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Properties of inactive Photosystem II centers

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Abstract

A fraction (usually in the range of 10–25%) of PS II centers is unable to transfer electrons from the primary quinone acceptor Q_A to the secondary acceptor Q_B. These centers are inactive with respect to O_2 evolution since their reopening after photochemical charge separation to the $S_2Q_A^-$ state involves predominantly a back reaction to S_1Q_A in the few seconds time range (slower phases are also occurring). Several properties of these centers are analyzed by fluorescence and absorption change experiments. The initial rise phase F_o–F_{pl} of fluorescence induction under weak illumination reflects both the closure of inactive centers and the modulation of the fluorescence yield by the S-states of the oxygen-evolving system: We estimate typical relative amplitudes of these contributions as, respectively, 65 and 35% of the F_o–F_{pl} amplitude. The half-rise time of this phase is significantly shorter than for the fluorescence induction in the presence of DCMU (in which all centers are involved). This finding is shown to be consistent with inactive centers sharing the same light-harvesting antenna as normal centers, a view which is also supported by comparing the dependence of the fluorescence yield on the amount of closed active or inactive centers estimated through absorption changes. It is argued that the exponential kinetics of the F_o–F_{pl} phase does not indicate absence of excitation energy transfer between the antennas of inactive and active centers. We show that the acceptor dichlorobenzoquinone does not restore electron transfer in inactive centers, in disagreement with previous suggestions. We confirm, however, the enhancement of steady-state electron flow caused by this quinone and suggest that it acts by relieving a blocking step involved in the reoxidation of a fraction of the plastoquinone pool. Part of the discrepancies between the present results and those from previous literature may arise from the confusion of inactive centers characterized on a single turnover basis and PS II centers that become blocked under steady-state conditions because of deficient reoxidation of their secondary acceptors.

Abbreviations: DCBQ – 2,6-dichloro-*p*-benzoquinone; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMQ – 2,5-dimethyl-*p*-benzoquinone; PS – photosystem

Introduction

The first indication for the presence of a fraction of PS II centers that are inactive for oxygen evolution dates back to the fluorescence experiments of Joliot et al. (1971). These authors showed that the reoxidation of the primary quinonic acceptor Q_A includes a minor slow phase in the range of several hundred ms, in addition to the major fast phase (in the hundreds

of μs range) associated with electron transfer to the secondary quinone Q_B. This slow phase was interpreted as a back reaction in centers that are unable to transfer electrons to Q_B. It was distinguished from another slower contribution to the decay of the fluorescence yield, which was shown to reflect the deactivation of the oxidized states (S_2 and S_3) of the oxygen evolving system. Correlatively, the authors indicated that both contributions account for the first phase of the fluorescence rise curve under continuous illumination from the dark-adapted F_o level to a plateau level F_{pl}.

The occurrence of a fraction of PS II centers that do not reopen in the ms range, but 3 orders of magnitude slower, has been the object of renewed attention in the past years, mainly for two reasons. First, the contribution of these centers may have to be taken into account when estimating the relative amounts of PS II and PS I centers in chloroplasts. Second, the problem of the physiological significance of these centers was addressed in the context of the processes of photoinhibitory degradation and repair of PS II centers. The papers which are at the origin of this renewed interest are those of Melis (1985), Graan and Ort (1986) in which the term of 'inactive centers' (for oxygen evolution) was proposed, and Chylla et al. (1987). In these and later reports, several new ideas emerged. The inactive centers were proposed to be located in the non-appressed (stromal) part of the thylakoid membrane, where PS I is segregated and were identified with 'β-type centers' (Melis 1985) with a lower light-harvesting antenna than the active, 'α-type', centers present in the appressed (granal) region. It was also suggested that these centers could be reactivated by providing them with a suitable exogenous acceptor (DCBQ) that would restore their electron transfer and oxygen-evolution capability (Graan and Ort 1986, Cao and Govindjee 1990, Nedbal et al. 1992). In the present paper, we will reexamine these aspects and argue against some of the these conclusions. An important point is to distinguish between inactive centers characterized, as above, on a single turnover basis and the blocking of a fraction of PS II centers under steady-state illumination due to inefficient draining of electrons from their accessible plastoquinone pool.

The presence of inactive centers is easily observed when dark-adapted material is illuminated by a train of single turnover flashes spaced in the 10 ms–500 ms range. In fluorescence or absorption change experiments they appear as a specific offset occurring on the first flash. Furthermore, the flash sequence procedure is convenient for identifying signals related to the oxygen-evolving system so that their contribution on the first flash can be extrapolated and subtracted. This is basically what was done in previous work (Lavergne 1987) studying absorption changes associated with the S-transitions. This allowed a determination of the light minus dark spectrum of the inactive centers that appeared similar to that of the $S_1Q_A \rightarrow S_2Q_A^-$ transition in normal centers. The latter spectrum is easily obtained as the light minus dark change in the presence of DCMU where the contribution or 'active' (now inhibited) centers is predominant. The absorption changes of the inactive centers possess both the C-550 and UV changes characteristic of Q_A^- and the UV contribution of S_2. However, due to the limited experimental accuracy of these experiments in the blue region, some ambiguity persisted that was dissipated when studying the kinetics of the recovery of inactive centers. This recovery (or a part of it, as discussed below) occurs through charge recombination in the few seconds range, similarly to DCMU-inhibited centers. The spectrum of this decay phase was shown to be identical, blue region included, to that observed in the presence of DCMU (Lavergne 1991). Another feature of the inactive centers is that their charge separation contributes the delocalized membrane potential difference that can be measured through electrochromic changes in the green region (Chylla et al. 1987, Lavergne 1987, 1991). Thus, in all respects so far, the inactive centers behave like DCMU-inhibited normal centers, suggesting that their impairment consists basically in a blocking of the $Q_A^- \rightarrow Q_B$ electron transfer. Their denomination as 'non-Q_B transferring' is thus fully justified, although this is at the origin of some confusion with the 'non-B' acceptors previously described (Lavergne 1982a,b), which, as will be argued in the Discussion, are a quite different matter.

Materials and methods

Algae and chloroplasts

Two strains of the green alga Chlorella Sorokiniana were grown and used as previously described (Lavergne et al. 1984). S-11 is a single mutant devoid of PS I centers, while S-56 is a double mutant lacking both PS I and part of the chlorophyll-protein antenna complexes (see Lacambra et al. 1984, for mutant selection and biochemical characterization). The light-harvesting efficiency of PS II is decreased about twofold in S-56 compared with S-11. The cells were used in fluorescence or absorption change experiments after treating them with *p*-benzoquinone: 200 μM of this substance was added to the culture which was immediately centrifuged for 5 min, resuspended in a quinone free medium and pelleted again, the whole procedure being carried out under dim light. The resuspension medium (used in absorption or fluorescence experiments) was 50 mM KCl and 50 mM phosphate buffer, pH 6.5. The benzoquinone treatment blocks the dark reduction of the plastoquinone pool that takes place in living algae, allowing a fully oxidized state of PS II acceptors in the dark-adapted treated cells.

The experiments of Figs. 1 and 4–7 were done with frozen thylakoids from pea or spinach (as indicated in the legends), prepared as follows. The leaves were ground in a medium containing 0.3 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM Na ascorbate, 2.6 g/l BSA and 50 mM tricine, pH 7.5. The slurry was filtered, pelleted and resuspended in the same medium without ascorbate and BSA. The preparation was stored frozen in the presence of 5% DMSO. In fluorescence experiments the thylakoids were diluted to 4 μg/ml of chlorophyll in a medium containing 0.3 M sucrose, 5 mM $MgCl_2$, 10 mM NaCl and 50 mM MES, pH 6.5.

It was checked that the results obtained with frozen thylakoids remained valid in freshly prepared material. This is illustrated by the experiments of Figs. 2, 9 and 10 using spinach thylakoids prepared as described by Graan and Ort (1981). Other conditions are indicated in the text.

The experiments using DCBQ were done with recrystallized 2,6-DCBQ; however, similar results were obtained when using non-recrystallized 2,6- or 2,5-DCBQ (Eastman-Kodak).

