Published in final edited form as:

Biochemistry. 2009 February 17; 48(6): 1381–1389. doi:10.1021/bi801774f.

Photosynthetic oxygen evolution is not reversed at high oxygen pressures:

mechanistic consequences for the water-oxidizing complex†

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Abstract

We investigated the effects of elevated O₂ pressure on the production of O₂ by photosynthetic organisms in several species of plants, algae, and a cyanobacterium. Using a non-invasive fluorometry technique to monitor sequential turnover of the Photosystem II (PSII) reaction center as a function of O₂ pressures, we showed that none of the reactions of water oxidation are affected by elevated O₂ pressures up to 50-fold greater than atmospheric conditions. Thus, the terminal step of O₂ release from the water oxidation complex ($S_4 \rightarrow S_0 + O_2 + nH^+$) is not reversible in whole cells, leaves, or isolated thylakoid membranes containing PSII, in contrast to reports using detergent-extracted PSII complexes. This implies that there is no thermodynamically accessible intermediate that can be populated by preventing or reversing the O₂ release step with atmospheric O₂. To assess the sensitivity of PSII charge recombination to O₂ pressure we quantitatively modeled the consequences of two putative perturbations to the catalytic cycle of water oxidation within the framework of the Kok model. Based on the breadth of oxygenic phototrophs examined in this study, we conclude that O₂ accumulation in cells or the atmosphere does not suppress photosynthetic productivity through the reversal of water oxidation in contemporary phototrophs, and would have been unlikely to influence the evolution of oxygenic photosynthesis.

Oxygenic photosynthesis is the most successful form of biological energy metabolism on Earth today. In terms of biomass, it dominates our planet, being represented by all species of higher plants, algae and cyanobacteria. Recent publications (1-3), letters (4-6) have addressed a fundamental question of energy metabolism in these organisms: Is the production of O₂ by photosynthesis slowed or prevented at atmospheric pressures of O₂ that are or were formerly present on Earth? If the answer is yes, the consequences for the evolution of life on earth are significant. Moreover, this question addresses the fundamental understanding of the chemistry of water oxidation by photosynthesis, which serves as model for creating biomimetic catalysts for solar-to-chemical energy conversion.

Clausen *et al.* (1,2), have given recent evidence that the last step in the four-step water oxidation reaction has an intrinsically low thermodynamic driving force (ΔG) and thus, can be prevented by application of low O₂ pressure (50% suppression at 2.3 bar with an observable suppression beginning at atmospheric pressure). A consequence of their conclusion is that the rate of photosynthetic energy storage (proton-driven ATP production) would slow even at partial solar flux owing to charge recombination in O₂-blocked centers. This postulated loss of solar energy

†This work was supported by grants from the NIH GM-39932 and NSF-IDBR # DBI-0138012.

D.R.J.K. was supported from the Camille and Henry Dreyfus Postdoctoral Fellowship in Environmental Chemistry and Donors of the American Chemical Society Petroleum Research Alternative Energy Fellowship.

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conversion efficiency could have slowed the evolution of oxygenic photosynthesis on early Earth (7). Other lines of historical evidence, which have been recently reviewed, have concluded that atmospheric O₂ has little if any effect on photosynthetic productivity under current atmospheric conditions (3). In contrast, inhibition of photosynthetic biomass accumulation due to illumination at elevated O₂ pressure does occur in photobioreactors (8). Although, the origin of this loss is believed to be dominated by O₂-sensitive photoinactivation rather than blocking of O₂ evolution (9).

To understand how O₂ pressure might possibly influence the chemistry of O₂ production, we must examine the catalytic site, the O₂-evolving complex (OEC), located within a domain of the Photosystem II reaction center protein complex (PSII). In the overall reaction, four electrons and four protons are stripped from two water molecules to produce O₂. It has been known for 36 years that O₂ production requires a catalyst that accumulates four electron vacancies (oxidation equivalents or holes) formed upon four sequential light-driven charge separation reactions (10,11). The yield of O₂ formed by the OEC upon exposure to individual short flashes is described by the standard Kok model. This model is a simple Markov chain that passes through five sequential oxidation states (S₀, S₁, S₂, S₃, S₄) that are populated by photon absorption. After S₄ forms, it spontaneously decays in the dark to form the initial state, S₀, by releasing O₂ (Fig. 1A). The Kok model is shown in Fig. 1A and includes two additional postulated intermediates between S₃ and S₀ that have been proposed recently (see below).

