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Study of the PS I acceptor side by double and triple flash experiments

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Electron transfer from P-700 to the PS I electron acceptors has been studied by flash absorption spectroscopy after double or triple excitation at room temperature, in the presence of glycerol, in pea chloroplasts and in PS I particles supplemented with purified plastocyanin. After two flashes, the reduction of P-700$^+$ occurs with $t_{1/2}$ = 250 μs when the excitations are separated by less than 10 ms. This reduction is attributed to the recombination reaction between P-700$^+$ and the reduced iron-sulfur center X. Under conditions where bound plastocyanin can rapidly reduce P-700$^+$ after the second excitation, a third flash induces an oxidation of P-700 which decays with a $t_{1/2}$ of 30 μs. This new kinetic phase is tentatively attributed to a back-reaction between P-700$^+$ and an electron acceptor more primary than the iron-sulfur center X. Addition of glycerol to the samples is shown to inhibit the reduction of the iron-sulfur center A at room temperature. Data are compared with results obtained under background illumination and reducing conditions.

Introduction

The structure of the Photosystem I reaction center is complex. At least five spectroscopically different PS I electron acceptors have been described in the literature. Results concerning their relative amount and the sequence of electron transfer are still conflicting (for reviews see Refs. 1 and 2). It is generally agreed that secondary acceptors are iron-sulfur centers (Fe-S_A, Fe-S_B and Fe-S_X), but their functional organization is still in debate. Fe-S_A and Fe-S_B are proposed to be the terminal electron acceptors of PS I, but it is not clear whether their arrangement is sequential or if they operate in parallel under physiological conditions. There is still little direct evidence for the role of Fe-S_X as direct electron donor to Fe-S_A and Fe-S_B. The optically detected species A_2 and Fe-S_X (detected by EPR) have been proposed to be the same electron acceptor [3], a proposal which received strong support from recent studies [4,5]. Concerning the more primary electron acceptors, A_1 has been tentatively identified as phylloquinone (or vitamin K-1) [6–8]. Whether there is one or two quinone molecules accepting electrons in PS I is still under discussion. The fifth species is the primary acceptor A_0, presumably a specialized molecule of chlorophyll *a*. Due to the use of EPR spectroscopy and also to the low redox potential of the molecules involved, many studies on the PS I acceptor side have been performed at cryogenic temperatures, often under highly reducing and anaerobic conditions, at high pH values. Results

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Abbreviations: ΔA, absorption change; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPIP, dichlorophenolindophenol; Mes, 4-morpholineethanesulfonic acid; PS I, Photosystem I; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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obtained under such experimental conditions are difficult to extrapolate to room temperature and thus, electron transfer within the PS I acceptors under physiological conditions is very poorly understood.

Here, we present results dealing with the PS I electron acceptor side, obtained on chloroplasts and on PS I particles, at neutral pH and at room temperature. A large effect of glycerol on PS I electron transfer reactions is reported. Two microsecond kinetic phases of P-700$^+$ reduction are tentatively attributed to back-reactions originating from two different electron acceptors. Results are compared to those performed under strongly reducing conditions. It has been previously shown that it is possible to photo-oxidize and rereduce P-700 twice within a very short time (100 μs) in the presence of an exogenous electron acceptor [9]. Kinetic analysis of the P-700$^+$ reduction after each excitation indicates that this behavior is due to the binding of two plastocyanin molecules to the PS I reaction center, and that when the first plastocyanin ('close' bound plastocyanin) has reduced P-700$^+$ (with a $t_{1/2} = 12$ μs), it is replaced or re-reduced by the second one with a $t_{1/2} = 110$ μs. The 12 μs P-700$^+$ reduction phase is also observed in the presence of 60% (v/v) glycerol. Under such conditions, the 12-μs reduction phase reappears after the second flash with a half-time of about 10 ms (instead of 110 μs in the absence of glycerol). Glycerol is also known to interact with the PS I electron acceptors and to be likely to modify the electron transfer pathway between them [10].

The rationale of our experiments is as follows. In the absence of any artificial electron acceptor and in the presence of glycerol, a first saturating flash will reduce a terminal electron acceptor and the fast reduction of P-700$^+$ by plastocyanin will stabilize it. If a second flash is given before plastocyanin is again able to reduce P-700$^+$, a more primary electron acceptor will be reduced, but P-700$^+$ will tend to be re-reduced by back-reaction with that acceptor. If the second flash is given after a time sufficient for plastocyanin to be again able to reduce P-700$^+$ rapidly, a second electron acceptor will be stably reduced, and thus a third flash will yield information on the recombination reaction between P-700$^+$ and a still more primary PS I electron acceptor.

