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Fundamental differences between period-4 oscillations of the oxygen and fluorescence yield induced by flash excitation in inside-out thylakoids

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The period-4 oscillation pattern of the chlorophyll *a* fluorescence induced by flash excitation of dark-adapted inside-out thylakoids was compared to the oxygen-yield pattern obtained under the same conditions of flash energy and adaptation to dark. For a quantitative comparison, a least-square best fitting method was used. In the Kok model, besides the miss and the double hit parameters α and β, it has been necessary to introduce a new variable, z, which describes a decrease of the apparent number of active centers by a factor z at each flash of a series. We demonstrate that under similar flash conditions, the oscillation pattern of fluorescence yield (measured 80 ms after each of a series of flashes) fundamentally differs from that of the O_2 yield. After a 3 h dark-adaptation period, the oscillation pattern of the oxygen yield presents a change of phase as a function of flash number, associated with the damping coming from misses ($\alpha \geqslant 0.12$). In contrast, under the same conditions, the fluorescence oscillation showed no phase change. The same oscillation pattern was repeated every four flashes with a decreasing amplitude. The numerical fitting of the oscillation pattern of fluorescence yields no misses, no double hits, but a progressive vanishing of fluorescent centers in a proportion $1 - z_F$. The higher the flask energy the faster the decrease of the oscillating part of fluorescence. The product of flash energy, I, by the flash number necessary to decrease the fluorescence oscillation by half, $N_{1/2}$, was found to be independent of the exciting flash energy. The initial amplitude of the fluorescence oscillation was not light saturated in our experimental conditions, though the flash energy used was at least 5-times higher than necessary to saturate the transitions S_i of O_2 evolution (except $S_2 \rightarrow S_3$). All these results show that the fluorescence oscillation with period 4 is not related to the S_i states of most O_2-evolving centers. The existence of a 4-step reaction series (the S_i' states), functioning in parallel to the S_i states, is postulated. The S_i' states seen by fluorescence are controlled by the limited amount of a component stored in the dark, which is exhausted after each flash of a series in a quantity proportional to flash energy.

Introduction

The oxygen-evolving complex of plant Photosystem II carries out the four-step photooxidation of water to molecular oxygen. This complex can exist in five oxidation states denoted S_i ($i = 0,\ldots,4$) [1]. Attempts to identify the successive oxidation steps have not until now been very rewarding. The transition metal manganese appears to be integral to the oxygen-evolving complex [2,3]. An Mn(III) Mn(IV) dimer appears to be consistent with the multiline EPR signal associated with the S_2 state of the oxygen-evolving complex [4].

Abbreviations: PS II, Photosystem II; Mes, 4-morpholineethanesulfonic acid.

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In darkness, the S_0 and S_1 states are stable, S_2 and S_3 revert to S_1 in a few minutes, and the unstable S_4 releases oxygen and returns to the S_0 state. As a result, the system relaxes largely to S_1 in darkness and subsequent illumination by a series of flashes induces an oscillation of the oxygen yield with a periodicity of 4 [1].

A period-4 oscillation has also been detected in the fluorescence measured some milliseconds after each flash of a series of flashes, and was reported to be correlated with the sum of the S_2 and S_3 state centers [5,6]. In this paper, we have extended previous observations [5–8] by using a large range of flash energies. The simplest model explaining the period 4 of fluorescence oscillation is that each S_i state modifies the fluorescence yield with a relative weight f_i. This assumption predicts that the intrinsic parameters of the oscillation, i.e. misses α, and double hits β, must be the same for oxygen and fluorescence, in the same sample, under all experimental conditions, particularly as a function of flash energy. We analyzed the oscillation patterns of the oxygen yield and fluorescence, and were unable to fit these patterns with the same parameters. For this reason, we have concluded that the S_i states of most centers observed in the oxygen-yield pattern are not strictly identical to the four states contributing to fluorescence that we have called S_i' states to differentiate them clearly from the S_i states.

Materials and Methods

Inside-out vesicles were prepared from pea chloroplasts according to Åkerlund and Anderson [9] and possessed mainly PS II properties as previously described [10]. The suspension used was a medium containing 300 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 40 mM Mes-NaOH buffered at pH 6.5.

Flash excitation was provided hby Stroboslave General Radio flash lamps (half fall, 3 μs). Relative flash energy was measured with a photodiode (HP 2-4207).

