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# Absorption studies of primary reactions in Photosystem I. Yield and rate of formation of the P-700 triplet state

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**The quantum yield of P-700 triplet state formation has been found from flash-induced absorption studies in the microsecond time range to be 0.45 and 0.35 at 294 K and 6–20 K, respectively, in CP1-SDS particles which lack the secondary acceptors. From these quantum yield measurements, yields of formation of the P-700 triplet state from the primary biradical ($P\text{-}700^{+}\text{–}A_0^{-}$) were calculated to be around 0.6 at both temperatures, whereas double-laser flash experiments allowed us to derive upper limits for this yield (0.84 at 294 K and 0.79 at 20 K). These values agree with the high values that have been previously calculated from an earlier absorption study (Sétif, P., Hervo, G. and Mathis, P. (1981) Biochim. Biophys. Acta 638, 257–267) but appear significantly higher than the yield calculated from EPR experiments (5–10%) (Gast, P., Swarthoff, T., Ebskamp, F.C.R. and Hoff, A.J. (1983) Biochim. Biophys. Acta 722, 163–175). Possible explanations for this discrepancy are discussed. From absorption studies in the submicrosecond time range as well as from double-laser flash experiments, the lifetime of the biradical ($P\text{-}700^{+}\text{–}A_0^{-}$), from which the P-700 triplet state is formed by recombination, has been measured to increase from 30–50 ns at room temperature up to 120–130 ns between 10 and 110K.**

## Introduction

In all kinds of photosynthetic reaction centers that have been studied so far, the formation of the triplet state of the primary donor has been shown to occur when normal electron transfer is blocked by removing or reducing the secondary acceptor(s) [1–4]. In all cases, the first clear evidence for this state came from EPR experiments at low temperature where, under illumination, a (bacterio)chlorophyll triplet signal was observed, with a peculiar spin polarization resulting from the recombination process which follows the primary charge separation [5].

In PS I, the formation of the primary donor triplet state, $^{3}$P-700, has recently been shown to result from the recombination between $P\text{-}700^{+}$ and the reduced primary acceptor $A_0^{-}$, a species which exhibits an EPR signal around $g = 2.002$ [6,7]. This primary acceptor, which is thought to be a chlorophyll molecule [8] precedes four different membraneous acceptors named $A_1$, of unknown chemical nature, and $F_X$, $F_B$ and $F_A$, all of which are probably iron-sulfur proteins. The P-700 triplet state can be observed when these secondary acceptors are either reduced before illumination [2,6] or destroyed by LDS [9,10] or SDS treatment [11,12].

There is a good correlation between the decay kinetics at low temperature (10–30 K) of the spin-polarized P-700 triplet signal detected by EPR [7,12] and the decay kinetics of a large flash-ab-

Abbreviations: PS I, Photosystem I; Chl, chlorophyll; DCIP, dichlorophenolindophenol; EPR, electron paramagnetic resonance, ADMR, absorption-detected magnetic resonance.

sorption signal ($t_{1/2} = 800$ μs) observed in CP1 particles prepared with SDS [11,12]. This kinetic correlation at low temperature, together with the spectral features of the absorption signal, prompted us to attribute the flash-absorption signal to the formation of the P-700 triplet state. Similar spectral features observed in the near infrared region led us to ascribe the same P-700 triplet nature to a flash-absorption signal which lasts for a few microseconds at room temperature [11]. These assumptions were recently confirmed for both temperature ranges: a very detailed absorption spectrum, measured by ADMR, and which can be attributed to the ($^3$P-700–P-700) difference [10] has been recently obtained with spectral features very similar to the features observed in Ref. 11; at room temperature, the P-700 triplet nature of a 10 μs flash-induced absorption signal was also recently confirmed [13].

Among this general agreement, there is however at least one major discrepancy between our absorption results [11] and the EPR work on the P-700 triplet state. This discrepancy concerns the amplitude of the P-700 triplet signal and has been stressed recently by Gast and coworkers [7] who calculated that only 5–10% of the PS I reaction centers give an EPR triplet signal at 5 K, in Triton as well as in LDS particles. A quantitative evaluation of the EPR P-700 triplet signal induced by a saturating flash, after extrapolation to time zero and based on the simulation of the EPR signal, shows also that this signal corresponds to 5–10% of the reaction centers in CP1-SDS particles (Vänngård, T. and Sétif, P., unpublished data), assuming that only the $T_0$ sublevel of the triplet is populated in the magnetic field of the EPR experiment. These low values contradict the estimation that could be made from flash-absorption experiments for the yield of formation of $^3$P-700 from the primary biradical [11]. These optical signals are indeed fairly large and P-700 triplet yields of 0.5 and 0.7 were derived at room temperature and at 10 K, respectively [11].

The study presented in this paper was undertaken to evaluate more precisely the yield of P-700 triplet state formation, by two different optical methods: a direct measurement of the quantum yield of $^3$P-700 formation at low exciting light intensities and double laser flash experiments. Additionally, this last experiment allowed us to estimate the lifetime of the biradical state ($P\text{-}700^+ \ldots A_0^-$) that we have also measured directly by submicrosecond absorption measurements. Quantum yields of formation of the chlorophyll *a* triplet in vitro and of charge separation in PS I at different temperatures were also measured for the sake of comparison with $^3$P-700 measurements and appear as side-products of this study.

