Charge Recombination Reactions in Photosystem II. 2. Transient Absorbance Difference Spectra and Their Temperature Dependence†

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ABSTRACT: Absorbance difference spectra of the transient states in photosystem II (PS II) have been examined in the Q_y absorption region between 660 and 700 nm. The $P680^+Pheo^-$/P680Pheo, $^3P680$/P680, and $P680^+Q_A^-$/$P680Q_A$ spectra were measured in O_2-evolving PS II core complexes from *Synechococcus* and PS II-enriched membrane fragments from spinach. The low-temperature absorbance difference spectra vary only slightly between both PS II preparations. The $^3P680$/P680 spectrum is characterized by a bleaching at 685 nm at 25 K and indicates weak exciton coupling with neighboring pigment(s). We conclude that P680 absorbs at 685 nm in more intact PS II preparations at cryogenic temperature. The difference spectra of the radical pairs are strongly temperature dependent. At low temperature the $P680^+Q_A^-$/$P680Q_A^-$ spectrum exhibits the strongest bleaching at 675 nm whereas the $P680^+Pheo^-$/P680Pheo spectra show two distinct bleaching bands at 674 and 684 nm. It is suggested that an electrochronic red shift resulting in a bleaching at 675 nm and an absorbance increase at about 682 nm dominates the spectral features of the charge-separated states. On the basis of the present results and those in the literature, we conclude that the interactions between the pigments and especially the organization of the primary donor must be quite different in PS II compared to bacterial reaction centers, although the basic structural arrangement of the pigments might be similar. Spectral data obtained with samples in the presence of singly and doubly reduced Q_A indicate that the primary photochemistry in PS II is not strongly influenced by the redox state of Q_A at low temperature and confirm the results of the accompanying paper [Van Mieghem, F. J. E., Brettel, K., Hillmann, B., Kamlowski, A., Rutherford, A. W., & Schlodder, E. (1995) *Biochemistry 34*, 4798−4813]. The spectra of the primary radical pair and the reaction center triplet obtained with more intact PS II preparations differ widely from those of D1/D2/cyt *b*-559 complexes. In the latter sample, where $^3P680$ formation results in a bleaching at 680 nm, the $P680^+Pheo^-$/P680Pheo spectrum shows only one broad bleaching band at about 680 nm, and the main bleaching due to photoaccumulation of $Pheo^-$ at 77 K appears at 682 nm instead of 685 nm in PS II core complexes. This indicates that the removal of the core antenna which is accompanied by the loss of Q_A causes also structural changes of the reaction center.

In photosystem II (PS II),[1] the light-induced charge separation across the membrane leads to the oxidation of water and the reduction of quinone. Absorption of light by antenna pigments is followed by energy transfer to a special Chl *a* molecule, P680, the primary electron donor of PS II. From its lowest excited singlet state, P680 transfers an electron to the primary electron acceptor, a pheophytin *a* (Pheo). The charge separation is stabilized by further electron transfer from the $Pheo^-$ to a special plastoquinone, Q_A. $P680^+$ is subsequently rereduced by a redox-active tyrosine (Tyr_Z) of the D1 protein, which itself accepts electrons from the oxygen-evolving complex. The Q_A^- delivers the electron to a second quinone acceptor, Q_B. At temperatures below 150 K, this step and the rereduction of $P680^+$ by TyrZ are prevented, and therefore charge recombination of the secondary radical pair, $P680^+Q_A^-$, occurs [for reviews see Mathis and Rutherford (1987), Hansson and Wydrzynski (1990), and Renger (1992)].

Electron transfer can be blocked at the level of the primary radical pair, $P680^+Pheo^-$, when Q_A is prereduced or removed. Under these conditions, $P680^+Pheo^-$ decays by charge recombination either to the ground state (directly or via the excited singlet state) or to the triplet state, $^3P680$. This triplet state is formed after singlet−triplet mixing in

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[1] Abbreviations: ΔA, absorbance change; ADMR, absorbance-detected magnetic resonance; BChl, bacteriochlorophyll; BV, benzyl viologen; CD, circular dichroism; Chl, chlorophyll; *d*, optical path for the measuring light; EPR, electron paramagnetic resonance; FWHM, full width at half-maximum; LD, linear dichroism; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; P680, primary donor of PS II; Pheo, pheophytin, PS II, photosystem II; Q_A and Q_B, first and second quinone-type electron acceptors; Tyr_Z, tyrosine residue, electron donor to $P680^+$.

