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KINETICS OF REDUCTION OF THE OXIDIZED PRIMARY ELECTRON DONOR OF PHOTOSYSTEM II IN SPINACH CHLOROPLASTS AND IN *CHLORELLA* CELLS IN THE MICROSECOND AND NANOSECOND TIME RANGES FOLLOWING FLASH EXCITATION

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Summary

Absorption changes (ΔA) at 820 nm, following laser flash excitation of spinach chloroplasts and *Chlorella* cells, were studied in order to obtain information on the reduction time of the photooxidized primary donor of Photosystem II at physiological temperatures.

In the microsecond time range the difference spectrum of ΔA between 750 and 900 nm represents a peak at 820 nm, attributable to a radical-cation of chlorophyll *a*. In untreated dark-adapted material the signal can be attributed solely to P^+-700; it decays in a polyphasic manner with half-times of 17 μs, 210 μs and over 1 ms. The oxidized primary donor of Photosystem II (P^+_{II}) is not detected with a time resolution of 3 μs. After treatment with 3—10 mM hydroxylamine, which inhibits the donor side of Photosystem II, P^+_{II} is observed and decays biphasically (a major phase with $t_{1/2}$ = 20—40 μs, and a minor phase with $t_{1/2} \simeq 200$ μs), probably by reduction by an accessory electron donor.

In the nanosecond range, which was made accessible by a new fast-response flash photometer operating at 820 nm, it was found the P^+_{II} is reduced with a half-time of 25—45 ns in untreated dark-adapted chloroplasts. It is assumed that the normal secondary electron donor is responsible for this fast reduction.

Introduction

Major advances have been made during the last years in the understanding of photosynthetic reactions in bacteria and in higher organisms. However photo-

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Abbreviation: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

synthetic oxygen evolution remains a major process whose mechanism has not been elucidated. It is initiated by a photochemical electron transfer from the primary electron donor P_{II}, presumably a specialized chlorophyll *a* molecule, to the stable primary acceptor A_1 (often named Q) which probably is a plastoquinone molecule. A major problem has been to identify the donor P_{II} and to measure the rate at which P_{II}^+, resulting from the primary electron transfer reaction, is reduced by secondary donors. These donors, whose number and chemical identity remain unknown, are normally connected to an enzymatic complex responsible for oxygen evolution.

Three methods have been used for the determination of the rate of reduction of P^+ in photosynthetic structures. It has been directly measured by kinetic absorption spectroscopy at the red absorption maximum of P_{II} (around 690 nm) [1—3] and at the near infra-red absorption maximum of P_{II}^+ (around 820 nm) [4—6]. A second method relies on the hypothesis that P_{II}^+ is a quencher of chlorophyll fluorescence [7] so that its reduction will be measurable as a fluorescence induction following flash excitation [8—13]. Thirdly some information has been derived from luminescence (delayed light emission) measurements on the assumption that luminescence results from a back-reaction between P_{II}^+ and the reduced primary acceptor [14—16].

A presently widely accepted view is that P_{II}^+ can be reduced in at least three kinetic paths:

(i) A back reaction between P_{II}^+ and A_1^-. This back-reaction has been well characterized at low temperature from a parallel decay ($t_{1/2} \simeq 3$ ms) of P_{II}^+ detected by EPR [17] and by absorption spectroscopy [4], and of A_1^- detected by flash absorption spectroscopy as C-550 [18] or as a plastoquinone radical anion [19]. At room temperature the back-reaction ($t_{1/2} \simeq 100$—200 μs) has been studied in materials in which normal reactions on the donor side were inhibited by a low pH or by treatment with Tris [5,6,20—22]. It may correspond to the 200 μs phase reported for Chl a_{II} or X-320 [1,2,23,24].

(ii) Reduction by a donor with $t_{1/2} \simeq 35$ μs. This phase has been reported to occur in untreated chloroplasts [3], specially after two preilluminating flashes or at a low pH of the intrathylakoid space [10,25,26]. It is also much larger after inhibition of the donor side of Photosystem II with hydroxylamine or Tris [6,9,10,12,22]. A similar phase has been observed in measurements of luminescence decay following a short flash [12,14,27,28].

