Photosynthesis Research (2005) 84: 355–365

Regular paper

How fast can Photosystem II split water? Kinetic performance at high and low frequencies★

Gennady Ananyev & G. Charles Dismukes*
*Department of Chemistry and Princeton Environmental Institute, Princeton University, Princeton, NJ 08544, USA; *Author for correspondence (e-mail: dismukes@princeton.edu; fax: +1-609-258-1980)*

Received 11 October 2004; accepted 2 December 2004

Key words: Arthrospira, *Chlorella*, Kok S-states; oxygen evolution, photosynthesis, Photosystem II, *Spirulina*, variable fluorescence, water oxidation

Abstract

Molecular oxygen evolution from water is a universal signature of oxygenic photosynthesis. Detection of the presence, speed and efficiency of the enzymatic machinery that catalyzes this process *in vivo* has been limited. We describe a laser-based fast repetition rate fluorometer (FRRF) that allows highly accurate and rapid measurements of these properties via the kinetics of Chl-*a* variable fluorescence yield (Fv) in living cells and leaves at repetition rates up to 10 kHz. Application to the detection of quenching of Fv is described and compared to flash-induced O_2 yield data. Period-four oscillations in both Fv and O_2, caused by stimulation of primary charge recombination by the O_2-evolving complex (WOC) within Photosystem II (PS II), are directly compared. The first quantitative calculations of the enzymatic parameters of the Kok model (α – miss; β – double hit; S-state populations) are reported from Fv data over a 5 kHz range of flash frequencies that is 100-fold wider than previously examined. Comparison of a few examples of cyanobacteria, green algae and spinach reveals that *Arthrospira m.*, a cyanobacterium that thrives in alkaline carbonate lakes, exhibits the fastest water-splitting rates ever observed thus far *in vivo*. In all oxygenic phototrophs examined thus far, an unprecedented large increase in the Kok α and β parameters occur at both high and low flash frequencies, which together with their strong correlation, indicates that PS II-WOC centers split water at remarkably lower efficiencies and possibly by different mechanisms at these extreme flash frequencies. Revisions to the classic Kok model are anticipated.

Abbreviations: α, β – miss and double hit parameters in Kok model; Chl – chlorophyll; FRRF – fast repetition rate fluorometer; Fv – variable fluorescence; PS II – Photosystem II; T_{dark} – dark interval between single turnover flashes; STF – single turnover flash with saturated light intensity; $Y_1 \ldots Y_n$ – yield of quantum efficiency Fv/Fm on 1…*n*th single turnover flashes; WOC – water-oxidizing complex; Y_Z – redox active tyrosine on the D1 protein

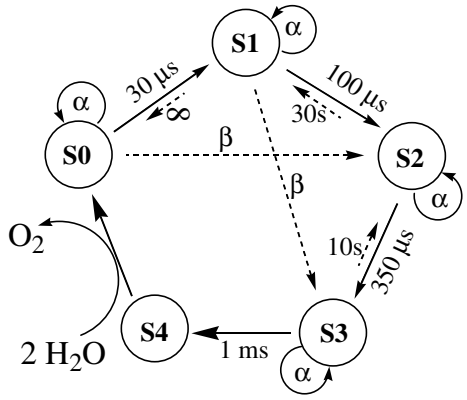
Introduction

It is our great pleasure to contribute to this special issue in honor of Professor Norio Murata. He has made historical contributions to the applications and understanding of fluorescence phenomena in photosynthetic systems, including the analysis of fluorescence emission and excitation spectra of Chl-*a* in chloroplast organelles and the influence of environmental factors. These works form part of the foundation of this paper.

★ Dedicated to Professor Norio Murata on the occasion of his retirement.

Fluorescence emission from the photooxidizable Chl-*a* molecule, P680, within Photosystem II (PS II) has served for more than 70 years as a sensitive probe of the bioenergetics processes that modulate the charge recombination reactions between $P680^+$ and photoreduced electron acceptors (Govindjee 1995; Schreiber et al. 1995). The yield of PS II variable fluorescence, denoted Fv, rises under saturating illumination to a maximum (Fm) and decays in the dark, reflecting events that fill the $P680^+$ hole and block the forward flow of electrons out of the reduced primary acceptor, plastosemiquinone-A, or Q_A^-. Total Chl-*a* fluorescence typically represents a loss of about 2–6% of the absorbed energy, while typically more than 95% of the total Chl-*a* fluorescence originates from PS II (Lazar 1999).

Quenching of Fv fluorescence occurs by interaction with the $[S_2] + [S_3]$ oxidation states of the oxygen-evolving (or water-oxidizing) complex of PS II (WOC), depicted in Scheme 1 (Delosme 1971). This interaction gives rise to an oscillation in Fv fluorescence yield with periodicity of four, when using saturating single turnover flashes. This modulation has served for more than 33 years as an internal monitor of the cycling in oxidation state of the WOC with enormous potential for non-invasive studies on intact organisms (Renger and Govindjee 1993; Delosme and Joliot 2002). However, applications of this technique to understanding the WOC both *in vivo* and *in vitro* have been very limited, stemming from the low level of quenching of Fv fluorescence by the WOC seen thus far, and experimental difficulties in detecting it compared to the greater instability of high power lasers and flash tube sources used to produce saturating flashes.



Scheme 1. Modified Kok model for flash-induced O_2 evolution yield showing the fitting parameters α (misses) and β (double hits) representative time constants for the forward and back (deactivation) reactions are shown from typical PSII complex in higher plants or green algae. The population of individual S-states depends on the pre-incubation dark time. At $T_{dark} = 30$ min, $[S0] = 0.25$ and $[S1] = 0.75$ (Kok et al. 1970). Increase of α leads to a significant reduction of the sharpness of the oscillation pattern and the increase of β leads to disappearance of oscillations and the increase of the amplitude of oxygen yield after the second flash (Shinkarev 2003).

