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# PRIMARY AND SECONDARY ELECTRON DONORS IN PHOTOSYSTEM II OF CHLOROPLASTS

## RATES OF ELECTRON TRANSFER AND LOCATION IN THE MEMBRANE

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## Summary

Absorption changes at 820 or 515 nm after a short laser flash were studied comparatively in untreated chloroplasts and in chloroplasts in which oxygen evolution is inhibited.

In chloroplasts pre-treated with Tris, the primary donor of Photosystem II ($P$-680) is oxidized by the flash, as observed by an absorption increase at 820 nm. After the first flash it is re-reduced in a biphasic manner with half-times of 6 $\mu$s (major phase) and 22 $\mu$s. After the second flash, the 6 $\mu$s phase is nearly absent and $P$-680$^+$ decays with half-times of 130 $\mu$s (major phase) and 22 $\mu$s. Exogenous electron donors ($MnCl_2$ or reduced phenylenediamine) have no direct influence on the kinetics of $P$-680$^+$.

In untreated chloroplasts the 6 and 22 $\mu$s phases are of very small amplitude, either at the 1st, 2nd or 3rd flash given after dark-adaptation. They are observed, however, after incubation with 10 mM hydroxylamine.

These results are interpreted in terms of multiple pathways for the reduction of $P$-680$^+$: a rapid reduction (<1 $\mu$s) by the physiological donor $D_1$; a slower reduction (6 and 22 $\mu$s) by donor $D_1'$, operative when $O_2$ evolution is inhibited; a back-reaction (130 $\mu$s) when $D_1'$ is oxidized by the pre-illumination in inhibited chloroplasts. In Tris-treated chloroplasts the donor system to $P$-680$^+$ has the capacity to deliver only one electron.

The absorption change at 515 nm (electrochromic absorption shift) has been measured in parallel. It is shown that the change linked to Photosystem II activity has nearly the same magnitude in untreated chloroplasts or in chloro-

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

plasts treated with hydroxylamine or with Tris (first and subsequent flashes). Thus we conclude that all the donors ($P$-680, $D_1$, $D'_1$) are located at the internal side of the thylakoid membrane.

## Introduction

In green plants the primary reactions at the Photosystem II reaction center lead to the reduction of a primary electron acceptor and to the oxidation of the primary donor, a specialized complex of chlorophyll molecules ($P$-680). In subsequent reactions $P$-680$^+$ is reduced by the first secondary electron donor, which is connected more or less directly to the complex responsible of oxygen evolution. The organisation of the so-called 'donor side' of Photosystem II is still poorly understood, largely because of a lack of techniques which would permit a direct access to its constituants.

Conflicting results have been obtained concerning the location of the donors in the thylakoid membrane. The primary donor $P$-680 has been located either at the internal side [1] or closer to the outside [2,3]. Secondary donors are more consistently located at the internal side, although there are indications for an access from the outside (see ref. 4 for a review). Conflicting results have also been obtained on the rate at which $P$-680$^+$ is reduced and it is difficult to incorporate the existing data into a simple scheme. It may be considered as well established that $P$-680$^+$ is reduced very rapidly after a short flash given to dark-adapted chloroplasts: less than one microsecond according to some measurements [5—8], about 30 ns according to others [9], a value which would also account for a rapid fluorescence rise observed by Mauzerall in Chlorella cells [10]. It is not clear, however, whether this rapid phase also occurs in preilluminated chloroplasts since slower phases (half-times of about 6 $\mu$s and 30 $\mu$s, according to different authors and methods of measurement) have been shown to be more important in that case [11—15]. These phases have also been found, with a large magnitude, after inhibition of oxygen evolution by treatment of the chloroplasts with high concentrations of Tris or hydroxylamine [5—9,12, 15—18].

When the donor side of Photosystem II is severely damaged by treatment at pH 4.0, $P$-680 is disconnected from its donors and $P$-680$^+$ is reduced largely by back-reaction with the primary acceptor [18,19], with a half-time of about 120 $\mu$s. This back-reaction has also been shown to occur in Tris-treated chloroplasts when the electron donating capaciting of the secondary donors is exhausted [8,15,19,20], although there is some discrepancy as to the size of the electron pool. The hypothesis of a direct back-reaction can also account for the component recovering with $t_{1/2} \approx 100$—$200$ $\mu$s in the bleaching around 690 nm observed in repetitive flash absorption spectroscopy [21—25].

