Photosynthesis Research **51:** 43–49, 1997.

Regular paper

Kinetics of the oxygen evolution step in plants determined from flash-induced chlorophyll *a* fluorescence

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Received 2 October 1996; accepted in revised form 4 December 1996

Key words: Photosystem II, oxygen evolution, S-states, fluorescence

Abstract

Photosystem II (PS II) of plants and cyanobacteria, which catalyzes the light-induced splitting of water and the release of oxygen, is the primary source of oxygen in the earth atmosphere. When activated by short light flashes, oxygen release in PS II occurs periodically with maxima after the third and the seventh flashes. Many other processes, including chlorophyll (Chl) *a* fluorescence, are also modulated with period of four, reflecting their sensitivity to the activity of Photosystem II. A new approach has been developed for the analysis of the flash-induced fluorescence of Chl *a* in plants, which is based on the use of the generalized Stern–Volmer equation for multiple quenchers. When applied to spinach thylakoids, this analysis reveals the presence of a new quencher of fluorescence whose amplitude is characterized by a periodicity of four with maxima after the third and the seventh flashes, in phase with oxygen release. The quencher appears with a delay of $\approx$ 0.5 ms followed by a rise time of 1.2–2 ms at pH 7, also in agreement with the expected time for oxygen evolution. It is concluded that the quencher is a product of the reaction leading to the oxygen evolution in PS II. The same quenching activity, maximal after the third flash, could be seen in dark adapted leaves, and provides the first fully time-resolved measurement of the kinetics of the oxygen evolution step in the leaf. Thus, the non-invasive probe of Chl *a* fluorescence provides a new and sensitive method for measuring the kinetics of oxygen evolution with potential for use in plants and cyanobacteria *in vivo*.

Introduction

Among the various photosystems in photosynthetic organisms, Photosystem II (PS II) from higher plants, algae and cyanobacteria is unique in having the oxidizing power to oxidize water to molecular oxygen. The reaction center photopigment, P680, drives the light-induced splitting of water (reviewed in Britt 1996; Debus 1992; Diner and Babcock 1996; Rutherford et al. 1992):

$$2H_2O \xrightarrow{4h\nu} O_2 + 4H^+ + 4e^- \qquad (1)$$

with oxygen escaping to the outside and protons being deposited inside the lumen of the thylakoids. In a series of saturating light flashes, the amount of oxygen released exhibits a periodicity of four (Joliot et al. 1969). This periodicity was explained by introducing the concept of charge accumulation in the S-states (S_n, with n = 0, 1, 2, 3, 4) of an oxygen evolving complex, where S_n reflects different numbers of stored oxidizing equivalents (Kok et al. 1970).

Depending on the experimental conditions, one can observe a periodicity of four in the fast (Delosme 1971; Zankel 1973) (μs) as well as the slow (Joliot and Joliot 1971) (ms and s) components of the chlorophyll (Chl) *a* fluorescence yield decay after light flashes. In the sub-millisecond to millisecond time range after flashes, the yield of variable Chl *a* fluorescence also exhibits a periodicity of two, arising from differences in the

rate of electron flow on the electron acceptor side of PS II: from the reduced first plastoquinone acceptor Q_A^- to the second plastoquinone acceptor (Q_B or Q_B^-) (Bowes and Crofts 1980). Because the amplitude of the Chl *a* fluorescence yield is substantially modulated by period four (about 25% at 1 ms), one can use the fluorescence yield kinetics to extract the characteristic times of the S-state transitions.