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the primary radical pair and gives rise to a spin-polarized EPR signal (Rutherford et al., 1981). There was some discussion which raised the possibility that the triplet state could be transferred from P680 to a nearby Chl (Rutherford et al., 1981; Van Mieghem et al., 1991). However, there is now accumulating evidence that the triplet remains on P680 (Tang et al., 1990; Noguchi et al., 1993; Kwa et al., 1994b; see Discussion).

The ^{3}P680 EPR signal could not be detected in spinach PS II membranes in the presence of Q_A^-, but it appeared with high yield when the quinone was doubly reduced (Van Mieghem et al., 1989). This result was explained by the assumption that the formation of the radical pair itself is suppressed because of the electrostatic repulsion between the $Pheo^-$ and the semiquinone anion. In the double-reduced form, protonation of Q_A^{2-} was assumed to occur, neutralizing the negative charges and allowing the formation of the primary radical pair with high yield.

In the preceding paper (Van Mieghem et al., 1995), we have shown by correlating time-resolved absorbance difference spectroscopy and EPR that at low temperature the yields of both the primary radical pair and ^{3}P680 are largely independent of whether Q_A is doubly or singly reduced but that the decay of ^{3}P680 is more than 100 times faster in the presence of singley reduced Q_A, explaining why it was not detected under continuous illumination by standard EPR. In this work, we examine the absorbance difference spectra of the transient states associated with the primary photochemistry in PS II in the wavelength range around 680 nm in order to obtain information on the spectral and structural properties of the cofactors in PS II.

Spectroscopic studies indicate that the electron acceptor side components and their protein environments are very similar in PS II and the purple bacterial reaction center (Rutherford & Mathis, 1987; Hansson & Wydrzynski, 1990). Knowning the crystal structure of the reaction center of *Rhodopseudomonas viridis* (Deisenhofer et al., 1985), a folding model for the reaction center of PS II was proposed on the basis of similarities between the amino acid sequences of the D1 and D2 subunits of PS II and the L and M subunits of bacterial reaction centers (Trebst, 1986; Michel & Deisenhofer, 1988). The fact that the two histidine residues coordinating the special pair (L173 and M200 ⇒ D1-198 and D2-198) are conserved suggests that the structure of the primary donor P680 in PS II resembles closely that of the special pair in bacterial reaction centers. From the X-ray structure of bacterial reaction centers, it is known that the special pair is formed by two BChls that are oriented with their ring planes almost perpendicular to the membrane plane (Deisenhofer et al., 1985; Allen et al., 1987). In PS II, however, the orientation dependence of the ^{3}P680 EPR signal suggests that this Chl is oriented nearly parallel to the membrane plane, and the zero-field splitting parameters of ^{3}P680 agree well with those of monomeric Chl *a* (Rutherford et al., 1981; Rutherford, 1985; Van Mieghem et al., 1991). Another obvious difference between P680 and the primary donor of purple bacteria is the much higher redox potential of $P680^+$ which must be sufficient to drive water oxidation. Data from optical spectroscopy show that the primary donor in PS II is not organized as a strongly coupled dimer, but the spectral features of P680, i.e., the location of the absorbance bands at low temperature and the extent of the excitonic coupling, are still under discussion (Den Blanken et al., 1983; Tetenkin et al., 1989; Durrant et al., 1990; Braun et al., 1990; Van Kan et al., 1990; Otte et al., 1992; Van der Vos et al., 1993). In addition, the location of the Q_y band of the primary acceptor Pheo is controversial (Ganago et al., 1982; Nuijs et al., 1986; Schlodder & Brettel, 1988; Breton, 1990; Braun et al., 1990; Van Kan et al., 1990; Van der Vos et al., 1993). Most of these studies were performed using PS II reaction center preparations (D1/D2/cyt *b*-559 complexes). These complexes are considerably modified on the donor and on the acceptor side. The light-induced charge separation is restricted to the formation of the primary radical pair. It is not yet clear how far the spectroscopic properties of the D1/D2/cyt *b*-559 complexes are altered compared with intact PS II. Furthermore, the interpretation of their optical spectrum is controversial and is made more difficult because the complex is sensitive to the duration of detergent treatment and to exposure to light and oxygen, resulting in spectral changes [for a recent review see Seibert (1993)].