In this work we reexamine the kinetics of re-reduction of $P$-680$^+$ after a short flash by following the recovery of the absorption increase at 820 nm, with chloroplasts either untreated or treated by Tris or hydroxylamine. The effect of a first, second or third flash has been studied. This study has been prompted by the availability of a sensitive fast-response apparatus (effective time resolution: 1 $\mu$s) which permits rather good signal-to-noise ratio [9]. Once the state of oxidation of the donors was known with some confidence it became possible to measure the carotenoid absorption change at 515 nm in well defined conditions and thus to get some information on the localisation of the donors in the membrane.

## Materials and Methods

### *Chloroplasts preparation*

Spinach leaves were ground for 10 s in 0.4 M sucrose, 10 mM NaCl, 20 mM Tris (pH 7.8). The brei was filtered on a nylon mesh (10-$\mu$m openings) and the chloroplasts pelleted by centrifugation. Tris-treatment was performed with 0.2 M Tris at pH 9.0 (treatment Tris pH 9) [26] or with 0.8 M Tris at pH 8.0 as described by Blankenship and Sauer [27] (treatment Tris pH 8). As indicated in the Results section, both treatments led to the same results. The pellets were either kept on ice and suspended in 200 ml of buffer (50 mM tricine pH 7.6, 10 mM KCl, 2 mM $MgCl_2$, with or without 0.4 M sucrose) for experiments performed with a flow system [8,19,28] or homogenized in a minimum amount of buffer, kept on ice and diluted just before doing experiments in a regular 10 X 10 mm cuvette. No significant differences were observed between the results obtained with or without sucrose, except that the latter condition permitted a better chemical oxidation of *P*-700 by ferricyanide.

### *Measurement of absorption changes*

The kinetics of flash-induced absorption changes ($\Delta A$) were measured with an apparatus which can explore the time range from 1 $\mu$s to 1 ms [9]. The measuring light was provided by a pulsed tungsten lamp and filtered, before entering the cuvette, by an interference filter (820 nm, $\Delta\lambda = 10$ nm or 515 nm, $\Delta\lambda = 3$ nm) or by absorbing filters (Schott RG 715-3 nm plus 3 X Wratten No. 64) which allowed irradiation of the cuvette by a background of far-red light at wavelengths above 720 nm. The photodetector was protected by an interference filter (820 or 515 nm).

The square cuvette was excited at right angle by the preilluminating xenon flashes (Stroboslave, 5 $\mu$s duration at half-height) and by the actinic flash. For measurements of $\Delta A$ at 820 nm we used a ruby laser, and for $\Delta A$ at 515 nm we used the ruby laser or a dye laser, in the same conditions as in Ref. 9. Except when mentioned, all flashes were of saturating intensity for Photosystem II reactions.

For the measurement of the rise-time of $\Delta A$ at 515 nm a different apparatus was used. The measuring light was provided by a xenon lamp, pulsed during a few milliseconds (made by Applied Photophysics), coupled with a monochromator. Lamp pulsing was synchronized to fall within the opening time of a shutter. The intensity of the measuring light was not stable in the submicrosecond time range, and for that reason it was divided into two parts, one of them passing through the sample and the other serving as reference. Each beam fall on a photomultiplier (RCA, type 1 P 21). The photomultiplier outputs were connected to a differential amplifier (Tektronix, type 7A13) of the digitizer).

For experiments involving the flow system the suspension was at +9°C in the cuvette. For other experiments it was at +21°C.

## Results

### *Absorption changes at 820 nm in untreated chloroplasts*

Excitation by a flash of dark-adapted untreated spinach chloroplasts leads to an absorption increase at 820 nm. The results of such an experiment is shown in Fig. 1a, in which the time scan is limited to 50 μs. In previous work it has been shown that the decay could be approximated by three exponential phases with half-times of 10—17 μs, 210 μs and over 1 ms and that, to a first approximation, the $\Delta A$ was due solely to $P$-700$^+$ [9,18]. We confirmed that the signal was not affected by a 2 flash preillumination in the presence of 10 μM DCMU and 100 μM hydroxylamine, provided that DPIP and ascorbate were added to keep $P$-700 in the reduced state. In the absence of inhibitors, as shown in Fig. 1, the first and the third flashes bring about nearly the same $\Delta A$, with little difference either in amplitude or kinetics. The fast decaying phase has a half-time of ≈11 μs in both cases. The same data were obtained with no addition, with 1 mM ascorbate or 1 mM ascorbate plus 10 μM DCIP. These experiments have been repeated in the presence of ferricyanide and a background of far-red light, a treatment intended to oxidize $P$-700 and its donors prior to the actinic flash. It is shown in Fig. 1 that the first flash induces a much reduced $\Delta A$ which decays biphasically. The slow phase has a $t_{1/2}$ greater than 1 ms and is probably due to $P$-700$^+$. The fast phase ($t_{1/2} \approx 6$ μs) is not due to $P$-700$^+$ (compare Ref. 29); it is probably due to a fraction of $P$-680$^+$, evaluated to 20—25%.