## Material and Methods

*Biological material*

CP1 particles were purified by polyacrylamide gel electrophoresis in the presence of SDS, as previously described [14]. PS I particles, containing about 110 chlorophylls per P-700, were prepared according to [15]. This procedure involves the solubilization of thylakoid membranes by digitonin and a one-step fractionation by polyacrylamide gel electrophoresis in the presence of deoxycholate. A slight modification of this procedure permits the preparation of PS I particles containing about 65 chlorophylls per P-700 just by increasing the digitonin to chlorophyll ratio and allowing solubilization at room temperature for 2 h. These two kinds of PS I particles will be referred to as PS $I_{110}$ and PS $I_{65}$. The PS I particles (including CP1) were dialyzed against buffer, and then centrifuged at $5000 \times g$ for 15 min in order to eliminate the larger aggregates so as to minimize light scattering by the sample.

Bacterial reaction centers from the carotenoidless mutant R26 of *Rhodopseudomonas sphaeroides* were kindly provided to us by Dr. D. Tiede. Chlorophyll *a* was extracted from spinach leaves, purified by chromatography on sucrose powder and kindly provided to us by J. Kléo. For the experiments, some dry chlorophyll *a* was dissolved in ether and introduced in a $10 \times 10$ mm cuvette which was degassed by three cycles of freezing and thawing.

*Absorption measurements*

*Quantum yield measurements.* The general set-up for flash-induced absorption changes in the microsecond time-range was the same as described previously [16], using a silicon photodiode and a laboratory-made amplifier [17]. For measurements

at 294 K, the material was contained in a square cuvette (10 × 10 mm). For measurements at low temperature, the PS I particles were introduced in flat plexiglas cuvette which was inserted in a cryostat cooled with helium gas [18,19] and positionned at 45° to the mutually perpendicular exciting and measuring beams. Excitation was provided by a YAG-pumped dye laser (duration, 20 ns; broadband emission, around 594 nm). In order to homogenize the exciting light, the laser beam was passed trough a piece of opal glass; the light was then collected by a lens which gave a parallel beam, which could be attenuated by neutral density filters and filtered by an interference filter (590 nm) before falling on the cuvette. The resulting exciting light had a wavelength of 591.5 nm (bandwidth at half intensity, 4.5 nm).

For room temperature measurements, a diaphragm was placed just in front of the cuvette and the pulse energy was measured with an energy-meter (Laser Precision corp. RjP 7200, measuring cell RjP 735) placed after the cuvette. Without the cuvette and with no attenuation, the laser energy measured after the cuvette holder was about 2 mJ. The diaphragm was such that the size of the laser beam was smaller than the area of the energy meter and that within the cuvette holder, the exciting beam was all within the measuring beam. The transmitted light intensity was measured under three different conditions: without the cuvette, with the cuvette containing the solvent or buffer, and with the cuvette filled with the sample. The energy absorbed by the sample was calculated using the expression derived in Ref. 20 to take into account the multiple internal reflections in the cuvette. For reaction center complexes, the transmitted light energy in the presence of the sample, which enters this expression, was modified (increased) so as to take into account a small light scattering by the sample. From the absorption spectra measured on a Cary 17 spectrophotometer, this light scattering was roughly estimated to increase the apparent absorption of the sample by about 10% for bacterial reaction centers and by 10–20% for PS I particles at the wavelength of excitation. Interference filters were placed on the roughly parallel measuring beam before and after the cuvette. The quantum yields of formation of the various species were calculated from the number of absorbed photons and from the total number of photoproduced (triplet or cation) molecules. This last number was calculated from the absorption change, giving an average concentration of photoinduced states which was multiplied by the effective volume probed by the measuring beam. The diameter of the diaphragm, which determines the diameter of the parallel exciting laser beam, has been changed by a factor of 2 without affecting the calculated quantum yield (3–6 mm). The same is true for the width of the measuring beam which has been varied from 2 to 9 mm. In all cases, the absorption of the sample at 591.5 nm was less than 0.2, in order to get as much as possible an homogeneous excitation of the sample along the direction of the exciting beam.

For low temperature measurements, the set-up was nearly the same and quantum yields at low temperature could be compared from one sample to another. Experiments with PS $I_{110}$ particles were conducted with the low temperature set-up (cryostat and flat cuvette), at room temperature as well as at low temperature, in order to compare the yields obtained in the two temperature conditions.

Absorption change signals were accumulated at a rate of one flash every 2 s with a Biomation 2805 transient digitizer coupled to a Tracor TN 1710 multichannel analyzer. It was checked that this repetition rate was low enough to allow for complete relaxation of the system between two successive flashes.

*Double-flash experiments.* These flash-absorption experiments were done with a set-up which has been described previously [16]. This set-up could be modified for low-temperature experiments as described in the preceding paragraph. Excitation was provided by two ruby laser flashes (maximum energy of each flash, 40 mJ; duration of each flash, 6 ns; $\lambda = 694.3$ nm). The delay between the two independent flashes could be varied from 10 ns to several ms.