Material and Methods

Intact chloroplasts were isolated from 10–15-day-old pea plants as described by Nakatani and Barber [11]. The chloroplasts were osmotically shocked in the measuring cuvette and the final composition of the suspension medium was 0.33 M sorbitol, 20 mM Tricine-NaOH (pH 7.5), 10 mM KCl, 10 mM $MgCl_2$, 1 mM sodium ascorbate, 10 μM DCMU, 40 μM hydroxylamine and 60% (v/v) glycerol, unless otherwise indicated. In experiments performed in the presence of sodium dithionite, sodium ascorbate and hydroxylamine were omitted and dithionite was 10 mM.

Digitonin-solubilized PS I particles from spinach (D-144 particles) were prepared according to Boardman [12]. The particles were suspended in 50 mM Mes-NaOH (pH 6.0), 10 mM $MgCl_2$, 2 mM sodium ascorbate and 160 μM spinach plastocyanin. In experiments performed without addition of plastocyanin, the particles were suspended in either 100 mM Tricine-NaOH (pH 7.5) or in 400 mM glycine-NaOH pH 10. When indicated, 60% (v/v) glycerol was added. Spinach plastocyanin was purified and assayed as described by Bottin and Mathis [13].

Flash-induced absorption changes at 820 nm were measured, as described by Bottin and Mathis [9,13], with an apparatus previously described by Van Best and Mathis [14]. Excitation was provided by one or two similar ruby laser flashes (Quantel; $\lambda = 694.3$ nm; $t_{1/2} = 6$ ns; $E_{max} = 40$ mJ) and a dye laser (Electrophotonics; $\lambda = 600$ nm broad band; $t_{1/2} = 1$ μs). The laser flashes were attenuated with neutral density filters to be just saturating and homogenized by a piece of ground glass. The delay between the three laser flashes was varied from 30 μs to 100 ms. Absorption changes were recorded with a transient digitizer (Biomation, Model 1010) and transferred to a signal averager (Tracor Northern, TN 1710). The kinetics resulting from 4 to 16 flash excitations were added in the signal averager. The time between two flashes (or groups of two or three flashes) was 20 s. Data acquisition was initiated either by the first, the second or the third laser flash. In some experiments, continuous background illumination was provided by the analysis light, in replacing the 820 nm interference filter, placed between the iodine-tungsten lamp and the cuvette, by a 5 mm thick RG 715 Schott filter.

EPR spectra were recorded at 15 K with a Bruker ER 200D-SRC X-band spectrometer equipped with an Oxford Instruments ESR 900 liquid helium cryostat. The standard cavity (TE 102 mode) was used. Continuous illumination was provided by a 800 W tungsten-iodine lamp. The light was filtered for infrared (water cuvette + Calflex filter) and concentrated onto the cavity window by a plexiglas light pipe. Freezing under illumination was performed with the same lamp and a B-VT 1000 Bruker temperature unit for temperature control. The EPR sample tubes contained pea chloroplasts at a chlorophyll concentration of about 0.9 mg/ml. Chloroplasts were osmotically shocked in distilled water, then suspended in 0.33 M sorbitol, 20 mM Tricine-NaOH (pH 7.5), 10 mM KCl, 10 mM $MgCl_2$ and 50% (v/v) glycerol. Dithionite was added to the samples either in darkness or under a weak illumination. Its final concentration was 10 mM. Samples were frozen in an ethanol/solid CO_2 bath (200 K) and then transferred into liquid nitrogen.

Other experimental methods and computer analysis of kinetic data were essentially as described earlier [13].

Results

Double and triple flash experiments on pea chloroplasts and on PS I particles

In the presence of glycerol (60% v/v), the reduction kinetics of P-700$^+$ after one excitation flash have already been described [13]. Spectroscopic measurements performed at 820 nm with chloroplasts and PS I particles show that after a very fast absorption increase due to P-700 oxidation, the signal decays in a biphasic manner. In pea chloroplasts, about 50% of the total absorption increase at 820 nm returns with a $t_{1/2} = 12$ μs, as in the absence of glycerol. The remaining absorption decays very slowly, with a $t_{1/2}$ longer than 5 ms (Fig. 1a). The decay of this slow component cannot be measured with our apparatus since the amplifier of the photodiode is Ac-coupled. When glycerol is present, the 200 μs phase attributed to the reduction of P-700$^+$ by diffusing plastocyanin is no longer observed.

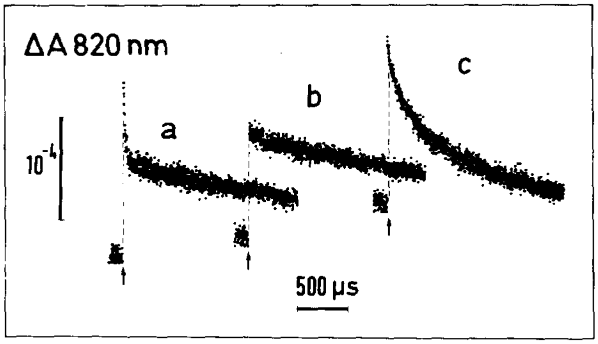

Fig. 1. Kinetics of flash-induced absorption change at 820 nm in pea chloroplasts at a concentration of 25 μg chlorophyll/ml, at 20 °C. Pea chloroplasts were osmotically shocked in the cuvette in distilled water. The suspension medium was then adjusted to 20 mM Tricine-NaOH (pH 7.5), 0.33 M sorbitol, 10 mM KCl, 10 mM $MgCl_2$, 1 mM sodium ascorbate, 10 μM DCMU, 1 mM methyl viologen, 40 μM hydroxylamine and 60% (v/v) glycerol. Cuvette optical path: 10 mm. Average of 16 experiments. (a) Signal induced by a single laser flash. (b) Signal induced by the second of two identical laser flashes separated by 5 ms. (c) Same conditions as (b), but in the absence of methyl viologen. The vertical arrows indicate the time of the laser flashes.

