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Characterization of two types of oxygen-evolving Photosystem II reaction center by the flash-induced oxygen and fluorescence yield

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In freshly prepared inside-out thylakoids, the periodicity of four of the oscillation patterns of fluorescence yield differs fundamentally from that of the oxygen yield (Delrieu, M.J. and Rosengard, F. (1987) Biochim. Biophys. Acta 892, 163–171). In this paper, further investigation of the oxygen and fluorescence patterns provides information on the origin of this discrepancy, i.e., the heterogeneity of the oxygen-evolving centers. The following results are reported. (1) The oscillation pattern of fluorescence yield was characteristic of an S_2 state, having maxima after one and five flashes, showing that the decay of the flash-induced fluorescence yield matches the deactivation of S_2 (half-time, 40 s), after a stability time of 2 s. (2) Under the conditions to be described, large changes in the oscillation pattern of the oxygen yield were observed when the fluorescence pattern remained the same. In freshly prepared inside-out thylakoids, a large miss (17.5%) was detected in the oxygen-yield pattern, even after addition of ferricyanide which could relieve the acceptor side of a large number of centers. In contrast, when the sample was stored with 30% ethylene glycol in liquid nitrogen and then thawed, the oscillation pattern of the oxygen yield was characterized by a progressive decrease in the mean value of the oxygen yield as a function of flash number, even in the presence of ferricyanide, but the true miss value did not exceed 7%. In this stored sample the oscillation patterns of the oxygen and fluorescence yield look very similar. Consequently, a functionally homogeneous population of oxygen-evolving centers was isolated, i.e., those which induce period-4 fluorescence oscillations. These centers, related to the plastoquinone pool, could be identified with the α centers. During flash illumination the reducing equivalents accumulated in the plastoquinone pool, through the secondary acceptor Q_B^{2-}, prevent the centers from being reactivated between the flashes, and thus are responsible for the disappearence of a small number of centers after each flash of a series. The other types of center observed in freshly prepared samples, characterized by large misses, are not related to the plastoquinone pool and could thus be the β centers.

Introduction

The notion of functionally and structurally distinct populations of PS II centers within the thylakoid membranes of chloroplasts is well established in the literature. The supporting evidence for the existence of PS II heterogeneity comes primarily from the interpretation of the

Abbreviations: PS II, Photosystem II; Mes, 4-morpholineethanesulfonic acid; DCBQ, 2,5-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1′-dimethylurea; DMBQ, 2,5-dimethylbenzoquinone; PPBQ, phenyl-*p*-benzoquinone; PQ, plastoquinone; Q_B secondary quinone acceptor of PS II.

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fluorescence induction of chlorophyll measured in DCMU-poisoned chloroplasts. An analysis of the growth of the area over the fluorescence induction curve yields two components, a fast α-component and a relatively slow β-component [1]. These two components have been attributed to the two forms of PS II, termed PS IIα and PS IIβ, respectively. The most significant differences among PS II centers were found to be in their location on thylakoid membranes [2], in the size of the chlorophyll antennae [1,2] and in their ability to reduce plastoquinone [3,4]. Far less information exists on how such heterogeneities might affect the oxygen-evolving properties of the two types of PSII center when measured in repetitive turnovers. Here, by studying the oscillation patterns of the oxygen and fluorescence yield, we show that two types of oxygen-evolving PS II center are indeed functionally distinct and, furthermore, that differences exist in the oxygen-evolving complex itself.

Photosynthetic oxidation of water requires the generation of four oxidizing equivalents per molecule of oxygen evolved. It is generally accepted that these oxidizing equivalents are produced by single charge-separation events in PS II, and are stored in an oxygen-evolving complex. Five intermediate oxidation states are produced in the process. These states are denoted S_i ($i = 0$–4), where i is the number of electrons removed from the oxygen-evolving complex. Oxygen is released during the conversion of the unstable S_4 to S_0 [5,6]. The discovery by Dismukes and Siderer [7] of a multiline S_2-state EPR signal which is characteristic of an exchange-coupled manganese complex, suggests that the catalytic site of the oxygen-evolving complex may consist of as many as four manganese ions in close magnetic interaction. In addition to the so-called multiline signal, the S_2 state exhibits a more recently discovered EPR signal centered around $g = 4.1$ [8,9]. Both signals arise from the same site in the S_2 oxidation state [10], and their spectroscopic differences probably reflect structural changes in the Mn active site [10,11]. On the oxidizing side of PS II, another component, D, is present, which, in its oxidizing form, gives rise to an EPR signal called SII_{slow} [12]. Component D has been shown to interact magnetically with the Mn cluster [13]. In its reduced form, D could reduce the S_2 state [14].

