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Changes in the S_0 and S_1 properties during dark adaptation in oxygen-evolving Photosystem-II-enriched thylakoid membranes

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Analysis of oxygen and fluorescence flash yield patterns reveal two types of period 4 behavior. Two types of oxygen-evolving center have been characterized, those with few misses which induce fluorescence oscillations (however, instead of misses a small proportion of active centers, about 10%, are lost after each flash of a series) and the others responsible for the highly damped oxygen-yield oscillations (Delrieu, M.J. and Rosengard, F. (1988) Biochim. Biophys. Acta, 936, 39–49). In this paper, further investigation of the fluorescence yield patterns shows that in the oxygen-evolving centers responsible for the period 4 fluorescence oscillations (and only in these centers), a change in the S_0 and S_1 properties develops during dark adaptation, affecting the S state advancement only on the first flash of a series. The miss factor (around 1–4%) was greatly increased on the first flash of a series for the $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ transitions (1) when the relatively high flash energy was decreased, (2) during the (short) dark period that followed one or three pre-flashes, (3) after partial Cl^- or Ca^{2+} depletion. There are two possible explanations of the misses on the first flash only: (a) either after the first flash no reaction occurs in a percentage of S_0 and S_1 state centers and the miss factors are therefore α_0 and α_1, or (b) a reaction occurs after the first flash, and the reaction results in the formation of inactive S_0, S_1 and S_2 state centers (the miss factors are then α_0, α_1 and α_2). The centers studied represent a minor fraction of the O_2-evolving centers at an ambient temperature. In these centers, a structural reorganization in the S_0 and S_1 states during dark adaptation could account for misses. This process is generally reversed by one flash.

Introduction

The main function of the O_2-evolving core complex of photosynthesis is to extract electrons and protons from water on one side of the membrane and to reduce quinone molecules on the other side. The active site in the water-splitting enzyme complex is an entity consisting of 2–4 Mn atoms, presumably organized in a cluster [1–3]. Successive charge separations in the reaction center result in the accumulation of highly oxidizing equivalents at least partially on the Mn-cluster. The intermediate redox states are termed S_0 to S_4 [4]. S_0 is the most reduced state, while S_1, S_2 and S_3 represent higher oxidation states. The oxygen molecule is released in the $S_3 \rightarrow S_0$ transition, in which S_4 is a transient state. In darkness, the S_0 and S_1 states are stable; S_2 and S_3 revert to S_1 in a few minutes. A multiline EPR signal, first reported by Dismukes and Siderer [5], provided the first evidence that Mn is involved in S-state transitions. Based on its dependence on the flash number, the multiline EPR signal has been assigned to the S_2 state. Another EPR signal at $g = 4.1$ is also associated with the S_2 state [6–8]. Recently, a broad EPR signal correlated with the S_1 state has been observed using the parallel polarization technique, indicating that the O_2-evolving complex in the S_1 state is also paramagnetic [9]. An EPR signal attributed to a formal S_3 state has also been discovered in Ca^{2+} inhibited membranes [10]. The 'Mn cluster' interacts chemically and magnetically with a component D, which in its oxidized form, D^+, gives rise to an EPR signal called Signal II_s [11]. D^+ has recently been shown to be a tyrosyl radical [12]. D in its reduced form can reduce at least the S_2 state [11,13,14], while D^+ during dark adaptation accepts an

Abbreviations: PS, Photosystem; Q_B, secondary quinone acceptor of PS II; Mes, (2[*N*-morpholino]ethanesulfonic acid; Taps, ([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)-1-propanesulfonic acid).

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electron from S_0 in a slow reaction [15]. The O_2-evolving complex is connected to the photochemical reaction center of PS II via a component Z which, when oxidized, has an EPR spectrum very similar to signal II_s but with fast reduction kinetics [16].

In the past, a variety of spectroscopic signals that correlate with the period 4 behavior have been reported; these include EPR, optical absorption, prompt and delayed fluorescence and thermoluminescence measurements. The period 4 oscillations detected in the fluorescence yield measured some milliseconds after each flash of a series have been reported to be correlated with the formation of the S_2 state [17]. It has been shown that when the time between the flashes of a series is varied, the oscillation patterns are characteristic of an S_2 state assuming S_2 and S_3 decay with half-times equal to 43 s and 77 s, respectively, at pH 6.5 [17]. Furthermore, the oscillation pattern of fluorescence yield has appeared very similar to the experimental oscillation pattern of the S_2 state multiline EPR signal reported by Styring and Rutherford [15]. These authors have shown that, after large multiline signals on the first and the second flash, the signals on the third and fourth flash are practically zero. This fact suggests that misses and double hits should be few, which was what we found in the centers responsible for the fluorescence oscillations [18,17]. Studies on the period 4 fluorescence oscillations have shown that fluorescence originates from a type of O_2-evolving center characterized by individual properties. For example, after each flash of a sequence, a small number of these centers become inactive and are not reactivated until dark adapted. This was explained by the amount of reduced plastoquinone molecules of the pool and the slow exchange between reaction centers, which prevent the complete reoxidation of Q_B in the doubly reduced state during the dark interval between the flashes of a sequence, 630 ms [17,19]. As previously stated, these centers are characterized by a low miss percentage (less than 1% under the best conditions in inside-out thylakoids), in contrast to the other type of O_2-evolving centers responsible for the greatly damped oxygen-yield oscillations. In this paper, we show that in these low-miss centers a large miss does occur, but only on the first flash of a series. This was found indirectly from the anomalous changes of the apparent S_0 and S_3 state fractions calculated in the dark. On the basis of these results it is suggested that during dark adaptation a structural change in the Mn site occurs which is removed by one flash.

Materials and Methods

Inside-out thylakoids were obtained by mechanical disintegration of pea chloroplast thylakoids, followed by phase partitioning according to Akerlund and Andersson [20]. 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. In order to partially depleted Cl^- from the inside-out thylakoids, the sample was exposed for 40 s to a Cl^--free medium of pH 8.5 (40 mM Taps, 300 mM sorbitol) and stored under Cl-free conditions at pH 6.5 (40 mM Mes, 300 mM sorbitol). PS II membranes can be depleted of Ca^{2+} by a treatment at low pH [21]. The sample was only suspended in a medium containing 300 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 40 mM succinate-NaOH buffered at pH 4.5.

Flash excitation was provided by a Stroboslave General Radio flash lamp (3 μs at half-peak height). Two identical Stroboslave flash lamps were used for the experiment described in Fig. 1B.

