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Photosynthesis Research **52:** 147–155, 1997.

Regular paper

Donor side capacity of Photosystem II probed by chlorophyll *a* fluorescence transients

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Received 31 October 1996; accepted in revised form 8 April 1997

Key words: donor side, heat stress, oxygen-evolving complex, S-state, fluorescence

Abstract

The chlorophyll *a* fluorescence transient measured under high light shows a typical O-J-I-P polyphasic rise. However, under certain stress situations such as heat or drought stress, a rapid phase with a maximum around 300 μs has been observed and called K (Guissé et al. (1995a) Arch Sci Genève 48: 147–160). Here, we show that under various conditions, the appearance of the K-step and the following dip, as well as the lowered maximum fluorescence level (F_M) attainable, can be explained by an imbalance between the electron flow leaving the RC to the acceptor side and the electron flow coming to the RC from the donor side. This leads to a stable oxidation of the secondary electron donor, the tyrosine Z (Y_Z), and possibly to the accumulation of $P680^+$. In the case of heat stress, we confirm that this situation is caused by an inhibition of electron donation to Y_Z, which is due to a damaged oxygen evolving complex (OEC). Finally, we present a model which includes the OEC, Y_Z, P680, Q_A and Q_B which is in good agreement with the experimental data. The appearance of the K-step, under natural conditions, can now be used as a convenient stress indicator and specifically attributed to a damage on the electron donor side.

Abbreviations: Chl *a* – chlorophyll *a*; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; K, J, I – intermediate steps of Chl *a* fluorescence rise between F_o and F_M; F_o, F_M – initial and maximum Chl *a* fluorescence; OEC – oxygen evolving complex; P680 – primary electron donor of PSII; Pheo – pheophytin *a*, the primary electron acceptor of PS II; PS II – Photosystem II; Q_A – primary quinone acceptor of PS II; Q_B – secondary quinone acceptor of PS II; RC – reaction center; Y_Z – Tyr161 of the D1 protein, the secondary electron donor of PS II

Introduction

The chlorophyll *a* fluorescence transient is one of the most often used tools in photosynthesis research (Dau 1994; Govindjee 1996) to probe the Photosystem II (PS II) reactions. In response to high-intensity actinic light (typically 600 W m^{-2}), the fluorescence yield shows a polyphasic rise (Neubauer and Schreiber 1987) called O-J-I-P transient (Strasser et al. 1995). The step J occurs around 2 ms, and the step I around 30 ms, the exact times being dependent upon light intensity. The O-J rise has been attributed to partial closure of the RC, the J-I to the closure of the remaining centers, and the I-P rise to the removal of plastoquinone quenching (Vernotte et al. 1979; Strasser et al. 1995). However, under various stress conditions such as heat stress (Srivastava et al. 1995; Srivastava and Strasser 1996; Srivastava et al. 1997) or drought stress (Guissé et al. 1995a,b) an early step can be seen around 300 μs which has been labeled K, in alphabetical order after I and J. It has been suggested that the K-step is related to an inhibition of the OEC, and to the accumulation of a precursor of Q_A having a high fluorescence yield (Guissé et al. 1995a,b; Srivastava et al. 1995; Srivastava and Strasser 1996).

Here, we show that the appearance of the K-step, under various conditions, can be explained by an imbalance between the electron flow leaving the RC

to the acceptor side and the electron flow coming to the RC from the donor side. Whenever the electron transfer from the OEC to Y_Z is slower than the electron transfer from P680 to Q_A and beyond, the K-step will appear. We attribute the fluorescence rise to the K-step to the first reduction of Q_A, which occurs after the light-induced charge separation between P680 and Pheo, followed by the reduction of Q_A. The first reduction of Q_A and the subsequent reduction of $P680^+$ requires one electron which is normally present on Y_Z (Diener and Babcock 1996); and there is no necessary involvement of the OEC. We propose that the dip after the K-step is caused by oxidation of Q_A^-, which either can not be re-reduced due to a deficient electron donor side (Guissé et al. 1995a) or which is reduced but with a concomitant accumulation of $P680^+$. In order to test this hypothesis, we have varied the capacity for electron donation by using different treatments. First, we used high temperature treatment (44 °C), which is known to inactivate the OEC (Nash et al. 1985; Brudvig et al. 1989). Second, we investigated the effect of millimolar concentration of hydroxylamine, which is known to cause Mn release from the OEC (Debus 1992). Third, we have preilluminated the sample with two single turn-over flashes in order to get an OEC in the S_3 state (Joliot et al. 1971), in which the electron donation rate to the RC is the slowest of all S-states. The rate of Q_A^- reduction was modulated by using different light intensities.

