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Picosecond absorbance difference spectroscopy on the primary reactions and the antenna-excited states in Photosystem I particles

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Absorbance difference spectra at various delay times, and kinetics of absorbance changes induced by a 35 ps excitation pulse at 532 nm, were measured of relatively intact Photosystem I particles from spinach containing about 70 chlorophyll *a* molecules per photoactive primary electron donor P-700. The excitation pulse produced absorbance changes due to the formation of singlet- and triplet-excited antenna chlorophyll *a*, and, in the case of active reaction centers, also those due to the oxidation of P-700. The formation of excited chlorophyll *a* was accompanied by the bleaching of the Q_y ground state absorption band and by the appearance of a rather flat absorption increase in the region 550–900 nm. The lifetime of singlet-excited chlorophyll *a* was found to be 40 ± 5 ps. When the iron–sulfur centers were prereduced (photo)chemically, the formation of a radical pair consisting of P-700$^+$ and a chlorophyllous anion was observed. The absorbance–difference spectrum calculated for the reduction of the acceptor was similar to that measured earlier (Shuvalov, V.A., Klevanik, A.V., Sharkov, A.V., Kryukov, P.G. and Ke, B. (1979) FEBS Lett. 107, 313–316), and indicated that the acceptor is a chlorophyll *a* species absorbing around 693 nm. The lifetime of the radical pair was at least 25 ns. If, however, the acceptor complex was in the oxidized state before the flash, only the oxidation of P-700 was observed. No direct evidence was obtained for the reduction of the chlorophyllous acceptor, implying that if such an anion is formed, it must be reoxidized within 50 ps.

Introduction

After the absorption of a photon by the antenna of Photosystem I and the subsequent transfer of the excitation from the antenna chlorophyll to the reaction center a charge separation takes place between the primary electron donor, P-700, and an acceptor complex which ultimately reduces soluble ferredoxin. EPR measurements under progressively decreasing redox potentials have provided evidence that three different iron–sulfur centers, F_X, F_B and F_A, act as sequential secondary electron acceptors in the complex (see Ref. 1 for a review). The participation of iron–sulfur acceptors is further evidenced by optical spectroscopy [2,3]. Under conditions where the iron-sulfur centers were reduced or removed, formation of the spin-polarized triplet state of P-700 was still observed [4], indicating the existence of an electron acceptor, designated 'A_1', preceding F_X. Both

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Abbreviations: P-700, primary electron donor chlorophyll 700; F_X, F_B, F_A, iron–sulfur centers; A_0, A_1, 'A_1', C, primary electron acceptors; Chl, chlorophyll; PMS, *N*-methylphenazonium methosulfate; PS, Photosystem; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

optical and ENDOR data have suggested that 'A_1' is a chlorophyll *a* anion [5,6]. A careful analysis of the EPR spectra accompanying the progressive reduction of 'A_1' at cryogenic temperatures has shown that 'A_1' in fact consists of two components, A_1 and an earlier acceptor A_0 [7,8]. The characteristics of the A_0^- EPR signal indicate that A_0 is a chlorophyll monomer [7], which conclusion is supported by recent optical measurements [9]. It was demonstrated in this work that accumulation of A_0^- induced a bleaching around 670 nm, similar to the absorbance changes observed earlier by Swarthoff et al. [5], upon reduction of 'A_1'. The nature of A_1, the other constituent of 'A_1', is still unclear. No absorbance changes were detected in the visible region upon accumulation of A_1^- [9]. Q-band EPR spectra are compatible with the identification of A_1^- as a semiquinone [10].

Direct picosecond absorption measurements on small PS I particles led Shuvalov et al. [11] to the conclusion that 'A_1' is a chlorophyll dimer absorbing around 694 nm. Their 'A_1^-' spectrum is clearly distinct from the A_0^- spectrum induced by continuous illumination. No explanation for the apparent discrepancy has been proposed yet. The data on the acceptor chain of PS I are reviewed in Ref. 1.

In this paper we report a picosecond absorption study on relative intact PS I isolated from spinach. The data are consistent with the idea that the primary acceptor is a chlorophyll *a* species absorbing around 693 nm.