Several other approaches have been used to monitor the turnover of the WOC by single turnover illumination: (1) amperometric detection of extra-cellular O_2 concentration (Joliot et al. 1969; Joliot 2003); (2) tyrosine-Z radical (Y_Z) reduction kinetics by EPR (Babcock et al. 1976); (3) H^+ evolution kinetics (Junge et al. 1977); (4) EPR signal from the Mn cluster (Dismukes and Siderer 1980); (5) optical absorbance changes at 290–320 nm from Mn (Dekker et al. 1984; Renger and Weiss 1986); (6) UV absorbance of $P680^+$ (Koike et al. 1987). The first of these approaches leads to the now iconic S-state model of Kok and coworkers (Scheme 1), which provided a phenomenological description of the oscillations in O_2 concentration (Kok et al. 1970). These approaches have contributed significantly to our understanding of the mechanism of water oxidation, but are either too slow for investigation of the upper limit of WOC turnover, or not applicable to whole organisms or chloroplasts that scatter light. Moreover, most have been applied to only a narrow range of oxygenic phototrophs, typically, the easily cultured or those with high PS II content. In order to search for possible diversity in the types of enzymes that perform oxygenic photosynthesis, there is a real need for new tools that permit quick and accurate detection of the speed and efficiency of WOC turnover in whole cells and leaves at both the high and low limits of turnover that occur *in vivo*.

The classical method for monitoring period-four oscillations of PS II-WOC turnover is based on the O_2 uptake rate from a thin mono-layer of cells placed directly on an open platinum electrode polarized to −650 mV (Joliot et al. 1968; Joliot 2003). The main limitations of this approach are: (1) the slow time resolution (owing to O_2 diffusion) which restricts the repetition rate to less than 10 Hz; (2) the consumption of O_2 produces both anaerobic conditions and harmful H_2O_2 in the

sample; (3) only changes in the rate of O_2 reduction are measured (absolute O_2 concentration is not known); and (4) the signal level arises from net extra-cellular O_2 output in competition with O_2 uptake processes. Multi-cellular species (like *Spirulina*, etc.) or leaves greatly retard the time resolution and generally cannot be studied by this approach. Chl-*a* fluorescence measurements overcome all of these limitations. The classical methods for Chl-*a* fluorescence measurements include xenon flash lamp as excitation source (Delosme 1971; Zankel 1973) or Q-switched lasers (typically at wavelength 532 nm), coupled with a modulated measuring light source that induces fluorescence, typically a blue light-emitting diode (Reifarth et al. 1997; de Wijn and van Gorkom 2002).

Currently there exists two advanced fluorescence methods for photosynthetic studies that could contribute to resolving these issues: (1) pulse amplitude modulated (PAM) fluorometry (Schreiber et al. 1993, 1996) and a double-modulation version of fluorometer (Trtilek et al. 1997; Nedbal et al. 1999); and (2) fast repetition rate fluorometry (FRRF) (Kolber et al. 1998). The PAM and double-modulation method uses two light sources: a lower intensity-modulated measuring source and a high power actinic source to induce photochemistry. These methods typically utilize ultra-bright light-emitting diodes or xenon flash lamp as sources. The FRRF method uses only one light source to do both the functions. Importantly, the FRRF method permits complete dark-adaptation of the sample and has also an intrinsically higher S/N ratio if operated at high flash frequencies where time-based noise discrimination is possible. The recent availability of high-power laser diodes having high frequency switching capability, excellent power stability and a range of wavelengths, now permits the development of improved benchtop FRRF instruments suitable for revealing even more subtle and short-lived fluorescence phenomena (Kolber et al. 1998) and can be adapted to remote sensing (Osmond et al. 2004).

Herein, we describe the design of a second-generation FRRF based on a powerful and stable laser diode source that achieves considerably higher intensity, sensitivity and time resolution and can be used on whole cells and leaves. We illustrate its capabilities with an application to the detection of period-four oscillations of Fv fluorescence yield in whole cells of cyanobacteria and green algae. This instrument allows measurement of the turnover rate of the water-splitting center of PS II over a greatly expanded frequency range, that extends the upper frequency limit by 100-fold. For the first time we can answer the question: how fast can water oxidation occur *in vivo* at high solar driving rates? By analysis of the data using the classic Kok model, we report very large changes in the photochemical misses and double hits, characterizing the inefficiencies in the water oxidation center as a function of the flash excitation rate. Revisions to the classical Kok model are proposed.

Materials and methods

The filamentous cyanobacterium *Arthrospira maxima* (*Spirulina maxima*, strain LB 2342) was obtained from UTEX, The Culture Collection of Algae at the University of Texas at Austin. It was grown on complete Zarrouk media at initial pH 9.0 and light intensity of 40 $\mu E\ m^{-2}\ s^{-1}$ (Vonshak et al. 1982). *Spirulina m.* is an alkalophile that originates from soda lakes containing 0.4–1.2 M carbonate and pH 10–11. The original source, Lake Natron, Chad, Africa, is noted for the exceptionally high abundance of *Spirulina m.*, and gives rise to the pigmentation of the pink flamingo which dines on *Spirulina m.* as its principal food source. The green algae *Chlorella pirenoidosa* and *Euglena sociabilis* were grown on standard BG11 media. The samples of cyanobacterial cells (50 µl at 50 µg of Chl/ml) were placed in a quartz cuvette 6 mm in diameter and 1.2 mm in depth.

A laser fluorometer was constructed that extends the LED-based configuration described by Kolber and coworkers (Kolber et al. 1998). A higher and wider range of flash repetition frequencies was achieved by several improvements to the laser source and flash controller. The excitation source was a laser diode (model BLI-CW-9M-C1-655-0.5M-PD, Boston Laser, Inc. USA) with emission at $\lambda_{max} = 655$ nm and maximum continuous optical power of 0.5 W. The light intensity incident on the sample was adjusted to a maximum of about 32,000 $\mu E\ m^{-2}\ s^{-1}$ at a driving current of 1.2 A by means of a focusing lens. A spot of uniform intensity was created using a solid light guide 6 mm in diameter with transmittance 75%

(Edmund Scientific, USA). This light intensity was sufficient to saturate the fluorescence emission within 10–60 μs in a variety of oxygenic phototrophs. A home-built electronic driver provides pulse currents up to 2.0 A at 5 MHz repetition rate. The maximum frequency for square optical pulse output is 500 kHz. Using a bank of buffer capacitors (48,000 μF) decreased the fluctuations in the exciting light intensity to less than 1%. As a photodetector, we used a cooled large area (16 mm) avalanche photodiode (APD, Advanced Photonix, Inc., USA) operating at 1.9 kV and with bandwidth from DC to 20 MHz. The quantum efficiency of the APD at 680 nm is 65–70%, or 10-fold higher than a typical red-sensitive photomultiplier tube.