Finally, we present a kinetic model, which includes the OEC, Y_Z, P680, Q_A and Q_B and correctly predicts the K-step in the case of inhibited electron transfer from the OEC to Y_Z^+.

The appearance of the K-step under natural conditions, can now be specifically attributed to damage on the donor side. This rapid step can be used as a convenient stress indicator, for any stress reducing the PS II donor side capacity.

Materials and methods

The unicellular green algae *Scenedesmus obliquus* was grown autotrophically at 30 °C in a liquid inorganic culture medium (Bishop and Senger 1971). The culture was aerated with 3% CO_2 in air and illuminated continuously with a combination of fluorescent lamps (Osram-L 40W/15-1 and Osram-L 40W/25-1; Osram, München, Germany). The light intensity at the level of the culture was 20 Wm^{-2}. The chlorophyll content was determined after hot methanol extraction according to Senger et al. (1993). All measurements performed with a cell suspension were done at a final chlorophyll concentration of 20 μg Chl ml^{-1}.

Chl *a* fluorescence transients were measured by a plant efficiency analyzer (PEA; Hansatech Ltd., King's Lynn, Northfolk, UK) using various excitation light intensities. Light was provided by an array of six light-emitting diodes (650 nm), focused on the sample surface to provide homogeneous illumination over the exposed area of the sample.

Chl *a* fluorescence signals were detected using a PIN photocell after passing through a long pass filter (50% transmission at 720 nm). The experiments were performed with 500 μl cell suspension in 1 cm diameters vials. The optical pathlength of the sample was 5 mm and the diameter of the sample irradiated was 1 cm. For some measurements (see text) the cell suspension had been filtrated with a syringe on a Millipore filter (pore size, 8 μm) to get a higher signal-to-noise ratio in the fluorescence measurements, and a higher light intensity at the surface of the filter. The diameter of the irradiated sample area was 4 mm. Chl *a* fluorescence signals were recorded in a time span from 10 μs to 10 seconds, with a data acquisition rate of 10 μs for the first 2 ms and 12 bit resolution. The fluorescence signal at 50 μs was considered as a true F_o, as the fluorescence yield at this time was shown to be independent of the light intensity. Using a high speed digital oscilloscope (Nicolet # 410), we verified that at 50 μs the light intensity of the LEDs was over 99% of their final value.

The single turn-over flashes were provided by a home built xenon flash lamp filtered by a Corning 9782 CS-4-96 blue filter. The electrical flash energy was 2.2 J. Saturation by the flash was assumed since a 50% flash intensity resulted in the same fluorescence rise in the presence of DCMU. The half-peak width of the flash was 3.5 μs. The flash and the fluorometer were triggered by a Digital Delay/ Pulse Generator (DG535 Stanford Research Systems, Inc.). The flash rate was 1 Hz, and the measurement started 500 ms after the last flash.

The cells were dark adapted for at least 2 hours. DCMU ($<$ 0.1% ethanol at final concentration) and hydroxylamine addition were done in complete darkness, one minute before the measurement.

For the heat treatment, the cell suspension was immersed in a water bath at 44 °C during one hour. Then, the cell suspension was left one more hour at room temperature. The treatment was performed in complete darkness.