(iii) A very fast reduction has been inferred from the measurements of fluorescence induction [9,10] as well as from the inability to detect the full amount of P_{II}^+ in flash absorption spectroscopy with microsecond resolution [6,25]. This fast donation, by a donor which we will name D_1, may account for a very fast phase of fluorescence induction (25 ns) observed by Mauzerall in dark-adapted *Chlorella* cells [29].

In this work, as previously, we studied the kinetics of reduction of P_{II}^+ formed by a short flash, making use of the weak absorption band of that species at 820 nm. Confirming and extending previous observations, P_{II}^+ was not detected in untreated dark-adapted material (spinach chloroplasts and *Chlorella*) with a microsecond time resolution. It appeared with full amplitude, however, after treatment with hydroxylamine. We also made use of a new flash absorption photometer, with nanosecond time resolution, which permitted us

to measure a presently unreported very rapid phase of reduction of P_{11}^{+} (about 30 ns) in dark-adapted chloroplasts.

Materials and Methods

Biological material

Chloroplasts from freshly picked spinach leaves were prepared as described previously [30]. For the measurements in the nanosecond range, in order to reduce light scattering, the chloroplasts were suspended in a hypotonic buffer (50 mM Tricine, pH 7.6, plus 1% w/w bovine serum albumin); the suspension was briefly centrifuged (30 s between the start and the stop of the centrifuge, type Serval Superspeed) and the supernatant was collected. Some experiments were performed on *Chlorella pyrenoidosa* cells, kindly provided by Dr. J. Lavorel (CNRS, Gif-Sur-Yvette), and diluted in 10 mM phosphate buffer (pH 6.8) with 10 mM KCl.

The suspension (100—250 ml) was kept in darkness in a cooled reservoir connected to the cuvette (10 × 10 mm section, height: 35 mm) with blackened catheter tubing. The content of the cuvette was renewed after each actinic flash with a magnetic valve located after the cuvette. The suspension was recycled when necessary. It was used in this way for a time never exceeding 15 min, after which a fresh suspension was used. The temperature in the cuvette was about 9° C; in other parts of the circuit it varied between 0 and 9° C.

Apparatus for measurement in the microsecond range

The apparatus for the measurement of the kinetics of absorption changes from about 1 μs to 1 ms after a laser flash is shown schematically in Fig. 1A. The measuring light was provided by a tungsten halogen lamp (220 V, 800 W) whose DC power supply, normally maintained at 50 V, was increased to 220 V just before the measurement. This procedure minimized the heating by the tungsten lamp. The filter F inserted between the lamp and the cuvette included a 10 cm water filter, a Schott RG 715/3 mm filter and an additional blocking (Wratten 87) allowing broad band near infra-red light to fall on the cuvette. The measuring wavelength was selected from this broad incident band by a 500 mm Bausch and Lomb monochromator located after the cuvette. This optical arrangement was chosen in order to minimize fluorescence and stray light artifacts.

The monitoring light was measured with a silicon photodiode (UDT, type PIN-10, active area: 1 cm^2). The changes in the photocurrent of the photodiode were amplified by a specially constructed low-noise AC amplifier and measured with a transient digitizer (Tektronix R-7912, with a 7A22 amplifier) whose output was connected to a signal averager (Intertechnique, Didac 4000) through a homemade interface. The electronic bandwidth covered the range from 0.3 kHz to 1 MHz.

Actinic excitation of the sample was achieved by saturating flashes from a dye laser (electrophotonics; $\lambda \simeq 605$ nm; $t_{1/2} = 0.6$ μs; 10 mJ) or a Q-switched ruby laser (Quantel; $\lambda = 694$ nm; $t_{1/2} = 8$ ns; 100 mJ). The laser beam was incident on an opal glass and then collected by a solid plastic light pipe whose exit