The fluorescence signal was filtered in the wavelength interval from 680 to 690 nm by an interference filter centered at 685 nm with off-peak rejection = 4.0 O.D. (Intor, Inc., USA) and by a glass filter RG-695 (Schott North America, Inc., USA). The fluorescence signal was sampled at a rate of 20 MHz (50 ns/sample) using a 12-bit A/D data acquisition board (PCI-DAS4020/12 by Computing Measurements, Inc.). A programmable sequence of pulses for controlling the laser driver was generated from two 32-bit counters (PCI-6602, National Instruments, Inc.). The excitation pulse sequence employed in this study consists of a train of 25 pulses (fixed at 1 μs light and 1 μs dark) denoted as a single turnover flash (STF). Secondary illumination which selectively excites PS I at wavelength centered at $\lambda_{max} = 735$ nm was provided by a cluster of infrared LEDs at 0–5 mW optical power (Model QDDH75502, Quantum Devices, Inc.). Optical power measurements were made using a light meter (Model 1815-C, Newport Research Corp.). The photon flux density (PFD) was measured by a light meter (model LI-189) equipped with quantum sensor (LI-190SA, Li-Cor, Inc.).

Results

Chl-a *fluorescence induction by individual STF train*

Figure 1 illustrates the Chl-*a* fluorescence response from *Spirulina m*. cells to a train of 25 pulses (one laser-STF) of light duration 25 μs and total duration 50 μs. The use of a 500-kHz pulse rate and digital sampling allows superb rejection of noise from both ambient light and electrical sources, as well as interference from continuous background illumination. Because the sampling rate is 20 MHz, the Fo level can be measured reliably within 0.2–0.3 μs of the beginning of the first pulse and before induction of the fluorescence rise. The excitation wavelength of 655 nm is appropriate for both Chl-*a* + Chl-*b* containing organisms and the cyanobacteria which contain Chl-*a* + phycobilins instead of Chl-*b*.

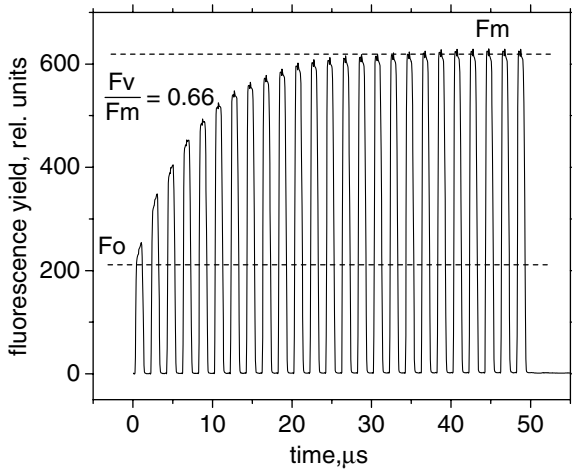


Figure 1. Experimental fluorescence emission intensity excited by a train of 25 laser pulses at $\lambda = 655$ nm. Each pulse has identical intensity and 1 μs duration and is separated by 1-μs dark period for a total illumination period of 25 μs. This train of pulses is called a single turnover flash (laser-STF) because it induces the fluorescence yield to rise between a minimum dark adapted level (Fo) and a saturated level (Fm). This train can be repeated at any frequency from 0 Hz to 10 KHz and with data sampling rate at 20 MHz. The sample is dark adapted *Spirulina maxima* filaments (50 μl) at a concentration of 50 μg Chl/ml.

The high power laser diode source produces very stable and sharp pulses which have several advantages. Fluctuations in the exciting light intensity were found to be very small, less than 1% and at a frequency which has no effect on the Fv/Fm ratio. Compared to a xenon flash lamp, the laser diode has no measurable light tail (Figure 1) which eliminates the probability of doing an inadvertent double turnover (contributes to β double hit Kok parameter). Compared to high power Q-switched lasers the light intensity is one order of magnitude more stable, which enables the detection of Fv/Fm changes as small as 0.003 (0.6%) at a Fv/Fm level = 0.5 for a single measurement. Pulses also lack the temporal structure

associated with Q-switched and mode-locked lasers, which can lead to the generation of emission quenchers in LHCII and PS II (Barzda et al. 2000). The present results show that the laser STF method provides the highest sensitivity and most reproducible measurement of Fv/Fm fluorescence.

In principle it is possible to create even shorter excitation pulses capable of saturating the PS II photochemistry by adjustment of the focal distance to create even higher optical power density on the sample. 100% yield of Q_A photoreduction can be attained in 2–5 μs. However, such fast Q_A reduction has no benefit for studies of the period-four oscillations in Fv from PS II because: (1) an increase in the digitizing error for shorter pulses and weaker signals due to the smaller excitation volume; (2) photobleaching of Chl fluorescence due to quencher generation from over excitation (Barzda et al. 2000; Chekalyuk et al. 2000); (3) the α miss and β double hit parameters of the Kok model do not change significantly (data not shown).

Transition from single to multiple turnovers

Figure 2 shows the Fv/Fm signal from *Spirulina* filaments obtained at two laser-STF repetition rates of 125 and 200 Hz. The average optical power of the laser-STF at 200 Hz repetition rate was converted to the average photon flux density (PFD) and is equal to 160 μE m^{-2} s^{-1}. In general, the Fv/Fm level exhibits a complex induction curve that consists of a sequential series of distinct phases previously described in the literature and called the Kautsky induction curve after its discoverer (Govindjee 1995; Lazar 1999). Figure 2 demonstrates that at least five distinct phases in the time evolution of Fv/Fm can be resolved following dark pre-incubation. In this report, we focus only on the initial transient response.