The rate electrode used for oxygen-flash yield measurements has been previously described [17]. The relative O_2 yield was measured at the maximum amplitude of the signal.

Fluorescence experiments were performed using the apparatus described in Ref. 19. The printing of the fluorescence decays (12 ms), from 73 ms after each flash of a sequence of 16 flashes were obtained, as well as the printing of the fluorescence yield values 80 ms after each flash, F_v. The fluorescence after darkness, F_0, previously measured was then subtracted from the fluorescence yield after each flash, F_v.

The fluorescence yield patterns induced by 16 flashes after a variable time of dark adaptation were fitted to fluorescence change values calculated according to the model of Forbush et al. [22] using varying values for misses α, double hits β and z, a term which was introduced [17,18] to take into account a loss of active centers after each flash of a sequence. z is the proportion of centers that remain active after each flash of a sequence. The oscillating fluorescence yield changes are assumed to be proportional to the S_1 state prior to the flash. In the model which simulates transition parameters on the first flash different from those on the other flashes of the sequence, the first flash fluorescence changes was equal to $S_1(0)$ multiplied by $(z - \alpha_1 - \beta^1)$ whereas the other flash fluorescence changes were equal to S_1 $(n-1)$ multiplied by $(z - \alpha - \beta)$ (n, flash number; α_1, β^1, miss on the $S_1 \rightarrow S_2$ transition and double hit, respectively, on the first flash).

The method used to find the appropriate values of α_0, α_1 (misses according to the Forbush et al. model) and β^1 on the first flash of a series, is based on the generalized form of the recurrence law of Forbush et al., which allows the calculation of the S-state concentrations after the $(n+1)$th flash from those after the nth flash [18]. The S_{0-3} concentrations after the first flash were obtained by the fitting of the experimental oscillation pattern of fluorescence yield with the first flash yield omitted. Assuming the S_{0-3} concentrations after 0

flash, we determined the miss and double hit values on the first flash only.

Results

Flash energy dependence

Fig. 1 shows that the flash pattern of fluorescence yield depends on the flash energy. However, the expected non-saturating pattern was not observed when the flash energy was decreased from 100% to 30%. The least-squares fitting of the experimental data reveals that the miss parameter, α, was small: around 0.02, 0.03, even with a relative flash energy of 30%. Apparently, the important change with flash energy occurs essentially on the first flash as suggested by the large decrease of the fluorescence yield on the first flash of

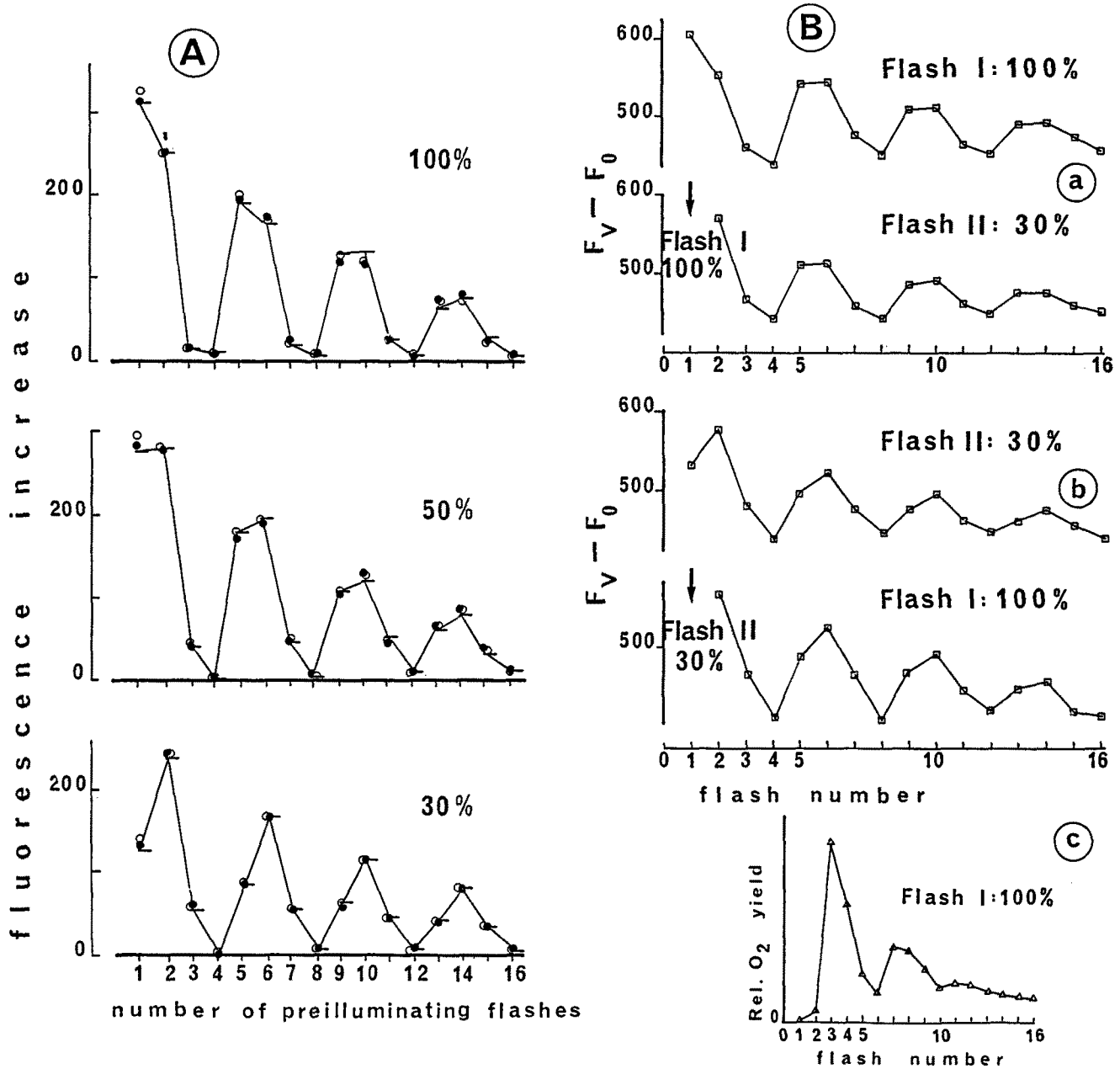

Fig. 1. Effects of flash energy on the experimental pattern of fluorescence yield, in dark adapted (10 min) inside-out thylakoids at pH 6.5 in the presence of 0.5 mM FeCy. The flash interval was 630 ms. Fluorescence yield was measured 80 ms after each flash of a series of 16 flashes. (A) The flash energy was uniformly attenuated from 100% to 30%. A constant level of fluorescence was subtracted from the fluorescence yield values. This level was ajusted to obtain the best fit. The fitting results are shown in Table I. Experimental data are represented by dashes, theoretical data by filled circles. Open circles indicate simulation of the fluorescence patterns assuming 25% S_0, 75% S_1 in the dark and different α_0, α_1, β^1 values (in Table I) on the first flash of the series. (B) The energy of the first flash differed from the energy of the subsequent flashes. (a) The energy of the first flash (Flash I) is kept at 100% and the energy of the subsequent flashes is reduced to 30% (Flash II). (b) The energy of the first flash is reduced to 30% (Flash II) and the following flashes are kept constant at 100% (Flash I). (c) Effect of the 100% flash energy (Flash I) on the oscillation pattern of the O_2 yield under similar conditions.

