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PHOTOSYSTEM I PHOTOCHEMISTRY AT LOW TEMPERATURE

HETEROGENEITY IN PATHWAYS FOR ELECTRON TRANSFER TO THE SECONDARY ACCEPTORS AND FOR RECOMBINATION PROCESSES

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Electron transport has been studied by flash absorption and EPR spectroscopies at 10–30 K in Photosystem I particles prepared with digitonin under different redox conditions. In the presence of ascorbate, an irreversible charge separation is progressively induced at 10 K between P-700 and iron-sulfur center A by successive laser flashes, up to a maximum which corresponds to about two-thirds of the reaction centers. In these centers, heterogeneity of the rate for center A reduction is also shown. In the other third of reaction centers, the charge separation is reversible and relaxes with a $t_{1/2} \approx 120$ μs. When the iron-sulfur centers A and B are prereduced, the 120 μs relaxation becomes the dominant process (70–80% of the reaction centers), while a slow component ($t_{1/2} = 50$–400 ms) reflecting the recombination between P-700$^+$ and center X$^-$ occurs in a minority of reaction centers (10–15%). Flash absorption and EPR experiments show that the partner of P-700$^+$ in the 120 μs recombination is neither X nor a chlorophyll but more probably the acceptor A_1^- as defined by Bonnerjea and Evans (Bonnerjea, J. and Evans, M.C.W. (1982) FEBS Lett. 148, 313–316). The role of center X in low-temperature electron flow is also discussed.

Introduction

Cryogenic temperatures have long been used in studies of electron transfer among the membrane-bound acceptors in PS I. Low temperature EPR experiments allowed the identification of the secondary acceptors A, B and X, which are probably all iron-sulfur proteins (for recent reviews, see Refs. 1 and 2). Recently, two more primary acceptors named A_0 and A_1 were reported in Refs. 3–5. A_0 may be a chlorophyll molecule, but the *g*-value of A_1^-, around 2.005 [3–5], seems too high for a similar identification. According to the redox potential of these acceptors in spinach, a linear scheme can thus be written as: A_0–A_1–X–B–A. However, the linearity of this sequence has been questioned in two different aspects: A and B may function in parallel [1,2] and X may be on a sidepath of the electron flow [6].

The detailed dynamics of PS I photochemistry at low temperature are still poorly understood. Different kinds of experimental approaches give different results, which have not been interpreted up to now in a simple unique scheme. When all the acceptors are initially oxidized, illumination at low temperature of chloroplasts or PS I particles induces a charge separation between P-700 and the

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Abbreviations: PS I, Photosystem I; Chl, chlorophyll; ΔA, absorption change; DCIP, dichlorophenolindophenol; CIDEP, chemically induced dynamic electron polarization.

iron-sulfur center A [1,2]. This reaction is quasi-irreversible on a time scale of 1 h, at temperature below 20–30 K, although some charge recombination was observed in the same 10–30 K temperature range [7–10]. The latter studies were done with light pulses of a long duration and did not provide relative yields for charge stabilisation and recombination. Additionally, the partner of $P\text{-}700^+$ in the back-reaction was generally not identified, with the exception of a weak X^- signal decaying with $t_{1/2} \approx 0.8$ s [11].

Using laser flash excitation, a fast relaxation ($t_{1/2} \approx 120$ μs) of light-induced absorption changes was observed in chloroplasts [12] and PS I particles [13] at 10 K. It was interpreted as a back-reaction between $P\text{-}700^+$ and an unidentified acceptor. This observation was recently corroborated by dual laser-flash EPR experiments [6]. The 120 μs halftime does not fit with the kinetics of the following PS I relaxation processes at 10 K: (i) when A and B are chemically prereduced, charge separation at 10 K is followed by a slow back-reaction with $t_{1/2} = 130$–800 ms [14–16]; (ii) in PS I particles poised at a very low reduction potential or treated with SDS, illumination at low temperature induces the formation of the triplet state of P-700 [17,18], which decays with a mean half-time of about 800 μs [19]; and (iii) is probably the result of a submicrosecond recombination [19,20] between $P\text{-}700^+$ and the reduced primary acceptor, designated now A_0^- [3,4].

We shall here present evidence that the 120 μs phase reflects a recombination between $P\text{-}700^+$ and a reduced acceptor molecule, which is probably neither a chlorophyll molecule, nor X. It is also shown that the Photosystem I photochemistry is heterogeneous at 10 K: at a moderate reduction potential, about one-third of the reaction centers undergo only the 120 μs recombination whereas in the other two thirds, this reaction competes with irreversible electron transfer to the iron-sulfur center A. When the centers A and B are prereduced, the 120 μs phase becomes the dominant process; electron transfer to X and subsequent slow relaxation occurs only in a small minority of centers.

Material and Methods

Biological material

Chloroplast membranes were obtained from spinach leaves as in [21]. PS I particles were prepared according to [22] (hereafter named PS I particles without specificiation). This procedure basically involves the solubilization of thylakoïd membranes by digitonin and a one-step fractionation by polyacrylamide gel electrophoresis in the presence of deoxycholate. The chlorophyll to P-700 ratio of these particles is 90–110. Some other PS I particles were also prepared, using digitonin [23] or Triton [24,25] as a detergent.

