Plant Physiol. (1989) 90, 765–772
0032-0889/89/90/0765/08/$01.00/0

Received for publication October 24, 1988
and in revised form February 14, 1989

Inactive Photosystem II Complexes in Leaves[1]

Turnover Rate and Quantitation

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ABSTRACT

The flash-induced electrochromic shift, measured by the amplitude of the rapid absorbance increase at 518 nanometers (ΔA518), was used to determine the amount of charge separation within photosystems II and I in spinach (*Spinacia oleracea* L.) leaves. The recovery time of the reaction centers was determined by comparing the amplitudes of ΔA518 induced by two flashes separated by a variable time interval. The recovery of the ΔA518 on the second flash revealed that 20% of the reaction centers exhibited a recovery half-time of 1.7 ± 0.3 seconds, which is 1000 times slower than normally active reaction centers. Measurements using isolated thylakoid membranes showed that photosystem I constituted 38% of the total number of reaction centers, and that the photosystem I reaction centers were nearly fully active, indicating that the slowly turning over reaction centers were due solely to photosystem II. The results demonstrate that in spinach leaves approximately 32% of the photosystem II complexes are effectively inactive, in that their contribution to energy conversion is negligible. Additional evidence for inactive photosystem II complexes in spinach leaves was provided by fluorescence induction measurements, used to monitor the oxidation kinetics of the primary quinone acceptor of photosystem II, Q_A, after a short flash. The measurements showed that in a fraction of the photosystem II complexes the oxidation of Q_A^- was slow, displaying a half-time of 1.5 ± 0.3 seconds. The kinetics of Q_A^- oxidation were virtually identical to the kinetics of the recovery of photosystem II determined from the electrochromic shift. The key difference between active and inactive photosystem II centers is that in the inactive centers the oxidation rate of Q_A^- is slow compared to active centers. Measurements of the electrochromic shift in detached leaves from several different species of plants revealed a significant fraction of slowly turning over reaction centers, raising the possibility that reaction centers that are inefficient in energy conversion may be a common feature in plants.

In normally functioning PSII complexes, bound plastoquinone is reduced by electrons from water, and subsequently released into the thylakoid membrane (reviewed in Crofts and Wraight [4]). The reduction of plastoquinone requires two sequential turnovers of the PSII reaction center and involves two bound plastoquinone molecules, Q_A[2] and Q_B, operating in series. Q_A acts as a single electron carrier and is permanently bound in PSII. The plastoquinone molecule at the Q_B site differs from Q_A in that it becomes fully reduced to plastoquinol after two turnovers of the reaction center, and it exchanges rapidly with the freely mobile plastoquinone in the membrane. In reaction centers in which Q_A and Q_B are initially oxidized, the first light reaction drives an electron from P680, the primary donor of PSII, to pheophytin, which in turn reduces Q_A. The electron is then transferred from Q_A to Q_B, enabling the reaction center to turn over a second time. In the second light reaction an electron is transferred over the same path to Q_B^-, and together with two protons results in the reduction of Q_B^- to QH_2. QH_2 then debinds from PSII, leaving the Q_B site empty for plastoquinone to bind, thereby enabling repetition of the cycle. The turnover rate of active PSII complexes operating in leaves or in isolated thylakoid membranes in continuous saturating light is commonly 200 to 300 e^-/s at 18 to 24°C (e.g., 15).

In addition to the normally functioning PSII complexes, several lines of evidence indicate that a significant fraction of PSII complexes is impaired in the ability to transfer electrons into the plastoquinone pool (2, 7, 13–15, 19, 21, 26). Recently, we investigated (2) the turnover rate of PSII reaction centers by measuring the electrochromic shift, which is a monitor of transmembrane charge transfer within the reaction centers, and the fluorescence induction, which is a monitor of the redox state of Q_A. In thylakoid membranes isolated from spinach the results indicate that 32% of the PSII reaction centers have turnover rates that are approximately 1000 times slower than normally active reaction centers. The slow turnover rate of the inactive centers is due to the oxidation of Q_A^-, which has a half-time of approximately 2 s. A key question that remained unanswered was whether the membrane isolation procedure contributed to the inactive PSII complexes.

In this work we investigate the activity of PSII complexes in leaves. The objective is to determine the concentration and turnover rate of slowly turning over PSII complexes by meas-

[1] This work was supported in part by the Photosynthesis Program of the Competitive Grants Office of the U.S. Department of Agriculture (grant Nos. 86-CRCR-1-1987 and 88-37130-3366).