the oscillation patterns. In Table I, the distribution of S states after dark adaptation reported as a function of flash energy indicates an increase of the $S_0/(S_0 + S_1)$ ratio and of the S_3 fraction, whereas the S_2 fraction diminishes with the decrease of flash energy. A large dependence of the S_0 and the S_3 state fractions after dark adaptation (10 min) on flash energy was obtained, assuming that the fluorescence oscillations reflect the S_2 state concentration generated by each flash of the sequence. It is difficult to understand that the real S_0 fraction (and the S_3 fraction) after dark adaptation depends on flash energy. However, the apparent S fractions in the dark have actually been calculated under the assumption that the transition parameters are not a function of flash number. One possible explanation of this anomalous result, is a larger miss on the first flash than on the other flashes of the sequence, a miss which depends on flash energy. The calculated S concentrations in the dark were obtained with α close to 0 on all the flashes of a series. Therefore if a miss on the $S_1 \rightarrow S_2$ transition (α_1 according to the model of Forbush et al.) occurs on the first flash, the remaining $\alpha_1 S_1$ centers after this first flash cannot be considered as missing centers because $\alpha \simeq 0$, but as coming from S_0 before the first flash, i.e., after dark adaptation. Consequently, the calculated S_0 fraction will be larger than the real S_0 fraction after dark adaptation, and the apparent S_1 fraction smaller than the real S_1 fraction. Similarly, if on the first flash, a large miss occurs on the $S_0 \rightarrow S_1$ transition (α_0 according to the Forbush et al. model), the remaining amount of S_0 after the first flash will be considered as issuing from the S_3 concentration in the dark. Thus, a miss, α_0, on the first flash will increase the apparent S_3 concentration in the dark and decrease the S_0 one. On the other hand, a double hit on the first flash will increase the apparent S_2-state fraction in the dark, as found for a 100% flash energy. In order to show that the apparent change of the S_0 and S_3 state concentrations in the dark with flash energy can really result from greater misses on the first flash of the sequence, the experimental data were fitted to flash yields calculated assuming a different set of α_0, α_1, β^1 on the first flash only and S_0, S_1 in the dark (see Material and Methods for details). After a short dark-adaptation period (10 min), the distribution of S states is probably close to 25% S_0 and 75% S_1 in accordance with the model of Kok et al. [4]. Therefore in Table I, the S_0 and S_1 concentration in the dark are assumed to be the same whatever the flash energy and equal to 25%, 75%, respectively. As shown in Fig. 1 and Table I, the change of the fluorescence yield patterns with flash energy may be explained by increasing miss values α_1 and α_0 on the first flash of series when the flash energy is decreased. In spite of uncertainties about the real values of S_0 and S_1 in the dark, these experimental results show that during darkness a short time after light (10 min), a phenomenon occurs at the level of S_0 and S_1, possibly a structural change, which could be responsible for large misses mainly on the first flash of the sequence. If this assumption is correct, that the effect relates only to the first flash of the sequence within the flash energy range 100–30%, a given energy of the first flash should induce the same following oscillation pattern whatever the energy of the subsequent flashes may be. The results of such an experiment are shown in Fig. 1B. The energy of the first flash was kept at 100% and the energy of the subsequent flashes was reduced to 30% (Fig. 1Ba). Conversely, only the energy of the first flash was reduced to 30% (fig. 1Bb). The expected results were obtained. The oscillations of fluorescence yield showed no change in periodicity: as a function of flash number, the same oscillation pattern was repeated every four flashes with a decreasing amplitude. The change was induced by the first flash only.

During a long dark-adaptation period (2 h), the miss on the $S_1 \rightarrow S_2$ transition decreased on the first flash ($\alpha_1 = 0.19$ instead of 0.4 in Table II) while α_0, the miss on the $S_0 \rightarrow S_1$ transition remained constant. This result indicates that the dark-adaptation process which occurs in the S_1 state appears to be independent of that developed in the S_0 state.

Short dark adaptation time dependence

The oscillation pattern of fluorescence yield induced by 16 flashes was studied after one pre-flash and after three pre-flashes followed by a variable time of dark adaptation period, from 30 s to 10 min. Some of the oscillation patterns obtained are shown in Figs. 2(2) and

TABLE I

Effects of flash energy on the least-squares fitting results of experimental pattern of fluorescence yield (Fig. 1)

The samples were dark adapted for 10 min. z is the percentage of centers remaining active after each flash of the series. (a) The S_{0-3} concentrations in the dark directly calculated in %. (b) The S_{0-3} concentrations in the dark are assumed to be identical as a function of flash energy and equal to 25, 75, 0, 0%. The fitting of the experimental data were obtained with different transition parameters on the first flash only.

Relative flash energy (%)	z	α	β	A [a]				B [b]		
				S_0	S_1	S_2	S_3	α_0^1	α_1^1	β^1
100	0.91	0.025	0	45.2	51.5	2.7	0.6	0.067	0.254	0.033
50	0.91	0.030	0	49.1	44.7	0.5	5.7	0.259	0.366	$5 \cdot 10^{-4}$
30	0.92	0.020	0	57.9	28.7	0	13.4	0.518	0.556	0

TABLE II

Effects of the dark-adaptation time on the least-squares fitting results of experimental patterns of fluorescence yield (Figs. 2 and 3)

(a) The S_{0-3} concentrations in the dark directly calculated in %. (b) The fitting of the experimental data were obtained with different transition parameters on the first flash, α_0^1, α_1^1 and β^1, and expected concentrations S_0, S_1 in the dark. (c) The S_2 state concentration in the dark of 3% is assumed instead of a double hit on the first flash.

