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Regulation of thermal dissipation of absorbed excitation energy and violaxanthin deepoxidation in the thylakoids of *Lactuca sativa*. Photoprotective mechanism of a population of photosystem II centers

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Abstract

Non-photochemical quenching of chlorophyll *a* fluorescence is thought to be mainly associated with thermal dissipation of excitation energy taking place within the antenna and reaction center of PS II. In this report, non-photochemical fluorescence quenching was investigated in the fluorescence yields induced by a series of short and high-energy flashes after dark adaptation. The observation of period four fluorescence oscillations with increasing flash number indicates functioning O_2 evolving centers. It was found that these PS II centers could not be identical to all the O_2 evolving centers. Appreciable differences in antenna size and the number of centers were observed between the PS II centers contributing to fluorescence oscillations and the PS II centers that evolve the flash-induced steady-state O_2 yield. Direct evidence for non-photochemical fluorescence quenching was provided by the numerical fitting of the fluorescence oscillations. This procedure revealed that a proportion of the centers exhibiting oscillating fluorescence yields, converted into quenching centers after each flash of a series (7% in February; 17% in June). The observed quenching could not be related to a dissipative process inside the reaction center. Instead, it was attributed to a change in the organization of some PS II centers in the membrane, possibly a conversion of PS II dimers into PS II monomers, resulting in a decreased absorption cross-section for these centers. Quenching resulting from energy de-excitation in the antenna was also observed. This was a light-initiated process, but the modification of the antenna occurred in the dark on a time scale of a few minutes. After this dark period and only on the first flash of a series, antenna quenching was revealed by a smaller absorption cross-section of the PS II centers involved in fluorescence oscillations. This process was reversed on the following flashes. The same period of darkness after illumination was necessary to allow maximum zeaxanthin formation to occur in the dark at a higher pH than the pH for optimum violaxanthin deepoxidation in the absence of preillumination. To explain this effect, comparable to that referred to as light activation for non-photochemical quenching (Ruban and Horton, Aust. J. Plant Physiol. 22 (1995) 221–230), we propose that upon preillumination (before darkness), the protons released in response to a net positive charge in these PS II centers, have access to proton binding groups acting in a cooperative way in LHC II. This accounts for the proton

Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCCD, *n*,*n*′-dicyclohexylcarbodiimide; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; LHC II, light-harvesting chlorophyll *a*/*b* antenna of photosystem II; PS, photosystem; Q, plastoquinone

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cooperativity as can be deduced from the pH dependence of the rate constant of violaxanthin deepoxidation (Hill coefficient n from 2 to 6).

Keywords: Photosystem II; Oxygen evolution; Chlorophyll fluorescence; Nonphotochemical quenching; Zeaxanthin; Photoprotection

1. Introduction

The photosystem II (PS II) reaction center in higher plants is susceptible to photoinhibitory molecular damage of its components upon exposure to excess light. It is generally accepted that the main mechanism that protects PS II against such damage consists of dissipation of the excess absorbed light energy in the form of heat, accompanied by a decline in chlorophyll (Chl) *a* fluorescence yield called the non-photochemical quenching of chlorophyll fluorescence (for reviews, see Refs. [1,2]). The dissipation of excitation energy has been proposed to be localized, either in the reaction center [3–5], or in the light-harvesting chlorophyll *a/b* antenna of PS II (LHC II) prior to trapping in the reaction center [6,7]. This process was particularly correlated with the conversion of special pigments of the xanthophyll cycle [8,9].

On the one hand, results on non-photochemical quenching of variable Chl *a* fluorescence induced by low pH in PS II-enriched thylakoid membranes, suggest a mechanism for non-photochemical quenching based on direct quenching by $P680^+$ inside the reaction center [5]. On the other hand, the effective antenna size of PS II is believed to be actively regulated. Two factors, the acidification of the lumen (low pH) and the formation of zeaxanthin by deepoxidation of the carotenoid violaxanthin, appear to promote energy dissipation in LHC II, i.e., the antenna of PS II. The LHC II proteins can be manipulated in vitro, so as to give rise to fluorescence quenching, and it has been proposed that non-photochemical quenching occurs by the same mechanism in the LHC II in vivo [10]. Protonation of LHC II complexes in vitro, leads to dramatic changes in protein–protein interactions, resulting in the aggregation of these complexes. The spectroscopic changes accompanying such aggregation have been reported to have the same features as those observed during the induction of non-photochemical quenching in thylakoids and leaves [11–13]. It has been inferred that a reorganization of the light-harvesting pigment–protein complexes is activated by the protonation of specific amino acid residues [14].

Evidence has also been obtained to support the view that the xanthophyll cycle carotenoids exert control over LHC II structure [11,15,16]. It was found in vitro that zeaxanthin stimulated the formation of LHC II aggregates with reduced fluorescence yield, whereas violaxanthin caused the inhibition of such aggregation and an elevation of fluorescence. In vivo, the xanthophyll cycle consists of the conversion of the diepoxide compound violaxanthin to the epoxide-free zeaxanthin, with anteraxanthin as the intermediate, and of the back reaction via an independent pathway. It is probably completely contained within LHC II, possibly in the minor complexes [11,17]. The deepoxidase reaction is light-dependent under physiological conditions, but can be driven in the dark at acidic pH [18–20]. This observation represents an important step in the comprehension of the regulation of the violaxanthin deepoxidation reaction.

Since much of the total non-photochemical quenching is observed during induction of photosynthesis [21], the origin of quenching was investigated by a series of saturating flashes after dark adaptation. The flashes were short enough to elicit only single turnovers of the PS II centers. Under a flash series, the photosynthetic system is susceptible to light-induced damage or photoinhibition as under continuous moderate light intensity. The question of concern herein is whether non-photochemical quenching is observed in the changes of the flash-induced fluorescence yield with increasing flash number during photosynthetic induction. If this is the case, is there a relationship between non-photochemical quenching induced by flashes and the photoprotective response associated with the deepoxidation of violaxanthin to zeaxanthin?

It is generally considered that the fluorescence yield is mainly determined by photochemical quenching mechanisms. The maximum unquenched level (F_m) observed under a continuous saturating light, is

related to the closure of the PS II centers, i.e., the reduction of the primary quinone electron acceptor Q_A. F_m may be up to five times as high as the minimum quenched level (F_0), when the PS II centers are open. By a series of saturating single turnover flashes given to dark-adapted chloroplasts, it has been revealed that active O_2 evolving centers could give rise to fluorescence oscillations with period four, instead of quenching fluorescence [22–25]. This observation shows that for the same increase in fluorescence yield, the PS II centers may be in different states: either the PS II centers are closed (or inactive) containing Q_A^-, or the centers are active and participate in the transitory increase of the period four fluorescence oscillations. The oscillations indicate that the probability of photon excitation trapping is decreased in these active centers for some S state(s). It has been reported that the patterns of fluorescence yield and electrochromic absorbance transients were oscillating in a similar way with period four [26]. Since the fluorescence yield shows a susceptibility to an external electrical field [27,28], both measurements were related to the effect of a local electric field induced by the creation of a non-compensated positive charge in the water-oxidizing complex during the S_1–S_2 transition [29–31]. However, comparison of the oscillation patterns of the oxygen yield and fluorescence yield under the same conditions of flash energy and adaptation to dark, has revealed two different period four behaviors [23,24]. The different oscillations were intrinsically not the same, and could not be described by the same transition parameters: misses, double hits, etc. The damping of the O_2 yield oscillations was essentially due to misses, which introduce a phase delay in the oscillations without a change in the total number of active centers. In contrast, the fluorescence oscillations were characterized by an exact periodicity of four, and a progressive decrease in the total number of the centers implicated in these fluorescence oscillations [23]. This has led us to suggest that the O_2 evolving centers contributing to fluorescence oscillations may not be strictly identical to most of the O_2 evolving centers [23–26].

It is well established that plants have the ability to adapt their photosynthetic apparatus to different light environments [32,33]. At low irradiance, thylakoids apparently have larger photosynthetic units with more LHC II complexes, and fewer PS II reaction centers to maximize light-harvesting, while at high irradiance, there are more PS II reaction centers, with less LHC II complexes, and smaller antennae to minimize the deleterious effects of photoinhibition. Here, studies on thylakoids extracted from differently acclimatized lettuces show that the PS II centers exhibiting fluorescence oscillations are characterized by a large antenna size. These PS II centers did not follow the same adaptation to light environment as the other PS II centers. To explain non-photochemical fluorescence quenching observed after each flash of a series, we present a new mechanism that significantly contributes to the protection of these PS II centers without increasing thermal dissipation of absorbed excitation energy.