*Submicrosecond absorption experiments.* These experiments were done at room temperature as well as at low temperature with an apparatus described previously [21], which makes use of a lser diode ($\lambda = 815$ nm; AEG-Telefunken) as source of measuring light and an avalanche photodiode as detector. Excitation was provided by one

ruby laser flash as described above. The time response of the apparatus was about 20 ns. For low temperature experiments, the cuvette holder was replaced by the cryostat, as used in other experiments.

## Results

*Quantum yield measurements under low intensity excitation. PS I particles*

Three different kinds of PS I particles were studied at 294 K and at 20 K. CP1-SDS particles are likely to retain only a functional primary acceptor $A_0$ [7,11,12] whereas PS I particles (65 and 110) contain all the membraneous PS I acceptors $A_0$, $A_1$, $F_X$, $F_B$ and $F_A$ [22]. In these experiments, the flash-induced absorption changes were measured at 820 nm with a strongly attenuated (from one to three hundreds times) laser flash, so that the signal size was linear versus excitation energy. For each kind of particle studied at low temperature, it was checked that the signal size under low intensity excitation remains constant between 20 K and the minimum temperature attainable with our cryostat (about 6 K).

*CP1 particles.* At room temperature, the relaxation in the microsecond time domain of the flash-induced absorption changes of CP1 particles (Fig. 1) is polyphasic, with a major fast phase ($t_{1/2} = 6$ μs; 60–70% of the total signal) [11,23]. This signal has been previously attributed to the triplet state of P-700 [11]. Assuming that the triplet state of chlorophyll *a* has a somewhat lower absorbance than the cation species at the wavelength of the red maximum of chlorophyll *a* [24,25] and taking an absorption coefficient of 64000 $M^{-1} \cdot cm^{-1}$ for the difference (P-700$^+$–P-700) [26], an absorption coefficient of 75000–80000 $M^{-1} \cdot cm^{-1}$ can be estimated for the difference ($^3$P-700–P-700) at 700 nm. With tnis last value and a ratio of 13 between the absorption changes at 700 and 820 nm due to the P-700 triplet state [11], an absorption coefficient of 6000 $M^{-1} \cdot cm^{-1}$ at 820 nm was chosen to calculate the quantum yield of $^3$P-700 formation, as described in the Methods section.

For low temperature experiments, CP1 particles, poised with sodium ascorbate, and frozen in the dark down to 20 K, were first submitted to a bright continuous illumination for a few seconds.

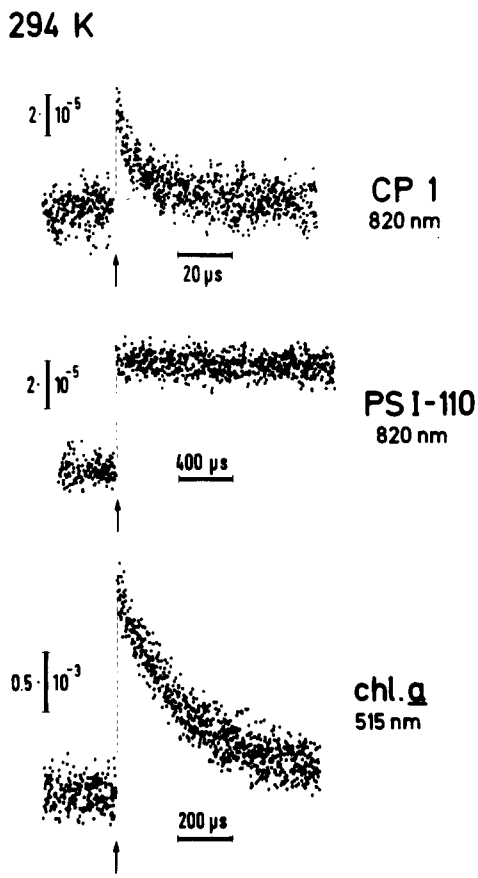


Fig. 1. Absorption transients induced at 294 K by a strongly attenuated dye laser flash ($\lambda_{exc} = 591.5$ nm) given at room temperature. CP1 and PS $I_{110}$ particles in Tris buffer 50 mM (pH 8); addition of sodium ascorbate (1 mM) and DCIP (20 μM). Upper trace: CP1 particles: $A_{591.5\ nm} = 0.09$; incident energy corresponding to $1.1 \cdot 10^{14}$ photons/cm$^2$ per pulse; average of 160 experiments. Middle trace, PS $I_{110}$ particles; $A_{591.5\ nm} = 0.12$; incident energy corresponding to $5 \cdot 10^{13}$ photons/cm$^2$ per pulse; average of 120 experiments. Lower trace: chlorophyll *a* in ether; $A_{591.5\ nm} = 0.15$; incident energy corresponding to $7 \cdot 10^{14}$ photons/cm$^2$ per pulse, average of 40 experiments.)

This light treatment induces a stable charge separation in about 50% of the reaction centers, between P-700 and an unidentified electron acceptor [12]. In order to avoid a progressive change of the sample during the course of the quantum yield measurements, this light treatment was carried out first. Afterwards, flash-absorption experiments were conducted under low intensity excitation. The CP1 particles exhibit an absorption change

which decays quasi-monoexponentially ($t_{1/2}$ = 800 μs) [11] and which was ascribed to the P-700 triplet state [12]. To calculate the quantum yield of $^3$P-700 formation, the same ε of 6000 $M^{-1} \cdot cm^{-1}$ was used as at room temperature and the fraction of reaction centers were P-700 is irreversibly oxidized (50%) was taken into account, i.e., half of the total-absorbed exciting energy was used for the calculation.