[2] Abbreviations: Q_A, primary quinone acceptor in photosystem II; FL1, FL2, actinic flash 1 and 2, respectively; Q_B, secondary quinone acceptor in photosystem II; P680, primary donor in photosystem II; P700, primary donor in photosystem I; Inactive PSII, photosystem II centers that require greater than 50 ms to recover after a saturating flash.

uring the electrochromic shift and the fluorescence induction *in vivo*. The results reveal that over 30% of PSII reaction centers in spinach leaves turn over at rates of 0.3 e^-/s, which is slow compared to normally active centers. Experiments done on detached leaves of corn, cotton, cowpeas, peas, sorghum, soybean, and sunflower, reveal a significant fraction of inactive reaction centers, raising the as yet untested possibility that 20% or more of the PSII reaction complexes in plants may be inactive with regard to photosynthetic energy conversion.

A preliminary account of this work has been presented earlier (3).

MATERIALS AND METHODS

Spinach plants (*Spinacia oleracea*) used in this study were grown under two different conditions. (a) Plants were grown in a 2:1:1 mixture of soil, peat, and perlite from seed (Hybrid No. 424, Ferry-Morse Seed Co., Mountain View, CA)[3] in controlled environment chambers (Conviron PGW 36, Controlled Environments, Pembina, ND, or EGC model 31-15 growth chamber, Chagrin Falls, OH). The conditions were: light 12 h, temperature 20 ± 2°C, relative humidity 45%; dark 12 h, temperature 15 ± 2°C, and relative humidity 85%. The growth light intensity was between 400 and 600 $\mu E/m^2$ s measured between 400 and 700 nm. The soil mixture was fertilized with Osmocote (14N:14K:14P, Sierra Chemical Co., Milpitas, CA) during seed planting and the plants were watered daily. White growth light was provided by a combination of VHO cool-white fluorescent lamps and incandescent lamps. Plants were used for experiments 4 to 6 weeks after planting. (b) Plants were grown hydroponically in a controlled environment chamber as described elsewhere (24). Plants grown under the two different conditions were indistinguishable in this study.

Pea leaves (*Pisum sativum*) were harvested from plants grown in a controlled environment chamber as described elsewhere (15). Leaves from corn (*Zea mays*), cotton (*Gossypium hirsutum*), cowpeas (*Vigna sinensis*), sorghum (*Sorghum bicolor*), soybean (*Glycine max*), and sunflower (*Helianthus annuus*), were taken from the top of the canopy from field grown mature plants. Detached leaves were placed with their cut ends in water and used for measurements within 3 h. There were no visible signs of water loss.

Thylakoid membranes were isolated from spinach leaves (grown as described above or purchased from local markets) as described elsewhere (28). The Chl concentration was determined in 80% (v/v) acetone/H_2O using the specific absorption coefficients at 664 and 647 nm for Chl *a* and *b* determined by Ziegler and Egle (30).

Flash-induced absorbance changes were measured using a laboratory-built single beam spectrophotometer (?8). The apparatus was equipped with a leaf chamber that oriented the leaf perpendicular to both the measuring beam and actinic flash. A bifurcated light guide was placed against the top of the leaf. One arm of the light guide carried the transmitted measuring light to the photomultiplier tube, while the other arm carried the actinic flashes to the leaf. The fiber optic elements of the light guides used in this study were randomized to ensure that the measuring and actinic light were exposed over the same leaf area. To prevent reaction center turnover induced by the measuring beam, the measuring beam was blocked by an electronic shutter (Uniblitz, Vincent Associates, Rochester, NY) except during and 100 to 200 ms prior to recording a kinetic trace. Short actinic flashes (half-peak width = 6 μs) were produced by Xenon flash lamps (FX193 or FX200, EG & G Electro-Optics, Salem, MA) filtered by a red blocking filter (CS 2-58, Corning Glass Works, Corning, NY). Each actinic flash in a sequence of flashes was shown to have the same time duration and integrated intensity. The actinic flashes were saturating in that a 70% attenuation of the flash resulted in 1% or less reduction in the extent of the flash-induced absorbance change at 518 nm. Further details of the instrument are described elsewhere (2).

PSI turnover in thylakoid membranes was determined by measuring the flash-induced reduction of methyl purple as described in Graan and Ort (6). Methyl purple is a quinoneimide redox dye which can serve as an electron acceptor for PSI. Upon reduction by PSI methyl purple undergoes a bleaching that can be quantitated by the absorbance decrease at 590 nm (6).

Fluorescence measurements were done using a trifurcated light guide placed against the top of the leaf. One arm of the light guide carried continuous green light to the leaf that excited the fluorescence. The light was produced by a 250 W tungsten lamp and filtered by heat filters, a DT Gruen interference filter (Balzers, Rolyn Optics, Arcadia, CA), and a blocking filter (CS 4-96, Corning). Illumination by the exciting light was controlled by an electronic shutter. The second arm of the light guide carried the fluorescence emission from the leaf to a photomultiplier tube (EMI 9558 QA, Thorn-EMI Corp., Holliston, NY) that was protected from the exciting light by a red blocking filter (CS 2-58, Corning). The third arm of the light guide carried a blue actinic flash to the leaf (Xenon flash lamp as described above blocked by a Corning, CS 4-96 filter).