The inset in Figure 2 shows an expanded scale of the fluorescence induction during the first 30 STF trains, illustrating that transient oscillations occur with a periodicity of four. The initial amplitude of the oscillations amounts to about 16% of the steady-state Fv/Fm level provided the pre-illumination dark period is at least 5 min. The transient amplitude decreases until it reaches a value equal to an S/N ratio of 1 after 11 oscillation periods (Figure 3). The transient oscillations arise owing to the imbalance in populations favoring the S_0 and S_1 states in the dark, as they disappear completely if the sample is not dark adapted prior to illumination.

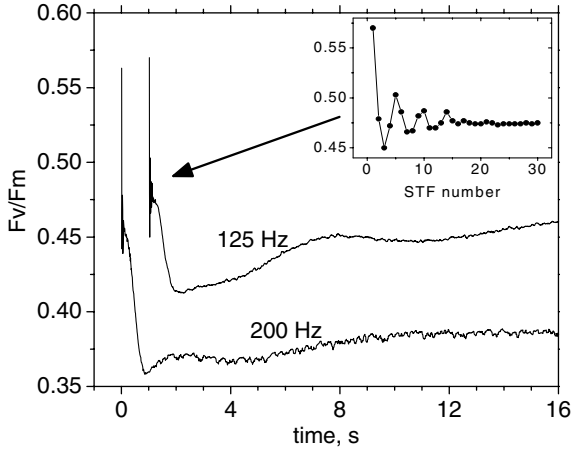


Figure 2. The yield of Chl-*a* variable fluorescence from *Spirulina m.* filaments (Fv/Fm), also known as PSII quantum efficiency, caused by STF-laser pulse trains at frequency equal at 125 Hz and 200 Hz (each STF train fired every 8 and 5 ms, respectively). Dark adaptation time prior to illumination is 5 min; the STF train (Figure 1) consists of 25 pulses of 1 μs each and total STF duration period of 50 μs. Inset shows the expanded initial Fv/Fm at 125 Hz.

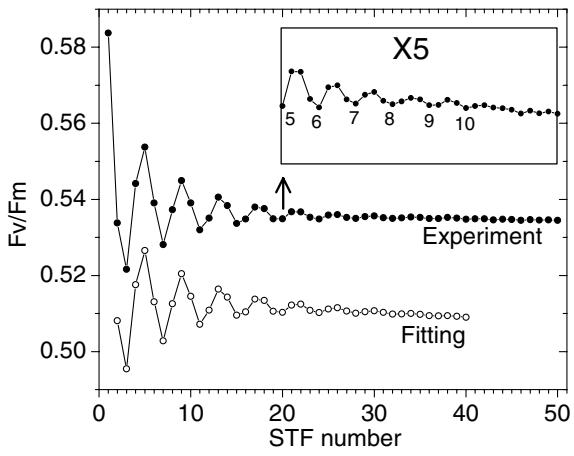


Figure 3. Fitting of the initial transient Fv/Fm oscillations to the modified Kok model given in Scheme 1. Young culture (4 days old) of *Spirulina m.* filaments recorded using 50 μs duration STF train repeated at 100 Hz (10 ms dark interval between STF); dark incubation time is equal to 1 min; data set is an average of 40 measurements. Fits were made by minimizing the residuals between the experimental data and the model using a program written by V. P. Shinkarev (Shinkarev 2003). Parameters of the fit: $\alpha = 0.099$, $\beta = 0.031$, reduced $\chi^2 = 1.2 \times 10^{-6}$.

Other experimental observations concerning the behavior of Fv/Fm (data not shown): (1) the first STF train applied to a dark-adapted photosynthetic sample always produces a higher Fv/Fm value than all remaining STF trains ($Y_1 > Y_{n+1}$); (2) this high initial value of Fv/Fm(Y_1) is reduced by pre-illumination with weak near IR light at 735 nm (0.6 mW optical power), which photooxidizes P700 and thus removes electrons from the plastoquinone pool; (3) the latter illumination at 735 nm (< 3 mW) increases the oscillation amplitude by about 20% (at 100 Hz STF trains) in green algae and higher plants (containing Chl-*a* + Chl-*b* pigments), but not in cyanobacteria (containing phycobilins but no Chl-*b* pigment). These observations indicate that the emission on the first STF train involves a contribution from a fluorescent Chl pigment that does not contribute to the subsequent oscillations with period four.

Modeling of the transient Fv/Fm oscillations

The oscillations in Fv/Fm seen in Figure 3 extend for 10 periods (see inset). The experimental data (excluding Y_1, see above) were fitted to the classic Kok model given in Scheme 1 using the analytical solutions described by Shinkarev (Shinkarev 2003). The best fit to the data is given by the simulated Fv/Fm curve shown in Figure 3 and gives a regression fit of $R^2 = 0.974$. The fitted parameters for the model are the miss coefficient $\alpha = 0.099$, the double hit coefficient $\beta = 0.031$, and the initial S-state populations in the dark [S0] : [S1] : [S2] = 0.24 : 0.70 : 0.06. The quality factor (Q) of the PS II oscillations can be described by the damping of oscillations: $Q = 1/(\alpha + \beta) = 7.75$.