Recent evidence suggests that the terminal step in O₂ evolution in which the O-O bond forms, S₄ → S₀ + n H⁺ + O₂, may proceed through an intermediate (5,12). Time-resolved “pump-probe” X-ray absorption spectroscopy (XAS) capable of tracking Mn X-ray fluorescence changes with a time resolution of 10 μs has detected a transient intermediate that forms after photoexcitation of S₃ and before O-O bond formation (12). This intermediate was proposed to be a deprotonated S₃-state (denoted S₄[′]). It is important to note that the XAS data that supports the existence of this intermediate at ambient pressure does not show evidence for or against a second intermediate (12), which was recently a point of contention (4-6).

Clausen and Junge proposed the formation of a different intermediate during S₄ → S₀, an S₂-peroxo intermediate denoted S₂^{*}(H₂O₂), in which the substrate water has been partially oxidized by two electrons (1,2). This was postulated to form at elevated O₂ pressure and unlike native S₄, was speculated to decay to the native S₂ state with release of H₂O₂, e.g., S₄ → S₂^{*}(H₂O₂) → S₂ + H₂O₂. This hypothesis was based on a decrease in the manganese UV absorbance change following photo-generation of S₄ under elevated O₂ pressures. To explain this result, it was proposed that an S₂^{*}(H₂O₂) intermediate builds up at elevated O₂ pressure as a result of a shift in the equilibrium of the terminal reaction. It was also speculated that the S₂^{*}(H₂O₂) intermediate could decay to S₂ upon liberation of H₂O₂ (1). Formation and decay of an intermediate was later supported by delayed fluorescence work done by Clausen *et al.* (2), although H₂O₂ was not detected as a product. Both of these possibilities have been included in the modified S-state cycle model represented in Fig. 1A. The observed effect reached 50% of maximum at 2.3 bar and saturated between 14 and 30 bar. Their study was performed with detergent-extracted PSII core complexes from the cyanobacterium *Synechocystis* sp. PCC6803, and was supported by independent measurements of delayed chlorophyll-*a* fluorescence using detergent-extracted PSII-enriched thylakoid membranes isolated from spinach (2). The formation of a native peroxide-like intermediate at S₂ has been postulated before (13-16) and H₂O₂ production has been observed from blocked PSII centers incapable of O₂ production or normal S-state turnover (17,18). However, there has been no direct experimental support for a peroxo intermediate or O₂-bound product following S₄ in any PSII complex until the reports by Clausen *et al.*

In the present study, we have investigated O₂ production in all three major branches of oxygen-producing phototrophs at elevated O₂ pressures up to 10.3 bar (and up to 43 bar in *A. maxima*) using a non-invasive method that monitors chlorophyll fluorescence from intact cells (microalgae and cyanobacteria), plant leaves, and isolated thylakoid membranes. Our results show that none of the reactions of water oxidation, especially the terminal step ($S_4 \rightarrow S_0 + O_2 + nH^+$), are mechanistically affected by elevated O₂ pressures up to 50-fold greater than atmospheric conditions. Thus, the terminal reaction has no thermodynamically accessible peroxo or O₂-bound intermediate that can be populated by reversing the O₂ release step using elevated levels of O₂. The consequences for the mechanism of water oxidation are examined.

EXPERIMENTAL PROCEDURES

Sample Preparation

Subchloroplast membrane fragments (thylakoids) were isolated from market spinach following Berthold *et al.* (19) with minor modifications as follows: a commercial juicing machine was employed for initial mechanical homogenization of the sample; material isolated after the second centrifugation step was used in the experiments. *A. maxima* cells were grown at 30°C in complete Zarrouk medium at initial pH 9.0 and light intensity 40 $\mu E\ m^{-2}\ s^{-1}$, and harvested just after the logarithmic phase of growth (6-8 days, pH 8.6). CO₂ bubbling was provided to replace inorganic carbon depleted from the growth medium through carbon fixation. Samples were suspended at 50 $\mu g\ Chl/ml$ and placed in shallow sample cuvette (1.0 mm deep, 50 μL total volume). A “moat” surrounding the sample cuvette was filled with water to prevent sample dehydration during long measurement periods. For simultaneous O₂ concentration and fluorescence yield experiments, whole cells of *A. maxima* were layered on a membrane-covered Clark-type Pt electrode at a thickness 0.3 mm and dark adapted for 5 min before flash illumination (20). *Dunaliella* sp. CCMP 1320 was grown at 25°C in f/2 medium that was not supplemented with silica (<http://www-cyanosite.bio.purdue.edu/media/table/f2.html>).