Measurement of absorption changes and fluorescence kinetics

Both types of measurement were carried out in the same apparatus, previously described by Joliot et al. (1980) and Joliot and Joliot (1984). The principle of this machine is to use monochromatic microsecond flashes as a measuring light, which provides a high peak intensity (and consequently good signal to noise ratio) while keeping the overall illumination energy below the actinic range. The intensity of each detecting flash is measured in a reference path and used for normalizing the transmitted or fluorescence light signal of the measurement path. The timing of the detecting flashes is programmed at will, with a minimum interval of 2 ms between successive triggerings. When measuring absorption changes, the light-detecting diodes were protected from scattered actinic illumination and from fluorescence by a broad band blue filter (Corning BG-38). Fluorescence was measured in the same geometry (180°) as absorption changes, modifying only the filter on the detecting photodiode in the measurement path (a Wratten 96 was used in combination with two low fluorescence filters, Schott KV-550 and Ulano rubylith). In this mode, the excitation wavelength (detecting flashes) was set at 480 nm. A small fluorescence from the analysis filters (excited by transmitted and scattered light from the detecting flash) was subtracted in order to obtain the true zero of chlorophyll fluorescence. Actinic flash illumination was provided by a xenon flash of 2 μs width at half intensity, filtered by broad red (Schott RG-665) or blue (Corning BG-38) filters in, respectively, absorption change or fluorescence measurements. The flash was checked to be fully saturating under all experimental conditions used here. The actinic continuous illumination was provided by two arrays of nine red light-emitting diodes (Toshiba TLRA-180x, peak wavelength around 660 nm) which illuminate opposite faces of the cuvette. Keeping the chlorophyll concentration low ($<10\ \mu$g/ml), the actinic illumination is essentially homogeneous

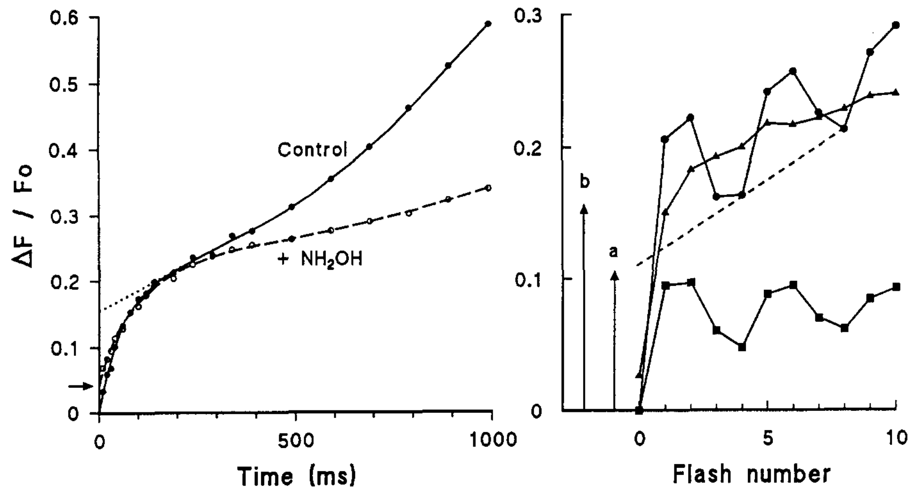

Fig. 1. A comparison of the fluorescence kinetics under continuous or flashing illumination (pea thylakoids). *Left panel*: rapid phase (F_o–F_{pl}) of the induction under continuous illumination in the absence (closed circles) or presence (open circles) of 2 mM hydroxylamine. The origin and unit of the vertical scale is the dark-adapted level F_o of the control; the arrow indicates the corresponding level in the presence of hydroxylamine. The dotted line indicates extrapolation of the slow phase (in the absence of hydroxylamine). *Right-hand panel*: Fluorescence during a series of short saturating flashes in the absence (circles and squares) or presence (triangles) of hydroxylamine. The datapoints indicate the level recorded immediately before firing the next flash. The spacing between flashes was 100 ms (circles and triangles) or 5 s (squares). The dashed line shows extrapolation of the 4th and 8th flash levels to the level indicated by arrow (a). Arrow (b) indicates extrapolation to the origin of the dotted line in the left-hand panel.

across the sample. This source was used at an intensity corresponding to one photoact per center per 50–100 ms (as may be estimated, e.g. from the fluorescence induction kinetics in the presence of DCMU). In the fluorescence measurement mode, the direct contribution to the signal of the fluorescence excited by the actinic beam was negligible, because of the high peak intensity of the detecting flashes and because lower frequencies are rejected electronically.

Results

Observation of inactive centers in fluorescence experiments

When dark-adapted chloroplasts are illuminated with continuous light, the concentration of reduced Q_A increases in a biphasic manner and so does the fluorescence yield which is an increasing, although not linear, function of Q_A^- (a typical fluorescence kinetics is shown in the inset of Fig. 2). The small rapid phase (from the dark-adapted fluorescence yield F_o to a plateau level generally denoted F_{pl}: Forbush and Kok 1968) corresponds basically to a single photoact; the plateau level reflects the (quasi) steady-state where the photoreduction and reoxidation rates of Q_A are balanced. Depending on the efficiency of electron transfer to and through PS I, electrons accumulate in the pool of secondary acceptors, causing a decrease of the rate of Q_A^- reoxidation and thus build-up of Q_A^-; when the electron flow through PS I is slow (e.g. due to the absence of efficient acceptor), total reduction of Q_A takes place progressively and the fluorescence rises from F_{pl} to the maximum F_m level (see Joliot et al. 1992 and Lavergne et al. 1992, for a detailed analysis of this process). Under sufficiently weak illumination intensity, the photochemical turnover rate is slow compared with the sub-ms reoxidation of Q_A^- in active centers and the F_o–F_{pl} phase should reflect only the closure of inactive centers. However, as pointed out by Joliot et al. (1971), the fluorescence yield is also slightly modulated by the state of the oxygen evolving system, so that the transition from the dark-adapted state (predominantly S_1) to the steady-state equipartition of $S_0 \ldots S_3$ contributes the F_o–F_{pl} phase.

Since fluorescence is a sensitive and widely

used method for observing the inactive centers, it is of interest to determine their specific contribution to the F_o–F_{pl} phase by subtracting the interference from the oxygen evolving system. Figure 1 shows a comparison between the F_o–F_{pl} phase under weak continuous illumination (left) and a flash series experiment (right) on the same material. In the latter experiment, the flashes were spaced 100 ms (circles) or 5 s (squares) apart and fluorescence was sampled immediately before each flash. The 100 ms period corresponds to a similar average photochemical efficiency as in the continuous light experiment. A pronounced oscillation with periodicity of four flashes is present, reflecting a slightly higher fluorescence yield in states S_2 and S_3 with respect to states S_0 and S_1 (Joliot et al. 1971, Delosme 1971). A more precise analysis (not shown) using the methodology described in Lavergne (1991) confirmed that the yields are about equal for S_2 and S_3 on the one hand or S_0 and S_1 on the other hand (in disagreement with Delrieu 1988, who suggested a higher yield on the sole S_2 state). This pattern is similar to that observed for local electrochromic changes (Saygin and Witt 1985, Lavergne 1987) that respond to changes in the net charge of the S-system in relation with proton release (Rappaport and Lavergne 1991). The minima of the oscillation occur on flashes 0 (dark-adapted state), 4, 8 . . . , where the population of states S_0 and S_1 is maximal. Thus, a crude approximation of the specific offset caused by inactive centers from the first flash onward may be obtained by taking the difference between the level reached after the fourth flash and the dark-adapted level. This neglects two effects, however, namely the damping of the Kok sequence (less S_0 and S_1 are present on the fourth flash than in the dark-adapted state) and the progressive rise of the 'plateau' level accompanying the reduction of the plastoquinone pool. Thus, a better estimate is obtained by extrapolating the datapoints at flashes 4 and 8, as shown by the dashed line (right-hand panel). The arrow (a) gives the extrapolated amplitude at the origin. For simplicity we used a straight line which probably leads to overestimate the length of this arrow. The induction curve (control) in the left-hand panel shows the F_o–F_{pl} phase, in which both contributions are present (with strong damping of the S-dependent oscillation) and the initial part of the slow phase corresponding to pool reduction. The amplitude of the F_o–F_{pl} phase, extrapolated as shown by the dotted line was plotted as arrow (b) in the right-hand panel and matches well the average value of the oscillating sequence. The amplitude of (a) is about 65% of that of (b), indicating that the F_{pl} level overestimates the inactive centers contribution by the 35% contribution of the S-related fluorescence rise. These figures are typical, although they may vary significantly depending on the material. The experiment with 5 s spacing between flashes (right hand, squares) shows a similar oscillating pattern with marked decrease of the offset due to inactive centers. This is consistent with a recovery of most of these centers in the few seconds range, as further examined below, while the deactivation of the $S_{2,3}$ states is a slower process. When the induction curve is recorded by starting the illumination at 100 ms after the last flash of a series (not shown), the first phase consists of a decay (after one or two flashes) or a rise (after three or four flashes) towards the plateau level.

It is possible to eliminate the interference of the S states by adding hydroxylamine that extracts the Mn atoms of the oxygen evolving system and acts as an electron donor to PS II. The effect of this addition in both types of experiment is shown in Fig. 1. The dark-adapted level is slightly increased (another example of modulation of the fluorescence yield by the donor side of PS II). The flash oscillations disappear as expected, but the amplitude of the fast induction phase (or the rise caused by the first flash), although diminished, remains slightly larger than the extrapolated amplitude (a) in the control. This suggests that hydroxylamine may increase somewhat the amount of inactive centers (a similar indication was obtained by monitoring the C-550 absorption change in algae – not shown).