Dark-adaptation period	z	α	β	A [a]				B [b]				
				S_0	S_1	S_2	S_3	α_0^1	α_1^1	β^1	S_0	S_1
2 h	0.875	0.03	0	19.6	59.7	18.5	2.2	0.44	0.19	0.175	5.5	94.5
10 min. preceded by one flash	0.90	0.03	0	40.1	52.7	3.3	3.8	0.46	0.384	0 [c]	10	87
10 min. preceded by three flashes	0.925	0.06	0	38.7	43.3	0	18	0.58	0.376	0	30	69.5

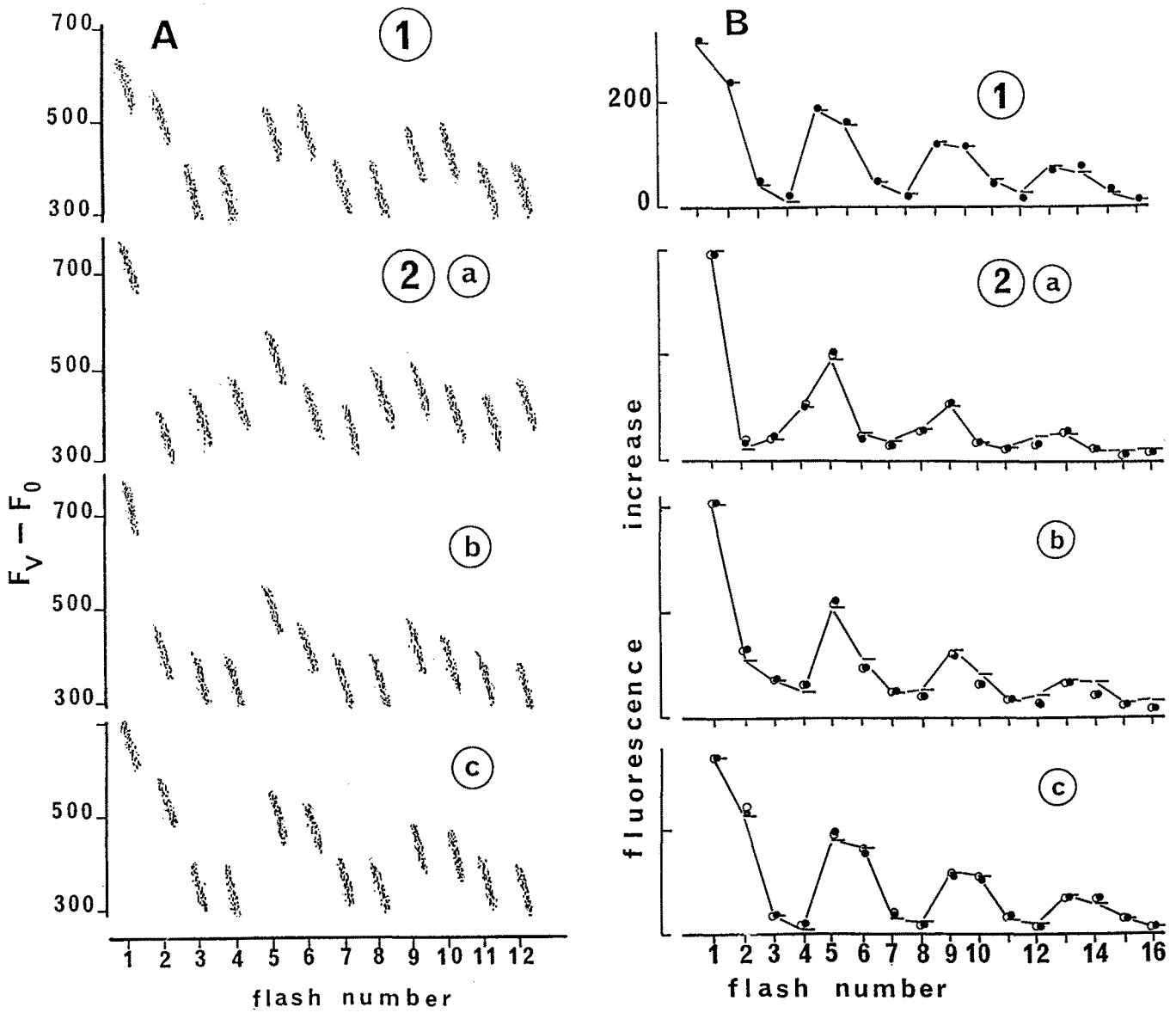

Fig. 2. Experimental fluorescence yield decays (from 73 to 85 ms) (A), and best fitting of the fluorescence yield measured 80 ms after each flash of a series of 16 flashes (B), after various dark adaptation conditions: (1) A series of 16 flashes followed by a 5 min dark adaptation period. (2) One preflash followed by variable dark-adaptation periods: (a) 30 s, (b) 2 min, (c) 10 min. 0.5 mM FeCy was added. The time interval was 630 ms. Experimental data in B) are represented by dashes, theoretical data by closed circles. The least-squares fitting yields: (1) $z = 0.91$, $\alpha = 0.03$, $\beta = 0$. (2) z varied from 0.86 (30 s) to 0.89 (10 min), $\alpha = 0.03$, $\beta = 0$. The calculated S_{0-3} concentrations in the dark are those in Fig. 4A. Open circles: simulation of the fluorescence patterns assuming S_{0-3} concentrations and different α_0 and α_1 miss values on the first flash of the series as in Fig. 4B.

