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FLASH-INDUCED ABSORPTION CHANGES IN PHOTOSYSTEM I

RADICAL PAIR OR TRIPLET STATE FORMATION?

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Absorption changes induced in chlorophyll protein (CP 1) particles by short laser flashes have been analyzed in order to decide whether a state lasting for a few microseconds at 21°C or 800 μs at 10 K corresponds to the biradical *P*-700$^+$ ... A_1^- (A_1 being a chlorophyll *a*) or to a triplet state produced in a submicrosecond recombination of the preceding state. At 21°C the spectrum of the flash-induced ΔA (720–870 nm) presents a flat-topped band from 740 to 820 nm, clearly different from that of *P*-700$^+$. A saturation curve (ΔA vs. laser energy), obtained with a 2 or 10 ns laser pulse, indicates that ΔA saturates at a value 2- or 3-times smaller than that expected on the basis of the chemical oxidation of *P*-700. At 21°C the size of the flash-induced ΔA is slightly decreased (5–15%) when the sample is subjected to a 400 G magnetic field. The kinetics of decay are not affected; they are not affected either by the oxygen concentration. At 10 K the spectrum of the flash-induced ΔA has been measured between 650 and 1700 nm. Between 650 and 720 nm, the spectrum presents only one major negative peak at 702 nm; it is quite different from that due to the chemical oxidation of *P*-700 (which has additional peaks at 688 and 677 nm). Between 720 and 870 nm, the spectrum is identical to that obtained at 21°C. Above 870 nm, the spectrum includes a broad band around 1250 nm, which is absent in *P*-700$^+$. A saturation curve leads to a maximum ΔA greater than that at 21°C and which is also greater with a 1 μs dye laser flash than with a 10 ns ruby laser flash. An analysis of the spectral data indicates that these do not fit correctly with the hypothesis of a contribution of *P*-700$^+$ and of a chlorophyll *a* anion radical. They fit more closely with the hypothesis of a triplet state of *P*-700, a hypothesis which is discussed in relation to other experimental data.

Introduction

Among photosynthetic reaction centers, PS I is characterized by the low redox potential of its electron acceptors, and this renders the study of these acceptors difficult. Earlier studies led to the conclusion that iron-sulfur centers play an active role as electron acceptors [1–4]. These include the 'bound ferredoxins' or centers A and B (probably corresponding to the species named *P*-430 in absorption studies), and a species termed 'X' from EPR studies at low temperature [5–7], probably identical to the acceptor called A_2 in absorption experiments at physiological temperatures [8–10]. When all these acceptors are reduced by poising the medium at a very low redox potential [8–10], or are absent, as in PS I particles prepared with SDS [11,12], a flash-induced oxidation of *P*-700 can still be elicited, which reverses in 3–10 μs at 21°C. It was thus concluded [8] that the electron carriers in PS I are arranged as follows: *P*-700 ... A_1 ... A_2(X) ... *P*-430 (centers A and B).

It has been proposed that the primary electron acceptor A_1 is a molecule of Chl *a*, since this species has a low redox potential (about –0.9 V, in vitro) compatible with that of A_1 [13]. This proposal has

Abbreviations: Chl, chlorophyll; DCIP, dichlorophenolindophenol; SDS, sodium dodecyl sulfate; PS I, Photosystem I; CP, chlorophyll protein.

been widely accepted, with occasional variations, such as the idea that A_1 is dimeric Chl *a*, on the basis of experimental data obtained by subnanosecond [14,16] or microsecond [10,12,17] absorption spectroscopy, EPR studies of A_1^- trapped at low temperature [18,19], and CIDEP * measurements [20,21]. A critical examination of all these data indicates that they are often contradictory or of little help in the chemical identification of A_1 [22]. The Chl. *a* hypothesis can nevertheless be considered as the best one for the moment.

The first experimental evidence for the existence of A_1 [8–11] was unambiguous in its requirement for a new, early acceptor associated with *P*-700. Nevertheless it was impossible to decide whether the state lasting for a few microseconds was a radical pair ($P\text{-}700^+ \ldots A_1^-$) or a triplet state of *P*-700 formed in a submicrosecond recombination of the radical pair. A polarized triplet state probably originating in such a recombination has recently been observed in PS I by EPR spectroscopy [23,24]. In this work we undertook a detailed examination of the absorption properties of the reaction center of PS I, in CP 1 particles prepared by SDS-polyacrylamide gel electrophoresis, in an attempt to decide between the two hypotheses. We conclude that the data do not fit well with the radical-pair hypothesis if A_1 is a Chl *a* molecule, and that the state observed by flash absorption spectroscopy in CP 1 is more probably a triplet state.

Materials and Methods

Biological material. CP 1 particles were prepared and purified by polyacrylamide gel electrophoresis in the presence of SDS as described in Ref. 25. Other PS I particles were prepared after treatment with Triton X-100 according to Ref. 26. Both types of particles were dialyzed against Tris-HCl buffer (50 mM, pH 8.0), and were further diluted with the same buffer for study. For low-temperature experiments, redox reagents (see text) were added, and the sample was then mixed with 2 vol. glycerol.

Absorption measurements. Flash-induced absorption changes were measured as described previously [8], using a silicon photodiode and a laboratory-made amplifier [27]. For measurements at 21°C the material was contained in a square cuvette (10 × 10 mm). For measurements at low temperature, the material plus 60% glycerol was placed in a plexiglass cuvette, which was then inserted in a cryostat cooled with cold helium gas (as described in Refs. 28 and 29). The measuring light was filtered with a narrow-band filter ($\Delta\lambda$ = 3 nm below 750 nm, 6–10 nm above 750 nm) before reaching the cuvette. The sample was excited either by a ruby laser (pulse duration 2 or 10 ns) or by a dye laser (broad-band emission at about 600 nm; pulse duration 1 μs).