Simultaneous α and β coefficients from oxygen and fluorescence data

In Figure 4 we present data for the simultaneous measurement of flash-induced O_2 concentration (using a modified Clark type electrode) (Ananyev and Dismukes 1996) and Fv/Fm using the same laser STF train. Measurements were done at a slow repetition frequency of 0.5 Hz. A special cell was fabricated to facilitate simultaneous measurements on the same sample. Both data sets (excluding Y_1 for Fv/Fm, see above) were fitted by regression analysis to the classic Kok model in Scheme 1 using Shinkarev's analytical solution

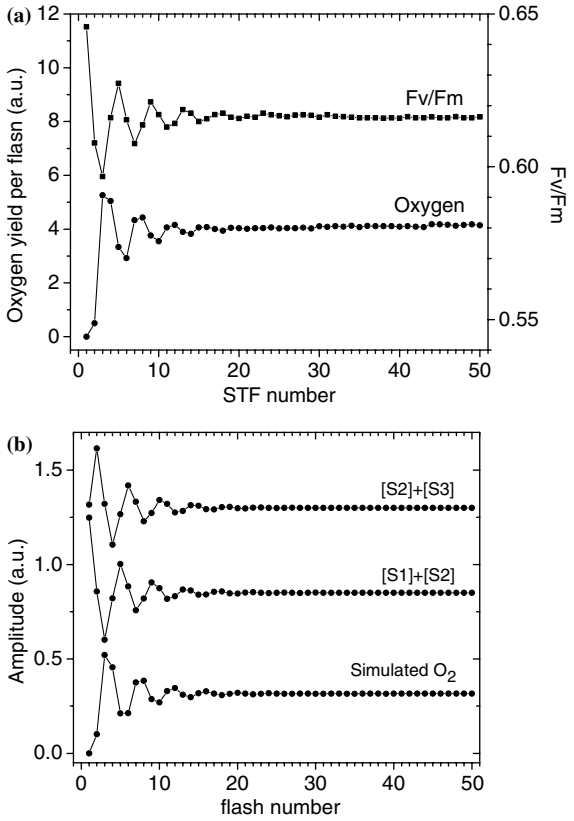


Figure 4. (a) Comparison of simultaneous measurement of dissolved oxygen concentration and Chl-*a* variable fluorescence (Fv/Fm) upon illumination by laser-STF train. *Spirulina m.* filaments were layered on top of a membrane-covered Pt electrode at a thickness of about 0.3 mm and dark adapted for 5 min. The filament suspension was exposed to air from above. The duration of the dark interval between STF trains is 2 s. Other conditions as is in Figure 3. (b) Simulation of flash oxygen kinetic based on Kok parameters obtained from experimental oxygen kinetic in (a): $\alpha = 0.130$, $\beta = 0.080$, [S0] = 0.45, [S1] = 0.55. $R^2 = 0.98$ $\chi^2 = 1.3 \times 10^{-6}$.

(Shinkarev 2003). Results for O_2 concentration give $\alpha = 0.13$ and $\beta = 0.08$; *versus* Fv/Fm: $\alpha = 0.13$ and $\beta = 0.07$. The Goodness of Fit is $R^2 = 0.98$ and the reduced $\chi^2 = 2.9 \times 10^{-7}$ (normalized least-squares residual) for the best fit of the data to the model. We may conclude that practically identical values for the α and β coefficients are obtained using either the external O_2 concentration measured by the Clark electrode or the internal Chl-*a* variable fluorescence yield. However, unlike the slow response time of the Clark electrode, the fluorescence approach used here can be applied at STF frequencies up to 10 kHz. In Figure 4, we show that the Fv/Fm

oscillations correlate most closely with the sum of populations $[S_1] + [S_2]$. The populations on each flash were calculated from the initial (dark) S-state populations taken from fits of the model to the O_2 concentration data, followed by propagating the populations using the model and the α and β coefficients derived from the fluorescence data.

Frequency dependence of PS II water oxidation at high turnover rates

Figure 5a shows that the amplitude of the period-four oscillations in Fv/Fm disappears at a repetition rate of 1 kHz (dark interval 1 ms) and is reduced by 50% between 4 and 5 ms dark interval (250–200 Hz). This is more clearly seen in Figure 5b which gives a plot of the Fourier transform of the data in Figure 5a. As expected, the steady-state level of Fv/Fm also decreases sharply at high repetition frequency, from a maximum of 0.54 decreasing by 55% to a value of 0.24 at 1 kHz. Because the steady-state level of Fv/Fm is proportional to the fraction of open PS II centers with Q_A oxidized (see review Lazar 1999; Maxwell and Johnson 2000), we may conclude that recombination with reduced Q_A^-, although necessary for Fv fluorescence, is not responsible for the loss of the transient period-four oscillations, as the latter disappears at 2–4-fold lower frequency. In a later section we show that this loss is due to intrinsic inefficiencies in the WOC (α and β).

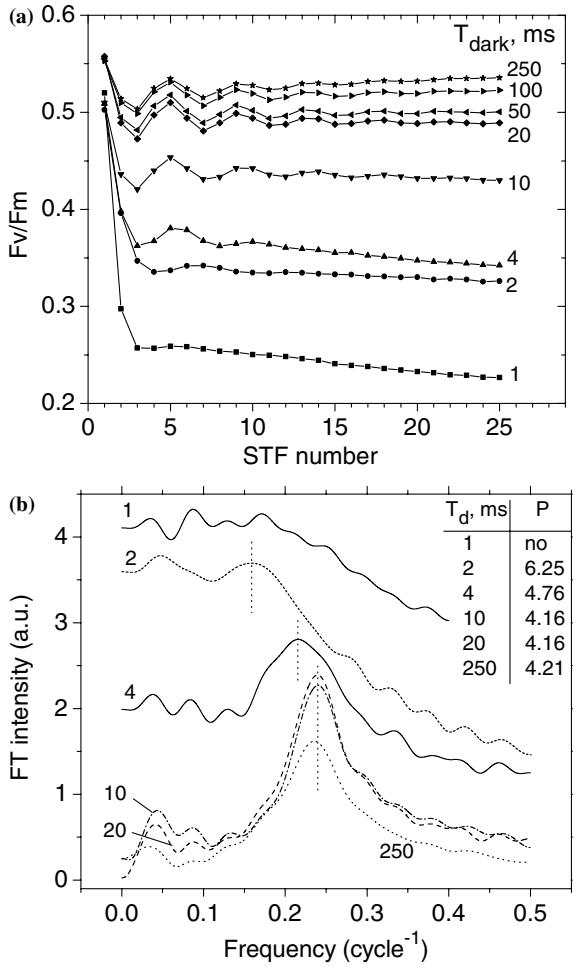


Figure 5. (a) Dependence of Chl-*a* variable fluorescence intensity (Fv/Fm) on repetition rate of the STF trains. The dark interval between STF trains was varied between $T_{dark} = 1$ and 250 ms. For details see Figure 1. The pre-incubation time in the dark is 1 min; each data set is an average of 40 measurements; (b) Fourier transformation of data presented in (a). Columns in right corner of (b) give T_{dark} (ms) *versus* period of oscillations (P). Period of oscillations is given as $P = (cycle)^{-1}$.