Spinach plants were dark-adapted for at least 20 min before the onset of measurements. The leaf area illuminated was 1 cm^2, which received no more than 100 actinic flashes. The bottom of the leaf was exposed to atmospheric levels of CO_2. Blowing air over the bottom of the leaf did not alter the results. Measurements using thylakoid membranes were done as described elsewhere (2).

We quantitated both the rapid absorbance change induced by a flash, and the fluorescence levels F_i and F_o by a linear regression analysis of the digitized data. The extent of the stable, rapid absorbance change was determined by taking the difference between a straight line based on the data over the time range from approximately 1 to 30 ms and extrapolated to time $t = 0$, and a straight line extrapolated through the base line to $t = 0$. The software used to perform this curve fit was based on the method of linear least squares. Unless stated

[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or the University of Illinois and does not imply its approval to the exclusion of other products or vendors which may be suitable.

otherwise, all the uncertainties shown are standard deviations.

RESULTS

Electrochromic Shift (ΔA518)

The electrochromic shift, monitored by the light-induced absorbance increase at 518 nm (Fig. 1), is the result of several reactions occurring in the thylakoid membrane (reviewed in Witt [29]). The first step is the light-driven transfer of an electron in PSII and PSI reaction centers that results in a stable charge separation across the membrane. In PSII an electron is transferred from the primary donor, P680, located near the lumenal side of the membrane, to the first stable quinone acceptor, Q_A, located near the stromal side of the membrane (18). In PSI an electron is transferred in the same direction across the membrane from the primary donor, P700, to a special Chl *a* molecule (25). Charge separation at the two reaction centers creates an electric dipole field that is subsequently delocalized laterally over the membrane by ion movements. The delocalization occurs within a few microseconds or faster and results in a uniform transmembrane electric field. The electric field across the membrane causes a shift in the absolute absorption spectrum of antenna pigments (primarily carotenoids and Chl *b*) that results in an absorbance change with a maximum increase near 518 nm (29). In addition to the rapid electrochromic shift, there is a slow electrochromic shift associated with plastoquinol oxidation and reduction by the Cyt *b/f* complex that has a rise time of a few milliseconds or slower (11). Two features of the rapid electrochromic shift make it useful for probing the turnover time of the reaction centers. One is that the rise of the flash-induced absorbance increase at 518 nm is rapid (<10 μs) and is easily distinguishable from slower flash-induced absorbance changes that occur in the millisecond time range or slower. The second is that the extent of the electrochromic shift is directly proportional to the number of electrons transferred across the membrane (29). Thus, if charge separation within the reaction center crosses the membrane, then the amplitude of the rapid ΔA518 is a direct measure of the number of reaction centers turning over in a short saturating flash.

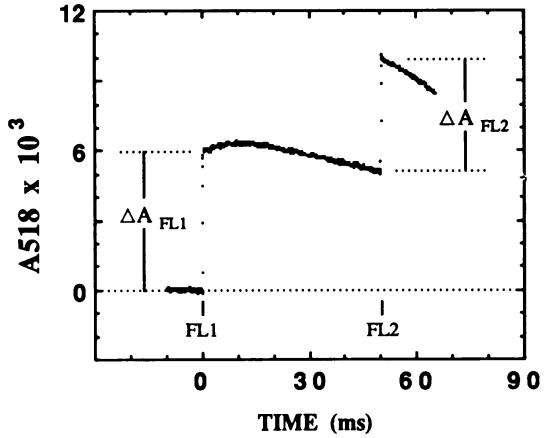

Figure 1. Absorbance changes measured *in vivo* at 518 nm in spinach leaves induced by two actinic flashes given 50 ms apart. The absorbance change induced by the first flash (FL1) was measured after a 50 s dark period. The absorbance change induced by the second flash (FL2) was measured 50 ms after the first flash. Both flashes were saturating by the criteria described in "Materials and Methods." The trace is the average of 48 runs, measured at an instrument response time of 50 μs and a measuring beam half-band width of 4 nm. The measurements were done at room temperature (22–25°C). Further details are given in the text.

The photochemical activity of the reaction centers in intact leaves was determined by comparing the amplitudes of the rapid absorbance increase at 518 nm induced by two saturating flashes separated by a variable time interval (Fig. 1). ΔA_{FL1} is the extent of the flash-induced ΔA518 in a leaf that had been dark adapted for 50 s. ΔA_{FL2} is the extent of the absorbance change created by a flash given 50 ms after the first flash. As discussed above, the rapid flash-induced rise reflects the absorbance increase due to the electrochromic shift, and is assumed to be directly proportional to the number of reaction centers turning over.