The rate electrode used for oxygen-flash yield measurements has been previously described [112,12]. External acceptors were added in the circulating medium of the upper chamber of the electrode.

The fluorescence yield of chlorophyll *a* was measured with an optical pathlength of 2 mm and a chlorophyll concentration of 125 μg/ml. A red 2-64 Corning filter was placed before the photomultiplier tube (EMI 9558-B). Fluorescence detecting light was provided by electroluminescent diodes (Hewlett Packard HLMP 3519; dominant wavelength, 565 nm) emitting very weak light. A photomultiplier gating circuit was used in order to protect the photomultiplier tube during flash illumination. In this paper, fluorescence yield was measured 80 ms after each flash of a series of 16 flashes (spacing: 500 ms). Flashes and measuring light (15 ms in Fig. 2, 6 ms in Fig. 5) were triggered by a microcomputer (Apple II +). After each flash of the same series, chlorophyll *a* fluorescence signal was first amplified, then recorded in the transient waveform recorder (Physical Data Incorporation 523-a, 10 bits, 10 MHz) and partially stored in the microcomputer through a fast parallel interface. This method allows the accurate recording of the fluorescence decays observed after all the flashes of a same sequence. Finally, the fluorescence yield 80 ms after each flash of a series, F_V, was printed out and subtracted from F_0, the fluorescence yield after darkness [13].

Mathematical method for the numerical fitting of the oscillation pattern

Forbush et al. [14] have explained the damping of the oscillation pattern of the oxygen yield, by assuming the occurence of misses, α, and double hits, β.

This model assumes that the total number of active centers remains constant during the whole series of flashes. However, in our experiments the mean oxygen yield was not perfectly constant as a function of flash number [10]. We have introduced another variable, called z, allowing a decrease of the total number of active centers by a factor z at each flash.

We have used a generalized form of the Forbush et al. [14] recurrence law, which calculates the S_i state concentrations after the $(n+1)$th flash from those after the nth flash:

$$S_i^{n+1} = \gamma S_{(i-1)}^n + \alpha S_i^n + \beta S_{(i-2)}^n$$

(For $i = 0$, $i - 1 = 3$ according to the cyclic per-

mutation of the four S_i states). γ is the single-advancement probability, α the miss probability, β the double-advancement probability. The variation of the total number of centers from one flash to another changes in the ratio: $\Sigma_i S_i^{n+1}/\Sigma_i S_i^n = z = \gamma + \alpha + \beta$.The factor z is the sum of transition probabilities. For $z = 1$, the total number of centers is constant as a function of flash number. For $z < 1$, the number of centers decreases by a factor z at each flash. Thus, $\gamma = z - \alpha - \beta$. In the unequal miss model [11,15] ($\beta = 0$), the equation is:

$$S_i^{n+1} = (z - \alpha_{(i-1)})S_{(i-1)}^n + \alpha_i S_i^n$$

The least-square fitting method used in this work is the same as already presented [15]. We have used the recurrence equation giving the oxygen yield Y_{n+4} as a function of the four preceding Y_{n+i} ($n = 0$–3):

$$Y_{n+4} - \sigma_1 Y_{n+3} + \sigma_2 Y_{n+2} - \sigma_3 Y_{n+1} + \sigma_4 y_n = 0$$

This equation corresponds to the remark that the S_i states are not measurable experimentally at this time. Thus, when the different relations giving Y_{n+i} as a function of the S_i states, these S_i states are algebraically eliminated, the preceding recurrence equation is obtained, involving the experimentally measured flash yield Y_i and the σ_i's, which are only functions of the transition parameters [15,16]. The new parameter z also changes the σ_4 expression given in Ref. 15. In the equal miss model the equation is $\sigma_4 = \alpha^4 + \beta^4 - \gamma^4 + 4\gamma^2\alpha\beta - 2\alpha^2\beta^2$.