By contrast, the amplitudes of both the transient oscillations and the steady-state level of Fv/Fm decrease more rapidly in the green algae. Both *Chlorella p*. and *Euglena s*. exhibit complete loss of oscillations at a frequency of 250–300 Hz and a 50% loss of amplitude occurs at about 70 Hz (Supporting Information).

Inefficiencies in water oxidation at high PS II turnover frequencies

Figure 6 compares the experimentally determined values of the Kok miss (α) and double hit (β) parameters (Scheme 1) for *Spirulina* filaments and *Chlorella* cells over a 2500-fold range of flash repetition rates, corresponding to dark intervals between STF trains between $T_{dark} = 2$ and 5000 ms. Both species have an overlapping region of repetition frequencies where α (β) is relatively flat, but the range is about 5-fold wider for *Spirulina*. For *Chlorella* this region is between $T_{dark} = 40$ and 500 ms (40–1000 ms), while for *Spirulina* this region is between $T_{dark} = 8$ and 500 ms (8–1000 ms). The mean values in these regions are very similar in both *Spirulina* and *Chlorella*, with $\alpha = 10\%$ for both, while $\beta = 3\%$ and 4%, respectively. When T_{dark} is either greater or lesser than these values, both α and β increase rapidly. Between $T_{dark} = 8$ and 2 ms, α rises from 10.5% to 21% for *Spirulina,* while it increases to 16% at $T_{dark} = 8$ ms for *Chlorella* (upper limit of detection). For *Spirulina* at high frequencies β

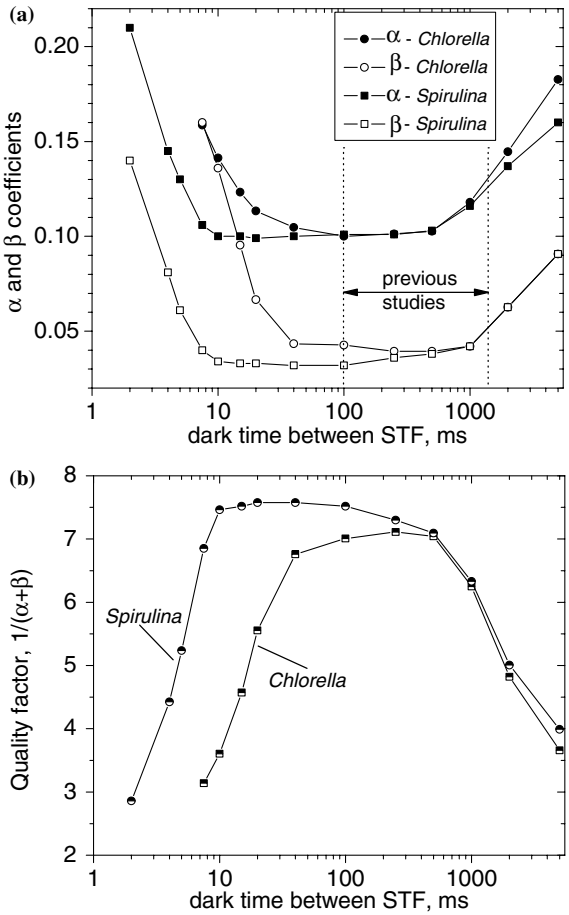


Figure 6. (a) Dependence of the Kok fitting parameters α misses) and β (double turnovers) on the dark time between STF trains for *Spirulina* filaments and *Chlorella* cells. The double headed arrow shows the dark time region used in most previous flash O_2 studies. (b) Dependence of quality factor, $Q = 1/(\alpha + \beta)$, on the dark time between STF trains. Pre-incubation dark time before measurement is 1 min; each data set is an average of 40 measurements.

increases by 4-fold between $T_{dark} = 10$ and 2 ms, which is the same rate (slope) as α. For *Chlorella* at high frequency, β increases even more rapidly than does α (greater slope). The differences in the Kok parameters between the cyanobacterium and the green alga are much smaller or zero at low flash frequencies; α rises to 16% and 18%, respectively, at 5 s flash interval; while β increases to 9% and there is no difference between *Spirulina* and *Chlorella*. For comparison, Figure 6 (double-headed arrow) shows the range of flash rates for which α and β have been measured by most other methods.

These data reveal that the transit time through the Kok cycle in *Spirulina* filaments is the fastest and produces by far the fewest misses and double hits *versus* the green algae, both *Chlorella* (Figure 6) and *Euglena* (data not shown). Analogous measurements of higher plants (spinach) show that they perform about the same as the green algae (data not shown). The relationship between this high kinetic activity in *Spirulina* and the physiological requirement for bicarbonate for functioning of the WOC is currently under investigation.