Induction kinetics of inactive centers

Figure 2 shows fluorescence induction curves in the absence (bottom) or presence (top) of DCMU, with (open symbols) or without (closed symbols) hydroxylamine. The half-rise times for each curve are indicated by vertical arrows. In

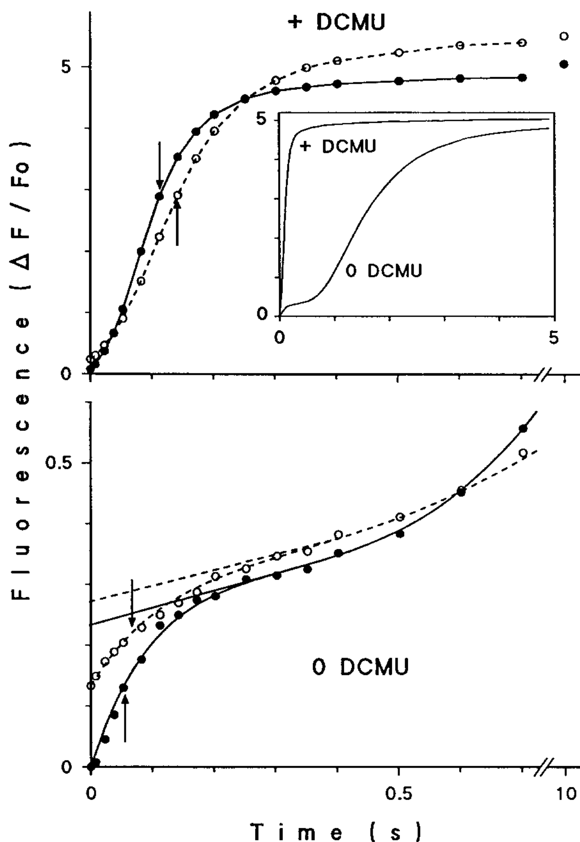

Fig. 2. Fluorescence induction curves in a fresh preparation of spinach chloroplasts. The thylakoids were used at 7.5 μg chlorophyll per ml in a medium containing 0.4 M sorbitol, 2 mM $MgCl_2$, 20 mM KCl, 25 mM MES (pH 6.5) and 1 μM gramicidin. *Bottom panel*: F_o–F_{pl} phase with no addition (solid circles) or 2 mM hydroxylamine (open circles). The straight lines indicate extrapolation of the plateau level, used to estimate the half-times indicated by vertical arrows. *Top panel*: 20 μM DCMU, without (solid circles) or with (open circles) 2 mM hydroxylamine. The datapoints on the right indicate the asymptote levels measured at 10 s used for estimating the overall half times indicated by vertical arrows. The origin (and unit) of the vertical scale is the dark-adapted level F_o with no addition. The inset shows the induction curves (± DCMU, no hydroxylamine) on a longer time scale.

the bottom figure, the estimates of these times relies upon the determination of the asymptotes (extrapolated straight lines), which is somewhat arbitrary. This inaccuracy is, however, of little importance for the qualitative point we want to make, namely that the half-time of the F_o–F_{pl} rise is *markedly shorter* that that of the induction curve with DCMU present. The F_o–F_{pl} phase can be better isolated from the secondary rise by adding acceptors (see Figs. 5 and 9) and the more accurate half-times determined under such conditions also agree with this conclusion.

As explained above, the relevant curve for the kinetics of inactive centers is that obtained in the presence of hydroxylamine (bottom panel, open symbols), where the S-dependent contribution is eliminated. For comparison with the curves obtained in the presence of DCMU (which reflect the photochemical closing of the bulk of PS II centers), it may be more appropriate to consider the kinetics obtained in the absence of hydroxylamine (top panel, solid symbols), because the photochemical efficiency is severely diminished in the combined presence of hydroxylamine and DCMU. In agreement with previous reports (Etienne 1974, Melis and Homann 1976, Joliot and Joliot 1977, Lavergne 1982b) the fluorescence rise caused by a saturating flash under these conditions is markedly smaller than the total variable fluorescence (less than half at pH 6.5). This decreased efficiency accounts for the slower kinetics of the hydroxylamine curve in the top panel. On the other hand, the efficiency of a saturating flash for closing inactive centers in the absence of DCMU remains high in the presence of hydroxylamine (see Fig. 1, right). Thus, the most appropriate comparison of the fluorescence kinetics of inactive and active centers is probably between, respectively, the hydroxylamine experiment with no DCMU and the DCMU experiment with no hydroxylamine. This minimizes the difference between the half-times for which we obtain a ratio of about 0.6.

As explained in the Discussion and Appendix, this finding is qualitatively consistent with the view that inactive centers and the bulk of active (α-type) centers share the same light-harvesting antenna. In order to test this possibility, we investigated the relationship between the fluorescence yield and the amount of closed centers in two strains of algae with marked (about twofold) difference in their PS II antenna complement. In the experiments shown in Fig. 3, the variable fluorescence yield was plotted as a function of the amount of reduced Q_A during a photoreduction experiment using a train of short saturating flashes spaced 75 ms apart. Both algal strains are

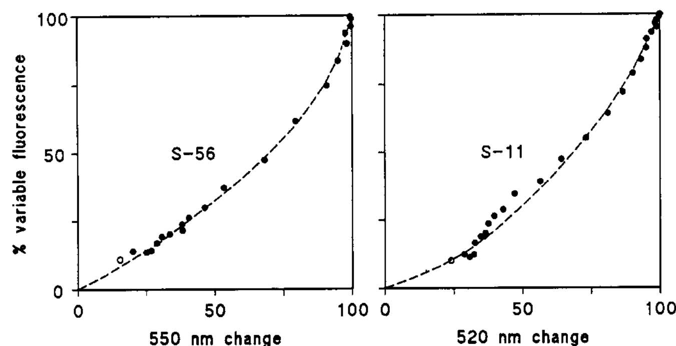

Fig. 3. A plot of the relative variable fluorescence yield against the fraction of closed centers in two strains of *C. Sorokiniana* devoid of PS I centers. The algae were illuminated with a train of 50 short saturating flashes spaced 50 ms apart that completes reduction of the acceptor pool. The ionophore dicyclohexyl-18-crown-6 (1.5 mM) was added in order to prevent accumulation of the membrane potential. The fluorescence level was recorded immediately before each flash. The fraction of closed centers was determined from the 550 nm (indicating Q_A^-) or 520 nm (field-indicating) change. The 550 nm change was recorded with respect to the initial dark-adapted baseline and sampled immediately before the following flash (as for fluorescence). The field-indicating signal was taken as the change at 75 μs after each flash with respect to the level immediately before the flash (a small residual signal remaining after 30 flashes – see Fig. 8 – was subtracted). This gives the relative amount (q) of open centers (the horizontal scale is $1 - q$). The two indicators (550 nm, 520 nm) give basically the same information; however, the 550 nm change is less easily measured in the normally pigmented S-11 and the 520 nm change is smaller and deviates slightly from linearity in the less pigmented S-56. The first 10 flashes were plotted as individual datapoints (with an open symbol for the first flash) and then every second flash. See Fig. 8 for a direct kinetic plot of the 520 nm changes in S-11.

lacking PS I centers with either a normal antenna complement in S-11 (right-hand panel) or lacking part of the chlorophyll-protein complexes (S-56, left panel). Q_A^- was measured either by the 550 nm change or by the field-indicating change at 520 nm. The inactive centers become closed on the first flash (open symbol), whereas the subsequent flashes cause photoreduction of the plastoquinone pool and associated progressive accumulation of Q_A^- in normal centers. It may be seen that in both experiments, no significant break is observed in the hyperbolic relationship between fluorescence and Q_A^-, that would reflect specific fluorescence characteristics of the inactive centers. The fluorescence level on the first flash was not corrected from the S-contribution and is thus slightly overestimated. The relative amounts of inactive centers in these particular experiments are 16% in S-56 and 24% in S-11.

Recovery of inactive centers

As previously reported (Lavergne 1991), the inactive centers or a fraction of them decay with kinetic and spectral characteristics which are similar to 'active' centers with inhibited $Q_A^- \rightarrow Q_B$ transfer through addition of DCMU or similar inhibitors, thus suggesting an $S_2Q_A^- \rightarrow S_1Q_A$ recombination process. As a consequence, one expects that addition of millimolar hydroxylamine, replacing the oxygen evolving complex as a donor, will block the Q_A^- decay by removing the oxidizing substrate, as observed for normal centers in the presence of DCMU (Bennoun 1970). Figure 4 shows the decay kinetics of fluorescence in the absence or presence of hydroxylamine. In order to minimize the S-dependent contribution in the control (as discussed above for Fig. 1), the illumination consisted of a group of four flashes.

In the absence of hydroxylamine, the larger part (70%) of the decay beyond 50 ms takes place with $t_{1/2} \approx 800$ ms. The slower component ($t_{1/2}$ in the tens of seconds) partly reflects deactivation of the small fraction of S_2 and S_3 states present after four flashes. Nevertheless, its amplitude is larger than expected if this were the only contribution, suggesting that a part of the inactive centers does not recombine (a broad spread of the recovery rate was also reported previously by Chylla et al. 1987). We observed this heterogeneous recovery in algae (mutants devoid of PS I centers) as well, by monitoring the decay of the C-550 signal associated with inactive

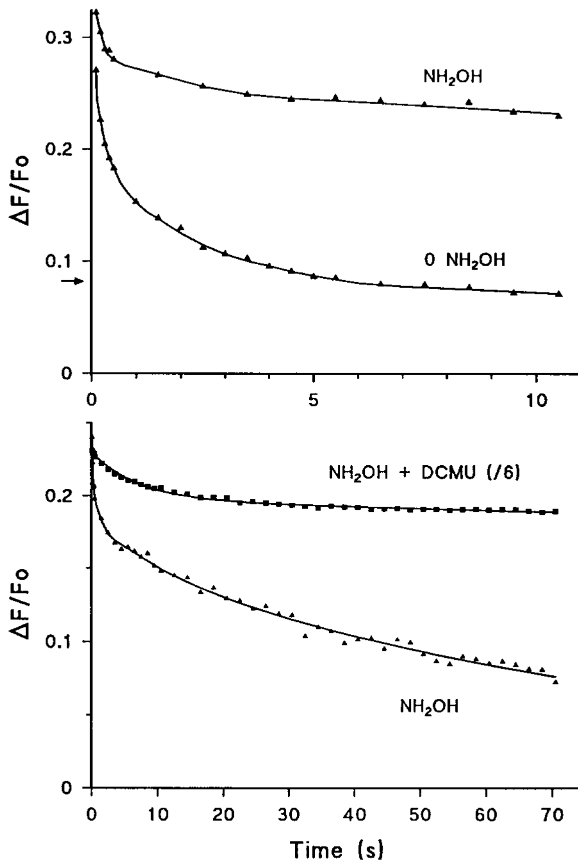

Fig. 4. Kinetics of fluorescence recovery after a group of 4 flashes, 50 ms apart, in spinach thylakoids. *Top*: a comparison between data in the absence or presence of 2 mM hydroxylamine. The origin of the vertical scale is the F_o level in the absence of hydroxylamine; the arrow indicates the modified F_o in its presence. *Bottom*: a comparison between data in the presence of 2 mM hydroxylamine, in the absence or presence of 20 μM DCMU. The time scale is longer than in the top panel and the origin of the vertical scale is now the individual F_o in each experiment; the trace in the presence of DCMU is reduced 6-fold.

centers or the recovery of the field-indicating change (not shown). Tentatively, the non-recombining fraction may be ascribed to inactive centers in the S_0 state in the dark that are photoconverted to $S_1Q_A^-$.