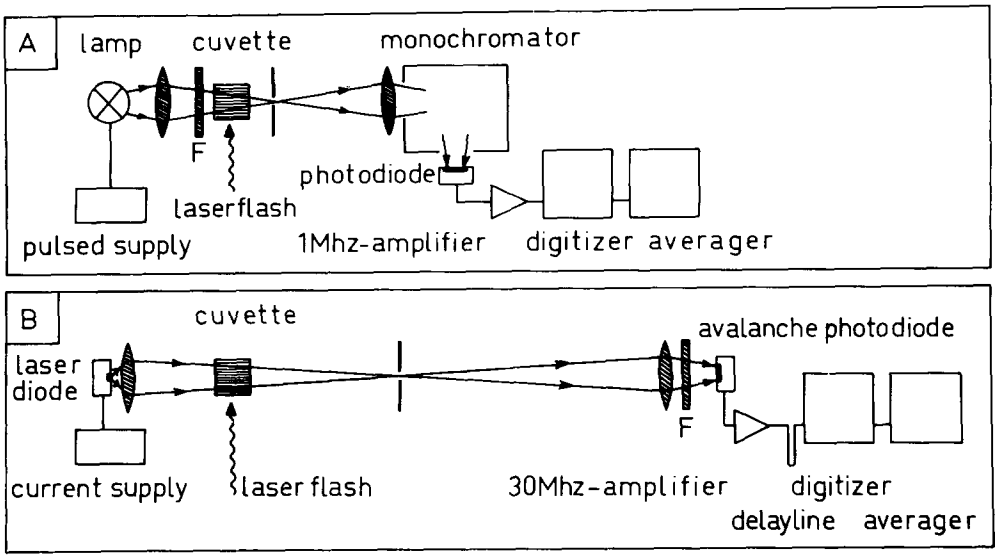


Fig. 1. Apparatus for the measurement of the kinetics of absorption changes after a laser flash. (A) Time range: 1 μs to 1.5 ms, measuring light source: filament lamp. (B) Time range: 20 ns to 10 μs, measuring light source: laser diode (820 nm), F: filters.

end was cut to the shape of the cuvette face. In some cases the laser flash was preceeded, at an adjustable time interval, by a saturating white light xenon flash (General Radio, type Stroboslave; $t_{1/2} \simeq 5$ μs).

Apparatus for measurement in the nanosecond range

With the preceeding apparatus the main limitation for rapid measurements around 820 nm is due to the flash-induced fluorescence from the sample. This fluorescence, even at 820 nm, is large enough to perturb the photodetector and the amplifier. With the following apparatus (see Fig. 1B) the solid angle of light collection by the detector was greatly reduced, but the measurement was limited to 820 nm. However, this apparatus permitted the measurement of absorption changes from about 30 ns to several μs after a laser flash.

The measuring light source was a current-driven aluminium-gallium-arsenide injection laser diode (RCA, type C 30127) which emits 10 mW of continuous light at 820 nm (HBW: 2 nm) when driven with 350 mA. The diode was turned on 2 s before each measurement. The light emitting area (12 × 2 μm) was imaged by a microscope lens at a 1.5 × 0.6 mm diaphragm placed about 1 m behind the cuvette. This optical arrangement greatly reduced the flash-induced fluorescence artifact. The laser diode beam was further focused by a lens on the active area (≈7 mm²) of a silicon avalanche photodiode (RCA type C 30872), protected by a narrow band interference filter. The detector was chosen because of its high quantum efficiency (85%) at 820 nm, of its low-noise current amplification (≈60 at 330 V) and its fast rise and fall time (2 ns, without a post-impulsion "tail").

The amplified photocurrent of the avalanche photodiode was fed into a low noise 30 MHz AC-amplifier (Van Best, J.A., manuscript in preparation) and, via a delay line (23 m of 50 ohm cable, resulting in a delay of ≈120 ns)

to the digitizer (with a Tektronix 7A13 plug-in unit) and to the signal averager. In order to minimize the effect of time jitter in the ruby laser flash, the recording procedure was started by a photodiode which monitored part of the flash. The delay line permitted the recording of the signal level before the flash.

