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Primary-charge separation and excitation of chlorophyll *a* in Photosystem II particles from spinach as studied by picosecond absorbance-difference spectroscopy

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Photosystem II particles from spinach, containing about 80 chlorophyll *a* molecules per reaction center, have been investigated with picosecond absorbance-difference spectroscopy. The 35 ps excitation pulse at 532 nm produced absorbance changes due to the formation of singlet excited antenna chlorophyll *a* and to the primary-charge separation in the reaction centers. The appearance of excited chlorophyll *a* was accompanied by the bleaching of the ground state Q_y absorption band and by the formation of a rather flat absorption band in the region 550–900 nm. At high flash intensity its average lifetime was found to be several tens of picoseconds. In the reaction center charge separation was observed between the primary electron donor P-680 and pheophytin *a*. Reduction of pheophytin *a* was accompanied by an absorbance increase between 640 and 675 nm and a bleaching around 685 nm. Electron transfer to a secondary acceptor occurred with a time constant of 250–300 ps. If this secondary acceptor was reduced chemically, the primary radical pair decayed by charge recombination in about 2 ns.

Introduction

The most primary products of the photochemical reaction in system II of photosynthesis (PS II) that have actually been measured under physiological conditions are the oxidized reaction center chlorophyll *a*, P-680$^+$, and the plastosemiquinone anion Q_A^- (see Ref. 1 for a review on electron transfer in PS II). In photosynthetic purple bacteria a bacteriopheophytin molecule is known to act as an intermediary electron acceptor between the primary donor and Q, a ubiquinone molecule [2]. By analogy, when illumination of PS II at low redox potential was found to cause the reduction of a pheophytin *a* (Phe *a*) molecule, Klimov et al. [3] postulated that this molecule acts as an intermediary acceptor between P-680 and Q_A. Shuvalov et al. demonstrated the presence of flash-induced absorbance changes in the region 400–600 nm after reduction of Q_A with dithionite [4]. These changes, which had a decay time of about 4 ns, proved to be spectrally similar to the sum of the differential absorption spectra for oxidation of P-680 and reduction of Phe *a*. This finding strongly supported Klimov's proposal, but a transient reduction of Phe *a* during normal PS II turnover remained to be demonstrated (see Ref. 5 for a review on the role of Phe *a* as a primary electron

Abbreviations: Chl, chlorophyll; P-680, primary electron donor chlorophyll; Phe *a*, pheophytin *a*; I, primary electron acceptor; Q_A, plastoquinone.

acceptor in PS II). On the basis of chlorophyll fluorescence lifetime measurements the reoxidation of Phe a^- by Q_A is expected to occur in a few hundred picoseconds [6]. No picosecond absorbance difference measurements on the primary charge separation and consecutive electron transport in reaction centers of PS II have been reported yet. The large number of antenna chlorophyll *a* molecules absorbing at the same wavelength as P-680 and Phe *a* seriously complicates such measurements.

Recently, we reported the application of sensitive picosecond absorbance difference spectroscopy to membrane preparations of the green sulfur bacterium *Prosthecochloris aestuarii,* which still contain about 80 bacteriochlorophyll molecules per reaction center [7]. In this paper we report results obtained with the same apparatus on spinach PS II particles with a chlorophyll *a*/reaction center ratio of about 80. The formation of singlet-excited chlorophyll *a* and of a charge separation in the reaction centers was observed. Our results confirm the suggestion that pheophytin *a* acts as a primary electron acceptor in PS II. Reoxidation of Phe a^- occurred with a time constant of 250–300 ps. When Q_A was chemically reduced before excitation, the lifetime of the radical pair P-680$^+$Phe a^- was about 2 ns.