It should be noted that the two-flash technique of measuring the electrochromic shift to determine the recovery time of the reaction centers is insensitive to the decay rate of the electric field. The rate of decay of the electric field is due to the movement of unpaired charge across the membrane (29), which is strongly affected by the activation state of the ATPase (16). The focus of our experiment is only on the relative amplitudes of the rapid absorbance increases ΔA_{FL1} and ΔA_{FL2}, which are easy to determine because the decay of the electrochromic shift is much slower than the rise time (Fig. 1).

As shown in Figure 1, the amplitude of the fast rise of the ΔA518 produced on the second flash (ΔA_{FL2}) is 80% of the amplitude produced on the first (ΔA_{FL1}). This indicates that 20% of the reaction centers (PSII + PSI) that produced a stable charge separation on the first flash (after a 50 s dark period) were incapable of producing a stable charge separation on a second flash 50 ms later. In other words, 20% of the reaction centers had a recovery time greater than 50 ms. To determine the kinetics of the recovery of the slowly turning over reaction centers in the time range from 50 ms to 500 s, we varied the dark time interval between the flashes, and measured the ratio of the flash-induced absorbance changes. In Figure 2 the amplitude of the absorbance change at 518 nm created by the second flash, divided by the amplitude of the absorbance change produced by the first flash (*i.e.* $\Delta A_{FL2}/\Delta A_{FL1}$), is shown as a function of the time between the two flashes. The recovery kinetics of the ratio $\Delta A_{FL2}/\Delta A_{FL1}$ indicates that 24% of the reaction centers recovered slower than 50 ms and that the half-time of the recovery was 1.6 s. The remaining 76% of the reaction centers recovered rapidly, in less than 50 ms.

In vivo experiments done using different spinach plants are summarized in Table I. The average extent of the slowly recovering reaction centers was 20 ± 4%, and the average recovery half-time was 1.7 ± 0.3 s. The average amplitude of the rapid absorbance increase at 518 nm on the first flash was 0.0056 ± 0.0008 (average data from 62 different leaves). Experiments done using detached leaves gave the same results, as did experiments done using market spinach leaves. The spectra of the rapid absorbance changes due to the first and

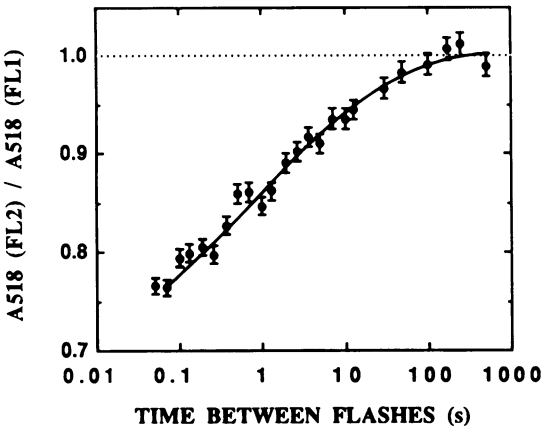

Figure 2. Recovery of the flash-induced absorbance change measured *in vivo* at 518 nm in spinach leaves as a function of the time between the first and the second flash. The recovery is defined as the ratio of the amplitude of the rapid absorbance increase at 518 nm produced by the second flash (A_{518} [FL2]) to the amplitude of the rapid absorbance increase produced by the first flash (A_{518} [FL1]). The ratios are normalized to the average extent of the rapid absorbance increase at 518 nm measured after a 100 s or greater dark period (dashed line). The measurements were done as shown in Figure 1, except the time between the flashes was varied. The error bars are the standard error of the mean. Further details are given in the text.

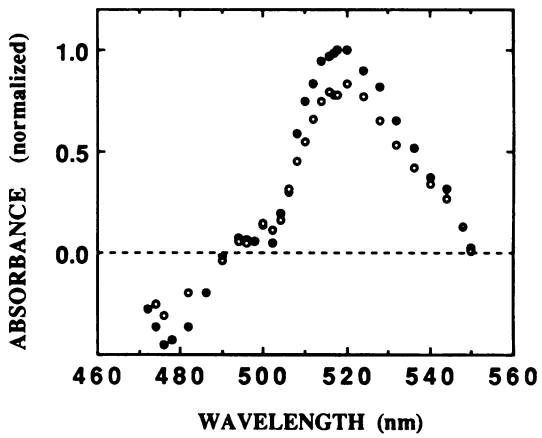

Figure 3. Flash-induced absorbance change measured *in vivo* in spinach leaves as a function of wavelength. The closed circles represent the spectrum of the absorbance change due to the first flash measured after a 30 s dark period. The open circles represent the spectrum of the absorbance change due to the second flash measured after a 100 ms dark period. The absorbance changes shown are normalized by dividing by the absorbance change measured at 518 nm due to the first flash. The average absorbance increase due to the first flash was 0.0051. Experimental conditions were otherwise as described in Figure 1.