The laser beams were homogenized by a piece of opal glass. Their relative energies were measured with a silicon photodiode; they could be varied with neutral density filters (Schott, type NG). The absorption changes at wavelengths greater than 1000 nm were measured with a germanium detector cooled to 77 K as described in Ref. 30.

Difference spectra due to the chemical oxidation of *P*-700 were measured in a Cary 17 spectrophotometer. Difference spectra at 21°C were simply recorded with an oxidized-sample cuvette and a reduced-sample reference cuvette. For the spectra at low temperature, the cryostat was inserted in the sample compartment; absorption spectra of an oxidized-sample cuvette and of a reduced-sample cuvette were recorded separately and then subtracted.

Results

Absorption changes at 21°C

The CP 1 particles used in this work were prepared by a method slightly different from that used in Ref. 11. The flash-induced absorption changes, however, behave practically identically. At 820 nm an immediate absorption increase is followed by a multiphasic decay including a major fast phase ($t_{1/2} \simeq 6$ μs), an intermediate phase, and a signal (10–15%) which appears irreversible on a 2 ms time scale. A bleaching with similar kinetic characteristics takes place at about 700 nm (Fig. 1). The decay at 820 nm is practically unaffected by the presence of oxygen in the medium (Fig. 1): the signal decaying between 0 and 100 μs has a $t_{1/2}$ of 7.2 μs under argon, and 6.5 μs under pure O_2. A small acceleration by O_2 was reproducibly observed, but it is not

* CIDEP, chemically induced dynamic electron polarization.

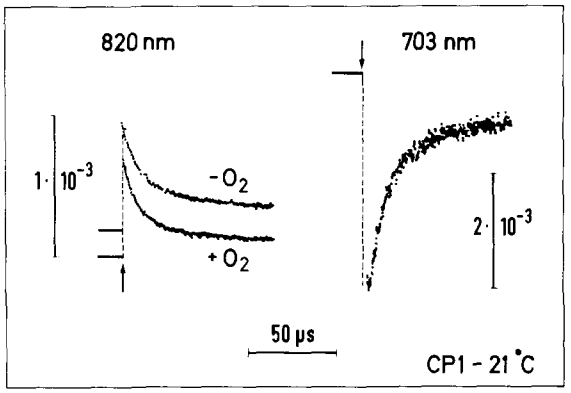

Fig. 1. Kinetics of ΔA induced by a 10 ns ruby laser pulse in CP 1 particles at 21°C (A_{678} = 0.88). Addition of 1 mM ascorbate and 20 µM DCIP. Electrical bandwidth: 1 MHz. Average of five experiments at 703 nm (cuvette under air) and 10 experiments at 820 nm (cuvette under argon or under pure O_2 at 1 atm).

clear whether it represents an acceleration of the fast phase, or a relative increase in the magnitude of the fast phase as compared to slower phases.

The spectrum of the flash-induced ΔA, from 730 to 870 nm, is plotted in Fig. 2. It is very flat between 740 and 800 nm, and 820 nm is located on the decreasing side. The same graph shows the signal measured in the presence of ferricyanide when *P*-700 is oxidized prior to the flash. The small signals representing 7–15% of the maximum ΔA at 820 nm are poorly resolved kinetically but, as in reducing conditions, they decay with a $t_{1/2}$ of 5–10 µs; we attribute them to the formation of the triplet state of Chl *a*, which has a broad band around 760 nm [30]. We have checked that the triplet state of carotenoids does not absorb in that spectral region. For the sake of comparison, the difference spectrum due to the chemical oxidation of *P*-700 is also plotted in Fig. 2 (bottom). This spectrum was obtained with the same material and differs significantly from the flash-induced spectrum. The chemically induced spectrum was obtained by poising the redox potential with ferri- and ferrocyanide, at a molar ratio of 40; under these conditions, the difference spectrum in the red (640–710 nm) did not indicate any chemical oxidation of the bulk chlorophyll. The bottom curve of Fig. 2 leads to a Chl *a*/*P*-700$^+$ ratio of 45, which was obtained reproducibly (taking a molar absorptivity

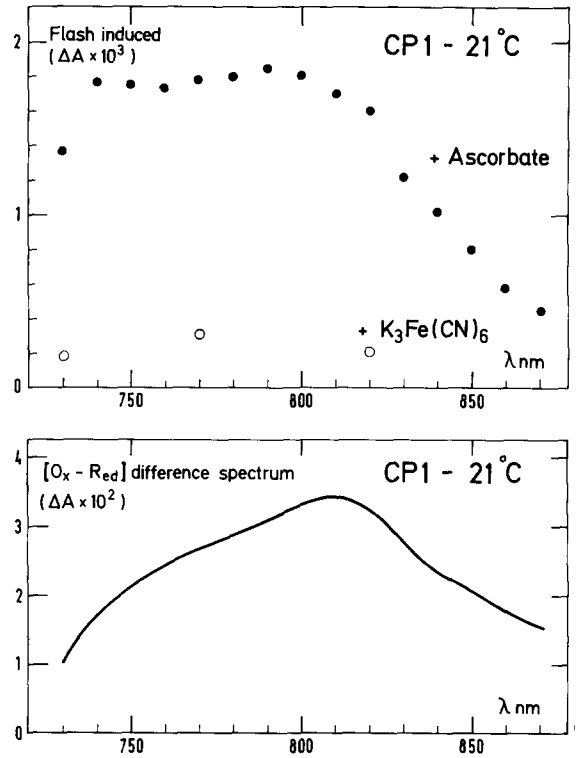

Fig. 2. Difference spectra obtained with CP 1 particles at 21°C, in the near infrared. Top: ΔA induced by a nonsaturating 10 ns ruby laser flash with particles (A_{678} = 3.2) supplemented with 1 mM ascorbate and 20 µM DCIP (•) or with 1 mM potassium ferricyanide (○). Bottom: difference spectrum between a sample cuvette (addition of 0.1 mM ferro- and 4 mM ferricyanide) and a reference cuvette (addition of 2 mM ascorbate and 40 µM DCIP) both with an equal amount of CP 1 (A_{678} = 15.5).

value of 6500 $M^{-1} \cdot cm^{-1}$ for *P*-700$^+$ at 820 nm [30]). The top curve of Fig. 2 is not directly comparable, since it was obtained with nonsaturating flashes.