Materials and Methods

PS II particles were isolated from spinach chloroplasts according to the method of Berthold et al. [8] with modifications as in Ref. 9. These particles were used as starting material for the isolation of reaction center enriched PS II preparations via an adaptation of the methods described by Mullet and Arntzen [10] and Lam et al. [11]. The starting material was resuspended in 20 mM tricine-NaOH (pH = 7.8) to 1.0 mg Chl/ml, and incubated with digitonin (10 mg/mg Chl) and *n*-octyl-β-D-glucopyranoside (4 mg/mg Chl) for 1 h at 4°C in the dark, followed by centrifugation for 20 min at $18000 \times g$. The supernatant was loaded (6 ml per tube) on a 0.2–1.0 M sucrose linear density gradient containing 20 mM Tricine-NaOH (pH = 7.8) and 0.1% (w/v) sodiumcholate. The gradient centrifugation steps were carried out for 1.5 h in a Beckman VTi 50 rotor at $196000 \times g$. After centrifugation the gradient showed two chlorophyll-containing bands of which the lower one contained PS II. After collection the purified PS II preparations were dialyzed overnight at 4°C against 20 mM Tricine-NaOH (pH = 7.8), 0.1% (w/v) sodiumcholate, and concentrated in an Amicon ultrafiltration cell with a PM 30 Diaflo membrane. This preparation was incubated with 2% β-octylglucoside for 45 min before loading on a second gradient of the same type. After dialysis and concentration the reaction-center-enriched PS II particles were collected. The particles were characterized by an inactive oxygen evolving complex, a Chl *a*/*b* ratio of more than 7 and contained approx. 80 Chl *a* molecules per reaction center. The redox condition of the PS II particles was controlled by the addition of either dithionite or ferricyanide. This latter redox compound served as an electron carrier from Q_A^- to P-680$^+$ in the millisecond time range [12], and thus ensured, by inducing cyclic electron transport, the reopening of the reaction centers between successive flashes. The light-harvesting chlorophyll *a*/*b*-protein complex was isolated according to Lam et al. [11].

The picosecond absorbance difference measurements were performed with the apparatus briefly described in Refs. 7 and 13. The samples were excited at 532 nm with a 35 ps pulse from a frequency-doubled mode-locked Nd-Yag laser (maximum excitation energy density, about 2.5 mJ/cm^2). The 35 ps probe pulse was generated by focussing the 1064 nm radiation that remained after frequency doubling into a 15 cm water cell. The wavelength of the probe pulse (full width at half maximum, about 3 nm) was varied between 550 and 900 nm. Schott KV 550 colored glass and suitable interference filters were used to prevent stray excitation light and fluorescence from reaching the detectors. In some experiments a part of the excitation pulse was deflected by a beam splitter onto the sample via a short-cut to serve as a 'preflash', to close most or all of the reaction centers before the arrival of the excitation pulse. The repetition rate of the pulses was 0.5 Hz. By averaging over about 25 laser flashes the best resolution obtained, depending on the wavelength, was about $2 \cdot 10^{-4}$ units of absorbance. All measurements were performed at room temperature.

Results and Interpretation

As will be shown, excitation of the PS II particles at 532 nm induced absorbance changes that could be ascribed both to the formation of excited states of antenna chlorophyll (Chl) and to charge separation in the reaction center. In order to distinguish between these phenomena we monitored the absorbance changes that occurred in the antenna separately in both the isolated light-harvesting Chl *a/b* protein and in PS II particles in which the reaction centers had been photooxidized by a preflash.

Fig. 1a shows the absorbance difference spectrum of the PS II particles in the region 550–900 nm with coincident excitation and probe pulses (denoted as 0 ps) at 5 ns after a preflash. At this time the reaction centers are presumably closed in the state P-680$^+$Q$_A^-$. The spectrum shows broad increases in absorption in the regions 550–660 and 750–900 nm and a bleaching centered at about 685 nm. The same features can be observed in the 0 ps absorbance difference spectrum of the isolated light-harvesting Chl *a/b* protein (Fig. 1b). We ascribe the bleaching in both spectra to the disappearance of Chl *a* ground states due to excitation of the antenna molecules. A bleaching of antenna absorption bands has also been observed upon formation of excited states in a number of photosynthetic bacteria [7,14,15]. In further support of this assignment, Fig. 1c shows the absorbance difference spectrum of Chl *a* dissolved in methanol at 25 ps after the excitation pulse. The bleaching, centered at about 665 nm, coincides with the absorption band of the solution, and indicates the disappearance of Chl *a* ground states. Also the shallow trough at 600 nm corresponds to a ground-state absorption maximum. A comparison of the difference spectrum with the absorption spectrum of Chl *a* in methanol shows that singlet-excited Chl *a* (Chl *a**) probably has a rather flat absorption spectrum in the region 550–900 nm. The kinetics at 665 nm (not shown) indicated only a partial decay of the bleaching on a time scale of 5 ns, as could be expected on basis of a fluorescence lifetime of 5 ns and a chlorophyll triplet yield of about 70%. A comparison of Figs. 1a and b with Fig. 1c now shows that the spectra of the PS II particles and the Chl *a/b* protein at 0 ps can be fully ascribed to the bleaching of Chl *a* ground-state bands and the formation of absorption bands of Chl *a**. The shallow trough at about 650 nm in the difference spectrum of Fig. 1b is lacking in Fig. 1a and c, and thus may indicate the presence of some singlet excitations on Chl *b* of the Chl *a/b*-protein complex.