In freshly prepared inside-out thylakoids, the period-4 oscillations detected in the fluorescence yield measured some milliseconds after each flash of a series were found to be different from the oscillations of the oxygen yield detected under the same conditions [15]. The amplitude of the oxygen-yield oscillations was largely damped by misses, which induce a phase retardation in oscillations. On the other hand, the fluorescence pattern showed strictly no change of phase and was thus characterized by the same oscillation pattern repeated every four flashes with a decreasing amplitude. This difference could be explained by assuming the existence of two different charge accumulation systems leading to oxygen evolution, the S_i and S_i' states. However, there is no direct evidence of the participation of two types of center in oxygen evolution. In this paper, this assumption is confirmed. By changing the conditions (lowering the temperature, storing with 30% ethylene glycol in liquid nitrogen), it is possible to observe almost the same pattern but shifted by two flashes for the flash-induced oxygen yield (S_4) and the flash-induced fluorescence yield (as an S_2 state). Thus, a functionally homogeneous population of oxygen evolving centers was obtained, devoid of most of PS II centers responsible for the greatly damped oxygen-yield oscillations.

Generally, the feature of the oscillation patterns with period 4 of the oxygen or fluorescence yield is explained by assuming the occurrence of misses and double hits [6]. On any one flash, a percentage of centers is not converted (misses) and another percentage may induce two conversions (double hits). However, this simple definition does not explain the mechansim for misses. On one flash some centers do not react, but on a subsequent flash they may do so. For example, large misses appear under non-saturating flashes. In this case, the number of excitations is smaller than the number of centers. On one flash, some centers do not receive any excitation, but on a subsequent flash they may receive one and react. The number of potentially active centers remains the same throughout the whole flash sequence.

In inside-out thylakoids, the mean value of the oxygen yield slightly decreases as a function of flash number, even in the presence of efficient external acceptors [15,16]. The least-squares best

fit is considerably improved, assuming that the total number of active centers progressively decreases after each flash of the series [15]. Thus, in the model used, the number of active centers after each flash is equal to that before each flash, multiplied by $z < 1$. The factor $1 - z$ represents the percentage of active centers that disappear after each flash. But, in contrast to the centers with misses, described above, the inactive centers remain non-functional during the entire flash sequence (see Ref. 15 for the introduction of z in the least-squares fitting method). When the number of active centers remains constant as a function of flash number, this variable, z, is equal to 1. Thus, this factor does not change the model of Forbush et al. [6], but it does test the validity of the assumption of a constant number of centers, implicitly made in usual fits. The analysis of the oscillation pattern of the oxygen yield that does not take account of this factor of the loss of centers, z, generally overestimates the misses [15]. Graan and Ort [4] have also pointed out the existence of a small proportion of PS II centers which have negligible turnovers upon flash illumination.

Material and Methods

Inside-out thylakoids were obtained by the mechanical desintegration (Yeda press) of pea chloroplast thylakoids, followed by phase partition according to Akerlund and Andersson [17]. 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. The chlorophyll concentrations and the chlorophyll a/b ratios were determined according to Arnon [18]. The chlorophyll a/b ratios of inside out vesicles and unfractionated control were about 1.87 and 2.40, respectively.

White flash excitation was provided by Strobolave General Radio flash lamps (3 μs at half-peak height). All flashes in the experiments to be described were saturating.

The rate electrode used for oxygen flash yield measurements has been previously described [19,20]. External acceptors were added in the circulating medium of the upper chamber of the electrode.

The fluorescence yield of chlorophyll a, was measured essentially as described previously [15]. The thickness of the suspension was 2 mm and the chlorophyll concentration 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 emitting a weak green light. Fluorescence yield was measured 80 ms after each flash of a series of 16 flashes. Flashes and measuring light (12 ms) were triggered by a microcomputer (Apple II+). After each flash of the same series, the chlorophyll a fluorescence signal was first amplified, recorded in the transient waveform recorder (10 bits, 10 MHz), and then partially stored in the microcomputer through a fast parallel interface. By this method, a record of the fluorescence decays after all the flashes of a particular sequence was obtained. Finally, the fluorescence yields 80 ms after each flash, F_V, were printed out. F_0, the fluorescence yields after darkness, measured just before the flash series, were subtracted from F_V.