Table I. *Summary of the Quantitation and Turnover Time of Inactive Reaction Centers in Attached Spinach Leaves*

The amount of slowly turning over reaction centers was determined by measuring the ratio of the amplitude of the ΔA518 on the second flash (FL2) divided by the amplitude of the ΔA518 on the first flash (FL1) (see Fig. 1). The second flash was given 100 ms after the first. The amount of inactive reaction centers [Inactive Reaction Centers]/[PSII + PSI] is defined as $1 - (\Delta A518[FL2]/\Delta A518[FL1])$. The experiments were done as described in Figure 1. The recovery half-time of inactive centers was determined as described in Figures 2 and 5. Each measurement was done using a different leaf. The percent of inactive reaction centers is the average of 52 measurements. The recovery half-time based on the ΔA518 and fluorescence induction is the average of three and five measurements, respectively. SD is the standard deviation.

	Measurement	Average	SD
Percent of inactive reaction centers	$\left[1 - \frac{\Delta A518\ (FL2)}{\Delta A518\ (FL1)}\right] \times 100$	20%	±4%
Recovery half-time	ΔA518 recovery	1.7 s	±0.3 s
Recovery half-time	Fluorescence induction	1.5 s	±0.3 s
Percent of inactive PSII centers[a]	ΔA518 recovery	32%	±6%

[a] The fraction of inactive PSII was calculated based on measurements in attached leaves and in isolated thylakoid membranes as described in the text.

second flashes are shown in Figure 3. The similarity between these two spectra, and the electrochromic shift spectrum measured in thylakoid membranes (29), indicates that the rapid absorbance increase at 518 nm in leaves was due primarily to the electrochromic shift and that contributions of other flash-induced absorbance changes were minimal. This is consistent with the results in isolated thylakoid membranes, in which 85 to 95% of the flash-induced ΔA518 is due to the electrochromic shift (2).

These data indicate that approximately 20% of the reaction centers (PSII + PSI) in spinach leaves turn over slowly, but do not reveal the proportion of PSII and PSI in the slow-recovering fraction. This question was addressed in a previous study (2) using isolated thylakoid membranes that demonstrated the slowly turning over fraction to be almost exclusively PSII reaction centers. Here, further evidence is provided that PSI reaction centers in isolated thylakoid membranes are fully active. The recovery time of PSI was determined using thylakoid membranes in the presence of methyl purple (6) by measuring the amplitude of the absorbance decrease produced by two flashes separated by 100 ms. The amplitude of the absorbance decrease due to the second flash was 96 ± 1.5% of the amplitude produced by the first flash, indicating that the recovery of PSI in thylakoid membranes was nearly complete within 100 ms. Based on these results we argue that the slowly turning over reaction centers in leaves revealed by the recovery of the ΔA518 belong to PSII. The fluorescence induction measurements shown in the next section provide independent evidence supporting this conclusion.

Efforts to determine the possible contribution of PSI to the slowly turning over reaction centers in leaves by measuring the recovery after a flash of $P700^+$ at 702 nm did not provide sufficiently quantitative results to be of use. The problems were: (a) that we could not resolve a fast component of $P700^+$ rereduction (in the 20 μs range) because of a fluorescent artifact due to the actinic flash, so the two-flash technique is not readily applicable, and (b) at 700 nm there appears to be a contribution to the absorbance change due to the electrochromic shift (29).

These data indicate that 32 ± 6% of the total amount of PSII is kinetically inactive with respect to light-saturated rates of photosynthetic electron transport. This calculation is based on the following data and assumptions: (a) the inactive centers in leaves are PSII (discussed above and in the next section), (b) 20 ± 4% of the reaction centers in leaves are inactive (Table I), and (c) the amount of PSI reaction centers in leaves is the same as in thylakoid membranes, in which we showed 38 ± 3% of the total number of reaction centers to be PSI (2). Thus, 42% of the reaction centers are active PSII complexes. It is important to note that this technique can detect only reaction centers that undergo light-driven transmembrane charge separation that is stabilized for more than 10 μs. Reaction centers that are not photochemically active, or that undergo a rapid back reaction (<10 μs), are not detected. Based on these calculations, the estimated ratio of active PSII to inactive PSII to PSI was 1.1:0.5:1.0.

