

# Preferential Deactivation of the $S_3$ State of the Water-Oxidizing Complex, Favoured by Plastoquinone Reduction in Barley Chloroplasts

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The flash patterns of oxygen evolution after pre-illumination by continuous light or by a flash sequence were compared in etiochloroplasts and mature chloroplasts of barley. In both types of plastids sub-saturating continuous illumination of some seconds strongly affected the  $S_3$  but not the  $S_2$  state of the oxygen-evolving complex. This result is ascribed to efficient charge recombination of  $S_3$  with the acceptor side of photosystem II, favoured by partial reduction of the plastoquinone pool. The increase of  $S_3$  observed in the presence of dichlorophenolindophenol in etiochloroplasts confirms this interpretation. These observations strengthen the recent hypothesis of a conformational change during the transition from  $S_2$  to  $S_3$  recently proposed to interpret the different susceptibility of these two states to hydroxylamine and hydrazine (F. Frank and G. H. Schmid, *Biochim. Biophys. Acta* **977**, 215–218 (1989); J. Messinger, U. Wacker, and G. Renger, *Biochemistry* **30**, 7852–7862 (1991)).

## Introduction

Water oxidation by photosynthetic organisms involves the stepwise accumulation of four oxidizing equivalents at the donor side of photosystem II, followed by the oxidation of two water molecules and the release of molecular oxygen. It is widely accepted that the accumulation of positive charges takes place through oxidation of a cluster of manganese atoms close to the PS II reaction center, but the molecular mechanism of water oxidation in relation to manganese oxidation states is unknown [1, 2]. The reaction is formally described in terms of light induced transitions of successive oxidation states of a water-oxidizing complex or  $S_n$  states (the Mn cluster), where  $n$  is the number of positive charges [3, 4]. This mechanism can be experimentally investigated by measuring the oxygen produced by dark-adapted chloroplasts under short

light flashes, each of which generates a transition from  $S_n$  to  $S_{n+1}$ . Since  $S_1$  is formed in darkness and is therefore predominant in dark-adapted chloroplasts [3], oxygen is released after the third flash. A maximum in the flash-induced oxygen production is then found every four flashes.

Reductants like  $NH_2OH$  or  $NH_2NH_2$  have the general property to reduce the higher  $S_n$  states to  $S_0$  and to a formal  $S_{-1}$  [5]. Using etiochloroplasts which have more stable  $S_3$  than mature chloroplasts [6], we showed in a previous paper [7] that  $S_2$  exhibits a peculiar sensitivity to  $NH_2OH$  when compared to  $S_2$  and  $S_3$ . This was confirmed by Messinger and Renger [8] and Messinger *et al.* [9] who extended this type of investigation to mature chloroplasts and found the same result using either  $NH_2OH$  or  $NH_2NH_2$ . The higher susceptibility of  $S_2$ , or the relative insensitivity of  $S_3$ , towards these reductants could indicate that a structural change takes place during the transition from  $S_2$  to  $S_3$  [9].

In the absence of exogenous reductants,  $S_2$  and  $S_3$  are spontaneously reduced to  $S_2$  and  $S_1$ . This process, known as deactivation, involves the reduction of  $S_2$  and  $S_3$  by endogenous reductants in PS II. Thermoluminescence experiments have shown that  $S_2$  and  $S_3$  deactivate through charge recombination with reduced QB (the exchangeable

**Abbreviations:** DPIP, 2,6-dichlorophenol indophenol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; QA, QB, the primary and the secondary quinone acceptors of photosystem II.

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plastoquinone in the PS II reaction center) after flash illumination [10, 11]. The long lifetimes of S<sub>2</sub> and S<sub>3</sub> observed in isolated PS II particles supplied with an exogenous electron acceptor has confirmed the role of the PS II reducing side in deactivation processes [12]. The Tyr-160 electron donor of the D<sub>2</sub>-peptide of PS II may also be involved in S<sub>2</sub> and S<sub>3</sub> reduction [13, 14] as well as another unidentified reductant [15].

Having in mind a plausible structural change during the S<sub>2</sub>→S<sub>3</sub> transition, we have investigated the effect of the reduction level of the plastoquinone pool on the deactivation of S<sub>2</sub> and S<sub>3</sub>. For this purpose the modifications of the S<sub>n</sub> population induced by continuous illumination with or without PS II electron acceptors were studied in etiochloroplasts and in mature barley.