EPR measurements

EPR spectroscopy was carried out with a Bruker ER200TT X-band spectrometer equipped with an Oxford Instruments ESR 900 liquid helium cryostat. The minimum response time of the spectrometer was reduced to 10 μs. Two different cavities were used: a standard cavity (TE 102 mode) and an optical transmission cavity. The temperature was checked with an Allen-Bradley carbon resistor calibrated at 4.2 K and 77 K and inserted in a sample tube. For kinetic experiments, the sample was excited by a xenon flash-lamp (10 μs half-width; 20 J; pulse frequency about 0.4 Hz). In some experiments the output of the spectrometer was fed into a Biomation 1010 transient recorder and the contents of the Biomation were transferred to a Tracor TN 1710 multichannel analyser. In other experiments made on a longer time scale, the output of the spectrometer was directly fed to the analyser (minimum dwell-time per channel 10 μs).

For non-kinetic experiments, the sample could be excited inside the cavity by a YAG-laser which was frequency doubled ($\lambda = 532$ nm; 11 ns). Continuous illumination was provided by a 800 W tungsten-iodine lamp whose light was filtered for infrared (water cuvette + Calflex filter) and concentrated onto the cavity window by a plexiglas light pipe. For continuous illumination at 200–230 K or 4°C and freezing under illumination, the same lamp was used with a B-VT 1000 Bruker temperature unit for temperature control.

The EPR sample tubes were calibrated (i.d. 3 mm) and contained PS I particles with a typical

chlorophyll concentration of 200 μg per ml. The maximum extent of reduction of the PS I acceptors was obtained by poising the PS I particles at pH 10 with sodium dithionite and freezing after and during illumination. The PS I particles thus treated exhibit, besides a fully developed (A^-, B^-) signal, the EPR signals of iron-sulfur center X and some radical signals probably due to the reduced primary acceptors, A_0 and A_1 (Fig. 1). A less reduced redox state (X oxidized, A^-, B^-) has been reproducibly obtained with and without the presence of glycerol. Without glycerol, the sample was fully reduced as described above, then thawed and maintained at room temperature for 20 min and frozen in the dark. Under these conditions, the state (X, A^-, B^-) is obtained (Fig. 1). Centers A and B are fully reduced as shown by the disappearance of the high-field peak of A^- at $g = 1.86$ which is replaced by the $g = 1.89$ signal of the coupled (A^-, B^-) centers. A minor X^- signal remains sometimes, which represents at most 10% of the maximum X^- signal observed under fully reducing conditions. With glycerol, a weak one-minute illumination at 220 K followed by a dark adaptation at the same temperature before immersion into liquid nitrogen provides the same redox state with a freshly prepared sample (pH 10 + dithionite).

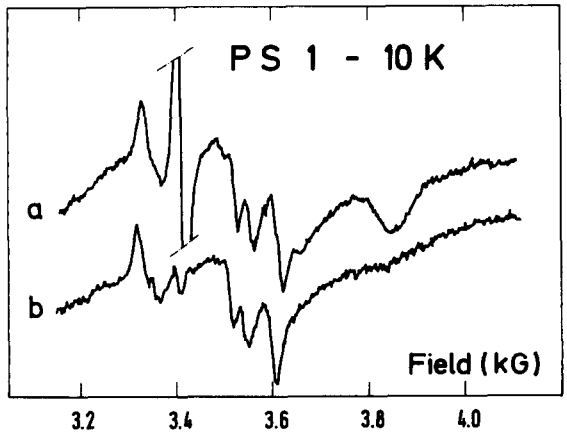

Fig. 1. EPR spectra of the reduced PS I acceptors in PS I particles; 0.2 M glycine buffer (pH 10); 20 mM sodium dithionite. (a) Frozen after and during illumination. (b) The same after 20 min in the dark at room temperature and then frozen in the dark. Instrument settings: temperature, 10 K; microwave power, 20 mW; frequency, 9.47 GHz; modulation amplitude, 20 G.

Absorption measurements

The PS I particles or chloroplast membranes were contained in a plexiglas cuvette which was inserted in a cryostat cooled with helium gas (as described in Refs. 12 and 26) and positioned at 45° of the mutually perpendicular exciting and measuring beams. Excitation was provided by a YAG-pumped dye laser (duration 20 ns; broadband emission around 600 nm). On a millisecond time-scale, the kinetics of flash-induced ΔA were measured with an apparatus described previously [27], using a silicon photodiode and a laboratory made a.c.-coupled amplifier [28]. On a longer time-scale (typically 0.5 s), the output of a PIN 10 photodiode was fed to a d.c. amplifier before entering a Didac 4000 (Intertechnique) or Tracor TN 1710 multichannel analyzer for signal averaging and subtraction of the artifact due to the actinic light. Light pulses of long duration were obtained with an electronic shutter placed in front of a 800 W projector. The measuring light was filtered before entering the cuvette either by an interference filter or by a red filter (RG 630-Schott). To get a spectrum of flash-induced ΔA in the red region, the red filter was used with a constant intensity of the measuring light to ensure an identical actinic effect of the measuring light at all wavelengths. Before detection, the light transmitted by the sample was filtered either by an interference filter or was focused on the entrance slit of a Bausch and Lomb grating monochromator with 3 nm (in the red region) or 10 nm (in the near infra-red region) bandpass.

Difference spectra due to illumination at low temperature were measured in a Cary 17 spectrophotometer ($\Delta\lambda = 0.2$ nm). The output of the spectrophotometer was fed into a Tracor TN 1710 analyzer for accumulation and processing of the data. Illumination was provided by a 800 W projector.

