*Photosynthesis Research* **38**: 309–314, 1993.

*Regular paper*

# High misses after odd flashes in oxygen evolution in thoroughly dark-adapted thylakoids from pea and *Chenopodium album*

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Received 8 April 1993; accepted in revised form 20 July 1993

*Key words:* chloroplasts, oxygen evolving complex, Photosystem II, quinone acceptors, S-states

## Abstract

In Photosystem II (PS II), water is oxidized to molecular oxygen and plastoquinone is reduced to plastoquinol. The oxidation of water requires the accumulation of four oxidizing equivalents, through the so-called S-states of the oxygen evolving complex; the production of plastoquinol requires the accumulation of two reducing equivalents on a bound plastoquinone, $Q_B$. It has been generally believed that during the flash-induced transition of each of the S-states ($S_n \rightarrow S_{n+1}$, where n = 0, 1, 2 and 3), a certain small but equal fraction of the PS II reaction centers are unable to function and, thus, 'miss' being turned over. We used thoroughly dark-adapted thylakoids from peas (*Pisum sativum*) and *Chenopodium album* (susceptible and resistant to atrazine) starting with 100% of the oxygen evolving complex in the $S_1$ state. Thylakoids were illuminated with saturating flashes, providing a double hit parameter of about 0.07. Our experimental data on flash-number dependent oscillations in the amount of oxygen per flash fit very well with a binary pattern of misses: 0, 0.2, 0, 0.4 during $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions. Addition of 2 mM ferricyanide appears to shift this pattern by one flash. These results are consistent with the 'bicycle' model recently proposed by V. P. Shinkarev and C. A. Wraight (Oxygen evolution in photosynthesis: From unicycle to bicycle, 1993, Proc Natl Acad Sci USA 90: 1834–1838), where misses are due to the presence of $P^+$ or $Q_A^-$ among the various equilibrium states of PS II centers.

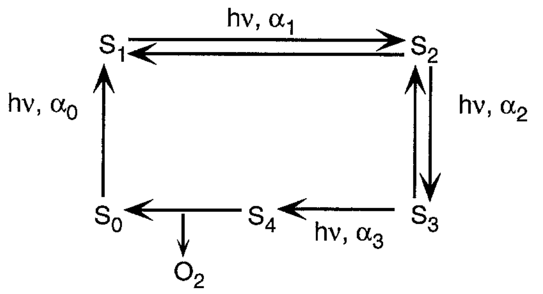
*Abbreviations:* $\alpha$ – miss parameter; $\beta$ – double hit parameter; PS II – Photosystem II; $Q_A$ – primary one-electron acceptor of PS II, a plastoquinone molecule; $Q_B$ – secondary plastoquinone two-electron acceptor of PS II; S-states ($S_n$, where n = 0, 1, 2, 3 or 4) – redox states of the oxygen evolving complex

## Introduction

In oxygenic photosynthesis, light quanta are used to induce transmembrane electron transport from water to plastoquinone through Photosystem II (PS II). The process begins with charge separation at the PS II reaction center, producing $P680^+Pheo^-$, where P680 is the primary electron donor, and pheophytin (Pheo) is the primary electron acceptor. This process occurs within a few ps (Govindjee and Wasielewski 1989). The electron on $Pheo^-$ is transferred to the primary plastoquinone one-electron acceptor $Q_A$ within 200 ps (Eckert et al. 1988). The electron on $Q_A^-$ is transferred to the secondary plastoquinone two-electron acceptor $Q_B$ on a 100–200 $\mu$s timescale (Crofts and Wraight 1983). A double reduction of $Q_B$ leads to the formation of $Q_B^{2-}$, which is then protonated to form $Q_BH_2$. This is what is known as the two electron gate mechanism, discovered by Bouges-Bocquet (1973) and Velthuys and Amesz

(1974). On the other hand, P680$^+$ transfers its oxidizing equivalent to a charge accumulator, the so-called S-states via an intermediate Z (tyrosine; Debus et al. 1988, Metz et al. 1989). The oxidation of the oxygen evolving complex occurs in a 4-step mechanism and involves redox changes in manganese (see Debus 1992).