3(2). The increasing of the dark adaptation period after one pre-flash, induced an important change in the ratio between the second fluorescence flash yield and the first, which is a qualitative measurement of the apparent S_0/S_1 ratio. The quantitative analysis of these oscillation patterns provides the apparent S state distribution just before the flash sequence. The miss percentage on all 16 flashes was found low: $\alpha = 0.03$. Fig. 4A shows the different changes of the calculated S states during the dark period following one pre-flash. The results seem difficult to explain. After one pre-flash, the apparent S_0 fraction which is small but not quite zero, decreased, then after 30 s of darkness (or 1 min depending on sample) increased rapidly up to 40%. The apparent S_3 fraction increased in the dark up to around 10% and then decreased. Only the decay of the S_2 state after one flash with a half-time of 45 s agreed with the experimental values already measured under the same conditions (Refs. 17,7 (in BBY)). One possible explanation of the strange results shown in Fig. 4A, is to assume the occurrence of misses only on the first flash of a series which depend on the dark time after one pre-flash. Misses α_1 and α_0 (according to the Forbush et al. model) on the first flash of a series only, lead to an overestimation of the S_0 and S_3 concentrations in the dark when the fitting method yields a miss close to 0, as explained previously. The fact that the apparent S_3 concentration increased up to around 10% in the dark after one pre-flash, which is unrealistic (Fig. 4A), indicates that on the first flash of the series an increasing

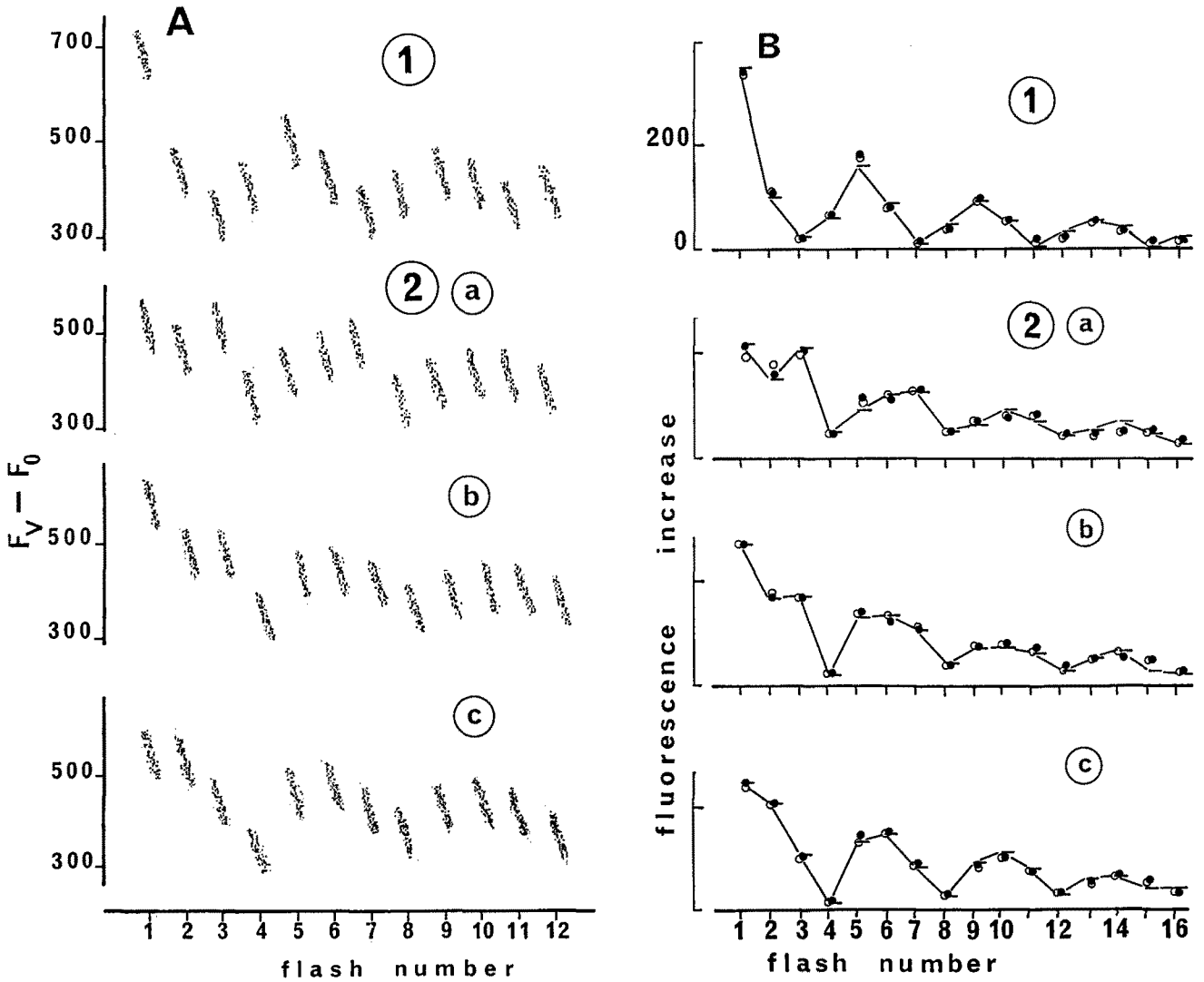

Fig. 3. Experimental fluorescence yield decays (from 73 to 85 ms) (A) and best fitting of the fluorescence yield measured 80 ms after each flash of a series of 16 flashes (B), after various dark adaptation conditions different from those in Fig. 2: (1) 2 h dark adaptation period; (2) three preflashes followed by variable dark-adaptation periods: (a) 30 s, (b) 2 min, (c) 10 min. 0.5 mM FeCy was added. The time interval was 630 ms. Experimental data in (B) are represented by dashes, theoretical data by closed circles. The least-squares fitting yields: (1) $z = 0.875$, $\alpha = 0.03$, $\beta = 0$. (2) z varied from 0.91 (30 s) to 0.925 (10 min), $\alpha = 0.06$, $\beta = 0$. The calculated S_{0-3} concentrations in the dark are those in Fig. 5A. Open circles: simulation of the fluorescence yield patterns assuming S_{0-3} concentrations and different α_0 and α_1 miss values on the first flash of the series as in Fig. 5B.

miss on the $S_0 \rightarrow S_1$ is probably responsible for the apparent S_3 state concentration in the dark, and that the true S_0 concentration after the pre-flash cannot be lower than this concentration (10%) in our sample (this may be the remaining S_0 concentration due to α_0 during the preflash). The different fluorescence patterns that followed one pre-flash were fitted with different values of α_0 and α_1 on the first flash and expected S_{0-3} values in the dark (in Fig. 2B(2) and Fig. 4B and C). A constant concentration of S_0 was assumed in the dark after one pre-flash because, apparently, the oxygen evolving complex undergoes conversions during the dark-adaptation process that populate the S_1 state (and not the S_0 state) in the dark [15]. As shown in Fig. 4C, it is likely that a miss α_0 on the first flash of the series increases progressively after one pre-flash up to its maximum value and then decreases after 5 min of darkness. At the same time, the other miss, α_1, does not increase immediately as α_0 and there is a lag of 30 s,