Fluorescence Measurements

A stainless steel measurement cell, capable of holding pressures up to 40 bar for 24-48 hrs, was built to adapt the existing FRR fluorometer (21) for high pressure measurements. The home-built FRR fluorometer is an accurate, versatile instrument that enables a wide range of chlorophyll fluorescence measurements. A laser diode excitation source ($\lambda_{max} = 655\ nm$) provides sharp, stable pulses at a maximum light intensity of 32,000 $\mu E\ m^{-2}\ s^{-1}$. In typical single turnover excitation experiments, a train of 25 identical pulses, each 1 μs in duration followed by a 1 μs dark interval, induces chlorophyll-*a* fluorescence at 680-690 nm to rise from its initial level after dark adaption (F_0) to a saturated state (F_m); the flash train thus constitutes a single-turnover flash in which 100% of the primary acceptor Q_A is reduced to Q_A^- . Repeating single-turnover flashes at 10-100 Hz produces transient period-four oscillations in the yield of variable fluorescence (F_v/F_m), indicating modulation from the donor side S-state cycle.

Analysis of Fv/Fm Data

Signal averaging of flash sequences was performed to improve S/N ratio. Dark adaptation between signal co-additions was chosen to ensure recovery of the initial S-state populations characteristic of the dark stable populations. Averaging was done over 2 to 24 hours for atmospheric conditions, while 2 hours was the upper limit used for elevated O₂ conditions. We compared the rate of damping of Fv/Fm oscillations that were produced by 50 saturating flashes starting with dark-adapted samples by using the following analysis (the procedure is demonstrated in Fig. 2): For the purpose of analyzing the oscillations, it is standard practice to remove the first flash response from all samples as this includes non-cycling PSII centers (21). Then, the non-oscillating baseline was subtracted from all traces using a cubic spline fit.

To compare the effect of elevated O_2 on the rate of damping of the Fv/Fm oscillations between samples, it was essential to compensate for the signal loss arising from inactivation of centers caused by superoxide in the samples that builds up upon exposure to elevated O_2 and light (this slow cumulative effect is included due to the 2-hr window used for averaging). This was accomplished by normalizing the trace from the oxygen-treated sample to the atmospheric-pressure sample by using the 3-to-5 flash amplitude. Finally, the Fast Fourier Transformation algorithm was then performed to obtain the frequency components responsible for the decay in amplitude from dephasing of S states. The lowest frequency attainable by our analysis is 0.5 flash number⁻¹, which corresponds to a period of 2 flashes. This is a mathematical limitation and is due to the fact that it is impossible to use a flash number below 2 in the experiment. Regardless, if a component of period 2 were present in our data, it would be observable as a shoulder at period 2. Data were plotted in Origin 7.0 and Fourier transformations were performed in software written by Sergei Astashkin (University of Arizona, Tucson). Kok model fittings were performed in Origin 7.0 using an algorithm reported by Shinkarev (22).

Traditionally, S-state cycling data are analyzed using a Kok model (11,22,23). However, deviations from progressing through four sequential S-states (e.g. skipping S_1 with an S_2^* (H_2O_2)→ S_2 shunt, Fig. 1A) would cause the symmetry in the model to be broken and require additional parameters in an already underdetermined model (22-24). To bypass these difficulties, FRRF data were analyzed by fast Fourier transform to characterize the frequency spectrum of the variable fluorescence yield (Fv/Fm) upon S-state cycling. This model-independent approach eliminates the assumptions implicit in the Kok model, as it simply relies on measuring the different intensities of the oscillating components of Fv/Fm produced during S-state cycling.

Simulation of Fv/Fm Data

A simple Markov model was implemented in MatLab 7.4.0 to generate the simulation. For the Kok model, the following equation was used:

$$\begin{bmatrix} S_1 \\ S_2 \\ S_3 \\ S_0 \end{bmatrix} = \begin{bmatrix} S_0 & S_3 & S_1 \\ S_1 & S_0 & S_2 \\ S_2 & S_1 & S_3 \\ S_3 & S_2 & S_0 \end{bmatrix} * \begin{bmatrix} 1 - \alpha - \beta \\ \beta \\ \alpha \end{bmatrix} - \begin{bmatrix} 0 \\ 0 \\ 0 \\ 0 \end{bmatrix}$$

where, α and β represent misses and double hits, respectively.

For the shunt model, the following equations were used:

$$\begin{bmatrix} S_1 \\ S_2 \\ S_3 \\ S_0 \end{bmatrix} = \begin{bmatrix} S_0 & S_3 & S_1 \\ S_1 & S_0 & S_2 \\ S_2 & S_1 & S_3 \\ S_3 & S_2 & S_0 \end{bmatrix} * \begin{bmatrix} 1 - \alpha - \beta \\ \beta \\ \alpha \end{bmatrix} - \begin{bmatrix} 0 \\ -S_3 * (1 - \alpha - \beta) * \delta - S_2 * \beta * \delta \\ 0 \\ S_3 * (1 - \alpha - \beta) * \delta + S_2 * \beta * \delta \end{bmatrix}$$

where, α and β represent misses and double hits, respectively; and δ represents the percentage of centers undergoing the $S_3 \rightarrow S_2$ shunt.