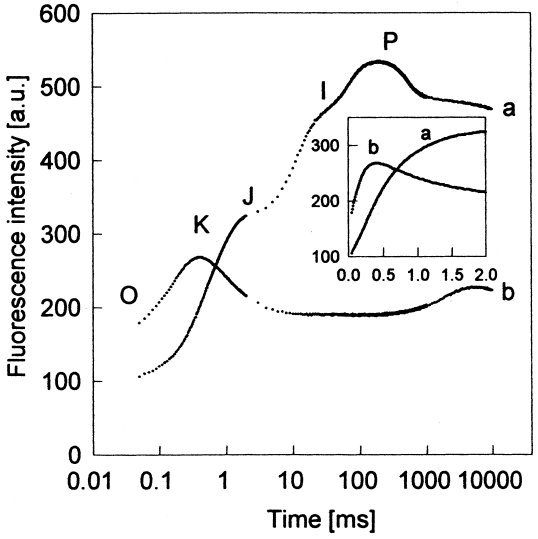

Figure 1. The effect of heat treatment on the Chl *a* fluorescence transient of a *Scenedesmus obliquus* cell suspension. Light intensity: 100 W m^{-2}. *(a)*: control *(b)*: after heat treatment (1 h at 44 °C). Insert: The first 2 ms presented on a linear time scale.

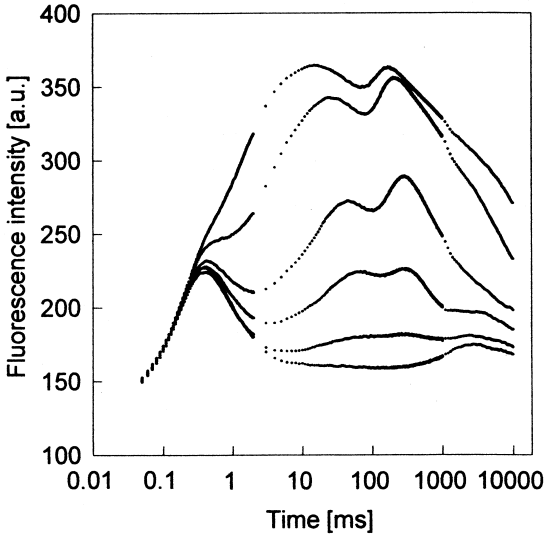

Figure 2. The effect of increasing concentrations of hydroxylamine on the Chl *a* fluorescence transient of a heat treated *Scenedesmus obliquus* cell suspension. Light intensity: 100 W m^{-2}. From top to bottom: 100 mM, 40 mM, 10 mM, 4 mM, 1 mM, 0 mM.

Results and discussion

Heat treatment can cause inhibition of the electron transfer from the oxygen-evolving complex (OEC) to tyrosine Z (Y_Z), due to OEC destruction by release of two Mn (Nash et al. 1985; Brudvig et al. 1989). It has also been shown that in leaves (Srivastava et al. 1995; Srivastava and Strasser 1996; Srivastava et al. 1997; Guissé et al. 1995a,b) heat treatment can induce a rapid rise in the polyphasic Chl *a* fluorescence transient. This rapid phase, with a maximum around 300 μs has been labeled K, and is the fastest phase observed in the O-J-I-P transient which, consequently, becomes an O-K-J-I-P transient. The K phase can be followed by a pronounced dip. Here, we present evidence that the K-step arises when the electron flow to the acceptor side exceeds the electron flow from the donor side, leading to an oxidation of the RC. Thus, injury of the OEC, due to heat stress for example, induces the K-step, by inhibiting efficient electron donation to the RC.

In order to test this hypothesis, we have studied heat treated (44 °C for 1 h) green algae (*Scenedesmus obliquus*) which show a well pronounced K-step, a drastically decreased F_M, an increased F_o (Figure 1), and complete suppression of oxygen evolution (data not shown). The K-step appears very clearly and consists of a rapid rise to a maximum (at 300 μs) followed by a decrease to level close to F_o. All other steps, J and I are absent from the transient. A comparable decrease of the variable fluorescence (F_M–F_o), mainly due to a large decrease in F_M, has been reported for higher plants (Weis 1981; Havaux 1993; Klinkovsky and Naus 1994). One can also note an increase in F_o which could either be due to a decrease in the sum of all deexitation rate constants (Havaux et al. 1991) or to the presence of free chlorophyll and uncoupled antennas proteins induced by the heat stress (Briantais et al. 1996). Depending on the duration and temperature of treatment, a significant increase in the initial fluorescence rise, can be observed, as in Figure 1. At lower temperatures, and for shorter duration of treatment, the K-step is present without any increase in the initial fluorescence rise. Therefore, this independent phenomenon is not addressed here, and we will focus on the appearance of the K-step solely. The increase in the initial fluorescence rise has been investigated by Srivastatva et al. (1997) who attribute it to a change in the PS II antenna architecture. In conclusion, the heat-effect on the fluorescence transient of *Scenedesmus obliquus* resembles closely the respective effect on higher plants.

