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Photosystem I photochemistry under highly reducing conditions: study of the P700 triplet state formation from the secondary radical pair ($P700^+$-A_1^-)

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The photochemistry of spinach Photosystem I reaction centers has been studied under conditions of prereduced iron-sulfur centers Fe-S_A, Fe-S_B and Fe-S_X by flash absorption spectroscopy in the UV, blue and near-IR regions. Two kinetic phases are observed under such conditions, with $t_{1/2} \approx 250$ ns and 4–5 μs. From the spectral analysis of these two phases including the comparison with flash-induced absorption data obtained under moderately and strongly reducing conditions, the following conclusions were derived: the 250 ns phase corresponds to a recombination reaction between $P700^+$-A_1^- forming $^3P700$ with a yield close to one. This triplet state then decays with $t_{1/2} \approx 4$–5 μs. These results provide direct evidence for the reaction scheme: $P700^+$-$A_1^- \rightarrow {}^3P700$ under highly reducing conditions. The same reaction was proposed to occur in a preceding paper (Sétif, P. and Bottin, H. (1989) Biochemistry 28, 2689–2697), but the halftime of the recombination reaction was then overestimated (750 ns). It is also found that the same reaction is occurring in reaction centers from the two cyanobacteria *Synechococcus* sp. and *Synechocystis* 6803.

Introduction

The electron transport chain of Photosystem I (PS I) comprises the primary electron donor P700 and five membrane-bound electron acceptors: the primary electron acceptor A_0, which is a chlorophyll molecule, a secondary acceptor A_1 and three iron-sulfur centers Fe-S_A, Fe-S_B and Fe-S_X which act as tertiary electron acceptors (for reviews see Refs. 1 and 2). The chemical nature of the intermediate electron acceptor A_1 has been recently a matter of controversy, although most experimental data support its identification with vitamin K-1 (see Refs. 3,4 and references therein).

The kinetics of forward electron transfer reactions involving A_1 at room temperature are still scarce. The lifetime of A_0^- reoxidation has been measured around 30 ps [5]. This reaction was tentatively ascribed to electron transfer from A_0^- to A_1. The reoxidation of A_1^- during forward electron transfer has been measured with halftimes of approx. 15 ns [6] and 200 ns [7,8] in PS I particles of spinach and of the cyanobacterium *Synechococcus* respectively.

Recombination reactions between $P700^+$ and A_1^- have been thoroughly characterized at low temperature by absorption [9,10] and by EPR spectroscopy (see Ref. 11 and refs. therein) but have been evidenced at room temperature only recently. When the iron-sulfur centers Fe-S_A and Fe-S_B are prereduced, it is well known that $P700^+$ decays by recombination with a reduced acceptor with $t_{1/2} \approx 250$ μs [12]. The partner of $P700^+$ in this recombination reaction was generally considered to be Fe-S_X^-, although the spectroscopic information in favor of this attribution was rather poor. In complete contrast with that view, results derived both from flash-absorption [13] and EPR spectroscopies [14] rather suggest that this partner is A_1^-, at least in the cyanobacterium *Synechococcus* (deviating observations were made for some PS I preparations from spinach (Ref. 6 and Brettel, K. and Sétif, P., unpublished)). In the case where Fe-S_A and Fe-S_B are removed by chaotropic agents or by detergent treatments, a recombination reaction with $t_{1/2} \approx 1.2$ ms was observed and attributed to the pair ($P700^+$ Fe-S_X^-) [15,16].

Under conditions where Fe-S_X is maintained in the reduced state (weak background illumination under

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Abbreviations: PS, Photosystem; DPIP, 2,6-dichlorophenolindophenol; Fe-S, iron-sulfur center.

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highly reducing conditions), it has been reported that the triplet state of P700, as detected in the near-IR, is formed with $t_{1/2} \approx 750$ ns and an efficiency approaching 100%, supposedly by charge recombination in the radical pair ($P700^+$-A_1^-) [17]. In the same study, the P700 triplet state, formed by this reaction, was found to decay with a halftime of approx. 4 μs. We report here more recent data obtained by flash absorption spectroscopy in the UV, blue and near-IR regions with a better time resolution in PS I particles from spinach and from the two cyanobacteria *Synechococcus* and *Synechocystis*. These data provide direct evidence for the reaction: $P700^+$-$A_1^- \rightarrow {}^3P700$ under conditions of prereduced Fe-S_X. The halftime of this recombination turned out to be only approx. 250 ns. The results presented here also confirm that during the recombination reaction, the triplet state of P700 is formed with a yield approaching unity.