When oxygen evolution induced by short single-turnover saturating flashes is measured, an oscillation pattern with a period of 4 is observed (Joliot et al. 1969), with the first maximum at flash 3:



The oscillation pattern can be explained with a model in which the system starts both in the $S_0$ (25%) and the $S_1$ state (75%) in the dark. With successive flashes, $S_0$ (and $S_1$) are converted to $S_1$ (and $S_2$; 1st flash), $S_2$ (and $S_3$; 2nd flash) and $S_3$ (and $S_4$; 3rd flash). State $S_4$ (also written by some as $S_3Z^+$) is reduced to $S_0$, accompanied by $O_2$ evolution (Kok et al. 1970). After the first cycle, a periodicity of 4 is evident, but then there is a damping in the oscillations. A complete explanation requires the introduction of additional parameters: misses (labeled as $\alpha$) and double hits ($\beta$). A certain small fraction of the reaction centers is unable to make a turnover, and thus gets out of phase with the bulk of the centers; this is what is called misses. Another small fraction turns over twice (called the double hits, Joliot and Kok 1975). Since the misses occur even at saturating flash intensities, a mechanism other than not exciting the antennae pigments must be responsible. It was suggested by Shinkarev and Wraight (1993) that certain misses are determined by reaction centers that are inactive at the time of the flash due to the presence of either P680$^+$ or $Q_A^-$, according to the electron transfer equilibria on the donor and acceptor sides of the reaction center and the back reaction of $Q_A^-$ with P680$^+$ competing with forward electron transport.

The $\alpha$-parameter has been assumed by most authors to be independent of the flash number (Kok et al. 1970) and, thus, for individual S-state transitions. However, Delrieu (1974, 1983) has assumed that $\alpha$ is unequal for each S-transition. She suggested a rather large miss ($\alpha_2 = 0.5 - 0.7$) for the $S_2$ to $S_3$ transition, but none after all other S-state transitions. This conclusion has been difficult to reconcile with any known mechanism and has not been generally accepted.

Vermaas et al. (1984) and Naber (1989) showed that after thorough dark adaptation (more than 20 min) the system starts in 100% $S_1$, simplifying the analysis of $O_2$ evolution data. Naber (1989) also introduced the concept of unequal values of miss parameters with a binary oscillation. Here, we present data and analysis of oxygen yield per flash as a function of flash number in such a system, and show that misses are high after odd and low after even flashes. Further, ferricyanide shifts this pattern by one flash. These data provide experimental support for the 'bicycle' model as proposed by Shinkarev and Wraight (1993).

## Materials and methods

Pea (*Pisum sativum*) and two biotypes of *Chenopodium album* L., the wild-type (S) and a triazine-resistant mutant (R), were grown in a growth chamber as described earlier (Jansen et al. 1986). Thylakoid membranes were isolated from the leaves as described elsewhere (Van Rensen et al. 1978). The isolation medium contained 0.3 M sorbitol, 50 mM Tricine-NaOH (pH 7.5), 100 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$ and 2 mM $MnCl_2$. Chloroplasts were broken in a medium containing 10 mM Tricine-NaOH (pH 7.6), 10 mM NaCl and 5 mM $MgCl_2$. Thylakoids were stored at −80 °C. Before use they were thawed and kept on ice. Samples were incubated in the dark on the electrode surface to allow the thylakoids to settle down. The thylakoids were dark adapted for at least 1 h and used for measurements not later than 6 h after thawing. Such a long dark-adaptation ensures that the system starts with 100% $S_1$ (Vermaas et al. 1984).

The flash-induced oxygen production was measured with a Joliot-type of electrode (Joliot 1972). Flashes with a duration of about 8 $\mu$s were fired with a Xenon lamp. A Schott BG23 blue filter was used in order to minimize excessive red tailing effects. The flash train used for measuring oxygen production consisted of a series of 10 flashes spaced 0.1 to 4 s apart. For data recording and processing a Nicolet digital storage oscilloscope was used in combination with a HP-86 computer.