Fig. 2 shows the absorbance difference spectra in the region 550–900 nm of PS II particles with open reaction centers (no preflash). Ferricyanide was added to ensure reopening of the centers between flashes. At 1 ns after the excitation pulse (a) the spectrum shows bleachings around 630 and 680 nm, and absorbance increases between 550 and 600 nm, and between 710 and 900 nm, and is quite similar to the difference spectra observed by Döring et al. [16] and by Van Gorkom et al. [17], which spectra were attributed to the oxidation of the primary electron donor P-680 [17]. The spectrum at 0 ps (c) resembles that of Fig. 1a, which was measured after prior photooxidation of P-680 with a preflash, and thus can largely be ascribed to the formation of singlet-excited antenna Chl *a*. The spectrum of Fig. 2b shows the absorbance changes at 200 ps after the flash. The contribution

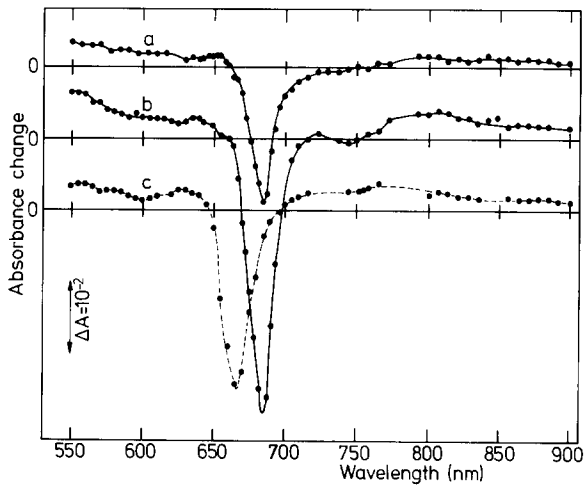

Fig. 1. Absorbance difference spectra with coincident excitation and probe pulse (0 ps). (a) PS II particles 5 ns after a 35 ps preflash at 532 nm; 2.5 mM ferricyanide added; the extinction at 675 nm was 1.4 in a 2 mm cell. (b) Light-harvesting Chl *a/b*-protein; the extinction at 675 nm was 1.0. (c) Chl *a* dissolved in methanol with an extinction of 1.0 at 665 nm. The excitation-energy density was about 0.7 mJ/cm^2.

of Chl a^* to this spectrum is probably small, as can be judged from the absence of a significant absorbance increase in the region 550–630 nm (compare with Figs. 1a and 2c). A comparison with the difference spectrum after 1 ns (Fig. 2a) indicates that the 200 ps spectrum contains, in addition to contributions from the oxidation of P-680, an absorbance increase in the region 640–665 nm and above 730 nm, and a bleaching between 665 and 730 nm. As will be discussed below, these additional absorbance changes are largely due to reduction of the primary electron acceptor, I.

Fig. 3 shows the absorbance difference spectra of PS II particles in the presence of dithionite to reduce Q_A chemically. At this condition it is expected that a recombination of the radical pair $P\text{-}680^+I^-$ takes place with a time constant of 2–4 ns [4]. A comparison with Fig. 2a appears to confirm the presence of $P\text{-}680^+$, both at 1 ns (Fig. 3a) and at 200 ps (Fig. 3b) after the flash. In addition an absorbance increase between 640 and 665 nm, and an extra bleaching at the long-wavelength side of 680 nm can be observed in both spectra. Again, a significant contribution from Chl