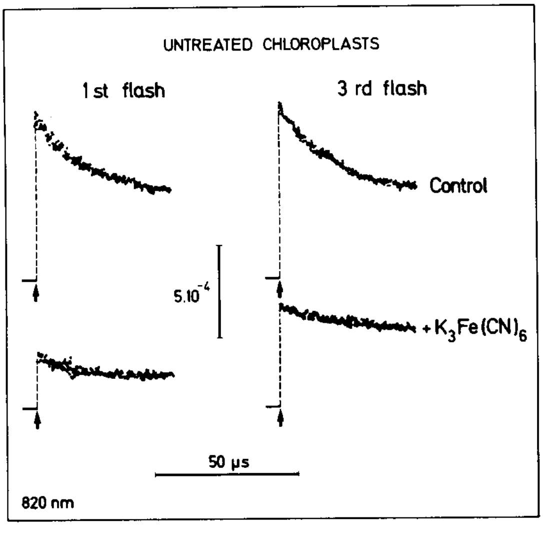


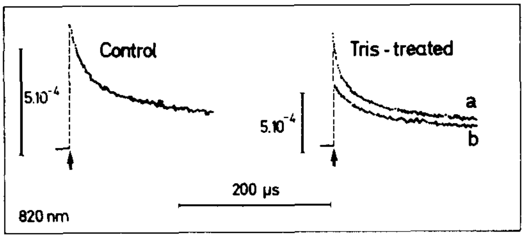
Fig. 1. Absorption changes at 820 nm induced in untreated chloroplasts (buffer without sucrose; 21°C) by a ruby laser flash. The chloroplasts were dark-adapted for 2 min and the laser flash was given alone (1st flash) or was preceded by two xenon flashes (100 and 200 ms before). The medium contained only the buffer (no addition; a 820 nm filter was placed on the measuring beam before the cuvette), or 6 mM ferricyanide (a background of far red light was provided). Chlorophyll concentration: 63 · $10^{-6}$ M (no addition) and 58 · $10^{-6}$ M (+ferricyanide). Average of 2 experiments.

We cannot decide whether this signal of $P$-680$^+$ is induced by the ferricyanide treatment or if it was already present in the control. In the same conditions the third flash induces a much larger $\Delta A$, probably because $P$-700$^+$ is largely reduced by electrons coming from Photosystem-II under the effect of the two preilluminating flashes (see Ref. 30). The fraction which decays in 50 $\mu$s is rather small (it would again represent 20—25% of $P$-680$^+$) and the decay is somewhat slower than after the 1st flash ($t_{1/2}$ included between 7 and 15 $\mu$s).

From these data we conclude that in physiological conditions $P$-680$^+$ is reduced in less than 1 $\mu$s, probably in 30 ns [9], either at the 1st, 2nd (data not shown) or 3rd flash, and thus that the S state of the oxygen evolving system has no obvious effect on its rate of reduction. Mauzerall reported that a fast phase of fluorescence induction was observed after the first flash given to dark-adapted *Chlorella* cells, but not after subsequent flashes [10]. This fast induction is attributable to the disappearance of the fluorescence quenching of $P$-680$^+$, since $P$-680$^+$ is reduced with a comparable half-time in dark-adapted chloroplasts [9]. More recent experiments by Mauzerall (personal communication) indicate, however, that the fast phase of induction is not limited to the first flash.

### *Absorption changes at 820 nm in Tris-treated chloroplasts*

In Tris-treated chloroplasts the first flash following dark-adaptation induces a larger $\Delta A$ at 820 nm, 1.6 to 1.9X the signal in the control (Fig. 2). If the suspension (supplemented with DPIP and ascorbate) is preilluminated by two flashes in the presence of 10 $\mu$M DCMU the signal is decreased to about its level in untreated chloroplasts (Fig. 2b). In view of the inhibitory effect of DCMU on Photosystem II, this remaining $\Delta A$ is attributed to $P$-700$^+$ and the