For flash-induced ΔA measurements, samples in the redox state (X, A^-, B^-) were prepared with and without glycerol in the same conditions as for EPR measurements.

Results

Photochemistry in PS I at moderate reduction potential

PS I particles, poised with sodium ascorbate

and frozen in the dark, show no EPR signal at 20 K before illumination (Fig. 2). Laser flashes, given on the same sample at 20 K, induce the appearance of the EPR signals of P-700$^+$ (Signal 1) and of iron-sulfur center A$^-$ (Fig. 2: $g = 1.86$ and 1.94). Minor signals attributable to B$^-$ also appear (Fig. 2: $g = 1.88$), probably due to the presence of glycerol [29]. A greater extent of light-induced irreversible charge separation is produced by an intense continuous illumination (Fig. 2). The laser flashes were saturating, as we checked in parallel experiments with an identical sample and attenuated flashes.

The flash-induced ΔA at 820 nm was also measured with PS I particles prepared in identical conditions (Fig. 3). The initial instrument-limited rise is followed by a multiphasic relaxation. For the first saturating flashes, a significant fraction of the ΔA remains after 1 ms (Fig. 3a and b). The initial signal size decreases according to the number of the flash down to a minimum level which is achieved after a continuous illumination (Fig. 2e). This last signal relaxes nearly completely within 1 ms after the flash with one major fast phase (80–90%; $t_{1/2} \approx 120$ μs) and its size represents about 40% of the first flash signal. A similar kinetic behaviour is observed for a flash-induced bleaching at 703 nm (Fig. 3, inset).

The EPR and absorption data can be correlated. If the decrease in ΔA at 820 nm induced by successive flashes results from the progressive population of reaction centers which are trapped in the state (P-700$^+$ – A$^+$), then the flash-induced

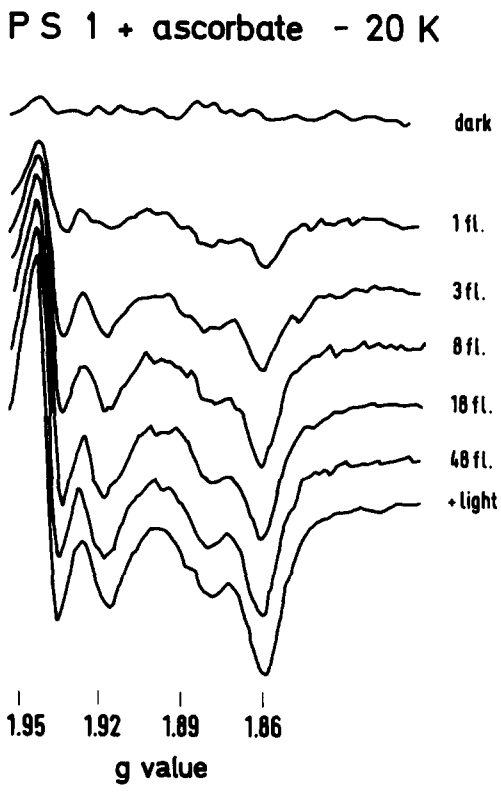

Fig. 2. Iron-sulfur centers A and B photoreduction induced at 20 K by successive laser flashes and a subsequent continuous illumination in PS I particles poised at pH 8 with sodium ascorbate (5 mM) and DCIP (200 μM) in the presence of 600% glycerol and frozen in the dark. Laser flashes (frequency-doubled YAG-laser: λ = 532 nm; 11 ns) were saturating. Instrument settings as in Fig. 1, except temperature (20 K).

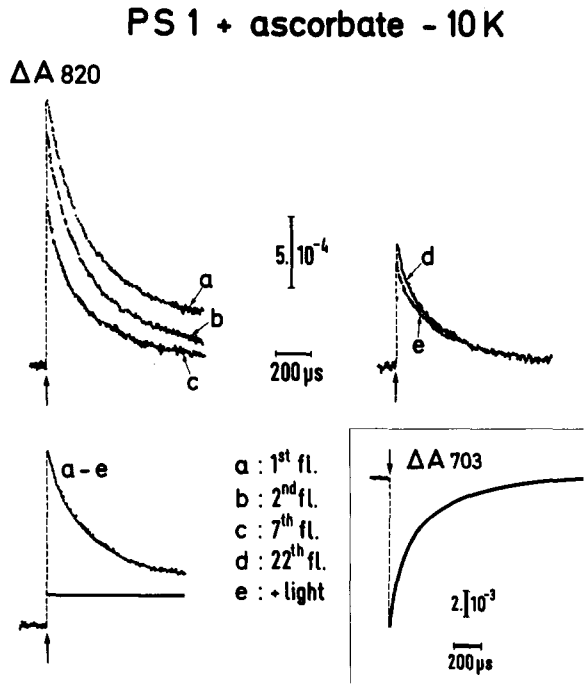

Fig. 3. Absorption transients induced by a saturating dye laser flash given at 10 K to PS I particles in a mixture of glycerol and Tris buffer (pH = 8.0). Addition of ascorbate (1 mM) and DCIP (20 μM). The sample was dark-adapted before freezing in complete darkness. $A_{679\,nm}$ (45°) = 1.71. (a)–(d): ΔA at 820 nm induced by the first, second, seventh and twenty-second flash, respectively. (e): ΔA at 820 nm induced by a flash after a continuous illumination given at 10 K; inset: idem at 703 nm. Bottom left: difference between traces (a) and (e). The horizontal line drawn after the flash corresponds to the zero-time difference between traces (a) and (b).

appearance of the EPR A^- signal after the nth flash should parallel the decrease in initial size of ΔA at 820 nm for the $(n+1)$th flash compared to the first one. This is shown to be approximately the case in Fig. 4, where these two quantities are plotted versus the flash number, after being normalized to their maximum extent obtained after continuous illumination. These observations corroborate a previous quantitative EPR study [30], in which Williams-Smith et al. found approximately the same number of spins for the EPR signals of P-700$^+$ and iron-sulfur center A^- photoinduced at low temperature.

