Biochimica et Biophysica Acta, 369 (1975) 371–381
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BBA 46970

CHLOROPHYLL RADICAL CATION IN PHOTOSYSTEM II OF CHLOROPLASTS. MILLISECOND DECAY AT LOW TEMPERATURE

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(Received March 3rd, 1975)

SUMMARY

We compare the absorption changes, in the near infrared and in the green part of the spectrum, induced in spinach chloroplast suspensions, at − 170 °C, by continuous light and by flashes.

(1) Following flash excitation, an absorption increase peaking at 825 nm which reverses rapidly ($t_{\frac{1}{2}} = 3.0$ ms) is not affected by ferricyanide; it is suppressed when chloroplasts are preilluminated in the presence of 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea (DCMU) and hydroxylamine. The reversion of that signal is simultaneous with a partial back reoxidation of *C*-550 (fully reduced by the flash) and with partial (about 25 %) oxidation of cytochrome b_{559}. The magnitude of the signal peaking at 825 nm (that we attribute to the radical cation of the trap chlorophyll of Photosystem II, acting as a primary electron donor) decreases progressively within a series of successive flashes.

(2) An absorption increase (40 % of which is slowly reversible) with a broad peak around 810 nm is induced by continuous light or by a flash. It is suppressed by pretreatment with ferricyanide, but it is little affected by the treatment with 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea and hydroxylamine. We attribute it to oxidized P_{700}.

(3) With chloroplasts pretreated with 10 mM ferricyanide, an absorption increase, whose magnitude is nearly independent of wavelength between 790 and 870 nm, can be induced by continuous light. One saturating flash produces only 20 % of the signal. This absorption change (20 % of which is reversible in 30 s) might be due to a secondary donor of Photosystem II.

INTRODUCTION

It is usually assumed that the primary photochemical step in photosynthesis is an electron donation from the excited trap chlorophyll (or bacteriochlorophyll) to a primary acceptor. In the case of Photosystem I of green plants, some studies by ESR

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

and by optical absorption spectroscopy [1–4] firmly establish that P_{700} is the primary donor, whose oxidized form ($P^{+}{}_{700}$) is a chlorophyll cation radical, very similar to the π-cation radical produced in vitro by various methods [5–8]. This last species presents a distinctive absorption maximum around 830 nm, both at room temperature [6] and at liquid N_2 temperature [7], with a molar extinction coefficient of about 7000 $M^{-1}cm^{-1}$. Detailed studies have also been successfully performed regarding bacterial photosynthesis (see e.g. ref. 9).

In the case of Photosystem II of green plants, precise evidence is still lacking. By techniques of absorption spectroscopy, the primary acceptor [10–13] has been identified as *C*-550 or *X*-320 (possibly a plastoquinone), whereas a bleaching of the chlorophyll absorption band (chlorophyll a_2, P_{680}) has been interpreted as an oxidation of the primary electron donor [14, 15]. By ESR it has been possible to obtain a photoinduced radical which is probably due to oxidized chlorophyll, but only in the presence of strong oxidizing agents [16–19]. It is still questionable, however, whether both types of results actually concern the primary electron donor. Several difficulties arise in the observation of absorption changes in the chlorophyll absorption bands: possible artefacts due to changes in chlorophyll fluorescence yield [20], important screening due to the antenna chlorophyll molecules, possible distortions due to local field effects [21]. Moreover, no precise kinetic correlation has been done between the primary acceptor and the primary donor, as it has been done with Photosystem I [22, 23].

We found it useful to make a comparative study of well-known low-temperature constituents (*C*-550, cytochrome *b*559) and of chlorophyll cation radicals, assuming that these radicals would absorb in the near infrared, as has been found in vitro [6–8]. In previous experiments we observed that, at − 170 °C, the primary acceptor of Photosystem II is fully reduced by one flash and largely reoxidized in a few milliseconds [24]. This result was interpreted by a partial back-reaction between the reduced primary acceptor (C-550^{-}) and the oxidized primary donor. We now propose to establish that a species absorbing at 825 nm is the oxidized primary donor of Photosystem II at low temperature (a chlorophyll cation radical, $Chl^{+}{}_{II}$) and that it behaves according to the back-reaction hypothesis [24–26]. It is discriminated from absorption changes due to $P^{+}{}_{700}$ and from those possibly due to a secondary electron donor of Photosystem II in ferricyanide-treated chloroplasts.