In the presence of an electron acceptor such as methyl viologen, a second laser flash given to the chloroplasts suspension induces the following features. For a delay time between the two excitations longer than 60 ms, the reduction of P-700$^+$ is identical after the first or the second flash (50% of 12 μs phase, 50% of slow phase). For delay times shorter than 10 ms, no fast reduction is observed after the second flash and P-700$^+$ is reduced only through a slow phase (Fig. 1b). When the delay is increased, the 12 μs phase progressively reappears, with a half-time of reappearance of about 9 ms (Fig. 2) [9].

Similar experiments performed also in the presence of glycerol but in the absence of any exogenous PS I electron acceptor gave significantly different results. For time intervals between the flashes longer than 60 ms, results were identical to those obtained in the presence of methyl viologen, i.e., same kinetics of P-700$^+$ reduction after the first and the second flash. On the other hand, for delays shorter than 10 ms, the P-700$^+$ re-reduction was not slow, but occurred mainly through a reaction of $t_{1/2} = 250$ μs (Fig. 1c). This kinetic phase represented about 96% of the total decay of

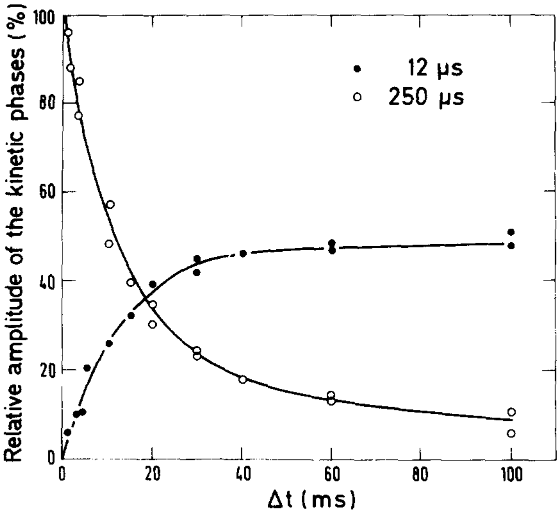

Fig. 2. Effect of the time interval between flashes on the relative amplitudes of the kinetic phases observed after the second laser flash (same conditions as in Fig. 1). Effect of the delay between the flashes on the relative amplitude of the 12 μs reduction phase of P-700$^+$ (solid circles) and on the relative amplitude of the 250 μs decay (open circles).

the signal induced by the second flash for a delay of 500 μs. When the delay was increased, the relative amplitude of this 250 μs phase decreased and the time delay corresponding to its half-disappearance was about 8.5 ms (Fig. 2). This value is similar to the delay needed for the half-reappearance of the 12 μs phase after the second flash (Fig. 2). The fact that the disappearance of the 250 μs phase and the reappearance of the 12 μs phase after the second flash occur with the same characteristic time most likely indicates that both phenomena are related.

Double flash experiments performed with PS I particles (D-144) supplemented with purified spinach plastocyanin, have been shown to give similar results as chloroplasts, in the presence of methyl viologen [13]. In this material, glycerol addition modifies the P-700$^+$ reduction kinetics as in pea chloroplasts. Double excitation experiments performed on this reconstituted material, in the absence of exogenous electron acceptor, gave qualitatively the same results as those obtained with pea chloroplasts. For delays between the flashes longer than 60 ms, P-700$^+$ reduction kinetics after the first and the second flash were identical. For shorter delays, P-700$^+$ was reduced after the second flash, through a major kinetic phase of $t_{1/2} = 250$ μs. When the delay was increased, the amplitude of the 250 μs phase decreased together with the increase of the amplitude of the 12 μs phase, both with a half-time of about 10 ms.

Addition of methyl viologen or benzyl viologen totally suppressed the 250-μs decay in both kinds of material (Fig. 1b). The acceptor concentration giving the half-suppression of the 250 μs decay was about 100 μM for either methyl viologen or benzyl viologen. The total disappearance of this kinetic phase was achieved in the presence of a concentration of electron acceptor (600 μM) much higher than the concentration commonly used in electron transfer studies (about 10 μM). This is most likely due to the high viscosity of the suspension medium, affecting the rate of electron transfer from PS I to the viologen.