Materials and methods

Spinach (*Spinacia oleracea)* thylakoids were isolated as previously described (Eaton-Rye and Govindjee 1988). Chl *a* fluorescence yields after single-turnover saturating flashes were measured with an instrument described by Eaton-Rye and Govindjee (1988). The experimental protocol was as follows. Each measurement was done with a fresh, dark-adapted 2 ml sample taken from a 100 ml vat of thylakoids that were first dark adapted for 10 min. F_0 was measured with a weak blue, short measuring flash, exciting only 1% of the reaction centers. This was followed by exposure of the sample to one or more saturating 2.5 μs blue flashes and measurements of Chl *a* fluorescence yield, again with weak measuring flashes, beginning at 70 μs after the last strong flash. Chl *a* fluorescence after single-turnover saturating flashes was also measured by an instrument similar to that described by Kramer et al. (1990). The fluorescence was measured using a photodiode (Hamamatsu S3590-01) covered by Schott RG695 and Kodak Wratten 89B filters. The fluorescence was tested with weak (exciting less than 1% of reaction centers), short (5 μs) flashes from 4 LEDs (HLMP-8104, peak emission at 637 nm) passed through a 650 nm interference filter (Oriel, bandpass 10 nm), before and after blue (Corning 4-96), saturating, short ($\approx$5 μs at half intensity) flashes. The results obtained by the two instruments are almost identical. Flash-induced oxygen evolution by thylakoids was measured with a bare platinum electrode using a home-made AC amplifier (Meunier and Popovic 1988). All calculations were made on the software 'GIM' (DR.ACHEV DEVELOPMENT, Tempe, AZ).

Results

Figure 1A shows the kinetics of Chl *a* fluorescence yield $(F - F_0)/F_0$ (F = measured fluorescence intensity at time t after the flash; F_0 = fluorescence intensity

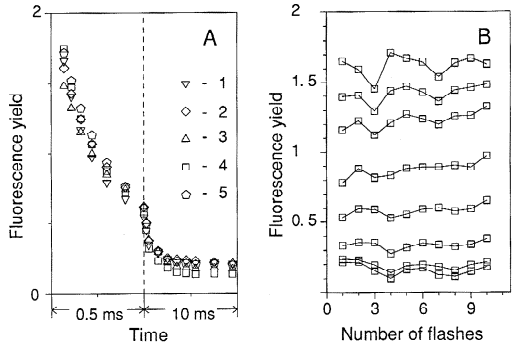

Figure 1. (A) Kinetics of the Chl *a* variable fluorescence yield $(= (F - F_0)/F_0)$ decay, following a series of single-turnover actinic flashes in spinach thylakoids, on two time scales and two vertical scales. Dark time between flashes, 1 s. Suspension medium: 20 mM HEPES (pH 7), 0.4 M sorbitol, 50 mM NaCl, 2 mM $MgCl_2$, 1 nm gramicidin. F = Chl *a* fluorescence intensity at time *t* after an actinic flash, and F_0 = Chl *a* fluorescence intensity before the flash. The concentration of Chl in the sample was 10 μM as determined according to Porra et al. (1989). (B) Oscillation of the yield of variable Chl *a* fluorescence after each of a series of ten saturating flashes measured at different times after the flash. Conditions as in (A). The measurements were at (from top to bottom) 70 μs, 110 μs, 160 μs, 300 μs, 500 μs, 1 ms, 3 ms and 5 ms after the actinic flash.

before the flash), induced by 1 to 5 actinic flashes. For short times after the flashes ($t < 0.25$ ms), the amplitude of the Chl *a* fluorescence yield induced by the third flash is smallest while for longer times ($t >$ 0.5 ms), the smallest amplitude is after the fourth flash. A plot of $(F - F_0)/F_0$ as a function of flash number reveals a period 4 behavior reflecting the involvement of the oxygen evolving complex charge accumulator in the modulation of the observed Chl *a* fluorescence yield. However, the source of this modulation in the Chl *a* fluorescence yield still needs to be clarified (see below).