The ruby laser was used as the actinic light source. The oscillator laser cavity was Q-switched by a Pockels cell. The Q-switched laser pulse was then shortened to less than 10 ns by a second Pockels cell, before being amplified by a second ruby rod. This provides a clean pulse, devoid of any tail. The relative energy of each laser shot was measured by means of an integrating photodiode and of a storage oscilloscope. The occasional appearance of extra laser pulses was thus detected and measurements under such conditions as well as those at too low or too high laser energies were rejected. The laser flash intensity was approximately saturating (its reduction by a factor of two led to an about 20% decrease of the signal of P_{II}^{+} in chloroplasts treated with hydroxylamine).

Measuring procedure

The signals from a number of separate experiments were added in the signal averager. A same number of experiments were also made without the measuring light and subtracted from the previous ones in order to compensate for deviations in the baseline of the digitizer and for fluorescence artifacts.

The response of the photodetector and of the amplifier (rapidity, linearity and amplification) under different illuminations was checked by measuring the small repetitive signal of a light emitting diode.

The fluorescence increase of the chloroplasts suspension after a laser flash was routinely measured to check for a good functioning of Photosystem II [12]. The fluorescence was excited by a weak xenon flash ($t_{1/2} \simeq 1$ μs) given before or 80 μs after the laser flash. It was measured by a silicon photodiode and a storage oscilloscope.

Results and Interpretation

Measurements in the microsecond range

Excitation of a suspension of spinach chloroplasts by a saturating laser flash induces an immediate absorption increase at 820 nm (Fig. 2A). A semi-log plot of the decay after the first flash does not indicate a single first-order process (Fig. 3A). In this particular experiment the decay can be accounted for in terms of three exponential phases with half-times of 17 μs, 210 μs and 1.1 ms (this last value is a very rough approximation) and with respective weights of 45, 29 and 26 (drawn curve in Fig. 3A). For unknown reasons the relative magnitude of the three components were rather variable from one batch of chloroplasts to another. The signals of Figs. 2A and 3A are attributed primarily to oxidized *P*-700. This assignment is based on observations that the signal is little affected by treatments with block Photosystem II (but not Photosystem I) but is almost completely suppressed by treatments which oxidize *P*-700 prior to the laser flash (see ref. 6). The kinetic phases that we observed at 820 nm are also in fair agreement with those reported previously for *P*-700 [31,32].