Results

Properties of the S_i states as revealed by the oscillation pattern of fluorescence yield

Fig. 1 shows fluorescence decays (73–85 ms) induced by each flash of a series of flashes in dark-adapted inside-out thylakoids. In the freshly prepared material, the pattern is hardly different when the flash spacing varies from 650 ms to 2.9 s. Thus, the S_i state (s) giving rise to fluorescence oscillations, remains stable for quite a long time (2 s) before the decay. This stability is shown to be independent of the presence or absence of an external acceptor such as ferricyanide, as also shown in Fig. 1. These measurements were made after a 45 min total darkness equilibration. We checked that, under these conditions, the EPR Signal II_{slow} was still identical in the dark adapted and illuminated samples (experiment not shown, performed with a Bruker B ER 420 spectrometer). This reveals that D was in its oxidized form, and thus could not interact with the S_2 state [21,14].

Fig. 1b also reveals that the minimum value of F_V is always the same on the 3rd-4th, 7th-8th and 11th-12th flashes in the oscillation pattern of fluorescence yield. So, as shown in Fig. 2, once sub-

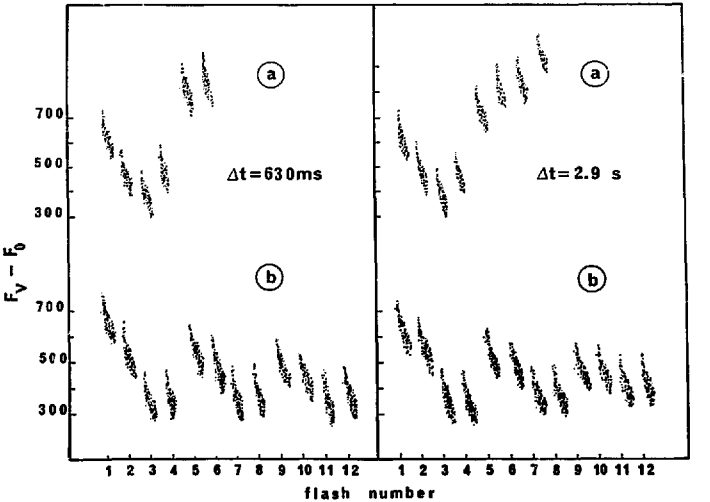

Fig. 1. Fluorescence yield decays (73–85 ms) induced by each of the first flashes of a series of 16 flashes, in freshly prepared inside-out thylakoids adapted to 45 min darkness, at pH 6.5, in the absence (a) and in the presence (b) of 0.5 mM ferricyanide. The flash interval is 630 ms (left) and 2.9 s (right). (a) Six flashes (left) and eight flashes (right); (b) 12 flashes.

tracted from a constant fluorescence level, the oscillation patterns of fluorescence yield have very few misses (about 1%) and no double hits. The only important factor for the fitting is z, which allows the variation of the total number of centers from one flash to another. We found $z = 0.90$, representing an exponential decrease of 10% of the number of centers after each flash. However, as already explained in Ref. 15, there is another way to fit the fluorescence oscillation, because the reference level (or level for zero state) of the fluorescence oscillation amplitude is not known with certainty. If it is assumed that the total number of active centers remains constant during the whole series of flashes, such patterns may be fitted with an equal percentage of misses and double hits of around 7–8% [15]. In the literature, oscillation patterns similar to those shown in Fig. 1 or 2, were also fitted with an equal percentage of misses and double hits. Dekker et al. [22] found 9 % misses, 9% double hits for the oscillation pattern of flash-induced absorbance change at 350 nm. Forster and Junge, found 6% misses, 5% double hits [23] and 10% misses, 10% double hits in another work [24] for the same flash-induced proton release on the donor side. The physical mechanism leading to this constant fortuitous equality of misses and double hits in various experimental cases is difficult to explain.

With regard to the experiments of Graan and Ort [4], who found that only about 60% of PS II centers are able to transfer electrons to PS I at an appreciable rate, the assumption of a constant number of active centers all along the flash series is questionable. Furthermore, the oscillation pattern of fluorescence yield in Fig. 2, simulated with a decreasing proportion of active centers on each flash of the series, appears very similar to the