Figure 1B shows the pattern of flash-induced Chl *a* fluorescence oscillations in spinach thylakoids at pH 7 for different times after each actinic flash. The Chl *a* fluorescence yield measured at 70 μs after each flash has a periodicity of four, with minima after the third and seventh flashes. At longer times (ms), the period four oscillations show minima shifted to the fourth and the eighth flashes. This modulation pattern for the Chl *a* fluorescence yield is in agreement with the results of earlier investigators (Delosme 1971; Zankel 1973; Eaton-Rye and Govindjee 1988; Kramer et al. 1990). In general, the observed periodicities are explicable in familiar terms. The period four modulation at short times ($t < 0.1$ ms) is likely due to S-state dependent periodic changes in the concentration of the oxidized

form of $P680^+$, which is a well known quencher of Chl *a* fluorescence (Mauzerall 1972; Sonneveld et al. 1980; Kramer et al. 1990). The period four oscillations arise from S-state dependent changes in the equilibrium and rate constants for electron transfer between $P680^+$ and its electron donor tyrosine, Y_Z (Witt 1991; Shinkarev and Wraight 1993a). A binary oscillation component is frequently seen on the time scale of electron transfer between Q_A and Q_B and can be adequately explained by the two electron gate function of Q_B (Bowes and Crofts 1980).

In principle, the period four oscillations observed here at longer times ($1 < t < 10$ ms) could also be indicative of the differences in the equilibrium constant, K_{nP}, between the n-th S-state and P680, although the equilibrium concentration of $P680^+$ is expected to be close to zero [$P680^+$ is almost fully reduced to P680 at 1 ms (Witt 1991)]. However, in order to account for the depth and phase of the oscillations, the requisite equilibrium constant, K_{0P} or K_{3P}, would have to be much less than any of the published values for the equilibrium constants of electron transfer between S-states and P680: $K_{0P} \approx 300$, $K_{1P} \approx 270$, $K_{2P} \approx 10$–25, $K_{3P} \approx 130$–325 (reviewed in Shinkarev and Wraight 1993b). Hence, there must exist other quenchers of Chl *a* fluorescence, also changing with period of four. Oxygen and intrathylakoid protons created during water oxidation are both possible candidates (see 'Discussion' below).

To quantify the nature of the period four modulation of the Chl *a* fluorescence, we will assume that the following generalization of the Stern–Volmer equation for many quenchers is valid:

$$1/\phi = a + b[X] + c[Y] + \ldots \qquad (2)$$

where ϕ is the quantum yield of fluorescence, [X], [Y], etc., are concentrations of different quenchers of Chl *a* fluorescence, and *a, b, c,...* are constants.

The applicability of the Stern–Volmer equation to Chl *a* fluorescence under modulation by states of the PS II reaction center implies a matrix or 'lake' model of energy transfer (Duysens 1979; Lavergne and Trissl 1995). The validity of a linear relationship between the reciprocal of the fluorescence and quencher concentration is somewhat controversial. Parallel measurements of flash-induced changes of fluorescence and UV absorbance of the primary semiquinone, showed perfect linearity between Q_A^- and reciprocal fluorescence (Duysens 1979), but many others have found a hyperbolic dependence, and it is routinely presented in that way (see e.g. Lavergne and Leci 1993). In the recent analysis of Lavergne and Trissl (1995), an analytical continuum between these behaviors is established on model/theoretical grounds. Nevertheless, the relationship between reciprocal quantum yield of fluorescence and quencher is indicated to be linear at relatively small concentrations of Q_A^- (see Figure 6 of Lavergne and Trissl 1995). In our analyses of the quenching at times greater than 0.5 ms, the variable fluorescence yield has decayed to about 25% or less of the initial value and a linear relationship applies well in all situations.