The amplitudes of the 10 signals were used for the fitting procedure. First, the calculations were done using 6 variable parameters: $S_0$, $\beta$, $\alpha_0$, $\alpha_1$, $\alpha_2$ and $\alpha_3$. When it appeared that $\alpha_0$ and $\alpha_2$ approached zero, these parameters were fixed at 0.0. Final calculations were then performed using only four variable parameters: $S_0$, $\beta$, $\alpha_1$ and $\alpha_3$. These were calculated from the amplitudes of the 10 signals from at least 7 series of experiments with flash sequences of 0.25, 0.5, 1, 2, 4 and 10 cycles per second, fitted to the smallest sum of quadratic differences between experimental and theoretical data.

## Results and discussion

Prior to the calculation of the miss parameters as a function of flash number, the goal of this paper, it was necessary to establish the flash pattern of oxygen evolution under different experimental conditions. Oxygen yield per flash of a series of 10 saturating flashes in three different conditions (Joliot and Kok 1975) are shown in Fig. 1. All data were normalized to a steady state oxygen production of an arbitrary unit of 1.

For trace A, two preflashes were given, spaced at 0.2 s, and flash 1 of the flash train was fired 0.2 s after the pretreatment. Here, the preflashes converted $S_1$ to $S_2$, and $S_2$ to $S_3$, and the first flash of the flash sequence produced $O_2$ by converting $S_3$ to $S_0$. For trace B, two preflashes spaced at a time interval Δt of 10 s were given, and flash 1 of the flash train was fired 0.2 s after the pretreatment. Here, the long dark time (Δt) led to reduction of some of the $S_2$ states to $S_1$, by the slow donor D (Vermaas et al. 1984). In the experiment of trace C, two preflashes spaced at 0.2 s were given, and the first flash of the flash train was fired after a time interval Δt of 10 s. In this case, some of the $S_3$ states, the precursor of the $S_4$ state(s), deactivated before the first flash was fired.

By varying the time interval Δt in experiment B, the time of the decay of $S_2 \rightarrow S_1$ was calculated. By varying the time interval Δt between the last of the two preflashes and the flash train (Fig. 1, trace C), the back reaction of $S_3 \rightarrow S_2$ was calculated. The deactivation of both $S_2$ and $S_3$ states can be explained by suggesting that they are reduced by donor D. Donor D was assumed to cause an exponential

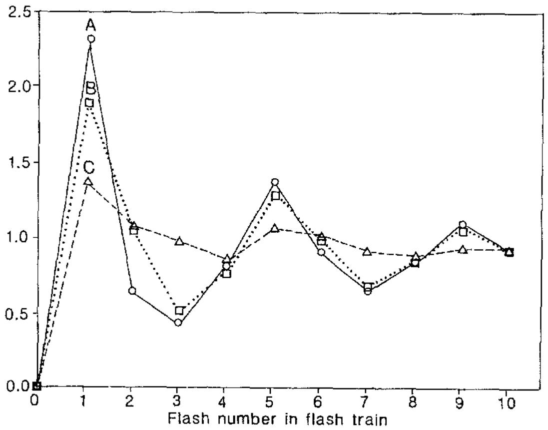


*Fig. 1.* Oxygen production (in arbitrary units) per flash as a function of light flash number from pea thylakoids after illumination with 2 preflashes. A (○): preflashes 1 and 2 (spaced at 0.2 s), then 0.2 s before the flash sequence; B (□): preflashes 1 and 2, spaced at Δt = 10 s, then 0.2 s before the flash sequence; C (△): preflashes 1 and 2 (spaced at 0.2 s),then Δt = 10 s before the flash sequence. Flash frequency in the flash train, 1 cycle per second.

