Pope and Berger have reported that exposure to high oxygen pressure diminishes the rate of oxygen production in the cyanobacterium *Anacystis nidulans* (Pope and Berger 1973). In the cyanobacterium *Spirulina platensis*, a sevenfold increase of oxygen pressure over standard reduces the F_v/F_m ratio and the electron transport rate (Torzillo et al. 1998) and fourfold increase of the oxygen concentration over ambient significantly reduces the maximum quantum yield F_v/F_m, the relative electron transport rate, and the overall photosynthetic efficiency in various other algal cultures (McMinn et al. 2005). However, it has remained an open question to what extent these results represent an *indirect* inhibition, e.g., due to photooxidative damage or Rubisco-inhibition, or a *direct* inhibition of the oxygen producing reaction cascade based on the law of mass action. Our previous work on the inhibitory effects of increased oxygen pressure was carried out on the minimal unit capable to evolve oxygen, photosystem II-core particles of the cyanobacterium *Synechocystis* sp. PCC 6803, and here, in particular, the rate limiting "millisecond phase" of the terminal reaction cascade (Clausen and Junge 2004). This allowed us to discriminate the direct action of oxygen on this cascade proper from indirect effects on photosynthesis in general.

Water oxidation to yield dioxygen and protons takes place in the Mn_4CaY_Z-Cluster at the oxidizing side of photosystem II. Its structure is emerging (Zouni et al. 2001; Kamiya and Shen 2003; Ferreira et al. 2004; Loll et al. 2005; Yano et al. 2006; Kern et al. 2007). Upon excitation with short flashes of light, the catalytic Mn_4CaY_Z-complex is cycled through a series of redox-states, coined S_n, where n indicates the number of stored oxidizing equivalents ($n = 0$–4) (Kok et al. 1970). Only after reaching the fourth oxidation state, S_4, oxygen is released and the Mn_4CaY_Z-complex is reset to S_0. UV transients at 360 nm have been taken as diagnostic for the transitions between the S_n-state transitions and oxygen-evolving reaction (Saygin and Witt 1987; Dekker 1992; Lavergne 1991), and a millisecond component apparent after firing of the third flash in a row, in particular, was taken to reflect the rate limiting step of the terminal reaction cascade leading to the release of oxygen (Clausen and Junge 2004). We have monitored the transition from the highest oxidized state $S_4 = S_3Y_Z^{ox}$ to the most reduced state S_0 under variation of the oxygen pressure (up to 28 bar) by time-resolved UV spectroscopy at 360 nm (Clausen and Junge 2004), and as an independent approach, by delayed fluorescence (Clausen et al. 2005). At pH 6.7, the terminal reaction is half-suppressed at a partial oxygen pressure of 2.3 bar oxygen, only 10-fold over ambient. It has been attributed to an equilibrium shift of the catalytic cascade stabilizing a redox intermediate (state S_2* with bound peroxide). It is conceivable that some of the above-mentioned inhibitory effects of elevated oxygen concentration on cyanobacterial and algal cells as reported by other authors are also attributable to this direct inhibition of the oxygen-releasing reaction.

In our previous work, the involvement of protons was not studied in detail. This prompted us to reinvestigate the interplay of increased oxygen pressure and acidification. Except for the abundant water, being both substrate and solvent, oxygen and protons are the only soluble reaction partners in the reaction cascade, such that their concentration determines the equilibrium of partial reactions. Grossly speaking, we expected a similar effect on the equilibrium of increasing the concentration of either product, oxygen and proton. The inhibition should then depend on the product $[O_2]^*[H^+]^n$, wherein "n" denotes the H^+/O_2-stoichiometry. In this sense, lowering the pH was expected to mimic elevating the oxygen partial pressure.

Materials and methods

Cells of the modified "wild-type" (WT*) (Hays et al. 1999) of *Synechocystis* sp. PCC 6803 were cultivated as described in Chu et al. (1995), 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 (Clausen et al. 2001).

UV-Flash-spectrophotometric measurements (Junge 1976) with PSII core particles at 360 nm were performed as described in Clausen and Junge (2004). Three batch preparations of core particles were pooled. Long time dark adapted samples (8 μM Chl) were excited by a series of six laser flashes of a Nd:YAG laser (532 nm, FWHM ~9 ns, 100 ms between flashes). The optical pathlength was 1 cm. The accuracy of pressure application was ±0.2 bar. Photometric transients were digitized on a Nicolet Pro92 transient recorder. The time resolution was 100 μs/address.