The effect of flash intensity on the magnitude of ΔA at 820 nm is plotted in Fig. 3. The curves are identical for a pulse duration of 2 or 10 ns. They also indicate a clear saturation, at which level the ratio $A_{678}/\Delta A_{820}$ is 1150 (several experiments led to values ranging from 850 to 1200). If the ΔA at 820 nm were due to *P*-700$^+$ this would give a Chl *a*/*P*-700$^+$ ratio of 115, well above the value obtained by chemical oxidation (a submicrosecond decay would not have been detected in these experiments). A larger flash-induced ΔA was obtained with some preparations (up to $A_{678}/\Delta A_{820}$ = 500) but this was

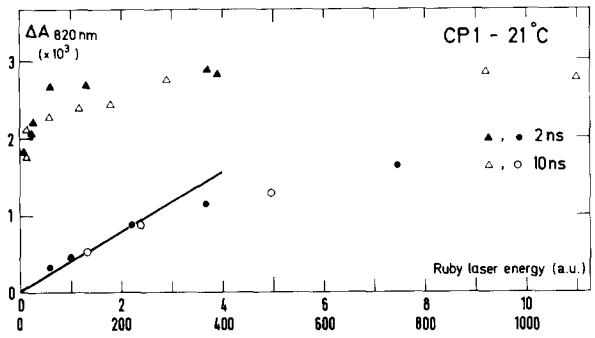

Fig. 3. A plot of ΔA at 820 nm induced in CP 1 particles (A_{678} = 3.0) at 21°C by ruby laser pulses of 2 or 10 ns, and of variable energy. The energy is in arbitary units (an energy of 1000 corresponds to approx. 100 mJ), but a given unit corresponds to the same energy for either pulse duration. The circles correspond to the low-energy scale and the triangles to the high-energy scale.

correlated with a large ΔA remaining in the presence of ferricyanide (up to 35% at high laser intensity). We assumed that this indicated significant formation of the bulk chlorophyll triplet state and the data obtained with those preparations have not been taken further into consideration.

The maximum flash-induced bleaching was obtained at 700 nm (other filters available were at 695 and 703 nm). After subtraction of the ΔA remaining in the presence of ferricyanide, we calculated the following values (the ΔA values were obtained with a saturating ruby laser pulse of 2 ns): $A_{678}/\Delta A_{820}$ = 900–1300, $A_{678}/\Delta A_{700}$ = 100–150 and $A_{700}/\Delta A_{820}$ = 8.5 ± 0.5.

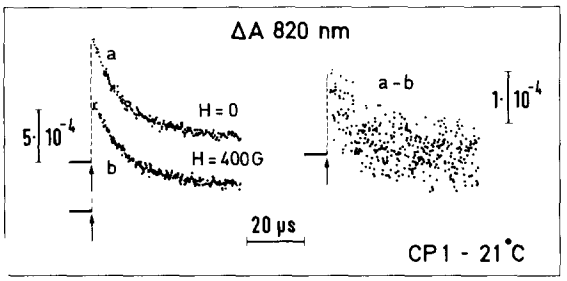

Fig. 4. Kinetics of ΔA at 820 nm induced in CP 1 particles (A_{678} = 3.0) by a subsaturating 2 ns ruby laser pulse, at 21°C. a, without magnetic field; b, H = 400 G; a–b: difference between the two former traces. Each trace is the average of 12 experiments.

The size of the flash-induced ΔA is slightly decreased when a magnetic field is applied to the particles (Fig. 4). In these experiments a permanent magnet was placed at 45° to the mutually perpendicular measuring and exciting beams. A 2 ns ruby laser pulse was used, and its energy was reduced so that the ΔA was linear in laser energy. The ΔA at 820 nm decreases by 5–15% (in different experiments) at H = 400 G; the effect is about half as large at H = 200 G. We checked that the magnetic field had no effect on the measuring device. In another control the CP 1 particles were replaced by D-144 * PS I particles; the magnetic field had no significant effect on the flash-induced ΔA due to P-700$^+$ in these particles.

Effect of temperature on the flash-induced absorption changes

The influence of temperature on the kinetics of absorption recovery at 820 nm, following flash excitation, were studied in the range 5–294 K. The pattern is highly complex. At intermediate temperatures, as at 21°C, a minimum of three exponential components is necessary for a correct description of the decay kinetics. The most conspicuous effect of lowering the temperature is that the very slow phase progressively disappears; below 10 K the decay is nearly exponential and a phase with $t_{1/2} \simeq 0.8$ ms represents about 90% of the decay. The experiments to be described below were all performed at 10 K or slightly below, where the decay is nearly exponential.

Absorption changes at 10 K

The spectrum of flash-induced ΔA, with CP 1 at 10 K, was measured between 650 and 1700 nm (Fig. 5). In order to obtain reasonable accuracy the spectrum was divided into three ranges (650–720, 720–870 and 870–1700 nm), with appropriate conditions of concentrations and of detection. The results obtained in the three spectral regions were normalized by comparing, under identical conditions, ΔA at 700 and 820 nm, and then ΔA at 820 and 1230 nm. From several experiments we found that $\Delta A_{700}/\Delta A_{820}$ = 12 ± 1 and $\Delta A_{820}/\Delta A_{1230}$ = 5 ± 0.5. In the range 720–870 nm, the spectrum is similar to that measured at 21°C. In the two other regions the spec-

* D-144, PS I particles obtained on treatment with digitonin and centrifugation at 144 000 × g.