Discussion

The total transit time through the Kok cycle has been measured by several methods (reviewed in Razeghifard and Pace 1997): EPR spectroscopy was found to be about 1.6 ms for spinach chloroplasts (Babcock et al. 1976), optical absorbance was about 1.6–1.8 ms for spinach chloroplasts and isolated PS II membranes, 1.7 ms for isolated PS II membranes from a thermophillic cyanobacterium (1 ms at 50 °C), and longer times for PS II core particles and PS II core particles lacking the extrinsic subunits of the WOC (Qian et al. 1997). The rate-limiting step in this process is O_2 evolution on the S3 $\rightarrow$ S0 transition which has a time constant of about 1–1.5 ms (Joliot and Joliot 1968; Joliot et al. 1969; Clausen et al. 2004). Comparison to the multiple turnover results reported here indicates that the half-time for loss of the oscillation amplitude is 1 ms (1 kHz) for *Spirulina* and about 4 ms (250 Hz) for *Chlorella*. For *Spirulina*, the upper turnover rate of the WOC under these multiple turnover conditions is determined by intrinsic inefficiencies, characterized by the Kok α and β parameters, rather than by the rate of oxidation of Q_A^-. To the best of our knowledge, this is the first report of the transit time through the Kok cycle at high repetition rates above 10 Hz and at rates slower than 0.5 Hz. We were unable to find any systematic studies in the literature by comparing the total transit time through the Kok cycle for different photosynthetic species at any repetition rate. *Why do oscillations in fluorescence disappear at high flash frequency?* The duration of the oscillations of Fv can be characterized by a quantity from resonator theory called the quality factor (Q). We define $Q = 1/(\alpha + \beta)$ and plot it as a function of STF frequency in Figure 6. There is a strong correlation between the frequency dependence of Q and the frequency

dependence for the disappearance of the oscillation amplitude (Fourier transform in Figure 5b). We may conclude that the same mechanisms that give rise to the increase in α and β at high flash repetition frequencies are the dominant sources for the loss of oscillation amplitude. This is not surprising, but it does reassure us that the classic Kok model does a reasonable job in accounting for the frequency dependence of the oscillation amplitude through 10 cycles.

The present work provides the first systematic study of α and β from fluorescence measurements. In Figure 4 we found that the Fv/Fm oscillations correlate most closely with the sum of populations $[S_1] + [S_2]$. This result differs from the original measurements based on Fv which found a closer correlation with the populations $[S_2] + [S_3]$ in chloroplasts (Delosme and Joliot 2002; Schlodder et al. 1985). The reason for this difference is unclear. However, two possible sources may be: (1) auto-reduction of the S-states that occurs in the dark to different extents in chloroplasts *versus* cyanobacteria and, (2) chemical reduction from H_2O_2 produced either internally via respiration in the dark, or from an external source such as leaking from the membrane-covered Pt electrode used for O_2 concentration measurements. This is a subject for future research.

What is most surprising to us is that very large increase in both α and β occur at both high and low frequencies and that they are *strongly correlated with one another*. We were unable to find reports in the literature giving these parameters other than in a narrow 10-fold range of frequencies in any organism. Our data show that double turnover parameter is an intrinsic parameter that plays an equally important role as the miss parameter in determining PS II-WOC photochemistry. In the previous works, β has been largely ignored, as it was small, constant, and thought to arise from experimental problems due to the use of too long excitation pulse (xenon flash tubes) or from unstable Q-switched lasers. These problems do not occur in the laser diode FRRF approach.

The strong correlation between α and β at both high and low frequencies implies that there is a common mechanism. This cannot be true if β characterizes the probability of making a double turnover, e.g., double photooxidation, which is the classic interpretation of β based on O_2 measurements at slow flash rate (Kok et al. 1970). However, the Kok model in Scheme 1 is a cyclic model in which β can be interpreted either as a double turnover or a double deactivation (Shinkarev 2003). At present, we have no direct data which answers which of these two possibilities is correct. In fact, β may have contributions from both mechanisms with their relative contributions differing as a function of flash frequency. For example, we speculate that double deactivation may occur at high flash rates, while double turnovers are known to occur at low flash rates, based on O_2 yield data (Kolber et al. 1998; Shinkarev 2003). Double turnovers at low flash frequency might be expected if cooperativity in charge separation is possible between monomers within the known dimeric PS II complex (Ferreira et al. 2004). Cooperative photoprotection of PS II centers is observed to increase during photoinactivation, possibly by a related mechanism as suggested here (He and Chow 2003). Double deactivation with aborted production of H_2O_2 might be expected at high flash frequencies, if relaxation of the transient S-states cannot keep up with the electron transfer events, perhaps owing to slower proton evolution from the WOC. It is known that H_2O_2 formation occurs in modified WOC centers depleted of chloride or inhibited by fluoride, that are defective in proton evolution (reviewed in Dasgupta et al. 2004). In future studies we hope to address these provocative questions.

In conclusion, we have provided measurements of α and β over a 5000-fold range of flash frequencies within living cells using a novel fluorescence method. The strong deviation of these parameters seen at high and low flash frequencies and their strong correlation indicate that PS II-WOC centers split water at remarkably lower efficiencies and possibly by different mechanisms. Among the several oxygenic phototrophs examined thus far, the cyanobacteria derived from alkaline soda lakes are capable of the fastest water splitting rates *in vivo*.

Acknowledgements

We thank Drs A. Tyryshkin, M. Gorbunov, D. Klimov, Z. Kolber, V. Shinkarev, P. Falkowski, T. Wydrzysnki and V.V. Klimov for their support and advice. We thank Drs M. Kagawa and T. Nguyen for cultivation of *Spirulina*. Funds

for construction of instrumentation (NSF-DBI-0138012), microbial library and culturing (Human Frontiers Science Program) and personnel (National Institutes of Health, GM-39932) are gratefully acknowledged.

References

Ananyev GM and Dismukes GC (1996) High-resolution kinetic studies of the reassembly of the tetra-manganese cluster of photosynthetic water oxidation: proton equilibrium, cations, and electrostatics. Biochemistry 35: 14608–14617

Babcock GT, Blankenship RE and Sauer K (1976) Reaction-kinetics for positive charge accumulation on water side of chloroplast Photosystem 2. FEBS Lett 61: 286–289

Barzda V, Vengris M, Valkunas L, van Grondelle R and van Amerongen H (2000) Generation of fluorescence quenchers from the triplet states of chlorophylls in the major light-harvesting complex II from green plants. Biochemistry 39: 10468–10477

Chekalyuk AM, Hoge FE, Wright CW and Swift RN (2000) Short-pulse pump-and-probe technique for airborne laser assessment of Photosystem II photochemical characteristics. Photosynth Res 66: 33–44