MATERIAL AND METHODS

Biological material

Chloroplasts were isolated from spinach leaves, in an isotonic buffer (0.4 M sucrose/0.01 M NaCl/0.02 M Tris, at pH 7.8), and then allowed to stay for 5 min in a ten-fold diluted buffer, at 4 °C, in some cases with 10 mM potassium ferricyanide. After centrifugation the pellet was homogenized with a mixture of buffer and of glycerol (40/60, v/v), with (in some experiments) addition of 10 mM potassium ferricyanide or of DCMU and hydroxylamine. The chloroplast suspension was then poured into small tubes and kept in liquid N_2.

Measurement of absorbance changes

The chloroplast suspension was thawed and transferred to a cuvette (thickness

1 mm) made of lucite windows inserted in a copper frame. The suspension was dark-adapted (1 min at 20 °C); it then received two saturating flashes of white light (2 μs duration, separated by 0.5 s) and then (after 2 s) was dipped for 1 min in liquid N_2. The cell was then transferred to a partly unsilvered Dewar flask, containing liquid N_2. The temperature in the cuvette was — 170 °C. The measurements of absorbance changes were effected after the cuvette stayed for 5 min in the Dewar. The actinic and measuring beams, perpendicular to each other, were at 45 °C of the plane of the cuvette.

In flash experiments the actinic beam was provided by a Q-switch ruby laser (8 ns, 300 mJ). In order to homogenize and attenuate the beam, a ground grass plate was inserted on its path, 5 cm from the cuvette. For the measurement of absorption changes (ΔA) induced by continuous light, we used the arrangement described in ref. 27.

The measuring beam was provided by a 800 W tungsten-iodine lamp mounted on a Bausch and Lomb 500 mm monochromator. For measurements in the near infra-red the lamp was operated at full power and the monochromator slits were of 3 mm. The following filters were used: a Wratten 99 between the monochromator and the cuvette; in front of the photomultiplier: an MTO interference filter ($\Delta\lambda$ about 15 nm; wavelengths: 802, 822, 840, 867 nm), two Wratten 48 A and one Wratten 87. In the visible range, the lamp was operated at 250 W and the slits were of 0.8 mm. The filters were as in ref. 27. We checked for the absence of actinic influence of the measuring beam.

The photomultiplier (EMI 9659 B) was operated at constant anode current (3 μA). The required electrical bandwidth was obtained by variable load resistors and capacitors. The amplified signal was measured with a multichannel analyzer which allowed for an improved signal/noise ratio by storage of several signals (see refs 24, 27 and 28).

The measuring beam was allowed to fall on the cuvette for about 10 s before a measurement. In the case of flash-induced ΔA, we stored in different sub-groups of the memory the signals due to the first flash and to the subsequent flashes; then the cuvette was illuminated for 8 s by saturating continuous light, and we stored the signals due to the subsequent flashes. The laser flashes were saturating and separated by 30 s. We usually subtracted the adequate number of laser artefacts (obtained with the same photomultiplier voltage and no measuring beam). In the case of ΔA induced by continuous light, we stored in 2 sub-groups the signals due to a first illumination and to a second illumination given 30 s later. A small fluorescence artefact (in the near infra-red) was then subtracted. The presence of this artefact precludes any precise evaluation of the kinetics of light-inducted ΔA (in the near infra-red), but it does not affect the maximum level of ΔA.

Each experiment was repeated identically with a few (2–30) cuvettes and the results were summed for averaging. The number of averaged experiments is indicated in the figure legends.