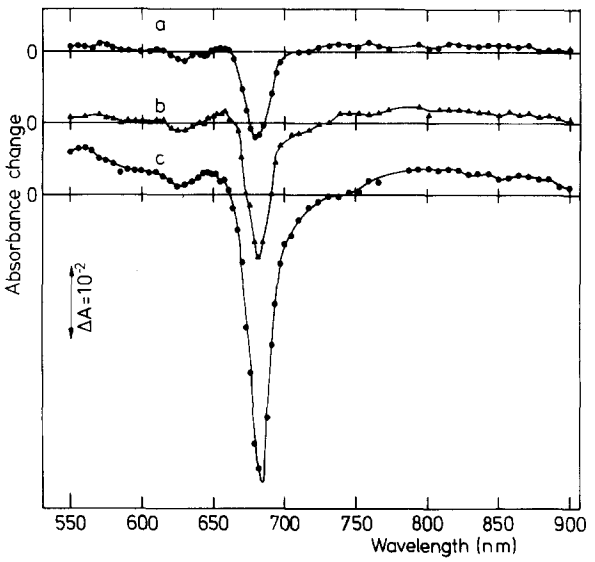

Fig. 2. Absorbance difference spectra of PS II particles in the presence of 2.5 mM ferricyanide, at an excitation energy density of 1.1 mJ/cm^2. (a) 1 ns; (b) 200 ps; (c) 0 ps. The extinction of the sample was 1.4 at 675 nm in a 2 mm cell.

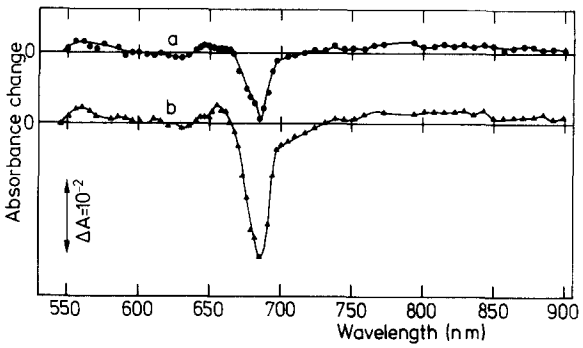

Fig. 3. Absorbance difference spectra of PS II particles in the presence of dithionite. (a) 1 ns; (b) 200 ps. Further conditions, as for Fig. 2.

a^* to the spectra of Fig. 3 can be excluded on the basis of the relatively small absorbance changes in the region 550–640 nm. It thus appears that these additional changes can be ascribed to the reduction of the primary electron acceptor, and that the lifetime of I^- in the chemically reduced sample is lengthened with respect to samples with normal electron transport. Furthermore, it follows that under reducing conditions the decay of I^- is accompanied by that of $P\text{-}680^+$ as can be concluded from a comparison of the amplitudes of the absorbance changes of the $P\text{-}680^+$ spectrum (Fig. 2a) and of the spectra of Fig. 3.

From the above considerations it follows that the 200 ps spectra of Figs. 2 and 3 can be largely ascribed to $P\text{-}680^+$ and I^-, and the 1 ns spectrum in the presence of ferricyanide (Fig. 2a) to $P\text{-}680^+$. Since, as we will show below, the lifetime of $P\text{-}680^+$ is about 2 ns in reduced particles and much longer in particles with uninhibited electron transport, the same amount of $P\text{-}680^+$ as observed in fig. 2a should be present in both the 200 ps spectra. Thus, subtraction of the spectrum of Fig. 2a from the 200 ps spectra should yield mainly the absorbance difference spectra of $I^- - I$. The result of this subtraction in the region 600–750 nm is shown in Fig. 4. Inspection of the figure indicates that the spectra are very similar and thus that the same acceptor can be photoreduced in chemically reduced particles (open circles) and in particles with normal electron transport (solid circles). The nature of this acceptor will be discussed under Discussion.

To determine the lifetime of I^- we have mea-

sured the kinetics at 655 nm at which wavelength an absorbance increase due to formation of I^- is expected (Fig. 4). The results are shown in Fig. 5. In the presence of both ferricyanide (open circles) and dithionite (solid circles) an absorbance increase is observed. The rise-time of the increase is mainly determined by the temporal profile of the exciting and probe pulses. The first 200 ps of the decay are also distorted due to convolution with the shape of the pulses. The absorbance changes in this time range for the larger part represent the decay of Chl *a**, which absorbs more than the ground state at this wavelength (Fig. 1a). In the kinetics with ferricyanide the residual increase is observed to decay with a time constant of 250–300 ps. Its lifetime is 2 ns or more in the kinetics with dithionite. These observations allow to conclude that in PS II particles with uninhibited electron transport the reoxidation of I^- occurs with a time-constant of 250–300 ps.