If hydroxylamine (3 to 10 mM) is added and allowed to incubate for about

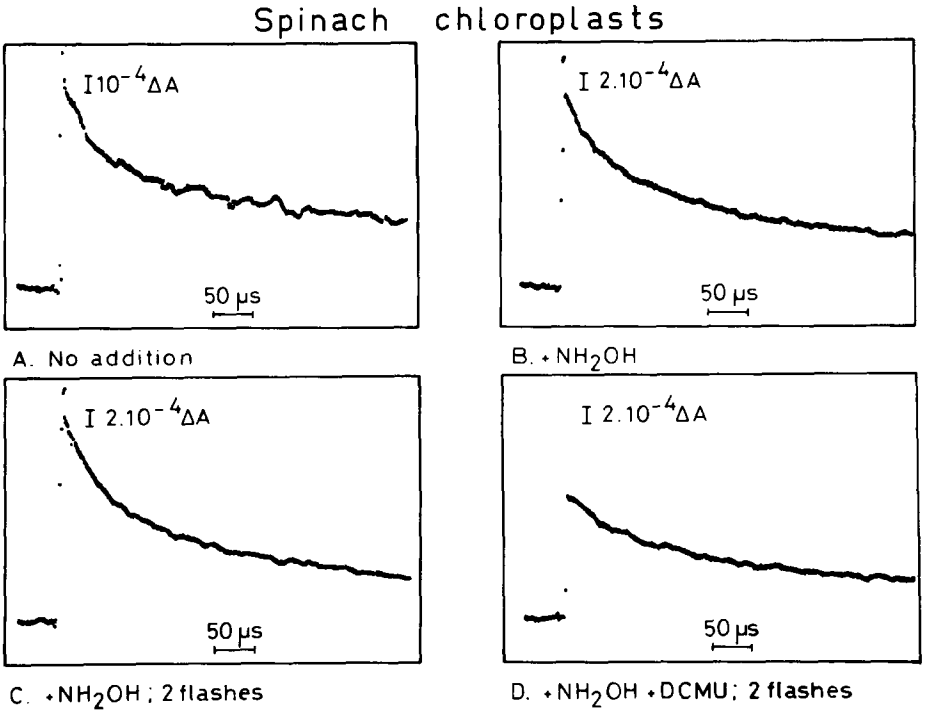


Fig. 2. Absorption change kinetics at 820 nm in a sample of dark-adapted spinach chloroplasts after a saturating dye laser flash. (A) No addition, (B) after incubation with 10 mM hydroxylamine, (C) as (B) but the laser flash was preceded by a saturating xenon flash (Δt = 0.1 s), (D) as (C) after further addition of 10 µM DCMU, average of 4 experiments. Chlorophyll concentration: $5 \cdot 10^{-5}$ M.

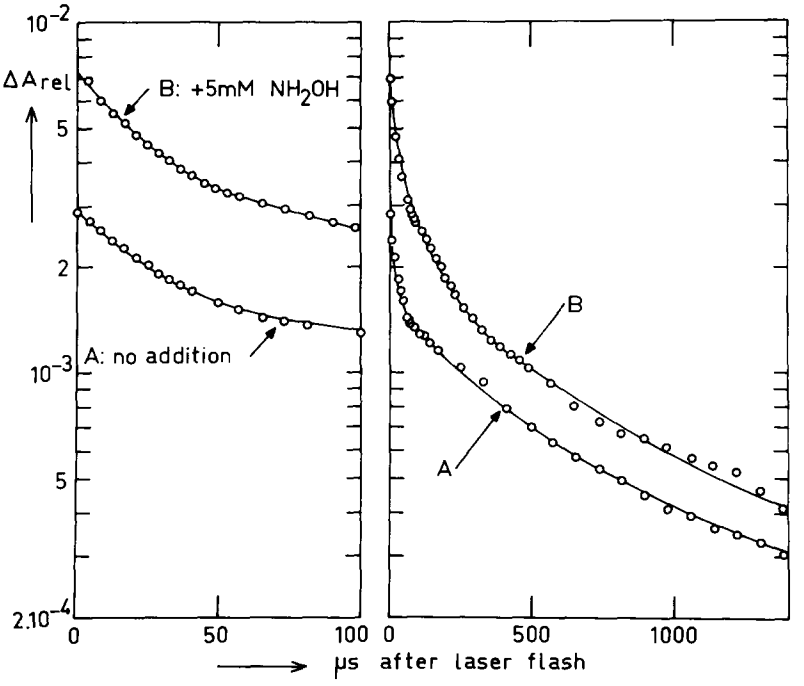


Fig. 3. Semi-log plot of the kinetics of decay of the absorption change at 820 nm induced by a saturating dye laser flash in a suspension of dark-adapted chloroplasts. Curve A: no addition, curve B: addition of 5 mM hydroxylamine. Average of 5 experiments.

5 min prior to the laser flash, the absorbance change at 820 nm is about twice as great (Fig. 2B). Upon further addition of 5 μM DCMU and preillumination by two flashes the ΔA is reduced to a value hardly greater than in the control (Fig. 2D), although this preillumination has no effect without DCMU (Fig. 2C). This result is interpreted as the appearance, after hydroxylamine treatment, of the ΔA due to the oxidized primary donor of Photosystem II (P_{II}^+), similarly as it has been observed previously after treatment at a low pH [5,6]. With hydroxylamine the decay of ΔA following a flash is also rather complex (Fig. 3B). The difference between the signal with hydroxylamine minus the control can be decomposed in two exponential phases (Fig. 4) with respective half-times of 22 μs and 160 μs, and with relative weigths of 53 and 47 (drawn curve). A relatively large fluctuation was observed for the half-time of the fast phase (20 to 40 μs), as well as for its magnitude (50 to 80% of the total).