In the present work, we used oxygen-evolving PS II core complexes of *Synechococcus* and PS II-enriched membrane fragments of spinach for recording the difference spectra of the primary radical pair, $P680^+Pheo^-$/P680Pheo, and of the reaction center triplet state, ^{3}P680/P680. For direct comparison these difference spectra were also measured in Q_A-depleted reaction center complexes of *Synechococcus*, which are comparable to D1/D2/cyt *b*-559 complexes. The intact preparations offer additionally the advantage that the difference spectrum of the secondary radical pair, $P680^+Q_A^-$/$P680Q_A$, is available, which gives further information about the spectral properties of the primary donor, P680. For the spectral analysis in this paper, we focus mainly on data from the *Synechococcus* core preparation because, unlike the spinach membranes, they do not show the flattening effect (Duysens, 1956) and are therefore not prone to spectral distortions due to a potentially imperfect flattening correction [see the accompanying paper (Van Mieghem et al., 1995)]. In addition, the *Synechococcus* core preparation, having less chlorophyll per reaction center and being optically clear, allows a better signal to noise ratio.

We have also examined the temperature dependence of the difference spectra. From these data it was possible to distinguish the absorbance changes associated with the oxidation and reduction of the components from those that may result from electrochromic effects. With the whole set of difference spectra a clearer assignment of the bands to the components of primary photochemistry is achieved, and the consequences for the structure of the primary donor and the pigment arrangement will be discussed.

In order to analyze the effect of the redox state of Q_A on the primary photochemistry in PS II, samples were used with Q_A either singly or doubly reduced as monitored by EPR. The higher quality spectral data obtained in *Synechococcus* core preparations confirm the results in the accompanying paper (Van Mieghem et al., 1995) in terms of yield and reaction pathways of the primary radical pair as a function of the state of Q_A.

Some of the results have been presented at the Fifth Congress of the European Society for Photobiology, Sept 19−26, 1993, and by Van Mieghem (1994).

MATERIALS AND METHODS

Sample Preparations. O_2-evolving PS II core complexes were prepared from the cyanobacterium *Synechococcus* sp.

according to Schatz and Witt (1984) and subsequently purified by sucrose density centrifugation (Rögner et al., 1987). The PS II core complexes were finally obtained in 20 mM MES/NaOH (pH = 6.5), 10 mM $MgCl_2$, 20 mM $CaCl_2$, and about 1 M sucrose. They were stored at 200 K.

In order to remove completely the phycobiliproteins, these preparations were diluted 5-fold with buffer A [containing 20 mM MES/NaOH (pH = 6.5), 200 mM sucrose, 500 μM $CaCl_2$, 25 mM $MgCl_2$, 25 mM $MgSO_4$, and 0.025% *n*-dodecyl β-D-maltoside) and loaded onto a Q-Sepharose column (Pharmacia FF) which was equilibrated with buffer A. The column was washed with buffer A until the eluate became colorless. To elute the PS II core, 75 mM $MgSO_4$ was added to buffer A. The purified PS II core complexes contained one photoreducible Q_A per 40 Chls. For the isolation of Q_A-depleted D1/D2/cyt *b*-559-type PS II reaction centers, the PS II complexes were precipitated by 30% PEG and centrifuged at 16000*g* for 15 min. The pellet was resuspended in buffer A, except that 5 mM $MgSO_4$ was present, and treated as described by Van Leeuwen et al. (1991). During the treatment, the secondary acceptor, Q_A, is lost. Less than one photoreducible Q_A per 250 Chls was determined from the flash-induced absorbance changes at 320 nm. The amount of cyt *b*-559 was determined from the dithionite-reduced minus ferricyanide-oxidized difference in absorbance at 559 nm using an extinction coefficient of 15 000 M^{-1} cm^{-1}. The Chl *a*:cyt *b*-559 ratio varied from 6 to 14 depending on the incubation time with Triton X-100. As known already from the literature (Ikeuchi et al., 1989) cyanobacteria are more resistant to detergent treatment, and so the preparations still contain some CP47 protein. The preparations were stored in buffer A with 100 mM $MgSO_4$ at 248 K until used.