Materials and Methods

Biological material

PS I particles were prepared from spinach and from the cyanobacteria *Synechococcus* sp. and *Synechocystis* 6803 as described respectively in Refs. 18, 19 and 3. The chlorophyll to P700 ratios of these particles were estimated at 65, 80 and 120, respectively. For study under low redox potential conditions, the particles were suspended in a buffer containing 0.2 M glycine NaOH at pH 10 and sodium dithionite was added with a final concentration of 25 mM. Cyanobacterial particles were suspended in the presence of 0.04% (w/w) *β*-dodecyl-D-maltoside.

Parallel EPR and absorption measurements

Spinach PS I particles were used at a concentration of 300 μg chlorophyll/ml for both kinds of measurements. EPR spectroscopy was carried out at 10 K using a standard cavity (TE 102 mode) with a Bruker ER200D spectrometer equipped with an Oxford ESR 900 helium cryostat. Absorption measurements at 820 nm with a μs time resolution were performed as described in Ref. 9. The sample was contained in a plexiglas cuvette (1 mm wide) positioned at 45° of the mutually perpendicular exciting and measuring beams. Before the spectroscopic measurements, the samples in the EPR tube and the plexiglas cuvette were preilluminated with a 800 W tungsten-iodine lamp whose beam was filtered to remove infrared light (water cuvette + Calflex filters). The light flux onto the samples was about 30 mW/cm^2.

Absorption measurements

Control measurements at 700 nm were performed as described in Ref. 7 (time resolution of 30 μs) in order to estimate the fraction of centers where Fe-S_X was not yet prereduced (these centers exhibit a 250 μs recombination). Nanosecond absorption changes from 337 to 495 nm and at 980 nm were performed at room temperature with the set-up described in Ref. 20, using repetitive (1.7 Hz) excitation by 532 nm laser flashes of 3 ns duration, approx. 2 mJ/cm^2. The bandwidth (FWHM) of the measuring beam was usually between 7 and 10 nm, except for measurements centered at 450 and 488 nm where it was only 3 nm. For measurements at 1064 nm, a CW Nd/YAG laser (ALC 1064-50P from AMOCO) was incorporated as measuring light source into the set-up described in Ref. 21. During the experiments, the sample was submitted to a weak background illumination (white light, approx. 2 mW/cm^2). Under such conditions, it was not possible to avoid a long term evolution of the sample corresponding to some irreversible changes and the sample had to be changed from time to time. These irreversible changes were identified by the appearance of a fast phase with $t_{1/2} \approx 30$ ns, which is typical of a recombination reaction between $P700^+$ and A_0^-, and were ascribed to the double reduction of A_1 [17]. The ^{3}P700 decay was also measured at a few wavelengths with a μs time resolution with the set-up described in Ref. 9. The bandwidth (FWHM) of the measuring beam was 5 nm at 405 and 450 nm and 4 nm at 430 nm. The sample was first preilluminated as described above with a strong light (250 mW/cm^2). A square cuvette was used (1 × 1 cm) and the flash repetition rate was 1 Hz. Numerical data analysis was performed by means of least squares curve fitting programs [21].

Results

Correlation between the extent of Fe-S_X reduction and a decay phase with $t_{1/2} \approx 4$ μs

The correlation between the prereduction of Fe-S_X and the 4 μs relaxation phase of flash-induced absorption changes (attributed to the decay of ^{3}P700 [17]) was shown by the following experiment: PS I particles were illuminated for 5 min at 3°C with a weak light intensity under highly reducing conditions (excess of dithionite at pH 10). The light intensity was adjusted in order to provide a significant extent of Fe-S_X reduction without inducing irreversible effects which have been previously ascribed to the double reduction of A_1 [17]. In one series of experiments, flash-induced absorption transients were measured at 820 nm at different times Δt after the end of the illumination. In another series of experiments, PS I samples that had been pretreated in a similar way were frozen after different times Δt of dark adaptation for EPR measurements at low temperature. The amount of reduced Fe-S_X has been estimated from the size of the $g \approx 1.77$ peak. The results of these parallel experiments are shown in Fig. 1 for four different times Δt of dark adaptation. For $\Delta t = 1$ min, comparison with a fully reduced EPR sample (not shown)