### Materials and Methods

Etiolated barley seedlings (*Hordeum vulgare* var. Avilion) were grown in darkness at 23 °C on vermiculite and tap water during 6 days. They were then illuminated by white light (800 lux) during 3 h. Plastid isolation was performed according to [6], but without serum-albumine in the medium. For isolation of chloroplasts from mature leaves, the same method was applied to barley grown in a greenhouse during 14 days.

Oxygen measurements were performed by polarography using the three-electrode system described by Schmid and Thibault [16]. The assay suspension contained undisrupted plastids in  $25 \times 10^{-3}$  M Hepes buffer (pH 7.5) with 0.4 M sucrose,  $5 \times 10^{-3}$  M MgCl<sub>2</sub> and  $15 \times 10^{-2}$  M KCl. The total chlorophyll content of the sample (total volume 0.35 ml) was approx. 40 µg with etiochloroplasts and 60 µg with mature chloroplasts. Flash sequences at 3.3 Hz frequency were used. Calculations of oxygen production at flash N ( $Y_N$ ) were made with a home-made computer program. Continuous white light ( $165 \mu\text{E} \times \text{m}^{-2} \times \text{s}^{-1}$ ) was provided by a projector. Red light ( $30 \mu\text{E} \times \text{m}^{-2} \times \text{s}^{-1}$ ) was obtained using a cut-off red filter transmitting light above 640 nm.

### Results

Etiochloroplasts isolated from etiolated leaves after a 3 h greening period showed the well-known oscillation of flash-induced oxygen evolution with

a maximum under the third flash in the dark-adapted state (Fig. 1A, sequence 1). In these experiments,  $Y_N$  stands for the normalized oxygen production at flash N.

At this early stage of chloroplast development, the lifetime of S<sub>3</sub> was longer than in mature chloroplast [6]. This resulted in high oxygen yields under the first flash ( $Y_1$ ) when short dark-adaptation times were used. The flash pattern obtained 30 s after a pre-illumination sequence of 15 flashes is shown in Fig. 1A, sequence 2. It showed only weak variations from one flash to another. In that case an almost equipartition of the S-states was observed. This is in sharp contrast with the situation encountered 30 s after a 10 s continuous illumination by red light. When a sequence was recorded after this pre-treatment, the  $Y_N$  oscillations were partly recovered (Fig. 1B, sequence 1).  $Y_1$  was much lower 30 s after 10 s of continuous red light than 30 s after a flash sequence. A much smaller decrease of  $Y_2$  was found. This result points to a particular effect of continuous light, when compared to flashes, on the deactivation rates of the higher S-states, especially S<sub>3</sub>. The concomitant increase of the oxygen yield under the

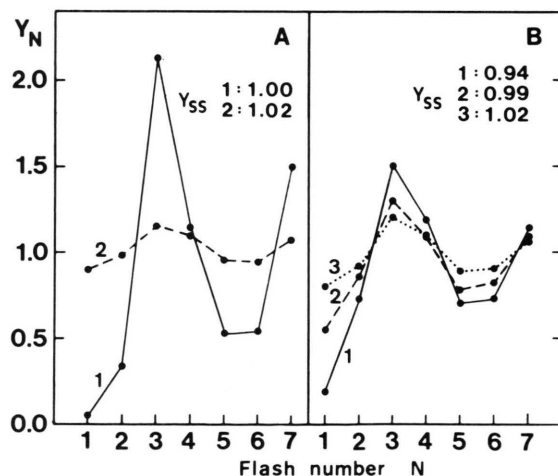


Fig. 1.  $Y_N$  sequences of etiochloroplasts in the dark-adapted state (A, 1), 30 s after 10 s continuous red light (B, 1) and in subsequent flash sequences given every 30 s (B, 2 and B, 3). The intensity of the continuous red light was  $30 \mu\text{E} \times \text{m}^{-2} \times \text{s}^{-1}$ . The  $Y_N$  values have been normalized on the steady-state oxygen evolution ( $Y_{ss}$ ) measured as the average  $Y_N$  at flashes 12 to 15. Actual  $Y_{ss}$  values are indicated with  $Y_{ss}$  arbitrarily set to 1.00 for the dark-adapted sample.

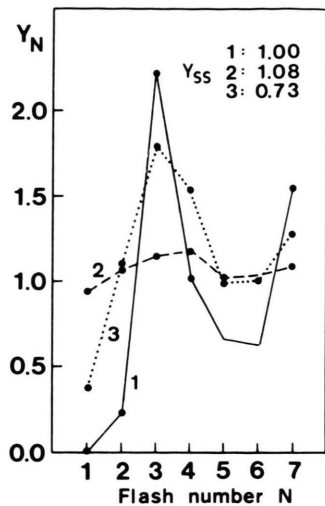


Fig. 2.  $Y_N$  sequences of mature chloroplasts in the dark-adapted state (1), 10 s after a first 15 flash sequence (2) and 10 s after 3 s of continuous red light (3).

third flash ( $Y_3$ ) shows that  $S_3$  deactivates to  $S_1$  under these circumstances.