The results indicate that the 250 μs decay probably originates from a recombination reaction between P-700$^+$ and a PS I electron acceptor. Such a 250 μs phase has already been observed by Sauer et al. [3] and was attributed to the back-reaction between A_2^- (probably Fe-S_X^-) and P-700$^+$, occurring after the reduction of both centers Fe-S_A and Fe-S_B. If one considers that the 250 μs phase observed in this work is also due to this back-reaction, then it must be assumed that in the presence of glycerol, only one electron can be transferred from P-700 toward either Fe-S_A or Fe-S_B, at least during the time of the experiment (about 100 ms). The next electron arriving from the photochemical reaction will then not have the possibility to go further than Fe-S_X. As already mentioned, another effect of glycerol is to increase the delay needed after the first flash for the reappearance of the 12 μs reduction phase of P-700$^+$ after the second flash.

Further investigation of the state of electron acceptors after two flash excitations was performed by triple flash excitation under similar conditions. The first two flashes were separated by a variable delay (Δt_1). Then, a third flash was given shortly after the second one ($\Delta t_1 = 40$ μs). For Δt_1 shorter than 10 ms, the absorption increase at 820 nm induced by the third flash decayed mainly through a 250 μs phase. So, for short delays, the kinetics following the second and the third flash are similar. This result brings further evidence for the attribution of the 250 μs phase to a back-reaction. In the present case, as Δt_1 and $\Delta t_1 + \Delta t_2$ are not long enough for plastocyanin to reduce P-700$^+$ either after the second or the third flash, then the charge separation states induced by the second and the third flashes are similar and decay by charge recombination. For longer values of Δt_1 (40 ms in Fig. 3), the absorption increase at 820 nm induced by the third excitation decayed with a major exponential phase of $t_{1/2} = 30$ μs (60% of the total amplitude) and an additional 250 μs component of smaller amplitude (20%). Fig. 4 shows the effect of the delay between the two first flashes (Δt_1) on the relative amplitude of the 30 μs and 250 μs phases observed after the third excitation, when the delay between the second and the third flashes was kept constant ($\Delta t_2 = 40$ μs). When Δt_1 was shorter than 10 ms, the relative amplitude of the 30 μs phase was small and P-700$^+$ decayed mainly with a half-time of 250 μs. Upon increasing Δt_1, the amplitude of the 30 μs phase increased at the expense of the 250 μs phase and reached a maximum for $\Delta t_1 = 40$ ms. The delay between the two first flashes giving 50% of the maximum amplitude for the 30 μs and the 250 μs phases observed after the third flash was 8 to 10 ms. This time is very similar to the half-delay measured for the reappearance of the 12 μs reduction phase after the second flash. This result can be simply interpreted by assuming that the 30 μs decay is observed only when the charge separation state formed on the second flash has been stabilized. The charge stabilization can occur when plastocyanin is able to reduce P-700$^+$ rapidly. This requires that the 12 μs phase must have reappeared after the second flash, avoiding any 250 μs recombination reaction. So, as long as plastocyanin has not reduced P-700$^+$ after the second flash, a 250 μs decay is observed after the second and the third flashes (Fig. 2 and Fig. 4b). Fig. 4a also shows that if Δt_1 was greater than 40 ms, the relative amplitude of the 30 μs phase was decreased. The half-disappearance corresponded approximately to a Δt_1 of 320 ms. This can be attributed to the fact that the reduced state formed on the second flash is only stable on a 100 ms time scale and that it disappears through an electron leak. This can be due to a slow electron transfer to an exogenous molecule, or to a slow internal transfer toward another electron acceptor. Considering that sequences of three flashes given to a sample every 20 s elicited similar kinetics, it can be assumed that the reduced state formed after three excitations totally relaxes in less than 20 s.

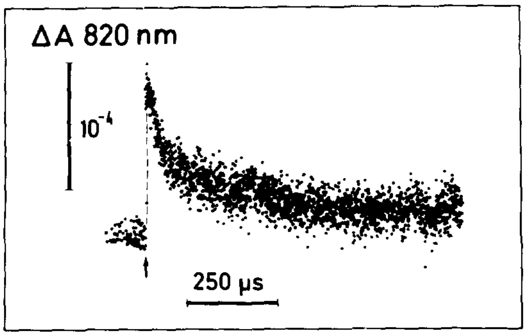

Fig. 3. Time-course of flash-induced ΔA at 820 nm. Same conditions as in Fig. 1, without methyl viologen. Pea chloroplasts at a chlorophyll concentration of 35 μg/ml. Average of 12 experiments. Signal induced by a third laser flash when the delay between the first and the second flashes, and between the second and the third flashes are 40 ms and 40 μs, respectively. The effects of the first two flashes are not seen on this time-scale.