For the inactivation model, the following equations were used:

$$\begin{bmatrix} S_1 \\ S_2 \\ S_3 \\ S_0 \end{bmatrix} = \begin{bmatrix} S_0 & S_3 & S_1 \\ S_1 & S_0 & S_2 \\ S_2 & S_1 & S_3 \\ S_3 & S_2 & S_0 \end{bmatrix} * \begin{bmatrix} 1 - \alpha - \beta \\ \beta \\ \alpha \end{bmatrix} - \begin{bmatrix} 0 \\ 0 \\ 0 \\ S_3 * (1 - \alpha - \beta) * \gamma + S_2 * \beta * \gamma \end{bmatrix}$$

where, α and β represent misses and double hits, respectively; and γ represents the percentage of centers that during the $S_3 \rightarrow S_0$ step, undergo an inactivation.

The sum of the S_1 and S_0 populations was then determined per theoretical flash. These populations were subjected to an iterative process where results from one theoretical flash were used to generate results for the next theoretical flash.

RESULTS

The light-intensity-dependent chlorophyll-*a* fluorescence yield, called variable fluorescence (Fv), is sensitive to both the redox state of the Mn_4Ca cluster (i.e., the S state) and the redox state of the primary quinone acceptor (Q_A) (25-27). This sensitivity has been widely used to monitor the yield and kinetics of turnover of PSII (28,29). Using laser-based fast-repetition-rate fluorometry (FRRF) (21), we monitored the yield of prompt chlorophyll-*a* fluorescence arising from photon emission using short (single turnover) flashes that saturate the yield of charge separation. Starting with dark-adapted samples, the fluorescence yield on each flash rises from a low level observed in samples with open PSII centers (F_o), to a maximum level in closed centers (F_m). The ratio of the variable to maximum fluorescence yields, (F_m-F_o)/F_m, denoted by Fv/Fm, is proportional to the PSII charge-separation quantum efficiency (21, 29). A representative example is shown in Fig. 2A. It is useful to note that Fv/Fm is an intensive variable and thus, is independent of sample size.

The accuracy with which FRRF can monitor the S-state cycle is illustrated in Figure 1B, which shows that when a series of flashes is given to a dark-adapted sample, Fv/Fm oscillates 180° out of phase with the O₂ yield (measured electrochemically via a fast Clark electrode) (21). The approximately period-four oscillation arises because the resulting fluorescence from the reaction center depends upon the S-state of the OEC (26). Laser-based FRRF thus provides an alternative method to monitor S-state cycling that works well at very high flash repetition rates and with intact phototrophs, unlike oximetry which relies on O₂ diffusion to an external electrode, and thus is limited to about 5 hertz flashing rates and protein or cellular suspensions. O₂ detection using a bare Pt electrode and thin film samples also has fast resolution, but requires electrochemical biasing of the sample at a reducing potential (Joliot-type electrode). This biasing produces H₂O₂ which alters the S-state distribution and eventually inactivates the sample (30). Another benefit of FRRF, and essential to the basis of this paper, is that high levels of O₂ do not directly interfere with the detection of chlorophyll-*a* fluorescence as opposed to electrochemical detection of O₂. Oxygen electrodes are quickly saturated at the O₂ pressures used within this study.

High-pressure O₂ Data

To record the intrinsic effect that O₂ pressure has on water oxidation chemistry *in vivo*, we examined living cells and leaves under otherwise physiologically relevant conditions following dark adaptation to achieve population of the dark stable S-states. Samples consisted of a cyanobacterium, *Arthrospira maxima* CS328, a marine alga, *Dunaliella* sp. CCMP 1320, a freshwater alga *Chlamydomonas reinhardtii*, and several higher plant species: spinach, white clover, ginkgo, *Euonymus* sp., and sugar maple (Fig. 2, Fig. 3, and Table 1). We also obtained data from a subcellular sample of native spinach thylakoid membranes which were isolated

without using detergents (Fig. 3 and Table 1). We were unable to obtain high-quality Fv/Fm data on detergent-extracted PSII protein and detergent-fractionated PSII-enriched thylakoid membranes (like those used in references (1,2), respectively) due to higher background chlorophyll-*a* fluorescence from free or uncoupled chlorophyll.