If successive trains of flashes were given 30 s after the 10 s pre-illumination by continuous light, the  $Y_N$  were progressively modified to approach the control sequences recorded 30 s after a first flash sequence in a dark-adapted sample. This is seen by comparing sequences 1 to 3 of Fig. 1B, where it is again evident that the main effect lies on  $S_3$ , as shown by the progressive restoration of  $Y_1$ .

Results similar to those described above were obtained when using chloroplasts of mature green leaves instead of etiochloroplasts. In that case however, a shorter dark period was used since the deactivation of  $S_3$  after flashes is more rapid than in etiochloroplasts. An example is shown in Fig. 2, with continuous red light pre-illumination of 3 s and a dark period of 10 s. Again, when compared to flashes, continuous light induced a marked decrease of  $Y_1$  ( $S_3$ ), together with an increase of  $Y_3$  ( $S_1$ ). The progressive reversal of this effect upon further flash sequences was also found (not shown).

Since illumination of isolated plastids by continuous light should lead to a more rapid reduction of the plastoquinone pool than flash illumination, we attributed the rapid deactivation of  $S_3$  to a charge recombination between  $S_3$  and  $QB^{2-}$ , favoured by the larger extent of reduction of the plastoquinone

pool. In order to verify this hypothesis, we used various PS II electron acceptors and attempted to measure their effect on oxygen flash sequences in pre-illuminated chloroplasts or etiochloroplasts. Using chloroplasts we found that FeCN, *p*-benzoquinone and DPIP, even at low concentrations, induced an oxygen uptake under the flashes which were superimposed on oxygen evolution, and made  $Y_N$  calculations difficult [6]. In etiochloroplasts however, low concentrations of DPIP (15 to  $25 \times 10^{-6}$  M) induced only a weak oxygen uptake when the same red light pre-illumination as above was used.

Fig. 3A shows the effect of  $25 \times 10^{-6}$  M DPIP, added to dark-adapted etiochloroplasts, on the oxygen flash pattern measured 20 s after a 10 s pulse of continuous red light. A significant ( $\sim 40\%$ ) enhancement effect on  $Y_1$  was observed, whereas  $Y_2$  was practically unchanged. The increase of  $Y_1$  had its counterpart in a decrease of  $Y_3$  and, to a lesser extent, of  $Y_4$ . This effect of DPIP confirms that the accelerated deactivation of  $S_3$  upon continuous illumination is due to a preferential charge recombination of this state with the reducing side of PS II. Taking into account 10% of misses under flashes (which gave good fits with experimental sequences) we calculated the initial  $S_n$  distributions corresponding to the sequences of Fig. 3A. The

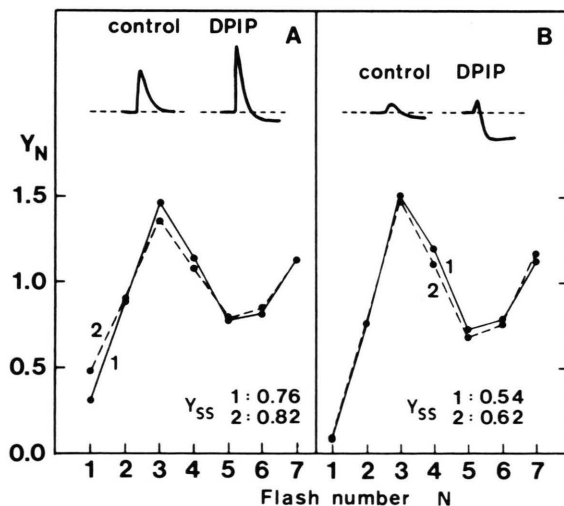


Fig. 3.  $Y_N$  sequence of etiochloroplasts measured 20 s after 10 s red (A) or white (B) light of 30 and  $165 \mu E \times m^{-2} \times s^{-1}$  respectively, without (1) or with (2)  $25 \times 10^{-6}$  M DPIP. The first flash signals are shown in the upper part of the figure.

difference between the two calculated distributions (DPIP minus control) is shown in Fig. 4. The increase of S<sub>1</sub> at the expense of S<sub>3</sub> induced by DPIP reflects the effect of the electron acceptor on the deactivation of S<sub>3</sub>.