RESULTS

(1) *Absorption changes around 825 nm. Comparison with C-550 and cytochrome* b_{559}

A comparative study of absorption changes in the near infrared and in the

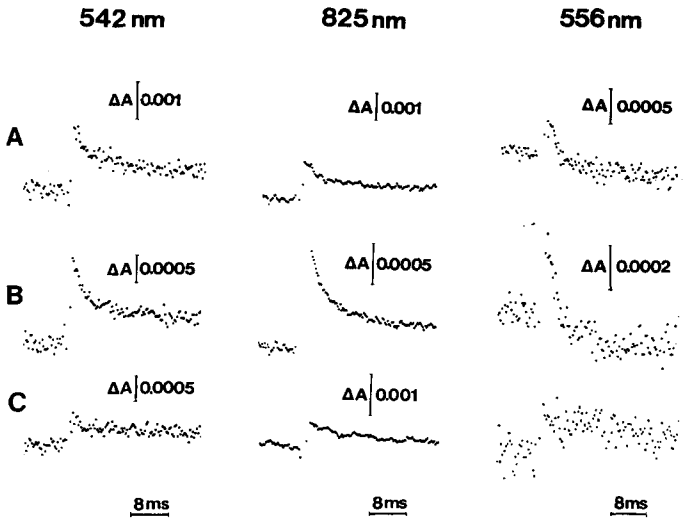

Fig. 1. Kinetics of absorption changes induced by a laser flash in chloroplast suspensions at −170 °C (chlorophyll concentration: 0.79 mg · ml^{-1}) at three wavelengths. A laser artefact (measuring beam off) has been subtracted. A: first flash. B: average of the first three successive flashes. C: average of the first three successive flashes, 30 s after a saturating illumination by continuous light (at 556 nm the absorbance scale is the same as for B). Number of averaged experiments: 8 at 542 nm, 6 at 825 nm, 30 at 556 nm.

Fig. 2. Absorption changes induced by continuous light in chloroplast suspensions at −170 °C (same batch as for fig. 1). Upper curves: first illumination. Lower curves (542 and 825 nm): second illumination (30 s after the first). At 825 nm an artefact due to the actinic beam has been subtracted; its magnitude is nearly equal to the signal due to the second illumination. Number of averaged experiments: 4 at 542 nm, 2 at 825 and at 556 nm.

green part of the spectrum has been performed with chloroplast suspensions excited by flashes or by continuous light. Some results are presented in Figs 1 and 2.

At 825 nm an absorption increase occurs within less than 120 μs; part of the signal decays with $t_{\frac{1}{2}} = 3.0 \pm 0.3$ ms and part is not reversible on our time scale (fig. 1A, 825 nm). Within a series of successive flashes (separated by 30 s) the amplitude of the rapidly decaying phase decreases progressively whereas the apparently non-reversible signal has a maximum amplitude after the first flash and the same amplitude (around 40 % of the first) on all subsequent flashes. When the suspension is first excited by a saturating illumination with continuous light, the rapidly decaying

phase is very much decreased (about 25 % of the effect of the first flash for non-illuminated chloroplasts); the apparently irreversible phase, however, has the same 40 % amplitude as in the precedent case (Fig. 1C, 825 nm). The amplitude of absorption change induced by saturating continuous light (Fig. 2) is about the same as the irreversible phase due to the first saturating flash; a second illumination (30 s after the first) induces an absorption increase which is about 40 % of that induced by the first.

The difference spectra of absorbance changes occurring in the near infrared are depicted in Fig. 3 (left), for the two phases in flash excitation and for the effect of continuous illumination. The spectrum of the rapidly decaying phase presents a distinctive peak around 825 nm, whereas the two others are very similar and are much broader.