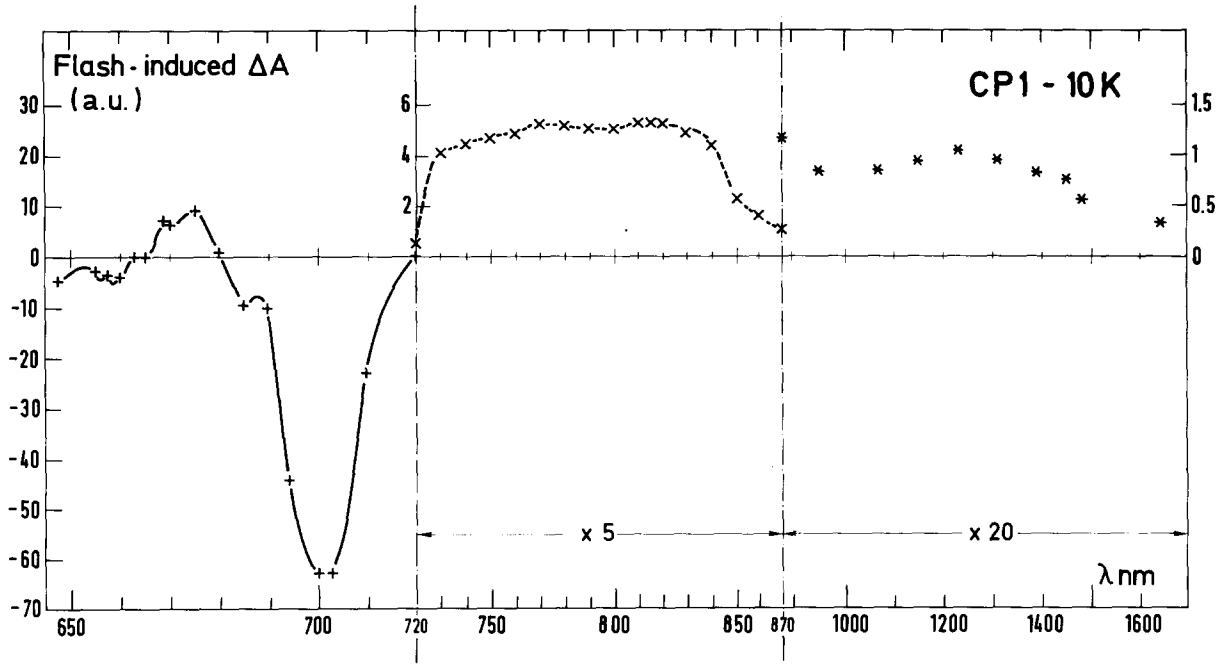

Fig. 5. Difference spectrum of dye laser flash-induced ΔA at 10 K, in CP 1 particles. The concentration was different in the three spectral ranges: A_{678} (following the direction of the measuring beam): 0.77 (650–720 nm; average of two to six experiments), 1.1 (720–870 nm; average of four experiments), 2.2 (870–1700 nm; average of eight experiments, except 32 at 1640 nm). The different segments of spectrum have been normalized as indicated in the text. At 700 nm the actual ΔA was $11 \cdot 10^{-3}$. At 870 nm the points have been plotted in the two vertical scales.

trum could not be measured at 21°C. Around 1250 nm a broad positive band is apparent. Between 650 and 720 nm, the spectrum resembles very much that obtained previously with comparable particles [11]. When potassium ferricyanide (1 mM) was added to the cuvette prior to cooling, a small flash-induced signal remained present at 10 K, with approximately the same kinetics as with ascorbate. The spectrum of this signal resembles grossly that obtained with ascorbate but is 5–7-times smaller. The signal remaining with ferricyanide is due either to reaction centers in which *P*-700 has not been oxidized or, more probably, to the triplet state of long-wavelength antenna Chl *a*.

The size of the flash-induced ΔA at 820 nm, at 10 K, was measured versus laser intensity (dye laser or ruby laser pulse). Identical results were obtained with a 2 or 10 ns duration of the ruby laser (Fig. 6). The energies of the two types of laser were normalized so that the curves coincide in the linear region, at low energy. As shown in Fig. 6, the two curves deviate progressively so that, at the highest energy available with the dye laser, ΔA is about 30% greater than with the ruby laser. With both lasers the ΔA remaining with ferricyanide represents about 20% at the maximum laser energy; the deviation between the two curves cannot be attributed to this remaining signal. At the highest energies available, the signal appears to be practically saturated with the ruby laser, but obviously not with the dye laser. Under these conditions $\Delta A_{678}/\Delta A_{820}$ values of 650 and 500 were obtained for the ruby and dye lasers, respectively, corresponding to 800 and 600 after subtraction of the signal remaining with ferricyanide. The latter figures showed little variation from one preparation of particles to another.

The P-700⁺/P-700 difference spectrum

In order to evaluate the significance of the flash-induced absorption transients in CP 1, we attempted to measure the difference spectrum corresponding to the oxidation of *P*-700 under comparable conditions.