The 4-step seen by fluorescence can be different from the S_i states seen by oxygen measurements. So, we call S_i' the states seen by fluorescence. In the simplest model, the oscillating part of the fluorescence yield is a linear superposition with weight f_i of the 4 S_i' states, $F_n = \Sigma_i f_i S_i'$. After algebraic elimination of the unknown S_i' states, the same recurrence law is found for fluorescence F_n:

$$F_{n+4} - \sigma_1 F_{n+3} + \sigma_2 F_{n+2} - \sigma_3 F_{n+1} + \sigma_4 F_n = 0$$

The weight f_i for F_n does not appear in this recurrence relation, due to the general properties of linear relations. The preceding recurrence law is quite general to any process like oxygen or fluorescence, which is described by a linear superposition of states changing from one flash to another by a linear relation (or matrix) with constant coefficients. The numerical analysis of the best fit gives the σ_i's, which allows the determination of the parameters α, β, z (or α_2, z in the unequal miss model [11,15]) whatever the weight f_i of each S_i' states. The f_i can be determined if assumptions are made on the initial-values of the S_i' states in the dark.

If the same S_i states are involved in the oxygen yield and fluorescence oscillations (S_i states = S_i' states), we must find experimentally the same σ_i's and thus, the same corresponding damping (miss and double hit coefficients), the same z ($z_{O_2} = z_F$) within the experimental accuracy.

Results

In order to compare the oscillation pattern of the flash-induced oxygen yield with that of fluorescence measured 80 ms after each flash of a series, the measurements were carried out under identical conditions: same inside-out thylakoid preparation buffered at pH 6.5, same flashes, same flash energy and same spacing of 500 ms. In order to avoid the limitations of secondary electron transfer at the acceptor side, 1 mM ferricyanide was added. Without any addition, the fluorescence yield reaches a very high level in a few flashes. Ferricyanide efficiently decreases the fluorescence yield to a low constant level (after many flashes).

Fig. 1 shows the oscillation patterns of the oxygen yield obtained under different conditions of dark adaptation and flash energy. Whatever the conditions, the oscillation patterns of the oxygen yield present a change of phase as a function of flash number, corresponding to a phase retardation induced by misses. In inside-out thylakoids, the mean value of oxygen yield often slightly decreases as a function of flash number, even with efficient external acceptors such as ferricyanide or dichlorobenzoquinone [10]. The best fit is considerably improved assuming that the sum of transition probabilities after each flash is slightly less than 1 ($z = 0.94$–0.96 in Table I), indicating a small loss in the number of active centers after each flash (see Materials and Methods for the calculation method). With different flash energies

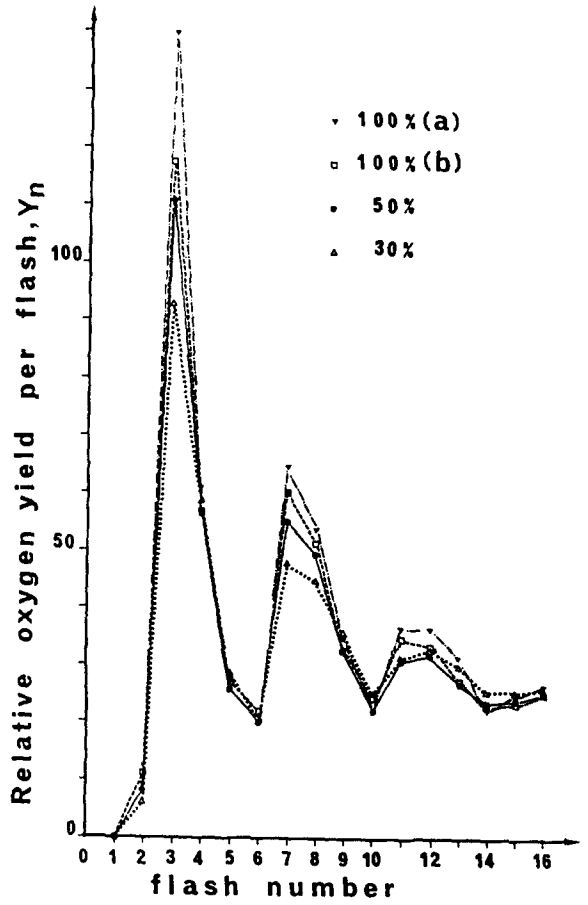

Fig. 1. Relative oxygen yields as a function of flash number in dark-adapted inside-out thylakoids at pH 6.5, in the presence of 1 mM FeCy, for different relative flash energies (100%, 50%, 30%). Dark adaptation: (a) at least 3 h; (b) and other patterns, 10 min.