The difference spectra of flash-induced ΔA are plotted in Fig. 5 for untreated chloroplasts and for chloroplasts incubated with hydroxylamine. They are attributed respectively to $P\text{-}700^+$ alone and to both $P\text{-}700^+$ and P_{II}^+. Both spectra are rather similar and present a well-defined peak at 820 nm, in agreement with their assignment as a radical cation of chlorophyll *a*. Assuming molar extinction coefficient of 7000 at 820 nm for this cation a concentration of one reaction center of Photosystem I and one of Photosystem II per 400—500 molecules of chlorophyll *a* has been derived.

Similar results were obtained with *Chlorella* cells (Fig. 6). A flash-induced ΔA at 820 nm is doubled upon addition of hydroxylamine, and returned to the starting level upon further addition of DCMU and preillumination (Fig. 6A, B, C). The signal which appears after incubation with hydroxylamine decays with a half-time of 40 μs (see ref. 22).

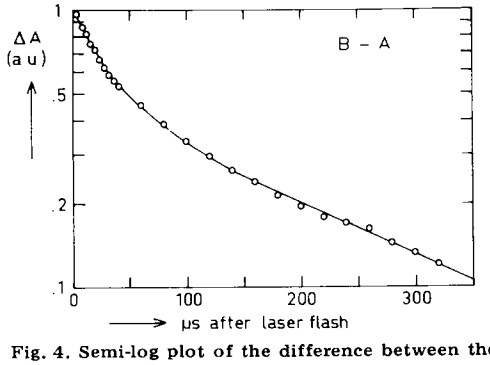


Fig. 4. Semi-log plot of the difference between the flash-induced absorption change at 820 nm in chloroplasts incubated with 5 mM hydroxylamine and in untreated chlroplasts. Average of 5 experiments.

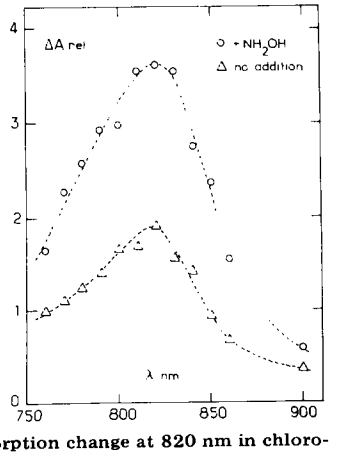


Fig. 5. Difference spectra of the absorption changes induced in dark-adapted chloroplasts by a dye laser flash (measurement 5 μs after the flash). Average of 5 experiments at each wavelength. Δ, no addition; ○, +5 mM hydroxylamine.

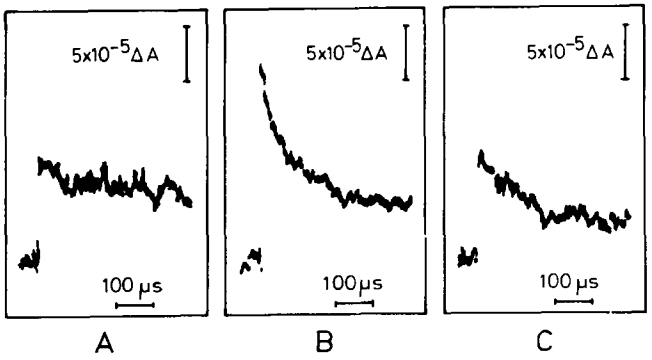


Fig. 6. Kinetics of absorption changes at 820 nm induced by a saturating dye laser flash in suspension of dark-adapted *Chlorella* cells. (A) No addition, (B) after incubation with 5 mM hydroxylamine, (C) after addition of 5 mM hydroxylamine, 5 μM DCMU and 2 preceding flashes (Δt = 0.1 s). Average of 10 experiments (A,C) or 20 experiments (B). Chlorophyll concentration: about $7 \cdot 10^{-6}$ M.

Measurements in the nanosecond range

From the previous measurements we concluded that P_{II}^{+} was probably reduced faster than our time resolution (≈3 μs) in untreated dark-adapted chloroplasts. In order to observe this fast phase of reduction we used the nanosecond equipment, with which 820 nm is the only available measuring wavelength. The signal/noise ratio with this apparatus is not as good because of the larger electrical bandwidth (30 MHz) and because of some noise intrinsic in the laser diode emission. For technical reasons the signals are inverted (positive ΔA downward).