Materials and Methods

Spinach PS I particles were prepared from the supernatant obtained after the Triton X-100 incubation of chloroplasts according to Dekker et al. [12]. This supernatant contained 5% (w/v) Triton X-100. Another 3.3% (w/v) Triton X-100 was added to this supernatant and the preparation was incubated for 45 min at 4°C. Then the supernatant was loaded (7 ml per tube) on a 20–45% (w/v) sucrose linear density gradient containing 20 mM Tricine (pH = 7.4), and 0.1% (w/v) Triton X-100. The centrifugation step was carried out for 16 h in a Beckman 70 Ti rotor at $220\,000 \times g$. After centrifugation the gradient showed three chlorophyll-containing bands of which the lower one contained PS I. The particles were characterized by a Chl *a*/Chl *b* ratio of more than 8 and contained approximately 70 Chl *a* molecules per reaction center as measured by the oxidation of P-700 under continuous light in the presence of 1 mM ascorbate.

Absorbance changes under continuous illumination were measured as in Ref. 13. Suitable interference and absorbance filters were used to select the actinic illumination and to protect the photomultiplier from stray actinic light. Picosecond absorbance difference measurements were performed by means of the apparatus described in Ref. 14. A 35 ps mode-locked ND/YAG laser was used as a light source. Part of the 1064 nm radiation was frequency-doubled to 532 nm to serve as an excitation pulse (maximum energy density, about 2.5 mJ/cm^2). The remaining 1064 nm radiation was focused in a water cell to generate a 'white' continuum. The 35 ps probe pulse was obtained from this continuum by means of a monochromator placed before the sample. All measurements were performed at room temperature.

Results

In order to distinguish the absorbance changes due to the charge separation in the reaction centers from those due to formation and decay of antenna excited states, we have studied the latter separately in PS I particles in which the reaction centers were kept in the closed state P-700$^+$ by means of ferricyanide and continuous background illumination.

Oxidized reaction centers

Fig. 1 shows the absorbance difference spectra in the region 550–900 nm of oxidized PS I particles at 40 ps (solid circles) and 200 ps (open circles) after the 35 ps excitation pulse at 532 nm. At 40 ps a bleaching around 685 nm is observed, flanked by increases in absorption between 550 and 660 nm and above 740 nm. We ascribe the bleaching to the disappearance of antenna chlorophyll *a* (Chl *a*) ground states due to excitation of the molecules and the increases in absorption to the absorbance of singlet excited Chl *a* (Chl* *a*). The spectrum is very similar to that measured

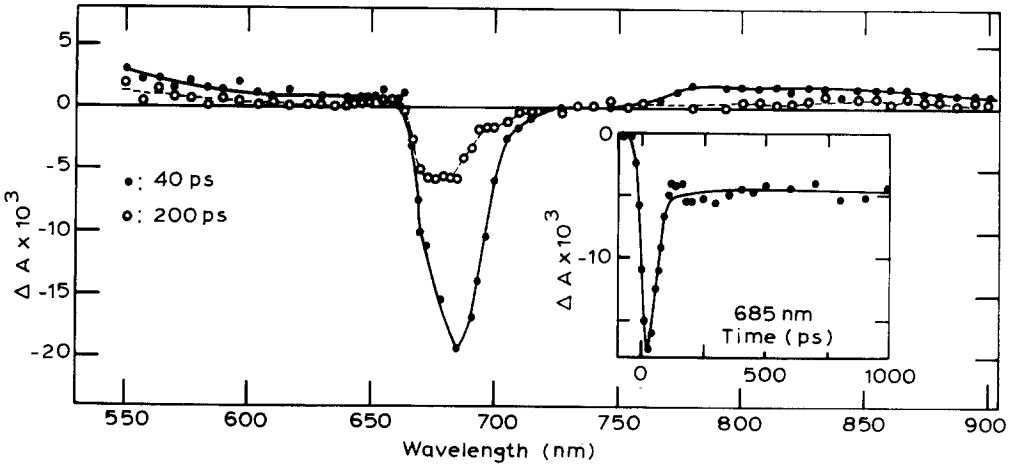

Fig. 1. Absorbance difference spectra of PS I particles from spinach at 40 ps (●) and 200 ps (○) after the 532 nm excitation pulse, in the presence of 3 mM ferricyanide and under continuous background illumination. The inset shows the kinetics at 685 nm. The exctinction of the sample was 1.4 at 675 nm in a 2 mm cell. The excitation energy density was about 0.9 mJ/cm^2.

upon excitation of Chl *a* in PS II particles from spinach [15]. The kinetics of the absorbance changes at 685 nm are shown in the inset of Fig. 1. The development and the first 100 ps of the decay of the bleaching represent the formation and disappearance of Chl* *a*. The kinetics in this time interval were deconvoluted using the Gaussian profile of the 35 ps excitation and probe pulses. The best fit was obtained using a 40 ps lifetime of Chl* *a*. From about 100 ps after the flash onwards a constant bleaching remains that does not decay during the first 4 ns (only the first nanosecond is shown in Fig. 1). This long-lived component probably reflects the formation of a triplet state of Chl *a* (Chl^T *a*). The spectrum of the long-lived component (open circles in Fig. 1) appears to be somewhat blueshifted with respect to the 40 ps Chl* *a* spectrum (solid circles). This indicates that Chl^T *a* and Chl* *a* are distributed differently over the various spectral forms of antenna chlorophyll.