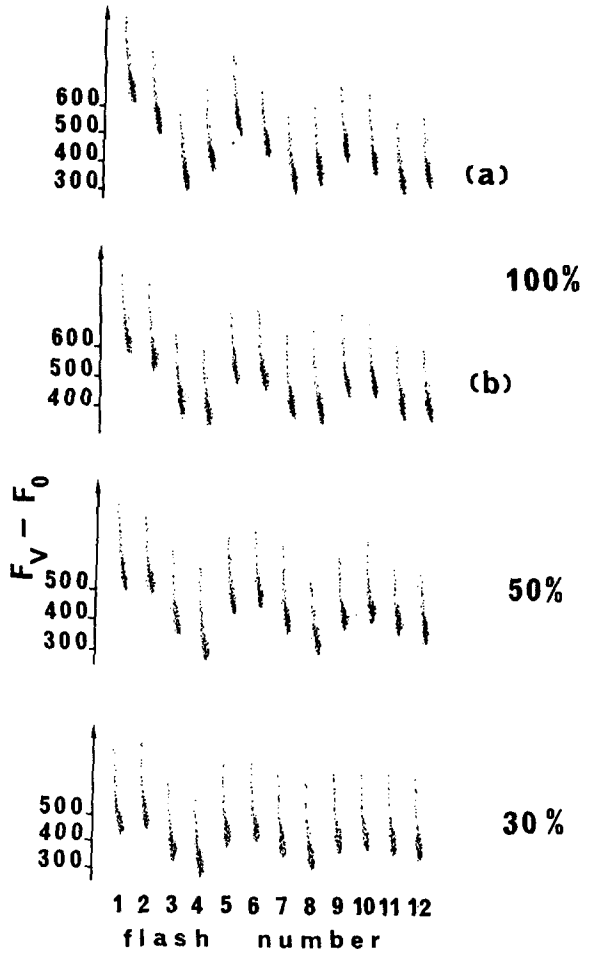

Fig. 2. Fluorescence yield traces induced by each of the first 12 flashes of a series of 16 flashes, from 75–90 ms, in dark-adapted inside-out thylakoids at pH 6.5 in the presence of 1 mM FeCy, for different relative flash energies (100%, 50%, 30%). Dark adaptation (a) at least 3 h, (b) and other recordings, 10 min. Time between flashes: 500 ms (same conditions as in Fig. 1). The flash-induced fluorescence yield, F_v, was subtracted from F_o, the fluorescence yield after darkness.

(from $I = 30\%-100\%$), the numerical fitting of the oxygen flash yield patterns of Fig. 1 indicates only small changes of the miss value, as shown in Table I. The miss percentage, varying from 12% to 16%, is in the range of that found in chloroplasts in the literature [17] (in the hypothesis of equal miss and equal double hit on each S_i state). The dark-adaptation time before flashing also has little effect on the damping of the oxygen yield pattern, as shown in Table I. As a matter of fact, only oxygen yield after the third flash in a sequence exhibits a large variation as a function of flash energy and dark-adaptation time (Fig. 1).

Fig. 2 shows the oscillation pattern of fluorescence under the same conditions as in Fig. 1. After a complete 3 h dark adaptation period, the features of the experimental fluorescence pattern were very typical. As a function of flash number, the same recurring oscillation pattern is repeated with a decreasing size (Fig. 2a, 100%). After only a 10 min dark period, the observed oscillation pattern was not strictly the same (Fig. 2b, 100%): the first oscillation minimum at flash 3 was displaced to flash 4. The period 4 of oscillation was nearly exact contrary to the oscillation pattern of the oxygen yield under the same conditions.

The more pronounced differences between the oxygen yield and fluorescence patterns were observed after a 3 h dark-adaptation period. In that case, the period 4 of the fluorescence oscillation was strictly exact (Fig. 2a). In contrast, with the increase in flash number, the maxima of the oxygen yield oscillation move on or two flashes to the right of the strict period 4 (Fig. 1).

Two kinds of numerical analyses were applied to the oscillation pattern of fluorescence.

(1) As shown in Fig. 2, the apparent mean value of the oscillating part of the fluorescence yield decreases as a function of flash number. Thus, assuming that the oscillation is symmetrical above and below the mean value, a positive drift was added to the experimental fluorescence yield values giving the patterns of Fig. 3A. The numerical fitting of these patterns (in Fig. 3A) yields nearly the same percentage of misses and double hits after each transition ($\alpha = 7\%$, $\beta = 7\%$) assuming there is a constant amount of active centers all along the whole sequence ($z_F = 1$). This result is not surprising because the phase retardation by α must counterbalance the phase advance by 3, to explain the observed perfect periodicity of 4.