The stability of the samples was monitored by integrating individual Fv/Fm flash sequences and plotting the sum of 50 flashes taken each 2 min, denoted integrated Fv/Fm, as a function of time over 24 hours (supporting information, Fig. S1). Samples under atmospheric conditions showed steady-state Fv/Fm (the non-oscillating component, which is approximately the Fv/Fm level during the final 10 flashes of the 50-flash measurements) that was either stable for 24 hours (other than random noise around some basal level), or had a small and very slow rate of decay reflecting a variety of intrinsic factors, most notably photoreduction of the finite pool of electron acceptors (21). All organisms showed a more rapid decay of the non-oscillating component of Fv/Fm over time when exposed to elevated levels of O₂ and light. Additionally, the rate of this decay was strongly species dependent. This decay was only observed when the samples were exposed to light and was not seen when samples were exposed to elevated levels of O₂ in the dark. Additional studies not reported here allowed us to attribute the decay of the non-oscillating component of Fv/Fm under elevated O₂ conditions to the production of oxygen induced radicals via PSI-mediated photoreduction of O₂ (i.e., the Mehler reaction) (Kolling, Brown, Ananyev, and Dismukes. Manuscript in preparation.). This decay of the fluorescence signal over 24 hours resulted in a relative loss of intensity of the oscillations in samples under elevated O₂ conditions versus those under atmospheric conditions (SI Figure S7). The decay of the oscillations begins to occur after 0 to 4 hours exposure to light and O₂, and depends upon the species (Fig. S2). This inactivation over hours is relatively slow compared to the damping of the oscillations. Although interesting, the focus of this study is the mechanism of water oxidation and not slow degradation of PSII turnover due to reactive oxygen species; therefore, here we focus on analyzing the changes in the oscillating component of Fv/Fm, which include changes in the rate of decay in the oscillations and the period.

Unlike the Kok model used for analyzing the S-state cycle (11), the Fourier transformation of the oscillations provides a model-independent approach to data presentation which eliminates the need for assumptions about the number of S-state cycle parameters and upon which S-states they act (22,23). An increase in the rate of damping of the oscillations (loss of amplitude during the 50-flash measurement used for sampling the S-state cycle) will show up as an increase in the Fourier period. A comparison is shown of measurements taken at 1 bar air vs. 43 bar oxygen for *A. maxima* in Fig. 2. The raw data reveal there is about 25% loss in amplitude of the steady-state Fv/Fm level over the two hour integration period, which is due to the aforementioned PSI-dependent Mehler reaction. All data were analyzed as shown in the example of *A. maxima* in Fig. 2 and described in the Experimental Procedures. Briefly, to directly compare the rate of damping of the oscillations, a spline fitting was first subtracted from both data sets, which removes the slowly decaying component of the steady-state Fv/Fm. Next, in order to compare the rate of damping of the oscillations, the data were normalized to the 3-to-5 peak intensity of the 1-bar air sample. Finally, a Fourier transformation is performed and for this sample there is a small, 3.2%, increase in the oscillation frequency and a small, -3%, loss in the Fourier amplitude at 43-bar oxygen versus 1 bar air (210x atmospheric pressure). This indicates that there is minimal difference in the rate of damping of oscillations under 43-bar oxygen. Measurements taken at 7 and 10 bars oxygen produce smaller shifts in the frequency equal to 0.2% and -1.2% with small changes in Fourier amplitude of 3% and 0%, respectively. From this, we learn that there are only small changes in the frequency of the S-state cycle, indicating minimal impact of oxygen pressure on the catalytic cycle of water oxidation for whole cells of *A. maxima*. In addition, oxygen pressure causes little, if any, changes in the Fourier amplitude.

Similar results were observed for seven other species of algae and higher plants as shown in Fig. 3 (raw data found in Fig. S3). Table 1 summarizes the relative changes in Fourier intensity and period of the oscillations (flash number). At 10 bar O₂ there are only small changes in the period of oscillations ranging from -3.7% to +3.9% with a comparable distribution of positive and negative examples (Fig. S4). Comparison of all samples exposed to atmospheric conditions and 10 bar O₂ shows that there may be a weak anticorrelation between the small changes in the Fourier amplitude and period. Specifically, the data show that a positive (negative) change in Fourier amplitude correlates with a negative (positive) shift in Fourier period (Fig. S4). We note that small nonsystematic differences in S-state cycle period are observed at atmospheric conditions which give a range of periods for any given type of sample (4.1-4.5 flash numbers). These variations arise from using field samples of different ages, physiological state, and prior history of the samples.

Interestingly, in the case of white clover, *Dunaliella* and *Euonimus*, 10 bar O₂ pressure actually *increased* the initial amplitude of Fv/Fm and lowered the rate of damping of oscillations prior to the onset of slow O₂ induced photoinactivation. The former is revealed by increases of 16, 9 and 4% in the Fourier amplitude, respectively. There is also slightly slower damping of these three samples (S-states retain synchronization for a greater number of flashes) which shows up as a small decrease of the Fourier period of -3.7, -0.7 and -2.3%, respectively. This decrease in period corresponds to centers that approach the idealized response of 4 flashes expected for an S-state cycle without damping (11). We may attribute these simultaneous increases in the non-oscillating Fv/Fm level, Fourier amplitude, and reduced damping of the oscillating Fv/Fm signal to O₂-induced oxidation of a partially reduced electron acceptor pool (plastoquinone). An increase in Fourier amplitude can also arise due to a shift in the dark distribution of S states from 25% S₀ + 75% S₁ in most dark adapted OECs to 100% S₁, caused by oxidation of the metastable S₀ state (11,31). In summary for these three samples, the OEC behaves as if elevated O₂ produces a more synchronized population of PSII centers with less damping.