Active PSII centers in the redox state $Q_A^-Q_B$ after the first flash would give rise to binary oscillations (4) and could be mistaken for inactive centers, since active centers in which Q_A is reduced would be unable to undergo a stable charge separation (2). To test this possibility, we measured the ΔA518 due to three saturating flashes spaced 50 ms apart. The experiment was designed to distinguish between slowly recovering PSII centers that exhibit the two electron reduction of Q_B, characteristic of normally active PSII, and those that can undergo only a single photochemical reaction, characteristic of the inactive centers. We found that the recovery of the ΔA518 on the second and third flashes was the same, 80 ± 1%. Thus, active PSII centers in the state $Q_A^-Q_B$ do not contribute significantly to the slowly turning over PSII complexes.

Measurements of the recovery of the ΔA518 on the second flash reveal inactive reaction centers in detached leaves from corn, cotton, cowpeas, peas, sorghum, soybean, and sunflower (Table II). In peas, based on measurements of the ΔA518 and the ratio of PSII to PSI we estimate that 33% of the PSII reaction centers are inactive (15). For the other plants we have not determined the ratio of PSII to PSI, so are unable to estimate the contribution of PSII to the inactive reaction centers. The fact that in the seven species we tested 9 to 18% of the reaction centers were inactive raises the possibility that inactive centers may be ubiquitous in higher plants.

Table II. *Percent of Inactive Reaction Centers in Detached Leaves of Several Plants*

The percent of inactive reaction centers ([Inactive Reaction Centers]/[PSII + PSI]) × 100 is defined as [1 − (ΔA518[FL2]/ΔA518[FL1]) × 100]. The experiments were done as described in Figure 1, except the first flash was given after a 30 s dark period, and the second flash was given 100 ms after the first flash. The estimated uncertainty in these measurements was ± 4%.

Plant	Percent of Inactive Reaction Centers
Corn	9
Cotton	14
Cowpeas	18
Peas	18
Sorghum	15
Soybean	18
Sunflower	15

Fluorescence Induction

Fluorescence induction measurements consist of exposing thylakoids to continuous actinic light and monitoring the time course of the Chl fluorescence intensity from the onset of the actinic illumination. The shape of the fluorescence induction curve is controlled primarily by the redox state of Q_A; when Q_A is oxidized the fluorescence is at a minimum, and when Q_A is reduced to the semiquinone anion, Q_A^-, the fluorescence is at a maximum (reviewed in Van Gorkom [27]). Early studies of the fluorescence induction showed that upon exposing thylakoid membranes to low intensity illumination, the fluorescence immediately rises to an initial level, F_o, followed by a small increase to a plateau level, F_{pl}, which is then followed by a large increase to F_{max} (Fig. 1 in ref. 5). The fluorescence increase to F_{max} has been widely used to investigate Q_A, Q_B, the plastoquinone pool, as well as the antenna size serving the PSII reaction centers (27). The initial induction phase, the fluorescence rise from F_o to F_{pl}, has received much less attention. Recently, it has been argued (19) that fluorescence induction from F_o to F_{pl} is due to $PSII_\beta$ centers that are disconnected from the plastoquinone pool. More recently, following the experimental procedure of Owens and Joliot (Fig. 8 in ref. 5), we determined (2) the effect of pretreating thylakoid membranes with a single turnover

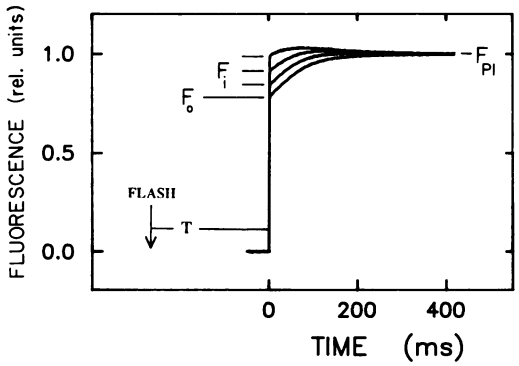

Figure 4. Fluorescence induction measured *in vivo* after an actinic flash in spinach leaves. The figure shows four superimposed kinetic traces. Each trace is a fluorescence induction transient produced by illumination with continuous light at varying dark time intervals (T) after an actinic flash. Continuous illumination was provided as green light at an intensity of 25 to 45 $\mu E/m^2$ s controlled by an electronic shutter. The traces were recorded after dark intervals of 0.1 s (a, top trace), 1.0 s (b, second from the top trace), 10 s (c, third from the top trace), and 50 s (d, bottom trace) after the actinic flash. The response time of the instrument was 0.1 ms. The leaves were oriented perpendicular to the continuous illumination and actinic flash. F_i is defined as the initial fluorescence intensity measured immediately after the opening of the shutter. F_o is defined as the control value of the initial fluorescence intensity, F_i, measured 50 s after the actinic flash (trace d). F_{pl} is defined as the fluorescence intensity measured between 300 and 400 ms after the opening of the shutter. The measurements were done at room temperature (23–25°C). Further details are given in the text.