In order to evaluate the extent of saturation achieved during red light pre-illumination, we recorded the oxygen evolution under a series of 15 flashes given during the pre-illumination itself. The amplitude of flash-induced oxygen production under such circumstances reflects the amount of S<sub>3</sub> with oxydized QA. Under the red light used in the above experiments, an almost constant production was found at each flash (Fig. 5, trace A). It represented 30% of the steady-adapted sample. Almost complete saturation could be achieved by removing the red filter between sample and light source, as shown by the practically undetectable flash-induced oxygen production under white light in Fig. 5, trace B. When a flash sequence was recorded 30 s after a 10 s pre-illumination by saturating white light, very low levels of S<sub>3</sub> were found even in the presence of  $25 \times 10^{-6}$  M DPIP (Fig. 3B). It must be noted however, that saturating white light enhanced the oxygen uptake under the first flash with DPIP (see first flash recordings in Fig. 3B). This uptake evidently caused an underestimation of Y<sub>1</sub>.

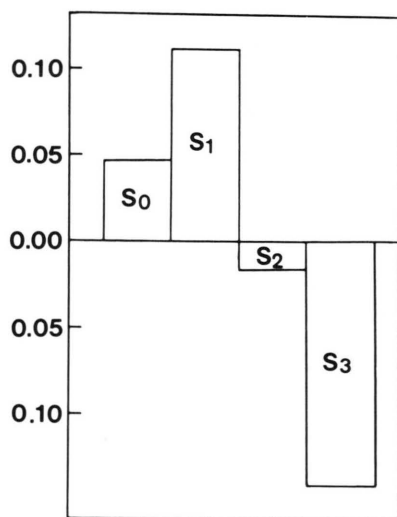


Fig. 4. Calculated difference in the S-state distribution 20 s after a 10 s red light pre-illumination with or without  $25 \times 10^{-6}$  M DPIP (DPIP minus control).

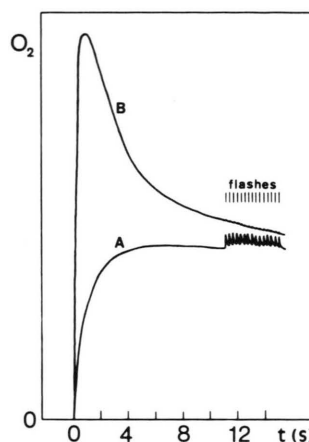


Fig. 5. Polarographic recordings of oxygen evolution during illumination of etiochloroplasts by red (A) or white (B) light of 30 and  $165 \mu \text{E} \times \text{m}^{-2} \times \text{s}^{-1}$  respectively. A sequence of 15 flashes was given at time 10 s.

The effect of continuous illumination on the deactivation of S<sub>3</sub> in etiochloroplasts was confirmed by comparing deactivation kinetics after a flash sequence and after 10 s red light pre-illumination (Fig. 6). The acceleration of the deactivation of S<sub>3</sub> after continuous illumination is evident.

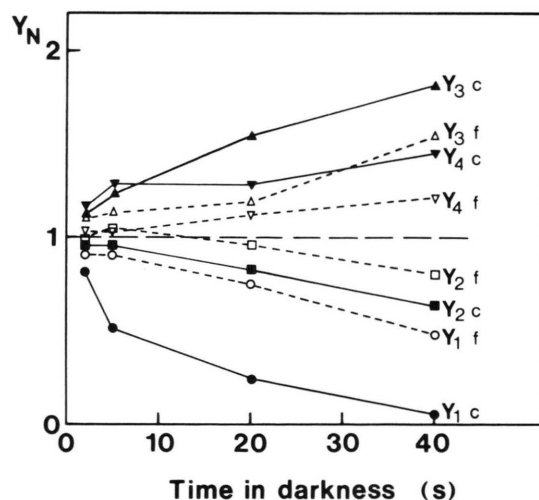


Fig. 6. Deactivation of the S-states in etiochloroplasts measured as the Y<sub>N</sub> oxygen flash yields (N = flash number) in a flash sequence given at increasing time in darkness after pre-illumination by 10 s continuous red light (closed symbols, c) or by a sequence of 25 flashes (open symbols, f) after dark-adaptation. Y<sub>N</sub> values were normalized on the steady-state flash yield (= 1).



Correspondingly, faster formations of S<sub>1</sub> (Y<sub>3</sub>) and, especially at short times after illumination, of S<sub>0</sub> (Y<sub>4</sub>) are found.