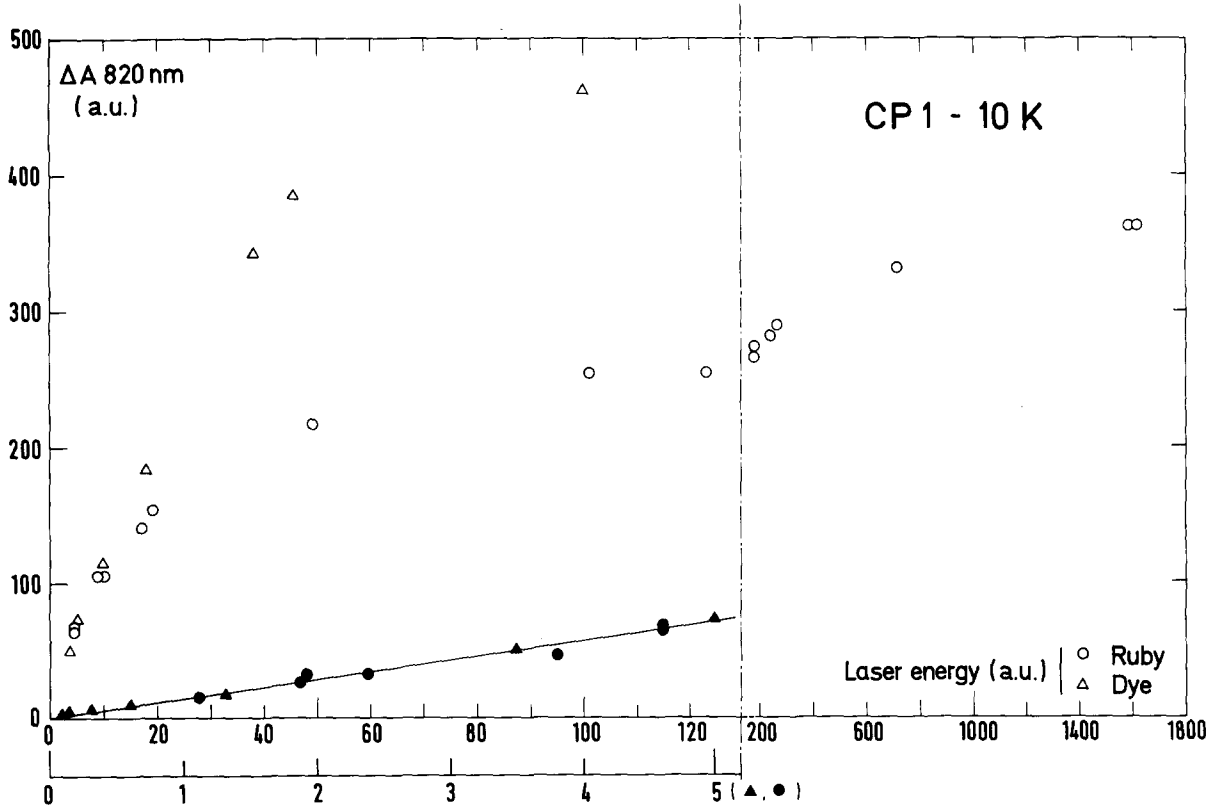

Fig. 6. A plot of ΔA at 820 nm induced in CP 1 ($A_{678} = 0.55$) at 10 K by ruby laser (10 ns) (circles) or dye laser (triangles) pulses of variable energy (in arbitrary units; the maximum energy is approx. 100 mJ for the ruby laser and 15 mJ for the dye laser). Average of two to eight experiments per point. Data points are circles for ruby laser excitation and triangles for dye laser excitation. Note that they are arranged according to two energy scales.

At 10 K, this could be done by chemical oxidation of *P*-700, for the range 650–720 nm (Fig. 7). This spectrum has been plotted with the assumption that $\Delta\epsilon$(700 nm) = 64 000 [31]. In addition to the large negative peak at 700 nm, it includes well defined peaks at 688 nm (positive) and 677 nm (negative), in agreement with the spectrum obtained by Lozier and Butler [32] with another material. Under the same conditions, the spectral region above 720 nm could not be safely covered, since the absorption variations are weak and broad, and thus very sensitive to any fluctuation of the baseline. In Fig. 7 we have also plotted the spectrum of flash-induced ΔA in PS I particles prepared with Triton X-100 measured at 21°C under conditions where the signals (decaying with $t_{1/2} \simeq 30$ ms) are attributed to the formation of the pair (P-700$^+$, P-430$^-$). P-430 is probably an iron-sulfur center; it is known that the reduction of these centers brings about only very weak absorption changes in the spectral region considered [33,34] (see also discussion in Ref. 30). The spectrum of Fig. 7 (720–1700 nm) includes a broad absorption peak around 800 nm, comparable to that reported in Fig. 2 (bottom) and practically no absorption change ($\Delta\epsilon <$ 300 $m^{-1} \cdot cm^{-1}$) above 1000 nm (see Ref. 30 for a discussion and a comparison with the Chl *a* cation radical). The spectrum was plotted with the assumption that $\Delta\epsilon_{820} = 6500\ M^{-1} \cdot cm^{-1}$ [30]. In the particles used, a ratio of $\Delta A_{700}/\Delta A_{820} = 10 \pm 0.5$ was measured.

For comparison, Fig. 7 also includes the spectrum of the triplet state of Chl *a*, from 730 to 1700 nm, in cyclohexanol at 21°C [30].