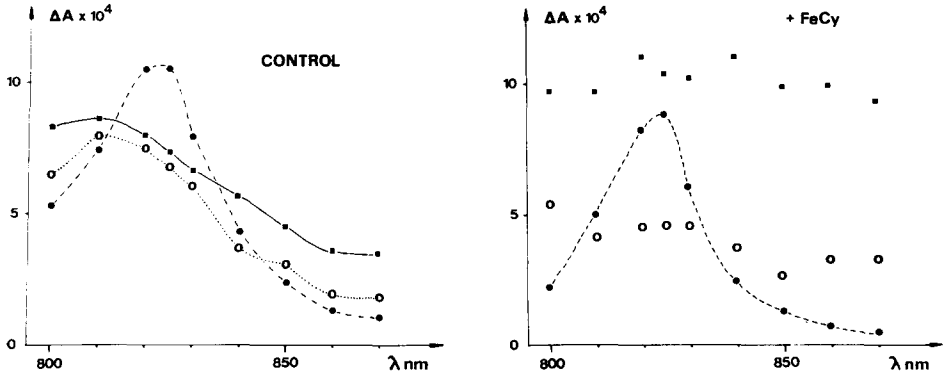

Fig. 3. Difference spectra of light-induced absorption increases in the near infra-red, without (control) or with 10 mM potassium ferricyanide (+FeCy). Chloroplast suspensions at 1 mg · ml^{-1} of chlorophyll. Solid circles: amplitude of the rapidly decaying phase induced by the first flash. Open circles: amplitude of the non- or slowly reversible phase induced by the first flash. Solid squares: absorption change induced by saturating continuous light.

In the green part of the spectrum, the absorption changes due to *C*-550 and to cytochrome b_{559} (Figs 1 and 2) are in agreement with our previous reports [24, 27]. At 542 nm the flash-induced signals are interpreted as a complete reduction of C-550 by the flash, followed by a partial backreaction; within a series of successive flashes the reaction centers are progressively photo-transformed to a stable state (with *C*-550 reduced and cytochrome b_{559} oxidized). After a saturating illumination by continuous light the remaining signal (Figs 1, 2) is interpreted as due partly to Photosystem I and partly to some slowly reversing Photosystem II reaction centers. In this series of experiments we found for the fast phase a half-time of 2.6±0.3 ms whereas in our previously reported experiments [24] the half-times were scattered around an average value of 4.2 ms. At 556 nm, the oxidation of cytochrome b_{559} by continuous light is detected as a slow absorption decrease (Fig. 2). In flash excitation, a small immediate positive absorption increase (already reported by Floyd et al.[15]) is followed by an absorption decrease ($t_{\frac{1}{2}} = 2.7 \pm 0.3$ ms) to a stable level (oxidation of cytochrome b_{559}). As for the rapid phase observed at 825 nm, the millisecond phase measured at 542 and at 556 nm has an amplitude which progressively decreases within a series of successive flashes. The first flash leads to the oxidation of about 25 % of the cyto-

chrome b_{559} which is oxidized by continuous light at − 170 °C. After a saturating illumination by continuous light, we observe at 556 nm a small flash-induced absorption increase (Fig. 1C, 556 nm); this positive signal, which will not be discussed below, is provisionally attributed to oxidized P_{700} (see ref. 2).

In these flash experiments the time-resolution was around 0.3 ms. Experiments performed with a time-resolution of 60 μs did not reveal any other absorption change at any of the studied wavelengths.

(2) Experiments with chloroplasts pretreated with DCMU and hydroxylamine

Chloroplast suspensions with DCMU (20 μM) and hydroxylamine (100 μM) received two flashes of white light at room temperature, before freezing. This procedure leads to a complete disappearance of absorption changes due to the reduction of *C*-550 and to the oxidation of cytochrome b_{559} [28]. Between 500 and 560 nm a remaining absorption increase has been attributed to Photosystem I[28]. Around 825 nm, the absorption changes induced by continuous light are practically unchanged (decreased by about 20 % compared to the control). In flash excitation the rapidly decaying phase is suppressed while the apparently non-reversible one is unchanged compared to the control (Fig. 4). These absorption changes which are not affected by the preillumination in the presence of DCMU and hydroxylamine are attributed to Photosystem I.

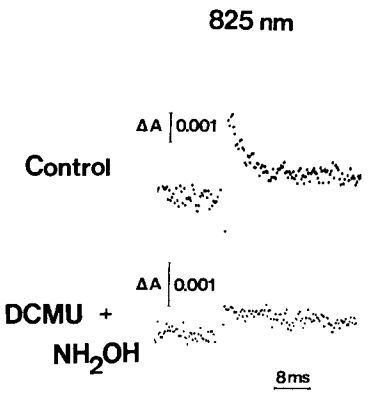

Fig. 4. Absorption changes at 825 nm induced by a laser flash in chloroplast suspensions at −170 °C (chlorophyll concentration: 0.9 mg · ml^{-1}) without inhibitor (control) or with DCMU (20 μM) and NH_2OH (100 μM). Average of the first five successive flashes in 2 experiments (control) or 3 experiments (DCMU+NH_2OH). The laser artefact has been subtracted.