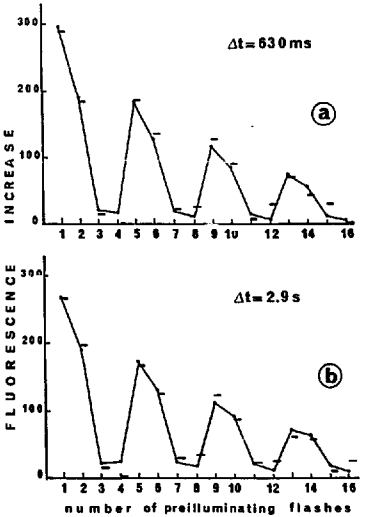

Fig. 2. Best least-squares fitting of the experimental oscillation patterns of fluorescence yield shown in fig. 1b. A constant level of fluorescence yield was subtracted from the fluorescence yield measured 80 ms after each flash of a series of 16 flashes. This level was adjusted in order to obtain the best fit. Experimental data are represented by dashes, theoretical data by dots. The best least-squares fitting yields a percentage of misses $\alpha = 0.01$, no percentage of double hits ($\beta = 0$), and a progressive decrease of 10% of the number of centers after each flash ($z = 0.9$) at a flash interval of 630 ms (a), and $\alpha = 0.015$, $\beta = 0$, $z = 0.91$ at a flash interval of 2.9 s (b).

experimental oscillation pattern of the S_2 state multiline signal reported by Styring and Rutherford [25] (in their Fig. 1). With relative accuracy, the results of these authors show that, after large multiline signals on the first and the second flash, the signals on the third and the fourth flash are practically zero. The same variations of the amplitude of the fluorescence oscillations are shown in Fig. 2, with nearly zero amplitude on the third and fourth flashes. These facts demonstrate that misses and double hits must be small. Assuming a zero concentration of the S_2 and S_3 states in the dark, the concentration of the S_2 state returns to zero after three and four flashes only in the case of no misses and no double hits. Thus, the centers that give rise to the multiline signal in the S_2 state [25],

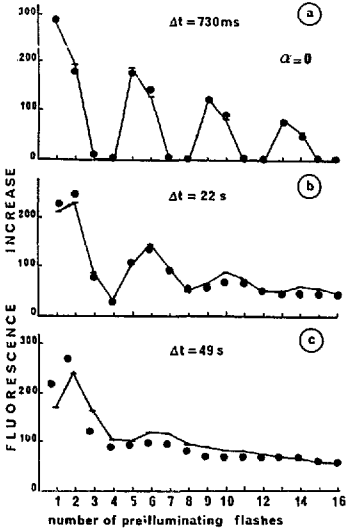

Fig. 3. Oscillation patterns of fluorescence yield induced by a series of 16 flashes in the presence of 0.5 mM ferricyanide. The time between flashes is (a) 730 ms, (b) 22 s, (c) 49 s. The freshly prepared inside-out thylakoids were adapted for 45 min before flashing. The dots represent the experimental amplitude of the fluorescence yield measured 80 ms after each flash, after substraction of a constant level of fluorescence yield. The small dashes are the theoretical data. The deactivation kinetics of S_3 ($S_3 \rightarrow S_2$), and S_2 ($S_2 \rightarrow S_1$) are assumed to be first-order reactions. The equations used are the following: $S_2(t) = S_2(0)\, e^{-v_2 t}$; $S_2(t) = S_2(0)\, e^{-v_2 t} + S_3(0)(v_3/(v_2 - v_3))[e^{-v_3 t} - e^{-v_2 t}]$; $S_1(t) = S_1(0) + S_2(0)\ [1 - e^{-v_2 t}] + S_3(0)[1 - e^{-v_3 t} - (v_3/(v_2 - v_3))(e^{-v_3 t} - e^{-v_2 t})]$; $S_i(t)$ is the concentration of the S_i state at time t after the flash; $S_i(0)$, the S_i state concentration at $t = 0$; v_2, v_3: rate constant of the kinetics of S_2 and S_3 found equal to 0.016 s^{-1} and 0.009 s^{-1} respectively. (a) $z = 0.9$; (b) $z = 0.93$; (c) $z = 0.94$. In all cases, the miss $\alpha = 0$. the calculated concentrations of S_0, S_1, S_2, S_3 in the dark are, in %, 42, 58, 0, 0, assuming that the fluorescence oscillations arise from the S_2 state.

as well as those inducing fluorescence oscillations, are characterized by very few real misses (at least up to the fifth flash for the multiline signal). All these facts also indicate that the fluorescence oscillations are likely to be related to the same S_2 state that induces the EPR multiline signal at 4 K.