In the presence of hydroxylamine, a drastic slowing down of the decay is observed (top panel). It remains nevertheless multiphasic, with a minor phase in the 100 ms range that is probably not related to inactive centers and another one in the few seconds range. The larger part decreases with $t_{1/2} \approx 70$ s, as may be seen in the bottom panel where the kinetics was plotted on a longer time scale. The decay observed in the presence of hydroxylamine and DCMU is also shown in this figure for comparison. It thus appears that the inactive centers are not as severely blocked as the bulk of active centers in the presence of DCMU.

Can DCBQ reactivate inactive centers?

Figure 5 shows a fluorescence induction experiment in the presence of increasing concentrations of DCBQ. For comparison, the bottom panel shows results obtained with DMQ. Addition of DCBQ at a few micromolar suffices to suppress the rise beyond the F_{pl} level, showing

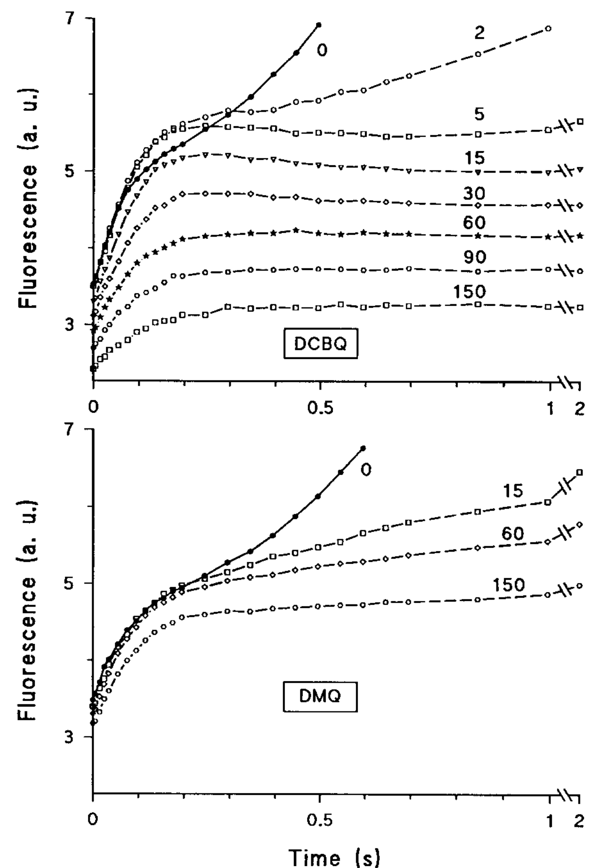

Fig. 5. F_o–F_{pl} phase in the presence of variable concentrations of DCBQ (top) or DMQ (bottom) in spinach thylakoids. The concentration of added quinone (μM) is indicated on each curve.

that it prevents efficiently the accumulation of reduced acceptors in the vicinity of PS II centers. In this respect, DMQ is less efficient and so is ferricyanide in the 100 μM range, as illustrated later in Fig. 9. The amplitude of the F_o–F_{pl} rise increases slightly at low DCBQ concentrations and then decreases according to an overall quenching effect discussed below. The presence of a small overshoot of the fluorescence curve, that was presumably masked by the secondary rise, becomes apparent. As explained in the Discussion, this overshoot is expected from the S-dependent contribution.

Hydroxylamine cannot be used in the presence of DCBQ, but the specific contribution of inactive centers can be estimated in a flash sequence experiment, as explained for Fig. 1. Results are shown in Fig. 6. DCBQ causes a small change in the oscillating pattern by decreasing the value recorded after the first flash and increasing that of the second flash while the rest of the pattern remains unmodified. We have presently no explanation for this finding. As explained earlier, the contribution of inactive centers can be approximately estimated as the level reached after the fourth flash (with respect to the dark-adapted level). As may be seen, no specific decrease of this level occurs in the 10 μM concentration range of DCBQ that was claimed to reactivate

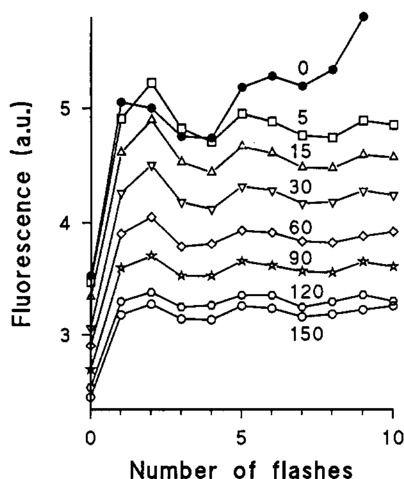

Fig. 6. Fluorescence yield during a series of saturating flashes 150 ms apart in the presence of variable concentrations of DCBQ (same material as in Fig. 4). The fluorescence level was sampled immediately before the next flash. The DCBQ concentration (μM) is indicated on each curve.

electron transfer in inactive centers (Graan and Ort 1986, Cao and Govindjee 1990, Nedbal et al. 1991). When increasing the DCBQ concentration all the fluorescence levels (F_o, F_{pl}, F_m in the presence of DCMU) are quenched, with no indication of a specific effect on the F_o–F_{pl} amplitude (we plotted ΔF_{4F}, the level detected after the fourth flash with respect to the dark-adapted level, as a more specific indicator of inactive centers), as may be seen in the plot of Fig. 7. It should be recalled that for a given concentration of a quencher molecule, the variable fluorescence is expected to decrease more than F_o, because of the hyperbolic Stern–Volmer dependence of fluorescence upon quencher concentration. The inset of Fig. 7 shows a Stern–Volmer plot (1/F vs. [DCBQ]) where the three lines (for F_o, F_m and F_{4F}, the latter characterizing the contribution of inactive centers) have the same slope, indicating an homogeneous quenching effect. These results, which contradict those of Cao and Govindjee (1990), were consistently observed under a variety of conditions (pea or spinach chloroplasts, varying the pH, using 2,5- or 2,6-DCBQ recrystallized or not).

Figure 8 shows the amplitude of the field-indicating change in mutant algae devoid of PS I centers during a series of saturating flashes. The pattern observed in the control is similar to an inverted fluorescence induction curve: The closing of inactive centers is reflected by the decrease between the first and second flash and the subsequent decrease expresses the accumulation of Q_A^- accompanying the pool reduction. This second phase is totally abolished by DCBQ, showing that this substance penetrates the algae and efficiently accepts electrons from PS II. The amplitude of the decrease between the first and second flash remains unaffected by DCBQ, which shows again that this quinone does not reactivate inactive centers. We have no explanation for the small decrease of the absorption change with respect to the first flashes in the control; we checked that it was not caused by non-saturation of the flashes due to quenching by DCBQ. The absence of effect of DCBQ on inactive centers can also be observed (not shown) in the LHC-deficient strain S-56 by monitoring the C-550 change during a train of flashes (as in Fig. 3; strain S-56 is convenient because its field

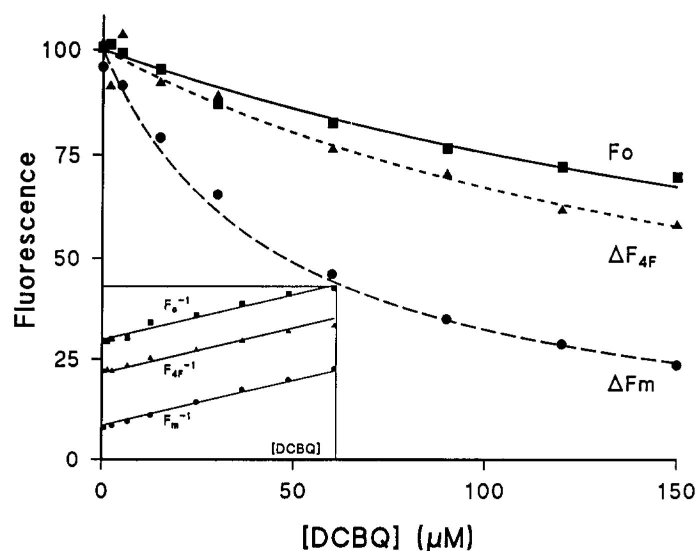

Fig. 7. A plot of various fluorescence levels as a function of DCBQ concentration, using the data of Fig. 6. ΔF_{4F} is the level after the 4th flash minus the dark adapted level. ΔF_m is the total variable fluorescence recorded in a separate experiment with 20 μM DCMU present. The inset is a Stern–Volmer plot of the reciprocal of the total fluorescence levels (measured from the zero) against the DCBQ concentration (the full horizontal scale is 150 μM).