Considering only the centers which can undergo an irreversible charge separation at 10–20 K, it appears that 20–30% of this process occurs after the first flash (Fig. 4). The relaxation of ΔA at 820 nm in these particular centers, can be obtained for the first flash by subtracting the signal after continuous illumination from the first flash signal. The difference (Fig. 3a–e) includes an irreversible component which comes from the centers undergoing a stable charge separation after the first flash. This component can be calculated from the initial difference bteween the first and second flash signals. For the other relaxing centers, the average $t_{1/2}$ of decay is 210 μs (Fig. 3: difference between (a)–(e) and the irreversible signal induced by the first flash). This decay is not monophasic; it probably includes a fast 120 μs component in addition to a slower component the halftime of which could not be derived from ΔA experiments with a millisecond time-scale.

The spectrum corresponding to the irreversible changes has been recorded at 10 K in the red region as a light-minus-dark difference spectrum (Fig. 5), which probably corresponds to the difference between the states (P-700$^+$...A^-) and (P-700...A). It exhibits three main peaks of nearly equal size, a positive maximum at 689 nm and two negative maxima at 685 and 703 nm. Flash-induced absorption changes at 10 K were also measured in the red region after a continuous illumination. These ΔA come from the reaction centers which do not undergo a stable charge separation. Fig. 5 includes a spectrum of the fast phase ($t_{1/2} \approx 120$ μs) of the decay, which accounts for 80–95% of the whole change at any wave-

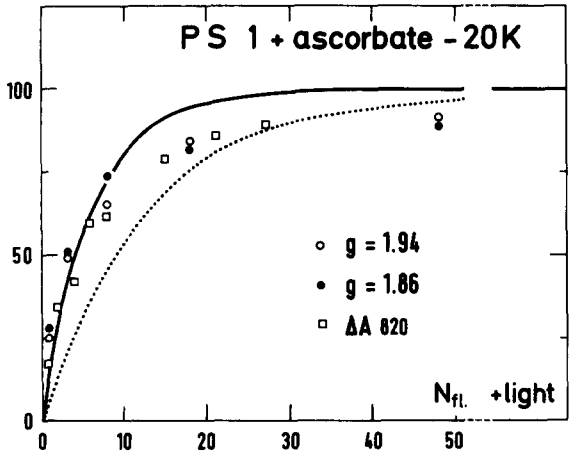

Fig. 4. A plot of: – the EPR signals of center A at $g = 1.86$ and $g = 1.94$ after the Nth flash – the decrease in ΔA at 820 nm for the $(N+1)$th flash compared to the first one, versus the number N of saturating laser flashes previously received by PS I particles poised with ascorbate and DCIP. These quantities were calculated in arbitrary units considering that their maximum value, which is obtained after a continuous illumination, was normalized to 100. The data shown here correspond to the experiments described in Figs. 2 and 3. The simulated curves (———) and (·····) were calculated for different yields (0.15 and 0.075, respectively) of the charge separation: P-700...center A → P-700$^+$...center A^- (see Discussion).

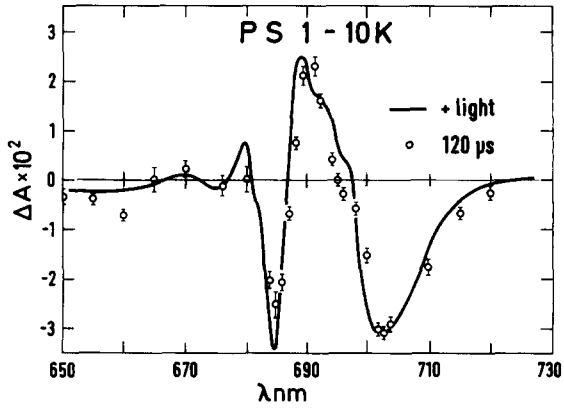

Fig. 5. Difference spectra of absorption changes induced at 10 K, in PS I particles poised with ascorbate (1 mM) and DCIP (20 μM). (1) Continuous line: after cooling in the dark, the spectrum was recorded before and after a continuous illumination (20 s) and the former was subtracted from the latter. $A_{679\,nm}$ (45°) = 2.2. (2) Circles: spectrum of flash-induced A was measured after a continuous illumination given at 10 K (120 μs decay phase). $A_{679\,nm} = 0.5$. The two spectra were normalized at 703 nm. The vertical scale corresponds to the spectrum in continuous line.