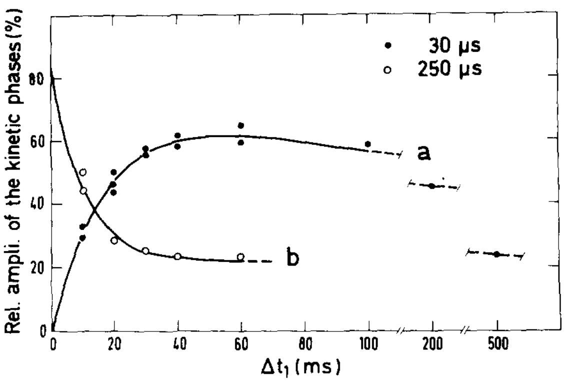

Fig. 4. Effect of the delay between the two first flashes (Δt_1) (a) on the relative amplitude of the 30 μs phase observed after the third flash, (b) on the relative amplitude of the 250 μs decay observed after the third flash. Same conditions as in Fig. 3. The delay between the second and the third flashes (Δt_2) was 40 μs.

Similar kinetic features were observed with D-144 particles supplemented with purified plastocyanine, in the presence of glycerol.

Addition of 1 mM methyl viologen to the samples (chloroplasts or PS-I particles) resulted in a total suppression of the 30 µs phase observed after the third flash as well as the 250 µs decay observed after the second flash. This can be simply interpreted by assuming that the stable reduction of the molecule responsible for the 250 µs decay is a prerequisite for the observation of the 30 µs phase. So, in the presence of methyl viologen, all the PS I electron acceptors are kept oxidized very efficiently.

Double flash experiments in the presence of dithionite

Double flash experiments performed with chloroplasts in the presence of glycerol under reducing conditions (10 mM sodium dithionite (pH 7.5)) gave the following results. Reduction of P-700$^+$ induced by a single laser flash still showed a 12 µs reduction phase representing 50% of the absorption increase. The decay of the remaining absorption was somewhat modified. It included a 250 µs component (30% of the total amplitude) in addition to the very slow kinetic phase observed in the absence of dithionite (Fig. 5a). When a second flash was fired on the sample, for delays longer than 20 ms, it induced a decay similar to the one observed after the first excitation. For delays shorter than 1 ms, P-700$^+$ induced by the second flash decayed through two major kinetic components: 60% of the total amplitude with $t_{1/2} = 30$ µs and 30% with $t_{1/2} = 250$ µs (Fig. 5b).

Upon increasing the delay, the relative amplitude of the 30 µs phase progressively decreased parallel to the increase of the relative amplitude of the 12 µs decay (Fig. 6). The relative amplitude of the 250 µs decay did not vary with the delay. The delays required for the half-disappearance of the 30 µs phase and for the half-reappearance of the 12 µs decay were similar ($t_{1/2} = 8$ ms). Thus, dithionite addition to the sample, in the presence of glycerol, results in the observation of the 30 µs decay after the second flash excitation and of the 250 µs decay after the first flash (instead of the third and second flashes, respectively, in the absence of dithionite). The occurrence of a 250 µs decay of constant amplitude after the second flash, is likely due to reaction centers devoid of 'close' bound plastocyanin. In these centers, P-700$^+$ is reduced by the 250 µs phase after both the first and the second flashes. The results can be interpreted by assuming that, as proposed above, glycerol inhibits the reduction of one of the iron-sulfur centers Fe-S$_A$ or Fe-S$_B$ and that under such conditions, dithionite can reduce or stabilize the iron-sulfur center which is not disconnected. It

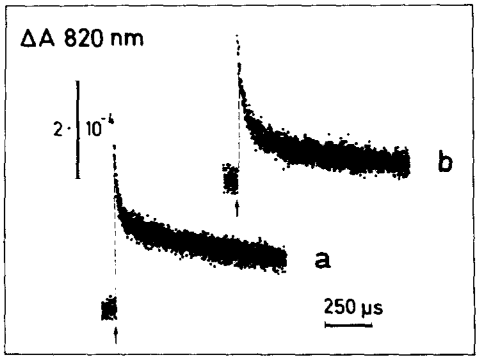

Fig. 5. Time-course of flash induced ΔA at 820 nm in pea chloroplasts. Same conditions as in Fig. 1, except that methylviologen and hydroxylamine were omitted and that 10 mM dithionite was added. Average of 16 experiments. (a) Effect of a single laser flash. (b) Effect of the second of two laser flashes separated by 1 ms. The ΔA induced by the first excitation is not seen on this time-scale.

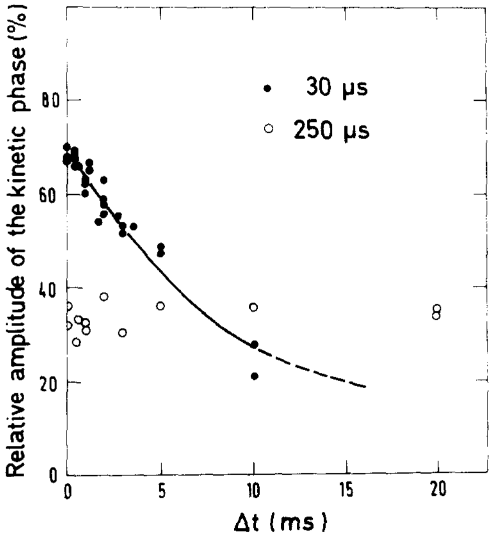

Fig. 6. Effect of the time interval between the two laser flash excitations on the relative amplitude of the 30 µs (solid circles) and 250 µs (open circles) decays following the second flash, in the presence of 60% (v/v) glycerol and 10 mM dithionite. Same conditions as in Fig. 5.

should be noted that the kinetics observed after the first and the following double flash groups were similar. The reduction state of the iron-sulfur centers of such samples has been investigated by EPR spectroscopy as described below.