For measurements of flash-induced absorbance changes at low temperatures, the sample was diluted by buffer B containing 20 mM MES/NaOH (pH = 6.5), 10 mM $MgCl_2$, 20 mM $CaCl_2$, and two parts (v/v) of glycerol to a final Chl concentration as given in the figure legends.

For measurements of the $P680^+Q_A^-P680Q_A$ spectra, the samples were incubated with 5 mM $K_3[Fe(CN)_6]$ for about 20 min before being frozen in the dark to oxidize the cyt *b*-559.

Measurements of the $P680^+Pheo^-$/P680Pheo difference spectra in intact PS II core complexes were performed with Q_A in the reduced state:

(1) Q_A was singly reduced in two ways: (a) by addition of 4 mM dithionite from a freshly prepared stock solution that contained 400 mM $Na_2S_2O_4$ and 1 M MES buffer or (b) the preillumination at 200 K. The sample was cooled to 200 K without further additions and illuminated with continuous white light (approximately 50 mW/cm^2) for 2 min. Afterward, the sample was frozen immediately to 77 K. By this treatment the state $S_2TyrZP680PheoQ_A^-$ can be trapped (De Paula et al., 1985).

(2) Double reduction of Q_A was achieved by illuminating the PS II core complexes in the presence of 100 μM BV and 40 mM dithionite for 6 min at room temperature. It is assumed that this treatment leads to the state $P680PheoQ_AH_2$ (Van Mieghem et al., 1989). Illumination at negative redox potentials is also known to cause $Pheo^-$ accumulation. Therefore, the sample was dark-adapted for 2 h before being frozen.

PS II-enriched membrane fragments from spinach were prepared and stored as described earlier (Van Mieghem et al., 1989). For absorbance measurements the samples were brought into a buffer containing 60% glycerol, 20 mM MOPS (pH = 7), 10 mM NaCl, 5 mM $MgCl_2$, 125 mM sucrose and, depending on the type of sample, 20 mM dithionite (Q_A singly reduced), 40 mM dithionite (Q_A doubly reduced), or 1 mM $K_3[Fe(CN)_6]$ (Q_A oxidized). For single reduction of Q_A, the sample from a concentrated stock solution was diluted with the dithionite-containing buffer under argon and incubated for 5 min in the dark before the sample was transferred to the precooled cryostat. Samples with doubly reduced Q_A were prepared by addition of 100 μM BV and 40 mM dithionite to the stock solution which contained also 200 mM formate and subsequent incubation (1.5 h) in the dark at room temperature. The disappearance of the Q_A^- state was monitored by EPR. The sample from the already doubly reduced stock was diluted in the buffer containing 40 mM dithionite and frozen in the dark. For further details see the accompanying paper (Van Mieghem et al., 1995).