(2) The numerical fitting is directly applied to the experimental fluorescence yield values without the addition of a positive drift. The results of the best least square fit are drawn in Fig. 3B. The fluorescence pattern obtained after a 3 h dark adaptation period (Fig. 2a and 3B, 100%) is described by zero misses and double hits ($\alpha = 0$, $\beta = 0$). The only variable is z_F, the factor that decreases the amplitude of the oscillating part of fluorescence as a function of flash number ($z_F = 0.87$). Surprisingly, a good fit is found, better than with the model used in Fig. 3A., so that the oscillation amplitude only decreases with the disappearance of a proportion $1 - z_F$ of fluorescent centers after each flash. In this fitting, many parameters can change: α, β, z_F. Finally only one, z_F, accurately describes the fluorescence oscillation. We have shown in a preliminary publication [18] that the fitting of oscillation patterns of fluorescence also yields no misses, no double hits for a flash energy of 50% and 30%, provided that flashing is preceded by a long period of darkness (for example 3 h). Misses are only detected for lower flash (energies less than 30%). In Fig. 3B, a small miss percentage (from 3% to 8%) is found in the fluorescence patterns obtained after a 10 min dark adaptation. The transition parameters found do not correspond to those of the oscillation patterns of the oxygen yield (Table I).

Different values of z are obtained from oxygen and fluorescence patterns. The origin of the small steady decrease of the oxygen yield as a function of flash number ($z_{O_2} = 0.94$–0.96 in Table I) is not known. This could be due to incomplete regeneration of the acceptor side in inside out vesicles. An increased susceptibility of the water-splitting enzyme complex to damage by light would also be likely. Anyhow, z_{O_2} is always much higher than

TABLE I

LEAST-SQUARE FITTING'S RESULTS OF EXPERIMENTAL O_2 YIELD PATTERN

The results as shown in Fig. 1 are according to the equal-miss and double-hit model [14] and to the unequal miss model ($\alpha_0 = \alpha_1 = \alpha_3 = 0$, $\beta = 0$) [15]. z is the percentage of centers remaining active after each flash of the series, found identical in both models. The S_0, S_1, S_2, S_3 values are the S_i concentrations in the dark, calculated in %.

Relative flash energy	z_{O_2} (%)	Equal miss model						Unequal miss model				
		$\bar{\alpha}$	$\bar{\beta}$	S_0	S_1	S_2	S_3	α_2	S_0	S_1	S_2	S_3
100% (3 h dark adaptation	0.94	0.12	0.03	12.3	85.4	1	1	0.44	0.4	0.93	6.2	0
100% (10 min dark adaptation	0.945	0.13	0.04	13.9	82.5	2.5	1	0.46	2.9	87	10	0
50% (10 min dark adaptation	0.95	0.14	0.04	13.2	85.6	0.6	0.6	0.47	3.6	89	7.4	0
30% (10 min dark adaptation	0.96	0.16	0.05	19.6	80	−11	0.4	0.52	9.2	84.6	6.2	0