Fig. 3 shows the behavior of the oscillation pattern of fluorescence yield when the time between the flashes of a series is varied. During the dark spacing between the flashes, the S_3 and S_2 state centers deactivate into S_2 and S_1, respectively, by charge recombination from the donor and acceptor side. In Fig. 3, the oscillation patterns are qualitatively characteristic of an S_2 state assuming S_3 and S_2 decay with half-times equal to 77 s and 43 s, respectively (see the model in Fig. 3 legend). The deactivation kinetics of the S_2 state observed by fluorescence were measured more precisely after one flash by varying the time between the first and the second flash of a series. Due to the four-step reaction series and the transition parameters, the S_2 state concentration on the fifth flash is proportional to the S_2 state concentration on the first flash. By increasing the spacing between the first and the second flash, the amount of S_2 state formed after the first flash and remaining at the second flash decays. This decay

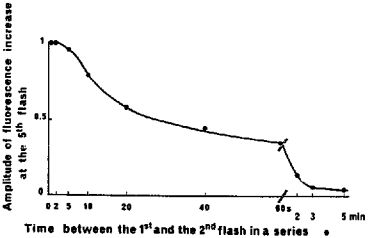

Fig. 4. Deactivation of the S_2 state observed by fluorescence. The amplitude of fluorescence yield subtracted from a constant level was measured on the fifth flash of a series of flashes (at an interval of 730 ms) as a function of the time spacing between the first and the second flash. Due to the four-step reaction series, after one cycle of reactions, the S_2 state concentration after the 5th flash is proportional to the S_2 state after the first flash of the series. By increasing the spacing between the first and the second flash, the S_2 state formed after the first flash deactivates. This deactivation is followed by the measurement of the amplitude on the fifth flash, which decays in the same proportion.

is measured by the change in the amplitude of fluorescence oscillation on the fifth flash, which is quite large (Fig. 4). Using this method, the half-

TABLE I

LEAST-SQUARES FITTING RESULTS OF EXPERIMENTAL O_2 YIELD PATTERN

z is the percentage of centers remaining active after each flash of the series. The S_0, S_1, S_2, S_3 values are the S_i concentration in the dark calculated in %.

			Δt	z	α	β	S_0	S_1	S_2	S_3
Freshly prepared inside-out thylakoids 45 min dark-adaptation period	without addition	22° C (Fig. 5)	430 ms	0.92	0.14	0.04	26	67.2	5	1.8
			2.25 s	0.95	0.19	0.05	30.5	68	1	0.5
		4° C (Fig. 6b)	650 ms	0.88	0.07	0.02	17	53	17	3
			2.25 s	0.88	0.11	0.03	29	56.3	9.4	5.2
	in the presence of 0.5 mM ferricyanide 22° C (Fig. 6a)		650 ms	0.97	0.175	0.05	27	72	0.5	0
			2.25 s	0.98	0.175	0.05	28.5	70.5	0.8	0
Inside-out thylakoids stored in liquid nitrogen in the presence of ethylene glycol	long dark adaptation period (Fig. 6c)		650 ms	0.91	0.06	0	12.4	79	8.7	0
			2.25 s	0.93	0.07	0.02	22.3	61	15	1.6
	10 min dark adaptation period (Fig. 6d)		650 ms	0.93	0.06	0	29	65	5	0
			2.25 s	0.96	0.08	0.01	27	62	7	4

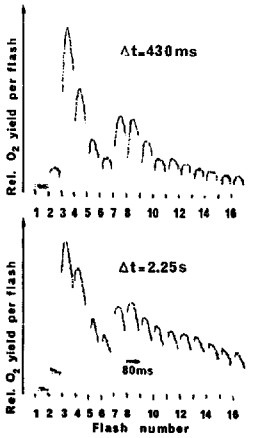

Fig. 5. Flash-induced oxygen-yield patterns in freshly prepared inside-out thylakoids at two different flash intervals, 430 ms and 2.25 s, at pH 6.5. Dark adaptation; 45 min. At each flash, recordings were made over a period of 80 ms including 8 ms just before the flash. The relative O_2 yield is measured by the maximum amplitude of the signal. See Table I for miss, double hit, z values and S_1 concentrations in the dark given by the best least-squares fitting method.

time of the S_2 state deactivation was found to be about 35–40 s, close to the value mentioned above. This value is of the same order as that of the EPR multiline signal after one flash in PS-II-enriched membranes [25].

Properties of the S_i states as revealed by the oscillation pattern of oxygen yield

Figs. 5 and 6a show the oscillation patterns of oxygen yield in freshly prepared inside-out thylakoids without and with 0.5 mM FeCy. The real damping was important in all cases (see Table I for the miss values), much larger than that detected in the fluorescence yield pattern (misses around 1%) under the same conditions. Without the addition of an external, acceptor (Fig. 5), comparison of the short-spaced flash sequence (650 ms) with a wider spaced one (2.25 s) clearly shows that for a time spacing short enough with respect to the usual stability of the states (650 ms), a relatively large percentage of PS II centers become apparently non-functional after many flashes. This fact is mathematically expressed by z, which is the percentage of centers remaining active from one flash to another. The factor z is smaller for a spacing of 650 ms ($z = 0.92$) than for a spacing of 2.25 s ($z = 0.96$). The larger the spacings, the more numerous are the centers able to turn over repeatedly.