Fig. 6 shows the kinetics of absorbance changes at 685 nm at three different conditions. The development and the first 200 ps of the decay of the bleaching are again distorted due to convolution with the shape of the pulses. The decay in this time-range mainly represents the recovery of Chl *a* ground-state absorption due to deactivation of Chl *a**. The initial decay indicates that the lifetime of Chl *a** is several tens of picoseconds under these conditions. The kinetics recorded in the presence of ferricyanide (open circles) show, in addition to this fast decay, a slower decay in the time range up to 1 ns, and a constant component from 1 ns onward. This last component is due to $P\text{-}680^+$ (Fig. 2a). We assign the intermediate component to the reoxidation of I^-, since this process takes place in the time region up to 1 ns (Fig. 5), and since reduction of I causes a bleaching at this wavelength (Fig. 4). This assignment is supported upon inspection of the kinetics monitored at the same excitation density and in the presence of dithionite (solid circles). The amplitude of the bleaching in the time interval from 100 to 600 ps in these kinetics is larger than that in the presence of ferricyanide, and increasingly smaller from 600 ps onward. These findings support the notion that the lifetime of I^- increases upon chemical reduction of Q_A and that its decay is accompanied by that of $P\text{-}680^+$. The time constant for the recombination between $P\text{-}680^+$ and I^- as estimated from Fig. 6 is about 2 ns. The kinetics monitored after a

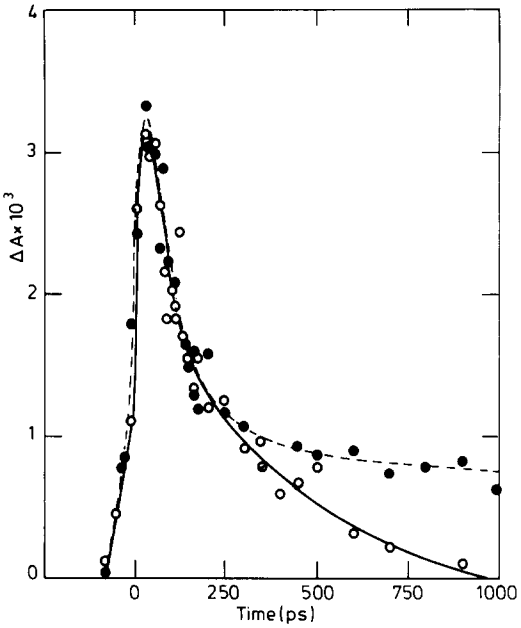

Fig. 5. Kinetics of absorbance changes at 655 nm in the presence of dithionite (●), and 2.5 mM ferricyanide (○). Further conditions, as for Fig. 2.

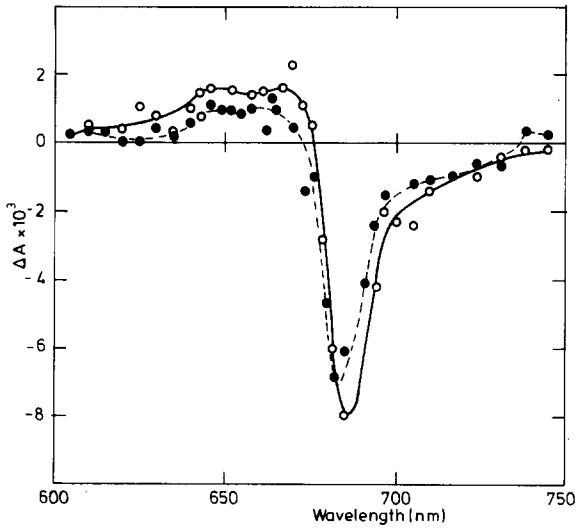

Fig. 4. Absorbance difference spectra of the PS II particles calculated by subtraction of the 1 ns spectrum of Fig. 2 from the 200 ps spectrum of Fig. 2 (●), and from the 200 ps spectrum of Fig. 3 (○). See text for details.