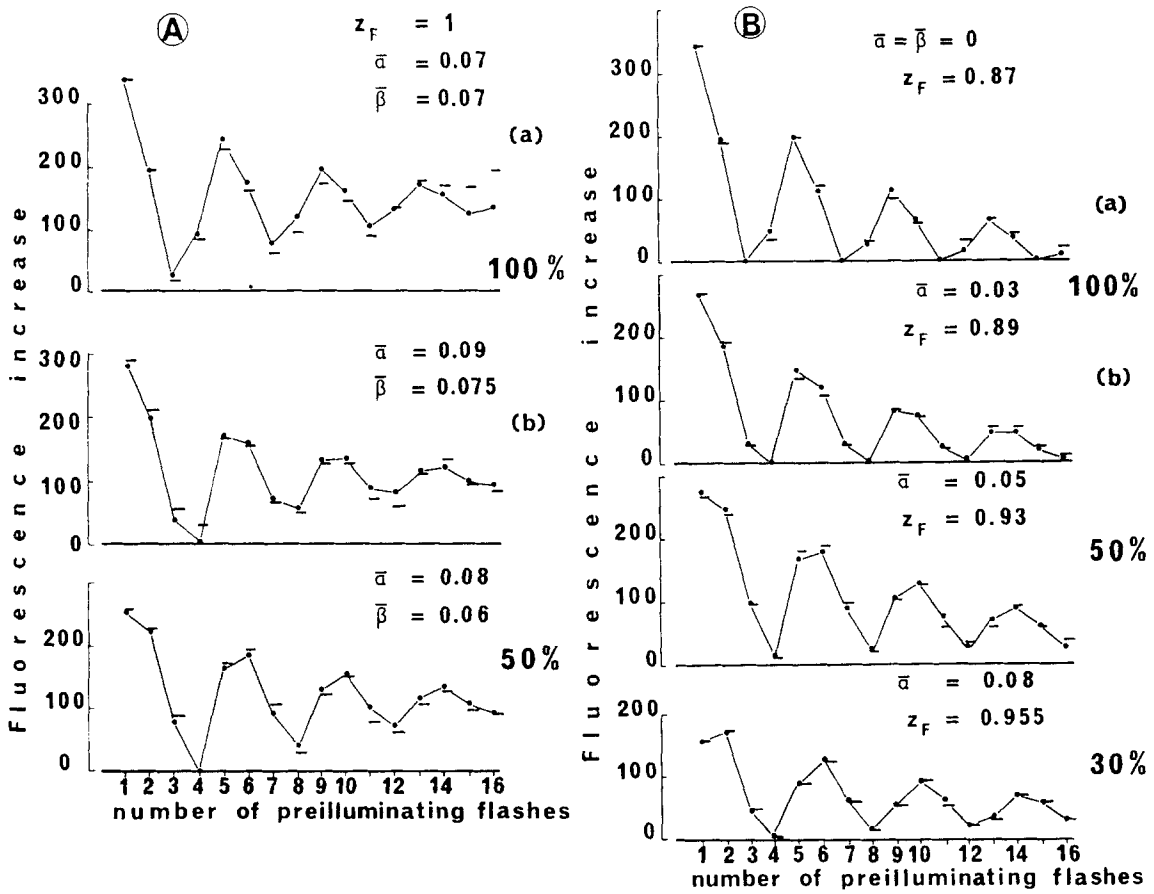

Fig. 3. Theoretical best fitting of the experimental oscillation patterns of fluorescence yield depicted in Fig. 2. Fluorescence yield was measured 80 ms after each flash of a series of 16 flashes. Experimental data are represented by dashes, theoretical data by dots. In any case, a constant level of fluorescence was subtracted from the fluorescence yield values. This level was adjusted in order to obtain the best fit. (A) A positive drift was added to the experimental values as a function of flash number, for the oscillation to be symmetrical above and below the mean value. $z_F = 1$ in all cases. $\alpha = 0.07$, $\beta = 0.07$ for a flash energy of $I = 100\%$ and a dark-adaptation period of 3 h, $\alpha = 0.09$, $\beta = 0.075$, for the same flash energy $I = 100\%$, but a dark adaptation period of 10 min, $\alpha = 0.08$, $\beta = 0.06$ for $I = 50\%$ and a dark adaptation period of 10 min. (B) Directly applied to the experimental data the best least-square fitting yields: $\alpha = 0$, $\beta = 0$, $z_F = 0.87$ for $I = 100\%$ and a dark-adaptation period of 3 h. For the following patterns, the dark adaptation period was 10 min: $\alpha = 0.03$, $\beta = 0$, $z_F = 0.89$ for $I = 100\%$; $\alpha = 0.05$, $\beta = 0$, $z_F = 0.93$ for I = 50%; $\alpha = 0.08$, $\beta = 0$, $z_F = 0.955$ for $I = 30\%$.

z_F. For example, in chloroplasts, a nearly steady-state level of oxygen yield was observed after many flashes ($z_{O_2} = 0.99$). Nevertheless, in the same preparation, the rate of decrease of the fluorescence oscillation amplitude after each flash (at high flash energy 100%) was much faster, $z_F =$ 0.89, i.e., 11-times faster (not shown). Thus, the decrease of fluorescence yield as a function of flash number is probably not related to the slight inhibition of PS II centers observed in the oxygen pattern.