Uncorrected quantum yields of 0.36 and 0.28 have thus been obtained for $^3$P-700 formation at 294 and 20 K (see Table I and below for corrections).

*PS I particles (PS $I_{110}$ and PS $I_{65}$).* Flash excitation at room temperature of PS I particles is known to lead to a charge separation (P-700$^+$...P-430$^-$) between P-700 and an iron-sulfur center named P-430 from its maximum of absorption change and which is presumably $F_A$. This charge separation is followed by a slow relaxation corresponding to a recombination reaction which takes place in about 30 ms [27] (Fig. 1). The contribution of P-430 at 820 nm is negligible, so that the quantum yield of P-700$^+$ formation can be easily deduced taking an ε of 6500 $M^{-1} \cdot cm^{-1}$ for P-700$^+$ at 820 nm [28]. Values of 0.59 and 0.50 were respectively obtained for PS $I_{65}$ and PS $I_{110}$ (see Table I). The slight difference between the quantum yields for these two PS I particles appears significant, as the signals were measured under identical conditions: the two samples exhibited the same absorption at the wavelength of excitation and a similar light scattering.

The situation is more complex at low temperature and has been described in detail in Ref. 22. PS $I_{110}$ particles, poised with sodium ascorbate and frozen in the dark, were first submitted at 20 K to a continuous illumination before performing the flash-induced absorption measurements. This continuous illumination induces a stable charge separation (P-700$^+$...$F_A^-$) in about two-third of the reaction centers. The last third of reaction centers exhibit, after the initial charge separation, a practically monophasic relaxation of absorption changes ($t_{1/2}$ = 120 μs). It has been proposed that this relaxation corresponds to the recombination between P-700$^+$ and the acceptor $A_1^-$ [22]. The contribution of $A_1^-$ in the near infra-red region appears negligible and an ε of 6500 $M^{-1} \cdot cm^{-1}$ was assumed for the difference ((P-700$^+$...$A_1^-$)-(P-700...$A_1$)). The quantum yield of the reversible formation of the state (P-700$^+$...$A_1^-$) was calculated to be 0.43 (see Table I), considering only one third of the total energy absorbed by the sample.

*Quantum yield measurements. Bacterial reaction centers and chlorophyll a in vitro*

As the quantum yield for charge separation in bacterial reaction centers and for chlorophyll *a* have already been obtained, these experiments were carried out in order to estimate the precision of our measurements and to calibrate our results (see Discussion).

TABLE I

QUANTUM YIELDS OF FORMATION OF SPECIES WHICH ARE PHOTOPRODUCED BY LASER FLASH EXCITATION

| Biological material | Temperature (K) | Photoinduced state | Measuring wavelength (nm) | Quantum yields (1) | (2) |
|---|---|---|---|---|---|
| CP1 | 294 | $^3$P-700 | 820 | 0.36 ± 0.07 | 0.45 |
| CP1 | 20 | $^3$P-700 | 820 | 0.28 ± 0.05 | 0.35 |
| PS $I_{65}$ | 294 | P-700$^+$...$F_A^-$ | 820 | 0.59 ± 0.06 | 0.73 |
| PS $I_{110}$ | 294 | P-700$^+$...$F_A^-$ | 820 | 0.50 ± 0.05 | 0.62 |
| PS $I_{110}$ | 20 | P-700$^+$...$A_1^-$ | 820 | 0.43 ± 0.07 | 0.53 |
| Chl *a* | 294 | $^3$Chl *a* | 515 | 0.61 ± 0.05 | 0.76 |
| Bacterial R26 centers | 294 | P-870$^+$...$Q^-$ | 870 | 0.78 ± 0.07 | 0.97 |

(1) calculated from the measurement of laser energy.

(2) calculated after normalization of the data, taking a quantum yield of 0.97 for the R26 reaction centers (20,35).

*Bacterial reaction centers.* Flash-induced absorption changes were studied at 870 nm at room temperature in reaction centers of the carotenoidless mutant R26 of the purple bacterium *Rhodopseudomonas sphaeroides*. Flash excitation of the reaction centers leads to a bleaching at 870 nm which corresponds to the charge separation betwen P-870 and a quinone acceptor and which relaxes with $t_{1/2} \gg 1$ ms (not shown; see Ref. 29). The 870 nm absorption changes were studied after excitation by a strongly attenuated laser flash (as for PS I particles) and care was taken to diminish the measuring light intensity so that it had no actinic effect. The calculation of the quantum yield of P-870 photooxidation was based on an extinction coefficient of 112000 $M^{-1} \cdot cm^{-1}$ for P-870 at 865 nm [30]. Without attenuation, the laser flash was saturating and elicited a signal corresponding to 95% of the signal which is expected on the basis of the preceding extinction coefficient, thus showing that nearly 100% of the reaction centers are functional in the preparation.

The value of the quantum yield thus calculated is 0.78 (see Table I).