If the large heat-induced decrease in the variable fluorescence, and the dip after the K-step were due to a lack of electron donation, then electron supply by an artificial donor should restore the fluorescence transient. We have chosen hydroxylamine (NH_2OH)

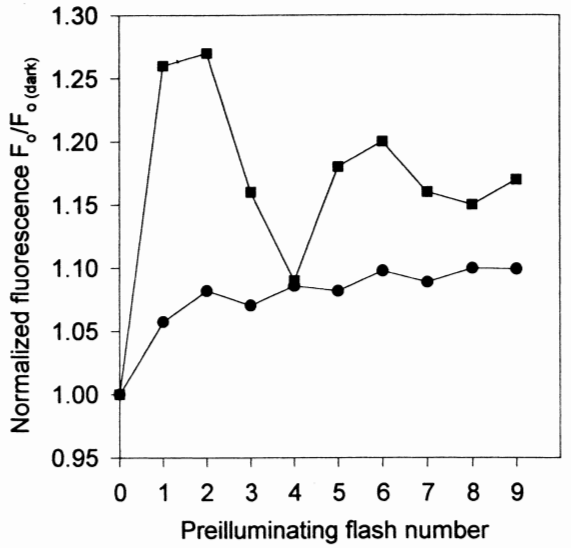

Figure 3. Flash number dependent oscillation in the Chl *a* fluorescence yield. Period four oscillations of F_o in a control sample (squares). Suppression of the period four oscillations of F_o in a heat treated sample (circles).

which is known to act as an electron donor to PS II RC (Trebst 1974, 1980; Debus 1992). The effect of increasing concentrations of hydroxylamine is shown in Figure 2. One can see a complete suppression of the dip after the K-step. Furthermore, the variable fluorescence is partially restored (Bennoun and Bouges 1971; Mohanty et al. 1971), and the I step appears clearly again. The J-step remains absent, but it is also absent from a non-heated sample treated with this concentration of hydroxylamine (data not shown). The restoration of the variable fluorescence indicates that the rise to the K-step is probably present in the control transient. However, the K-step is only made clearly apparent by the suppression of the subsequent fluorescence rise to the J level (at 2 ms in the control) which is replaced by a decrease to the F_o level, as it can be seen in the heat-treated sample.

In order to confirm that the K-step is related to damage of the OEC, we have studied fluorescence transients after different numbers of saturating single-turnover flashes (Hsu 1993). In non-heated samples, the F_o level shows period four oscillation (Figure 3, squares) characteristic of the S-state dependent modulation of the fluorescence yield (Delosme 1971; Joliot et al. 1971; Lavergne and Lecci 1995). After heat treatment, there is a complete suppression of the period-four oscillation (Figure 3, circles) for F_o.

We propose that the dip after the K-step is due to reopening of the centers by electron transfer from Q_A^- to Q_B, and eventually by a subsequent accumulation of centers with $P680^+$ which are known to have a fluorescence yield close to F_o (Mauzerall 1972; Sonnenvald et al. 1979; Deprez et al. 1983). The accumulation of $P680^+$ requires more than one turnover, since after the first turnover, $P680^+$ is rapidly re-reduced by Y_Z. It is only after Y_Z has been oxidized to Y_Z^+ that, after a second charge separation, $P680^+$ can accumulate (van Gorkom 1985; Britt 1996). However, using fluorescence measurements, we cannot distinguish between these two mechanism which result either in the accumulation of open centers ($P680\,Q_A Q_B^-$) or of closed centers but with $P680^+$ ($P680^+ Q_A^- Q_B^-$), since these two forms have a similar fluorescence yield. In any case, $P680^+$ is not likely to accumulate, since if it can not be rapidly reduced by Y_Z, it will recombine with Q_A^-. A scheme of the reaction sequence involved here (when the OEC is completely inactivated) is shown below.