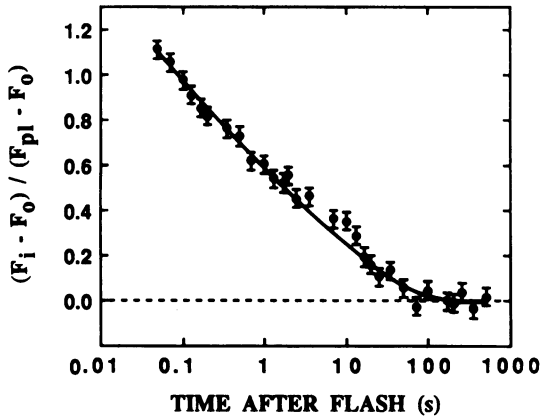

Figure 5. Decay of $(F_i - F_o)/(F_{pl} - F_o)$ measured *in vivo* in spinach leaves as a function of time (T) after an actinic flash. F_i, F_o, F_{pl}, and T are defined in the legend of Figure 4. Measurements were done and the experimental conditions were the same as described in Figure 4. The error bars are the standard error of the mean. Further details are given in the text.

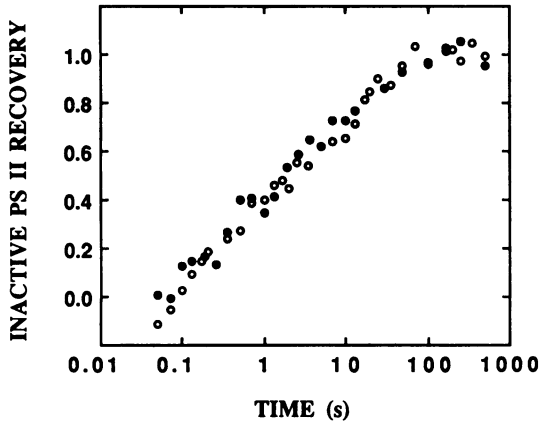

Figure 6. Recovery of inactive PSII *in vivo* in spinach leaves determined by the flash-induced absorbance change at 518 nm (closed circles) or by the fluorescence induction (open circles) as a function of time after an actinic flash). The recovery of the flash-induced absorbance changes at 518 nm is calculated using the data in Figure 2 by subtracting the contribution from the active reaction centers and normalizing by the total amount of inactive complexes. The recovery of the fluorescence induction is taken from the data in Figure 5. Further details are given in the text.

flash on the F_{pl} fluorescence induction kinetics. The objective was to determine the recovery time of the PSII centers giving rise to the F_{pl} induction phase. The measurements revealed a fraction of slowly turning over PSII centers, in which the reoxidation half-time of Q_A^- was approximately 3 s. Here, we apply the same technique to spinach leaves. It is important to note that the fluorescence rise from F_o to F_{pl} is due to slowly turning over PSII centers, and not to normally active centers. This is because the intensity of light driving the fluorescence induction is so low that in active PSII centers Q_A remains oxidized during the initial induction phase (5).

To determine the reoxidation kinetics of Q_A^- in leaves due to slowly turning over PSII centers, we measured the recovery of the F_{pl} induction phase at various times after a short saturating flash (Fig. 4). Trace d in Figure 4 shows the F_{pl} fluorescence induction for a leaf that was dark adapted for 50 s. The shape of the curve is the same for dark periods from 50 to 100 s (data not shown). However, at shorter dark periods the PSII centers giving rise to the F_{pl} fluorescence induction have not fully recovered. Traces a, b, and c show the recovery after a dark period of 0.1, 1, and 10 s. To relate the redox state of Q_A to the fluorescence parameters F_o, F_{pl}, and F_i we use the following equation (derived in refs. 10 and 17):

$$Q_A^-/(Q_A^- + Q_A) = (F_i - F_o)/(F_{pl} - F_o)$$

This equation, used elsewhere to determine the redox state of Q_A in active PSII centers (9, 12, 17, 23), assumes that the probability for excitation energy moving from a closed reaction center to another reaction center is zero (for discussion see refs. 5 and 10). This assumption implies that the rise of the fluorescence induction curve is first-order. A semilog plot of trace d in Figure 4 results in a straight line, indicating that the rise is first order (data not shown) and that excitation energy is not shared between inactive PSII reaction centers. The reoxidation kinetics of Q_A^- following a short flash is plotted in Figure 5 according to the above equation. The recovery curve indicates that the oxidation half time of Q_A^- in the slowly recovering PSII centers was 1.8 s. The average reoxidation half-time of Q_A^- in the slowly turning over PSII fraction was 1.5 ± 0.3 s (Table I).