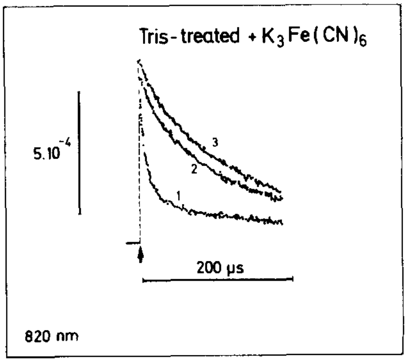


Fig. 2. Absorption changes at 820 nm induced in untreated chloroplasts (control) or Tris-treated chloroplasts (Tris, pH 9) (buffer with sucrose; +21°C) by a ruby laser flash. Control: dark adapted; addition of 15 $\mu$M DCIP and 1 mM ascorbate. Tris-treated: a (same as the control); b: further addition of 6 $\mu$M DCMU and preillumination by two xenon flashes (50 and 100 ms before the laser). Chlorophyll concentration: 56 · $10^{-6}$ M. Average of 4 experiments.

Fig. 3. Absorption changes at 820 nm induced in Tris-treated chloroplasts (Tris, pH 9) (buffer without sucrose; 21°C) by a ruby laser flash. Addition of 6 mM potassium ferricyanide. A background of far-red light was provided to the sample. 1: first flash. 2: 2nd flash (preillumination by one xenon flash, 50 ms before the laser). 3: 3rd flash (preillumination by two xenon flashes, 50 and 100 ms before the laser). Chlorophyll concentration: 46 · $10^{-6}$ M. Average of 4 experiments.

signal which has been supressed by preillumination in presence of DCMU is attributed to *P*-680$^+$. In Fig. 2a the kinetics of decay are highly complex. A good fit requires more than 4 exponential components, with half-times ranging from 4—6 μs to over 1 ms. This complexity is probably due to a multiphasic reduction of both *P*-680$^+$ and *P*-700$^+$. In order to greatly reduce the Photosystem I contribution, the chloroplast suspension was supplemented with potassium ferricyanide and a background of far-red light. Under these conditions the $\Delta A$ due to the first flash (Fig. 3) has a reduced amplitude and its decay (Fig. 4) can be approximated by two phases with half-times of 6 μs (50 to 70% of the amplitude at zero time), 22 μs (20 to 40%) and a very slow component ($t_{1/2}$ greater than 1 ms, 10 to 20%). After preillumination by one flash the decay is remarquably different (Fig. 3). The major phase has a $t_{1/2}$ of about 130 μs, the 22 μs phase is not much affected but the 6 μs phase is greatly reduced (less than 20%). After preillumination by two flashes (Figs. 3, 4) the 130 μs phase represents about 75% of the initial amplitude and the 22 μs phase about 25%. The 6 μs phase and the slowly decaying component are absent. It is worth pointing out that the larger $\Delta A$ observed after preillumination (Fig. 3) is a reproducible feature.

The dramatic change in the kinetics of decay after one preilluminating flash is not directly related to the presence of ferricyanide. It was also observed with

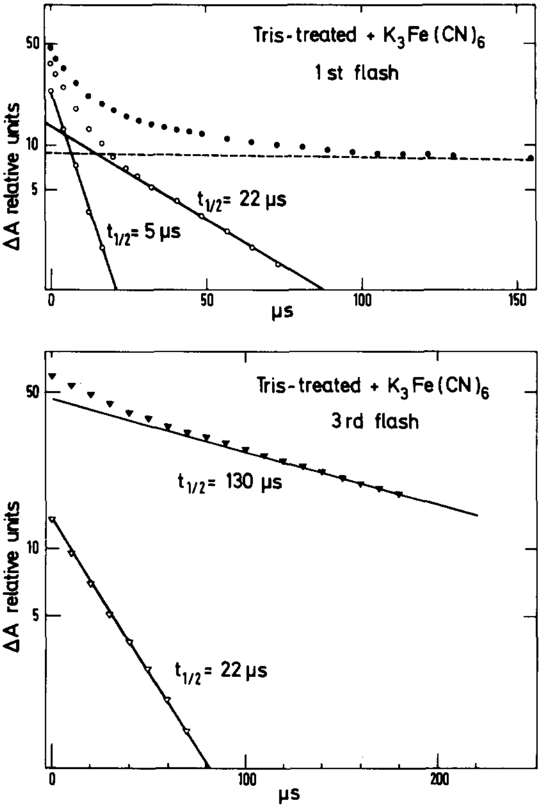


Fig. 4. Semi-log plot of the data of Fig. 3 for $\Delta A$ induced by the 1st and the 3rd flash.

chloroplasts without addition or with 1 mM ascorbate in experiments (not shown) at room temperature, with 10 ms delay between the preillumination and actinic flashes. The same behaviour was observed with chloroplasts treated with Tris at pH 8 or at pH 9. The effect was also observed with chloroplasts supplemented with ascorbate and DPIP or phenylenediamine. In this case, however, the effect of preillumination is apparently smaller, probably because of the occurrence of multiple phases of reduction of $P$-700$^+$ in addition to those of $P$-680$^+$.