*Chlorophyll a in ether.* Flash excitation of chlorophyll *a* dissolved in ether gives rise to absorption transients which decay monoexponentially with a $t_{1/2}$ of about 150–300 μs after degassing of the sample (Fig. 1). This kinetic behaviour is characteristic of the triplet state of chlorophyll *a*, the variation in $t_{1/2}$ from one sample to another reflecting probably the unequal quality of the degassing. Several wavelengths of the difference spectrum were studied: at 515 nm, in the red region, where the negative maximum was found at 662 nm, and in the near infra-red where the positive maximum due to the triplet absorption was found to lie at 710 nm, in contrast with a maximum at 750 nm that was found for chlorophyll *a* dissolved in cyclohexanol or in SDS [28]. Assuming an absorption coefficient for chlorophyll *a* in ether of 121000 $M^{-1} \cdot cm^{-1}$ at 428 nm [31], the absorption coefficient in the red maximum (662 nm) is found to be 87000 $M^{-1} \cdot cm^{-1}$, from which an extinction coefficient of 80000 $M^{-1} \cdot cm^{-1}$ was estimated at 662 nm for the difference ($^3$Chl *a*–Chl *a*) [24]. Considering this last coefficient, values of differential absorption coefficients at 710 and 515 nm were found to be respectively +6500 and +13100 $M^{-1} \cdot cm^{-1}$. The absorption signal becomes proportional to the exciting light energy for attenuation of the laser flash greater than 10. Under these conditions, the quantum yield of chlorophyll *a* triplet was derived to be 0.61 from absorption measurements at 515 nm and assuming a differential extinction coefficient of 13100 $M^{-1} \cdot cm^{-1}$.

*Absorption changes in the submicrosecond time-range*

Absorption changes at 815 nm, in the submicrosecond time range, were measured with CP1 particles, after addition of sodium ascorbate. After its initial rise, the signal decays polyphasically (Fig. 2). The fastest phase of the decay has been measured at different temperatures. Its half-time increases from 50 ns at room temperature to about 130 ns at 10 K (Fig. 2) (60 ns at 255 K, 120 ns at 145 K, and 130 ns between 10 and 110 K). After this fast decay phase, the flash-induced absorption change then relaxes in the microsecond time range [11,23] ($t_{1/2} = 7$ μs at 294 K; $t_{1/2} = 800$ μs at 20 K; see preceding paragraph), this relatively slowly decaying state being ascribed to the P-700 triplet state [11]. In these experiments, the laser flash was not far from saturation (for a three-fold attenuation of the laser flash, the signal decreased by about 30%). Without attenuation, the maximum signal corresponds to a ratio (maximum of absorption in the red region/absorption change at 815 nm) of about 600, with CP1 particles which contain one P-700 for about 45 chlorophylls. At any

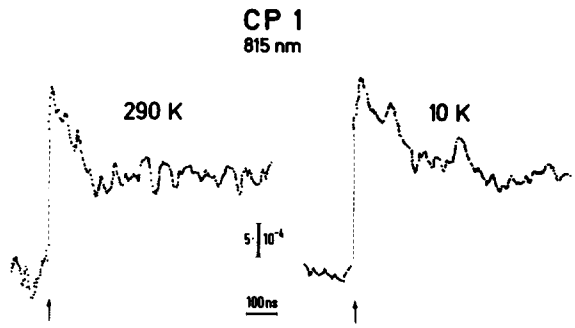


Fig. 2 Absorption transients induced at 815 nm by a ruby laser flash given to CP1 particles in a mixture of glycerol and Tris buffer 50 mM (pH 8) (50/50, v/v). Addition of sodium ascorbate (1 mM) and DCIP (20 μM). $A_{678\ nm}$ (45°) = 1.5. Average of 20 experiments. Left trace: 290 K. Right trace: 10 K.

temperature between 10 K and 290 K, the submicrosecond phase has approximately the same amplitude as the slower microsecond phase: the ratio between the fast and slow phases varies from 0.9 at 294 K to 1.2 at 10 K. Two different control experiments were done: in the presence of potassium ferricyanide, flash excitation does not induce any signal, either fast (submicrosecond) or slow, indicating that these two signals require that P-700 is reduced in the dark; in PS $I_{110}$ particles containing the secondary acceptors, a flash does not elicit any fast submicrosecond signal.

*Double laser flash experiments*

Assuming that the species which decays slowly, presumably the triplet state of P-700, is a product, with a yield of less than unity, of the state which decays in the submicrosecond time-range, presumably the biradical state ($P\text{-}700^{+}\ldots A_0^{-}$), it should be possible, after a first saturating flash excitation, to increase the amount of the slow phase by a second flash fired with an appropriate delay after the first one. Fig. 3 shows examples of such experiments which were conducted on CP1 particles poised with sodium ascorbate, at room temperature and at 20 K. Traces (a) correspond to a one-laser flash excitation whereas traces (b) correspond to a two-flash excitation (delay between the two flashes: 250 ns at 294 K and 20 μs at 20 K). As the laser flash was not completely saturating (90–95% of the signal for a two-fold attenuation), the laser was attenuated two-fold in the two-flashes experiments, but not in the single-flash experiments. Thus the energy received during single flash experiments (without attenuation) was identical to the energy received by the sample in two-flash experiments (with a 50% attenuation), where the two shots were simultaneous or spaced by a time interval $t$.