Recovery Kinetics of Inactive PSII Complexes

Last, we compare the recovery kinetics of the slowly recovering reaction centers by superimposing the kinetics determined by the electrochromic shift and by fluorescence induction (Fig. 6). Since the recovery kinetics measured by the fluorescence induction are due solely to PSII, the fact that two kinetics are the same within the experimental error provides additional evidence that the inactive centers revealed by the ΔA518 measurements are due to PSII. To assume that a fraction of PSI is inactive would require the unlikely circumstance that the recovery kinetics of inactive PSI centers be nearly identical to those of the inactive PSII centers.

DISCUSSION

Measurements of reaction center turnover lead us to conclude that PSII in spinach leaves exists as at least two distinct populations, active and inactive. Active PSII complexes oxidize water and reduce plastoquinone at turnover rates of 200 to 300 e^-/s that are consistent with measured rates of steady state electron transport (15), and comprise approximately 68% of the total. However, inactive complexes, comprising the remaining 32% of PSII, turn over at one-thousandth the rate of the active centers, 0.3 e^-/s. This is too slow to contribute significantly to the rate of steady state electron transport. As a consequence, their contribution to energy transduction would be negligible. The key different between active and inactive PSII complexes is the slow rate of electron transfer from Q_A^-. In inactive centers, the oxidation half-time of Q_A^- is approximately 1.6 s, compared to oxidation half-times of 0.5 to 2 ms in active centers. These conclusions are based on measurements of the electrochromic shift and fluorescence

induction, and are applicable to spinach leaves *in vivo* that have been dark adapted for 50 s or longer. The electrochromic shift demonstrates that 20% of the reaction centers (PSII + PSI) in leaves turns over slowly, with a half-time of 1.7 s. Measurements using thylakoid membranes indicate that the slowly turning over reaction centers are almost exclusively PSII. Independent evidence supporting this conclusion is provided by fluorescence induction measurements in leaves, which show that a fraction of PSII complexes turns over with a half-time of 1.5 s. The observation that the recovery kinetics of the inactive reaction centers determined by the two techniques are virtually identical provides additional evidence that the inactive reaction centers in leaves are primarily PSII.

The main functional difference between active and inactive centers is the interaction between Q_A and Q_B. To date, there is no evidence to suggest that primary photochemistry or oxygen evolution is impaired in inactive complexes. Measurements of the electrochromic shift show that electron transfer to Q_A in inactive centers is stable, and results in a transmembrane electric field (Figs. 1–3). Measurements using isolated thylakoid membranes indicate that the oxygen evolving components of inactive PSII complexes can function at physiological rates in the presence of exogenous quinone acceptors (7). Since the electron acceptors for Q_A^- oxidation in inactive centers remains unidentified, it is not known whether the Q_B site is occupied by plastoquinone (discussed in Chylla *et al.* [2]). However, it appears that the D1 protein (22) and the Q_B site are present in the inactive centers, since the affinity of certain PSII inhibitors for the Q_B site is essentially the same as in active centers (7). Elsewhere, we discussed the possibility that the physical difference between active and inactive complexes may be related to the heterogeneity exhibited by Cyt *b*559 and the Fe^{2+} atom near Q_A (2).

One of the physical differences between active and inactive complexes is their antenna system. The exponential rise of the fluorescence induction (Fig. 4) indicates that excitation energy is not shared between inactive centers (5). Furthermore, measurements of the light-saturation curve of the ΔA518 indicate that the inactive centers are served by a smaller antenna than active centers (our unpublished data). It is noteworthy that a small antenna size and exponential rise of the fluorescence induction is the basis for defining $PSII_\beta$ centers (20). However, despite the similarity between the antenna system of inactive PSII complexes and $PSII_\beta$ centers, they should not be identified as one and the same complex. Since inactive PSII complexes are defined by the interaction between Q_A and Q_B, whereas $PSII_\beta$ centers are defined by their antenna size, they represent two different criteria for classifying PSII complexes. This point is illustrated in recent work that suggests $PSII_\beta$ exists in both active and inactive forms (8). The relationship between inactive complexes, $PSII_\beta$ centers, and other heterogeneous components of PSII (1), remains to be elucidated.

Although there are two few data to answer the question of whether inactive centers serve a useful role in photosynthesis, for example in photoinhibition, development, or otherwise, it is noteworthy that in all the species we have surveyed to date we found evidence for inactive reaction centers, indicating that inactive PSII centers may be a common feature in higher plants.

ACKNOWLEDGMENTS

We thank Drs. Neil Baker and Don Ort for useful discussions, and Dr. John Hesketh for providing the field grown plants.

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