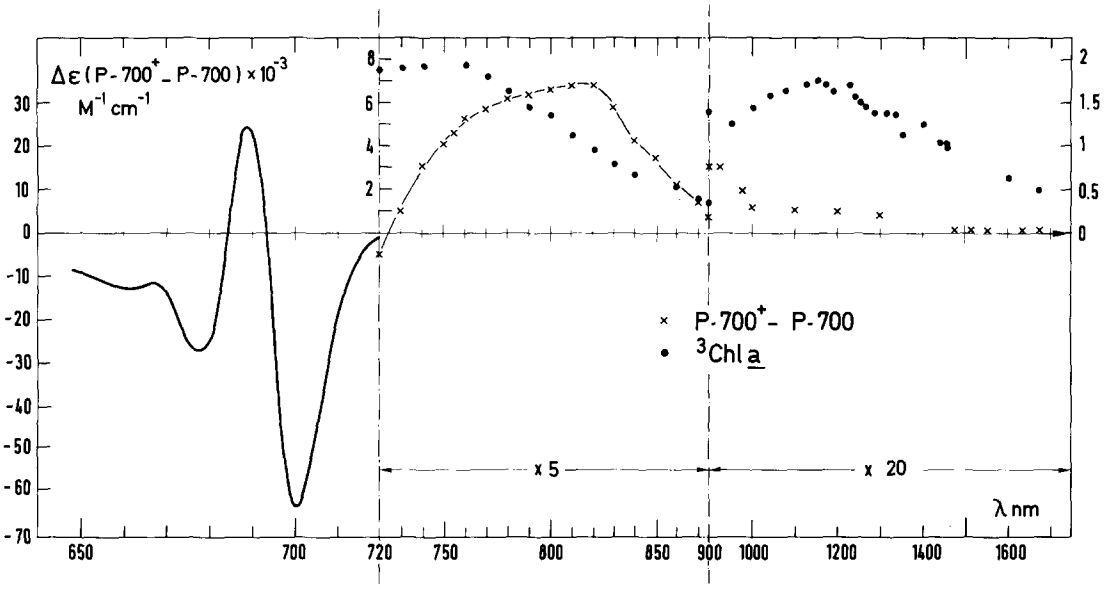

Fig. 7. Difference spectra in the red and near infrared regions. Left curve: difference spectrum, at 10 K, between a cuvette with CP 1 (A_{678} = 0.66) in which *P*-700 is oxidized by a mixture of ferricyanide (1 mM) and ferrocyanide (0.05 mM) and an identical cuvette with *P*-700 reduced by a mixture of ascorbate (1 mM) and DCIP (10 μM). Average of 10 spectra. ΔA 700 = $1.55 \cdot 10^{-2}$. Right curves (720–1700 nm): flash-induced ΔA in PS I particles prepared with Triton X-100 (crosses) (A_{678} = 2.4; addition of 1 mM ascorbate and 10 μM DCIP; average of four to eight experiments; $\Delta A_{820} = 4.8 \cdot 10^{-3}$) and the Chl *a* in vivo (circles; see Ref. 30). At 900 nm the points have been plotted in the two vertical scales.

Discussion

In a previous work [11] the absorption changes induced by flashes in CP 1 particles were interpreted as a charge separation between *P*-700 and a primary acceptor A_1. Another hypothesis, that the transients were due to a triplet state produced by a fast (less than 1 μs) back-reaction between *P*-700$^+$ and A_1^-, was provisionally discarded on the basis that the decay of the state is not accelerated by oxygen. In this discussion we shall consider our present results with reference to the two preceding hypotheses, that we shall name, for simplicity, 'biradical' and 'triplet' hypotheses. In the background of the discussion are the results obtained with photosynthetic bacterial reaction centers in which the primary charge separation (P^+I^-) is followed by a back-reaction (in the nanosecond time range) producing a variable amount of the triplet state ^3P (the yield is influenced by temperature and by magnetic fields) when the normal electron transfer is blocked [35]. In CP 1 particles the iron-sulfur centers are nonfunctional and forward electron transfer to them is thus impossible [36,37]. We shall also suppose that A_1 is a molecule of Chl *a* [13].

The spectrum of flash-induced ΔA obtained with CP 1 particles at 10 K (Fig. 5), as well as the partial spectrum obtained at 21°C (Fig. 2), is clearly different from that due to the oxidation of *P*-700 (Figs. 2 and 7). In the biradical hypothesis, the difference should be due to the reduction of A_1 (Chl → Chl$^-$), the difference spectrum of which is known from the work of Fujita et al. [13]. Basing our analysis on the more complete spectra obtained at 10 K, and assuming that the spectrum of *P*-700$^+$ above 720 nm is the same at 10 K as at 21°C, we are also faced with the problem that the maximum flash-induced ΔA at 820 nm ($A_{678}/\Delta A_{820}$ = 600) is smaller than that expected for *P*-700$^+$ alone (A_{678}/A_{820} = 470), and much smaller than that expected for the sum of *P*-700$^+$ (ϵ = 6500 $M^{-1} \cdot cm^{-1}$) and Chl$^-$ (ϵ = 5200 $M^{-1} \cdot cm^{-1}$) ($A_{678}/\Delta A_{820}$ = 260). In the biradical hypothesis we thus have to suppose that the highest energy flashes do not saturate the formation of the biradical.

From the spectrum of flash-induced ΔA (650–870 nm) (Fig. 5) we subtracted a variable amount of