Considering the above interpretation of this kind of experiments, the signal size after the second flash should be maximum when this flash is fired at a time when the biradical has completely relaxed (either to the P-700 triplet state or to the singlet ground state) and when the P-700 triplet state has not yet relaxed. This has been observed to be the case for a delay between the two flashes which is comprised between 150 and 600 ns at room temperature and between 1 and 50 μs at 10 K. The maximum increase due to the second flash has been measured to be 22% of the one-flash signal at 10 K, and 14 and 19% in two separate experiments at room temperature (Fig. 4).

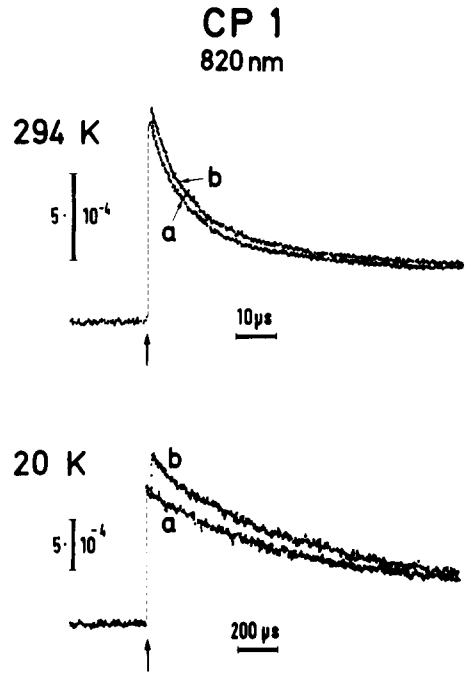


Fig. 3. Absorption transients induced at 815 nm by one or two nearly saturating ruby laser flashes given to CP1 particles. Addition of ascorbate (1 mM) and DCIP (20 μM). (a) One flash without attenuation; (b) two flashes with a two-fold attenuation. Upper part; temperature, 294 K; Tris buffer, 50 mM (pH 8); delay between the two flashes (trace b), 250 ns; $A_{678\ nm} = 1.04$. Lower part: temperature, 20 K; mixture of glycerol and Tris buffer; the cuvette was first preilluminated at 20 K with white light for a few seconds; delay between the two flashes (trace b) 20 μs; $A_{678\ nm}\ (45°) = 1.61$.

For longer delay times, the total signal after the second flash, measured at a constant time after the second flash, begins to decrease (not shown) due to the fact that the P-700 triplet had time to relax between the two flashes. More interestingly, it is possible to detect an increase of the total two-flash signal, when the delay between the two flashes increases from 0 to 150 ns at room temperature and from 0 to about 600 ns at 10 K (Fig. 4). The delay time for which half of the maximum increase is obtained is about 30 ns at room temperature and about 120 ns at 10 K.

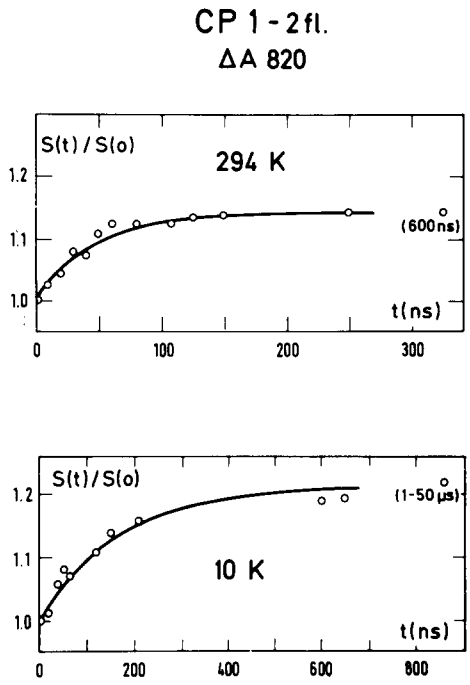


Fig. 4. A plot of the ratio $S(t)/S(0)$, where $S(t)$ is the total signal induced at 820 nm by two ruby laser flashes (two-fold attenuation) and $S(0)$ is the signal induced by one flash without attenuation, versus the delay $t$ between the two flashes.

## Discussion

*Yield of formation of the P-700 triplet state*

The double laser flash experiments show that the slowly decaying absorption signal (in the microsecond time domain) in CP 1 particles is due to a state which is formed with a yield of less than unity from a more rapidly (submicrosecond) decaying state and that this last state decays for another part to the ground state. Since the delay times corresponding to half of the maximum increase due to the second flash in double-laser flash experiments are in a fair agreement with the half-times provided by the submicrosecond absorption experiments, it can be concluded that the slowly decaying species is formed from the rapidly decaying species which is observed by submicrosecond absorption experiments. The most simple interpretation of these observations is that the P-700 triplet state, which corresponds to the slowly decaying signal, is formed through the fast submicrosecond recombination of the ($P\text{-}700^+ \ldots A_0^-$) biradical. The delay times between the two flashes which induce half of the maximum increase due to the second flash should therefore correspond to the half-time of repopulation of the P-700 ground state after the first flash.