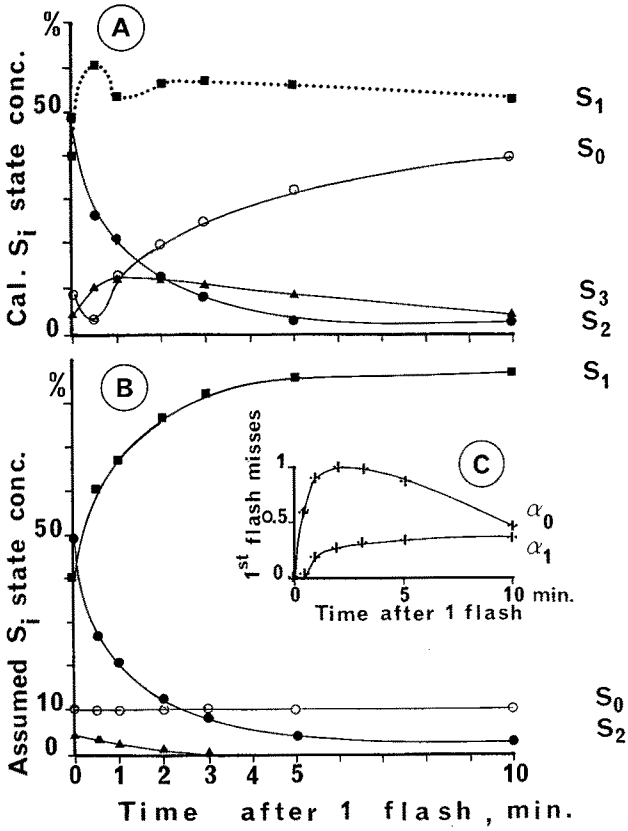

Fig. 4. Time-course of the S_{0-3} concentrations in the dark after one preflash. (A) The calculated S_{0-3} concentrations in the dark by fitting the oscillation patterns of fluorescence yield obtained a variable time after one preflash. (B) The expected S_{0-3} concentrations in the dark. The time course of the S_2 state remains similar to that in (A) with a $t_{1/2} = 45$ s. The S_0 concentration is assumed to be constant. The S_3 concentration decreases with a $t_{1/2}$ of 75 s. The S_1 concentration is equal to the complementary concentration of the other S states. (C) First flash misses, α_0 and α_1 as a function of time after one preflash assuming the S_{0-3} concentrations in (B). (S_0: open circles; S_1: squares; S_2: filled circles; S_3: triangles; α_1, α_0: crosses.)

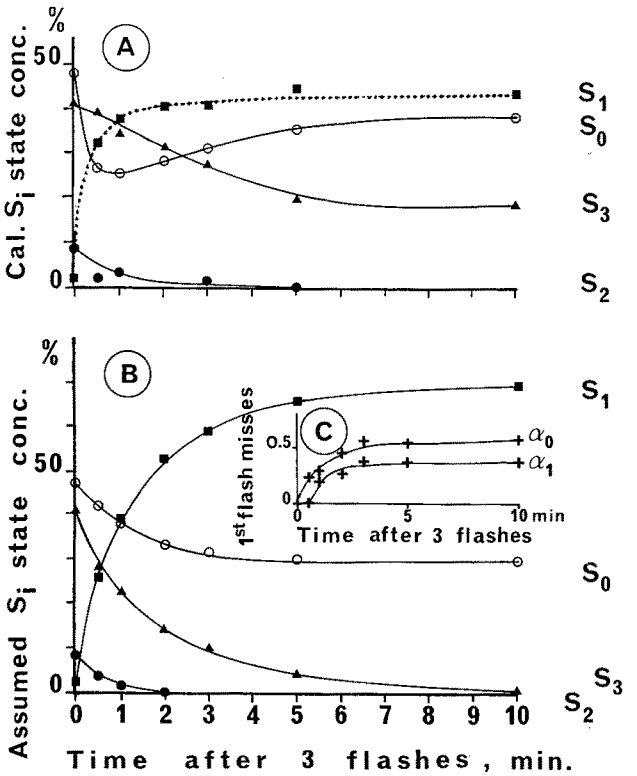

Fig. 5. Time-course of the S_{0-3} concentrations in the dark after three preflashes. As Fig. 4 except that three preflashes replace one preflash, and that the expected S_0 concentration in (B) is assumed to decrease to 70% with a $t_{1/2}$ of 50 s within the time range 0–10 min.

which is even longer in samples stored with ethylene glycol in liquid nitrogen.

The changes of the apparent S state concentrations after three flashes, in Fig. 5A, reveal almost the same anomalies as after one flash, i.e., essentially a decrease followed by an increase of S_0, and a large apparent S_3 concentration in the dark. Fig. 5B and C shows the expected real S_{0-3} state concentrations in the dark after three preflashes and the miss values on the first flash of the series α_0 and α_1 able to simulate the experimental oscillation patterns of fluorescence yield in Fig. 3B(2). The real S_3 state concentration is assumed to decay with a half-time equal to 75 s (value already found at pH 6.5 [15]). It has been shown that D^+ decays in the dark after three pre-flashes and that the subtrate of reduction of D^+ could be S_0 and that this is oxidized to S_1 in the reaction [15]. In PS-II-enriched membranes the decay half-time of SII_s is approx. 50 min after three pre-flashes [15]. On the other hand, short-term changes in S_0 after three pre-flashes have been recorded for its proton NMR relaxation properties [23]. The three flash relaxation rate response is a positive transient which decays with a half-time of approx. 50 s to a stable level. Such decay is assumed to be the same for real S_0 in inside-out thylakoids as shown in Fig. 5B. According to these assumptions, after three pre-flashes followed by a variable time of darkness (Fig. 5C), the misses α_0 and α_1 on

the first flash of a series increase as after one pre-flash, except that α_0 does not show a decrease after 2 min. This almost certainly occurs after any number of pre-flashes.

Fig. 5 also shows that the apparent S state concentrations in the dark calculated by the fitting method, may be very different from the real S state ones. So, the rapid decrease of the apparent S_0 concentration after three flashes (Fig. 5A) probably resulted from the progressive increase of a miss on the $S_0 \rightarrow S_1$ transition on the first flash of a series. It was only afterwards that a miss on the $S_1 \rightarrow S_2$ transition increased, on the same first flash of a series, inducing a slow apparent rise of S_0.