length. For the sake of comparison, the two spectra were normalized at 703 nm but the flash-induced change at 703 nm is in fact about half the size of the irreversible absorption change. The two spectra are very similar in their most important features. Considering that the difference spectrum due to the reduction of iron-sulfur center A per se contributes probably to a very weak extent in the red region, we assume that both spectra correspond essentially to the (P-700$^+$–P-700) difference at 10 K. Very large signals (negative around 685 nm and positive around 690 nm) have been already reported for the difference (P-700$^+$–P-700) [31,32] at low temperature. We observed that the size of these signals decrease together with ageing of the particles (data not shown). At least in part, they probably originate from an electrochromic shift of surrounding chlorophylls due to the positive charge on P-700$^+$ [33], and they vary from one type of particles to another. The minor differences between the two spectra of Fig. 5 (e.g., at 660, 680, 690 and 695 nm) may be the consequence of such minor electrochromic shifts due to a different reduced acceptor molecule in the two cases. They may also result from the wavelength bandwidths which are different in the two experiments. Thus the 120 μs spectrum strongly suggests that this phase corresponds to a back-reaction between P-700$^+$ and an acceptor molecule which does not contribute significantly in the red region. It is considerably different from the (^{3}P-700–P-700) triplet spectrum which presents no [34] or a little positive [35] signal at 690 nm. Studies in the near infra-red confirm this assignment: the 120 μs phase exhibits a progressive increase in the positive band from 730 to 820 nm characteristic of P-700$^+$ [36], contrary to the (^{3}P-700–P-700) spectrum which shows a flat positive band between 740 and 820 nm [34].

Basically the same results were obtained in absorption studies with D144 PS I particles and spinach chloroplasts. In this last case, besides a 120 μs phase with a similar spectrum as in Fig. 5 [12], a large slower component ($t_{1/2} \approx 1.7$ ms) is also present, which originates presumably from a PS II recombination between P-680$^+$ and the first quinone acceptor Q^- [37]. The EPR and absorption experiments depicted in Figs. 1 and 2 were repeated under different experimental conditions but none of the following factors led to a significant change in the results: presence or absence of glycerol, variation of the temperature between 10 and 30 K, and absence of magnetic field during the laser flash in the EPR experiment. It should be particularly noticed that the results presented in Figs. 2–4 are not related to the presence of glycerol since they were also observed, although with a poorer signal-to-noise ratio, in the absence of any cryoprotective agent.

Some flash-absorption experiments were also done with Triton PS I particles at 10 K. Relaxation of ΔA at 820 nm at any flash number is much slower than with the other PS I particles studied and than with chloroplasts [12]. The signal decreases also according to the flash number. However, after a continuous illumination the remaining signal exhibits a major 800 μs phase without faster observable decay in the μs time domain. The ΔA at 690 nm is very weak and the positive band from 750 to 820 nm is nearly flat. These features resemble closely those of the (^{3}P-700–P-700) spectrum. These observations suggest that the electron transfer is blocked at the level of the primary acceptor A_0 in those centers which do not undergo an irreversible charge separation and that ^{3}P-700 is produced from charge recombination between P-700$^+$ and A_0^-. Studies of the Triton particles were consequently not extended, as it seems that Triton disturbs the acceptor side of PS I which leads to electron transfer mechanisms at 10 K different from those observed in chloroplasts.

In PS I particles pretreated with a continuous illumination at 10 K, there is also, in the flash-induced absorption changes, a minor 800 μs component, which may be due to ^{3}P-700, besides the prominent 120 μs decay. At 820 nm, the proportion of this phase varies with different preparations of PS I particles from less than phase 5% to 20% of the total signal. We did not observe any clear positive correlation between the size of the 800 μs phase and ageing of the digitonin particles. The triplet most probably arises from a minority of damaged reaction centers, but it may be also the result of the 120 μs recombination, with a varying yield depending on the preparation.

PS I particles, treated as previously described (addition of ascorbate and illumination at 10 K), exhibit a weak transient EPR signal at $g = 1.77$ in

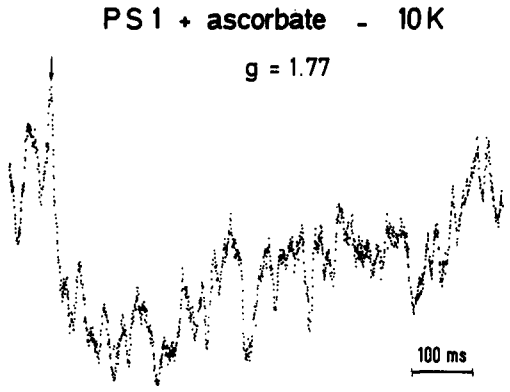

Fig. 6. Flash-induced EPR signal of iron-sulfur center X, recorded at $g = 1.77$ in PS I particles poised with ascorbate (5 mM) and DCIP (200 μM) in Tris buffer (pH = 8.0), cooled in the dark, and after a continuous illumination given at 10 K. Instrument settings as in Fig. 1. Time constant, 10 ms; average of 1000 experiments.