The attraction of this formalism is that the reciprocal fluorescence is directly proportional to the concentration of quenchers. These include open reaction centers (Duysens 1979) (in state PQ_A, where P stands for P680), oxidized $P680^+$ (Mauzerall 1972; Sonneveld et al. 1980), triplet states of carotenoids (Sonneveld et al. 1980), and probably the S-states themselves (Joliot et al. 1971). Since all our measurements are on the time scale of 50 μs – 50 ms, we can ignore the triplet state of carotenoid ($\tau < 10$ μs (Sonneveld et al. 1980)) as a quencher of Chl *a* fluorescence. From Equation (2), we obtain the difference of reciprocal fluorescence yields $1/\phi(i, t) - 1/\phi(j,\tau)$ for different flashes *i* and *j*, at times '*t*' and 'τ', as a linear combination of the differences of concentrations of all the quenchers:

$$\begin{aligned} &1/\phi(i,t) - 1/\phi(j,\tau) = \\ &b([PQ_A(i,t)] - [PQ_A(j,\tau)]) + c([P^+(i,t)] - [P^+(j,\tau)]) \\ &+ \sum_{n=0}^{4} d_n([s_n(i,t)] - [s_n(j,\tau)]) + \ldots \end{aligned} \qquad (3)$$

where $[PQ_A(i,t)]$ is the concentration of open reaction centers after flash '*i*' at time '*t*', etc. The differences between the reciprocals of the fluorescence yields at different times or after different flashes (Equation (3)) can reveal the kinetics and periodicity of natural quenchers in the thylakoids. When the concentrations of well known quenchers, such as PQ_A and P^+, do not significantly change on a given time scale under consideration, the kinetics of Chl *a* fluorescence can reveal the contribution and time evolution of additional less well-characterized quenchers, such as, the S-states or even oxygen. The difference $1/\phi(i,t)$-$1/\phi(j,\tau)$ then allows us to follow the concentration of such a quencher, *q*, in the thylakoids:

$$1/\phi(i,t) - 1/\phi(j,\tau) \propto ([q(i,t)] - [q(j,\tau)]) \qquad (4)$$

Figure 2A shows the difference $1/\phi(i, t) - 1/\phi(i, \tau)$ for times $t = 2$ ms and $\tau = 8$ ms as a function of

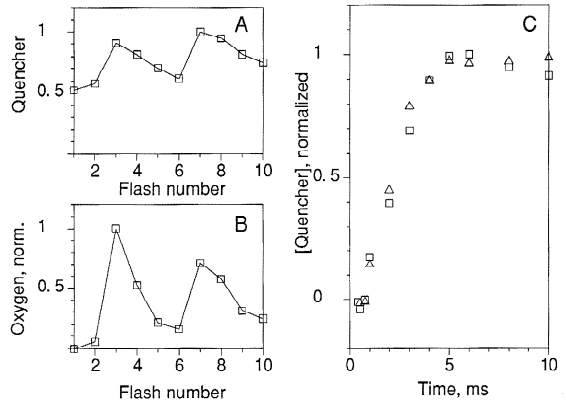

Figure 2. (A) Flash-number dependence of quenching of Chl *a* fluorescence, determined from the difference between the reciprocal of the fluorescence yield ($1/F \propto 1/\phi$) at 2 and 8 ms after an actinic flash. The initial offset of approximately 0.5 in is due to the main component of the fluorescence yield which continues in the period 2–8 ms (see Figure 1). (B) Oxygen evolution in spinach thylakoids measured by the bare platinum electrode. (C) Kinetics of the quencher of Chl *a* fluorescence as a function of time. The plot was constructed from the data of Figure 1, evaluated from the difference $1/\phi(1, t) - 1/\phi(3, t)$ (triangles) or by deconvolution of the component that oscillates in phase with oxygen evolution (squares) (see text).

flash number, '*i*', calculated from the data presented in Figure 1. This difference is modulated in phase with flash-induced oxygen evolution in thylakoids (Figure 2B), indicating an increase in quenching activity during the $S_3 \rightarrow S_0 + O_2$ transition. A similar correlation is observed for all times between 2 and 10 ms.