A comparison of intact spinach leaves vs. isolated thylakoid membranes reveals a loss of Fv/Fm intensity in thylakoids (Fig. S5). This arises from the limited electron acceptor capacity (plastoquinone pool size), which is partially lost upon isolation of thylakoids (32). The effect of 10 bar O₂ pressure on spinach leaves and thylakoids was distinguishable, but insignificant; Leaves and thylakoids exhibit small changes in Fourier period of +0.5% and -0.7%, respectively (Table 1).

DISCUSSION

Our studies show that there are only small direct effects of high O₂ pressure on the frequency and damping of the oscillations of the S-state cycle in intact cells and leaves sampled across cyanobacteria, algae and higher plants. This demonstrates that the mechanism of the OEC S-state cycle is unaffected in the presence of elevated levels of O₂. The weak anticorrelation between the small changes in the Fourier amplitude and Fourier period and the small elevated initial level of Fv/Fm at 10 bars O₂ can be attributed to known mechanisms that affect the electron acceptor pool or the distribution of native S₀ / S₁ states in the dark. In addition, we observed decreases in the non-oscillating component of the Fv/Fm signal intensity over time (on an hours-scale) due to build-up of reactive O₂ species produced by PSI through a light-dependent mechanism (Kolling, Brown, Ananyev, Dismukes. Manuscript in preparation). Our findings are consistent with several historical studies which show that atmospheric O₂ has little if any direct effect on photosynthetic electron transport and productivity other than secondary photoinactivation in some samples (3).

The absence of effect of 10 bar O₂ pressure on the S state cycle in spinach thylakoid membranes is noteworthy as membranes have reduced levels—if any at all—of respiratory enzymes that

could lower intracellular O₂ concentration. This observation contrasts with that made by Clausen *et al.* who found, using detergent-extracted PSII protein and protein-enriched membrane complexes, that they were unable to perform multiple complete S-state cycles at even lower O₂ pressures (1,2). In order to investigate this discrepancy further, we have performed simulations based upon two modified S state models that would be expected if an inactivation or shunt phenomenon were present, similar to the model of Clausen *et al.* Next we describe these simulations and compare them to the data.

Simulated vs. Real Photosynthetic Variable Fluorescence Data

Simulations were done using a simple Markov model that predicts S-state populations as a function of single-turnover flash number (see Experimental Procedures). Figure 4 shows the comparison between real and simulated Fv/Fm data as a function of single-turnover flashes for *A. maxima* at atmospheric pressure (Fig. S6 shows comparisons of simulations to Fv/Fm in other species). For comparison to the real data, the simulated data were processed by using the same method described in the Experimental Procedures and shown in Fig. 2; notably, this also includes the 3-to-5 flash amplitude normalization. The sum of the S₀- and S₁-state populations was found to accurately predict the Fv/Fm oscillations found in all of the samples, as previously described for *A. maxima* in air (21).

Shunt Simulation

Perturbations to the S-state populations in a normal Kok model were calculated by addition of a shunt with the S₃-state proceeding directly to the S₂-state. This model also includes the possibility of a shunt occurring from the S₄-state (see Fig. 1A); therefore, the shunt postulated by Clausen and Junge and later supported by Clausen *et al.* is also described by this shunt simulation (1,2). Figures 5A, 5C, and 5E show the results of the shunt simulation as a function of the % of the PSII centers participating in the shunt. The simulated data correspond to the optimal fitting of the real data using the analytical solution to the Kok model (parameters: $\alpha = 0.12$; $\beta = 0.03$; initial S₀ = 0.25; initial S₁ = 0.75). The rate of damping of the oscillations becomes greater during each 50-flash simulation and the Fourier amplitude of the period-four peak decreases, respectively. Upon 100% of photocenters experiencing the shunt, the signal is similar to a DCMU-treated sample (blocks Q_A⁻ reoxidation) in which most photocenters are blocked after one flash and no longer contribute to variable fluorescence (Fig. S7).