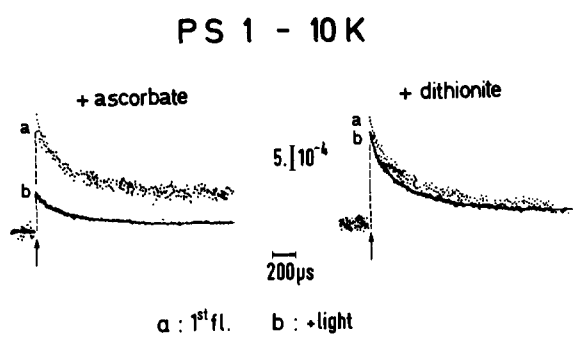

Fig. 7. Absorption changes induced at 820 nm by a saturating dye laser given at 10 K to PS I particles under different redox conditions in the presence of 60% glycerol; $A_{678\,nm}$ (45°) = 2.1. Left: addition of ascorbate (2 mM) and DCIP (20 μM) in Tris buffer (pH 8.0) before cooling in the dark. Right: addition of 40 mM sodium dithionite and light treatment at 220 K, providing centers A and B reduced and X oxidized (see Materials and Methods). (a) First flash signal; (b) Average of eight experiments after a continuous illumination at 10 K.

response to a saturating flash excitation at 10 K (Fig. 6). The decay has an approx. $t_{1/2}$ of 300 ms. The signal, recorded at the high field peak of center X^-, suggests that a back-reaction (P-700$^+$–X^- → P-700–X) occurs in some reaction centers. A weak P-700$^+$ signal (less than 5% of initial ΔA at 820 nm; $t_{1/2} > 5$ ms) has also been detected. This process occurs in a very small minority of the centers, as the EPR signal amounts to only 1–2% of the maximum X^- signal obtained under highly reducing conditions.

PS I photochemistry under reducing conditions

Fig. 7 shows the ΔA at 820 nm induced at 10 K by the first flash in PS I particles in which the centers A and B are fully reduced and the other acceptors (A_0, A_1 and X) oxidized, in the presence of glycerol. After a continuous illumination at 10 K, the flash-induced signal decreases by less than 10% (Fig. 7), showing that irreversible processes at 10 K are minor under these conditions. The same results were obtained in the absence of glycerol. For comparison, Fig. 7 also shows the results of similar experiments with the same PS I particles poised with ascorbate and having the same chlorophyll concentration. The first flash induces approximately the same ΔA with the two samples, suggesting that all P-700 is detected in both experiments. The size of these signals agrees with the concentration of reaction centers in these particles (Chls/P-700 ≈ 100), based on a $\Delta\varepsilon$ of 6.5 $mM^{-1} \cdot cm^{-1}$ for P-700$^+$ at 820 nm [36]. In the dithionite-reduced sample, three kinetic components appear in the decay of flash-induced ΔA at 820 nm on a 2 ms time scale (Fig. 7). The major phase has a $t_{1/2}$ of 120 μs and represents 70–80% of the whole signal. In the red region, its spectrum is very similar to the one previously described (Fig. 5, flash-induced ΔA). These observations strongly suggest that the same back-reaction between P-700$^+$ and an acceptor species takes place under both redox conditions. This reaction becomes more important when A and B are prereduced, as irreversible processes practically disappear. A 800 μs component contributes to the same relative extent at 820 nm as with ascorbate, that is 5–20% from one preparation to another.

A much slower decay, which amounts to 10–15% of the whole ΔA, has been studied on a different time-scale (Fig. 8, upper left). It can be satisfactorily decomposed in two phases of identical size with $t_{1/2} = 40$ and 400 ms. The decay of the flash-induced X^- EPR signal has been recorded at its high field peak (Fig. 8, bottom). It exhibits similar kinetics and the amount of X^- corresponding to this transient signal has been calculated to represent about 10% of the maximum X^- EPR signal. It was checked that the flash was saturating: the

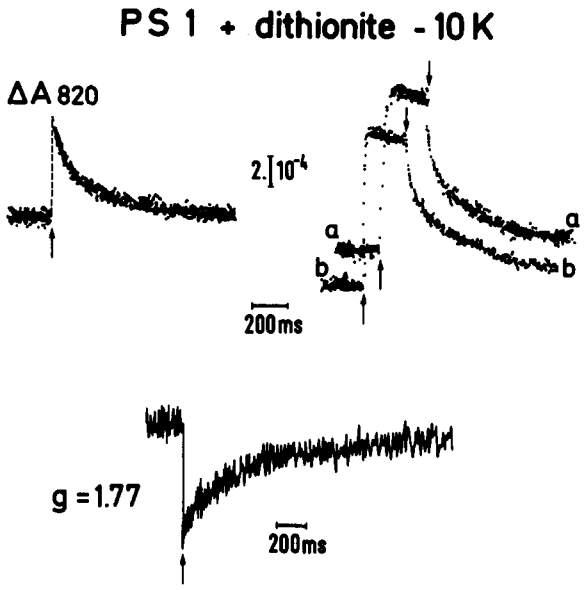

Fig. 8. Absorption and EPR signals photoinduced at 10 K in PS I particles under reducing conditions (centers A and B prereduced, center X oxidized) in the presence of 60% glycerol. Glycine buffer pH = 10.0; 40 mM dithionite. Top: ΔA at 820 nm recorded with a time-constant of 2 ms and induced by a saturating dye laser flash (left) or by a 250 ms pulse of intense white light (right); (a) without attenuation; (b) 50% attenuation. $A_{678\,nm}$ (45°) = 2.3. Bottom: EPR signal of iron-sulfur center X at $g = 1.77$ induced by a saturating xenon flash. The same signal was recorded without glycerol on a sample under identical redox conditions and with the same chlorophyll concentration. Instrument settings as in Fig. 1. Time constant 10 ms; average of 300 experiments.

same X^- signal is elicited with a 50% attenuation.