2. Materials and methods

2.1. Plant material and chloroplast isolation

Chloroplasts were extracted from market cabbage lettuce (*Lactuca sativa* L.). Forty grams of leaves were washed in ice-cold water and homogenized in a 100-ml grinding medium that contained 400 mM sorbitol, 5 mM $MgCl_2$ and 100 mM tricine/NaOH (pH 7.8) and 0.5 g/l of BSA. The slurry was filtered through a nylon mesh (20 μm) and then spun down at $1000 \times g$ for 10 min. The pelleted chloroplasts were resuspended in 10 mM NaCl and 5 mM $MgCl_2$, pH 6.5, and centrifuged at $3000 \times g$ for 10 min. The final pellet was diluted with 300 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 40 mM Mes/NaOH buffered at pH 6.5, yielding a stock solution with a chlorophyll concentration of about 2 mg/ml. The stock solution was stored on ice. Aliquots could be used for measurements during 5 h without appreciable ageing. For some experiments, the stock solution was dark-adapted for 3 h on ice.

2.2. Flash-induced oxygen and fluorescence yield measurements

Oxygen flash-yields were measured as previously shown [24] with a rate electrode [34]. The amperometric signal was differentiated electronically, and the amplitude of the spike of the resulting signal was taken proportional to the O_2 yield. The optical den-

sity of the layer was found equal to 0.17 in the red (680 nm) for a concentration of 500 μM Chl.

Fluorescence experiments were performed using the apparatus already described [24]. The Chl *a* fluorescence yield was measured with an optical path length of 2 mm and a Chl concentration of 50 μM. A red 2-64 Corning filter was placed before the photomultiplier tube (EMI 9558-B). Detecting light was provided by electroluminescent diodes (Hewlett Packard HLMP 3519), emitting very weak green light. In order to amplify the fluorescence signal $F_V - F_0$, the fluorescence yield after darkness F_0 was subtracted by offset. For this work, the fluorescence yield was measured either 80 ms or 800 ms after each flash of a sequence of 16 flashes. We could obtain the printing of these values, as well as the printing of fluorescence decay (of 12 ms), either from 73 ms or from 793 ms after each flash.

Flash excitation was provided by Stoboslave General Radio flashlamps (3 μs at half-peak height).

2.3. Violaxanthin deepoxidation

Violaxanthin deepoxidation was measured as the absorbance change at 505 nm, with 540 nm as the reference wavelength, in the dual-beam mode with an Aminco DW-2 spectrophotometer [35,19]. The band width of the measuring light was 3 nm. The Chl concentration was 10 μM. Prior to the measurements, 4 μM nigericin was added to increase the membrane ionic conductivity. Deepoxidation reactions were initiated in the dark by the addition of 30 mM sodium ascorbate. In isolated chloroplasts, the addition of ascorbate, which is presumably lost during isolation, is required as an essential and specific reductant for deepoxidase activity [18].

Two types of light treatment were used, either flash excitation from the same flashlamps as for O_2 and fluorescence measurements, or continuous light. In some of the experiments, light from a projection lamp was passed through a red glass filter, and guided by fiber optics to the cuvette.

The absorbance change at 505 nm can be used as a quantitative measurement for the violaxanthin to the zeaxanthin conversion. A linear relation between the ΔA_{515} values and the formation of antheraxanthin and zeaxanthin has already been observed [36]. Antheraxanthin formation, associated with a lower 505 nm change compared with complete deepoxidation [36,37], is responsible for minor deviations from this proportionality [17].

The absorbance difference at 505 nm as a function of time, $\Delta_{505}(t)$, were analysed in terms of the asymptote value, $\Delta_{505}\infty$ and the first-order rate constant ^1k. These parameters were estimated by fitting a single exponential curve to the actual data points. The fitting curves of the plot of ^1k versus pH were calculated according to the following equation: $^1k = [1 + 10^{n(\mathrm{pH} - \mathrm{p}K)}]^{-1}$, in which the activity of the violaxanthin deepoxidase is related to the protonation of the enzyme with n equivalent groups that can be deprotonated in strict cooperativity (n is the Hill coefficient).

2.4. Curve fitting

We used the function of Mathcad plus 6.0 *genfit* for performing regression. Using a nonlinear least-squares minimisation method, this function generates a curve so that the sum of squares of errors between itself and the data supplied is minimized.

The O_2 and fluorescence yield patterns induced by 16 flashes after dark adaptation were fitted to calculated patterns on the basis of a generalized form of the Kok model [23]. Misses (α), double-hits (β) and the z factor were determined by the same least-squares fitting method. The z factor is a term that has been introduced to take into account a loss of active centers after each flash of a sequence; z is the proportion of the centers that remain active after each flash of a sequence ($z < 1$ may indicate that there is no more active centers after a large number of flashes). For the fluorescence yield patterns, z is the proportion of the centers that remain involved in the fluorescence oscillations after each flash. Coefficients σ's [38] of the characteristic equation (giving the eigenvalues of the transition matrix between the S states after each flash) as a function of α, β and z [23] were used in the best fit.

3. Results

3.1. Properties of the oxygen evolving centers related to fluorescence oscillations

We used thylakoids extracted from lettuce leaves collected from mid-February to mid-July 1996. Sev-

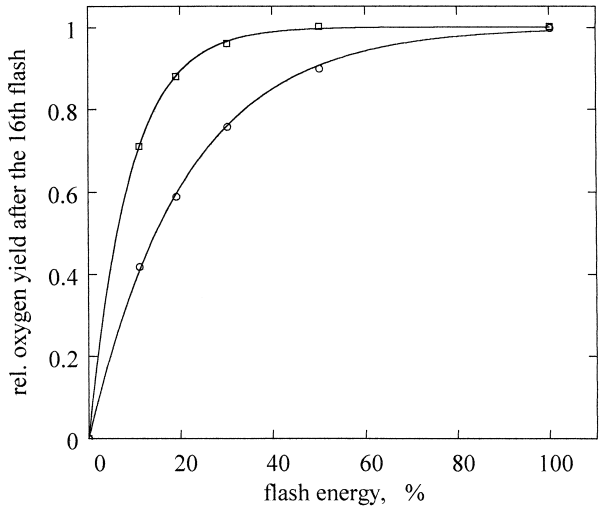

Fig. 1. Flash saturation curves of the oxygen yield after the 16th flash of a series of flashes from lettuce thylakoids extracted in February (squares) and in June (circles). The flash energy was varied by using neutral density filters. The sample was dark-adapted for 2 min before flashing. The flash spacing was 730 ms. The maximum steady-state O_2 yield values from both thylakoids are normalized to unity and the curves represent the fit of experimental data to a single exponential function: $(1-\exp(-I/I_{sat}))$. The characteristic saturation energy I_{sat}, which corresponds to $(1-1/e)$ of the maximum O_2 yield, was 9% flash energy in February and 21% flash energy in June.

eral series of short (half-height: 3 μs) and high-energy flashes spaced 730 ms apart were applied. Fig. 1 shows that the flash saturation curves of the O_2 yield measured after the 16th flash of a flash series in a 2-min dark-adapted sample, varied widely when differently light-acclimatized thylakoids are compared. It also reveals a more than two-fold smaller antenna size or absorption cross-section of the PS II units in thylakoids extracted in June than in thylakoids extracted in February, because the absorption cross-section (σ) is proportional to the reciprocal of the saturation energy I_{sat} ($\sigma = 1/I_{sat}$; see Fig. 1 caption). This is consistent with the well-established fact that photosynthesis of shade- or low-light acclimated plants becomes saturated with lower light levels than photosynthesis of sun or high-light acclimated plants [8]. Fig. 2 shows the pattern of the flash yield of O_2 evolution after a 2–3 h dark adaptation period in thylakoids extracted in February and in June. In the low-light grown thylakoids, we observed a proportionally higher O_2 yield on the third flash and less damping in the oscillations. The steady-state yield of flash-induced O_2 evolution reached a level that was at least two-fold higher in the summer samples than that in the winter ones, as would be expected from Fig. 1, on a chlorophyll basis.