Fourier transformation of the shunt model populations shows that as the effect of the shunt becomes greater, the period of the oscillations shift slightly to a greater number of flashes ($\Delta=0.09$ (2%) when 50% of the centers experience a shunt). In other words, as the shunt becomes more prevalent, more flashes are needed to cycle the system through the S₀-state. This positive shift is not large enough to use as a definitive metric for the presence of the shunt in actual systems, as the predicted shift generally falls within the range of shifts expected from other sources as described above. By contrast, the model predicts a large acceleration in the loss of the oscillating amplitude of Fv/Fm as more centers are affected by the shunt; this effect is significant and can be used as a metric (Fig. 5E). It should be clear that a system with a shunt should follow the trend predicted by the simulations.

This shunt simulation does not account for the possibility that an intermediate generated during the S₃-state to S₀-state transition would result in a higher level of fluorescence. It is reasonable to assume that the formation of S₂^{*}(H₂O₂) would lead to a higher level of fluorescence as the formation of H₂O₂ requires more energy than forming O₂; but the intensity of such change is difficult to predict, and its inclusion into the simulations would be problematic due to the vast number of trials that would have to be performed to explore this possibility. A noticeable change in the level of fluorescence due to this intermediate would lead to a shift in the period of the

oscillations and would be noticeable in our data. Since we did not observe any large shifts or formation of new peaks in our Fv/Fm data, there was no reason to attempt further simulations.

Inactivation Simulation

Simulations were performed to determine what would happen to S-state populations if an inactivation occurred following the S_3 -state. We modeled this inactivation by removing a percentage of PSII centers from the PSII population being measured after they reached the S_3 state. This simulation addresses the case, as suggested by Clausen and Junge and Clausen *et al.*, in which PSII centers in the hypothetical $S_2^*(H_2O_2)$ state are stabilized for the duration of the measurement (see Fig. 1). These centers would no longer contribute to the Fv/Fm measurement and hence, appear inactivated. It should also be noted that inactivation of the S-state cycle by trapping the S_3 -state, S_4 -state, or any intermediates during the terminal reaction ($S_4 \rightarrow S_0$) would be accurately described by this simulation.

Figures 5B, 5D, and 5F show the results of the inactivation at any point within $S_3 \rightarrow S_0$ on the S_0+S_1 populations and the corresponding Fourier transform, respectively; individual traces show conditions under which a certain percentage of centers are inactivated during a single PSII turnover. As a greater percentage of the PSII centers are subjected to an inactivation event, the intensity of the oscillations decreases as a simple first-order exponential decay function.

The Fourier transformation shows that as the % of centers contributing to the inactivation becomes greater, the period of the oscillations shift slightly to greater number of flashes ($\Delta = 0.02$ at 50% effect), but not as much as described previously for the period of the shunted model. By contrast, the predicted large decrease in Fourier amplitude can be used as a metric for this model.

Analysis of Models

Comparison of the models to the experimental results in Table 1 reveals that only data for Sugar Maple (10 bar), *C. reinhardtii* (10 bar) and *A. maxima* (43 bar) show both positive shift in Fourier period and loss of Fourier amplitude larger than random error, as predicted by both models. However, these changes are relatively small. *C. reinhardtii* showed the most pronounced decrease in its Fourier amplitude at 9%, accompanied by a very small 0.9% increase in period. The shunt model simulation predicts that a ~19% decrease in Fourier amplitude would occur for a 10% shunt effect. The inactivation model simulation predicts a 10% amplitude decrease for a 10% inactivation effect. Thus, at best these models could account for the response of only 5% and 9% of PSII centers in *C. reinhardtii*, respectively. None of the other seven distinct organisms exhibit blockage in S state cycling at elevated O_2 .

In the event of a shunt with the S_3 -state proceeding directly to the S_2 -state one might expect to see a period 2 oscillation as the PSII centers would only need two flashes to turnover. This effect was not observed in the simulations and is a result of the fact that one observes a high level of fluorescence on the S_0 -state to S_1 -state and S_1 -state to S_2 -state transition while fluorescence is largely quenched on the S_2 -state to S_3 -state and S_3 -state to S_0 -state transition (Fig. 1). The S_3 -state to S_2 -state shunt would result only in the third and fourth transitions of the S-state cycle, which would no longer result in an oscillation pattern.

Clausen and Junge and Clausen *et al* (1,2) determined that the O_2 induced blockage of S-state cycling in isolated PSII centers reached its half maximum point at 2.3 bar O_2 . Based on these works, we should expect to see a 75% blockage in our samples exposed to 10.3 bar O_2 . Even our measurements at 43 bar with whole cells of *A. maxima* failed to reveal a significant blockage in S state cycling. Our results do not resolve the origin of the effect they reported, although it may be a result their using detergent-extracted PSII particles.