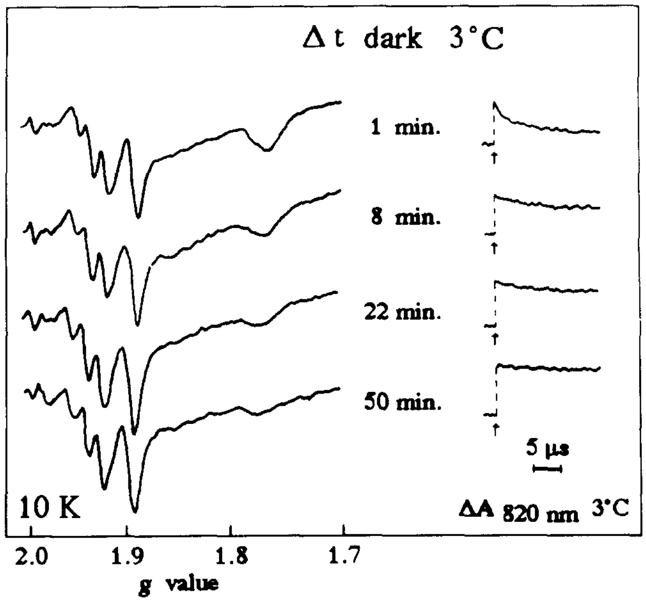

Fig. 1. Spectroscopic properties associated with different times Δt of dark adaptation (at 3°C) of spinach PS I particles which have been preilluminated with a weak background light under highly reducing conditions. Left: EPR spectrum recorded at 10 K (instruments settings: modulation amplitude, 10 G; microwave power, 20 mW). Right part: flash-induced absorption changes recorded at 820 nm and at 3°C. After a given time of dark adaptation, the EPR tube was rapidly frozen by immersion in a CO_2/ethanol bath. For both series of experiments: 300 µg chlorophyll/ml (other details in Materials and Methods).

indicates that about 70% of centers Fe-S_X are reduced. The same percentage is found in the µs absorption transient (Fig. 1, top) for the size of a 4 µs decay phase, which can be ascribed to the decay of the P700 triplet state (see Discussion). This decay phase is followed by a slower component with $t_{1/2} \approx 250$ µs (not shown) which corresponds to the reduction of $P700^+$ by a back-reaction when both Fe-S_A and Fe-S_B are prereduced. The amount of Fe-S_X^- decreases roughly in parallel with the extent of the 4 µs phase when Δt increases (see also Ref. 22). The initial magnitude of the absorption signal appears to be constant for the different times of dark adaptation (after extrapolation of the 4 µs phase to $t = 0$).

Flash-induced absorption changes under highly reducing conditions

Flash-induced absorption changes at room temperature have been measured with a time resolution of 5 ns in the region from 370 to 495 nm, at 980 and 1064 nm under highly reducing conditions, i.e., under weak background illumination in the presence of dithionite at pH 10. Under these conditions, Fe-S_X is maintained reduced in about 70% of the reaction centers. This extent of Fe-S_X reduction was estimated from the measurement of the size of the 250 µs phase at 700 nm which arises from centers where Fe-S_X is not reduced. This fraction of reduced Fe-S_X could be increased by increasing the background light intensity but this accelerated also the irreversible change of the sample (see Material and Methods). Kinetic traces in the nanosecond range are shown in Fig. 2 for some characteristic wavelengths. The four upper traces have been obtained with spinach particles. At 404 nm, a rapid instrument limited absorption increase is followed by the rise of a negative signal with $t_{1/2} \approx 250$ ns. A similar component is also observed at 450 nm and 980 nm. However, it is essentially absent at 430 nm where a small decay phase (less than 10% of the total signal) with $t_{1/2} \approx 30$ ns is present. On a 10 µs time scale, a subsequent decay with a main phase of $t_{1/2} \approx 5$ µs was observed, as shown in Fig. 3 for 430 and 450 nm. Results similar to those described so far for PS I particles from spinach were obtained with PS I from *Synechocystis* (not shown) and from *Synechococcus* (lower transients of Fig. 2), although the fast phase appears somewhat faster ($t_{1/2} \approx$ 150–200 ns).