The P-700 triplet state is probably formed by the radical pair mechanism, a process which has been much more thoroughly studied in bacterial reaction centers [34], and which is very briefly depicted in Fig. 5. Assuming that mixing of the singlet state with the triplet state of the biradical ($P\text{-}700^+ \ldots A_0^-$) is a homogeneous process and taking a yield $p$ of $^3$P-700 formation from the biradical state on a single turnover of the reaction centers, yields of $^3$P-700 formation should be $p$ and $p(1-p)$ on the first and second ruby-laser flashes (double-flash experiments) if the three next conditions are met. (i) A single flash induces a single turnover of the reaction centers. This should be approximately the case as the lifetime of the biradical (Fig. 2) appears to be much longer than the flash duration, and as the increase in the triplet signal in double flash experiments is only about 2% for a delay of 10 ns at 294 K, and about 1% for a delay of 25 ns at 20 K. (ii) Between the two laser flashes, the biradical has enough time to relax completely by recombination either to the singlet ground state or the the P-700 triplet state and. (iii) The $^3$P-700 state has not yet relaxed significantly. The existence of a plateau in the two curves of Fig.

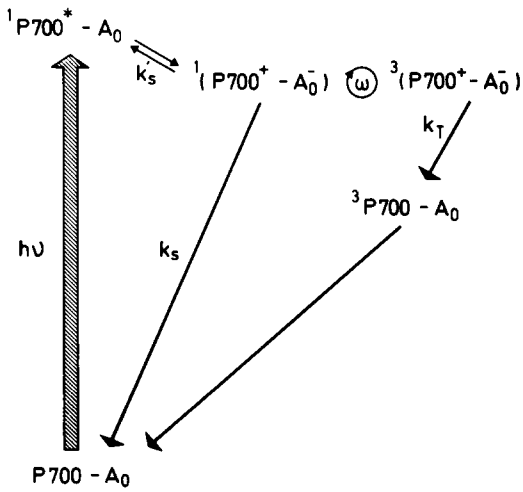


Fig. 5. Reaction scheme for PS I primary charge separation and recombination processes in the absence of secondary acceptors.

4 indicate that the conditions (ii) and (iii) are met for delay times between 150 and 600 ns at 294 K and between 1 and 50 μs at 10 K. Therefore, for the appropriate delays between the two flashes, the ratio of the signal induced by the second flash to the signal induced by the first one is $p(1-p):p$ or $1-p:1$, so:

at 294 K, $1-p=0.165$ (average of two separate experiments), i.e., $p=0.835$;

at 10 K, $1-p=0.21$, i.e., $p=0.79$.

However, mixing of the singlet and triplet states of the biradical should not be a homogeneous process in the frame of the radical pair mechanism, as it depends on the nuclear spins in $P\text{-}700^+$ and $A_0^-$. Reaction centers where the mixing is faster should give a higher triplet yield than reaction centers with a slower mixing. As the nuclear spins have no time to relax appreciably between the two successive flashes, the triplet yield is expected to be higher for the first flash than for the second one. Therefore the triplet yield on the second flash should be better written $p'(1-p)$ with $p'<p$, and the preceding values of $p$ are thus maximum values.

The quantum yields, which appear in the Results section, were calculated from the direct measurement of the laser energy with the energy-meter (Table I, column 1). However, this apparatus was only approximately calibrated, so that the energy measurements were only relative and were used to compare the quantum yields from one experiment to another. Calibration of our experiments in absolute quantum yields can be obtained by assuming a yield of 0.97 for $P\text{-}870^+$ formation in bacterial reaction centers R26 (average of a value of 0.96 obtained in Ref. 20 and a value of 0.98 obtained in Ref. 35 for excitation wavelengths of respectively 600 and 583.5 nm) at room temperature. The energy values given by our energy meter thus appear to have been overestimated, resulting in underestimated quantum yields. Quantum yields obtained with the appropriate correction factor of 0.97/0.78 are given in Table I, column 2. We do not claim a high precision for these quantum yield measurements as it can be seen from the error values (Table I, column 1). These error values are essentially due to the light scattering by the cuvette which renders the estimation of the absorbed energy inaccurate and to the uncertainty in the correction factor (due to the irreversible processes which occur in a fraction of the reaction centers) which is taken into account for low temperature measurements with CP1 and PS $I_{110}$ particles. Another source of inaccuracy comes, in the case of CP1 particles, from the uncertainty in the absorption coefficient of the ($^3$P-700 − P-700) difference. It can be noticed that the yield for chlorophyll *a* triplet state formation in ether (direct measurement, $p=0.61\pm0.03$; after correction, $p=0.76\pm0.10$) agrees with the value found in Ref. 31 ($p=0.64\pm0.09$) within the experimental uncertainties. On the other hand, the values obtained for the charge separation in PS I particles at room temperature are lower than the values previously found (around 1.0 in Ref. 32; at least 0.75 in Ref. 33). This may be due to the fact that some antenna chlorophylls have been disconnected by the detergent treatment in our PS I particles.

The quantum yield of $^3$P-700 formation can be expressed as: $p=p_1p_2$, where $p_1$ is the quantum yield of primary charge separation and $p_2$ is the yield of $^3$P-700 formation from the biradical ($P\text{-}700^+\ldots A_0^-$) state. Assuming that the quantum yield $p_1$ for the primary charge separation is the same in CP1 complexes as in PS $I_{65}$ particles (0.73 at 294 K, and about 0.60 at 20 K) and taking values of 0.45 and 0.35 for the quantum yields of $^3$P-700 formation at 294 K and 20 K (Table I, column 2), values for the yield of $^3$P-700 formation from the primary biradical state can be calculated:

at 294 K, $p_2=0.62$

at 20 K, $p_2=0.58$.