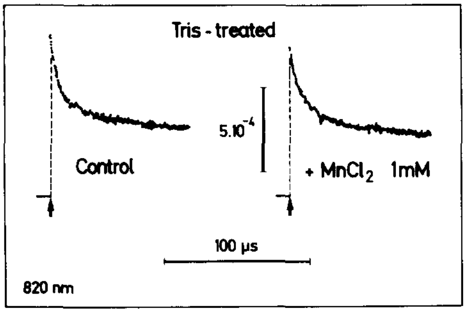
We have looked for an eventual effect of $Mn^{2+}$ on the kinetics. $MnCl_2$ was added either alone or with 1 mM ascorbate to chloroplasts pretreated with Tris at pH 8 or pH 9. We found no effect of this reductant on the decay following the 1st flash, even at the concentration of 1 mM (Fig. 5).

### *Absorption changes at 820 nm in the presence of hydroxylamine*

In previous articles [8,9,18] it has been reported that incubation of chloroplasts or Chlorella cells with 5—10 mM hydroxylamine led to an increased flash-induced $\Delta A$ at 820 nm, and that the additional signal decayed with $t_{1/2}$ = 20—40 $\mu$s. This experiment has been repeated (Fig. 6). As previously we found an increased $\Delta A$ (multiplied by 1.6—1.8), but the incremental signal induced by incubation with hydroxylamine appears to decay according to two exponential phases with half-times of 5—6 $\mu$s ($\approx$65%) and 20—25 $\mu$s ($\approx$35%). The difference between these values of half-times and the previously reported values [8,9, 18] is not yet understood.

### *Absorption changes at 515 nm*

In a few of the conditions at which absorption changes were measured at 820 nm, we also studied the flash-induced $\Delta A$ at 515 nm supposedly due to an electrochromic shift of the carotenoid absorption [31]. These experiments were done under air and thus the absorption change due to the triplet state of



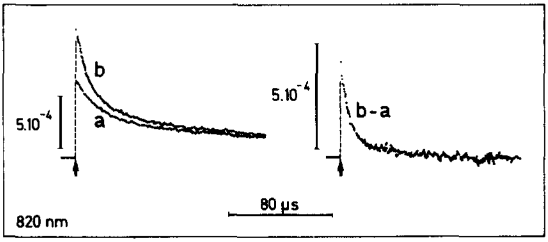


Fig. 5. Absorption changes at 820 nm induced in Tris-treated chloroplasts (Tris, pH 8) (buffer without sucrose; 21°C) by a ruby laser flash. The chloroplasts were dark-adapted for two minutes. The medium contained 1 mM ascorbate and no further addition (no addition) or 1 mM $MnCl_2$. Chlorophyll concentration: $34 \cdot 10^{-6}$ M. Average of 4 experiments.

Fig. 6. Absorption changes at 820 nm induced by a ruby laser flash in suspensions of dark-adapted chloroplasts (buffer without sucrose; 21°C). Addition of 15 $\mu$M DCIP and 1 mM ascorbate. a: untreated chloroplasts; b: incubation for 5 min with 10 mM hydroxylamine; b—a: numerical difference between signals in b and a. Chlorophyll concentration: $72 \cdot 10^{-6}$ M. Average of 4 experiments.

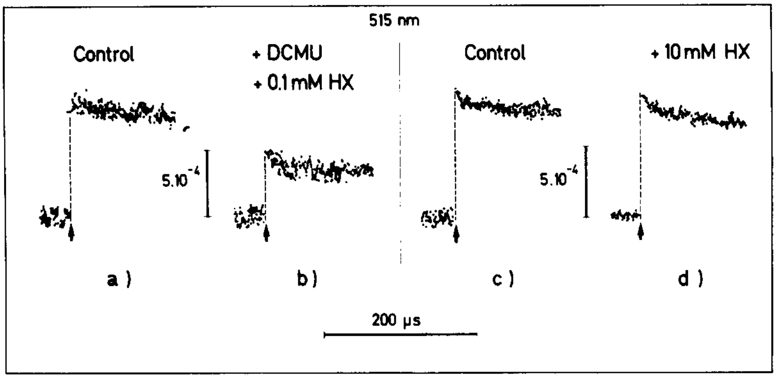
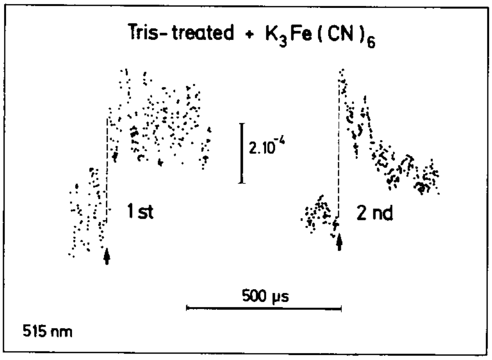