In the following, we use the set of flash-induced absorption transients obtained with PS I from spinach at different wavelengths in order to perform a spectral characterization of the state at the beginning of the 250 ns phase and of the state which is formed during this transition and decays subsequently with $t_{1/2} \approx 5$ µs.

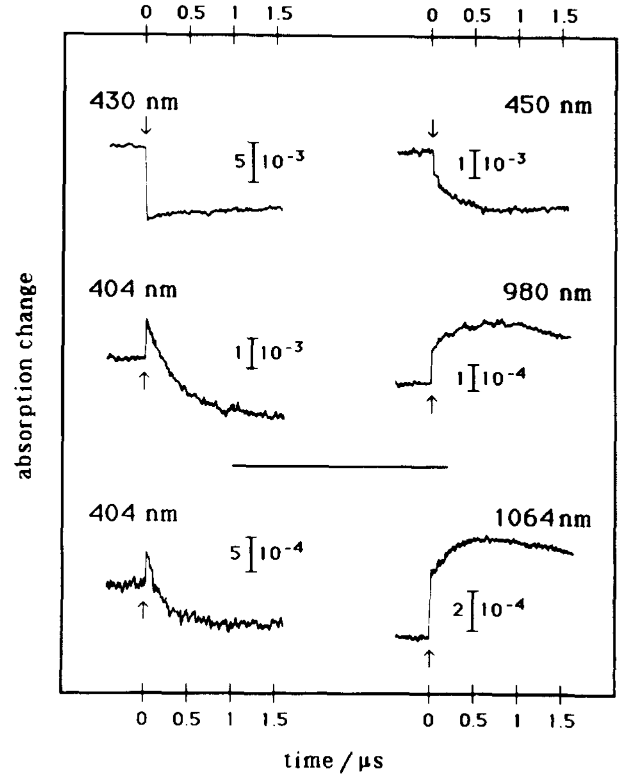

Fig. 2. Flash-induced absorption changes measured in PS I particles from spinach (four upper traces; $A_{678\ nm} = 1.16$) and from *Synechococcus* (two lower traces; $A_{679\ nm} = 0.92$ for left trace and 4.9 for right trace) under weak background illumination in the presence of sodium dithionite (25 mM). Average of 32 to 512 experiments.

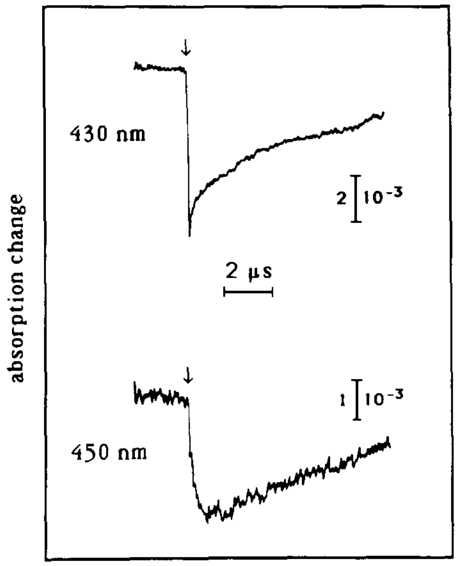

Fig. 3. Flash-induced absorption changes measured at 430 and 450 nm in spinach PS I particles on an extended timescale. Similar conditions as in Fig. 2. $A_{678\ nm} = 0.99$. Average of 64 and 256 experiments, respectively.

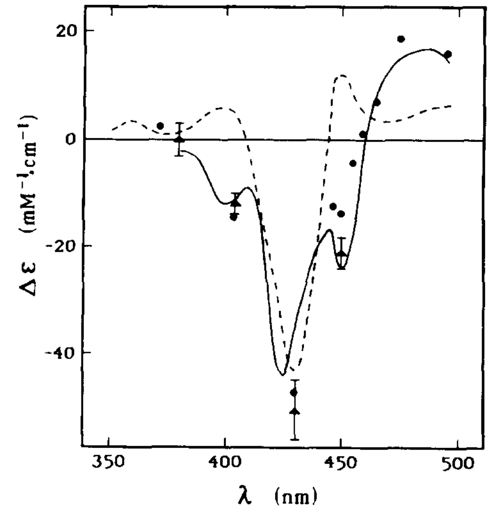

Fig. 5. Spectral characterization of the state which is formed during the 250 ns transition: circles, absorption changes decaying in the µs range, deduced from measurements as those depicted in Fig. 3; triangles, same for spinach particles which have been pretreated by a strong illumination under highly reducing conditions. Solid line: spectrum of the (^{3}P700-P700) difference at 1.2 K, redrawn from Ref. 24 and scaled arbitrarily. Broken line: P700$^+$-P700) difference at room temperature, redrawn from Ref. 23 (further details in the text).