The results of a series of 165 experiments is shown in Fig 7. The chloroplast suspension was replaced by a fresh one every 15 experiments (duration: about 10 min). A biphasic decay is clearly observed. The fast phase has a half-time included between 25 and 45 ns (we will refer to it as the 30 ns phase). In the average both phases have an equivalent magnitude. However, the magnitude of the 30 ns phase may be somewhat underestimated since we lose part of the signal during the time (about 20 ns) lost because of the fluorescence artifact.

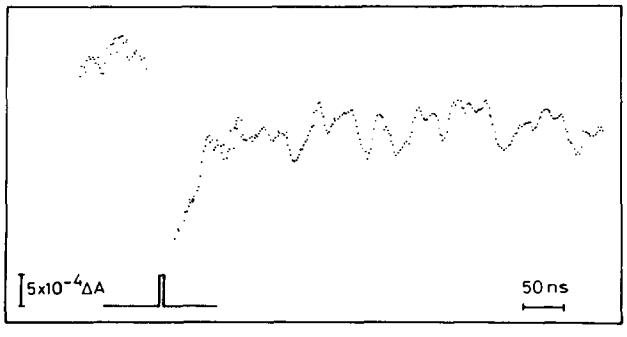
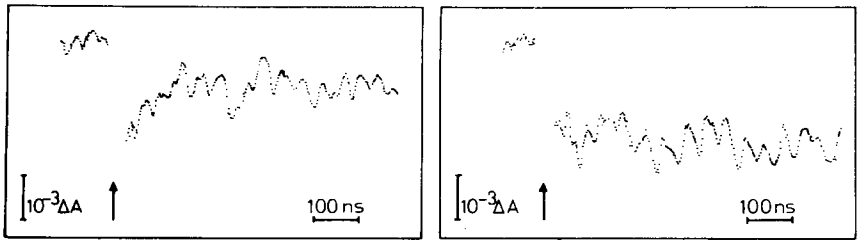


Fig. 7. Absorption spectra at 820 nm (positive downward) induced in a suspension of untreated chloroplasts by a saturating ruby laser flash (measurement with the nanosecond apparatus). Average of 165 experiments. Chlrophyll concentration: 10^{-4} M. The bottom insert gives the time positioning and the duration of the laser flash.

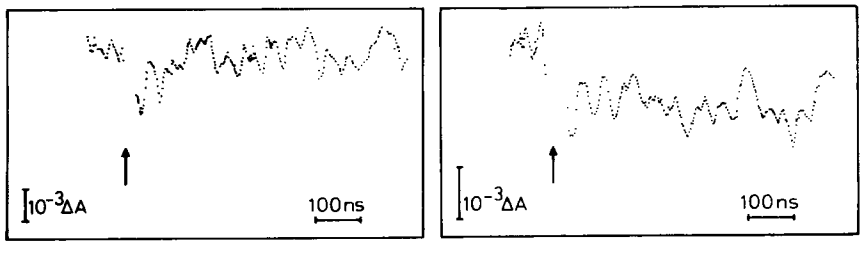


A. No addition B + NH_2OH

Fig. 8. Absorption changes at 820 nm (positive downward) induced in a suspension of dark-adapted chloroplasts with no addition (A) or with 8 mM hydroxylamine (B). Average of 80 experiments. Chlorophyll concentration: 10^{-4} M.

We compared the ΔA in chloroplasts before and after addition of hydroxylamine (Fig. 8). With each suspension 10 experiments were done without addition, then hydroxylamine was added and 10 more experiments were done and stored in a different memory of the averager. The process was then repeated with a fresh suspension. As shown in Fig. 8 the 30 ns phase is absent after incubation with hydroxylamine. The ΔA is about equal to the initial ΔA obtained with the control and also very close to the value measured 3 μs after the flash with the slower microsecond apparatus. It may be worth pointing out that addition of hydroxylamine induced a decrease by 50% of the light reaching the photodiode. This decrease has been compensated by increasing the bias voltage of the detector. It is attributed to a small change in the light scattering by the suspension whose effect is rather large because of the very small angle of collection of light by the photodetector.