In Fig. 3B (a), the larger amplitude of the fluorescence oscillation after the first flash, is associated with a faster decrease of the oscillation as a function of flash number (for a flash energy = 100%, $1 - z_F = 0.13$). In contrast, lower energy flashes (50%, 30%) give lower initial amplitude of the fluorescence oscillation, but the oscillation appears more sustained over more flashes than with high energy flashes. This behaviour fundamentally distinguishes the fluorescence oscillation from that of the oxygen yield. For example, the oxygen-yield

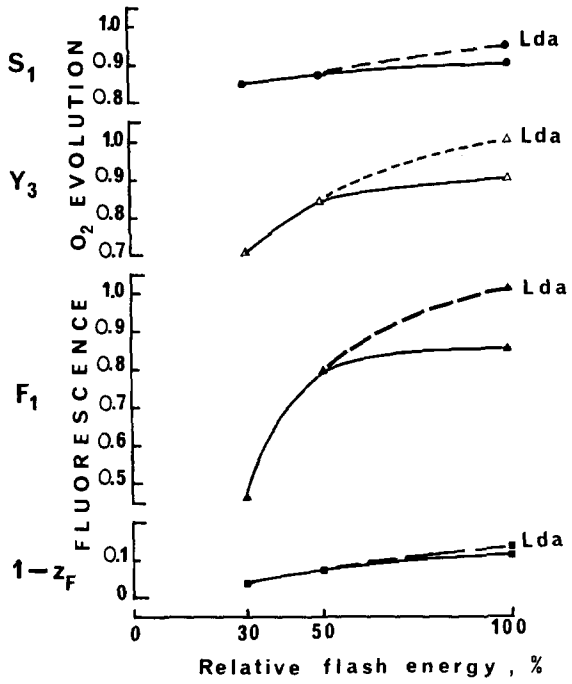

Fig. 4. As a function of flash energy, initial amplitude of fluorescence oscillation F_1, proportion of fluorescent centers lost after each flash of a series $1 - z_F$ (from Fig. 3B), O_2 yield evolved after the third flash of a series Y_3 and percentage of S_1 in the dark, calculated in Table I. (Lda: long (more than 3 h) dark-adapted preparation).

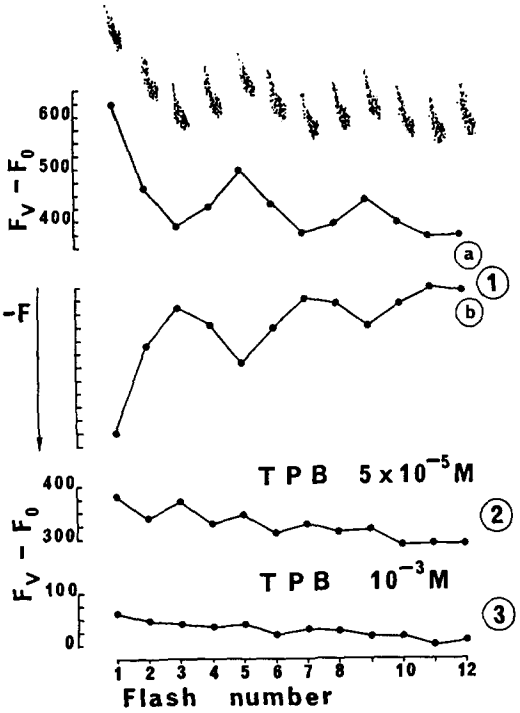

Fig. 5. Oscillation patterns of fluorescence yield measured 80 ms after each of a series of flashes (100%) at pH 6.5, in (1) (a) 3 h dark-adapted untreated inside-out thylakoid preparation, (b) same results as (a), except that the origin of ordinate is reversed; (2) after addition of 50 μM tetraphenylboron (TPB) (dark adaptation, 3 h) (3) after addition of 1 mM TPB.

oscillation vanishes more rapidly with low-energy flashes than with high-energy flashes in agreement with the damping model. The contrary is observed in the fluorescence oscillation which is preserved over more flashes when induced with low energy flashes (Fig. 3B, 30%). Thus, in the oscillation pattern of fluorescence, the proportion of fluorescent centers lost after each flash of a series, $1 - z_F$, increases with flash energy. As shown in Fig. 4, the experimentally found parameter, $1 - z_F$, qualitatively follows the formula:

$1 - z_F = KI$ (K is a constant; I the flash energy)

The amplitude of the fluorescence oscillations decreases exponentially as a function of flash number in Fig. 3B. Thus, the previous relation can be expressed differently by introducing the half flash number $N_{1/2}$. The half flash number is the number of flashes that decreases the oscillation amplitude by a factor $\frac{1}{2}$ ($N_{1/2} = \mathrm{Ln}2/1 - z$). Thus, the product $N_{1/2}I$ is found qualitatively constant and independent of the exciting flash energy.