(3) Experiments with chloroplasts pretreated with ferricyanide

The experiments described in paragraph 1 have been repeated with chloroplasts pretreated with 10 mM potassium ferricyanide, a treatment which oxidizes P_{700} and cytochrome b_{559}. As already reported [24, 28], this treatment abolishes the absorption changes at 556 nm and does not significantly affect the photoreduction of *C*-550 (both in continuous light and in flashes).

In the near infra-red we find that the rapidly decaying phase behaves for the control (Fig. 5), with $t_{\frac{1}{2}} = 2.6 \pm 0.3$ ms and a very similar difference spectrum (Fig.

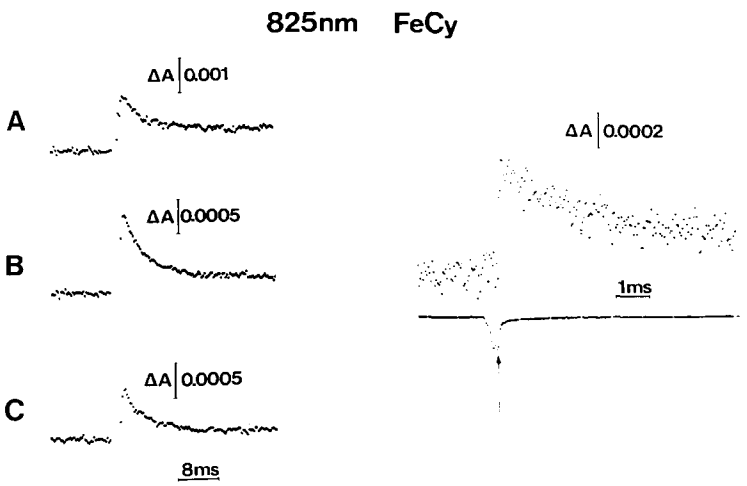

Fig. 5. Absorption changes at 825 nm induced by a laser flash in ferricyanide-treated chloroplast suspensions, at −170 °C. Left curves, A: first flash. B: average of the next five successive flashes. C: average of five flashes after a saturating illumination by continuous light. Average of 7 experiments. Laser artefact non subtracted. Chlorophyll concentration: 1.2 mg · ml^{-1}. Right curves, top: average of the first three successive flashes in 12 experiments at a greater time resolution. Laser artefact subtracted. Chlorophyll concentration: 0.3 mg · ml^{-1}. Bottom: laser artefact in identical experimental conditions. This artefact (downwards signal) is composed of a main electrical perturbation and of a superposed short optical perturbation (about two dots, i.e. two channels, at the arrow).

3, right). The non-reversible phase (in flash excitation) as well as the absorption changes induced by continuous light, however, are significantly affected by the ferricyanide treatment. At first their spectra, still similar between them, differ from their spectra in control chloroplasts, being practically flat (Fig. 3, right). Moreover, within a series of successive flashes, the amplitude of the non-reversible signal is relatively large after the first flash and then decreases very much (compare traces A and B in Fig. 5). After a saturating continuous illumination at − 170 °C, the remaining absorption changes induced 30 s later by continuous light (an appreciation of a slow reversion at the reaction centers) are smaller than for the control (20 % instead of 40 %, when compared to the effect of the first illumination).

An examination of the flash-induced ΔA with a better time-resolution (Fig. 5, right) indicates that the signal rise-time is not resolved at 825 nm ($t_{\frac{1}{2}}$ smaller than 40 μs). In the long-wavelength region (860 nm), however, we resolve the rise-time of a fraction of the signal; it is in the millisecond range, but the poor signal/noise ratio precludes any precise evaluation.

The kinetics of absorbance change induced by the same continuous light are about 1.5-times slower at 825 nm than at 542 nm. This comparison was not possible without ferricyanide because of the fluorescence artifact.