Fig. 3 displays the period four oscillations of the flash-induced fluorescence yield in lettuce chloroplasts successively extracted in February, April, May and June. The fluorescence yields were measured 80 ms after each flash of a series, at pH 6.5, after a dark

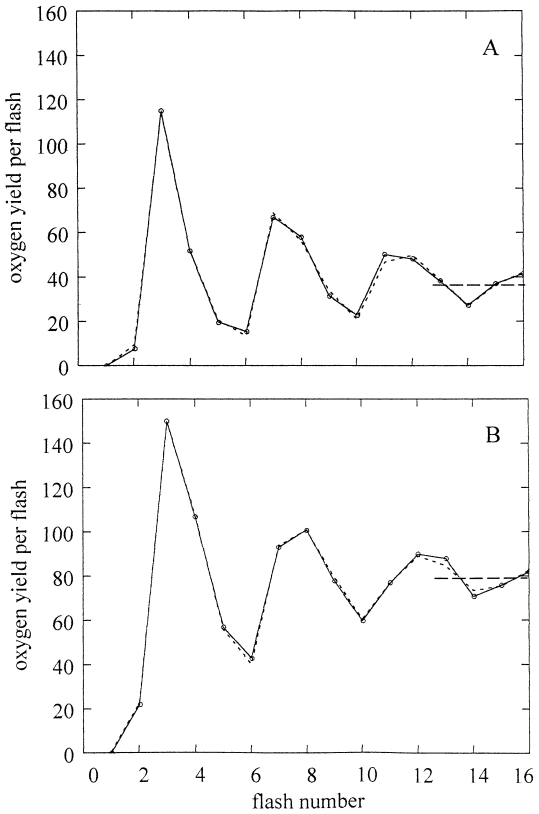

Fig. 2. Flash-number dependent oxygen yield in lettuce thylakoids extracted in February (A) and in June (B), at pH 6.5. Full line: experimental results; dotted line: best fitting simulation according to the hypothesis of unequal misses [39]. The sample was dark-adapted for 2–3 h. The flash interval was 730 ms. The extent of the steady-state O_2 yield is indicated by a dashed line. Assuming a unique miss on one of the S states α_3, double hits β and variations in z (proportion of the centers remaining active after each flash of a sequence), the results of the fit are: $\alpha_3 = 0.4$, $\beta = 0.009$, $z = 0.983$ in February, $\alpha_3 = 0.518$, $\beta = 0.001$, $z = 1$ in June (dotted line). Assuming the same miss on each S state, the results of the fit yield: $\alpha = 0.118$, $\beta = 0.027$, $z = 0.984$ in February; $\alpha = 0.163$, $\beta = 0.037$, $z = 1$ in June.

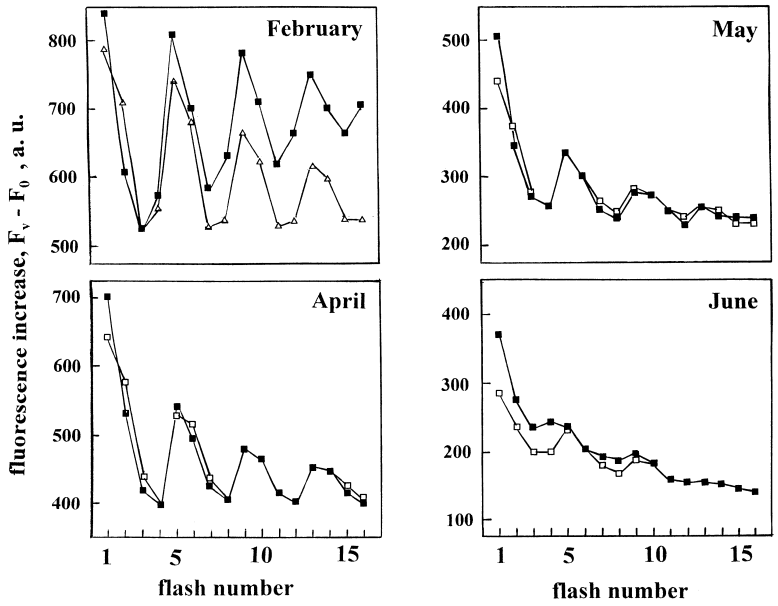

Fig. 3. Flash-number dependent Chl *a* fluorescence yield, F_V–F_0, measured 80 ms after each flash of a series, in lettuce thylakoids extracted in February, April, May and June, in the presence of 0.5 mM ferricyanide, at pH 6.5. The flash interval was 730 ms. The sample was either dark-adapted for 2–3 h before the flash series (filled squares), or dark-adapted for 5 min subsequent to a flash series (open squares), in the presence of 10 μM DCBQ (open triangles). F_0 measured around 20,000 arbitrary units (a.u.). Assuming misses α, double hits β, and variations in z (proportion of the centers remaining involved in the fluorescence oscillations after each flash of a sequence), the results of the fit for the oscillating part of fluorescence yield are $z = 0.93$, $\alpha = 0.01$, $\beta = 0$ in February; $z = 0.87$, $\alpha = 0.02$, $\beta = 0$ in April; $z = 0.83$, $\alpha = 0.03$, $\beta = 0$ in May; and $z = 0.83$, $\alpha = 0.03$, $\beta = 0$ in June.

adaptation period. It was necessary to add ferricyanide and sometimes DCBQ, to ensure the reoxidation of Q_A^- between the flashes. Otherwise, without the addition of these electron acceptors, the fluorescence level would progressively and markedly increase after each flash, because the flash interval (730 ms) was short compared with the reoxidation of Q_A^- in some PS II centers, thus, enlarging the proportion of closed-to-open PS II centers after each flash [24]. The effect of ferricyanide and DCBQ was to increase the rate at which Q_A^- is oxidized in a proportion of active centers [40].

As typically shown in thylakoids extracted in February in the presence of DCBQ and in April (Fig. 3), above the dark-adapted fluorescence yield F_0 level, the period four oscillations appear as if they were raised on a pedestal from the very first flash of the series, 80 ms after each flash. The difference between the minimum fluorescence level reached after the fourth flash and the dark-adapted level, F_0, was attributed to a constant amount of Q_A^- or 'inactive centers' [40]. Q_A^- at inactive centers is blocked possibly by obstruction of the Q_B pocket [40]. On the basis of similar fluorescence and 520 nm absorbance change kinetics following saturating flashes, it has also been suggested that the transmembrane electric field in the milliseconds to the seconds range could control the conversion of active PS II centers into inactive centers [41]. The number of 'inactive centers' was markedly reduced (80 ms after each flash) in June compared with the February results (Fig. 3).

The flash-induced fluorescence oscillations, either in low-light or in high-light grown thylakoids, were very characteristic. The oscillation period, exactly four here, was too short for the damping to be explained by misses. Misses introduce a delay in the phase of oscillations, which results in a periodicity becoming higher than four, as it is generally observed in the flash-induced O_2 yield oscillations (Fig. 2), even in the presence of ferricyanide [26]. In thylakoids extracted in February (with DCBQ) and in April (Fig. 3), the minimum fluorescence yields at flash 4, 8, 12 (or 3, 7, 11) were observed to be constant and the maximum at flash 1, 5, 9 to decrease

with increasing flash number. This cannot be explained by only misses and double hits that lead to a mean steady-state value with increasing flash number. The decreasing mean value of the fluorescence oscillations during a flash sequence indicates a progressive vanishing of the centers implicated in fluorescence oscillations after each flash. Previously [23–26], these oscillations have been described by a small proportion of active centers becoming inactive after each flash (called $1-z$), and remaining non-functional (i.e., being no longer involved in the fluorescence oscillations) until dark-adapted. In this model, the inactivity or non-functionality of these centers only refers to the ability to produce fluorescence oscillations. The non-functional centers are in a fluorescence state, so that the observed fluorescence is equal to that of the baseline or minimum level of the fluorescence oscillations at the end of the oscillations (after many flashes). This low fluorescence state is the same as that of the O_2 evolving centers without fluorescence oscillations. Thus, this model does not imply that the centers that vanished from the fluorescence oscillations after each flash of a series, are inactive for photosynthesis. The fitting method already presented [23] was applied to the fluorescence oscillations in Fig. 3. Slightly shifted constant baselines were tested until the best fit was obtained for the oscillations. The best fit yielded miss (α) and double hit (β) values close to zero, and a z value equal to 93% in thylakoids extracted in February, 83% in thylakoids extracted in May and June (as example of best fit, see Fig. 4). z is the proportion of the PS II centers remaining involved in the fluorescence oscillations after each flash.