In other laboratories, many studies on the recombination between P-700$^+$ and X^- were made with long light pulses [14,15]. We thus have undertaken similar absorption experiments to look for a possible accumulation of the state (P-700$^+$–X^-) during the light period. With an intense 250 ms light pulse, ΔA at 820 nm, after a fast rise is about 1.5-times larger than the signal elicited by a saturating laser flash (Fig. 8, upper right a). At the end of illumination, an unresolved fast relaxation component is also present. When the light pulse is attenuated to 50%, the same signal is observed (Fig. 8, upper right b). This suggests that at most 20% of the reaction centers in the digitonin particles can be accumulated in a slowly decaying (P-700$^+$–X^-) state.

We considered the possibility that, besides a slowly relaxing (P-700$^+$–X^-) state at 10 K, the same pair of radicals was responsible for the 120 μs decay. This would be consistent with a weak contribution of the acceptor species to the 120 μs spectrum in the red region, if X is an iron-sulfur center. A PS I sample, in the pure redox state (X, A^-, B^-), as checked by steady state EPR, was used to record the flash-induced EPR signal of X^- at $g = 1.77$. The kinetic experiments were carried out with a spectrometer response time of 20 μs in order to detect a possible 120 μs decay (Fig. 9). A large artefact is present during 200 μs after the flash, so that large fluctuations remain during this time period, even after the subtraction of an off-resonance signal. The amplitude of the component, which appears irreversible during the time-scale of the experiment in Fig. 9, fits approximately with the slowly decaying X^- signal that can be more easily determined with larger response time and time-scale (as in Fig. 8). The drawn line in Fig. 9 corresponds to a 120 μs decay followed by a very slow component, in the same relative proportions as in ΔA at 820 nm. Compari-

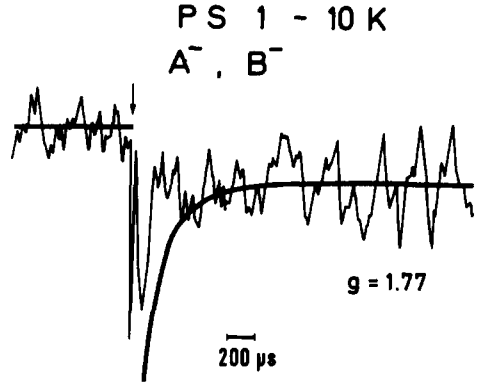

Fig. 9. EPR signal of iron-sulfur center X recorded at $g = 1.77$ after a xenon flash excitation (pulse frequency about 0.4 Hz) given to PS I particles under reducing conditions (A and B prereduced, X oxidized). Instrument settings: temperature, 10 K; microwave power, 10 mW; frequency, 9.47 GHz; modulation amplitude, 10 G; response time, 20 μs; average of 12000 experiments. The same number of off-resonance signals was subtracted from the signal at $g = 1.77$. The drawn line corresponds to a 120 μs decay followed by a very slow relaxation($t_{1/2} \gg 1$ ms; normalized to the slow experimental decay) in the same relative proportions as for ΔA at 820 nm under identical redox conditions. Chl concentration, 500 μg/ml.

son with the experimental curve suggests that there is no X^- transient decaying with $t_{1/2} \approx 120$ μs. This experiment was repeated three times with similar negative results. These data must be taken with caution, owing to the poor signal-to-noise ratio of the experiment, and to possible transient distortions of the EPR signal, which are not related to a chemical decay. However, we provisionally conclude that the 120 μs back-reaction is not between P-700$^+$ and X^-.

In the same PS I particles, fast EPR transient signals were also detected at 10 K in the radical region ($g = 2.0$) in the presence of ascorbate (Sétif, P., Mc Cracken, J.L. and Sauer, K., unpublished data). These signals are very similar, in their kinetics and spectrum, to the CIDEP signals reported by Mc Cracken et al. in spinach chloroplasts and D144 particles under reducing conditions (redox state X, A^-, B^-) [38] and ascribed to PS I reaction centers. Furthermore, the size of the signal with ascorbate is about half than when A and B are prereduced (Mc Cracken, J.L., personal communication). As this ratio of two corresponds to the ratio between the sizes of the 120 μs phase in the two different redox states, it is tempting to associate the two phenomena (see Discussion).

Discussion

Heterogeneity of the charge stabilisation: P-700.. center A → P-700$^+$... center A$^-$

Our absorption and EPR data indicate that, on the first flash, the proportion of A^- irreversible formation, is 0.2–0.3, when it is calculated with respect to the PS I reaction centers undergoing a stable charge separation after a continuous illumination. This is somewhat less than the yield of 0.4 found by Crowder and Bearden [6] for the charge stabilisation induced by the first flash at 25 K. If the reduction of center A competes with the 120 μs recombination, as it is suggested by Fig. 3a–e, a yield of about 0.25 would give a $t_{1/2} \geqslant 400$ μs for that reaction. This agrees with the data in [6], which show that center A is photoreduced in less than 1 ms after the first flash.

The yield of A^- formation decreases with the subsequent flashes. This is shown in Fig. 4 where two different curves are plotted for different yields of A^- formation. A high yield of 0.15 (still inferior to the first flash yield) mimics satisfactorily the A^- formation during the first few flashes, but the calculated curve deviates considerably from the experimental data for a large number of flashes and the converse is true for a low yield of 0.075. This shows that a distribution of yields for A^- formation has to be taken into account to simulate these data. The kinetic data obtained under reducing conditions (ΔA at 820 nm) suggest that a fast relaxation occurs in a large majority of the centers, with the same half-time of 120 μs. So a distribution of yields for A^- formation probably corresponds to a distribution of rates for forward electron transfer, leading to the stable state (P-700$^+$...A^-).