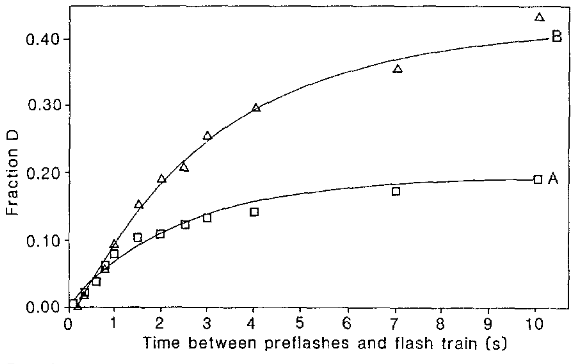


*Fig. 2.* Fraction $D_{red}$ as a function of the time interval Δt (varied from 0.2 to 10 s) between preflashes and flash train for pea thylakoids, as illustrated in Fig. 1. The lines are the results of exponential curve fitting of the experimental data points. In curve A the time interval was varied between both preflashes, whereas in curve B both preflashes (spaced at 0.2 s) were given before the time interval Δt. This means that in curve A the $S_2 \rightarrow S_1$ back reaction was measured, and in curve B the $S_3 \rightarrow S_2$ decay. The curves are drawn as the best fit through averaged values of 5 experiments.

decay of state $S_2 \rightarrow S_1$ or of $S_3 \rightarrow S_2$: $[D_{ox}]t = [D_{red}]*(1 - e^{-k_D*t})$. Here, $[D_{ox}]t$ is the fraction of the reaction centers coupled to an oxidized donor molecule at time t, $[D_{red}]$ is the fraction of the centers containing reduced D after dark adaptation and $k_D$ is the reaction constant for the electron donation. From Fig. 2 it can be calculated that 20% of the centers can reduce $S_2$ (curve A) and that 40% of the centers can reduce $S_3$ (curve B). This means that, on an average, 30% of the reaction centers were connected to reduced donor D after long dark adaptation. These are the centers that seem to be in state $S_0$ after a short dark adaptation period, because during flash illumination they are reduced once, and thus are apparently one turnover behind the bulk of the centers. However, in fact they were in state $S_1$ after long dark adaptation.

The fraction of reaction centers coupled to the water oxidation complex in state $S_0$ after thorough dark adaptation, the miss parameters $\alpha_n$ and the double hit parameter $\beta$ were calculated by fitting experimental data with theoretical oxygen evolution patterns using a least square fitting method. It turned out that, comparing many independent measurements, $S_0$, $\alpha_0$ and $\alpha_2$ approached 0 (Table 1).

The $S_0$-fraction after thorough dark adaptation was found to be 0 in all experiments. This confirms that indeed in our thoroughly dark-adapted samples, all centers are in the $S_1$-state. The parameter $\beta$ was found to be constant. The 2-electron gating mechanism in $Q_B$-reduction assures that upon a double turnover the quinone complex must cycle through both a semi-reduced and fully reduced state which is readily oxidized, starting in either of these states. This is the cause for an approximately constant value for $\beta$ during the flash train.

*Table 1.* Values of parameters to describe flash-induced oxygen evolution in thylakoids[1]

| | | Pea | *C. album* S | *C. album* R |
|---|---|---|---|---|
| | $S_0^a$ | 0 | 0 | 0 |
| | $\beta^b$ | 0.05 | 0.06 | 0.09 |
| $S_0 \rightarrow S_1$ | $\alpha_0^c$ | 0 | 0 | 0 |
| $S_1 \rightarrow S_2$ | $\alpha_1$ | 0.19 | 0.17 | 0.21 |
| $S_2 \rightarrow S_3$ | $\alpha_2$ | 0 | 0 | 0 |
| $S_3 \rightarrow S_0$ | $\alpha_3$ | 0.35 | 0.37 | 0.50 |

[1] Misses were calculated from mean values of at least 7 series of experiments with flash frequencies of 0.25, 0.5, 1, 2, 4 and 10 cycles per second. Sum of quadratic differences between experimental and theoretical data $S = \Sigma_{1-10}(Y_{exp} - Y_{TH})^2$ varied from 1 to 2.5%. $S_0$ = fraction of centers in the $S_0$-state after dark adaptation; $\beta$ = double hit parameter; $\alpha$ = miss parameter. [a]$[S_0]$ are 0 ± 0.01; [b]$\beta$ are values ± 0.005; [c]$\alpha$ are values ±0.005.