The UV transients after the third laser flash were normalized 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 the artificial electron acceptor 2,5-Dichloro-*p*-benzoquinone by procedures previously described (see Clausen and Junge 2004;

Clausen 2004). For presentation in Fig. 1, the original data were smoothed over 20 points.

Results

Previous experiments with PSII-core complexes have revealed the partial suppression of the millisecond phase of the transition from $S_3Y_Z^{ox}$ to S_0 from 100% at 1 bar air ($\triangleq$ 0.21 bar O_2) to 64.4% by lowering the pH from 6.7 to 5.7 within 1 min of incubation (Clausen 2004), foreshadowing a possible thermodynamic back pressure of the proton on oxygen evolution. Here, these measurements were expanded, standardized and compared to the oxygen evolution at various pH values.

Figure 1 shows the differences of UV-absorption transients at 360 nm representing the ms-phase of electron transfer into the catalytic centre. The black transient represents the assumed total reduction of $S_3Y_Z^{ox}$ to S_0 at pH 6.5 and ambient 0.21 bar oxygen pressure (air control). The red transient demonstrates the suppressive effect of a 10-fold increase of the proton concentration (from 6.5 to pH 5.5) at 0.21 bar oxygen after incubation for about 1 min. The extent of the millisecond-phase was reduced to 64.7%, corroborating our previous result. The green curve shows the remaining extent of ms phase (42.6%) after incubation for 30 min at pH 6.5 and 5 bar oxygen pressure. This was also in good agreement with the line of best fit (42.7%) previously published (Clausen and Junge 2004).

As mentioned above, half suppression of the ms phase was reached at 2.3 bar oxygen pressure at pH 6.7 (Clausen and Junge 2004, 2005; Clausen et al. 2005). Here, we studied the inhibitory interplay of protons and oxygen in the pH range from 5 to 6.5. The extent of the negative signal amplitude of the ms phase was analyzed in dependence on the pH at constant oxygen pressures of 0.21 bar (1 bar air) and 2.1 bar. The extent of the ms phase and the relative inhibition at 2.1 bar (red) and 0.21 bar (black) oxygen pressure are displayed in Fig. 2 in dependence of the pH. Starting from pH 6.5 and 0.21 bar O_2, 2.1 bar oxygen at the same pH lowered the extent of the signal to half-size, and lowering the pH diminished it further. While at pH 6.5, half-suppression (49.6%) was reached at 2.1 bar oxygen pressure (Fig. 2), and at pH 5.5, it occurred already at 0.21 bar (Fig. 2).

The observed suppression at low pH was not due to the removal of the extrinsic proteins, because this removal has only been observed at an even lower pH of 3 (Shen and Katoh 1991), two pH-units below the range covered in this work. Moreover, the inhibition we observed was reversible, as will be outlined in a forthcoming publication (submitted).

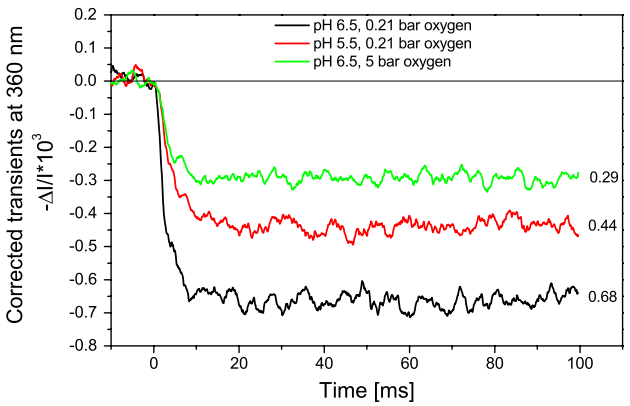

Fig. 1 Absorption transients at 360 nm representing the electron transfer into the catalytic Mn_4Ca-centre. Original transients upon the third flash in a row were corrected for events at the reducing side of PSII, as described elsewhere (Clausen and Junge 2004). Black: the ms phase under standard conditions (1 bar air [= 0.21 bar O_2], pH 6.5). Red: partial inhibition by a 10-fold increase of the proton concentration after 1 min of incubation. Green: partial inhibition by an about 25-fold increase of the oxygen pressure (5 bar). Four transients averaged. Original data were smoothed over 20 points

Discussion

Oxygen and protons are both soluble products of the water splitting reaction in photosystem II. Increase in concentration of either of these products was supposed to inhibit the terminal reaction step in the reaction sequence from $S_3Y_Z^{ox}$ to S_0 according to the law of mass action. This inhibitory interplay was demonstrated in the present work.