Spectroscopic Measurements. Measurements of flash-induced absorbance changes in the Q_y region with nanosecond time resolution were essentially performed as described previously (Schlodder et al., 1984) with the following modifications. Different laser diodes from Sony, Hitachi, and Toshiba with emission maxima between 670 and 690 nm at 293 K were used as measuring light sources. The emission wavelength of the laser diodes was tuned by controlling the temperature of the diodes by a laser diode control system from Profile (LDC 400) or from Liconix (LDD and LDC 201). In order to reduce the excitation of the sample by the measuring light, it was pulsed either by pulsing the laser diode or by using a combination of a chopper and a shutter. The pulse duration was approximately 70 μs. When the plateau of the pulse was not perfectly flat, e.g., at time scales exceeding 2 μs, a measurement without excitation was subtracted from an absorbance change measurement. The wavelength of the measuring light was controlled with a monochromator with a spectral resolution of about 1 nm. The laser light was focused on the active area of a silicon photodiode placed about 1.5 m behind the sample. By this setup and by interference filters and colored glasses in front of the detector, the flash-induced fluorescence artifact was very efficiently minimized.

After amplification (IV72A or IV86 amplifier from the Hahn-Meitner Institut, Berlin), the voltage corresponding to the plateau of the measuring light pulse was compensated. Signals were digitized and averaged by a transient recorder (DSA602A or TDS 540 from Tektronix or Biomation 6500) and transmitted to a personal computer. The minimal rise time of the detection system was about 2 ns.

For measurements on the millisecond time scale the apparatus described either by Van Mieghem et al. (1995) or by Gerken et al. (1989) was used. In the latter case the bandwidth of the monochromator was set to 2 nm for a better spectral resolution, and as transient recorder, a TDS 540 (Tekronix) was used.

If not otherwise stated, the sample was excited at 532 nm using frequency-doubled Nd:YAG lasers (Quantel) with a pulse duration at half-maximum amplitude of either 3 ns or 300 ps and pulse energies up to 20 mJ at a repetition of 1−5 Hz.

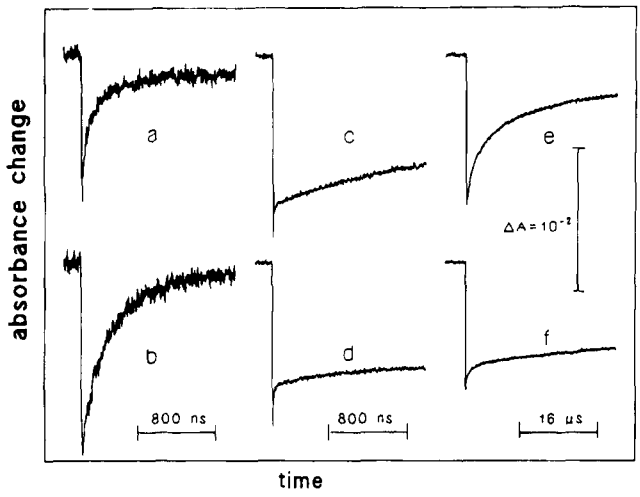

FIGURE 1: Time course of the flash-induced absorbance changes in PS II core complexes from *Synechococcus* in the presence of reduced Q_A at 25 K measured at 674 nm (left) and 684 nm (middle and right): upper row, Q_A singly reduced by addition of 4 mM dithionite; lower row, Q_A doubly reduced by preillumination with 40 mM dithionite and 100 μM BV at room temperature. The signals were normalized to [Chl] = 10 μM and d = 10 mm, with excitation by 532-nm laser flashes (FWHM, 300 ps; repetition rate, 5 Hz). The average energy per pulse corresponds to approximately 40% saturation. Note that traces e and f were measured on a longer time scale than traces a–d.

Low-temperature measurements were made using an Oxford Instruments DN 1704 liquid nitrogen cryostat (between 77 and 273 K) or an Oxford Instruments CF 1204 continuous flow cryostat (5–77 K when used with liquid helium). Alternatively, a SMC optical cryostat cooled with liquid helium was used.

The kinetics of the absorbance changes were obtained by fitting the time course to a (multi)exponential decay. Contributions, which were much longer lived than the time window of the measurement to be fitted, were taken into account by a constant or a slightly inclined straight line. The fitting procedure minimizes the sum of the unweighted least squares.