Initial spectrum of the absorption changes under highly reducing conditions and moderate redox conditions

Fig. 4 (circles) shows the "initial" absorption changes in PS I particles from spinach under highly reducing conditions. These data were obtained by an extrapolation to $t = 0$ of the 250 ns phase present in signals as those depicted in Fig. 2. Fig. 4 also exhibits the spectrum of the "initial" absorption changes observed when the same spinach PS I particles are supplemented with ascorbate and methyl viologen at pH 8 (crosses in Fig. 4). These values were obtained from an analysis allowing for two exponential phases with $t_{1/2} = 25$ and 150 ns (see Discussion). According to Refs. 6 and 7, we attribute this difference spectrum (crosses in Fig. 4) to the formation of the pair (P700$^+$A$_1^-$). The two spectra in Fig. 4 were normalized at 430 nm, assuming $\Delta\epsilon = -47000\ \mathrm{M^{-1} \cdot cm^{-1}}$ (value for (P700$^+$A$_1^-$/P700A$_1$) in Ref. 7). Evidently, the two spectra in Fig. 4 agree rather well. This is consistent with the assumption that the 250 ns reaction under highly reducing conditions starts from the state P700$^+$ A$_1^-$.

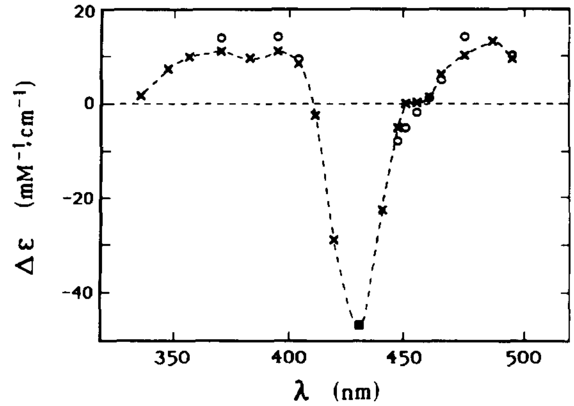

Fig. 4. Initial absorption difference spectra obtained under similar conditions as in Fig. 2 (circles) and under moderate redox conditions (4 mM ascorbate, 170 µM DPIP and 70 µM methyl viologen at pH 8 – crosses and broken line). Circles: the initial spectrum was deduced from measurements as those depicted in Fig. 2 by extrapolation to $t = 0$ of the 250 ns phase. Crosses: the initial spectrum was deduced by extrapolation to $t = 0$ with two exponential phases with $t_{1/2} = 25$ and 150 ns (see text for details). Both spectra were normalized at 430 nm, assuming $\Delta\epsilon = -47000\ \mathrm{M^{-1} \cdot cm^{-1}}$.

Spectrum of the absorption changes decaying in the µs range under highly reducing conditions

Fig. 5 (circles) shows the spectrum of the absorption changes in the µs range under highly reducing conditions (for examples, see Fig. 3), extrapolated to $t = 0$ and scaled as in Fig. 4. This difference spectrum is mainly due to the approx. 5 µs phase previously attributed to the decay of ^{3}P700 [17], but it contains also a contribution from the about 30% of centers with not yet reduced Fe-S$_X$ which exhibit the 250 µs recombination reaction (see Introduction). In order to establish the nature of the state which is formed during the 250 ns transition and decays with $t_{1/2} \approx 5$ µs, we compare our data with other relevant difference spectra, namely the (P700$^+$-P700) spectrum (broken line, redrawn from Ref. 23) and the (^{3}P700-P700) spectrum (solid line, redrawn from Ref. 24 and scaled arbitrarily; unfortunately, this difference spectrum has been measured only at 1.2 K in the blue region). Fig. 5 shows that the spectrum of the