The time dependence of the quencher or quenching state, revealed in Figure 2A, can be obtained from the flash-number dependence of the kinetics of the Chl *a* fluorescence yield. To do so we have used two methods: (i) the time evolution of the difference of reciprocal Chl *a* fluorescence after different actinic flashes; (ii) deconvolution of the observed reciprocal Chl *a* fluorescence yield into period four components and determination of a component that follows in exact phase with oxygen evolution.

Oxygen evolution is maximal after the third flash and minimal after the first and fifth flashes. Thus, one can approximately follow the kinetics of any component in phase with oxygen evolution by, for example, the differences:

$$1/\phi(3,t) - 1/\phi(1,t) \propto [O_2(3,t)] \qquad (5)$$

$$1/\phi(3,t) - 1/\phi(5,t) \propto [O_2(3,t)] \qquad (6)$$

The kinetics of the quencher of Chl *a* fluorescence evaluated from the difference $1/\phi(1, t) - 1/\phi(3, t)$, as a function of time, is shown in Figure 2C (triangles). The appearance of quenching, estimated from fluorescence data, is characterized by $\approx$ 2 ms rise time, in close agreement with earlier estimates for the $S_3 \rightarrow S_0 + O_2$ transition (Jursinic and Dennenberg 1990; Lavorel 1992; Dekker et al. 1984; Renger and Hanssum 1992). A lag of about 0.5–1 ms is also seen.

This difference approach is valid only for times between about 0.5 and 10 ms, where one can observe oscillations of amplitude in phase with oxygen evolution. For shorter times, the time dependence of the differences (Equation (5) and Equation (6)) includes significant changes in $[Q_A^-]$ and $[P^+]$. However, the presence of a component of the fluorescence yield decay which oscillates in phase with oxygen evolution, as shown by the differential method, could also be revealed through deconvolution of the reciprocal fluorescence with period four oscillations of the donor side of PS II (Vos et al. 1991). The kinetics of a component which oscillates in phase with oxygen evolution are shown in Figure 2C (squares). After a delay of about 0.5–1 ms, it rises with $\tau \sim 1.5$ ms.

Both methods (difference and deconvolution) provide identical results between 0.5 and 10 ms, although the method based on deconvolution is intrinsically more precise and extends the analysis to longer times. The fact that the quenching state after the flash appears, i.e. *increases*, in the time range 0.5–5 ms, is exceedingly unlikely behavior for $P680^+$ and effectively rules out this as the quenching species involved. However, the deconvolution procedure also indicates a fast decrease in reciprocal Chl *a* fluorescence in phase with O_2 evolution for times up to 200 μs (data not shown). This is consistent with the finding that the amplitude of the 35 μs component of $P680^+$ reduction has a periodicity of four (Shlodder et al. 1985).

Discussion

Differential quenching by the S-states has long been known. The Chl *a* fluorescence on the time scale of a few ms to tens of seconds is modulated in a series of flashes in reasonable agreement with the sum of $S_2 + S_3$ (Joliot et al. 1971), i.e. $S_0 + S_1$ ($= 1 - ([S_2] + [S_3])$) are relatively quenching (low fluorescence) compared to $S_2 + S_3$ (high fluorescence). The fluorescence level immediately after an actinic flash, corresponding to the closed reaction center state, is also modulated, showing that 'S_4' also exhibits some quenching activity (Delosme 1971). However, the relative quenching

efficiencies of individual S-states is not known and a contribution of P^+ (or Tyr^+) to the quenching activity of the 'S_4' state is likely.