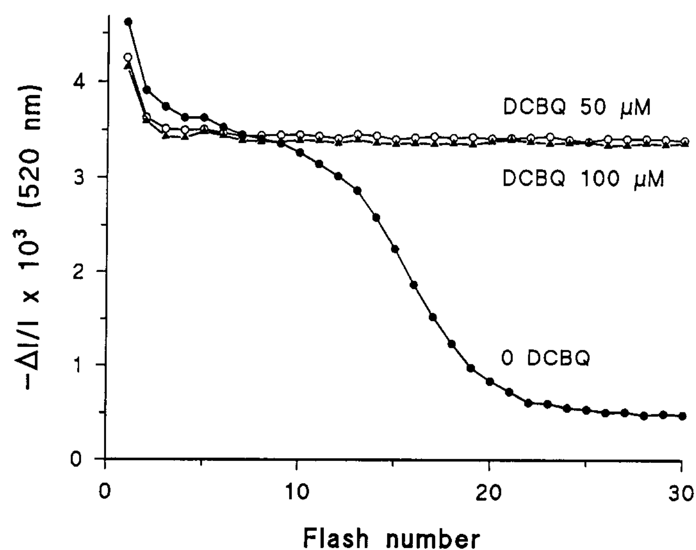

Fig. 8. A plot of the 520 nm change caused by each flash of a series (150 ms apart) in the S-11 strain of *C. Sorokiniana* in the presence of 1.5 mM dicyclohexyl-18-crown-6 (this ionophore prevents accumulation of membrane potential). Each datapoint is the change at 40 μs after a flash with respect to the absorption preceding the flash. Solid circles, control; open circles and closed triangles, 50 μM and 100 μM DCBQ, respectively.

indicating change is very weak in this region). The offset on the first flash was almost unaffected by DCBQ, while upon later flashes a photoreduction of cytochrome-*f* and plastocyanin was observed (at variance with the control in the absence of DCBQ).

Enhancement of steady-state electron flow by DCBQ

Most of the experiments that led to the conclusion of a reactivation of inactive centers by DCBQ were carried out under steady-state il-

lumination conditions. The technique used by Graan and Ort (1986) consists of a measurement (with a pH electrode) of the acidification caused by PS II during a steady-state train of saturating flashes (50 ms apart) with ferricyanide as a terminal electron acceptor. When adding DCBQ as a mediator between PS II and ferricyanide, the acidification slope was markedly increased (by 40% in Graan and Ort 1986). This enhancement was not observed with other quinones, such as DMQ. It should be realized that the results shown in Fig. 5 do imply a differential effect of DCBQ and DMQ on the steady state electron flow through PS II, since the secondary fluorescence rise was totally suppressed by a few micromolar of the former quinone, but not of the latter.

The experiments shown in Figs. 9 and 10 were designed to show that both the enhancement of electron flow by DCBQ *and* the absence of effect of this quinone on inactive centers can be observed in the same material (freshly prepared thylakoids according to the procedure of Graan and Ort 1981, were used in these experiments). Fluorescence induction curves are shown in Fig. 9, with no added acceptor (A), 100 μM ferricyanide (B), or 100 μM ferricyanide plus 10 μM DCBQ (C). The inset shows the fluorescence levels during a series of flashes in conditions (B) and (C). The contribution of inactive centers was estimated as above (extrapolation of the oscillation minima towards the origin) and shown by the vertical arrows. Identical values were obtained in (B) and (C), showing that in this material as well as in frozen thylakoids, DCBQ did not cause any decrease in the amount of inactive centers. It may also be noticed that the amplitude of the oscillating contribution does not increase as would be expected if the amount of O_2-evolving centers was increased by DCBQ. As mentioned earlier, a secondary fluorescence rise persists in the presence of ferricyanide, which is abolished by DCBQ. Thus, a fraction of PS II centers becomes blocked with reduced Q_A^- at steady-state in spite of the presence of ferricyanide and this block is relieved by DCBQ. Figure 10 shows how this state of things affects electron transfer rates. The oxidation of ferricyanide was monitored through the absorption decrease at 430 nm caused by a train of saturating flashes (75 ms apart). The steady-state slope is increased in the presence of DCBQ (the ratio

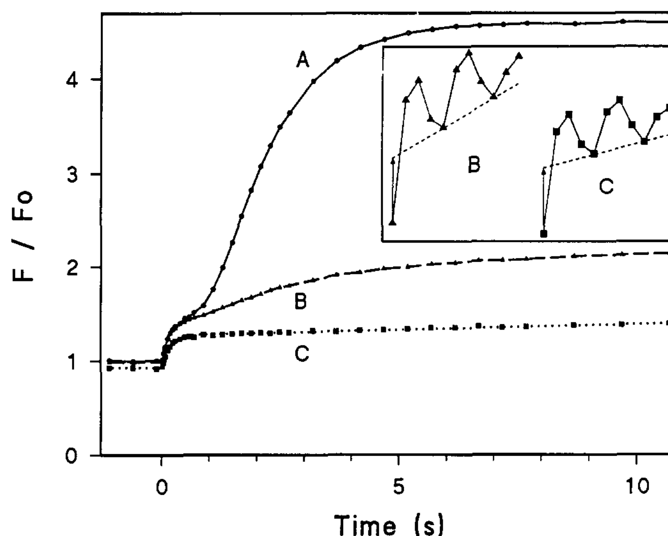

Fig. 9. Fluorescence induction curves using a fresh preparation of thylakoids. The material was diluted at 10 μg chlorophyll per ml in a medium containing 0.4 M sorbitol, 2 mM $MgCl_2$, 20 mM KCl, 25 mM MES (pH 6.5) and 1 μM gramicidin. (A) no addition; (B) and (C) 100 μM ferricyanide, with 10 μM DCBQ in (C). The inset shows a flash sequence experiment in samples (B) and (C) with a flash spacing of 75 ms. The fluorescence was sampled immediately before each flash (the first datapoint is the dark-adapted level). The vertical scale is enlarged 4-fold with respect to the main figure. The dashed lines indicate extrapolation of flashes 4 and 8 and the vertical arrows give an estimate of the contribution of inactive centers.

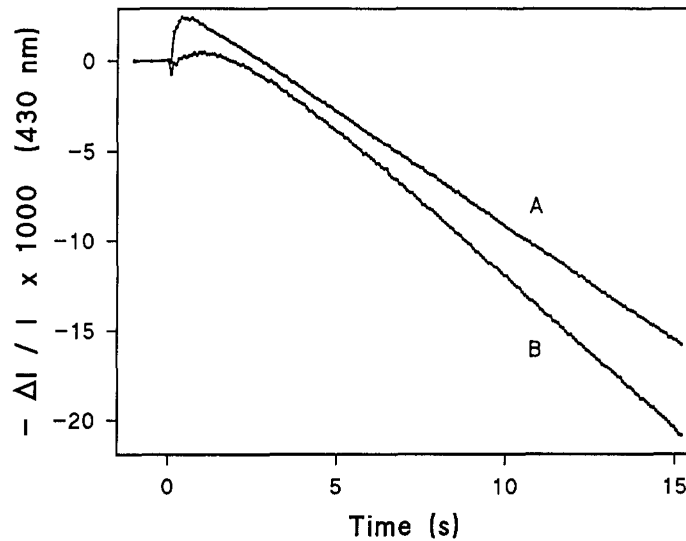

Fig. 10. Photoreduction of ferricyanide during a train of 200 saturating flashes spaced 75 ms apart. Same material and conditions as in Fig. 10, except the chlorophyll concentration was 17 μg/ml. 100 μM ferricyanide was present and, in (B), 10 μM DCBQ. The absorption of 430 nm was sampled immediately before each flash. A control in the absence of ferricyanide showed an absorption increase to a fixed level (4 units) terminated at 1.5 s.

of the slopes A/B is 74%), in qualitative agreement with Graan and Ort's finding.

Discussion

Antenna of inactive centers

As shown in Fig. 2, the kinetics of the F_o–F_{pl} fluorescence rise has, in our hands, a markedly shorter half-time than the overall kinetics measured in the presence of DCMU. For this comparison, one should consider the F_o–F_{pl} curve obtained with hydroxylamine present, which eliminates the S-dependent contribution from active centers. We obtained for the relative amplitude of the latter a figure of about 35% which is probably an underestimate because of the linear extrapolation used in Fig. 1. This ratio is not expected to be constant, since the amount of relative centers does vary depending on the material (see below). The half-time of the F_o–F_{pl} phase in the presence of hydroxylamine is slightly larger than in its absence, as expected from the distortion caused by the S-dependent kinetics (see the bottom inset of Fig. 11) because of the overshoot reflecting a transient maximum of $[S_2] + [S_3]$. Nevertheless, it is about 1.7-fold smaller than that of the DCMU curve. Clearly, this finding excludes that the antenna size of inactive centers could be of the β-type responsible for the slower phases of the DCMU curve.