The saturation behavior of the bleaching at 685 nm measured at 40 ps after the flash is shown in Fig. 2. The amplitude of the bleaching is nearly proportional to the flash excitation density. In several other photosynthetic preparations singlet–singlet annihilation has been observed to decrease both the fluorescence lifetime and the fluorescence yield when intense picosecond excitation pulses are used (see Ref. 16 and references therein). Recently we showed that also the amplitudes of the absorbance changes due to formation of (bacterio)chlorophyll singlet excited states are diminished when the lifetime of the excited states approaches or becomes less than the duration of the pulses [14]. The near-proportionality in Fig. 2 thus implies that the short lifetime of Chl* *a* in PS I is not much affected by singlet–singlet annihilation.

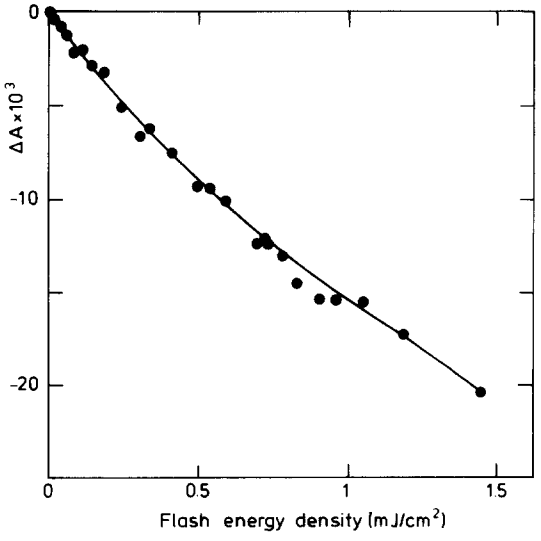

Fig. 2. Dependence of the bleaching at 685 nm at 40 ps after the flash upon the flash-energy density. Further conditions as for Fig. 1.

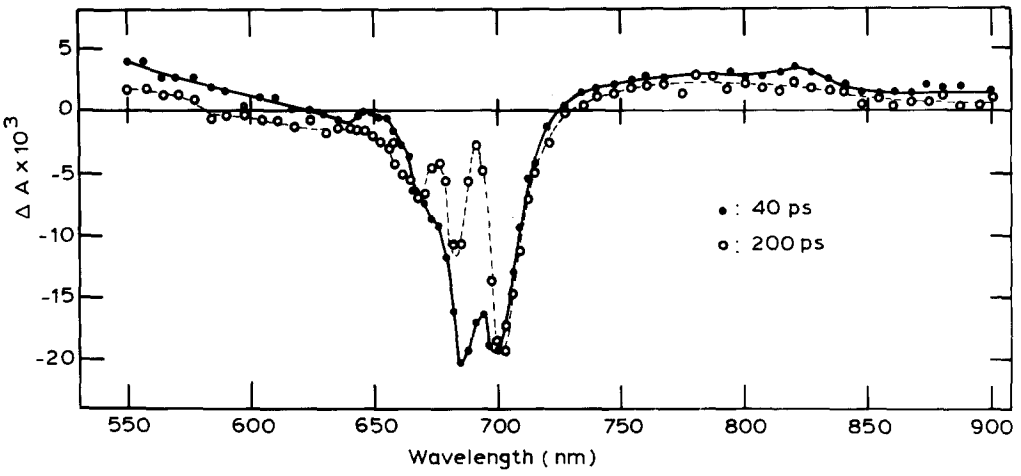

Fig. 3. Absorbance difference spectra of PS I particles at 40 ps (●) and 200 ps (○) in the presence of 20 μM PMS and 5 mM ascorbate. Further conditions as for Fig. 1.

Open reaction centers

The measurements described in this section were performed in the presence of PMS and ascorbate to ensure complete reduction of P-700$^+$ between successive flashes.