$$Y_Z\,P680\,Q_A\,Q_B \xrightarrow{k_L}$$
$$Y_Z\,P680^+\,Q_A^-\,Q_B \xrightarrow{k_{ZP}} Y_Z^+\,P680\,Q_A^-\,Q_B \underset{k_{BA}}{\overset{k_{AB}}{\leftrightarrow}}$$
$$Y_Z^+\,P680\,Q_A\,Q_B^- \underset{k_R}{\overset{k_L}{\leftrightarrow}} Y_Z^+\,P680^+\,Q_A^-\,Q_B^-$$

The effect of DCMU addition, which is known to block electron transfer between Q_A and Q_B (Trebst 1980), to the heat treated sample is shown in Figure 4. DCMU suppresses almost completely the dip after the K-step. Higher concentrations, up to 100 μm did not cause any further suppression (data not shown). We can conclude that the dip after the K-step requires a reopening of the RC by reoxidation of Q_A^-, and that there is a significant accumulation of Y_Z^+.

If the appearance of the K-step is due to a slowed rate of electron donation compared to the light-induced electron transport rate to the acceptor side, then it should be strongly dependent on the light intensity. In a heat treated sample, lowering the light intensity, which will lower the electron transport rate, results in a smaller amplitude of the K-step (Figure 5), and in a less pronounced decrease after the K-step. The relationship between the light intensity and the appearance of the K-step will be further substantiated in a later section (see below). The initial slope of the fluorescence rise is exactly proportional to the light intensity confirming the photochemical nature of this process (Strasser et al. 1995; Strasser and Strasser 1995).

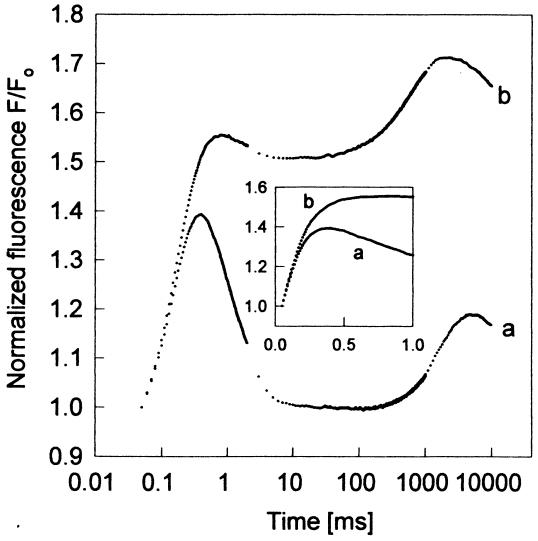

Figure 4. The effect of DCMU addition after heat treatment on the Chl *a* fluorescence transient of a *Scenedesmus obliquus* cell suspension. Light intensity: 100 W m^{-2}. *(a)*: heat-treated sample (1 h at 44 °C) *(b)*: heat treated sample + 50 μM DCMU. Insert: the first millisecond presented on a linear time scale.

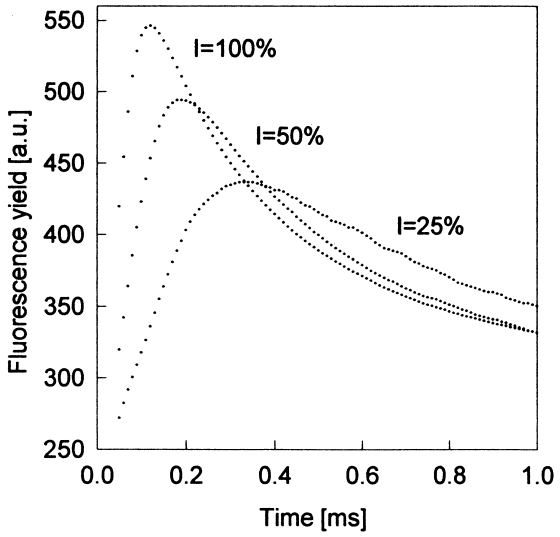

Figure 5. The light intensity dependence of the Chl *a* fluorescence transient of a heat treated *Scenedesmus obliquus* cell suspension. The different light intensities are given as a percentage of 100 W m^{-2}.