DISCUSSION

On the basis of difference spectra, kinetic properties and effects of inhibitors, we propose to classify the absorption changes that we observe in the near infra-red into

three types. We attribute them respectively to the cation radical of the trap chlorophyll *a* of Photosystem II (Chl_{II}^+) (rapidly decaying phase), to oxidized P_{700} ($P^+{}_{700}$) (non- or slowly reversible phase in control chloroplasts) and to an additional component of Photosystem II (non or slowly reversible phase in ferricyanide-treated chloroplasts). These will be examined successively.

(a) Chl_{II}^+

The rapidly decaying phase will be interpreted in the frame of the back-reaction model for Photosystem II at low temperature, a model initially proposed by Butler [25] and by Murata et al. [26] and which received strong support from our recent kinetic studies [24]. The model is expressed according to our present kinetic data, with the restriction that oxidation of cytochrome b_{559} (Cyt b_{559}) is probably not a straight-forward reaction [27, 29].

$$C\text{-}550 - Chl_{II} \ldots \text{Cyt } b_{559} \underset{K_r}{\overset{\text{light}}{\rightleftharpoons}} C\text{-}550^- - Chl_{II}^+ \ldots \text{Cyt } b_{559} \xrightarrow[K_b]{} C\text{-}550^- - Chl_{II} \ldots \text{Cyt } b_{559}^+$$

with $K_r = 200\ s^{-1}$ and $K_b = 50\ s^{-1}$

The hypothesis that the rapidly decaying phase is due to Chl_{II}^+ is fully coherent with the scheme: appearance immediately after the flash, disappearance in parallel with the reoxidation of *C*-550 and with the oxidation of Cyt b_{559}, absence in chloroplasts preilluminated in the presence of DCMU and hydroxylamine, resistance to ferricyanide. On the basis of $\Delta\varepsilon = 20\,000$ at 556 nm for cytochrome b_{559} [30] and of a 1 : 1 ratio between cytochrome b_{559} (oxidized at −170 °C by continuous light) and Chl_{II}^+ (formed on the first flash), we find for Chl_{II}^+, at 825 nm, $\varepsilon = 7000$. This value, as well as the shape of the spectrum, are in good agreement with the in vitro properties of Chlorophyll a^+ reported by Borg et al. [6] and by Seki et al. [7].

An alternative interpretation of the species absorbing at 825 nm would be to consider it as a secondary band of *C*-550. This hypothesis is worth being considered as *C*-550 is related with carotenoids [30, 32] and as the radical ions of these polyenes present an absorption band in the near infrared, around 900 nm for the cation [33], and at shorter wavelength for the anion (Land, E. J., personal communication). However, *C*-550 accumulates under the effect of continuous light, whereas the species absorbing at 825 nm does not accumulate. So it appears that this last species is a purely transient one, even at − 170 °C; we find it quite reasonable to assume that it is the radical cation of the trap chlorophyll *a*.

(b) $P^+{}_{700}$

We attribute to $P^+{}_{700}$ the absorption increase with a broad spectrum peaking around 810 nm (Fig. 3, left) which is non- or slowly reversible (in the 10 ms time range) in flash excitation and which can be induced by continuous light. Arguments for that attribution are the shape of the difference spectrum (similar to that reported by Ke [2] and by Inoue et al. [3] for oxidized P_{700}), the 40 % reversibility (previously found for oxidized P_{700}, at − 196 °C, by Lozier and Butler [21] and by Visser et al. [22]), and also the absence of influence of DCMU and hydroxylamine.

(c) An additional component of Photosystem II

In ferricyanide-treated chloroplasts, P_{700} is oxidized in the dark and cannot account for the absorption change whose magnitude is nearly independent of wavelength between 790 and 870 nm (Fig. 3, right). This one is not due either to the primary donor Chl_{II}^{+} which reverses with a half-time of 3 ms and has a very different absorption spectrum. It might be due to a secondary electron donor of Photosystem II (replacing cytochrome b_{559}, chemically oxidized by ferricyanide) or to a photoreaction not directly connected with the couple *C*-550, Chl_{II}.