Fig. 7. Absorption changes at 515 nm induced in chloroplast suspensions (buffer with sucrose; +9°C) by a dye laser flash. (a) Control: dark-adapted chloroplasts. (b) Addition of 10 $\mu$M DCMU and 100 $\mu$M hydroxylamine and preillumination by two xenon flashes (100 and 200 ms before the laser). The suspensions were supplemented with 15 $\mu$M DCIP and 1 mM ascorbate. Chlorophyll concentration: $20 \cdot 10^{-6}$ M. Average of 20 experiments. (c) Dark-adapted chloroplasts with no addition; (d) the same suspension after 5 min of incubation with 10 mM hydroxylamine. Chlorophyll concentration: $20 \cdot 10^{-6}$ M. Average of 10 (c) or 20 experiments (d).

carotenoids decay in a few microseconds [32] and does not interfere with the slowly decaying electrochromic shift. With untreated chloroplasts (Fig. 7a, b) the flash-induced $\Delta A$ is decreased by a factor of two when the activity of Photosystem II is blocked by pre-illumination in the presence of DCMU and a low concentration of hydroxylamine. Following incubation with 10 mM hydroxylamine, a treatment which inhibits oxygen evolution but leaves the Photosystem II reaction center in a photoactive state, the flash-induced $\Delta A$ is nearly the same as in the control (Fig. 7c, d).

In Tris-treated chloroplasts supplemented with ascorbate and DPIP the flash-



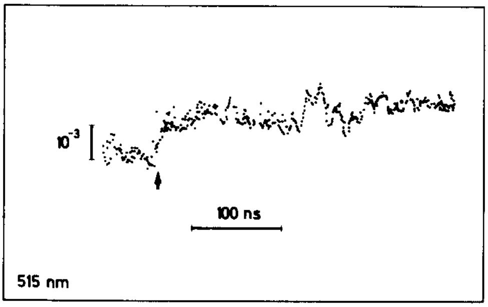


Fig. 8. Absorption changes at 515 nm induced in Tris-treated chloroplast suspensions (Tris, pH 9) (buffer without sucrose; 21°C) by a dye laser flash. Effect of the first flash after dark-adaptation (1st) or the second flash (preillumination by one xenon flash, 50 ms before the laser). Addition of 6 mM potassium ferricyanide. Chlorophyll concentration: $20 \cdot 10^{-6}$ M. Average of 20 experiments (left) and 40 experiments (right). The two signals were obtained with two different batches of chloroplasts.

Fig. 9. Absorption changes at 515 nm induced in untreated chloroplast suspensions (buffer with sucrose; 9°C) by a ruby laser flash. The flash was not saturating. Chlorophyll concentration: $35 \cdot 10^{-6}$ M. Average of 10 experiments.

induced $\Delta A$ is as large as in the control, but it amounts to only 40--50% of that value upon addition of 5 mM ferricyanide. In that case the signal induced by a first flash does not decay within 500 $\mu$s, whereas the $\Delta A$ induced by the 2nd or the 3rd flash decays with a half-time of 100—150 $\mu$s (Fig. 8). In the experiments reported in Fig. 8 the poor signal-to-noise ratio is due to the use of a reduced intensity of measuring light in order to avoid as much as possible a spurious actinic effect.

During that series of experiments we have also attempted to measure the rise-time of $\Delta A$ at 515 nm due to the electrochromic effect. In this experiment the laser was attenuated in such a way that the electrochromic effect was about 75% of its maximum value, in order to minimize the contribution from the triplet state of carotenoids. In despite of the poor signal-to-noise of the result (Fig. 9) it is possible to state that a large fraction (at least 75%) of the $\Delta A$ rises in less than our time resolution of 10 ns. This result, which confirms an earlier observation of Wolff et al. [33], will be discussed in the light of our other measurements at 820 and 515 nm. Experiments similar to that of Fig. 9 were also performed with a slower time scale and revealed that the absorption at 515 nm is constant between 6 and 100 $\mu$s after a flash.