The spectra of the absorbance changes in the region 550–900 nm at 40 ps (solid circles) and 200 ps (open circles) after the flash are shown in Fig. 3. The 200 ps spectrum is characterized by a bleaching in the region 590–730 nm, with minima around 668, 683 and 701 nm, and by a broad increase above 730 nm. This spectrum resembles the P-700$^+$ spectrum first measured by Kok [17],

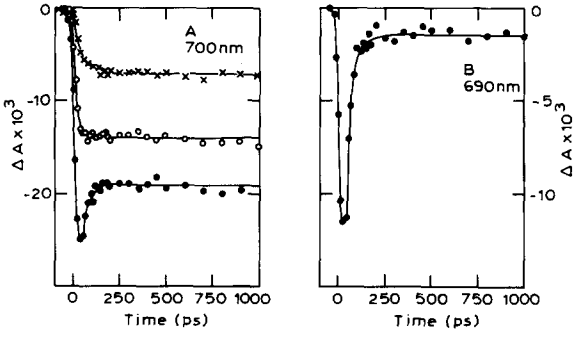

Fig. 4. Kinetics of absorbance changes at 700 nm (A) and 690 nm (B). (A) The excitation energy density was 0.13 mJ/cm^2 (×), 0.41 mJ/cm^2 (○) and 1.5 mJ/cm^2 (●). (B) The excitation energy density was 0.41 mJ/cm^2. Further conditions as for Fig. 3.

and it will be demonstrated in the Discussion that our 200 ps spectrum consists of contributions due to the formation of P-700$^+$ and of Chl^T *a*. At 40 ps (Fig. 3, solid circles) the spectrum shows bleaching bands around 685 and 699 nm and broad increases in absorption between 550 and 620 nm, and above 725 nm. The bleaching around 699 nm is predominantly due to the formation of P-700$^+$, whereas that at 685 nm is for the larger part caused by the excitation of antenna Chl *a*. It will be shown in the Discussion that the 40 ps spectrum can be approximated by a sum of the P-700$^+$ and Chl* *a* spectra, with little room left for significant contributions from a chlorophyllous acceptor.

The kinetics of the absorbance changes at 700 nm and 690 nm are given in Figs. 4A and B, respectively. At 690 nm a short-lived bleaching is observed, the formation and decay of which are largely determined by the temporal profiles of the excitation and probe pulses. We ascribe this bleaching predominantly to the formation of Chl* *a* (see Fig. 1, solid circles and inset). The small bleaching remaining after the decay of Chl* *a* is due to the presence of P-700$^+$ (cf. Fig. 3). The shape of the kinetics at 690 nm was almost independent of the excitation energy density (not shown). This was different for the kinetics at 700 nm (Fig. 4A). At a rather high excitation density (solid circles) the constant bleaching from about

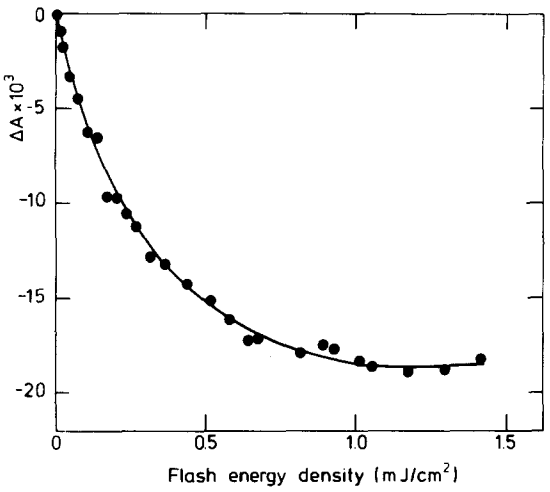

Fig. 5. Saturation behavior for the bleaching at 700 nm at 500 ps after the flash. Further conditions as for Fig. 3.

100 ps onwards is due to the formation of P-700$^+$. The short-lived initial bleaching can be ascribed to the excitation of Chl *a*. At lower energy densities the absorbance changes related to antenna excitation are less pronounced. At an intermediate energy density (open circles) the decay of Chl* *a* is counteracted by the formation of P-700$^+$, giving rise to no net short-lived decrease in absorbance. At an even lower energy density (crosses) an approx. 50 ps risetime for the bleaching can be observed which is largely determined by the risetime for the formation of P-700$^+$, with only minor distortions due to the formation and decay of Chl* *a*. Presumably this risetime represents the time needed for an excitation to reach the trap.