Considering the shorter half-time and faster initial slope (when amplitudes are normalized) of the fluorescence kinetics of inactive centers, one might be tempted to adopt the opposite view that inactive centers have a larger antenna size than even the α-type centers. On closer examination, it turns out that our data are in satisfactory agreement with the simple assumption that inactive centers share the same antenna as active centers (in majority of the α type) and compete with them for trapping excitation energy. The consequences of this assumption are analyzed in the Appendix, with results illustrated in Fig. 11. The theoretical predictions are that, provided inactive centers are a small fraction of the total centers, their fluorescence kinetics (curve B) should be exponential, with shorter half-time than the DCMU curve (A) and $(J + 1)$ times faster initial slope (for normalized amplitudes). J is a parameter, defined in the Appendix, which characterizes the exciton transfer in the α antenna (thus the degree of sigmoidicity of the DCMU curve). In stacked thylakoids, $J \approx 1.25$. At later times, curves A and B intersect, so that the final

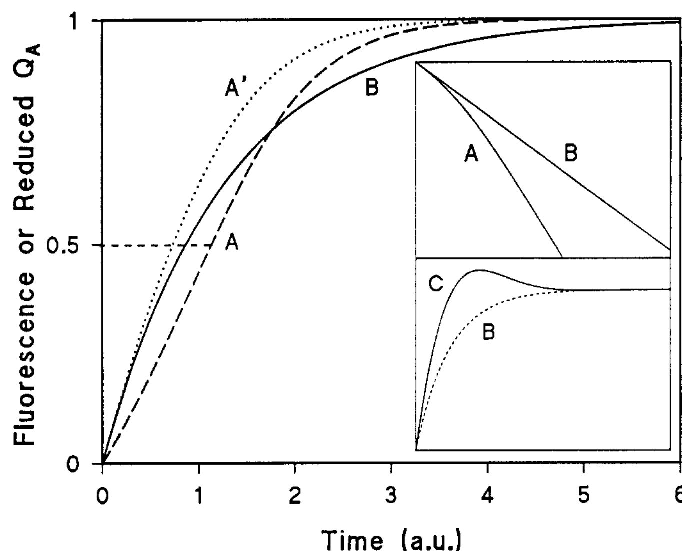

Fig. 11. A simulation of fluorescence or center closing kinetics. (A) is the fluorescence induction (a 'DCMU curve') computed with Eqs. (12) and (1) of the Appendix, with $J = 1.25$. (A') is the corresponding kinetics of the fraction of closed centers $(1 - q)$. (B) is the time course of fluorescence or amount of closed centers when a small fraction of ('inactive') centers is subject to photochemical closing in competition with the rest of the centers remaining permanent traps (Eq. (21)). All curves are normalized at 1 (vertical scale). *Top inset*: A semi-log plot of q(t) corresponding to curves (A') and (B), as indicated (the full horizontal scale is the same as that of the main figure). *Bottom inset*: A comparison between curve (B) and the time course of the sum $(S_2 + S_3)$ with initially 25% S_0, 75% S_1. This was computed from the evolution predicted by the Kok model for the S-states during a sequence of weak flashes (we used a miss parameter $\alpha = 0.98$). The relationship between the 'flashing frequency' ν and the rate constant ρI of B is: $\nu = \rho I/(1 - \alpha)$.

part of A is faster than that of B . The slower initial *fluorescence* kinetics of the DCMU curve does not mean a slower kinetics for the *amount* of centers getting closed (that can be obtained by integrating the fluorescence curve; for the F_o–F_{pl} phase the fluorescence kinetics is close to an exponential so that the integration is unnecessary), as may be seen by comparing curves A' and B. The simulations shown in Fig. 11 are only meant to illustrate the qualitative agreement with our results and omit the β-type contribution to the DCMU curve. When comparing the experimental half-times (Fig. 2), we simply took the overall half-time of the DCMU curve, thus including the slower phases: This probably accounts for the larger difference found experimentally than shown for curves A and B in Fig. 11.

The non-sigmoidal fluorescence kinetics of the inactive centers was generally interpreted as indicating separate photosynthetic units with no excitation transfer between them (Melis 1985, Hsu and Lee 1991). This conclusion is quite unnecessary, as shown in the Appendix and Fig. 11. An exponential time course is indeed expected when the inactive centers are a small fraction of the trapping centers so that closing a fraction of them modifies only little the photochemical rate of the remaining open centers. Deviation from the exponential character should only become significant when the fraction of inactive centers exceeds about 40%.

Consistently with the view that inactive centers do not have antenna heterogeneity with respect to the other centers, we found a smooth hyperbolic relationship between the fluorescence yield and amount of Q_A^-, in two strains of algae, with normal or deficient pigment complement, with no discontinuity corresponding to the F_o–F_{pl} phase. As explained in the Appendix, this should not be the case, except coincidentally, if inactive centers were organized in disconnected units – and the coincidence should not be maintained when altering the PS II antenna.

The initial slope of the relationship between fluorescence yield and amount of closed centers

is J + 1. As verified in algae, where the inactive centers were measured from absorption changes, this is the factor with which the relative amplitude of the F_o–F_{pl} rise (with the S-contribution eliminated) should be multiplied in order to obtain the relative amount of inactive centers. It is worth noting that, as shown in the Appendix (Eq. (26)), the complementary area of the F_o–F_{pl} phase *does not* yield the amount of inactive centers but (J + 1) times less, i.e., the same quantity as obtained directly from the fluorescence amplitude. Applying this method (fluorescence amplitude multiplied by $J + 1 \approx 2.25$), we obtained a range of 7–22% inactive centers in various preparations of pea or spinach thylakoids. In algae (mutant strains of *C. Sorokiniana* devoid of PS I centers), using either fluorescence or absorption changes, we found a somewhat larger fraction of inactive centers (15–30%) and more variability (according, presumably, to growth conditions): Figures as high as 50% were obtained in some instances (Lavergne 1987, Joliot et al. 1990). Our estimate for pea or spinach thylakoids (7–22%) is below the figures reported by other authors (40% in Graan and Ort 1986, 32% in Chylla et al. 1987), although in agreement (see below) with the fluorescence results of Hsu and Lee (1991). Possible reasons for this discrepancy will be suggested later.

Another argument against a preferential location of inactive centers in stromal regions is that the specific contribution of inactive centers (to an extent at least similar as in thylakoids) on the first flash of a series has been observed in granal membrane ('BBY', Berthold et al. 1981) preparations supplemented with DCBQ as an acceptor (Dekker et al. 1987, Lavergne 1991). An advantage of having the inactive centers sharing the antenna of normal centers is to limit their impact as to the light-harvesting efficiency of photosynthesis: A significant fraction (50–60%) of the excitons that visit a closed inactive center is still available for transfer to an active one.

Results such as those of Fig. 2, indicating a markedly faster initial kinetics of the F_o–F_{pl} rise compared with induction kinetics in the presence of DCMU, where, in our hands, totally reproducible, using a variety of thylakoid preparations or algae. Therefore, we have difficulty for suggesting possible explanations of the disagreement with other authors (e.g. Melis 1985) who reported the opposite result. Tentatively, we make the following remarks. The initial rate (and half-time) of the DCMU curve may be apparently accelerated when a significant fraction of Q_B^- is initially present and becomes converted to Q_A^- upon addition of DCMU (Velthuys and Amesz 1974): This causes truncation of the slower part of the sigmoid. Also, it may happen that the F_o–F_{pl} phase is not clearly separated from the secondary rise phases, so that its kinetics is mixed with slower events reflecting multiple turnover (this may also account for larger F_o–F_{pl} amplitudes than reported here). Another point is that, as noted above, when comparing the kinetics of the *amount* of closed centers, we do expect a slightly slower time course for inactive centers (but identical initial slope), as illustrated by curves B and A′ in Fig. 11. A detailed study of such kinetics was recently published by of Hsu and Lee (1991) who found equal initial slopes for inactive centers and the *α-type* centers (with (DCMU) in full agreement with our views (Hsu and Lee give the results of an experiment using Tris-washed thylakoids in which the S-contribution does not interfere). Another feature in this work which agrees with our analysis is that similar values (about 10%) were obtained for the relative amplitude of the F_o–F_{pl} rise and the relative complementary areas computed on this curve and on the DCMU curve (according to the previous discussion, the value of 10% corresponds to about 22% inactive centers).

However, there is evidence from other techniques supporting a small antenna size of the inactive centers. In the paper of Nedbal et al. (1991), the enhancement of steady-state electron flow by DCBQ compared with DMQ was shown to decrease at low intensity. This was interpreted as indicating a lower antenna size of the inactive centers that were assumed to be specifically reactivated by DCBQ. As explained later, we believe that the differential effect of DCBQ on steady-state electron flow is not related to inactive centers and that the meaning of the light saturation curves found by these authors should be reexamined accordingly. This remark does not apply, however, to the work of Chylla and Whitmarsh (1990) who studied the saturation curve of the field-indicating absorption change

on a first flash (all centers involved) or on a second flash following a saturating one (only active centers involved). These authors concluded that the antenna size of the inactive centers was about twofold smaller than that of active centers. We have no clear-cut objection against this method and can only suggest several possible problems. A difficulty is that when both photosystems are present, the interpretation of absorption changes becomes more complicated. A number of assumptions have to be made concerning the absence of extraneous signals interfering with the 518 nm measurement and the recovery and photochemical efficiency of PS I. Small distortions may affect a result which relies on the accurate measurement of the saturation curve of a 15% fraction of the total field-indicating change. Besides, a key hypothesis in this work is that the cross section of active centers is the same on the first or second flash. This is not expected to be so, however, if active and inactive centers share the same antenna, so that closing the inactive centers will increase the cross section of active centers on the second flash. However, we do not believe that this effect can fully account for the large difference in saturation curves that was reported. A possible additional distortion may arise from the build-up of the membrane potential caused by the 50 ms-spaced flashes. The membrane potential was reported by Diner and Joliot (1976) to induce a fluorescent, closed state of PS II centers. This effect would also increase the photochemical efficiency on the second flash (and might account for the large – compared with our estimates – fraction of inactive centers, 30%, reported in these experiments).