As it has already been explained [23], in our fitting procedure, the parameters α, β, and z do not depend on the weight or contribution of each S state in the oscillating part of the fluorescence yield. We used the linear recurrence relation between the successive experimental oscillating fluorescence yields (F_1, ..., F_{16}) and the constant coefficients called the σ's [38]. These coefficients are a function of the parameters α, β and z, and the iterative fitting procedure gives these coefficients using the σ's. For this reason, we did not use either the initial values of the S states in the dark, or the S state dependent weights. This method essentially fits the shape of the oscillations: damping ($\alpha+\beta$), period ($4(1+\alpha-\beta)$) and mean amplitude decrease (z). The quantitative outcome of the fit was also found independent of the time between flash and fluorescence measurement, when this time is increased, as shown later in this paper.

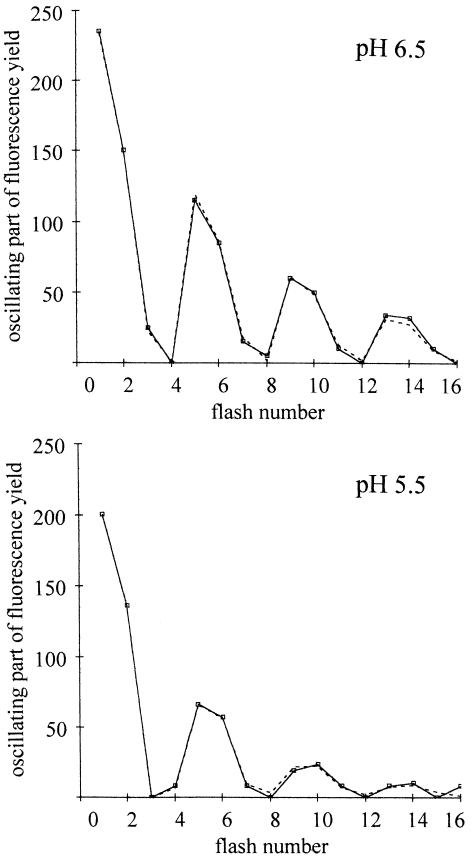

Fig. 4. Oscillating part of the flash-number dependent Chl *a* fluorescence yield, F_V-F_0, measured 80 ms after each flash of a series, in thylakoids extracted in May at pH 6.5 and pH 5.5 in the presence of 0.5 mM ferricyanide. The sample was dark-adapted for 5 min. The flash interval was 730 ms. The dotted line is the best fit. The fitted variables are $z=0.866$, $\alpha=0.02$, $\beta=0$ at pH 6.5; $z=0.80$, $\alpha=0.04$, $\beta=0$ at pH 5.5.

Thus, on the basis of the best fitting analysis of the fluorescence oscillations, we show that a proportion of quenching centers was formed from the PS II centers exhibiting oscillating fluorescence yields, after each flash of a series. This proportion changed from 7% in February to 17% in June (Fig. 3). Thus, the lumenal pH, which is probably lower in June than in February, could influence the proportion of quenching centers after each flash of a series. A more definitive proof of this point was obtained in thylakoids extracted in May at pH 6.5 and pH 5.5. In

this case, as indicated by the numerical fitting of the fluorescence oscillations in Fig. 4, when the pH was decreased, there was indeed an increase in the proportion of quenching centers after each flash of a series ($1-z=13.5\%$ at pH 6.5, 20% at pH 5.5). It must be noted that in thylakoids extracted in June (Fig. 3), the fluorescence oscillations were accompanied by a progressive decline of the baseline. This probably reflects the discharge of the membrane potential due to ion fluxes across the membrane [42] in leaky thylakoids.

From the flash-induced steady-state O_2 evolution in Figs. 1 and 2, we conclude that there were at least twice as many O_2 evolving centers in June as in February. As shown in Fig. 3, the size of the oscillating fluorescence on the first flash in February and in June was in a different ratio. The conditions were not exactly the same due to the presence of ferricyanide or DCBQ (in February) in the experiments on fluorescence oscillations. It has been shown that artificial electron acceptors mainly suppress the slow fluorescence rise with increasing flash number without appreciable changes in the shape of the fluorescence oscillation on the first flashes [24]. We consider these differences as small enough to justify the comparison of the number of the PS II centers exhibiting oscillating fluorescence yields, evaluated on the first flashes of a series in the presence of ferricyanide or DCBQ, with the number of steady-state O_2 evolving centers without ferricyanide or DCBQ. Thus, in Fig. 3, it can be observed that the number of the PS II centers exhibiting oscillating fluorescence yields remains almost constant from February to May, taking into account the first flash extent of the oscillating part of fluorescence yield, F_1–F_4 or F_1–F_3 (F_1, F_3 and F_4 are the fluorescence yield after the first, third and fourth flash, respectively). F_1–F_4 was twice as small in June as in February although the flashes were still saturating. In high-light grown thylakoids, the fewer O_2 evolving centers contributing to fluorescence oscillations, strongly contrasts with the more than doubled number of the O_2 evolving centers measured by the flash-induced steady-state O_2 yield.

To investigate whether preillumination has an effect on the antenna size of the O_2 evolving centers giving rise to fluorescence oscillations, the fluorescence emission at various degrees of light saturation was studied in moderate-light grown (May) thylakoids in the presence of ferricyanide, which fully reoxidized Q_A^- between the flashes. One problem is that the flash saturation curve of the PS II centers exhibiting oscillating fluorescence yields, and that of the fluorescent 'inactive centers', cannot be measured independently of each other under the previous measurement conditions. This was resolved by increasing the time between flash and measurement to 800 ms (the flash interval was 1.3 s), and by adding 1 μg/ml gramicidin. Under these conditions, the flash-induced fluorescence oscillations were observed to be very close to F_0, the fluorescence yield after darkness, since the flash-induced fluorescent 'inactive centers' were, for the most part, relaxed. The pattern of the fluorescence yield measured 800 ms after each flash of a series was found identical to that measured at 80 ms, as already shown [26]. Fig. 5 shows these flash-induced fluorescence oscillations as a function of the energy of the flashes in thylakoids extracted in May, adapted to dark for 2 h. The baseline level of the fluorescence oscillations (F_{bl}) was slightly shifted up in order to obtain the best fit (dotted line). The flash saturation curve of the fluorescence yield on the first flash (F_1–F_{bl}) derived from Fig. 5, reveals a characteristic saturation energy $I_{sat}=15$–16% flash energy (Fig. 6, squares), which is probably overestimated. When samples are kept in darkness for several hours, an oxidized active tyrosine residue Y_D, is reduced in two phases, in the 10-min (20–25%) and 1-h time range (75–80%) [43]. Thus, when long-term dark-adapted samples are illuminated, the formation of the S_2 state on the first flash is followed by its reduction to S_1 by Y_D [44]. Nevertheless, in May, the absorption cross-section of the 2-h dark-adapted samples ($I_{sat}=15\%$ in Fig. 6) was found much larger than that of the short-term (5 min) dark-adapted samples on which several series of 50 flashes fired at 3 Hz were previously applied, as shown in Fig. 7 (I_{sat} around 45% flash energy). These results indicate that after light exposure followed by a few minutes of darkness (5–10 min), at least on the first flash of a series, the effective absorption cross-section of the PS II centers involved in fluorescence oscillations becomes smaller. Fig. 7 also shows that the decreased antenna size was quite similar in thylakoids extracted in May and in June, although the size of the fluorescence oscillations was in a ratio of 2 to 1. A decrease in the effective