The saturation for the formation of P-700$^+$, monitored at 700 nm at 500 ps after the flash is shown in Fig. 5. The bleaching is observed to saturate at a value of about $18 \cdot 10^{-3}$ units of absorbance, which value equals that found under continuous illumination (not shown). It thus follows that even with such a short flash about all the reaction centers can be closed.

Reaction centers at low potential

The measurements described in this section were performed in the presence of dithionite at a pH value of 9.5 to reduce F_A and F_B chemically. Furthermore PMS was added and continuous background illumination was given to reduce F_X photochemically. The absorbance difference spectra monitored at 40 ps (solid circles) and 500 ps (open circles) after the flash are given in Fig. 6.

The 500 ps spectrum is characterized by a bleaching in the region 600–715 nm with minima around 670 and 697 nm, and a broad featureless increase in absorbance beyond 715 nm. It bears some resemblance to the 200 ps spectrum measured with non-reduced iron sulfur centers (Fig. 3, open circles). Apparently, however, the main

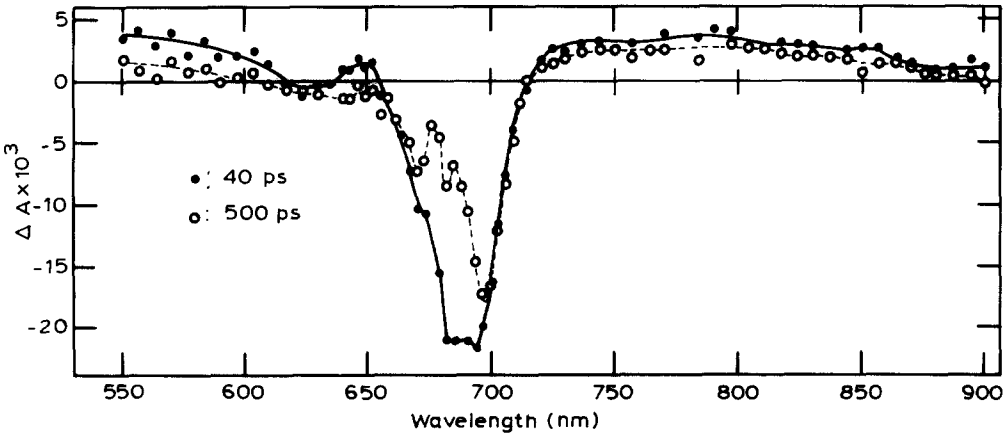

Fig. 6. Absorbance difference spectra of PS I particles at 40 ps (●) and 500 ps (○) at a pH value of 9.5 in the presence of 10 mM dithionite, 20 μM PMS and 5 mM ascorbate and under continuous background illumination. Glucose, catalase and glucose oxidase were added to maintain anaerobic conditions. The extinction of the sample was 1.4 at 675 nm in a 2 mm cell. The excitation energy density was about 1.1 mJ/cm^2.

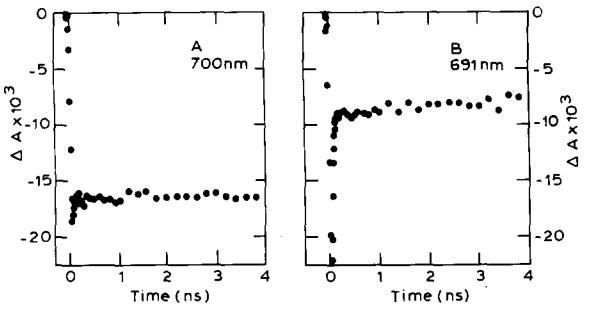

Fig. 7. Kinetics of absorbance changes at 700 nm (A) and 691 nm (B). Further conditions as for Fig. 6.

bleaching has shifted from 701 to 698 nm, and the steep increase around 690 nm has disappeared. Also note that the near-infrared isobestic point has shifted from 727 to 715 nm. It will be shown in the Discussion that the 500 ps spectrum, after being corrected for absorbance changes due to the formation of Chl^T *a*, is quite similar to that observed earlier by Shuvalov et al., which was attributed to the formation of a radical pair involving both $P\text{-}700^+$ and a chlorophyll anion [18]. The spectrum measured after 40 ps (Fig. 6, solid circles) is rather similar to that measured under non-reducing conditions and can likewise be decomposed into contributions from excited states in the antenna and reaction center processes (see below).

Fig. 7 presents the kinetics of the absorbance changes at 700 nm (A) and 691 nm (B). At both wavelengths a short-lived absorbance decrease is present, due to the formation and decay of Chl* *a*, and a residual bleaching that does not decay on a time scale of 4 ns, implying that the state of the reaction center characterized by the spectrum of Fig. 6 (open circles) should have a lifetime of at least 25 ns. The nature of this state will be discussed below.