In the presence of an electron acceptor (0.5 mM FeCy or 50 μM DCBQ, not shown), many more PS II centers were capable of oxidizing water after many flashes, even at short intervals, as shown in Fig. 6a ($z = 0.97$). Nevertheless, the percentage of misses did not decrease, but rather increased ($\alpha = 0.175$) with regard to the oxygen

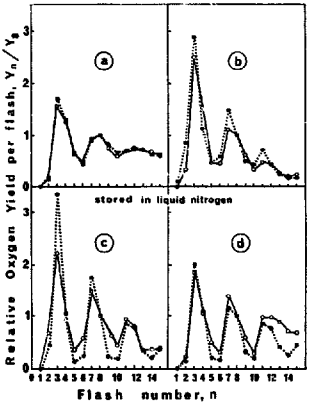

Fig. 6. Flash-induced oxygen yield patterns at two different flash intervals (·····) 650 ms, (———) 2.25 s, at pH 6.5: (a) in freshly prepared inside-out thylakoids in the presence of 0.5 mM ferricyanide; dark adaptation 45 min; (b) in freshly prepared inside-out thylakoids maintained at 4°C; dark adaptation 45 min; (c) in inside-out thylakoids stored in liquid nitrogen with 30% ethylene glycol, thawed at room temperature in complete darkness; (d) in the same sample as (c), but after at 10 min dark-adaptation period. See Table I for miss, double hit, z values, and S_1 concentrations in the dark given by the best least-squares fitting method.

yield pattern without an external accepter (α = 0.14 at short flash interval). This could indicate that the centers which donate electrons to FeCy are involved in the mechanism of misses for oxygen yield.

Evidence has been presented that changes in temperature can induce lateral rearrangements in the thylakoid membrane accompanied by a reversible conversion of PS IIα to PS IIβ [26,27]. An increase in the size of PS IIα has been induced by lowering the temperature [27]. Thus, in order to check the effect of temperature, inside-out-thylakoids were maintained at 4°C on the electrode. As shown in Fig. 6b and Table I, the decrease in temperature induced two effects. Firstly, the percentage of misses was much lower than at 20°C (see Table I). Secondly, at 4°C, the decrease of the number of centers after each flash of a sequence was much larger than at 20°C (z = 0.88, i.e., 12% of loss centers after each flash).

These changes were more apparent in samples frozen in liquid nitrogen in the presence of 30% ethylene glycol for conservation over a long period (in Fig. 6c and d). According to Zimmermann and

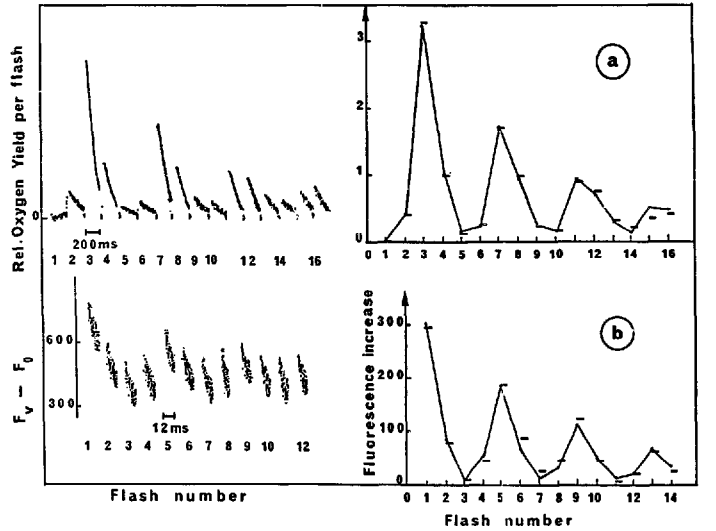

Fig. 7. Flash-induced O_2 yield pattern (a), in comparison with the flash-induced fluorescence yield pattern (b), in inside-out thylakoids stored in liquid nitrogen with ethylene glycol and thawed at room temperature in complete darkness. 0.5 mM ferricyanide were added (pH 6.5). The flash interval was 650 ms. For the O_2 yield pattern, at each flash, recordings were made for 200 ms, including 20 ms before the flash. The relative O_2 yield was measured by the maximum amplitude of the signal. For the fluorescence yield pattern, a constant level was subtracted from fluorescence yield measured 80 ms after each flash. Experimental data are represented by dashes, theoretical data by dots. The best least-squares fitting yields: α = 0.06, β = 0, z = 0.91, for the O_2 yield pattern; α = 0.025, β = 0, z = 0.90 for the fluorescence yield pattern. The S_0, S_1, S_2, S_3 concentrations in the dark calculated in % are respectively 12.5, 79, 8.5, 0 for the O_2 yield pattern, and 18, 63, 17, 2 for the fluorescence yield pattern assuming that the fluorescence oscillations arise from the S_2 state.