The S_i states seen by oxygen yields are practically light saturated in our experiments [18] (except $S_2 \rightarrow S_3$). Nevertheless, the initial amplitude of the oscillating part of fluorescence, F_1, increases with flash energy (Fig. 3b and Fig. 4). The photochemical character of the fluorescence oscillation (initial amplitude and decay rate) proves that the fluorescence oscillation is controlled by a finite quantity of a pool stored in the dark, called T, which is exhausted after each flash of a series proportionally to flash energy. A long adaptation period (3 h) is necessary for the pool T to be in the initial state.

Discussion

Fluorescence yield depends on several parameters, implying redox components at PS II donor

and acceptor side. After an actinic flash, the variation of fluorescence yield presents an initial increase (5 μs) followed by a decay, which corresponds to the reoxidation of Q_A by Q_B. The oscillation pattern of fluorescence yield measured 80 ms after each flash of a series closely resembles that measured a few milliseconds after the flashes, except that the pattern is superimposed on a lower background (see also Ref. 19 in algae). In the fluorescence experiments described in this paper, Q_B participated in the electron-transfer reactions on the acceptor side. As shown in Fig. 5, in the presence of ferricyanide as external acceptor, a very small period-2 oscillation was observed after addition of the electron donor tetraphenylboron (50 μM) [20,21]. Subtraction of this signal would not affect the conclusions. The presence of ferricyanide in the medium can induce a decrease in the amplitude of fluorescence yield on the first flash of a series [22]. The photoreduction by one flash of the ion Fe^{3+} to Fe^{2+} associated with the secondary acceptor Q_B [23] would be responsible for this effect. In our preparations we practically observed no effect of ferricyanide on the first flash of a series. Factors like hydrophobicity, protonation in the Q_B site may be involved in the formation of Fe^{3+} in the dark [23]. Factors modifying period-4 fluorescence oscillation must not be very important because in the literature, the oscillating properties of different types of measurement appear to be very similar to those of fluorescence yield, shown in this paper. Concerning the oscillation pattern of flash-induced absorbance change at 350 nm, Dekker et al. [24] have pointed out the repetition of a basic pattern with decreasing amplitude as a function of flash number. The same particular oscillation structure is also evident in the flash-induced absorbance change at 384 nm and at 700 nm in isolated oxygen-evolving PS II complexes, reported by Saygin and Witt [25] using short laser flashes. Proton release on the donor side also oscillates with a nearly exact period 4, with very few misses [26]. The low miss percentage given by Forster and Junge [26] ($\alpha = 6\%$) was never observed in any oxygen yield pattern so far published.

In the oxygen yield and fluorescence oscillations, the disappearance of the basic period-4 pattern as a function of flash number appears to be determined by completely different factors. The amplitude of the oxygen-yield oscillation is essentially damped by misses, which induce a phase retardation without a change in the total number of active centers $\Sigma_i S_i$. In contrast, the fluorescence oscillation is characterized by the factor z_F which decreases the total amount of fluorescence centers, but the period 4 of fluorescence oscillation remains exact. Therefore, the S_i states and the S_i' states seen by fluorescence probably originate from different centers.

The numerical fitting of the O_2 yield patterns gives average data on the damping. This method is not precise enough to detect a heterogeneity in centers. If the S_i' state centers were O_2-evolving centers, the fluorescence pattern having maxima after one five flashes would arise from the S_2' state. Consequently, in the dark, the S_2' and S_3' state concentration would be zero (or nearly zero) like the S_2 and S_3 states. The O_2-yield pattern of the S_i' state centers would be like that shown in Fig. 3B, but shifted by two flashes. We have checked that the least-square-fit method is unable to detect up to 30–50% of such S_i' state centers in the O_2-yield pattern. The addition of a theoretical S_i' state pattern in the previous proportion, to a damped pattern (assuming 13% α, 4% β, 0% S_0, 100% S_1) results in a pattern which is fitted equally well but with less misses (not shown). Thus, the assumption of two kinds of O_2-evolving centers (the S_i and S_i' sates) is not incompatible with our finding of two fundamentally different period-4 oscillations, found respectively in the O_2 yield and fluorescence patterns.

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