A center with high $\alpha$ means that it has a high probability not to undergo a turnover, i.e., a successful charge separation upon illumination. For $\alpha_n$ the best fits resulted in values approaching 0 for $\alpha_0$ and $\alpha_2$, while the sum of $\alpha_1$ and $\alpha_3$ was 0.54 for peas and *C. album*, wild type (Table 1). This means that the average is about 0.13 per flash, which is in agreement with the data of Vermaas et al. (1984) and Delrieu and Rosengard (1987) for spinach and pea, respectively. For triazine-resistant *C. album*, which has an altered rate of electron transport at the acceptor side, the sum of $\alpha_1$ and $\alpha_3$ was slightly higher as was the value of the $\beta$-parameter (Table 1). For a discussion of these deviations in the resistant biotype, see Naber (1989).

The $\alpha$ for even flashes is low, but those for odd flashes are high. This binary oscillation in the miss parameter is new. One might expect a binary oscillation because of the action of the binary electron gate $Q_B$. In the dark, the system starts mainly in $S_1Q_B$, but after one flash it is in $S_2Q_B^-$ state. Thus, $\alpha_1$, that measures the status of the 'dark' system, and $\alpha_2$, that measures the first flash status of the system, are expected to give low and high values, respectively, if the redox status of the electron acceptor side is the *only* contributing factor for the misses. This point becomes clear when one realizes that after one flash, $Q_B^-$ has a long lifetime and equilibrates with $Q_A$: $Q_AQ_B^- \rightarrow Q_A^-Q_B$; and the presence of $Q_A^-$ may cause 'misses'. Unfortunately, our data show contradictory results. Thus, other factors must be involved. One possibility is that under our experimental conditions, thylakoids may have been under anaerobic conditions and, indeed, many centers may start in $S_1Q_A^-Q_B \leftrightarrow S_1Q_AQ_B^-$. Addition of 2 mM ferricyanide showed a 1 flash shift in the misses (Naber 1989), supporting this possibility.

Recently, Shinkarev and Wraight (1993) presented a mechanistic interpretation of misses to be mainly determined by reaction centers that are inactive at the time of the flash due to the presence of *either* $P^+$ or $Q_A^-$. The high misses at odd flashes in thoroughly dark-adapted thylakoids, observed in this paper, are best explained by the higher equilibrium concentrations of $P^+$ and $Q_A^-$ before the odd flashes, as calculated by Shinkarev and Wraight (1993) from the published equilibrium constants on the donor as well as the acceptor sides of PS II. Shinkarev and Wraight (personal communication) suggest that $P^+$ and $Q_A^-$ may recombine more effectively after some rather than after other flashes due to differences in flash-number dependent kinetics of charge stabilization, and this could explain other patterns of misses under other experimental conditions.

Shinkarev and Wraight (1993) calculated misses that are different for each flash-induced reaction center turnover. They proposed two different reaction cycles of PS II with different turnover probabilities, producing an intrinsic heterogeneity of PS II activity. Both the cycles utilize four stable oxidation states of the donor side and two of the acceptor side. The two cycles were shown to have different period-four oscillations for the reaction centers of PS II functioning with different transition probabilities. The relative contributions of the two cycles depend on the initial conditions, i.e. in which cycle the reaction center is located before the flash series. When the system has both of the cycles operating, the binary oscillations tend to cancel out as was the case for the work of Forbush et al. (1971).

In our experiments, presented in Table 1, the thylakoids were dark-adapted for at least one hour. All reaction centers appeared to be in $S_1$ as the fraction of centers in $S_0$-state was found to be 0. Our finding of $\alpha_0$ and $\alpha_2$ to be 0, and $\alpha_1$ and $\alpha_3$ to be larger than 0.1 and its shift by 1 flash, with ferricyanide, provide the first experimental support for the existence of the proposed 'bicycle' model of Shinkarev and Wraight (1993).

## Acknowledgements

The authors thank W. J. M. Tonk and R. M. Bouma for constructing the Joliot electrode. We thank Vladimir Shinkarev and Colin Wraight for discussions. Govindjee thanks NSF (IBN 16838) for financial support.

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