Heat-treatment probably affects components of the photosynthetic apparatus other than the OEC (Schreiber and Berry 1977; Armond et al. 1978, 1980; Gounaris 1985). To confirm that the appearance of the K-step is solely related to the damage of the OEC, we

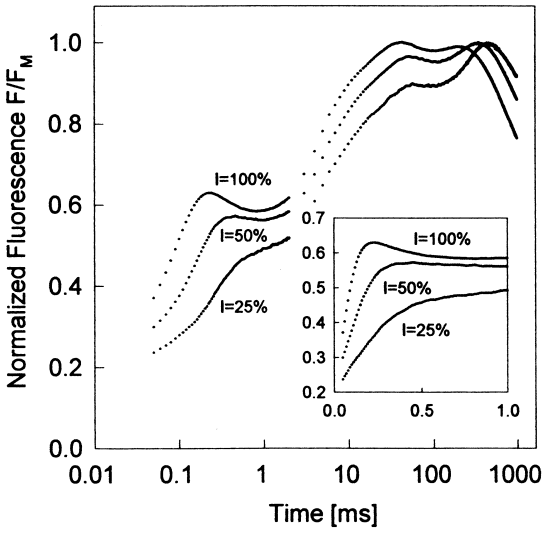

Figure 6. The effect 50 mM concentration hydroxylamine treatment on the Chl *a* fluorescence transient of *Scenedesmus obliquus* on filter, at various light intensities. The different light intensities are given as a percentage of 600 W m^{-2}. Insert: the first millisecond presented on a linear time scale.

have inactivated the OEC by hydroxylamine treatment of non-heated samples. It is known that millimolar concentration of hydroxylamine inhibits irreversibly oxygen evolution by extracting Mn of the OEC (Ghanotakis and Yocum 1990; Debus 1992). However, as shown above, hydroxylamine will also act as an electrons donor to the RC. In Figure 6 we show the light intensity dependence of the fluorescence transient after treatment with 50 mm hydroxylamine. The K-step is absent at 150 W m^{-2} light intensity. It becomes apparent at 300 W m^{-2} light intensity and becomes even more pronounced at 600 W m^{-2} light intensity.

In the heat-treated sample, where we expect a total absence of electron donation to the RC, moderate light intensities (25 W m^{-2}) are sufficient to make the K-step apparent. In non heat-treated samples, but after inactivation of the OEC by hydroxylamine, which is known to donate electrons to the RC at slow rate, the appearance of the K-step requires higher light intensities ($>$300 W m^{-2}). This confirms that the appearance of the K-step is not simply due to an absence of electron donation to the RC, but to an excessive electron flow from the RC to Q_A and beyond, leading to the accumulation of Y_Z^+, when compared to the electron donation capacity. Therefore, in a non-treated sample, with an intact OEC, the K-step should become apparent if much higher light intensities were used.

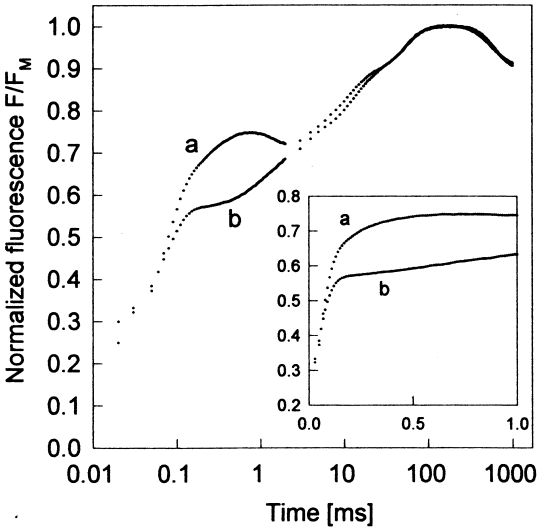

Figure 7. The effect of two single turn-over flashes on the Chl *a* fluorescence transient of *Scenedesmus obliquus* on filter paper. Light intensity: 600 W m^{-2}. *(a)*: dark adapted sample (maximal S_1 concentration) *(b)*: after 2 preilluminating flashes (maximal S_3 concentration). Insert: the first millisecond presented on a linear time scale.