Rutherford [11], ethylene glycol inhibits the formation of the EPR $g = 4.1$ signal. The centers which give rise to the $g = 4.1$ signal when in the S_2 state seem to be converted to centers giving rise to the multiline signal upon addition of ethylene glycol [11]. By using samples stored in liquid nitrogen in the presence of 30% ethylene glycol we tried to reproduce these conditions. After the samples were thawed to room temperature in total darkness, the oxygen yield sequence was very different from that observed in the same sample but freshly prepared (Fig. 6c, cf. Fig. 6a). The miss value did not exceed 7%, instead of 17.5% (see Table I). The oxygen yields on the 5th, 6th and also on the 9th, 10th flashes were very small. In the freshly prepared samples (Fig. 6a), the numerical fitting always gives a percentage of double hits (around 5%) associated with misses (see Table I). In the samples which had been stored in liquid nitrogen in the presence of ethylene glycol these double hits are no longer necessary for the fitting ($\beta = 0$ at short interval). The values of miss, double hit a z become close to that found for the oscillation pattern of fluorescence yield which remain almost constant in any material.

In Fig. 6, the oxygen-yield patterns obtained at two different flash intervals were either identical or very different, depending on the dark-adaptation conditions. According to several authors [14,21], D, which in its oxidized form gives rise to Signal II_{slow}, in its reduced form could reduce the S_2 state with a slow rate ($t_{1/2} = 1$–2 s) on the first flashes of a series. At low flash frequency, this phenomenon leads to a decrease in the oxygen yield on the third flash with regard to that on the fourth flash Y_3/Y_4, and to an apparent enhancement for the S_0 population in the dark [20]. As shown in Fig. 6 and Table I, when the short flash interval (650 ms) is replaced by a longer one (2.25 s), the Y_3/Y_4 and S_0/S_1 ratios change in two cases: after a long dark-adaptation period at 4°C, and after storage in liquid nitrogen. Under normal conditions, the redox state of D seems to be the oxidized form. Our results in Table I also show that the concentration of the S_2 state in the dark is negligible in freshly prepared samples or after a 10 min dark adaptation period. In contrast, after a long dark-adaptation period at 4°C or after storage in liquid nitrogen, the S_2 state concentration in the dark is around 15%. We suggest that in the latter case, D^+ is slowly reduced in the dark by oxidizing the S_1 state into S_2.

Fig. 7a and b shows the oscillation patterns of oxygen and fluorescence yield in the same sample stored in liquid nitrogen with ethylene glycol. After a shift of two flashes of the fluorescence pattern, these patterns look very similar. Both are fitted with nearly the same parameters (see Fig. 7 caption). The same ratios S_0/S_1 are also found qualitatively in the dark, assuming that the fluorescence yield pattern oscillates like the S_2 state. Ferricyanide does not affect the oxygen-yield pattern in this sample.

Discussion

In this paper, we present further evidence that two types of oxygen evolving center exist. This evidence is based on a comparative study of the oscillation pattern of the oxygen and fluorescence yield. These patterns were correlated in inside-out thylakoids previously stored in liquid nitrogen with ethylene glycol. Thus, a functionally homogeneous population of centers was isolated in this material. The S_2 state of this population induces fluorescence changes probably also gives rise to the EPR multiline signal at 4 K [11], in contrast to the other centers. This type of center is characterized by very few misses (maximum 6% in Fig. 7a), probably due to the stability of the S_2 state and S_3 state (more than 2 s). However, instead of misses, a small proportion of centers (9–10%) disappears after each flash of a series.