Chloride and calcium cofactor dependence

The S-state distribution in the dark was investigated in PS II centers that were inhibited by insufficient Cl^- or Ca^{2+} (see the decrease of the amplitude of the O_2 yields in Fig. 6C). As shown in Fig. 6A and B, Cl^- or Ca^{2+} depletion induced almost similar oscillation patterns of fluorescence yield. These oscillation patterns were relatively flattened as a consequence of the stabilization of the S_2 and S_3 state in the dark. A small

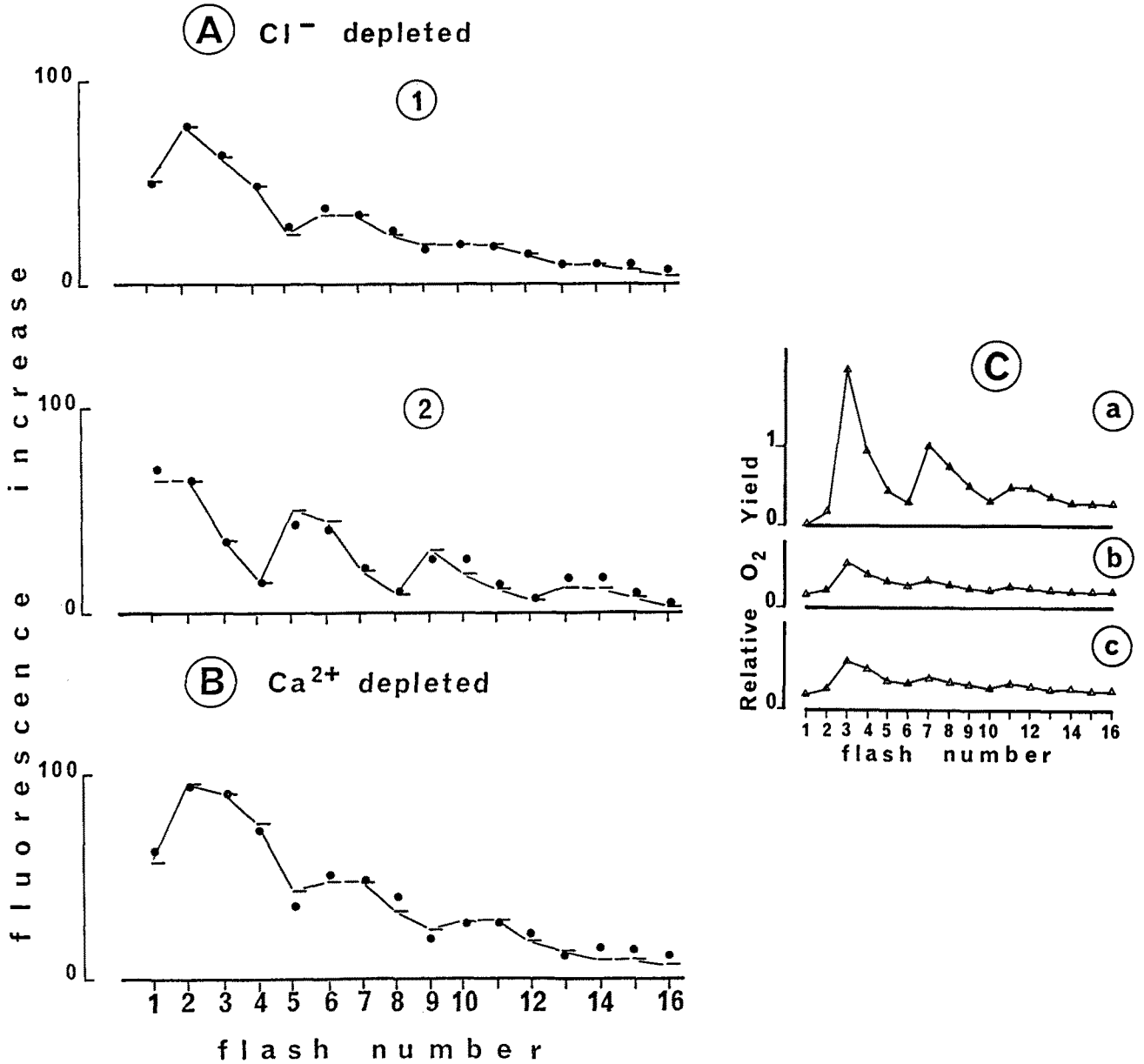

Fig. 6. Fitting of the oscillation patterns of fluorescence yield measured after each flash of a series of 16 flashes in the presence of 0.5 mM FeCy in Cl^- and Ca^{2+} depleted inside-out thylakoids. The sample was dark-adapted for 10 min. The flash interval was 630 ms. (A) Cl^--depleted sample: (1) without any Cl^- addition; (2) with 15 mM NaCl. (B) Ca^{2+}-depleted sample, at pH 4.5. The ordinate scale is extended with regard to Figs. 1, 2 and 3. Experimental data are represented by dashes, theoretical data by filled circles. The fitting yields: (A1) $z = 0.85$, $\alpha = 0.04$; (A2) $z = 0.89$, $\alpha = 0.01$; (B) $z = 0.86$; $\alpha = 0.01$. (C) The corresponding flash-induced O_2-yield pattern under similar conditions: (a) untreated inside-out thylakoids at pH 6.5; (b) Cl^--depleted; (c) Ca^{2+}-depleted.

apparent S_1 was found in the dark. This suggests that either the S_1 state is reduced to S_0 or, as already proposed above, the first flash of a series produces very few S-state advancements. In partially Cl^--depleted samples, the low level of fluorescence yield on the first flash can be increased after 15 mM NaCl addition (shown in Fig. 6A2). In Ca^{2+}-depleted samples, additional amounts of Ca^{2+} (20 mM $CaCl_2$) can also restore the original pattern observed without treatment (not shown). This suggests that the misses on the first flash depend on the presence of these cofactors. In Cl^-- or Ca^{2+}-depleted samples (Fig. 6A and 6B) the oscillation pattern is characterized by a larger than usual decrease in the amount of active centers after each flash of a series. Average z can be as low was 0.80 *. This result possibly reflects the inhibition of the advancement of the S state mechanism beyond S_2 [24] or S_3 (for Ca^{2+} depletion) [10] (maybe in an active form).