In conclusion, photosynthetic cells are known to be sensitive to damage caused by illumination in the presence of O₂. Oxygen accelerates the rate of damage to whole cells and leaves upon illumination at a site that involves superoxide formed at PSI and by reactive O₂ species production. However, the present results establish that S-state cycling within the OEC of native leaves and cells from both prokaryotic and eukaryotic oxygenic phototrophs is insensitive to O₂ pressure up to 50 times current atmospheric partial pressure (215x in *A. maxima*). We investigated two models for inactivation of S-state cycling which reveal distinctly different deactivation patterns of S-state populations, which were used to assess the sensitivity of the experimental method (14,15). From this we may conclude that the energetics of the terminal step, $S_3 \rightarrow S_0 + O_2 + e^- + nH^+$, are sufficiently exoergic that the release of O₂ cannot be prevented. This result does not mean that a peroxo species does not form during this transition, as there are sound energetic reasons for predicting this as intermediate (16,17), rather that it cannot be trapped by shifting the equilibrium. These results have implications for models of how oxygenic photosynthesis may have evolved in earlier geological epochs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank Paul Falkowski for ECCMP 1320 and Peter Wu for preliminary simulations.

ABBREVIATIONS

PSII, Photosystem II; OEC, O₂-evolving complex; FRRF, fast-repetition-rate fluorometry; XAS, X-ray absorption spectroscopy; DCMU, (3-(3,4-dichlorophenyl)-1,1-dimethylurea); *A. maxima*, *Arthrospira maxima*.

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Figure 1. The S-state cycle and how to monitor it

(A) Scheme of intermediates (S-states) in the reaction cycle of photosynthetic water oxidation (i.e., the Kok model). Lightning bolts represent photon absorbance events (hits) and wavy arrows represent fluorescence emission (arrow size correlates with fluorescence quantum yield). Proton release events are omitted in this scheme. Recently proposed intermediates of the terminal reaction of the cycle are indicated with a dashed boundary (5,12); also included is a hypothesized alternative pathway under elevated O_2 pressure (dashed arrows). (B) The PSII oxygen yield (—○—) and Fv/Fm (relative variable Chl-*a* fluorescence yield, —□—) obtained from single-turnover flashes given to cells of *A. maxima*. Note that the two methods produce approximately “period-four” oscillations, opposite in phase and with slightly different damping, reflecting S-state cycling.

Figure 2. Data analysis of *Arthrospira maxima* Fv/Fm traces under 1 bar air and 43 bar O₂
 Upper left panel of the figure shows Fv/Fm traces for *A. maxima* under atmospheric (□-) and elevated O₂ conditions (○-). The first point is removed from the trace and it is then subtracted by a cubic spline fitting to result in the traces in the upper right panel. For comparison of the oscillations, the O₂ trace is then normalized to the air trace at the 3-to-5 flash amplitude. Finally, the fast Fourier transformation of both traces over the 50 flash interval is taken and shown in the lower left panel. Comparison of these result shows that there is little change in the Fourier amplitude and the period of the cycle upon exposure to 43 bar O₂.

Figure 3. Fourier Transformation of Fv/Fm data for organisms at 1 bar air and 10.3 bar O₂
 Solid traces represent 1 bar air and dashed traces represent 10.3 bar O₂. All data were treated as described in Experimental Procedures and are on the same scale. Relative differences between the traces and respective normalization factors for each species are listed in Table 1.

Figure 4. A: Simulated Fv/Fm data versus Fv/Fm data of *A. maxima* at 43 bar O₂
A. maxima Fv/Fm data was baselined with a cubic spline fitting (-□-). Simulated Fv/Fm data were generated as described in Experimental Procedures (-○-).

Changes in Oscillating Component of Fv/Fm Fourier Amplitude and Fourier Period Relative to Atmospheric Conditions^a

Table 1

Organism	Normalization Factor ^c	Fourier Amplitude Change (%) ^b	Period	
			1 bar air	ΔPeriod
Higher Plants (leaves)				
<i>Ginkgo biloba</i>	1.05	-2	4.20	0.02
Sugar Maple	1.06	-4	4.40	0.17
<i>Euonimus</i> sp.	1.16	4	4.31	-0.1
White Clover	0.94	16	4.29	-0.16
Spinach	1.04	3	4.17	0.02
Spinach Thylakoids	1.41	0	4.14	-0.03
Algae				
<i>Dunalieta</i> sp. CCMP 1320	2.18	9	4.44	-0.03
<i>Chlamydomonas reinhardtii</i> cc124	1.92	-9	4.37	0.04
Cyanobacteria				
<i>Arthrospira maxima</i> (7 bar)	1.26	3	4.13	0.01
<i>Arthrospira maxima</i>	1.68	0	4.17	-0.05
<i>Arthrospira maxima</i> (43 bar)	1.22	-3	4.36	-0.14

^aData at 10.3 bars except as noted.

^bAmplitude changes follow normalization.

^c3-to-5 flash normalization factor applied to data taken under elevated O₂.