After 50 flashes, a supplementary irreversible charge separation is induced by a prolonged continuous illumination (Fig. 4). This suggests that, in some reaction centers, the flash-induced reduction of center A has a very low yield (less than 0.02). So the distribution in rates for A^- formation seems very large and there exists a possibility that this yield drops to zero in one-third of the centers. This would explain why one-third of the reaction centers do not undergo an irreversible charge separation at 10–30 K. An alternate possibility is that the iron-sulfur centers A and B are not present in one-third of the centers. Preliminary quantitative EPR experiments, undertaken to distinguish between the two hypotheses, gave ambiguous results.

The partner of P-700$^+$ in the 120 μs recombination

Our data strongly suggest that the 120 μs back-reaction is the dominant reversible process in PS I and 10 K, in ascorbate as well as when A and B are prereduced. Absorption data show that the partner of P-700$^+$ in this reaction is not a chlorophyll molecule, as the current primary acceptor A_0 is considered to be. Kinetic EPR experiments suggest that it is neither the iron-sulfur center X.

CIDEP data obtained on PS I at low temperature may help in the identification of this acceptor:

– Furrer and Thurnauer [39] observed CIDEP signals at room temperature attributed to PS I at a moderate redox potential in the cyanobacterium *Synechococcus lividus,* with a K-band as well as with an X-band spectrometer. Their results were unambiguous in the requirement for at least two

different radicals to account for the polarization pattern in the $g = 2.0$ region observed with the X and K-band spectrometers. They proposed that, besides the polarized spectrum of P-700$^+$, a species with a g-value comprised between 2.0048 and 2.0057 contributes to the overall-polarized spectrum. These room-temperature signals can be identified with low temperature CIDEP signals which were observed under different redox conditions [cf., e.g., 4,5,38 and references in Ref. 39].

– On the basis of orientation effects, McCracken and Sauer proposed a similar interpretation for the CIDEP signals obtained under reducing conditions (A^-, B^-) [5]: the EPR-polarized spectrum was interpreted as the sum of the polarized spectra of P-700$^+$ and of the acceptor A_1^- (g-value is approx. 2.005). Polarization of P-700$^+$ would be produced by the interactions between P-700$^+$ and the primary acceptor A_0^- during charge separation, whereas A_1^- is only polarized by the electron transfer from the A_0^- polarized radical. From the orientation dependence of the CIDEP signal, center X was concluded not to be involved as the partner of P-700$^+$, contrary to a previous interpretation [38].

– The CIDEP signals with ascorbate, are about half the size of those when A and B are prereduced (McCracken, personal communication). About the same ratio is found for the 120 μs phase in the two redox conditions. This is not case for the slowly relaxing (P-700$^+$–X$^-$) state which is produced with a very low yield at a moderate redox potential.

The interpretation of the CIDEP phenomena at low temperature, together with the absence of a $t_{1/2} \approx 120$ μs transient signal of X$^-$, strongly suggest that the partner of P-700$^+$ in the 120 μs recombination is the acceptor named A_1 [3–5] which has a g-value around 2.005 [3–5,39].

The chemical identity of A_1 is not yet known. It may be a quinone molecule, as the redox potential of quinone molecules can be decreased down to very low values by appropriate substitutions [40]. However E_m in vitro are not as low as the probable E_m of the A_1/A_1^- couple, which should be around or below that of the X/X^- couple (-705 mV; see Ref. 41).

The role of X in the electron flow

Our data suggest that, in the reaction centers that undergo an irreversible charge separation after the first saturating flash, center A is reduced with $t_{1/2} \geq 400$ μs. On the other hand, Crowder and Bearden found that this reaction occurs in less than 1 ms [6]. Assuming that A_1^- is the partner of P-700$^+$ in the 120 μs recombination (preceding paragraph), these two observations taken together do not leave much room for an intermediate between A_1 and center A. This would agree with the proposals that X is not on the linear electron flow from P-700 to center A [6] and that A and B function in parallel [1,2,42]: the electron could go directly from A_1^- to center A, unless we assume some fast ($t_{1/2} < 1$ ms) and irreversible electron-transfer reactions between the three iron-sulfur centers A, B and X at low temperature. Electron transfer does not seem to occur below 215 K between centers A and B [43], but it cannot be excluded from X$^-$ to center A. However, in that case, when centers A and B are prereduced, the proportion of reaction centers relaxing after a flash excitation via the slowly decaying state (P-700$^+$...X$^-$) should be greater (about 25%) than observed (10–15%).

Our data show that, when A and B are prereduced, the slowly relaxing (P-700$^+$–X$^-$) state, which is well documented in the literature [1,2,14–16] occurs in a minority of reaction centers in our PS I particles. A very wide distribution of rate constants for the reduction of X, in competition with a fast recombination with $t_{1/2} \approx 120$ μs, could explain the low yield of formation of the state (P-700$^+$–X$^-$) in PS I particles under reducing conditions.

Concluding remark

PS I reaction centers may be trapped in many different conformational states by freezing at low temperature. This could explain some large variations in rate constants that can be derived from our data for center A reduction and that may hold for center X reduction. An alternative explanation, not exclusive from the preceding one, would be that the low-temperature heterogeneity reflects a heterogeneity related to different physiological roles, for instance the cyclic and linear electron flows in PS I. However, much more data are necessary to choose between these possibilities.

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