Inactive centers and enhancement of electron flow by DCBQ

A specific effect of halogenated quinones, such as DCBQ, that would allow reoxidation of Q_A^- in inactive centers has been reported by a number of authors (Graan and Ort 1986, Cao and Govindjee 1990, Nedbal et al. 1991), but not confirmed by Chow et al. (1991). We could not find evidence supporting such an effect, monitoring either fluorescence or absorption changes (C-550 or field-indicating change) under a variety of conditions (material, pH, DCBQ type). Also in BBY particles with DCBQ in the 100 μM range, the presence of inactive centers was reported by Dekker et al. (1984) and Lavergne (1991).

Our results with DCBQ do not agree with the suggestion of Jursinic and Dennenberg (1988) of a significant amount of double turnovers when using this substance. For instance, we did not find an increase of the field-indicating change (but a slight decrease, Fig. 8). The oscillation pattern associated with the S-system (Fig. 6 and Dekker et al. 1984, Lavergne 1991) does not reveal an increased double hitting in the presence of DCBQ.

In order to discuss the discrepancy with authors who reported reactivation of inactive centers by DCBQ one should distinguish between two types of experiments involving either a single turnover measurement or steady-state electron flow. In the latter case, as explained below, our disagreement does not bear on the data but rather on their interpretation. The main evidence suggesting that inactive centers, characterized on a single turnover basis, can readily transfer electrons to DCBQ is the fluorescence experiment shown in Fig. 1 of the paper by Cao and Govindjee (1990). As illustrated in Figs. 5–7 and 9, we could not reproduce the result of these authors indicating total suppression of the F_o–F_{pl} rise at 15 μM DCBQ. Besides the disagreement on this effect, there are several features that we do not understand in these data, such as the sharp break occurring in the kinetics (instead of homothetic lowering) for non-saturating concentrations of DCBQ, or the absence of quenching of F_o by DCBQ. Above all, the suppression of the F_o–F_{pl} rise implies the unlikely result that the S-dependent contribution would be also cancelled by DCBQ. One actually expects that the reactivation of inactive centers should increase this contribution while decreasing that of inactive centers. This is why we took care to characterize independently the effect of DCBQ on each contribution (Figs. 6–7 and 9). Our feeling is that the true F_o–F_{pl} phase is not well resolved in the experiments of Cao and Govindjee, possibly because of slow shutter opening and use of a two high illumination intensity. Actually, their results could be reconciled with ours if we assume that the fluorescence rise they

measured is homologous to the secondary phase (ferricyanide- or DMQ-resistant) shown in Figs. 5 and 9, which is indeed quenched by DCBQ.

Concerning the reports indicating enhancement of steady-state electron flow by DCBQ, our view is that this effect should not be attributed to inactive centers, characterized as behaving like DCMU-inhibited centers in procedures that involve basically one photochemical turnover. It should be recalled that under steady-state illumination *even with limiting light intensity*, neither PS II nor PS I centers are 100% in an open state. This finding was described long ago by Joliot, Joliot and Kok (1968) under the term of a low (apparent) equilibrium constant (3–10) between the steady-state concentration of open PS I and PS II centers under illumination of limiting intensity and uncoupled conditions. The fraction of open PS II centers was only 42% under a 650 nm illumination, 75% at 686 nm. The full extent of open PS II centers was only obtained under a background illumination of $\lambda > 700$ nm. These results involved O_2 evolution measurements and thus concern the *active* centers. In agreement with this finding, Chow et al. (1991) stress the requirement of a weak far red background illumination in order to measure the full extent of PS II activity during a steady-state regime.

The experiments shown in Figs. 9 and 10 establish the link between the results of Joliot et al. (1968) and the enhancement of steady-state electron flow by DCBQ described by Graan and Ort. Acceptors such as ferricyanide or DMQ (see Fig. 5) or, for that matter, PS I acceptors such as methyl viologen, do not suppress a secondary fluorescence rise reflecting accumulation of Q_A^- in a fraction of PS II centers. On the other hand, DCBQ does suppress it, keeping the active centers fully open and enhancing accordingly the steady-state electron flow. The PS II centers in which Q_A becomes reduced in spite of ferricyanide being present are 'active' in the sense that they do transfer electrons to plastoquinones. The blocking of electron transfer appears to be due to a deficient *reoxidation* of (pool) plastoquinol rather than deficient plastoquinone *reduction*: Thus the distinction between inactive centers (on a single turnover basis) and steady-state accumulation of reduced Q_A in a fraction of active PS II centers is not purely semantic. The ability of DCBQ to prevent electron accumulation in these domains is in line with the finding (Lavergne 1991) that this quinone substitutes plastoquinone in the Q_B pocket, accepting electrons directly from Q_A^-.

The cause of the accumulation of reduced Q_A in a fraction of PS II centers in spite of the presence of ferricyanide and limiting illumination intensity is by no means straightforward. We believe this is related with the fact that global thermodynamic equilibration between PS II centers and plastoquinones is not achieved on a fast time-scale (Joliot et al. 1989, Lavergne and Joliot 1990, Joliot et al. 1992). In these papers, it was argued that fast equilibration only occurs on a local scale in isolated domains. In order to account for the occurrence of a fraction of totally reduced domains under steady-state illumination in the presence of ferricyanide (or other quinones than DCBQ), additional assumptions must be made concerning electron transfer to PS I. An interesting possibility is the hypothesis (Joliot and Joliot 1992) that some domains lack the b_6–f complex. However, this would not easily account for the wavelength dependence (the fraction of closed PS II centers under weak light illumination – adjusted at a constant PS II efficiency – depends on the relative absorption of PS I) found by Joliot et al. (1968) or Chow et al. (1991).

Inactive centers and 'non-B acceptors'

The literature on inactive centers often refers to previous work by one of us (Lavergne 1982a,b) concerning what he called 'non-B' acceptors. Although, clearly, the denomination of inactive centers as non-Q_B or non Q_B-accepting is fully justified, it should be made clear that the phenomena described in this earlier work have nothing to do with the inactive centers discussed in the present paper. The non-B acceptors were introduced to explain a number of findings. One of these (Lavergne and Etienne 1980) was the fact that the intensity of delayed light, when stimulated by membrane potential or ΔpH, displayed a marked S-dependence but no binary oscillation indicating Q_B^- as the reducing substrate (a fact that was rediscovered by Vos et al. 1991). This suggested that a fraction at least of

active centers (since turnover of the S-states was observed) could store an electron on their acceptor side with no modulation by the Q_B gate. Another finding was that less than half of the variable fluorescence was involved in the back transfer $Q_B^- \rightarrow Q_A$ induced by mixing with DCMU immediately after flash preillumination (Lavergne 1982a,b). In these experiments, the delay between preillumination and analysis was shorter than the recovery time of inactive centers, so that their contribution caused only a constant (and small) offset to the fluorescence levels (that was indeed observed). Part of these experiments were done in the presence of hydroxylamine, which blocks the recovery of inactive centers from the first flash on (see Fig. 4 in the present paper). Nevertheless, the large gap between the level induced by mixing with DCMU after one preillumination flash and the maximum level was still observed. From a number of arguments, it was inferred that the 'non-B' phenomenon involved a second turnover of the centers after reduction of Q_A, hence the term of non-B *acceptors*, rather than *centers*. Somewhat disturbingly for the author of this work, it must be recognized that in spite of considerable progress in our knowledge of the PS II center, no further insight has been gained on this matter – nor evidence for a basic flaw. At any rate, real or not, the non-B acceptors of 1978–1980 and inactive centers are two different things.

Origin of inactive centers

Several reasons may be imagined for explaining the presence of a fraction of inactive PS II centers, with a deficient or obstructed Q_B pocket. This may arise from a degradation process of the active centers attacking first this region of the complex. However, the impairment resulting from photoinhibition is characterized by a quenching form of the centers (Kyle et al. 1984, Krause 1988) which does not match the properties of inactive centers. Thus, it is more attractive to envisage these centers as precursors of active centers in a reparation process. One would expect, in this case, the final processing to occur in the stromal region, where the inactive centers should be stored (Guenther and Melis 1990). This view is not supported by our data showing that inactive centers are mainly present in grana regions. Another hypothesis was proposed in previous work (Lavergne and Joliot 1991, Lavergne et al. 1992), assuming an obstruction of the Q_B pocket resulting from the more or less random crowding of the membrane with integral proteins. In this case the inactive centers would not be a structurally and permanently impaired fraction of PS II centers, but reflect a statistical dynamic distribution (although the slow recovery, 70 s, obtained with hydroxylamine would require an unexpectedly slow rearrangement rate). A prediction and possible test of this model would be a decrease in the amount of inactive centers when diluting the protein concentration, e.g., by adding membrane lipids.