## Discussion

Flash-induced absorption changes at 820 nm in chloroplasts can be interpreted rather easily from the respective contributions of the two oxidized primary donors, $P$-680$^+$ and $P$-700$^+$ (see refs. 8, 9, 18, 19, 34). In the case of Tris-treated chloroplasts the experiments that we report here, and especially the effect of DCMU, of ferricyanide and of pre-illumination by flashes, indicate three phases of reduction of $P$-680$^+$ with $t_{1/2}$ of about 6, 22 and 130 $\mu$s. According to these experiments, observation of the 130 $\mu$s phase requires pre-illumination by one flash (i.e. probably accumulation of one positive charge on the donor side of $P$-680); it is absent when DCMU is added. It is always present when the donor side is totally inactivated by treatment at low pH, in which case it has been demonstrated that $P$-680$^+$ disappears by back-reaction with the reduced primary acceptor [18,19]. Thus we will consider that the 130 $\mu$s phase corresponds to a direct back-reaction. A similar view has been expressed explicitly by other authors [7,12,20,35], although there is a wide range of reported half-times (90--300 $\mu$s).

The 6 $\mu$s phase represents the major phase of reduction of $P$-680$^+$ following the first flash, in Tris-treated chloroplasts. This phase is absent or of small amplitude in untreated chloroplasts (Fig. 1) in which case $P$-680$^+$ is largely reduced in 30 ns by its physiological donor $D_1$ [9]. Thus the 6 $\mu$s donor ($D_{6\mu s}$) is an alternate donor or a modified state of $D_1$. It is still operative after incubation with ferricyanide so that its mid-point potential is above +500 mV. The 6 $\mu$s phase disappears practically after pre-illumination by one flash and is replaced by the 130 $\mu$s back-reaction, from what we conclude that $D_{6\mu s}$ has a one electron capacity. This result, in agreement with that of Pulles [20], contradicts previous reports by Jursinic and Govindjee [15] and by Haveman and Mathis [19]. It is worth mentioning, however, that the present data have been obtained in much better conditions than our previous results [19]. As shown in Fig. 3 the $\Delta A$ attributable to $P$-680$^+$ is greater after a pre-illumination that after the first flash, especially since the very slow part of $\Delta A$ induced by the first flash is probably due to $P$-700$^+$. We assume that this increase is due to a fraction of the rapid donor $D_1$ which remains in the fast donation state, although being disconnected from the oxygen evolving system. The $\Delta A$ induced by the 3rd flash, in the presence of ferricyanide, corresponds to one chlorophyll cation per Photosystem II reaction center (assuming one $P$-680 per 400 chlorophylls), even if the 3rd flash is given 3 ms after the 2nd. This result indicates that all reaction centers have a rapidly re-oxidizable electron acceptor. It contradicts previous conclusion from Pulles, who proposed that the primary acceptor was very slowly re-oxidized in about half of the Photosystem II centers in Tris-treated chloroplasts [20].

It is tempting to sketch a comparison between donor $D_{6\mu s}$ and the species giving rise to the EPR signal named Signal $II_f$ in Tris-treated chloroplasts [2,36]. This signal corresponds probably to the oxidized state of a high-potential donor, which lasts for hundred of milliseconds and is reduced by an exogenous lipophilic donor like phenylenediamine. Since $D_{6\mu s}$ is the major donor and has a one electron capacity, we can propose that its oxidized state gives rise to Signal $II_f$. From the recovery of the 6 $\mu$s phase at the expense of the back-reaction as a function of delay between flashes, the duration of $D^+_{6\mu s}$ has been estimated to be about 2 s in presence of 5 mM ferricyanide, and of about 10 ms or 100 ms in the presence of 100 $\mu$M or 10 $\mu$M reduced phenylenediamine, a series of values which are comparable to those reported by Babcock and Sauer [2,36]. These authors found that the magnitude of Signal $II_f$ was reduced upon addition of $Mn^{2+}$ and concluded that $Mn^{2+}$ donates electrons to $P$-680$^+$ or to an electron carrier located between $P$-680 and Signal $II_f$. Since there is probably no additional carrier (one electron capacity) and since we found that $Mn^{2+}$ does not donate to $P$-680$^+$ (Fig. 5), we are led to propose that the decrease in the magnitude of Signal $II_f$ is due to another effect of $Mn^{2+}$. This effect of $Mn^{2+}$ may be indirect, for example it may equalize the populations of different spin levels in Signal $II_f$ by enhancement of the rates of relaxation.