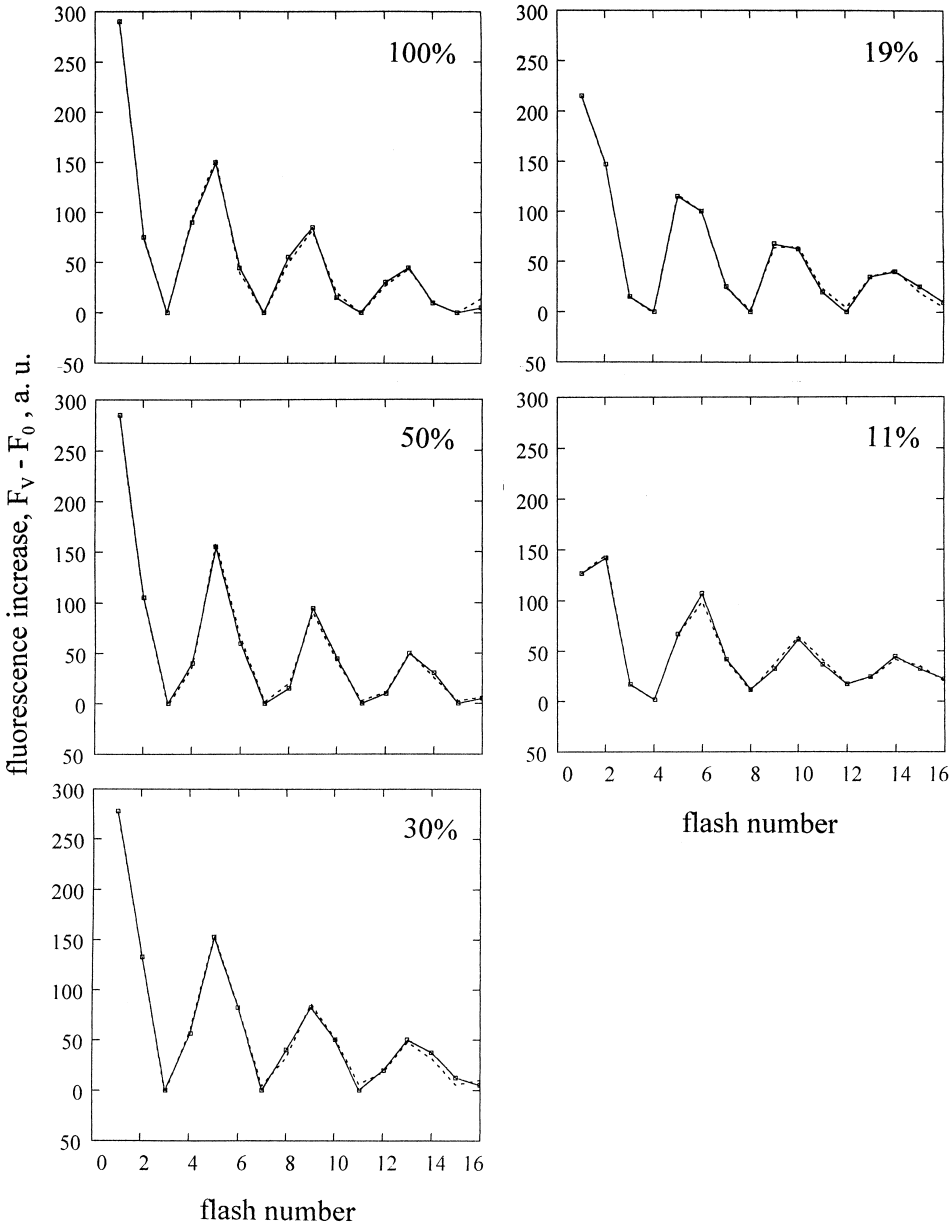

Fig. 5. Flash-number dependent Chl *a* fluorescence yield, measured 800 ms after each flash of a series, carried out at different flash energies (% transmittance of the neutral density filters as indicated in each frame) in moderate-light grown (May) lettuce thylakoids, in the presence of 0.5 mM ferricyanide and 1 μg/ml gramicidin, at pH 6.5. The flash interval was 1.3 s. The sample was dark-adapted for 2 h. The experimental F_0 level was between -50 and 0 of the ordinate, close to 0, the baseline of the fluorescence oscillations F_{bl}. The dotted line is the best fit. The fitted variables are: for 100% flash energy, $\alpha = 0$, $z = 0.853$; for 50% flash energy, $\alpha = 0.012$, $z = 0.876$; for 30% flash energy, $\alpha = 0.018$, $z = 0.877$; for 19% flash energy, $\alpha = 0.042$, $z = 0.901$; for 11% flash energy, $\alpha = 0.091$, $z = 0.946$ (for each case $\beta = 0$).

absorption cross-section is probably responsible for the decreased fluorescence yield on the first flash as shown in Fig. 3, when the series was preceded by a series of 16 flashes followed by 5 min of darkness.

However, though a large proportion of the O_2 evolving centers contributing to fluorescence did not react (misses) on the first flash in short-term (5–10 min) dark-adapted samples, these centers were efficient with no or few misses on the following flashes of a series [25]. This may indicate that on the follow-

ing flashes, most of the PS II centers exhibiting oscillating fluorescence yields no longer maintained their decreased absorption cross-section. The fact that the antenna size of these PS II centers progressively recovered upon illumination is demonstrated by the effect of one preflash on the fluorescence yield on the first flash of a series [25]. In Fig. 6, the flash-saturation curve of F_1-F_{bl}, when one preflash was given 1 min prior to the flash series (diamonds), indicates a much larger absorption cross-section ($I_{sat}=7.5\%$ flash energy) than after a longer dark adaptation period. One way to further test the hypothesis that the antenna size is being restored upon light exposure, is to evaluate the miss values from the fluorescence oscillations as a function of flash energy. Misses (α) or incomplete transitions of the S states are induced by insufficient flash energy. Thus, the plot of $1-\alpha$ versus flash energy provides information on the flash saturation energy of the fluorescence oscillations inside the region where the oscillations are damped,

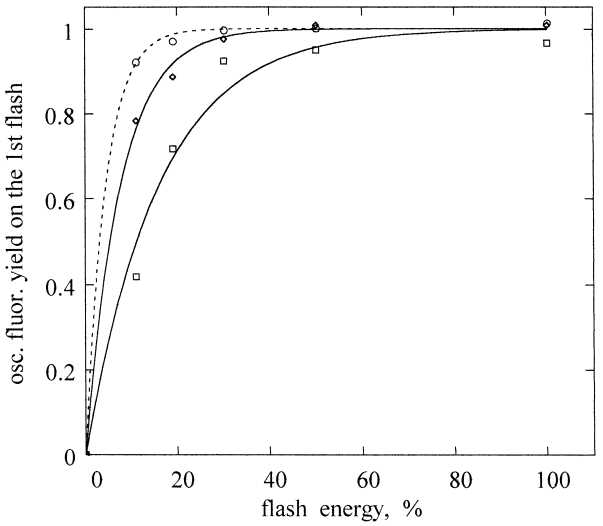

Fig. 6. Flash saturation curves of the oscillating part of the Chl *a* fluorescence yield measured 800 ms after the first flash in a series, F_1-F_{bl}, in lettuce thylakoids extracted in May, either dark-adapted for 2 h (squares): $I_{sat}=15\%$ flash energy, or dark-adapted for 1 min preceded by one preflash of the same energy as the flash series (diamonds): $I_{sat}=7.5\%$ flash energy. F_1 is the fluorescence yield after the first flash, F_{bl} is the baseline of the fluorescence oscillations. For comparison, $1-\alpha$ as a function of flash energy is shown, obtained from the miss values (α) in caption of Fig. 5 (dotted line, circles): $I_{sat}=4.5\%$ flash energy. Other experimental conditions were as in Fig. 5. The theoretical maximum values were normalized to unity and single exponential curves were fitted to the experimental data.

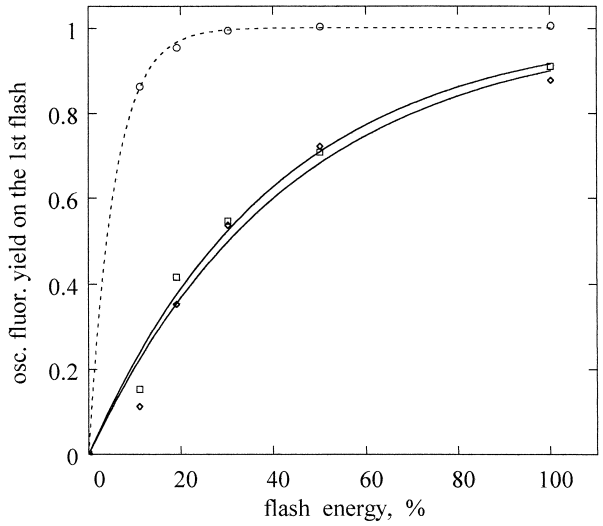

Fig. 7. Flash-saturation curves of the oscillating part of the Chl *a* fluorescence yield measured 800 ms after the first flash in a series, F_1-F_{bl}, in lettuce thylakoids extracted in May (squares): $I_{sat}=40.5\%$ flash energy, and in June (diamonds): $I_{sat}=43.5\%$ flash energy, after light treatment followed by 5-min dark adaptation. Light treatment consists of several flash series of 50 flashes fired at 3 Hz. The absolute values of F_1-F_{bl} were in the ratio 2 to 1, respectively. For comparison, $1-\alpha$ as a function of flash energy is shown, deduced from the miss values of the fit of patterns of fluorescence yield obtained at different flash energies in thylakoids extracted in May after light treatment followed by 5-min dark adaptation (dotted line, circles): $I_{sat}=5.5\%$ flash energy. The theoretical maximum values were normalized to unity, and single exponential curves were fitted to the experimental data.