By virtue of the difference method of our analysis, i.e. $1/\phi(3, t) - 1/\phi(1, t)$, the time dependent changes in P^+ and Q_A are minimized. Additional transitions of potential quenchers on the donor side include:

$$\text{flash 3} \qquad S_3 \Rightarrow S_4 \rightarrow\rightarrow S_0 + O_2$$
$$\text{flash 1} \qquad S_1 \Rightarrow S_2^{'} \rightarrow\rightarrow S_2$$

where $S_2^{'}$ indicates any transient states after activation. However, the kinetics of all S-state transitions except $S_3 \Rightarrow S_4 \rightarrow\rightarrow S_0$ are complete in less than 0.5 ms (reviewed in Diner and Babcock 1996). Thus, the evolution of a quenching state in the time range 0.5–10 ms, revealed by the difference $1/\phi(3, t) - 1/\phi(1, t)$, appears to correspond to the oxygen evolving process after flash 3 and implies that S_4 is not as strong a quencher as $S_0 + O_2$. The flash-number dependence of the quenching state clearly correlates it with this transition, and the rise time of 1–2 ms is certainly consistent with independent measurements of O_2 release, using polarographic methods. A lag phase has not been reported before in thylakoids, but Koike et al. (1987) observed a lag in O_2 evolving particles from *Synechococcus vulcanus*, resolvable only at sub-optimal temperatures for this thermophilic cyanobacterium, and Rappaport et al. (1994) have described a short lag (about 30 μs) in Photosystem II membranes (BBY preparations), which they associated with a deprotonation event preceding the electron transfer from S_3 to $Y_Z{}^+$.

Although the known quenching property of S_0 is qualitatively adequate to account for the state that appears in phase with oxygen evolution, quenching of Chl *a* fluorescence by oxygen itself is also well established *in vitro* and *in vivo*. In leaves, clear and distinct effects of O_2 pressures (but not N_2 pressures) are seen on the different levels of fluorescence achieved during prolonged induction in the light (Vidaver et al. 1981a,b). *In vitro*, in some solvents, the rate constant of oxygen interaction with the excited state of Chl *a* approaches a value of 10^{10} M^{-1} s^{-1} (Seely and Connolly 1986). Oxygen is much more soluble in hydrophobic phases than in water (Hildebrand and Scott 1964) and, if restricted to the membrane volume, the concentration of oxygen released after the third flash (1 O_2 per RC) would be 0.5 mM. However, the transient local concentration could be much higher, leading to a pseudo-first order rate constant of quenching that might be of the same order as the rate constant of normal prompt fluorescence.

A major contribution by oxygen to the quenching seen after the third flash, is not compatible with the stability of the quenching state, at least to 10 ms. Current understanding of the kinetics of the oxygen release in the thylakoids suggests that oxygen equilibration between local and bulk phases occurs in a few milliseconds (Lavergne 1990; Lavorel 1992). Thus, whatever locally high concentrations may be achieved transiently, they should decline drastically within 10 ms. We do not see a significant decrease in the concentration of the quencher on this timescale, although a decline of 20–30% is seen on a slightly longer time scale (20–50 ms) in some cases (not shown). Thus, a minor quenching effect by photosynthetic oxygen production cannot be ruled out.

A similar problem exists for another possible candidate for quenching – the intrathylakoid proton concentration, or some localized version of it. Since our measurements were carried out in the presence of gramicidin, the apparent lack of decay of the quencher argues against this. Furthermore, although some aspects of proton release in the lumen are poorly understood, the kinetics are known to be rapid (Lavergne and Junge 1993) and do not exhibit any lag. Thus, the clear existence of a 0.5 ms lag phase in the quenching component, as well as the observation of quencher in the presence of 20 mM buffer (HEPES) makes it unlikely that the intrathylakoid protons are responsible for the observed period four modulation of fluorescence.

The origin of the quenching activity of the S-states, especially S_0 and S_1, is unknown. Possibly, the paramagnetism of the S_0 state (suggested to be Mn^{3+} – Mn^{3+} – Mn^{3+} – Mn^{4+}) (Rutherford et al. 1992), and the close proximity of the manganese cluster to the RC or antenna pigments, provide conditions sufficient for quenching the Chl *a* fluorescence. Another such source of quenching might be the intervalence charge transfer band of the Mn complex (see e.g. Boussac et al. 1996). However, the action spectrum corresponding to this band, with maximum at 820 nm, has relatively small overlap with the emission spectrum of Chl *a*.