The different values of the P-700 triplet yield from the biradical state in CP 1 particles are shown in Table II, together with the yields that we have previously estimated (0.5 at 294 K, and 0.7 at 10 K) on the basis of the size of absorption changes induced in CP1 particles by a saturating 2 ns ruby laser flash [11].

In view of the uncertainties of the different estimations, these values are in a good agreement and the results presented in this paper confirm that large P-700 triplet yields are found in flash absorption experiments [11]. Possible origins for the low yields of $^3$P-700 formation that are derived from EPR experiments [7] are briefly discussed in the following, although there is clearly a need for further experimentation before any explanation

TABLE II

YIELD OF P-700 TRIPLET STATE FORMATION FROM THE PRIMARY BIRADICAL STATE ($P\text{-}700^+ \ldots A_0^-$)

| Temperature (K) | $^3$P-700 yield from double-flash absorption changes experiments | $^3$P-700 yield from low intensity flash-induced absorption changes | $^3$P-700 yield according to Ref. 11 |
|---|---|---|---|
| 294 | $p < 0.84$ | $p = 0.62$ | $p = 0.50$ |
| 20 | $p < 0.79$ | $p = 0.58$ | $p = 0.70$ |

can be safely proposed:

(i) The magnetic field of the EPR experiment (around 3300 G) might induce a large decrease of the P-700 triplet yield. This magnetic field effect has been largely documented in bacterial reaction centers [34], where a decrease by a factor larger than 2 has already been observed at room temperature. However, such a possibility appears very unlikely as preliminary absorption experiments indicate that a magnetic field of 400 G has no effect on $^3$P-700 formation at 10 K (not shown), whereas the same field induces only a 10% decrease on the microsecond signal at 294 K in CP1 particles [11];

(ii) the $T_{\pm 1}$ sublevels of the triplet state that is observed by EPR are also populated, but to a smaller extent than the $T_0$ sublevel (in order to account for spin polarization of the EPR signal). Such $T_{\pm 1}$ populations of the P-700 triplet state cannot be explained by the radical pair mechanism. However, a triplet excitation transfer to a neighboring chlorophyll molecule absorbing near 700 nm might be accompanied by a partial loss of polarization due for example to enhanced relaxation processes;

(iii) the state probed by EPR may not be the same as the state probed by absorption experiments. For example, absorption changes may correspond to two different states which are in equilibrium: a triplet state localized on P-700 which is probed by EPR and a charge-transfer triplet state between P-700 and an intermediate acceptor preceding $A_0$ (analogous to the bacteriochlorophyll intermediate acceptor in purple bacteria), whose EPR signal has not been observed. However, there is up to now no clear evidence for the contribution of an acceptor molecule to the spectrum of flash-induced absorption changes in CP1 particles [11];

(iv) the amplitude of the EPR triplet signal has been estimated from EPR transients induced by a flash of a long duration compared to the lifetime of the primary biradical (600 ns in Ref. 7; 10 μs in our experiments). The P-700 triplet state (or the triplet state of a neighboring chlorophyll that is probed by EPR) might be quenched by other excitons trapped by P-700 or by chlorophyll molecules surrounding P-700.

*Absorption changes due to the biradical ($P\text{-}700^+ \ldots A_0^-$)*

The life-time of the biradical that can be deduced from double-flash experiments agrees very well with the life-time found by submicrosecond absorption experiments at 20 K, but it is somewhat smaller at 294 K. This difference may be due to the response time of the apparatus for submicrosecond absorption changes measurements which increases the apparent half-time of the decay. However, this difference may also reflect the fact that the decay of the biradical is not monoexponential (see Fig. 5) so that the half-time for repopulation of the singlet ground state after the first flash is a little smaller than the half-time of decay of the biradical. Anyway, these halftimes appear in a fair agreement with the half-times of a delayed fluorescence component sensitive to a magnetic field and detected in PS I particles under highly reducing conditions (100 ns at room temperature and 200 ns at 77 K; see Ref. 36). It can be noticed that the half-time of the biradical at room temperature appears larger in PS I (30–50 ns), as in anaerobic green bacteria (20–35 ns; see Ref. 37), than in purple bacteria (about 10 ns, see Ref. 38) and in PS II (tentative assignment, 3 ns, see Ref. 39).

The maximum absorption signal in CP1 particles corresponds to about 60 chlorophylls per

P-700, if it is assumed that only P-700$^+$ contributes to the signal ($\epsilon = 6500\ M^{-1} \cdot cm^{-1}$). However, several limitations (response time of the apparatus, laser flash non entirely saturating for submicrosecond experiments, polyphasic decay of the biradical) impede to measure precisely the maximum absorption change due to the biradical just when it is formed. Thus our observations cannot allow to decide whether the primary acceptor molecule contributes significantly to the absorption change at 815 nm. Experiments at other wavelengths and (or) with a better time resolution should help in measuring transient absorption changes due to the primary acceptor.

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