i.e., roughly from the second to the 14th flash. Surprisingly, in both long-term (Fig. 6) and 5-min (Fig. 7) dark-adapted samples, the saturation energy I_{sat} for $1-\alpha$ (open circles) was unusually small, respectively, 4.5% and 5.5% flash energy, with regard to the other I_{sat}'s on the first flash. The validity of this conclusion is supported by the fact that the data fit quite closely the theoretical oscillations as in Fig. 5. The presence of an electron acceptor is not expected to change the antenna size of these PS II centers, since it has been reported that antenna sizes of PS II centers were practically the same both in the absence and in the presence of DCBQ [45]. The estimates of I_{sat} from the fluorescence oscillations were significantly smaller than expected based only on the I_{sat} of the steady-state O_2 yields (from 9% to 21% flash energy in Fig. 1). This result indicates that the absorption cross-section of the PS II centers exhibiting

oscillating fluorescence yields could be more than twice as large as that of the PS II centers that evolve steady-state O_2 evolution. This is consistent with a dimeric organization [46] of the O_2 evolving centers contributing to fluorescence oscillations. A heterogeneity in PS II centers based on differences in antenna size has already been suggested from the analysis of fluorescence induction curves in the presence of DCMU [47]. Recently, it has been shown that in thylakoids of intermittent light grown pea plants (lacking LHC II), such curves are fitted by assuming that the α-centers are dimers, and the β-centers monomers [48]. The more pronounced sigmoidicity of fluorescence induction curves for standard thylakoids has been interpreted in terms of dimers showing some degree of excitonic connectivity mediated by LHC II [49,48]. In contrast, the slower exponential induction kinetics reflect a barrier-to-energy transfer between the β-center monomers [47–49]. However, although the O_2 evolving centers contributing to fluorescence oscillations are likely to be the α-center dimers, our results indicate that in the absence of DCMU, a proportion (10–15%) of completely quenched centers are created after each flash, so that after about 20 flashes, the number of these PS II centers is almost exhausted. Therefore, if the PS II centers exhibiting fluorescence yields are the α-center dimers, their number is expected to be greatly reduced during the flash-induced steady-state O_2 evolution. This assumption may explain why none of the reported light saturation curves of the flash-induced steady-state O_2 yield in the literature [50], provide evidence for the α/β heterogeneity of Melis and Homann [47].

Thus, our data indicate that dark adaptation after a light-initiated process, causes a decrease in the absorption cross-section of the O_2 evolving centers contributing to fluorescence oscillations (which was large). This process was revealed at the beginning of a new illumination (on the first flash) and reversed on further illumination by a few flashes.

3.2. pH dependence of the deepoxidation of violaxanthin in low-light and high-light acclimatized lettuce thylakoids

The pH dependence of violaxanthin deepoxidation was determined in dark-adapted and preilluminated thylakoids by measuring the absorbance change at 505–540 nm [19]. In both cases, assays were carried out in the dark in the presence of an uncoupler. The reverse process of epoxidation characterized by a negative slope of the 505 nm absorbance change as a function of time was found to be negligible prior to the initiation of deepoxidation reactions by addition of 30 mM ascorbate. After addition of ascorbate, the initial part of the 505 nm kinetics, up to 25 min, roughly followed a first-order kinetics and confirmed previous results [19]. No deepoxidase activity was observed for pH values higher than 7. We studied only the fast kinetic-component of violaxanthin deepoxidation. The amount of violaxanthin that could be maximally deepoxidized from the rapidly converted pool $\Delta_{505}\infty$ was found to be relatively constant in the pH range of pH 5.2 to 6.4 for each sample and for the differently light-grown lettuce thylakoids. The estimated $\Delta A_{505}\infty$ values ranged between 0.8 and 1.3 (mg Chl ml)$^{-1}$. In contrast, the maximum first-order rate constants ^1k (from 1×10^{-3} s^{-1} to 6×10^{-3} s^{-1}) were smaller in thylakoids grown in low light than in those grown in higher light.

In low-light grown (February) thylakoids, preilluminated-type thylakoids were illuminated either by two saturating flashes 1 s apart (only one preflash was less efficient) or by a 65-μmol photons m^{-2} s^{-1} red actinic light for 1 min. A dark relaxation period of 5 min was inserted between preillumination and ascorbate addition. As shown in Fig. 8, the light pretreatment induced a change in the pH dependence of the rate constant of violaxanthin deepoxidation. In dark-adapted thylakoids, the optimum for deepoxidation was found at around pH 5, close to the first reported pH optima, at pH 4.8 in isolated chloroplasts, and at pH 5.2 in the isolated enzyme [51]. In preilluminated thylakoids, with decreasing pH between pH 6.3 and 6.1, there was a very steep rise ($pK_a = 6.2$) instead of a slow rise ($pK_a = 5.92$), and the enzyme was effectively saturated at pH 5.8. The fitted pH dependence curves yield Hill coefficients of 2.75 for dark-adapted thylakoids, and 5.8 for preilluminated thylakoids, as if a group of six protons could act in a hexacooperative way. Preillumination with a series of 16 flashes instead of two flashes 1 s apart gave equivalent results.

Fig. 9 shows that in high-light grown thylakoids (extracted in June), the pH dependence of the rate

constant ^1k hardly shifted towards high pH, either after a treatment similar to that applied to low-light grown thylakoids, or after a higher white light preillumination at 300 μmol photons m^{-2} s^{-1} for 10 s, followed by 5 min darkness before the addition of ascorbate. The curves fitted to the plot of ^1k versus pH, yield Hill coefficients of 3.75 and 5.3, respectively. The highest pK_a, at the inflexion point was at pH 6.05. Thus, compared with similar behavior observed in low-light grown thylakoids, the estimated pK_a's shifted towards a lower pH. A similar titration curve has already been reported [19]. Comparable observations have been described for the titration curves of non-photochemical quenching versus lumen proton concentration. After light treatment, the titration also shifted towards lower $[H^+]$ [52,53]. In the latter case, the estimated pK_a after light treatment was approximately 5.5–6.0, a value roughly equivalent to that found for the rate constant of violaxanthin deepoxidation in lettuce thylakoids extracted in June.

We observed that the rate of violaxanthin deepoxidation did not rise steeply immediately after light exposure. The maximum rate was reached after 6–7 min darkness with a half-time of 2–3 min (not shown). The final activated state of the violaxanthin deepoxidase was formed slowly, in the same way as the decrease in absorption cross-section of the PS II centers involved in fluorescence oscillations (Figs. 3 and 7). It should be noted that the activated state did not develop during light exposure (even >5 min). In low-light grown thylakoids, we also observed that after a longer period of darkness, the activated state of the violaxanthin deepoxidase relaxed with a half-time of about 20 min at a given pH (5.9). This was faster than generally described in whole leaves (30–60 min) [53].

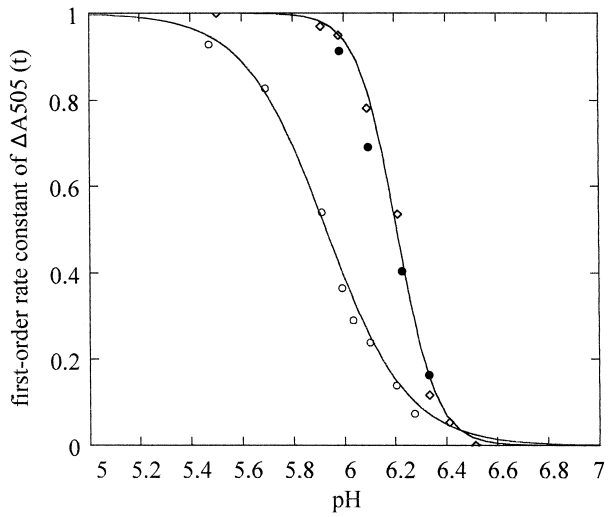

Fig. 8. pH dependence of the rate constant (^1k) of the initial part of the 505 nm absorbance change kinetics in low-light grown thylakoids (February), either dark-adapted for 3 h (open circles), or preilluminated: with 65 μmol photons m^{-2} s^{-1} red light for 1 min (diamonds), or with two flashes spaced 1 s apart (filled circles). Five minutes of darkness was inserted between preillumination and ascorbate addition. The fitting procedure yields pK_a values of 5.93 and 6.20, and Hill coefficients n of 2.7 and 5.8, respectively. The maximum rate constant was 1.3×10^{-3} s^{-1} at low pH.