Effect of continuous illumination on PS-I particles in the presence of dithionite

Sauer and co-workers [3] have shown that in Triton-solubilized PS I particles, when three flashes are given to the sample in the presence of an artificial electron donor to P-700$^+$, the third flash is followed by a 250 μs decay. This phase was attributed to the back-reaction between P-700$^+$ and Fe-S$_X^-$. Under highly reducing conditions (2 mg/ml dithionite (pH 10)), in the presence of an efficient electron donor and under continuous illumination, the absorption increase induced by a laser flash returns to the initial state with a $t_{1/2} = 3$ μs [3]. The 3 μs decay was then attributed to the back-reaction between P-700$^+$ and A$_1^-$, under conditions where Fe-S$_A$, Fe-S$_B$ and Fe-S$_X$ were all reduced prior to the flash. We have shown that when glycerol is present, the 250 μs decay does not occur after the third flash, but is observed either after the second flash or the first one (in the presence of dithionite). Furthermore, when the 250 μs phase is not observed, probably because of charge stabilization through P-700$^+$ fast reduction by plastocyanin, the following flash induces a 30 μs decay instead of the 3 μs component reported by Sauer et al. Experiments similar to those of Sauer and co-workers were performed with D-144 particles reduced by dithionite, either in the presence or in the absence of glycerol and at pH 7.5 or at pH 10. The four different conditions gave qualitatively similar results: a laser flash induced an absorption increase at 820 nm which decayed with a $t_{1/2} = 250$ μs (Fig. 7a). Under back-ground illumination, the 250 μs decay following the laser flash was partially transformed into a phase of $t_{1/2} = 4$ μs (Fig. 7b). Upon addition of DPIP, the amplitude of the 4 μs decay was increased at the expense of the 250 μs phase (Fig. 7c). No 30 μs kinetic component was detected. So, the occurrence of the 3 μs phase and the absence of the 30 μs decay in this type of experiment, are probably not related to the nature of the detergent used for membrane solubilization (digitonin in the present experiments). Furthermore, the absence of the 30 μs phase is related neither to the presence of glycerol nor to the pH of the suspension medium.

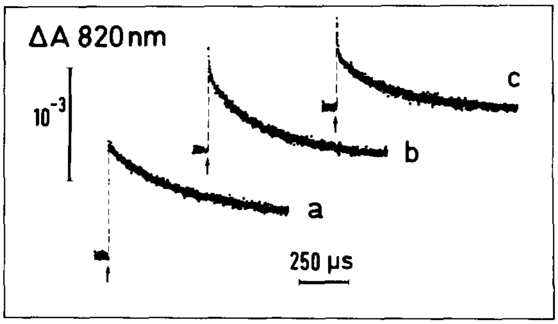

Fig. 7. Flash-induced absorption change at 820 nm in D-144 PS I particles. Particles at a chlorophyll concentration of 80 μg/ml were suspended in 200 mM Tricine-NaOH (pH 7.5), 60% (v/v) glycerol and 10 mM dithionite. Average of 12 excitation flashes. (a) No background illumination. (b) As in (a) but under far-red background illumination. (c) As in (b) except addition of 100 μM DPIP.

EPR measurements of the PS I electron acceptors

In order to interpret our optical experiments, we investigated the reduction state of the iron-sulfur centers by EPR spectroscopy. Pea chloroplasts in the presence of 50% (v/v) glycerol and incubated with 10 mM dithionite were rapidly frozen in an ethanol/solid CO$_2$ bath (200 K) and then transferred to liquid nitrogen in total darkness. EPR spectra of these samples are shown in Fig. 8. When dithionite was added to the sample in total darkness, no EPR signal attributable to reduced iron-sulfur center was detected in the spectrum (Fig. 8a). Low temperature (15 K) illumination of this sample resulted in the appearance of signals at $g = 1.86$, $g = 1.94$ and $g = 2.05$, indicating the reduction of center Fe-S$_A$ (Fig. 8b). If the addition of dithionite to the sample was done under room light, then the EPR spectrum showed signals indicating the reduction of Fe-S$_B$ alone ($g = 1.88$ and $g = 1.93$) (Fig. 8c). On this sample, low-temperature illumination resulted in the additional reduction of Fe-S$_A$ in the reaction centers where Fe-S$_B$ was already reduced (Fig. 8d). This is shown by the appearance of signals at $g = 1.89$, $g = 1.92$ and $g = 1.94$, characteristic of the presence of Fe-S$_A^-$ and Fe-S$_B^-$ in the same reaction center [1]. Considering that only one elec-