Clausen J, Debus RJ and Junge W (2004) Time-resolved oxygen production by PS II: chasing chemical intermediates. Biochim Biophys Acta Bioenerg 1655: 184–194

Dasgupta J, Willigen RTV and Dismukes GC (2004) Consequences of structural and biophysical studies for the molecular mechanism of photosynthetic oxygen evolution: functional roles for calcium and bicarbonate. Phys Chem Chem Phys 6: 4793–4802

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

de Wijn R and van Gorkom HJ (2002) S-state dependence of the miss probability in Photosystem II. Photosynth Res 72: 217–222

Delosme R (1971) Variations in fluorescence yield of chlorophyll *in-vivo* during very intense flashes. Comptes Rendus Hebdomadaires des Seances de L'Academic Academie des Sciences Serie D 272: 2828–2831

Delosme R and Joliot P (2002) Period four oscillations in chlorophyll a fluorescence. Photosynth Res 73: 165–168

Dismukes GC and Siderer Y (1980) EPR spectroscopic observations of a manganese center associated with water oxidation in spinach chloroplasts. FEBS Lett 121: 78–80

Ferreira KN, Iverson TM, Maghlaoui K, Barber J and Iwata S (2004) Architecture of the photosynthetic oxygen-evolving center. Science 303: 1831–1838

Govindjee (1995) 63 Years since Kautsky – chlorophyll-a fluorescence. Aust J Plant Physiol 22: 131–160

He J and Chow WS (2003) The rate coefficient of repair of Photosystem II after photoinactivation. Physiol Plant 118: 297–304

Joliot P (2003) Period-four oscillations of the flash-induced oxygen formation in photosynthesis. Photosynth Res 76: 65–72

Joliot P and Joliot A (1968) A polarographic method for detection of oxygen production and reduction of hill reagent by isolated chloroplasts. Biochim Biophys Acta 153: 625–638

Joliot P, Barbieri G and Chabaud R (1969) A new model of photochemical centers in Photosystem-II. Photochem Photobiol 10: 309–329

Junge W, Renger G and Auslander W (1977) Proton release into internal phase of thylakoids due to photosynthetic water oxidation – periodicity under flashing light. FEBS Lett 79: 155–159

Koike H, Hanssum B, Inoue Y and Renger G (1987) Temperature dependence of S-state transition in thermophilic cyanobacterium, *Synechococcus vulcanus* Copeland, measured by absorption changes in UV region. Biochim Biophys Acta 893: 524–533

Kok B, Forbush B and McGloin M (1970) Cooperation of charges in photosynthetic O2 evolution. I. A linear four-step mechanism. Photochem Photobiol 11: 457–475

Kolber ZS, Prasil O and Falkowski PG (1998) Measurements of variable chlorophyll fluorescence using fast repetition rate techniques: defining methodology and experimental protocols. Biochim Biophys Acta Bioenerg 1367: 88–106

Lazar D (1999) Chlorophyll a fluorescence induction. Biochim Biophys Acta Bioenerg 1412: 1–28

Maxwell K and Johnson GN (2000) Chlorophyll fluorescence – a practical guide. J Exp Bot 51: 659–668

Nedbal L, Trtilek M and Kaftan D (1999) Flash fluorescence induction: a novel method to study regulation of Photosystem II. J Photochem Photobiol 48: 154–157

Osmond B, Ananyev G, Berry J, Langdon C, Kolber Z, Lin GH, Monson R, Nichol C, Rascher U, Schurr U, Smith S and Yakir D (2004) Changing the way we think about global change research: scaling up in experimental ecosystem science. Global Change Biol 10: 393–407

Qian M, Al-Khaldi SF, Putnam-Evans C, Bricker TM and Burnap RL (1997) Photoassembly of the Photosystem II (Mn)4 cluster in site-directed mutants impaired in the binding of the manganese-stabilizing protein. Biochemistry 36: 15244–15252

Razeghifard MR and Pace RJ (1997) Electron paramagnetic resonance kinetic studies of the S states in spinach PS II membranes. Biochim Biophys Acta Bioenerg 1322: 141–150

Reifarth F, Christen G and Renger G (1997) Fluorometric equipment for monitoring P680+ reduction in PS II preparations and green leaves. Photosynth Res 51: 231–242

Renger G and Govindjee (1993) How plants and cyanobacteria make oxygen: 25 years of period four oscillations. Photosynth Res 38: 211–469

Renger B and Weiss W (1986) Functional and structural aspects of photosynthetic water oxidation. Biochem Soc Trans 14: 17–20

Schlodder E, Brettel K and Witt HT (1985) Relation between microsecond reduction kinetics of photooxidized Chl-a (P-680) and photosynthetic water oxidation. Biochim Biophys Acta 808: 123–131

Schreiber U, Neubauer C and Schliwa U (1993) PAM fluorometer based on medium-frequency pulsed Xe-flash measuring light – a highly sensitive new tool in basic and applied photosynthesis research. Photosynth Res 36: 65–72

Schreiber U, Endo T, Mi HL and Asada K (1995) Quenching analysis of chlorophyll fluorescence by the saturation pulse method – particular aspects relating to the study of eukaryotic algae and cyanobacteria. Plant Cell Physiol 36: 873–882

Schreiber U, Kuhl M, Klimant I and Reising H (1996) Measurement of chlorophyll fluorescence within leaves using

a modified PAM Fluorometer with a fiber-optic microprobe. Photosynth Res 47: 103–109

Shinkarev VP (2003) Oxygen evolution in photosynthesis: simple analytical solution for the Kok model. Biophys J 85: 435–441

Trtilek M, Kramer DM, Koblizek M and Nedbal L (1997) Dual-modulation LED kinetic fluorometer. J Lumin 72–74: 597–599

Vonshak A, Abeliovich A, Boussiba S, Arad S and Richmond A (1982) Production of *Spirulina* biomass – effects of environmental factors and population density. Biomass 2: 175–185

Zankel KL (1973) Rapid fluorescence changes observed in chloroplasts – their relationship to O2 evolving system. Biochim Biophys Acta 325: 138–148