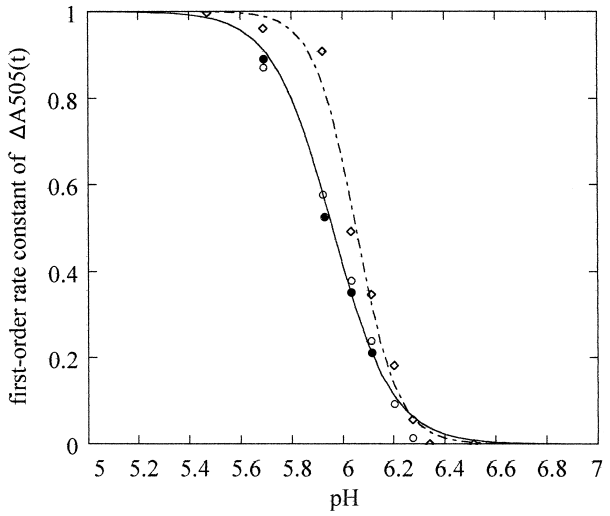

Fig. 9. pH dependence of the rate constant (^1k) of the initial part of the 505 nm kinetics in high-light grown thylakoids (June), either preilluminated with 65 μmol photons m^{-2} s^{-1} (open circles) or with two flashes 1 s apart (filled circles), or preilluminated: with 300 μmol photons m^{-2} s^{-1} white light for 10 s (diamonds). Five minutes of darkness was inserted between preillumination and ascorbate addition. The fitting procedure yields pK_a values of 5.96 and 6.05, and Hill coefficients n of 3.75 and 5.3, respectively. The maximum rate constant was 5.7×10^{-3} s^{-1} at low pH.

4. Discussion

When the dark-adapted photosynthetic apparatus is exposed to saturating light, a series of photoprotective mechanisms is expected to occur [11]. In this paper, the fluorescence yield is measured a long time

after saturating flashes (80 ms and 800 ms), much longer than the turnover time of normally functioning O_2 evolving PS II centers (a few ms). Thus, after this turnover time, the PS II centers able to evolve O_2 (with fully reoxidized Q_A) are expected to be open in a low fluorescence quenching state, but this is not observed. Instead, following saturating flashes, the fluorescence yield steadily decreases in the milliseconds to the seconds time range. The basic simple model is not sufficient to explain the kinetics following saturating flashes. In the observed fluorescence decay in the dark, two components in the fluorescence yield have been identified in more detailed experiments: the fluorescence part independent of the number of flashes (baseline), and the oscillatory part with period four. Even in the presence of artificial electron acceptors which fully reoxidize Q_A^- between the flashes, there are slowly turning-over reaction centers [40], as shown at a time as long as 80 ms after each flash of a series (Fig. 3). The very slow reoxidation kinetics of Q_A^- in these slowly turning-over reaction centers can explain [40,54] the observed fluorescence decrease. In this paper, the 'inactive centers' component was practically eliminated in moderate-light grown thylakoids and in the presence of gramicidin, when the fluorescence yield was measured 800 ms after each flash of a series. Gramicidin is known to induce an acceleration in the transmembrane electric field decay [42]; thus, roughly 500 ms after each flash, the slow fluorescence decay (in the absence of gramicidin) seems to be due to the electric field close to the PS II centers. One possibility, in line with Ref. [41], is that the electric field changes the probability of photon excitation trapping for these centers, even in an active state. A sufficient electric field can strongly decrease the probability of trapping, so that an active center unable to trap the excitations, appears to be an inactive center. Thus, the absence of 'inactive centers' allowed us to study the period four oscillations of the fluorescence yield, with a baseline just above F_0, the minimum fluorescence yield when all PS II centers are open after darkness.

Our results show that the period four fluorescence oscillations are associated with a distinct population of PS II centers. It has been established [32] that the photosynthetic capacities of plants are influenced by the light intensity the plant receives during growth. These past studies and our results show that the PS II centers that evolve the flash-induced steady-state O_2 yield, modulate their proportion in order to make the best use of the available quantum flux. In June, the antenna size was found to be two-fold smaller, and the number of PS II units two-fold higher than in February. In contrast, the relative abundance of the O_2 evolving centers exhibiting oscillating fluorescence yields decreased a little from February to May. The very few centers in June may indicate that these centers are inhibited under conditions of low lumenal pH.

The results of previous studies [23–26] with those in this paper provide information on the properties of these special O_2 evolving centers. After a long-term dark adaptation, the fluorescence maxima were observed after one, five and nine flashes in a series, i.e., the fluorescence yield was higher in the S_2 state than in the other S states, S_0, S_1, and S_3 [25]. Based on the resemblance between the S state dependent electrochromism transients and fluorescence yields, it has been proposed that the observed period four oscillations reflect net charge transients of the catalytic site of water oxidation in these centers [26]. The stable electrochromic shift [29–31] and the maximum fluorescence yield upon transition $S_1 \rightarrow S_2$ are indicative of a net excess of charge in the S_2 state that is neutralized upon $S_2 \rightarrow S_3$ and reduced upon the O_2 evolving transition $S_3 \rightarrow S_4 \rightarrow S_0$ [55].

If induced by saturating flashes, the PS II centers involved in fluorescence oscillations were characterized by exact period four oscillations, and thus, no or few misses. Each center generates a complete transition on each flash, or disappears as a center involved in fluorescence oscillations. This property helps these centers to be well synchronized, which is useful for a cooperative effect (for example, the net positive charges in the S_2 state of these centers are additive for the production of the electrostatic protons).

Our results also suggest that the O_2 evolving centers giving rise to fluorescence oscillations only exist during the induction period of photosynthesis, since their number progressively decreased after each flash of a series. In particular, on the basis of the numerical fitting of the fluorescence yield pattern, we show that these centers converted into a progressively increasing proportion $(1 - z^n)$ of quenching centers with increasing flash number n. Thus, our results

indicate that quenching mechanisms occur in the PS II centers exhibiting fluorescence oscillations. One possible interpretation is a direct quenching by $P680^+$ at low pH [5]. Such an assumption has been proposed to explain the low pH dependent non-photochemical quenching observed at the maximum fluorescence level F_m [5,7]. According to Bruce et al. [5], the quenching centers appear as 'open centers', because the excitation trapping by $P680^+$ and the subsequent relaxation to the ground state could occur on the same time scale as charge stabilization in open reaction centers. However, there are two arguments against this possibility in our experiments. (1) It is clear that the quenching observed at low pH after a saturating single-turnover flash is dramatic at time intervals less than 1 ms, but is much less at 5 ms [5]. (2) The patterns of the flash yield of O_2 evolution in this paper do not provide evidence for a progressive decrease in the total number of active O_2 evolving centers with increasing flash number (see Fig. 2 caption, z is close to 1). This raises the possibility that the origin of the quenching observed in our experiments results from a conversion of a type of active PS II centers that gives rise to fluorescence oscillations into another type of active PS II centers that maintains a low fluorescence state. As a result of the estimated antenna size in this study, the PS II centers involved in the fluorescence oscillations are likely to be the α-center dimers, whereas the β-center monomers seem to be mainly found in the steady-state O_2 yield after many flashes. A mechanism that is consistent with these results is that upon a series of flashes after darkness, dimeric PS II centers progressively undergo the conversion from dimer to monomer, accompanied by fluorescence quenching, so that only monomers (and a low fluorescence state) are present after many flashes. This monomerization, which involves a reorganization of a part of the PS II centers in the membrane, may be considered as a way of safely decreasing the absorption cross-section of the PS II dimers. The conversion from dimer to monomer (possibly faster at pH 5.5 than at pH 6.5, as suggested by Fig. 4) appears to be triggered by light exposure. In this regard, it has been reported that in vitro, isolated dimers convert into monomers when treated with photoinhibitory light [56]. The reverse conversion, i.e., the restoration of the PS II dimers, is expected to occur in the dark after light exposure.