μs decay in our samples (circles) shares the most distinctive qualitative features of the (^{3}P700-P700) spectrum at 1.2 K, i.e., bleaching around 400 and 450 nm and relatively strong absorption increase around 480 nm. The considerable absorption increase around 1000 nm during the 250 ns transition (see Fig. 2) is also in line with the formation of ^{3}P700 (cf. Ref. 17). As an additional check, we measured the room temperature absorption difference between ^{3}P700 and P700 at a few wavelengths in the blue region in the following experiments: the same spinach PS I particles were submitted to a prolonged and intense illumination under highly reducing conditions (250 mW/cm^2 for 5 min). After such a pretreatment, the forward electron transfer is inhibited after A_0^- and ^{3}P700 is formed from the primary radical pair (P700$^+$-A_0^-) with a yield smaller than 60% [5, 25–27]. ^{3}P700 then decays with $t_{1/2} \approx 4$ μs, and the (^{3}P700-P700) absorption difference can be evaluated from the amplitude of the 4 μs phase. These measurements were made alternately with control measurements at 820 nm which served as a reference wavelength. The ratio of absorption changes ($\Delta A_{\lambda_i}/\Delta A_{820\ \mathrm{nm}}$) for the difference (^{3}P700-P700) were 0.0 ± 0.5, -2.2 ± 0.4, -9.2 ± 1.0 and -3.8 ± 0.5 for $\lambda_i = 380$, 405, 430 and 450 nm, respectively. Assuming an absorption coefficient of 5500 $M^{-1} \cdot cm^{-1}$ for ^{3}P700 at 820 nm [28], these ratios correspond to differential absorption coefficients of 0 ± 3000 $M^{-1} \cdot cm^{-1}$, $-12\,000 \pm 2000$ $M^{-1} \cdot cm^{-1}$, $-50\,500 \pm 5500$ $M^{-1} \cdot cm^{-1}$ and $-21\,000 \pm 3000$ $M^{-1} \cdot cm^{-1}$ for (^{3}P700-P700) at respectively 380, 405, 430 and 450 nm. These data, shown as triangles in Fig. 5, corroborate the assignment of the state reached during the 250 ns reaction (characterized by the circles in Fig. 5) to ^{3}P700. The deviation around 450 nm may be explained by the positive contribution due to approx. 30% of the centers, which undergo the 250 μs recombination because Fe-S_X is not reduced (see above).

Discussion

It has been reported earlier that, when Fe-S_X is maintained in the reduced state by a weak background illumination under highly reducing conditions, the triplet state ^{3}P700 is formed with $t_{1/2} \approx 750$ ns (measured in *Synechocystis* particles) and with a yield approaching unity [17]. The following reaction scheme (1) was proposed:

$$\mathrm{P700^+\text{-}A_1^-\text{-}Fe\text{-}S_X^-} \xrightarrow{t_{1/2} \approx 250\ \mathrm{ns}} \mathrm{{}^3P700\text{-}Fe\text{-}S_X^-}$$

$$\xrightarrow{t_{1/2} \approx 4\text{–}5\ \mu\mathrm{s}} \mathrm{P700\text{-}Fe\text{-}S_X^-} \qquad (1)$$

Yield of ^{3}P700 formation = 1

This high yield of triplet formation stands in contrast with the low yield of ^{3}P-700 formation when it is formed at room temperature from the primary radical pair (P-700$^+$-A_0^-). In the latter case, yields comprised between 5 and 60% were found [5, 25–27]. In the results presented here which were obtained under similar conditions as in Ref. 17, a halftime of about 250 ns in PS I from spinach and 150–200 ns in PS I from *Synechococcus* was found instead of 750 ns. This is most probably due to a poor signal to noise ratio and a limited time resolution during the earlier experiments. The detailed results presented above provide direct evidence for the preceding interpretation (reaction scheme (1)) as discussed below:

That the 250 ns reaction starts from the pair (P700$^+$-A_1^-) is evident from the spectral data (circles in Fig. 4) characterizing the "initial" changes under highly reducing conditions: these data agree well with the spectrum which has been attributed to the formation of the state (P700$^+$-A_1^-) in *Synechococcus* particles [7] and also with the spectrum for the formation of (P700$^+$-A_1^-) in the same spinach particles under moderate redox conditions (crosses in Fig. 4). The most important common feature of these spectra is a positive signal between 340 and 410 nm where a significant contribution of the difference (A_1^-–A_1) has been put forward [7, 10, 13]. Some slight differences between the spectra appear around 450 nm where a contribution from electrochromic shifts due to the negative charge of A_1^- has been proposed [7]. Differences in the electrochromic shifts would not be surprising between the PS I particles from spinach and from *Synechococcus* , because of the different preparation procedures and origins of the samples. This does not hold for the slight difference around 450 nm between the two spectra depicted in Fig. 4. In this case, however, the presence of a negative charge on Fe-S_X under highly reducing conditions may be responsible for different electrochromic shifts, as both A_1 and Fe-S_X may lie in close proximity on one face of the PS I reaction center. In line with this observation, the difference spectra in the red region between the states (P700$^+$-A_1^-) and (P700-A_1) at 10 K have been reported to be different when center Fe-S_X is reduced or oxidized [29]. It should be mentioned that the absorption changes due to the forward electron transfer from A_1^- in the PS I particles from spinach under normal conditions required two exponential phases of $t_{1/2} \approx 25$ ns and 150 ns for a satisfactory description. A single exponential with $t_{1/2} \approx 200$ ns was sufficient, however, in PS I from *Synechococcus* [7] and from *Synechocystis* (P. Sétif and K. Brettel, unpublished). A detailed study of the differing behavior of these preparations is under way.

The results depicted in Fig. 5 demonstrate that the state which is formed during the 250 ns transition and decays with $t_{1/2} \approx 5$ μs is indeed ^{3}P700: the difference spectrum of this state compared to the "ground state"

($P700A_1Fe\text{-}S_X^-$) (circles in Fig. 5) exhibits features in the blue region which are characteristic of the ($^3P700$-P700) spectrum and which are not present in the difference ($P700^+$-P700) (bleachings around 400 and 450 nm). The halftime of 5 μs is very similar to what is found for the decay of $^3P700$ at room temperature in other types of PS I particles [5,27].

The correlation between the formation of $^3P700$ and the prereduction of $Fe\text{-}S_X$ is confirmed by the experiment depicted in Fig. 1.

The absence of a 250 ns phase at 430 nm reinforces the proposal that $^3P700$ is formed with a high yield. At 430 nm, the initial bleaching is followed by a fast decay with $t_{1/2} \approx 30$ ns which represents less than 10% of the initial signal and which is due to some recombination reaction between $P700^+$ and A_0^- in a minority of reaction centers (Fig. 2). This relaxation phase is followed by a major decay with $t_{1/2} \approx 5$ μs (decay of $^3P700$) (Fig. 3) but no 250 ns phase can be detected at this wavelength. Considering absorption coefficients of 47000 $M^{-1} \cdot cm^{-1}$ for the difference ($P700^+$-A_1^-)-(P700-A_1) [7] and of 50000 $M^{-1} \cdot cm^{-1}$ for ($^3P700$-P700) (see above), the absence of 250 ns phase agrees with a yield of $^3P700$ formation from the radical pair ($P700^+$-A_1^-) which is close to one.

The decay halftime of ($P700^+$-A_1^-) in reaction scheme (1) is similar to what was found for the reoxidation of A_1^- during forward electron transfer to the iron-sulfur centers in *Synechococcus* reaction centers ($t_{1/2} \approx 200$ ns; Ref. 7). However, the spectra of these two reactions exhibit very distinctive features. This is expected as the final states are different between these two reactions. Distinctive features are clearly seen for example at 404 and 450 nm. At these two wavelengths, the difference ($^3P700$ - P700) is clearly negative (see the signals in Fig. 2 at the end of the traces) whereas the absorption change is negligible at 404 nm and even positive at 450 nm at 1.6 μs after the flash during forward electron transfer (see Fig. 4 in Ref. 7).