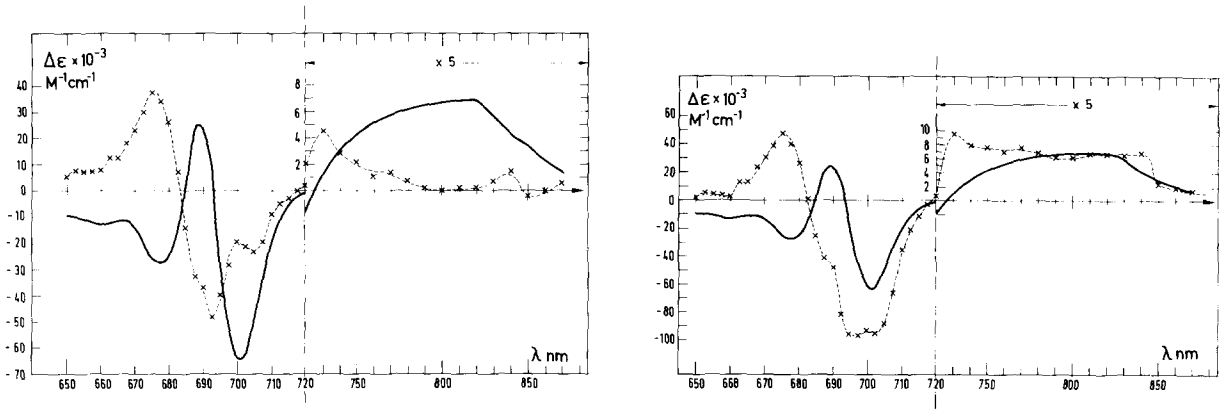

Fig. 8. Difference spectra obtained with PS I subchloroplast particles. Continuous line: difference spectrum due to the chemical oxidation of *P*-700 in CP 1 at 10 K (650–720 nm; redrawn from Fig. 7, assuming $\Delta\epsilon$ = 64 000 at 700 nm) or to the flash oxidation of *P*-700 in Triton X-100 suchloroplast paryicles at 21°C (720–870 nm; redrawn from Fig. 7, assuming $\Delta\epsilon$ = 6500 at 820 nm). Interrupted line: difference spectrum obtained by subtracting the preceding spectrum from the spectrum (Fig. 5) due to the flash excitation of CP 1 particles at 10 K. The (P-700$^+$ – P-700) spectrum has been supposed to contribute 75% (left part) or 40% (right part) of the flash-induced ΔA at 700 nm.

the difference spectrum due to the oxidation of *P*-700 (Fig. 8). The results have been shown for two limiting cases: in a first example (left curves), the remaining ΔA is zero at 800 nm, but the negative peak aroun 690 nm has the $\Delta\epsilon$ expected for the reduction of Chl *a* (approx. 42 000 [13]); for the curves on the right, the remaining ΔA at 800 nm corresponds to 6000 $M^{-1} \cdot cm^{-1}$ (a value of 8600 was reported in Ref. 13), but the contribution of *P*-700$^+$ at 700 nm is only 40%; the $\Delta\epsilon$ of the extra species is very high (95 000 $M^{-1} \cdot cm^{-1}$) and the peak is very broad. The conclusion of our analysis is thus that when a reasonable fit with Chl a^- is adopted around 690 nm, a very poor fit results around 800 nm; alternatively if we suppose an absorption around 800 nm for the acceptor compatible with that of Chl a^-, then an enormously high and broad peak is found around 690–700 nm.

In their study of TSF-I * particles at low redox potential at 5 K, Shuvalov et al. [10] studied a state very similar in kinetic and spectral properties to the one we studied here; they obtained a reasonable fit with the spectrum of *P*-700$^+$ and Chl a^-, but this was without considering the region above 700 nm. They also assumed that the difference spectrum due to the oxidation of *P*-700 is the same at 5 K as at 20°C, which is obviously not the case (compare the low-temperature spectra of Fig. 7 and of Ref. 32 with, e.g., those of Refs. 1 or 11). In our analysis a positive peak at 675 nm is affected little by these assumptions; its extinction coefficient (approx. 40 000 $M^{-1} \cdot cm^{-1}$) is about twice that reported for Chl a^- and is located at 35 nm to the red. A good fit between the flash-induced ΔA in CP 1 and the biradical hypothesis is not observed in the long-wavelength region either (Fig. 9, top). With the assumption that $\Delta\epsilon$ = 16 000 at 770 nm (cf. Refs. 30 and 13 for *P*-700$^+$ and Chl a^-, respectively), the flash-induced ΔA is much larger than that expected around 1000 nm, and the peak expected for Chl a^- around 1500 nm is not observable.

Most of our data, as well as previous observations are explained better by the triplet hypothesis, assuming that the biradical (*P*-700$^+$ ... Chl a^-) decays, in a time somewhat longer than 10 ns, into a triplet state of *P*-700, with a yield approaching 50%. The spectrum of ^{3}Chl *a* is well known in the visible [40–42] and was recently measured in the near infrared [30]. For monomeric Chl *a*, the triplet state presents a broad absorption around 1200 nm, which fits reasonably with the flash-induced ΔA in CP 1 (Fig. 9, bottom). Between 720 and 870 nm, ^{3}Chl *a* also has a broad band, with $\epsilon \approx 7000\ M^{-1} \cdot cm^{-1}$, which is red-

* TSF-I, Triton-fractionated PS I subchloroplast fragments.

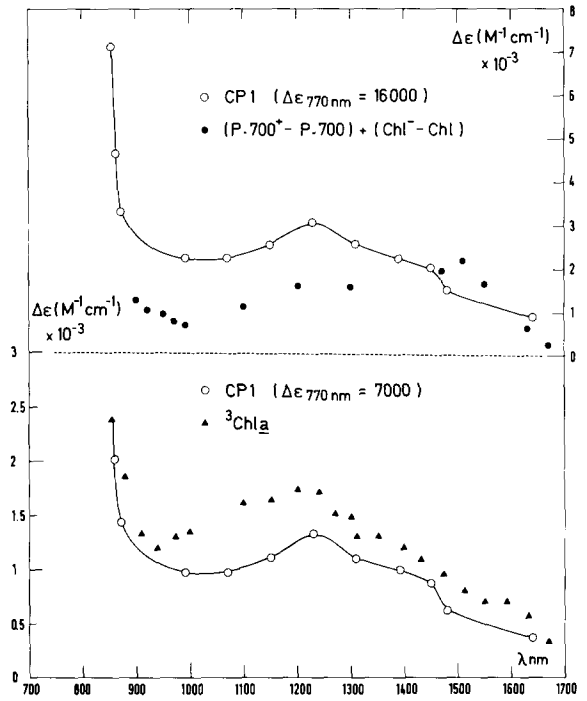

Fig. 9. Difference spectrum from 850 to 1700 nm due to flash excitation of CP 1 particles at 10 K (open circles (from Fig. 5) plotted in assuming a $\Delta\epsilon$ of 16 000 (top trace) or 7000 $M^{-1} \cdot cm^{-1}$ (bottom trace) at 770 nm, and compared to the difference spectra due to the oxidation of *P*-700 and the reduction of Chl *a* (obtained from Fig. 7 and Ref. 13, respectively) (full dots) and to the formation of the triplet state of Chl *a* at 21°C (from Ref. 30, triangles).