Our data in this paper also suggest that quenching due to de-excitation in the antenna occurs in the PS II centers exhibiting fluorescence oscillations (i.e., possibly the PS II dimers). After light exposure and a few minutes of darkness, the absorption cross-section of these PS II centers, measured on the first flash of a series, was found to be highly decreased. The decreased antenna size is probably responsible for the large misses observed on the first flash of a series under similar conditions [25]. These observations support the idea that in response to light, slow structural changes in the light-harvesting antenna system take place during darkness, so that on the first flash of a series, more absorbed light energy is dissipated as heat in the antenna, leading to a smaller effective absorption cross-section. However, our results also show that upon further illumination by several flashes, the antenna of these PS II centers rapidly undergoes the reverse conversion from a small size (fluorescence quenching pigment configuration) to a large size. Thus, increased heat emission in the antenna occurs apparently during a short time of illumination.

Low-light grown plants appear at a disadvantage in comparison with high-light grown plants with respect to the photoprotective responses to light exposure [57]. The light-initiated process responsible for antenna fluorescence quenching described above partially compensates for this handicap by allowing zeaxanthin formation to occur at a higher pH than usual in the dark, possibly in order to maintain photoprotection in the dark. More precisely, it has already been reported [52,53] that light preillumination allowed fluorescence quenching to occur at pH values higher than the pH for the same quenching in the absence of preillumination. This observation has led to the notion of 'light activation' of non-photochemical quenching. In this paper, we show that the effect referred to as light activation occurs for the violaxanthin deepoxidase activity. In low-light grown (February) thylakoids, two preflashes or a 65-μmol photons m^{-2} s^{-1} light preillumination followed by 5 min of darkness, resulted in an increased rate of violaxanthin deepoxidation at pH values (pH 6.0–6.2) when little zeaxanthin was formed in non-preilluminated thylakoids or immediately after preillumination. The shift of the titration curve of the violaxanthin deepoxidase activity towards higher pH was associated with an increased cooperativity between

proton binding sites (Fig. 8). In thylakoids extracted in June, this shift was less significant, for almost the same high proton cooperativity (Fig. 9). The fact that light activation induced almost comparable pH dependencies of non-photochemical fluorescence quenching [53] and violaxanthin deepoxidation, implies that the structural changes in the antenna and the increased rate of violaxanthin deepoxidation at high pH are interrelated. Our results also indicate a correlation in time (5–10 min darkness) between the formation of a decreased antenna size as determined by our fluorescence measurements and the increase in the proton binding site cooperativity in the antenna, as revealed by the pH dependence of the violaxanthin deepoxidase activity.

What mechanisms during illumination are able to induce a cooperative protonation in LHC II resulting in structural changes in the dark and formation of a fluorescence quenched state of the complex? At the catalytic site of water oxidation, in connection with the lumen, proton release primarily reflects the production of protons upon electron abstraction from water. This chemical production is the same on each transition of the four oxidation steps, one proton per active center [58], and does not depend on pH. However, recent studies on the pH dependence of the release of protons after the first flashes of a series have demonstrated that the chemical proton release from water oxidation is transiently covered by other protolytic reactions, due to the field of a net positive charge. pH-dependent proton release has been reported in O_2 evolving thylakoids [55] and in Mn-depleted PS II membranes in the presence of the electron donor, diphenylcarbazide (DPC) [59]. In both cases, the pattern of net proton release/uptake varies extremely as a function of pH. In O_2-evolving thylakoids [55], at low pH, the occupancy of a non-compensated positive charge during the transition $S_1 \rightarrow S_2$ (1st flash) induces a supplemented proton release, and the suppression of the positive charge during $S_3 \rightarrow S_0$ (3rd flash) a reduced proton release. No pH-dependent proton release/uptake is observed during $S_2 \rightarrow S_3$ (2nd flash). In Mn-depleted PS II membranes [59], excess/undershoot over one proton is induced by DPC oxidation and reduction, in a two-photostep process. Furthermore, the release/uptake of the pH-dependent protons has been found to be symmetrical and opposite at low and high pH [55,59]. These results are consistent with the suggestion that water molecules and dissociated ions H^+ and OH^- are attracted to a highly polarized catalytic site, allowing these ions to be the source of extra protons at low pH and of hydroxyl ions (OH^-) at high pH [59]. A plausible conclusion is that the net charge in the S_2 state and the properties of the catalytic site of water oxidation are responsible for transitory high levels of protons transferred into the lumen.

In thylakoids and PS II enriched membranes, the pattern of proton release oscillates strongly as a function of the S state transitions and varies as a function of pH. In contrast, in reaction center core preparations, it is featureless, close to 1:1:1:1 [60,61]. Nevertheless, in PS II core preparations, the pattern of local electrochromism is oscillatory [62]. This observation reveals that the LHC II proteins, which are absent in core preparations, could contribute to the transport of the electrostatically induced protons from the water-oxidation site to the lumen. Studies with the reagent DCCD, well-known to block proton channel activity in a variety of proteins, corroborate this view [63]. Furthermore, the covalent binding of DCCD to the minor LHC II species LHC IIa and LHC IIc, at glutamate residues in LHC IIc, has been correlated with the inhibition of non-photochemical quenching and the xanthophyll cycle [64,13,14]. These data, together with our studies, suggest that the function of the transported protons in LHC II, the concentration of which can be transiently high, is to activate structural changes in the LHC II species and stimulate the xanthophyll cycle deepoxidase enzyme.

The pH titration curve of the release of protons after the first flashes in a series has been reported to produce a sharp transition [55,59]. Increasing the pH results in the abolition of the pH-dependent proton release with a midpoint near pH 7 (followed by an increase of OH^- release [59]). The decline in proton release does not fit to a single pK_a-dependent event, instead it fits to a cooperative model, as if a certain number of protons (n) are buffered in a strictly cooperative manner ($n = 3$ in Fig. 4 of Ref. [55]; $n = 5$ in Fig. 8 of Ref. [59]). The above results reflect the proton fixation on proton binding groups which can act in a cooperative way. This also means that the pH-dependent protons have access to these groups.

It is likely that such a similar proton binding group is present in the violaxanthin deepoxidase and that

the number of the protons acting in a cooperative way, up to 6, modulates the enzyme activity. The sites of protonation are probably located in the highly charged domain of the violaxanthin deepoxidase, that includes a high concentration of glutamic acid residues as deduced from the primary structure [65]. Change in the protonation state of one of these residues is possibly associated with an allosteric transition of the enzyme [66]. An interesting property of the allosteric systems is also their capacity to mediate cooperative interactions. Most allosteric proteins are oligomers, involving several identical units [67]. Unfortunately, little is known about the structural organization of the components of the xanthophyll cycle in the membrane.

The comparable pH dependencies of non-photochemical fluorescence quenching [53] and violaxanthin deepoxidation indicate that the formation of a decreased antenna size and the increased rate constant of violaxanthin deepoxidation at high pH are controlled by the same physiological 'signal', the protons transported through LHC II. Most of these protons, electrostatic in nature, are produced by the PS II centers in which net charge transients occur, i.e., the O_2 evolving centers that induce period four fluorescence and electrochromic absorbance oscillations [26] (i.e., possibly the dimeric PS II centers). Antenna fluorescence quenching appears to be associated with a reorganization of the light-harvesting apparatus prompted by the cooperative protonation of specific amino acid residues or pigments. However, this does not seem to be sufficient, since our results indicate that the large antenna size is restored during illumination by several flashes. Therefore, fluorescence quenching due to de-excitation in the antenna appears to be only the result of the structural changes in the antenna accompanying the violaxanthin deepoxidase activity in the dark. Zeaxanthin formation at low pH during illumination and afterwards, at higher pH in the dark, mainly serves to protect the thylakoid membranes, probably against lipid peroxidation [68].

Our experiments show that upon exposure to a series of flashes in dark-adapted thylakoids, non-photochemical fluorescence quenching (80 ms or 800 ms after each flash) results from the O_2 evolving centers exhibiting oscillating fluorescence yields. The estimation of the antenna size for these PS II centers, supports a dimeric structure. Whether this structure accounts for the specific S state turnover properties of these centers, or whether a dimeric structure is a prerequisite for the slow structural modifications in the antenna pigment bed in the dark after illumination, requires further investigation.

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