When the suspension is illuminated by a background of far-red light in the presence of ferricyanide, a treatment which oxidizes *P*-700 prior to the actinic flash, the 30 ns phase is present but the slow phase is greatly decreased (Fig. 9A). A reverse effect (Fig. 9B) is observed when the suspension is supplemented with DCMU and preilluminated by three short xenon flashes prior to the ruby laser pulse (three flashes were given in order to insure a complete blocking of Photosystem II).



A. + ferricyanide + far red light B. + DCMU + flashes

Fig. 9. Absorption changes at 820 nm (positive downward) induced by a saturating ruby laser flash in suspensions of chloroplasts in two different conditions. (A) Addition of 5 mM ferricyanide and preillumination for 2 s by far-red light (20 mW · cm^{-2}, 740—900 nm). Average of 40 experiments. (B) Addition of 10 μM DCMU and preillumination by three white light xenon flashes 70 ms before the laser. Average of 60 experiments. Chlorophyll concentration: 1.1 · 10^{-4} M.

A straigthforward interpretation of these data is to propose that the 30 ns phase is due to the donation of electron by the first secondary donor to the oxidized form of the primary donor of Photosystem II. The slow phase is then due to the reduction of the primary donor of Photosystem I, except after hydroxylamine treatment where half of it is due to the 20—40 μs phase of reduction of P_{II}^+.

Conclusions

Taken together with the information previously drawn from fluorescence induction, luminescence and absorption spectroscopy (see Introduction), our present results may help presenting a coherent picture for early reactions on the donor side of Photosystem II. It appears that, in dark-adapted chloroplasts, P_{II}^+ is essentially reduced very fast ($\approx$30 ns) by the first secondary donor D_1. This donor is inactived by several treatments and, especially after treatment with hydroxylamine, an alternate donor D_1' reduces P_{II}^+ in 20—40 μs. We cannot exclude that D_1' is just a different state of D_1.

Trying a closer comparison with luminescence experiments, it appears that the 30—35 μs and the 200 μs phases, which are present in untreated material [10,12,27,28], were not observed in our experiments probably because they correspond to a minor phase of reduction of P_{II}^+. A similar behaviour has been observed at low pH [5,20]. Along the same lines a 30 ns phase may be expected to be present in luminescence decay curves, although its real observation may be technically difficult.

In our measurements of ΔA at 820 nm no decay was found in the 0.3—2 μs time range, either in untreated chloroplasts or in chloroplasts in which the primary acceptor of Photosystem II (A_1) was reduced by dithionite plus one preilluminating flash (data not shown). In these last conditions, however, a strong luminescence has been observed, with a decay time of 0.8 μs [12,33]. This time was attributed to the duration of the state ($A_1^- W^- P_{II}^+$), in which W is an additional acceptor operating when A_1 is reduced prior to the actinic flash. In our experimental conditions, in untreated chloroplasts, P_{II}^+ appears to be reduced in less than 100 ns after a flash, and thus it may be proposed that 0.8 μs is the duration of the state ($A_1 W^- P_{II} D_1^+$) which will disappear by a back-reaction via P_{II} (giving rise to luminescence) and by reduction of D_1^+ by the next electron carrier D_2.

In measurements of fluorescence induction, Mauzerall [29] found a fast phase (25 ns) after a flash given to dark-adapted *Chlorella* cells, but not after a flash given to cells preilluminated by one or several flashes. Our results are in good agreement with these data as far as dark-adapted material is concerned (chloroplasts in our experiments), provided we assume that P_{II}^+ is a quencher of chlorophyll fluorescence. Unfortunately we did not have the possibility of studying the 30 ns absorption change of P_{II}^+ in preilluminated chloroplasts and thus a more thorough comparison is not possible. With our "microsecond" equipment, however, we have found that, in spinach chloroplasts and in *Chlorella* cells, P_{II}^+ is essentially reduced in less than 3 μs after a flash given to a dark-adapted material as well as to a material preilluminated by two flashes ([6] and Van Best, J.A. and Mathis, P., unpublished results).

Acknowledgements

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