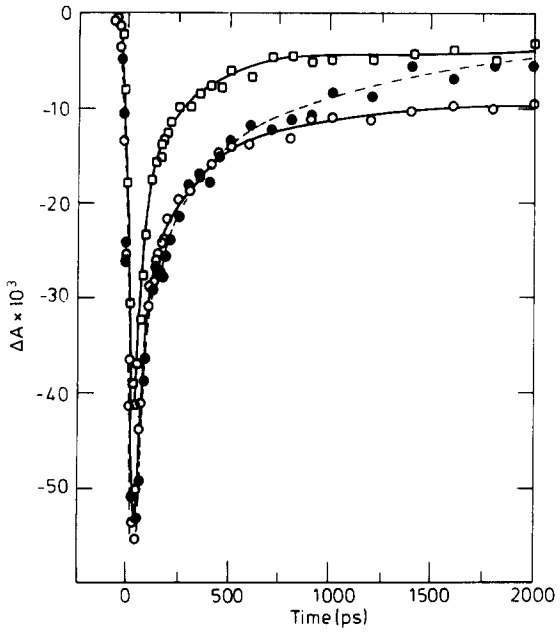

Fig. 6. Kinetics of absorbance changes at 685 nm. (□), about 5 ns after a preflash; (●) in the presence of dithionite, and (○) in the presence of 2.5 mM ferricyanide. Further conditions, as for Fig. 2.

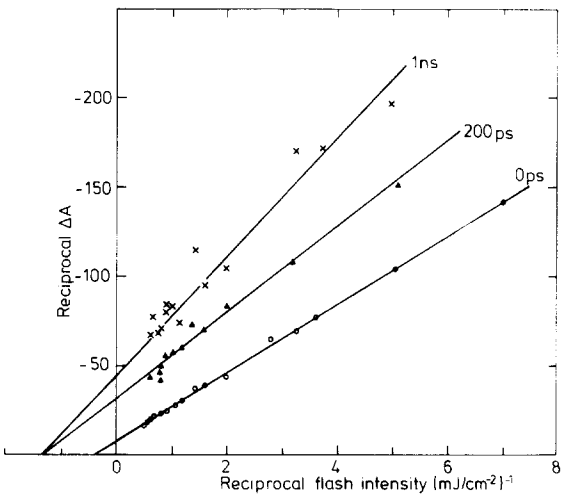

Fig. 7. Reciprocal absorbance changes at 680 nm of PS II particles in the presence of 2.5 mM ferricyanide as a function of the reciprocal flash energy density. (○) 0 ps; (▲) 200 ps; (×) 1 ns. The extinction of the sample was 1.4 at 675 nm.

preflash (Fig. 6, squares) indicate a much smaller constant component as compared with the kinetics measured with open reaction centers, in agreement with the idea that the preflash photooxidized most of the centers. Since a small component is, however, still observed, it follows that the preflash was not saturating with respect to primary photochemistry (see below). The component with the intermediate lifetime is also discernible in these kinetics and probably represents the reoxidation of I^- in the reaction centers that were not closed by the preflash.

Fig. 7 shows the reciprocal of the absorbance decrease at 680 nm as a function of the reciprocal of the flash-energy density for different delay-times with respect to the excitation pulse, in the presence of ferricyanide. The curve at 0 ps (circles), which is mainly due to the disappearance of Chl *a* ground states, indicates (after linear extrapolation) a maximal bleaching of about $120 \cdot 10^{-3}$. Since the maximal bleaching obtained is about $60 \cdot 10^{-3}$, formation of Chl *a** is only partially saturated at the highest flash energy density used. The formation of P-680$^+$ measured at 1 ns (crosses) shows a different saturation behaviour. The flash-energy density for half saturation of P-680$^+$ formation (about 0.8 mJ/cm^2) is clearly distinct from the corresponding value for Chl *a** formation (about 2.5 mJ/cm^2). More than 70% of the reaction centers can be closed at the highest flash intensity of 2 mJ/cm^2. The bleaching measured at 200 ps (triangles) points (after linear extrapolation) at a flash-energy density for half saturation about equal to that for P-680$^+$ formation alone. This suggests that the bleaching detected at 200 ps at 680 nm is mainly due to reaction-center constituents. A small contribution of Chl *a* excitation cannot be ruled out, however (see below).