It was found that both protons and oxygen, can suppress the oxygen-evolving reactions, broadly speaking as if the product $[O_2]^*[H^+]^n$ ($n \approx 1$) mattered. Lowering the pH

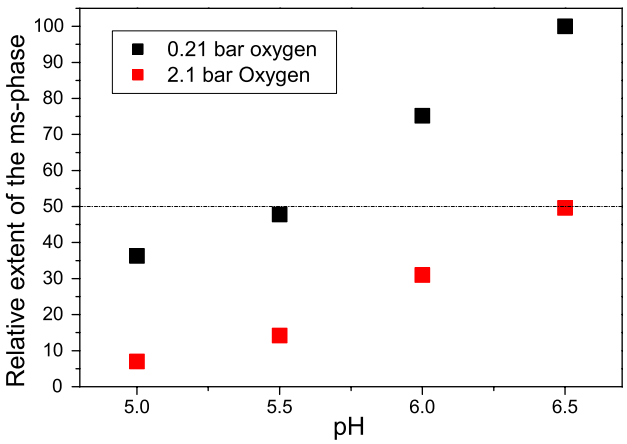

Fig. 2 Extent of the millisecond phase in dependence of the oxygen pressure (0.21 bar = red; 2.1 bar = black) and pH. At pH 6.5, half inhibition was reached by 2.1 bar oxygen, and at pH 5.5, by 0.21 bar oxygen

lowers the amount of oxygen necessary to half-suppress the water splitting reaction. A quantitation analysis of the inhibition by oxygen and proton concentrations in the pH range from 5 to 7.5 is in preparation. Preliminary data point to different reaction steps of their attack.

This inhibitory interplay has crucial consequences for the water splitting reaction in photosynthetic organisms, where photosystem II is localized in the thylakoid membranes of plants, algae, and cyanobacteria. Because its oxidizing side faces the lumen side of thylakoids, it contributes to the acidification of this phase. Previous estimates for the lumenal pH of chloroplasts from higher plants have yielded figures as low as pH 4 (Rumberg and Siggel 1969; Schuldiner et al. 1972; Rottenberg and Grunwald 1972; Rottenberg et al. 1972; Gräber and Witt 1976; Witt 1979). Such low pH values were, however, only reached in the presence of cofactors of cyclic electron flow. In whole giant cells of *Peperomia metallica*, the driving force for the synthesis of ATP, nearly 4 pH units, was mainly owed to the pH difference and less so to the voltage difference across the membrane (Vredenberg and Tonk 1975). According to our experiments on isolated PSII core complexes, such a low pH together with ambient oxygen pressure would result in a great suppression of the water-splitting activity. More recent experiments on plant leaves and intact cells revealed a rather different behavior, namely that a significant electric component of the total proton-motive driving force across the thylakoid membrane remains (8–50%), and the pH difference is less than was thought before, about two pH units, with the intra-thylakoid pH between 5.8 and 6.5 (Kramer and Sacksteder 1998; Kramer et al. 1999, 2003). In the same line, recent experiments with isolated chloroplasts have corroborated the relative high luminal pH of 5.7–6 (Tikhonov et al. 2008). Therefore, it appears as if the inhibition of oxygen evolution by the interplay of ambient oxygen levels and the second activity of PSII, namely, net proton pumping, is small under physiological conditions. However, it might become problematic under stress conditions (oversaturating light), when oxygen concentrations rise and/or pH drops (photoinhibition). Indeed, it was found that photoinhibition occurs in microbial mats, where the oxygen concentration is increased up to sevenfold over ambient (Jonkers et al. 2003).

The present work on the suppression of oxygen evolution by acid pH is in line with previous studies on the effect of high oxygen pressure (Clausen and Junge 2004; Clausen et al. 2005). Half-suppression occurred upon an only 10-fold increase over ambient of the respective concentration, both for oxygen and for proton. A concentration change by a factor of 10 implies a Free Energy change of only 5.6 kJ/mol. Energetically, the performance of photosystem II seems to be driven to the limits such that the remaining driving force for the terminal reaction cascade from water to dioxygen is rather small. Without further engineering, nature does not have the option to raise the present atmospheric oxygen level very much above the present one.

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