With respect to the energetics of the recombination: $P700^+\text{-}A_1^-\text{-}Fe\text{-}S_X^- \rightarrow {}^3P700\text{-}A_1\text{-}Fe\text{-}S_X$, it is of interest to determine the equilibrium constant K of this reaction. As the halftime of $^3P700$ formation is more than 10-times smaller than its decay halftime, the two species ($P700^+$ A_1^-) and $^3P700$ can be considered to be in quasi-equilibrium at 1.5 μs after the flash, which corresponds to 6-times the 250 ns halftime. Therefore, respective amounts of ($P700^+$ A_1^-) and $^3P700$ in this quasi-equilibrium can be deduced from the sizes of the absorption changes at 1.5 μs after the flash, at wavelengths where the absorption of these two states is different. E.g., at 404 nm, the differential absorption coefficients for ($P700^+$ A_1^--P700 A_1) and ($^3P700$-P700) are approx. 9000 $M^{-1} \cdot cm^{-1}$ and -12000 $M^{-1} \cdot cm^{-1}$, respectively, i.e., they have opposite signs. The signal at 1.5 μs is clearly negative. By comparison with the signal measured at 430 nm at the same time, and taking a value of -48500 $M^{-1} \cdot cm^{-1}$ for the differential absorption coefficient at this wavelength (($P700^+$ A_1^--P700 A_1) and ($^3P700$ - P700) having approx. the same coefficient at 430 nm), the 404 nm signal corresponds to a coefficient of approx. -11500 $M^{-1} \cdot cm^{-1}$, which is close to the triplet value. Taking into account the precision of the measurements and the roughly 30% of centers where $Fe\text{-}S_X$ was not prereduced, we estimate that ($P700^+$ A_1^- $Fe\text{-}S_X^-$) contributes less than 25% to the equilibrium, i.e., $K > 3$. This estimate is also compatible with the data at 450 nm. Hence the state ($P700^+$-A_1^--$Fe\text{-}S_X^-$) lies higher in free energy than the P700 triplet state by at least 30 meV at room temperature. This conclusion which was derived for the PS I particles from spinach holds also for PS I particles from *Synechococcus* (cf. Fig. 2, lowest trace, left). For PS I from *Synechocystis*, it has already been concluded from measurements with lower time resolution that ($P700^+$ A_1^- $Fe\text{-}S_X^-$) lies higher in free energy than ($^3P700$ A_1 $Fe\text{-}S_X^-$) [17].

We observed also that the reaction centers of *Synechocystis* behave similarly to the reaction centers of *Synechococcus* when only $Fe\text{-}S_A$ and $Fe\text{-}S_B$ are prereduced (unpublished data and Ref. 13): under these last conditions, the charge separation induces the appearance of the state ($P700^+$-A_1^-) which recombines with $t_{1/2} \approx 300$ μs, without indication of electron transfer to $Fe\text{-}S_X$. From this last observation, it could be expected that the prereduction of $Fe\text{-}S_X$ (with A_1 still oxidized) is precluded, at least in PS I from the two cyanobacterial species, by contrast to what has been inferred from the data presented here and in Ref. 17. This apparent contradiction can receive several explanations:

During charge separation, the coulombic attraction between $P700^+$ and A_1^- may be stronger than that between $P700^+$ and $Fe\text{-}S_X^-$, if A_1 lies closer to P700 than $Fe\text{-}S_X$. This effect could explain why, at room temperature, the free energy of (A_1^--$Fe\text{-}S_X$) is lower than the free energy of (A_1-$Fe\text{-}S_X^-$) when P700 is oxidized whereas the reverse is true when P700 is in the neutral state. The PS I reaction center could undergo some conformational changes under highly reducing conditions, during which the relative redox potentials of A_1 and $Fe\text{-}S_X$ can be reversed.

It appears that the recombination halftime of ($P700^+$-A_1^-) is strongly dependent upon the reduction state of $Fe\text{-}S_X$. The 250 μs halftime observed when $Fe\text{-}S_X$ is oxidized [13] is replaced by a 250 ns halftime when $Fe\text{-}S_X$ is initially reduced (these results). A possible explanation for this effect is that the triplet decay channel becomes open when $Fe\text{-}S_X$ is prereduced, whereas it is not available when $Fe\text{-}S_X$ is oxidized, as it would be uphill in energy under these last conditions. The free energy increase of the state ($P700^+$ A_1^-) when $Fe\text{-}S_X$ is prereduced can receive the same kinds of explanations as given just above, i.e., either a coulombic repulsion of the negative charge of Fe-S_X^- on the nearby A_1, or a conformational change.

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