Discussion

Application of picosecond time-resolved absorbance difference spectroscopy to relatively intact preparations of Photosystem II of spinach has permitted the observation of primary processes taking place in the antenna system and in the reaction center. Excitation with a 35 ps laser flash at 532 nm causes absorption changes due to formation and decay of singlet-excited antenna Chl *a* (Chl *a**), and due to the primary charge separation in the reaction center. The formation of Chl *a** is deduced from the bleaching of the

ground state Q_y absorption bands and from increases in absorption in the regions 550–600 nm and 730–900 nm. The bleaching is centered at about 685 nm, i.e., about 10 nm red shifted as compared to the absorption maximum of the preparation. Furthermore, the width of the bleaching at half maximum (about 16 nm) is appreciably smaller than the width of the absorption band (about 26 nm). Recently, evidence has been obtained that the singlet excitation of bacteriochlorophyll in the B880 antenna complex of the purple bacterium *Rhodospirillum rubrum* caused a bleaching of the 880 nm absorption band of the excited molecule and a blue shift, presumably due to changes of exciton interaction, of about six surrounding bacteriochlorophyll molecules, resulting in a difference spectrum in which the bleaching band was narrowed and shifted to longer wavelength [15]. Similar effects may have contributed to the shape of the difference spectrum observed for the antenna complex of the PS II particles, and presumably in addition to the red shift of the bleaching, as compared to the absorption maximum, due to a Boltzmann equilibrium between the main absorption band and the long-wavelength form of Chl *a*, that absorbs at 685 nm at 4 K [18].

The kinetics of Fig. 6 point at a lifetime for the main part of the singlet excitations on Chl *a* of only a few tens of picoseconds. It can be calculated from the incident excitation density and the extinction of the sample at 532 nm that, at the conditions at which the absorbance kinetics were monitored, about five singlet excitations were generated in the antenna per reaction center. At this high excitation density, non-linear contributions from singlet–singlet annihilation to the decay of Chl *a** are expected to occur, as has been observed in the dependence of the fluorescence yield of PS II particles on the intensity of a picosecond flash (see, for instance, Ref. 19 and references therein). Thus, a shortening of the average decay time of Chl *a** as measured under our conditions is expected as compared to the low-intensity fluorescence kinetics, which usually point at lifetimes of more than 100 ps [19].

An analogous effect of high-excitation intensities on absorbance kinetics has recently been demonstrated for the decay of bacteriochlorophyll *a** in the antenna of *Rhodospirillum rubrum* [15]. It has been shown for *R. rubrum* that following excitation with a ps pulse, singlet–singlet annihilation competes strongly with trapping of the excitations in the reaction center [20]. The slow saturation behaviour of P-680$^+$ formation (see Fig. 7) suggests that such a competition also occurs in the PS II particles.

Since at 1 ns after the flash in the presence of ferricyanide only absorbance changes due to P-680$^+$ are discerned (Fig. 2a), it follows that the decay of Chl *a** does not produce a measurable amount of triplet states of Chl *a* (Chl a^T), in contrast to the observations by Shuvalov et al. after excitation with a nanosecond pulse [4]. This difference can also be ascribed to the annihilation-induced shortening of the lifetime of Chl *a** in our experiments. Furthermore, it follows that singlet–singlet annihilation does not produce Chl a^T, which implies that the process occurs via the process $S_1 + S_1 \rightarrow S_1 + S_0 + \text{heat}$, in which S_0 and S_1 denote the ground and lowest-excited singlet state of Chl *a*, respectively.

As to charge separation in the reaction centers, our data support the hypothesis of an electron acceptor (I) between the primary donor P-680 and the traditional 'primary' plastoquinone acceptor Q_A. When the electron transport is not inhibited, reoxidation of I^- by the secondary acceptor, i.e. Q_A, occurs with a time constant of 250–300 ps. When Q_A is chemically reduced, the life-time of the radical pair is lengthened, and a recombination between P-680$^+$ and I^- takes place in about 2 ns. Such a recombination was also observed by Shuvalov et al. [4]. These authors applied nanosecond absorbance-difference spectroscopy in the region 400–600 nm to chemically reduced small PS II particles and found a time constant of about 4 ns for the decay of the radical pair. A recombination of the primary charge pair with a time constant of 2–4 ns had earlier been deduced by Klimov et al. from measurements of fluorescence emission of chemically reduced PS II particles [21]. Shuvalov et al. [4] confirmed the proposition put forward by Klimov et al. [3] that the primary acceptor is a pheophytin *a* (Phe *a*) molecule and estimated an upper limit of 400 ps for the forward electron transport from Phe *a* to Q_A. Information on the identity of the primary acceptor can also be