Discussion

A large apparent S_0/S_1 ratio in the dark was calculated under conditions of relatively low flash energy, Cl^- or Ca^{2+} deficiency. The occurrence of a large miss on the $S_1 \rightarrow S_2$ transition on the first flash of a series only, can explain this ratio by artificially increasing the S_0 concentration and decreasing the S_1 concentration in the dark. At pH 8.3, Plijter et al. [25] have observed that between one preflash and the measurement of flash-induced absorbance changes, by fitting the measurement they could obtain an apparent decrease of the percentage of reaction centers in the S_1 state. They have concluded that at pH 8.3, the S_1 state is reduced to S_0, though the change of the apparent S_1 concentration in the dark cannot be correlated with any redox change of D (the EPR signal II_s species). The assumption of a large miss on the first flash of a series, which is increased at pH 8.3 relative to that at pH 6.3, is an alternative proposal explaining the results of these authors. Renger and Hanssum [26] have already introduced the idea of a miss increase being responsible for the apparent increase of S_0 in the dark at alkaline pH. The inhibitory effect of alkaline pH on O_2 evolution may involve displacement of bound Cl^- by OH^- and thus, is probably induced by Cl^- depletion [27,28]. Ono et al. [24] have shown that the S_2-state multiline signal is not induced by illumination of the chloride-depleted particles, but that the signal is developed upon addition of chloride after illumination. This proves that there may be an inactive form of S_2 in the absence of chloride able to be converted to the active (modified) form in the presence of chloride in the dark. This has raised suggestions that in the Cl^--depleted samples and also in the untreated samples, the misses on the first flash can be explained by an alternative model to the model of Forbush et al. According to this alternative model, the miss can be due to the presence of an inactive S_i form in equilibrium with an active form of S_i [29,30]. The inactive S_i state after one flash may become an active S_i state after the following flash. After the first flash of a series, there may be an equilibrium between the active S_i state and the inactive S_i^* state, $S_{i-1} \rightarrow S_i^* \leftrightharpoons S_i$, and the miss would be equal to $\alpha_i = S_i^*/(S_i + S_i^*)$. According to this definition, the recurrence law is different from that of Forbush et al. [21]. The new recurrence law has already been published [30], and in its generalized form is:

$$S_i^{n+1} = (1 - \alpha_i)S_{i-1}^n + \alpha_i S_i^n \quad (n\text{: flash number}).$$

There are few changes in the formula with regard to that of Forbush et al. (essentially the miss number). Thus, it is possible that the misses α_0 and α_i according to the model of Forbush et al. were in fact α_0, α_1 and α_2, due to the presence of inactive forms of S_0, S_1 and S_2 respectively, after the first flash of a series. If this latter model is exact, the smaller amount of active S_2 fraction formed after the first flash (due to a proportion of inactive S_2) will be considered by the fitting method as issuing from a smaller S_1 concentration than the real S_1 in the dark. Consequently, the apparent S_0 and S_1 fractions in the dark are higher and lower than the real S_0 and S_1 fractions, respectively, as when the model of Forbush et al. is assumed.

Our results show that in the PS II centers studied, the mechanism of the S-state advancement is identical on each flash of a series except on the first flash. This excludes a possible miss α_3 (due to a proportion of inactive S_3 centers in equilibrium with the active S_3) after the second flash. However, this does not exclude the possibility that some S_3 centers remain inactive till after the flash sequence.

Beck et al. [31] have reported that the S_2 state multiline EPR signal intensity produced by illumination at 200 K after 6 min incubation in the dark at 0 °C is weak. Samples illuminated at 200 K after longer periods of dark adaptation produce much more intense S_2 state EPR signals. Despite a different dependence on illumination between the 6 min and 4 h dark-adapted samples which increase the difference in amplitude between the two different multiline signals when illumination occurs at 200 K, these authors clearly show that the 6 min dark-adapted samples produces a lower signal

* Since Cl^- and Ca^{2+} depletion affects a specific transition, a specific z for this transition should be used for the fitting of the data. This has been neglected because the S state concentrations oscillate slightly as a function of flash number (due to S_2 and S_3 in the dark). After each flash, the inhibition of all transitions in 10% centers is almost equivalent to the inhibition of a specific transition in 40% centers.

than the 4 h dark-adapted samples (in their Fig. 2). Therefore, our results can be correlated with theirs, as we found the same dependence on the length of dark adaptation of one of the miss on the first flash (Table II). They have also observed a different line-shape of the S_2 state multiline signal in the 6 min dark-adapted sample, characteristic of a modified S_2 state. These results can be accounted for if the O_2-evolving complex undergoes a structural reorganization during dark adaptation, affecting the properties of the S_0 and S_1 states [31]. In this paper, it is shown that this structural change is completely reversed by one flash. This reversion can take place partially on the first flash of a series provided that the flash energy is sufficiently high (Fig. 1). Beck et al. [31] have correlated the change detected in the Mn site that occurs during the dark adaptation period, with a change in the O_2 consumption activity of PS II. These authors have proposed that the O_2-evolving complex undergoes a conversion in the dark from an active state able to catalyse the reduction of O_2, to a resting state incapable of O_2 consumption. The reduced quinone, QH_2, may serve as the electron and proton donor to O_2. According to the results given in this paper, the terms 'active' and 'resting' state are ambiguous, as some active state centers for O_2 consumption in the dark (possibly S_1) appear to be converted into either the active modified S_2 or the S_2 inactive form after one flash. During darkness, the progressive structural changes in the S_0 and S_1 state centers are probably responsible for the slow change in the amount of the S_0, S_1 and S_2 inactive centers formed after the first flash of a series (according to the model of Ref. 30).

We show that the misses on the first flash depend on the Cl^- and Ca^{2+} cofactors. This is not surprising, as Cl^- and Ca^{2+} depletion induce effects on the S_2 state which are also of structural nature and which can be overcome by some physical factors, such as illumination temperature in the case of Ca^{2+} depletion [32].

The period 4 oscillations of fluorescence yield provide insight into the water splitting activity. However, the properties of the O_2-evolving centers reported in this paper make little contribution to O_2 yield data. As an example, a very small dependence of the apparent S_0 state fraction in the dark on flash energy has been found in O_2 yield data (Table I in Ref. 18). This supports the view that O_2 evolution is heterogeneous and that the O_2-evolving centers studied in this paper are fewer than the other centers. The miss increase on the first flash reported here appears to be hardly discernible in O_2-yield data. As already shown [17,18], most of the O_2-evolving centers described from O_2 yield data are characterized by important misses after each flash of the series (which induce a phase retardation in the period 4 oscillations), possibly arising from $S_2 \rightarrow S_3$ [33] and a steady-state O_2 yield after many flashes. These properties are absent in the O_2 evolving centers responsible for the period 4 fluorescence oscillations, essentially defined by a proportion of inactivated centers (10%) after each flash of a series until dark-adapted. On the basis of this observation, one may speculate that a local electric field, built up with the S_2 state, could affect some of these centers, resulting in their inactivation, as suggested by Joliot and Diner [34].

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