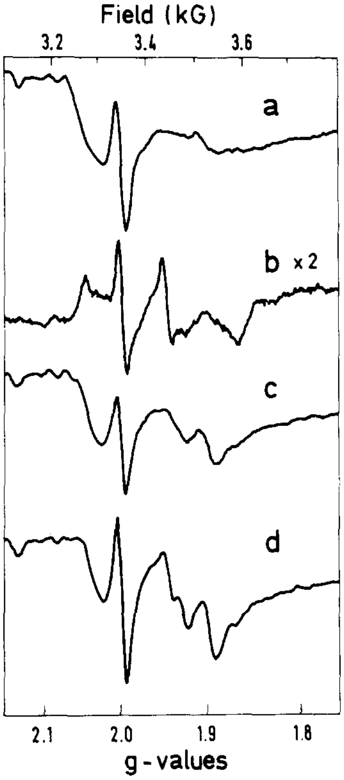

Fig. 8. EPR spectra of pea chloroplasts, 0.9 mg chlorophyll/ml in 0.33 M sorbitol, 20 mM Tricine-NaOH (pH 7.5), 10 mM KCl, 10 mM $MgCl_2$, 50% (v/v) glycerol with 10 mM dithionite. (a) Addition of dithionite and freezing of the sample in the dark. (b) Same as (a) but after low-temperature (15 K) illumination (light minus dark difference spectrum). (c) Addition of dithionite and freezing of the sample under room light. (d) Same as (c) but after low-temperature (15 K) illumination. Instrument settings: microwave power, 20 mW; frequency, 9.43 GHz; modulation amplitude, 10 G; receiver gain $2 \cdot 10^5$; temperature, 15 K.

tron can be transferred from P-700 toward the secondary electron acceptors upon low-temperature illumination, this result confirms that a majority of centers Fe-S_B were reduced prior to the low-temperature illumination. Thawing of this sample, followed by its freezing under illumination resulted in the appearance of signals indicating the reduction of both Fe-S_A and Fe-S_B (not shown). The amplitude of these signals was similar to that observed after low-temperature illumination. It indicates that the addition of dithionite under weak illumination followed by low-temperature illumination results in the almost complete reduction of both iron-sulfur centers, Fe-S_A and Fe-S_B. Similar experiments were also performed in the absence of glycerol. Under these conditions, no reduced iron-sulfur center was detected when dithionite was added either in total darkness or under room light. In the absence of glycerol, low-temperature illumination induced mainly the reduction of Fe-S_A as already reported [10]. Thus, EPR experiments show that, at room temperature, glycerol blocks the electron transfer from Fe-S_B^- to Fe-S_A. They also indicate that when glycerol is present, the effect of a weak illumination at room temperature in the presence of dithionite and at neutral pH results in the reduction of iron-sulfur centers Fe-S_B alone.

Discussion

EPR data clearly show that, in the presence of glycerol and dithionite, center Fe-S_B is easily reduced by a weak illumination at room temperature and that under such conditions, no Fe-S_A reduction occurs. This phenomenon is unambiguously related to the presence of glycerol. Strong effects of glycerol on the PS I electron acceptors have already been reported. Evans and Heathcote [10] measured significant increases in oxido-reduction potential for both iron-sulfur centers A and B after the addition of 50% glycerol. Furthermore, under these conditions, Fe-S_A and Fe-S_B were reduced under illumination at low temperature, whereas only center A was reduced in the absence of glycerol. However, this refers to results obtained at low temperature and, as pointed out by Chamorovsky and Cammack [15], the result of sample illumination is highly dependent on temperature. This parameter influences the nature of the iron-sulfur center which becomes photo-reduced and the stability of the reduced state. Chamorovski and Cammack [15] also showed that additions of glycerol tended to favor photoreduction of center Fe-S_B.

The EPR results help in the interpretation of the flash absorption kinetic data. They show that the observation of the 250 μs and the 30 μs decays after the first and the second excitation, respectively, in the presence of dithionite, instead of the second and the third flash when dithionite is omitted is due to the reduction of Fe-S_B by a weak illumination prior to the experiments. Thus the 250 μs kinetic component can safely be attributed to a recombination reaction for the following reasons. It is sensitive to the addition of the exogenous electron acceptors, methylviologen and benzylviologen. It is observed only under conditions where plastocyanin is not able to reduce rapidly P-700$^+$ and disappears in parallel with the reappearance of the fast reduction of P-700$^+$ by plastocyanin (Fig. 2). Finally, when plastocyanin is not effective, the 250 μs decay observed after the second flash is also detected after the third flash (Fig. 4b). A 250 μs phase has been observed in PS I particles by Sauer et al. [3] when centers Fe-S$_A$ and Fe-S$_B$ were pre-reduced. It was attributed to the back-reaction between P-700$^+$ and the electron acceptor A_2^-. Recently, the acceptor A_2 and the iron-sulfur center Fe-S$_X$ have been shown to be the same molecule [4,5]. We propose that, in our experiments, the 250 μs decay is also due to the recombination reaction between Fe-S_X^- and P-700$^+$.