If the lag in the quenching state that we observe in thylakoids is, indeed, significantly longer than reported for $S_3 \rightarrow S_0$ in Photosystem II particles (Lavergne et al. 1992; Rappaport et al. 1994), this may reflect the very different proton release patterns seen in different preparations, which are due to the sensitivity of the internal electrostatic interactions to the structure (Lavergne and Junge 1993).

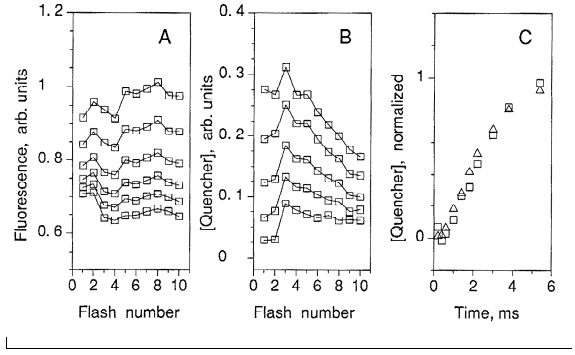

Figure 3. (A) Oscillation of the yield of variable Chl *a* fluorescence in geranium leaf (*Pelargonium* sp.) after each of a series of ten saturating flashes, measured at different times after the flash. The measurements were at (from top to bottom) 0.2, 0.4, 0.6, 1, 1.4 and 2.2 ms after the actinic flash. (B) Flash-number dependence of quenching of Chl *a* fluorescence, determined from the difference between the reciprocal of the fluorescence yield at (from top to bottom) 1.8 and 0.2 ms, 2.2 and 0.4 ms, 3.0 and 0.6 ms, 3.8 and 1 ms and 5.4 and 1.4 ms after an actinic flash. The decline in the signal and damping of the oscillations after the fifth flash is a well-known consequence of over-reduction of the plastoquinone pool prevalent in leaves (Groom et al. 1993). (C) Kinetics of the quencher of Chl *a* fluorescence evaluated from the difference $1/\phi(1, t) - 1/\phi(3, t)$ (triangles) or from the deconvolution of traces presented in panel (A) (squares).

The method developed in this paper provides reproducible and time resolved kinetics of the $S_3 \rightarrow S_0 + O_2$ transition in thylakoids. Of practical importance is the potential for using Chl *a* fluorescence to monitor the kinetics of $S_3 \rightarrow S_0 + O_2$ transition in intact leaves for which there are currently no time-resolved methods. Figure 3 shows measurements of flash-induced changes of the fluorescence in leaves of ornamental geranium (*Pelargonium* sp.), and similar results were obtained with spinach. One can see that the third flash induces the largest quenching effect and that the kinetics of the evolution of quenching are in agreement with those of spinach thylakoids. The current study therefore provides the first fully time-resolved measurements of the kinetics of the $S_3 \rightarrow S_0 + O_2$ transition in the leaf. Using a fast photoacoustic method, Mauzerall (1990) has also measured the kinetics of oxygen evolution in leaves. After correction for instrumental rise time he determined a lag phase of about 1 ms and a rise time of 5 ms. These numbers are in good agreement with our data if one takes into account that the photoacoustic measurement involves the diffusion of oxygen to the sensor, while the fluorescence assay is closely coupled to the kinetics of S-state transition. In particular, Mauzerall estimated that the lag observed by the photoacoustic assay was accountable by the diffusion time. Our measurements by fluorescence quenching, however, suggest that a substantial part of the lag may be intrinsic to the $S_4 \rightarrow S_0 + O_2$ reaction.

Acknowledgements

Authors are grateful to David Kramer and Tim Miller for valuable help in constructing the flash fluorimeter. This work was supported by a USDA grant 9401725 to VPS/CAW and a NSF grant 91-16838 (1994 supplement) to Govindjee.

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