Two types of experiments have been previously devised in order to characterize the primary donor of Photosystem II at low temperature: flash-absorption spectroscopy and ESR (in the case of ferricyanide-oxidized chloroplasts). Floyd et al. observed a 4-ms phase in low-temperature flash-induced absorption changes, which they attributed to P_{680}, the chlorophyll molecule acting as primary electron donor in Photosystem II [15]. Their observation, however, is not unambiguous. Indeed, Lozier and Butler [21] and Visser et al. [22] observed a shift in the red absorption band of chlorophyll, at − 196 °C, which seems to parallel the reduction of *C*-550. We previously reported [24], and we confirm here, that *C*-550 is largely reoxidized within a few milliseconds after a flash. We expect a similar millisecond transient in the chlorophyll absorption shift, so that some doubt remains as to whether Floyd et al. really observed the primary electron donor or the absorption shift described by Lozier and Butler [21] and by Visser et al. [22].

Chloroplasts pretreated with ferricyanide present a light-induced ESR signal which is stable at low temperature and has been attributed to P^{+}_{680}, the oxidized primary donor in Photosystem II [16–19]. This attribution has been contested by Lozier and Butler who found no light-induced stable absorption decrease in the red part of the spectrum that would be specific of ferricyanide-treated chloroplasts [21]. Our present observation of a back-reaction between the reduced primary acceptor (C-550^{-}) and the oxidized primary donor (Chl_{II}^{+}), both in control and ferricyanide-treated chloroplasts, is also in disagreement with the above interpretation of ESR signals. We observed a new absorption change (Fig. 3, right, flat spectrum) which shares several properties in common with the ESR signal: appearance in ferricyanide-treated chloroplasts, stability, reversibility of about 20 % in a fraction of a minute [19]. So we presume that the ESR signal and the absorption change with a flat spectrum originate in the same photoreaction, and possibly in the same compound. Visser and Rijgersberg [19] proposed that the stable ESR signal was due to a chlorophyll *a* molecule acting as a secondary electron donor in Photosystem II. Our preceeding remarks on the ESR signals probably apply also to the species studied by Van Gorkom et al. [17] and Van Gorkom [34], who present no strong argument for the assignment of their absorption changes and ESR signals to the primary donor chlorophyll.

This report indicates that it is possible to monitor three components taking part in the reaction center of Photosystem II at liquid N_2 temperature: *C*-550, Chl_{II} and cytochrome b_{559}. In the back-reaction model (see above) we expect that chemical oxidation of the secondary donor (cytochrome b_{559}) will affect the rate of disappearance of Chl^{+}_{II} produced by a flash (i. e. K_r+K_b and/or the rate of reduction of *C*-550 by continuous light (proportional to $(K_b/(K_r+K_b)$. The fact that both rates are unaffected by ferricyanide treatment (see also ref. 28) may indicate that cyto-

chrome b_{559} is not directly the electron donor to Chl_{II}^+ and that an unknown donor is located between cytochrome b_{559} and Chl_{II}. We previously proposed a more complex scheme which incorporates such a donor [28]. A secondary electron donor has been recently observed by ESR at room temperature [35].

Our observation of the chlorophyll cation radical in the primary reaction of Photosystem II could simplify the concepts related to the photochemistry of that photosystem. For example the recent proposals of a direct intervention of pheophytin *a* [36] (whose cation absorbs at 775 nm [8]) as well as a direct chlorophyll-sensitized photoreaction (without formation of the radical cation) [37] are not easy to reconcile with our results. It seems now that there is a great similarity between all photosynthetic primary reactions at low temperature. They result in a radical cation of chlorophyll (or bacteriochlorophyll) and they are followed by a more or less complete back reaction with variable half-times: 200 ms in Photosystem I [22], 3 ms in Photosystem II, and around 30 ms in bacteria [9]. In general the primary reactions in Photosystem II have revealed to be relatively difficult to study. We hope that further investigation of Chl_{II}^+ by its absorption band around 825 nm will be possible at room temperature and will provide information on Photosystem II reactions in physiological conditions.

ACKNOWLEDGEMENTS

We would like to thank A. Peronnard for his excellent technical contribution.

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