Discussion

Excitation of Photosystem I particles of spinach by means of a 35 ps, 532 nm pulse produced absorbance changes due to formation and decay of antenna Chl *a* excited states, and, in the case of active reaction centers, also those due to the charge separation.

Formation of antenna Chl* *a* is characterized by a bleaching around 685 nm, flanked by broad featureless increases in absorbance, as observed earlier in Photosystem II particles from spinach [15]. The shift of the position of maximal bleaching (685 nm) with respect to the absorption maximum of the preparation (675 nm) probably results from a thermal equilibrium of excitations on the spectrally inhomogeneous antenna of PS I, possibly combined with a shift in absorption of Chl *a* molecules adjacent to excited molecules. A similar explanation was given for PS II [15]. After the decay of Chl* *a* a longer lived state, probably Chl^Ta, can be observed, the formation of which induces an absorbance difference spectrum that is somewhat different from the one due to Chl* *a*. Apparently, the triplet states are distributed over the various spectral forms of antenna chlorophyll somewhat differently from the singlet excitations.

From the kinetics of the decay of Chl* *a* it was concluded that the lifetime of Chl* *a* is 40 ± 5 ps. Since this decay time was not determined by singlet–singlet annihilation, it must be the mono-excitation lifetime of Chl* *a* in this preparation. It is somewhat shorter then the reported fluorescence lifetimes of 50–100 ps (see Ref. 16 and references therein). Since the lifetime of Chl* *a* in the presence of $P\text{-}700^+$ (Fig. 1, inset) is about the same as the low-intensity risetime of $P\text{-}700^+$ formation when the reaction centers are initially open (Fig. 4A, crosses), it follows that $P\text{-}700^+$ traps excitations about as efficiently as the active reaction centers.

When the reaction centers are in the active state, and the iron–sulfur centers are not reduced, the oxidation of P-700 can be observed (Fig. 3, open circles) with a distortion due to the presence of Chl^Ta. In Fig. 8A the $P\text{-}700^+$ spectrum of the preparation induced by continuous illumination (dashed curve) is compared with that induced by the ps excitation pulse, which was corrected for the presence of Chl^Ta. The spectra are observed to be reasonably similar, which indicates that at 200 ps after the flash no reduced chlorophyllous acceptor is present.

Some years ago Shuvalov et al. obtained evidence from picosecond absorption spectroscopy that the primary acceptor is a chlorophyll *a* species, the reduction of which induced a bleach-