In a recent report [15], we studied the oscillating behavior of these centers as a function of flash energy by fluorescence measurements. With the increase in the flash energy, we observed an increase in fluorescence yield on the first flash, but, as a function of flash number, this larger amplitude was followed by a faster decay of the fluorescence oscillations. By varying the flash energy, we found that the product of flash energy, I, and the flash number necessary to decrease the fluorescence oscillations by half, $N_{1/2}$, is independent of the exciting flash energy. This relation suggests that the S_i states observed by fluorescence are controlled by the limited amount of a component, T, stored in the dark and which is exhausted after

each flash of a series in a quantity proportional to flash energy [15]. A possible candidate for T is the plastoquinone pool, which is slowly reoxidized in the dark. Thus, after darkness, successive flashes progressively reduce all the plastoquinone molecules of the pool. Consequently, on the donor side, the S_i state centers, i.e., those observed by fluorescence, appear to be in a finite quantity, restricted by the acceptor side limitation. If this is true, the number of flashes necessary to decrease the amplitude of the mean value of fluorescence oscillations by a factor $1/e$ is equal to the kinetic measurement of the plastoquinone pool, i.e., 7-times the number of centers in Fig. 2. Measuring the number of reducing equivalents stored in the intersystem pool subsequent to a series of single-turnover flashes, Graan and Ort [4] found a maximum of 11 electrons per P700 in spinach thylakoids. The existence of a pool of reducing equivalents subsequent to a flash sequence can be explained by the low rate of reoxidation of PQ with regard to the time between flashes generally used. As an example, in quinone-treated algae, a slow electron transfer from PS II to PS I is still observed in the 500 ms–5 s range [28]. In our material that contains few PS I, plastoquinone is probably more slowly reoxidized. Thus, flash illumination progressively reduces PQ molecules that remain reduced until the end of the flash series. On the acceptor side, the gating of electrons from the single electron turnover to the pair of electrons required to reduce plastoquinone, PQ, to plastoquinol, PQH_2, in the plastoquinone pool occurs as the result of the reduction of Q_B. At the Q_B site, plastoquinone and plastoquinol could be exchanging with this site [29]. However, on some centers, the absence of oxidized plastoquinone could leave Q_B in its reduced state, Q_B^{2-}, unable to be reoxidized either by charge recombination during the flash interval or by a following flash. Therefore, the accumulation of reducing equivalents in the plastoquinone pool could be responsible for the existence of the centers that disappear after each flash of a series. In our experiments, we have noticed a slight apparent increase of the plastoquinone pool when a flash interval of 2.9 s is given instead of 630 ms, indicating that a little more oxidized PQ is available for exchange with Q_B^{2-} (in Fig. 2, z increases from 0.90 to 0.91, and in Fig. 6 d, z increases from 0.93 to 0.96; in the latter case the pool related to z apparently increases from about 9 PQ to 11 PQ per center).

Actually, z should not be identical after each flash of a series, because centers with Q_B^- will have a higher z than the other centers. This fact is not included in our model, but this does not affect the conclusions.

Thus, the population of centers responsible for the fluorescence oscillations is connected to the plastoquinone pool. These centers cannot be induced to turnover by ferricyanide, as shown by the low value of z in the sample previously stored at 77 K in the presence of ethylene glycol (Fig. 7). Ferricyanide, being a one-electron acceptor, probably cannot compete with PQ for binding to the Q_B intermediate. In contrast, as reported in Ref. 30, high-potential benzoquinones such as DMBQ and PPBQ are able to occupy the Q_B site in PS II, using the bound ferrous iron for their photoreduction. According to Zimmermann and Rutherford [30], Q_A^- is reoxidized by PPBQ (possibly via Q_B). The semiquinone form of PPBQ thus produced is unstable and extracts an electron from Fe^{2+} to form Fe^{3+} and $PPBQH_2$.

Our results also show that another type of center is present in freshly prepared inside-out thylakoids (Fig. 6a). In contrast to the previous centers, these centers are characterized by large misses ($\alpha = 17.5\%$) and they do not seem to interact with the plastoquinone pool. These centers can be induced to turnover by ferricyanide, even at short flash intervals (650 ms in Fig. 6a). As reported by Thielen and Van Gorkom [31], the β centers are not related to the plastoquinone pool via Q_B. According to this definition, the centers not involved in the fluorescence oscillations can be identified with the β centers.

Table I shows that the miss percentage is not lowered by the addition of ferricyanide which, however, induces the β centers to turn over (with large misses). This suggests that the misses of the β centers do not originate from the acceptor side, due to incomplete reactions which would be shifted to completion in the presence of ferricyanide, but probably from the donor side. On the donor side, it is likely that the S_2 state of the β centers gives rise to the EPR signal centered at $g = 4.1$. The vanishing of this signal and of the β centers occurs

under similar conditions (in the presence of ethylene glycol at low temperature). According to Zimmermann and Rutherford [11], the centers in the S_2 state which give rise to the $g = 4.1$ signal appear to be more unstable than those which give rise to the multiline signal. Thus, an imperfect charge stabilization at the level of the S_2 state could be the origin (or one of the origins) of the damping of the oscillation pattern of the oxygen yield [32].

Further investigation are necessary to specify the principal function of each type of oxygen-evolving center.

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