Because of technical limitations this experiment could not be done. However, it is well known that the rate of electron donation by the OEC is S-state dependent, the $S_3 \rightarrow S_0$ reaction being the slowest of all (Dekker 1983; Hansson and Wydrzynski 1990; Britt 1996). We have therefore compared the fluorescence transients of dark-adapted samples preilluminated by 2 or 4 single turn-over flashes, at the highest available light intensity (600 W m^{-2}). Each flash should 'pump' the OEC into the next S-state (Joliot et al. 1971). Since in a dark adapted sample, we have mostly S_1 states (Vermaas et al. 1984; Styring and Rutherford 1987), after 2 flashes, we will have mostly S_3 states and after 4 flashes mostly S_1 states again.

As it can be seen in Figure 7 the K-step appears in the transient preilluminated with 2 flashes, but not in a dark adapted sample, or after 4 flashes. We expect a large fraction of S_3 states at the beginning of the transient preilluminated by 2 flashes, and the first S-state transition after the onset of continuous illumination will be the $S_3 \rightarrow S_4$ reaction. It is known that this reaction has a half time of about 1.5 ms which is about 15 times slower than the $S_1 \rightarrow S_2$ transition (Dekker et al. 1984; Hansson and Wydrzynki 1990; Britt 1996) expected to occur after 4 flashes, or in a dark-adapted sample. Here, in the presence of an intact OEC, we show that a slowed electron donation rate results in the appearance of a K-step.

We have simulated and fitted our experimental results from heat treated samples according to the following model.

$$\mathrm{Y_Z\,P680\,Q_A\,Q_B} \xrightarrow{k_L}$$
$$\mathrm{Y_Z\,P680^+\,Q_A^-\,Q_B} \xrightarrow{k_{ZP}} \mathrm{Y_Z^+\,P680\,Q_A^-\,Q_B} \underset{k_{BA}}{\overset{k_{AB}}{\leftrightarrows}}$$
$$\mathrm{Y_Z^+\,P680\,Q_A\,Q_B^-} \underset{k_R}{\overset{k_L}{\leftrightarrows}} \mathrm{Y_Z^+P680^+\,Q_A^-Q_B^-}$$

For clarity, the primary radical pair $P680^+Pheo^-$ is not explicitly considered since it rapidly recombines or transfers its electron to Q_A (Dau 1994). This model can be further simplified if we take in account that a closed RC with $P680^+$ has a fluorescence yield equal to that of an open RC (Mauzerall 1972; Sonnenvald et al. 1979; Deprez et al. 1983) or if $P680^+$ does not accumulate due to inefficient primary charge separation and recombination of $Q_A{}^-$ with $P680^+$ (van Gorkom 1985). The reduction of $P680^+$ by Y_Z can be omitted since it is much faster (20 ns) than the electron transfer (200 μs) from $Q_A{}^-$to Q_B (Dau 1994). Since we consider that the OEC is fully inactivated we need not to consider the reduction of $Y_Z{}^+$. The electron flow to the acceptor side being dependent on the rate constants k_L, k_{AB} and k_{BA} will evidently be larger that the electron flow from the donor side since the latter is equal to zero. The reactions can then be written as:

$$\mathrm{Y_Z\,P680\,Q_A\,Q_B} \xrightarrow{k_L} \mathrm{Y_Z^+\,P\,Q_A^-Q_B} \underset{k_{BA}}{\overset{k_{AB}}{\leftrightarrows}} \mathrm{Y_Z^+\,P\,Q_A\,Q_B^-}$$

The analytical solutions for the kinetics of these three components can be found in the Appendix.

Using these solutions, the best fit of the fluorescence transient from a heat treated sample is shown in Figure 8. Keeping the same parameters, but with k_L two or four fold smaller, we simulated the transients measured with 50% or 25% light intensity, respectively. As can be seen, the model gives a satisfactory simulation with the same parameter set under all light intensities. Furthermore, the rate constant obtained from the fit for the Q_A to Q_B electron transfer ($t_{1/2}$ = 300 μs) and for the reversed reaction ($t_{1/2}$ = 3.4 ms) are in good agreement with values reported in the literature (Robinson and Crofts 1983). The ratio of the fluorescence yield for closed and open centers was found to be about 5 which is in full agreement with experimental results. To obtain a better fitting, connectivity should also be considered, as it can be seen from the deviation between