An EPR signal similar to Signal $II_f$ has been observed in oxygen-evolving chloroplasts and named Signal $II_{vf}$ [37]. From the effect of the S state on the rate of disappearance of Signal $II_{vf}$, it was concluded that the species giving rise to Signal $II_{vf}$ is a carrier located between $P$-680 and the S state [38]. As a simple scheme for the main electron path on the donor side of Photosystem II we may thus consider that $P$-680$^+$ is normally reduced in 30 ns by the donor $D_1$ (whose oxidized state gives rise to Signal $_{vf}$), that Tris treatment modifies $D_1$ and the oxygen evolving complex, so that $D_1$ will now reduce $P$-680$^+$ in 6 $\mu$s and that $D_1^+$ ($D^+_{6\mu s}$) will stay oxidized a longer time and give rise to Signal $II_f$.

$P$-680 $\overset{30\text{ ns}}{\cdots\cdots\cdots}$ $\underset{(\text{Signal } II_{vf})}{D_1}$ ............ $O_2$ evolving complex physiological conditions

$P$-680 $\overset{6\ \mu s}{\cdots\cdots\cdots}$ $\underset{(\text{Signal } II_f)}{D_1}$ Tris-treated chloroplasts

Such a scheme presents obvious limitations (see e.g. refs. 39 and 40), but it might be worth being considered to represent a major fraction of different states of the reaction centers. The existence of two different states of the Photosystem II center in Tris-treated chloroplasts (and also with high concentrations of hydroxylamine) is probably the best way to account for the biphasic decay of $P$-680$^+$ (Figs. 3 and 4). In Tris-treated chloroplasts the two processes of donation (6 $\mu$s from $D_{6\mu s}$, 22 $\mu$s from $D_{22\mu s}$) do not co-exist at all the reaction centers since this would result in an increased rate of reduction of $P$-680$^+$ and a 2-electron capacity. The 22 $\mu$s phase appears to have nearly the same magnitude after the 1st, 2nd or 3rd flash. It thus behaves differently from the 6 $\mu$s phase; we presume that $D^+_{22\mu s}$ is re-reduced rather rapidly, either by back-reaction with the primary acceptor or by another high-potential donor. The effect of pre-illumination on $D_{22\mu s}$ could not be studied by our technique in reducing conditions (ascorbate + DPIP or phenylenediamine, hydroxylamine) because of interferences with a phase of reduction of $P$-700$^+$.

In a recent article, Renger et al. [41] reported the occurrence of a fast phase (2—5 $\mu$s) for the reduction of $P$-680$^+$ in untreated chloroplasts, but not after treatment with Tris or with hydroxylamine. In view of the present data the absence of that phase in inhibited chloroplasts is obviously a direct consequence of the repetitive technique utilized. We have no immediate explanation for the discrepancy between the results of Renger et al., who observed a large 2—5 $\mu$s phase in untreated chloroplasts, whereas we observed that at most 25% of $P$-680$^+$ is reduced in the time range from 2 to 50 $\mu$s, even after pre-illumination by two flashes (Fig. 1 and unpublished work in collaboration with T. Wydrzynski). The difference may stem from technical reasons (high repetition versus a few flashes) leading to a different pH inside the thylakoids, as proposed by Renger et al. [42].

In view of the preceding discussion the absorption changes at 515 nm may give some information on the location of the donors, if we suppose that the full $\Delta A$ at 515 nm is obtained when the oxidized donor considered is at the internal part of the thylakoid membrane. It is assumed that the primary acceptor is located at the outside of the membrane [4]. In untreated chloroplasts a maximum $\Delta A$ due to Photosystem II is indeed observed when the oxidized donor is $P$-680 (less than 20 ns after a flash, Fig. 9), $D_1$ (from 30 ns to $\simeq$100 $\mu$s after a flash, Figs. 7 and 9), or the oxygen-evolving complex (after 100 $\mu$s, Fig. 7). After treatment with Tris or hydroxylamine, the same maximum $\Delta A$ is observed when the oxidized donor is $P$-680 (Fig. 8, 2nd flash), $D_{6\mu s}$ or $D_{22\mu s}$ (Fig. 7, Fig. 8, 1st flash, after approx. 30 $\mu$s). It may thus be tentatively concluded that all the donors considered are located at the internal side of the membrane. This is in agreement with the results of Schliephake et al. [1] and on most data on the access of constituants of the donor side of Photosystem II to exogenous molecules [4,43]. It is worth mentioning, however, that Jursinic et al. [44] came to a different conclusion in using a different indirect approach.

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