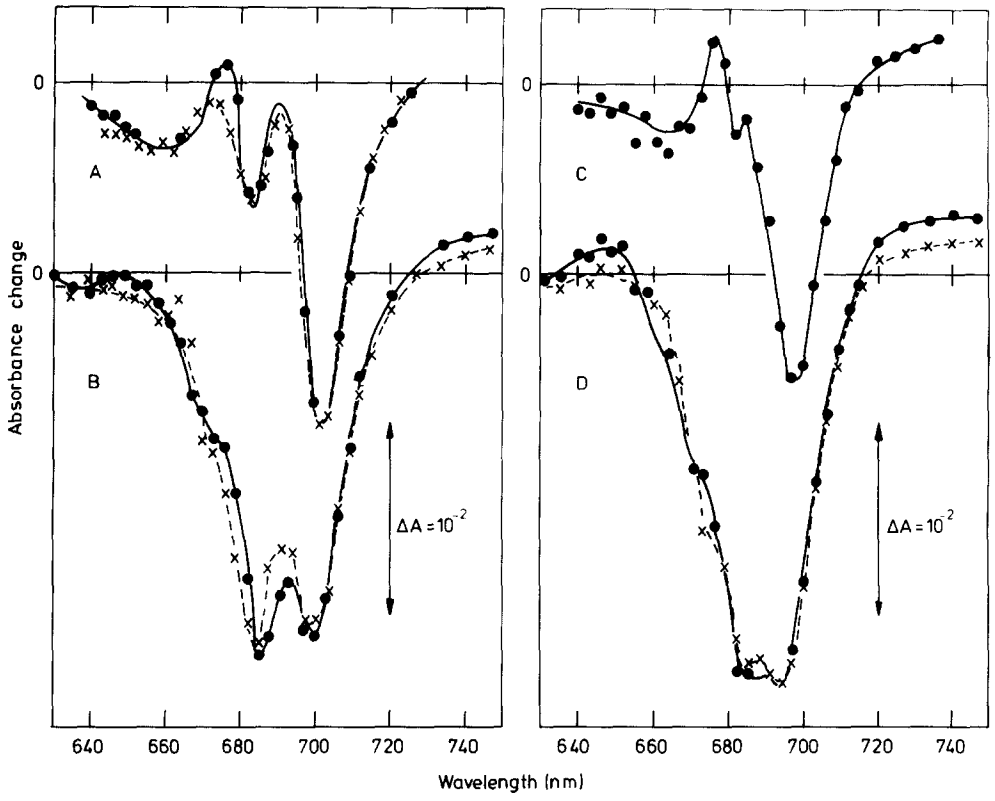

Fig. 8. (A) Comparison of the picosecond pulse-induced spectrum of P-700$^+$ formation (●) with the spectrum under continuous illumination (×). (B) The 40 ps spectrum under non-reducing conditions (●) simulated by a sum of the P-700$^+$ spectrum and the antenna Chl* *a* spectrum. The best fit (×) was obtained assuming the presence of 78% P-700$^+$ at 40 ps. (C) Spectrum at 500 ps under reducing conditions and corrected for absorbance changes in the antenna. (D) The 40 ps spectrum measured under reducing conditions (●) simulated by a sum of the 500 ps spectrum and the antenna Chl* *a* spectrum. The best fit (×) was obtained by assuming the presence of 72% of the long-lived reaction center component. See text for details.

ing around 694 nm and which transfers the electron to the next acceptor with a time constant of about 200 ps [11]. The kinetics at 690 and 700 nm (Fig. 4) exclude the presence of such a reduced acceptor with a lifetime of 200 ps in our preparation. The spectrum measured at 40 ps (Fig. 8B, solid circles) can be simulated reasonably well by a linear combination of the P-700$^+$ spectrum (Fig. 8A, dashed curve) and the Chl* *a* spectrum (Fig. 1, solid circles), although some small differences in the region 670–700 nm remain. These results imply that, if the acceptor observed by Shuvalov et al. [11] is photoreduced in our preparation as well, it must be reoxidized within 50 ps. Our data do not support the suggestion by Swarthoff et al. [5] and Mansfield and Evans [9] that a chlorophyll monomer absorbing at 670 nm acts as an electron acceptor, since our spectra can be reasonably well matched by P-700$^+$ and Chl* *a* alone, and since the absorbance increase around 690 nm accompanying the reduction of the 670 nm chlorophyll [5,9] did not show up in our kinetics. If such a monomer would be present as an electron acceptor, and if a differential extinction coefficient of 60 $mM^{-1} \cdot cm^{-1}$ at 670 nm is assumed for its reduction [5], then the lifetime of the anion must again be shorter than 50 ps.

When the iron–sulfur centers F_A, F_B and F_X are prereduced (photo)chemically, flash excitation still generates absorbance changes which, on the basis of their spectra and kinetics, are attributable to reaction center processes. When the spectrum, measured after 500 ps under these conditions, is corrected for the presence of Chl^T*a*, the spectrum

of Fig. 8C is obtained. This spectrum, which shows a bleaching around 698 nm, and a small increase in absorption around 677 nm, is rather similar to that obtained previously by Shuvalov et al. [11,18] at 150 ps after the flash under non-reducing conditions, and which was ascribed to the oxidation of P-700 and the reduction of a chlorophyll *a* species absorbing at 694 nm. Using the same hypothesis, we subtracted the absorbance changes due to the oxidation of P-700 (Fig. 8A, solid circles) from the spectrum of the radical pair (Fig. 8C), after normalization to the same absorbance of the sample. The resulting spectrum is shown in Fig. 9 and is attributed to the reduction of the Chl *a* acceptor. The spectrum shows a narrow bleaching band around 693 nm, and increases in absorption in the regions 650–685 nm and 702–750 nm, with a tail extending to 900 nm, and is very similar to that found by Shuvalov et al. [11] in PS I particles and by Fujita et al. [19] for the reduction of Chl *a* in vitro, apart from a red shift of our spectrum by about 25 nm. For the differential extinction coefficient at 693 nm upon reduction of the Chl *a* acceptor a value of 45 $mM^{-1} \cdot cm^{-1}$ is obtained taking a value of 64 $mM^{-1} \cdot cm^{-1}$ for the oxidation of P-700 at 700 nm [20]. The calculated extinction coefficient is very close to that of 46 $mM^{-1} \cdot cm^{-1}$ obtained earlier [11].