When the 250 μs back-reaction is short-circuited by P-700$^+$ fast reduction by 'close' bound plastocyanin, an additional flash excitation induces a P-700 oxidation which decays with a half-time of 30 μs. This kinetic component is observed only when the fast P-700$^+$ reduction by 'close' bound plastocyanin is operating after the second flash (long Δt_1). Double flash experiments in the presence of dithionite show that the 30 μs decay, like the 250 μs one, is in competition with P-700$^+$ reduction by plastocyanin. Both phases are absent when the 12 μs P-700$^+$ reduction phase reappears. So we propose that the 30 μs is also due to a back-reaction. Since it is observed under conditions where Fe-S$_X$ is probably stably reduced, it can be attributed to the recombination reaction between P-700$^+$ and an electron acceptor more primary than Fe-S$_X$. This attribution, however, raises serious difficulties. Sauer et al. [3] reported that, in PS I particles, when A_2 was reduced by continuous illumination in the presence of an efficient electron donor to P-700$^+$ and under strongly reducing conditions, laser excitation was followed by a back-reaction of $t_{1/2} = 3$ μs. We have shown (Fig. 7) that this discrepancy can be attributed neither to the presence of glycerol nor to the pH value of the sample. Furthermore, our data show that it is possible to observe the 30 μs decay in D-144 particles supplemented with plastocyanin in the presence of glycerol after three excitations, but with the same material, using conditions similar to Sauer et al. (10 mM dithionite, 200 mM glycine (pH 10) and 100 μM DPIP under continuous background illumination), a laser flash produced a biphasic decay of $t_{1/2} = 4$ μs and $t_{1/2} = 250$ μs, as already reported [3], and we have no evidence for a 30 μs component in this decay. Results also show that glycerol has probably no effect on the rates of the back-reactions in PS I.

From these conflicting results, and considering also that the attribution of the 3 μs decay observed by Sauer et al. [3] is still uncertain, we can propose at least two different hypotheses explaining the discrepancies described above. Firstly, if the 3 μs phase is due to the decay of the P-700 triplet state as proposed by Sétif et al. [16], thus the back-reaction between P-700$^+$ and A_1^- is not observed under the conditions used by Sauer et al. [3]. This can be due to an over-reduction of the PS I acceptors, leading to the reduction, or more likely the double reduction, of A_1 (no EPR radical signal attributable to A_1^- is detected under such conditions [17]) in the centers where Fe-S$_X$ is already reduced. In this hypothesis, we can conclude that the 30 μs decay is probably due to the recombination reaction between A_1^- and P-700$^+$. Secondly, if the 3 μs phase is due to the recombi-

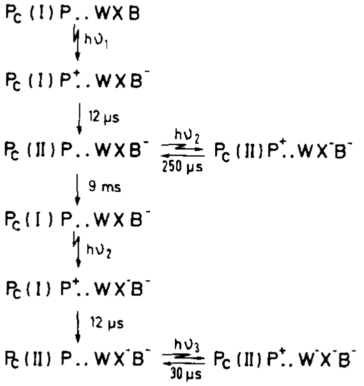

Fig. 9. Scheme of the electron transfer in PS I in the presence of glycerol in response to three flash excitations. Pc(I), Pc(II), P, B, X, W are bound reduced plastocyanin, bound oxidized plastocyanin, P-700, iron-sulfur center B (Fe-S$_B$), iron-sulfur center X (Fe-S$_X$) and the component responsible for the 30 μs decay, respectively. W is presumably not a new PS I component and is probably A_1.

nation reaction between P-700$^+$ and A_1^-, then the results presented here can be interpreted as follows. In multiple flash experiments, the 30 μs decay is induced very rapidly after the photo-reduction of Fe-S_X, and as the suspension medium is not reducing, all the electron acceptors rapidly return to the oxidized state after a flash sequence. In contrast, when the acceptors are slowly reduced under continuous illumination, Fe-S_X remains reduced for a longer time, allowing some modifications to occur in the proteins, such as a protonation or a conformational change. These modifications would then result in the acceleration of the reaction between P-700$^+$ and A_1^-. In this hypothesis, both 3 μs and 30 μs decays are attributed to the back-reaction between P-700$^+$ and A_1^-, but in two different states of the reaction center. Finally, one cannot exclude the possibility that the 30 μs phase originates from an electron acceptor different from A_1, such as the second iron-sulfur center Fe-S_X proposed by Bonnerjea and Evans [18].

The proposed electron transfer reactions occurring in double and triple flash experiments are summarized in Fig. 9. As discussed above, the component named W is a non-characterized PS I electron acceptor.

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