Acknowledgements

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Appendix

1. Fluorescence induction in the presence of DCMU

A simple expression relating the fluorescence yield to the amount of open centers is:

$$\frac{\phi_v}{\phi_v^m} = \frac{1-q}{1+Jq} \quad (1)$$

in which q is the fraction of open centers, ϕ_v the variable fluorescence yield, ϕ_v^m its maximal amplitude (when $q = 0$) and J a parameter characterizing exciton transfer. This expression was shown by Joliot and Joliot (1964) to fit the experimental data with J in the range of 1–1.5. It was derived by these authors by assuming that an exciton visiting a closed center has a probability p ($=0.55 \pm 0.05$) to visit another center. In this model, $J = p/(1-p)$.

It is worthwhile showing that expression (1) is valid under rather general assumptions. For example, let us assume that the exciton decay occurs through the following processes, with associated first or pseudo first order rate constants indicated in parentheses: Fluorescence (f), non-radiative deexcitation (d), photochemical trapping (φq). We also assume that the encounter with a closed center may cause deexcitation ($\varphi'(1-q)$), a fraction γ of this process being radiative. This pathway accounts phenomenologically for various possibilities such as trapping by a closed center, or energy barrier for an exciton to escape the longer wavelengths pigments surrounding the center ('funneling' of exciton transfer). One has:

$$\phi = \frac{f + \gamma\alpha\varphi(1-q)}{f + d + \alpha\varphi + (1-\alpha)\varphi q} \quad (2)$$

where $\alpha = \varphi'/\varphi$. For $q = 1$:

$$\phi_o = \frac{f}{f + d + \varphi} \quad (3)$$

and for $q = 0$:

$$\phi_m = \frac{f + \gamma\alpha\varphi}{f + d + \alpha\varphi} \quad (4)$$

The quantum yield of photochemistry for $q = 1$ is:

$$\rho = \frac{\varphi}{f + d + \varphi} \quad (5)$$

The photoreduction rate of Q_A in the presence of DCMU is:

$$-\frac{dq}{dt} = I \frac{\varphi q}{f + d + \alpha\varphi + (1-\alpha)\varphi q} \quad (6)$$

where I is the rate of exciton creation. From (2)–(4), one derives expression (1), with:

$$J = \frac{\phi_v^m}{\phi_o + \gamma \frac{\alpha}{1-\alpha}} \quad (7)$$

or, using Eq. (5):

$$J = \frac{\rho(1-\alpha)}{1-\rho(1-\alpha)} \quad (8)$$

Thus, Eq. (1) is valid in the whole range of models from isolated units ($\alpha = 1$, $J = 0$) to free exciton motion ($\alpha = 0$, $J = \rho/(1-\rho)$). The same conclusion was established by Paillotin (1976), using a different derivation.

One can similarly rewrite (6) as:

$$-\frac{dq}{dt} = \rho I \frac{\phi_v^m - \phi_v}{\phi_v^m} \quad (9)$$

which indicates how q can be computed by integrating the fluorescence curve (see Malkin an Kok 1966). Using Eq. (1), one has also:

$$-\frac{dq}{dt} = \rho I(1+J) \frac{q}{1+Jq} \quad (10)$$

Thus

$$-\rho I(1+J)\,dt = J\,dq + \frac{dq}{q} \quad (11)$$

which, as indicated by Joliot and Joliot (1964), is integrated as:

$$\rho I(1+J)t = J(1-q) - \mathrm{Ln}\,q \quad (12)$$

where the integration constant was taken so as to have $q = 1$ for $t = 0$. For each value of q one can compute the corresponding time using Eq. (12). The q(t) curve (A') in Fig. 11 was obtained in this manner and the ϕ(t) curve (A) deduced from q(t) using Eq. (1).

2. Inactive centers sharing a common antenna with active centers

We assume that the inactive centers represent a fraction x of the total amount of centers (which is kept equal to 1) and the normal centers $(1-x)$ are now considered as permanent traps (no DCMU), that compete with inactive centers for trapping excitation from a common antenna. Let β denote the fraction of open inactive centers. Equation (1) can be used with $q = 1 - x(1-\beta)$:

$$\frac{\phi_v}{\phi_v^m} = \frac{x(1-\beta)}{1 + J - Jx(1-\beta)} \quad (13)$$

The maximum fluorescence $\phi_v^{m'}$ when all inactive centers are closed ($\beta = 0$) is:

$$\frac{\phi_v^{m'}}{\phi_v^m} = \frac{x}{1 + J - Jx} \quad (14)$$

and (13) can be rewritten:

$$\frac{\phi_v}{\phi_v^{m'}} = \frac{1-\beta}{1+\beta\dfrac{Jx}{1+J-Jx}} \tag{15}$$

For small values of x, this is approximated as:

$$\frac{\phi_v}{\phi_v^{m'}} \simeq 1-\beta \tag{16}$$

In order to obtain the differential equation for the rate of closing of inactive centers ($d(\beta x)/dt$), we notice that the right-hand side of Eq. (10) still gives the rate of photochemical trapping. A fraction $\beta x/q$ of this rate is directed towards inactive centers, the rest ($(q-\beta x)/q$) is quenched by the permanent traps. Thus:

$$-x\frac{d\beta}{dt} = \rho I(1+J)\frac{q}{1+Jq}\frac{\beta x}{q} \tag{17}$$

Rearranging and using $q = 1 - x(1-\beta)$:

$$-\frac{d\beta}{dt} = \rho I(1+J)\frac{\beta}{1+J(1-x)+Jx\beta} \tag{18}$$

Using the same method as above, integration of Eq. (18) gives:

$$\rho I(1+J)t = Jx(1-\beta) - [1+J(1-x)]\,\mathrm{Ln}\,\beta \tag{19}$$

For small x, this gives $\rho It \approx -\mathrm{Ln}\,\beta$, thus:

$$\beta \simeq e^{-\rho It} \tag{20}$$

and, using Eq. (16):

$$\frac{\phi_v}{\phi_v^{m'}} \simeq 1 - e^{-\rho It} \tag{21}$$

The significance of these equations is that, when the fraction of inactive centers is small, they become closed according to an exponential kinetics (20) and the fluorescence kinetics is exponential as well because of the linear $\phi(\beta)$ relation (16). Computation of $\beta(t)$ using the (non-approximate) Eq. (19) shows that the deviation of the fluorescence curve from the exponential behavior will remain smaller than experimental accuracy for $x < 40\%$.

As shown in Fig. 11, the normalized fluorescence kinetics of inactive centers (B) computed from Eq. (21) is *initially faster* than that computed for active centers (with DCMU) using Eqs. (12) and (1). This may be checked by computing the initial slope of both curves. For the DCMU curve, one has:

$$\frac{d}{dt}\left(\frac{\phi_v}{\phi_v^m}\right) = \frac{d}{dq}\left(\frac{\phi_v}{\phi_v^m}\right)\frac{dq}{dt} \tag{22}$$

Using Eqs. (1) and (10), this gives:

$$\frac{d}{dt}\left(\frac{\phi_v}{\phi_v^m}\right) = \rho I\frac{(1+J)^2}{(1+Jq)^3}q \tag{23}$$

Thus, for $q = 1$ ($t = 0$):

$$\frac{d}{dt}\left(\frac{\phi_v}{\phi_v^m}\right) = \frac{\rho I}{1+J} \tag{24}$$

For inactive centers, using Eq. (21), the initial slope is just ρI, thus $J + 1 \approx 2.25$ times faster than that of the DCMU curve. However, the two curves intersect at a later time (see Fig. 11) and the kinetics of the *amount* of closed centers is in fact faster for active centers, with the same initial slope (ρI) for both $q(t)$ and $\beta(t)$.

It is of interest to examine the significance in this model of the complementary area (S) of the fluorescence rise due to inactive centers. One has:

$$S = \int_0^\infty \rho I\frac{\phi_v^{m'} - \phi_v}{\phi_v^m}dt = \rho I\frac{\phi_v^{m'}}{\phi_v^m}\int_0^\infty\left(1-\frac{\phi_v}{\phi_v^{m'}}\right)dt \tag{25}$$

For small x, we may use Eq. (21) and get:

$$S \simeq \frac{\phi_v^{m'}}{\phi_v^m} \simeq \frac{x}{1+J} \tag{26}$$

Thus, compared with the area of the DCMU curve, S does not give directly the relative amount of inactive centers, but $(J+1)$ times less, as does the ratio of variable fluorescence amplitudes (obtained from Eq. (14) for small x).

3. *Inactive centers with a specific antenna*

Two cases may be envisaged. First, inactive centers share a common antenna of their own, with energy transfer between them, but no competition from active centers. In this case

their fluorescence kinetics should be markedly sigmoidal, which is not observed.

The second possibility is to assume separate units for inactive centers. Their fluorescence kinetics should then be exponential, but we now have a contradiction with the results of Fig. 3. The maximum fluorescence for inactive centers is given by Eq. (14) in the case of a common antenna shared with active centers. Transposition to a separate unit model is easily obtained by setting $J = 0$, which gives $\phi_v^{m'}/\phi_v^m = x$. Thus, in the case of separate units, the datapoint for the first flash in Fig. 3, corresponding to $\phi_v^{m'}$, should lie on the diagonal, whereas it is found close to the $\phi(q)$ curve of the bulk of PS II. It could be argued that if inactive center units have specific trapping and fluorescence parameters, the above finding might be coincidental. However, the fact that this observation remains true when the antenna size of granal PS II centers is changed about two-fold (comparing strains S-11 and S-56) is a strong argument against such a coincidence.

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