The spectrum measured at 40 ps after the flash under reducing conditions (Fig. 8D, solid circles) can be fitted well by a combination of the radical pair spectrum and the Chl* *a* spectrum (see Fig. 8D, dashed curve), which suggests that the Chl *a* acceptor is reduced concomitantly with the oxidation of P-700.

No decay of the radical pair under reducing conditions can be observed during the first 4 ns (Fig. 7), implying that the lifetime of the pair is at least 25 ns. Recently, Sétif et al. [21] showed that in PS I particles from which the iron–sulfur acceptors were removed the flash-induced absorbance increase at 815 nm decayed biphasically with time-constants of 30–50 ns and of about 6 μs. These workers ascribed the fast phase to the recombination of the radical pair to the triplet state of P-700, $P\text{-}700^T$, and the slower phase in agreement with earlier work [22], to the decay of the triplet state.

Summarising, our data show that Chl *a* acts as an electron acceptor under conditions at which the iron-sulfur centers are reduced. As is suggested by the high yield and rapid formation of the radical pair under reducing conditions, Chl *a* probably is reduced during normal electron transport as well. It thus appears that the primary steps in the electron transfer in PS I can be schematically indicated as follows:

$$\text{Chl}^* \; a \rightarrow \text{P-700}^* \; C \; F_X \rightarrow \text{P-700}^+ \; C^- \; F_X \overset{< 50 \text{ ps}}{\rightarrow} \text{P-700}^+ \; C \; F_X^-$$

in which C is a Chl *a* species, with a Q_y band at 693 nm.

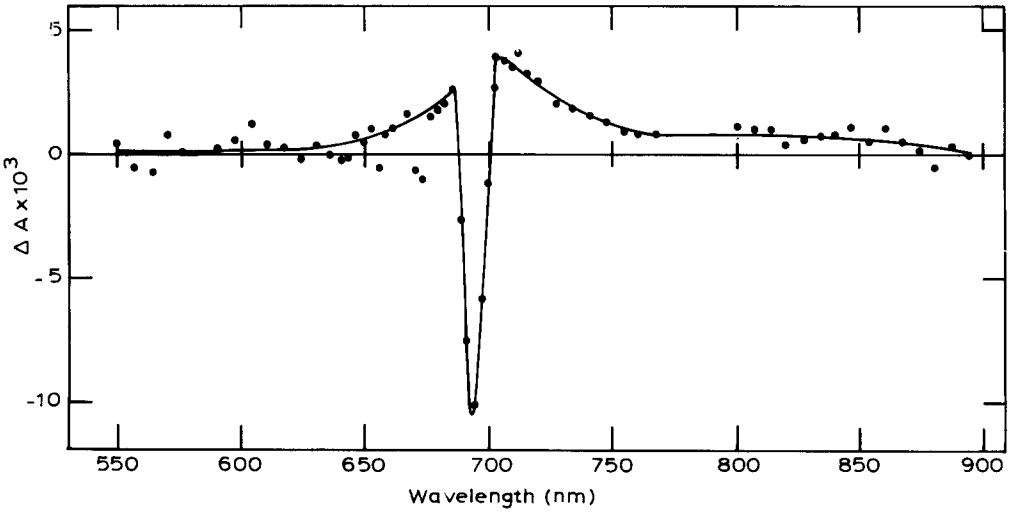

Fig. 9. Absorbance difference spectrum calculated for the reduction of the primary electron acceptor. See text for details.

Our data do not show the 200 ps step for the electron transfer from C^- to F_X, reported earlier by Shuvalov et al. [11]. Presumably this step occurs faster than 50 ps in our more native PS I particles. We have found no evidence for the participation in the acceptor chain of A_0 and A_1 which were reported to act as electron carriers between P-700 and F_X [7,9]. The reduction of these species was observed only under continuous illumination at low redox potential. Under these conditions excited states, especially triplet states of antenna pigments may react with dithionite in a way similar to model system reactions [23]. Therefore the results obtained by means of ps flash spectroscopy might be more valid.

A comparison of the picosecond absorption data on PS I with those on the photosystem of green sulfur bacteria shows that the rate of stabilization of the primary radical pair by secondary electron transfer is much higher in PS I than in the green sulfur bacteria, in which a bacteriochlorophyll *c*-like pigment transfers the electron in 500–600 ps to an iron–sulfur center [24,25].

Acknowledgements

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