shifted in an aggregate [30]. The spectrum obtained with CP 1 (Figs. 2 and 6) could well be due to the triplet state of *P*-700. In the red part of the spectrum, it can be expected that the formation of a triplet state will induce a bleaching of the *P*-700 band and the appearance of weak positive bands, in good agreement with the spectrum of Fig. 5. The large positive (688 nm) and negative (677 nm) peaks observed upon oxidation of *P*-700 (Fig. 7) may be due to an electrochromic shift, as discussed by Schaffernicht and Junge [38], and they are not expected to appear in the triplet spectrum. We also measured the spectrum of flash-induced ΔA between 400 and 650 nm (data not shown), which resembles that obtained by Shuvalov et al. [10]. However, the spectra of Chl a^+ (or *P*-700$^+$), of Chl a^- and of ^{3}Chl *a* are rather similar in the visible [1,13,39,42] and we do not think that this spectral region permits one to choose between the two hypotheses. The spectrum obtained by Baltimore and Malkin [17] in heat-treated PS I fragments could well be due to a triplet state.

The triplet hypothesis also allows one to account for the small size of the ΔA obtained with flashes of maximum energy, and for the larger ΔA obtained at 10 K with a dye laser flash. The longer duration of the dye laser flash (1 μs) may permit several cyclings of the reaction centers, compared to the ruby laser (2 or 10 ns), permitting a single hit. With the dye laser at maximum energy we obtained a ratio $A_{678}/\Delta A_{700} = 50$, which is nearly that expected for the bleaching of one *P*-700 per 45 chlorophylls. We have to suppose that the biradical has a lifetime between 10 ns and 1 μs, in better agreement with the biradical lifetime observed or inferred in bacteria (10 ns, see Ref. 43) or in PS II (approx. 4 ns [44]) than the previously assumed millisecond lifetime. The decay of the biradical into a triplet would also explain the result of Shuvalov et al. [16] on a signal decaying between the nanosecond and the microsecond time domain. From our data we can estimate that the yield of triplet formation, compared to the yield of biradical formation, with a 2 ns ruby laser pulse, is about 70% at 10 K and 50% at 21°C.

The triplet hypothesis permits one to understand the decrease in the flash-induced ΔA under the influence of a magnetic field (Fig. 4). A similar effect was reported in bacterial reaction centers and was interpreted in terms of singlet and triplet states of the biradical [45,46]. The effect here is small and seems to require a rather large field. These results are still preliminary, but they are in good agreement with a report by Voznyak et al. [47] on the stimulation by a magnetic field of the fluorescence in PS I at a low redox potential.

A spin-polarized triplet state was recently observed by EPR in PS I, when A_2 (or X) is absent or reduced [23,24]. We also observed this state in CP 1 particles at 5 K (Sétif, P., Quaegebeur, J.P. and Mathis, P., unpublished data). These was no kinetic information obtained on this triplet, but our results and their interpretation suggest that the same species (possibly the triplet state of *P*-700) is responsible for the flash-induced ΔA and for the EPR data (see the discussion by Rutherford and Mullet [24]). It should be noted,

however, that the EPR kinetic data of Shuvalov et al. [10] rather favor the biradical hypothesis.

The triplet hypothesis, which we favor on the basis of the preceding discussion, does not lead immediately to a better understanding of the kinetics of decay of the flash-induced ΔA. A plot of a first-order analysis of the kinetics [11] showed a striking similarity with the decay of the triplet state (P^R) in bacterial reaction centers and this similarity reinforces our interpretation. However, we have no good explanation for the multiphasic character of the decay. In our hypothesis, the triplet state ^3P-700 seems to be screened from oxygen, which does not accelerate the decay (Fig. 1); this screening may be a functional prerequisite, in order to avoid scavenging of the low-potential reductants by oxygen. In other photosynthetic structures from higher plants, the triplet state of Chl *a* is also efficiently quenched by carotenoids [48]. The CP 1 particles contain about six molecules of β-carotene per *P*-700 and we thus have to assume that none of them is located close enough to *P*-700 to quench its triplet state efficiently. The situation would thus be different from that encountered in bacterial reaction centers [49]. We measured the formation of the carotenoid triplet state in CP 1 at 77 and 10 K and found no influence of the redox state of *P*-700 on the triplet formation (data not shown). The triplet state with unusual spin polarization was observed by EPR in carotenoid-containing structures, at about 5 K [23,24]; in higher plants, the triplet-triplet transfer from ^{3}Chl *a* to carotenoids takes place at a very high rate at 5 K, and there is no energetic reason why low temperature should inhibit the rate of transfer from ^3P-700 to carotenoids. The EPR experiments are thus consistent with our results and with our interpretation of a spatial separation of *P*-700 and carotenoids.

In conclusion, the triplet hypothesis for the flash-induced absorption changes with CP 1 particles provides the best interpretation of the experimental data. The provisional rejection of the biradical hypothesis has been based on the assumption that the acceptor A_1 is a Chl *a* molecule, and this point remains to be firmly established. A conclusion analogous to ours was attained recently by Sonneveld et al. [50] who observed a luminescence in PS I which they attribute to the recombination between P-700$^+$ and A_1^-. The decay time (100–200 ns) is perfectly compatible with our data and their interpretation is also consistent with ours.

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