## Discussion

In whole plastids, illumination by a small number of saturating flashes leads only to limited reduction of the plastoquinone pool as shown by the high steady-state oxygen evolution under flashes. The oxidation of plastoquinol competes effectively with single electron reactions at each flash. However, a high extent of plastoquinone reduction can be achieved within a few s under continuous illumination of sufficient intensity if no electron acceptor is added [17]. Under this condition the cycling of the S<sub>n</sub> states is progressively impaired by the lack of reducible acceptors in PS II and charge recombination with reduced acceptors (QA and QB) is favoured. The probability to reach the higher S<sub>n</sub> during illumination therefore decreases, especially when approaching saturation.

Our main observation is that the deactivation of S<sub>3</sub> is greatly accelerated by pre-illumination in continuous light whereas S<sub>2</sub> is only weakly affected. This is demonstrated by the much lower Y<sub>1</sub> signal obtained after continuous pre-illumination when compared to that after a series of flashes both in etiochloroplasts and in mature chloroplasts, and by the relative stability of Y<sub>2</sub> under the same conditions. That this effect is due to the reduction of the plastoquinone pool is demonstrated, at least in the case of etiochloroplasts, by its sensitivity to DPIP which partly restores Y<sub>1</sub> but does not modify Y<sub>2</sub>. The decrease of S<sub>3</sub> after continuous pre-illumination can be therefore interpreted as a result of its recombination with reduced electron acceptors of the PS II reaction center (namely reduced QA and/or QB). The complementary behaviour of S<sub>1</sub> observed in all cases directly suggests recombination of the charge pair S<sub>3</sub>/QB<sup>2-</sup>.

The observed relative stability of S<sub>2</sub> suggests a relative insensitivity to the reduction status of the plastoquinone pool. This is in apparent contradiction to an earlier report by Sundblad *et al.* [18] who observed that in protoplasts both S<sub>2</sub> and S<sub>3</sub> rapidly deactivate after saturating white light pre-illumination unless it is followed by far-red light illumination. Most probably this discrepancy is due to the much higher intensity and the longer

duration of the pre-illumination used by these authors. An extensive study of S<sub>2</sub> and S<sub>3</sub> deactivation as a function of pre-illumination intensity and duration is certainly needed in order to appreciate in more detail the behaviour of these two states in response to various extents of reduction of the plastoquinone pool. The deactivation pattern obtained after partial reduction of the plastoquinone pool in this study does not support a trivial explanation of the relative constancy of S<sub>2</sub> after continuous pre-illumination due to deactivation of S<sub>3</sub> to S<sub>1</sub> via S<sub>2</sub>, since the deactivation of S<sub>2</sub> is only weakly affected over the entire period covering S<sub>3</sub> deactivation.

Our results show in any case a striking difference in the stability of S<sub>2</sub> and S<sub>3</sub> under pre-illumination conditions which induce partial or almost complete reduction of the plastoquinone pool (as shown by measurement of the additional flash-induced oxygen production during pre-illumination). The stability of S<sub>2</sub> found here after pre-illumination of moderate intensity is in agreement with earlier observations of the prevalence of S<sub>2</sub> during prolonged illumination by short flashes [15] or by sub-saturating continuous light [19] and also with the independence of S<sub>2</sub> deactivation towards the states I or II of the excitation energy distribution between the two photosystems in *Chlorella* [20]. An accumulation of S<sub>2</sub> during continuous illumination would also be favoured by an especially low probability of the S<sub>2</sub>→S<sub>3</sub> transition such as suggested by Delrieu [21].

Considering the recent finding [7–9] of a much greater susceptibility of S<sub>2</sub> to exogenous redox-active compounds when compared to S<sub>3</sub>, the inverse order of susceptibility to endogenous reductants (probably QB<sup>2-</sup>) found here suggests that if a conformational change takes place during the transition from S<sub>2</sub> to S<sub>3</sub> it modifies in an opposite way the probability of reduction of the S<sub>n</sub> by exogenous or endogenous reductants.

Considering the pronounced influence of plastoquinone on S<sub>3</sub> deactivation shown here, the gradual shortening of the S<sub>3</sub> lifetime in the course of greening [6] may be explained by a progressive decrease in the plastoquinone/chlorophyll ratio. Such a decrease most probably occurs during the first hours of greening since plastoquinone already exists in dark-grown plastids and slowly increases with illumination [22] whereas Chl is initially ab-

sent and rapidly accumulates during the first hours of greening.

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