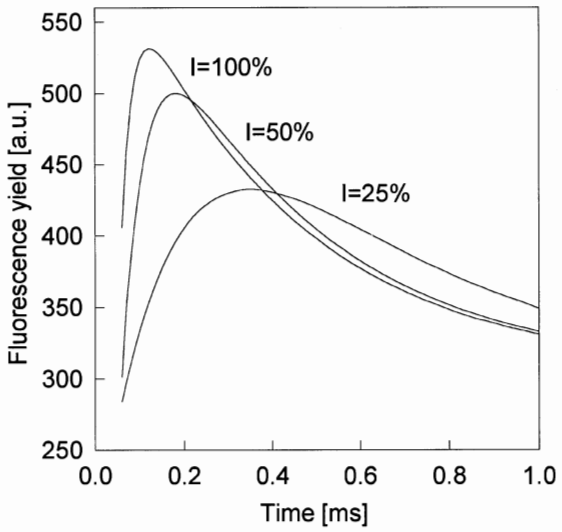

Figure 8. Curve fitting and simulation of the Chl *a* fluorescence transient of a heat treated *Scenedesmus obliquus* cell suspension at various light intensities (data of Figure 5). The different light intensities under which the experimental curves were measured are given as a percentage of 100 W m^{-2}.

the simulated curves and the experimental ones, at the beginning of the transient.

We conclude that the appearance of the K-step is satisfactorily explained by an imbalance between the electron flow to the acceptor side and the electron flow from the donor side leading to the accumulation of Y_Z^+. Thus, any treatment or stress condition which affects the donor side capacity will make the K-step apparent, if the electron flow to the acceptor side is sufficient. Therefore, the K-step can be used as a specific indicator of injury to the OEC.

Acknowledgments

Dr H. Dau, Prof. R.J. Strasser and his group are acknowledged for stimulating discussions, and B. Böhm for technical assistance. The experimental part of this work has been done in the lab of Prof. H Senger, Philipps-Universität, Marburg, with support by the ERASMUS program.

Appendix

For the simulations we used the following model:

$$Y_Z\,P680\,Q_A\,Q_B \xrightarrow{k_L} Y_Z^+\,P\,Q_A^-\,Q_B \underset{k_{BA}}{\overset{k_{AB}}{\leftrightarrow}} Y_Z^+\,P\,Q_A\,Q_B^-$$

Calling these three forms O, A and B, respectively,

$$O \xrightarrow{k_L} A \underset{k_{BA}}{\overset{k_{AB}}{\rightleftarrows}} B$$

the analytical solutions will be

$$O(t) = o_1 \cdot e^{-k_L \cdot t}$$

$$A(t) = {}_1 \cdot e^{-k_1 \cdot t} + a_2 \cdot e^{-(k_{AB}+k_{BA})\cdot t} + a_3$$

$$B(t) = b_1 \cdot e^{-k_L \cdot t} + b_2 \cdot e^{-(k_{AB}+k_{BA})\cdot t} + b_3$$

where the constants are chosen in order to have a starting concentration of 1 for O and of 0 for A and B.

$$o_1 = 1$$

$$a_1 = \frac{k_L - k_{BA}}{k_{AB} + k_{BA} - k_L}$$

$$a_2 - \frac{k_{AB} \cdot k_L}{(k_{AB} + k_{BA} - k_L) \cdot (k_{AB} + k_{BA})}$$

$$a_3 = \frac{k_{BA}}{k_{AB} + k_{BA}}$$

$$b_1 = \frac{k_{AB}}{k_{AB} + k_{BA} - k_L}$$

$$b_2 = \frac{k_{AB} \cdot k_L}{(k_{AB} + k_{BA} - k_L) \cdot (k_{AB} + k_{BA})}$$

$$b_3 = \frac{k_{AB}}{k_{AB} + k_{BA}}$$

The O and B states, representing open RC, and the A complex representing closed centers were associated with their respective fluorescence yields (F_o for an open RC, and F_M for a closed RC). Since the measured fluorescence is the sum of the fluorescence emitted by the open and the closed RC (Butler and Kitajiama 1975, Krause and Weis 1991), we get:

$$F(t) = [O(t) + B(t)]^* F_o + A(t)^* F_M$$

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