gained from our data on the region above 550 nm. The calculated absorbance difference spectra for the formation of I^- were shown in Fig. 4. The spectra appear to consists of an absorbance increase between about 630 and 675 nm, and a bleaching in the region 675–700 nm with a tail extending to about 740 nm. In the region 750–900 nm (not shown) a small absorbance increase is observed. The spectra compare reasonably well with that measured by Klimov et al. upon continuous illumination of PS II particles at low redox potential, which was ascribed to reduction of pheophytin *a* [3]. The tail in the bleaching from 700–740 nm in our spectra was not observed by Klimov et al. and might be due to a long-lived excited state of an artificially induced long-wavelength form of Chl *a* in our PS II preparation. The absence of significant changes below 630 nm shows that the contribution by native Chl *a** (cf. Fig. 1a) to the spectra of Fig. 4 is negligible. Upon inspection of Fig. 4 it can be concluded that Phe *a* is photoreduced both in chemically reduced particles (open circles) and in particles with normal electron transport (solid circles). The amplitude of the absorbance changes in the latter spectrum is reduced as a result of the reoxidation of Phe a^- at 200 ps after the flash in some of the reaction centers. The bleaching in the former spectrum appears to be shifted somewhat to the red as compared to the latter spectrum, and could be due to some remaining Chl *a**. A contribution to this red shift by the shift of the Phe *a* absorption bands observed upon reduction of Q_A [3] can be excluded, since this shift of Phe *a* occurs toward the blue. Our results are in accordance with the following scheme:

$$\text{Chl } a^* \overset{K_1}{\rightleftharpoons} \text{P-680}^* \overset{K_2}{\rightleftharpoons} \text{P-680}^+ \text{I}^- \overset{\approx 250\text{ ps}}{\rightarrow} \text{P-680}^+ \text{IQ}_A^- \qquad (1)$$

in which I denotes pheophytin *a*.

As judged from the absence of a noticeable amount of native Chl *a** in the 200 ps spectra (Figs. 2b and 3b), it follows that the equilibrium between Chl *a** and $P\text{-}680^+ I^-$ is drawn to the side of the radical pair. This appears to be in conflict with the suggestion that during the decay of $P\text{-}680^+ I^-$, about half of the excitations exist as Chl *a**, and the other half as the energy of the separated charges [1]. Our preparation contains only about 80 Chl *a* molecules per reaction center, however, and therefore the value for the equilibrium constant K_1 for our PS II particles in Eqn. 1, if not influenced by the presence of Triton X-100, is 1/80 instead of the 1/200 estimated for intact PS II. Also, the value for K_2 may have been underestimated.

As to the shape of the spectrum observed upon reduction of pheophytin *a*, it has been put forward by Ganago et al. [22] on the basis of linear-dichroism studies that the spectrum observed by Klimov et al. [3] upon reduction of Phe *a* under continuous illumination consists of the bleaching of a Phe *a* molecule absorbing at 680 nm, and of a blue shift of another molecule, perhaps P-680, together resulting in a maximal absorbance decrease at 685 nm. Since our I^- spectra (Fig. 4) resemble those by Klimov et al. reasonably well and do neither show the maximal bleaching at 680 nm, nor the zero transition at 665 nm, attributed to Phe *a* reduction by Ganago et al. [22], it is likely that a shift is included in our I^- spectra as well. Since in our experiments P-680 was oxidized concomitantly with the reduction of I the shift cannot be due to the primary donor, but must be ascribed to another chlorophyllous molecule close to the pheophytin *a* acceptor, and absorbing around 680 nm.

Summarizing, we may conclude that picosecond absorbance difference spectroscopy has allowed, for the first time, the observation of the primary reactions in PS II particles, and has demonstrated the role of Phe *a* as the primary electron acceptor during normal turnover of the PS II reaction centers.

Acknowledgements

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