RESULTS

The Primary Radical Pair $P680^+Pheo^-$. In the *Synechococcus* core preparations, the decay kinetics and the decay pathways of the primary radical pair have been analyzed at low temperature, when the first quinone acceptor, Q_A, is singly or doubly reduced. Figure 1 shows the time course of the absorbance changes at 674 nm (a,b) and 684 nm (c–f) for both types of sample (upper row for singly reduced Q_A and lower row for doubly reduced Q_A) measured at 25 K under otherwise identical conditions. The flash-induced absorbance decrease is attributed to the formation of $P680^+Pheo^-$ (see below for the absorbance difference spectra). The rise time is limited by the time resolution of the flash photometer ($t_r \geq 2$ ns).

At 674 nm, the bleaching decays almost completely in the nanosecond time range (Figure 1a,b) due to charge recombination. Clearly, the decay is slower in the sample which has been preilluminated at room temperature in the presence of dithionite and BV (Figure 1b). It is assumed that this treatment leads to the state $P680PheoQ_AH_2$ (van Mieghem et al., 1989). In this case, the time course fits reasonably well to a single exponential with a half-life of about 200 ns plus a small component which does not decay in the depicted time range. A slightly better fit can be obtained with two exponential phases for the nanosecond range with half-lives of about 100 ns (≈40%) and 300 ns (≈55%). In the sample with Q_A singly reduced, the decay kinetics are clearly multiexponential (Figure 1a). A satisfactory fit can be obtained using two exponentials with half-lives of about 20 ns (≈45%) and 150 ns (40%) plus a longer lived component represented in the fit by a slightly increasing straight line.

At 684 nm both in the sample with singly reduced Q_A (Figure 1c) and in the sample with doubly reduced Q_a (Figure 1d), the initial absorbance decrease decays only very slightly within the depicted time range (except for a very fast component with $t_{1/2} < 5$ ns). This is interpreted as being due to the primary radical pair decaying to form a longer lived state that has an absorbance similar to that of the primary radical pair itself at this wavelength. From its spectral features, the longer lived state can be identified with the triplet state $^3P680$ (see below).

The very fast component reflects the instrument response to unresolved absorbance changes and stimulated emission, probably caused by singlet-excited chlorophylls. It should be mentioned that we have not shown the full amplitude of the spike in Figure 1c,d. Because of the limited time resolution of the setup, this component was not further investigated.

The kinetics of the longer lived components have been analyzed on a microsecond and millisecond time scale. Figure 1e,f shows the time course of the absorbance changes at 684 nm up to 30 μs. In the presence of singly reduced Q_A, the bleaching decays mainly with half-lives of about 1.5 μs (45%) and 15 μs (45%) (Figure 1e). In the case of doubly reduced Q_A, the decay occurs in the millisecond time range with half-lives of 1.2 ms (85%) and 3.3 ms (15%), which are consistent with the lifetimes reported for the reaction center triplet, $^3P680$, formed by recombination of the radical pair (Takahashi et al., 1987; Durrant et al., 1990; Van Kan et al., 1990).

The spectra of the initial flash-induced absorbance changes, omitting the very fast component ($t_{1/2} < 5$ ns), are shown in Figures 2a and 3a for singly and doubly reduced Q_A, respectively. The signals measured at 25 K in the presence of singly reduced Q_A have been fitted in the time range between 5 ns and 1.5 μs at all wavelengths using two exponentials with fixed half-lives of 20 and 140 ns plus a slightly inclined straight line. The last component accounts for slow decay phases in the microsecond time domain. Contribution to the fast (20 ns) phase might result from energy transfer from (antenna) Chl triplet states to carotenoids, which is known to occur with about this half-life (Kramer & Mathis, 1980). Therefore, we looked for differences in the spectra of the two nanosecond components, but within the margin of error, they are not distinguishable (not shown). Furthermore, we analyzed the flash-induced absorbance changes at 676 nm in terms of the dependence on the excitation energy but did not observe any significant change in the kinetics up to saturating excitation. These results suggest that contributions of antenna Chl triplets to the spectrum shown in Figure 2 can be neglected. The absence of a 20-ns phase in the presence of doubly reduced Q_A is also consistent with this conclusion.

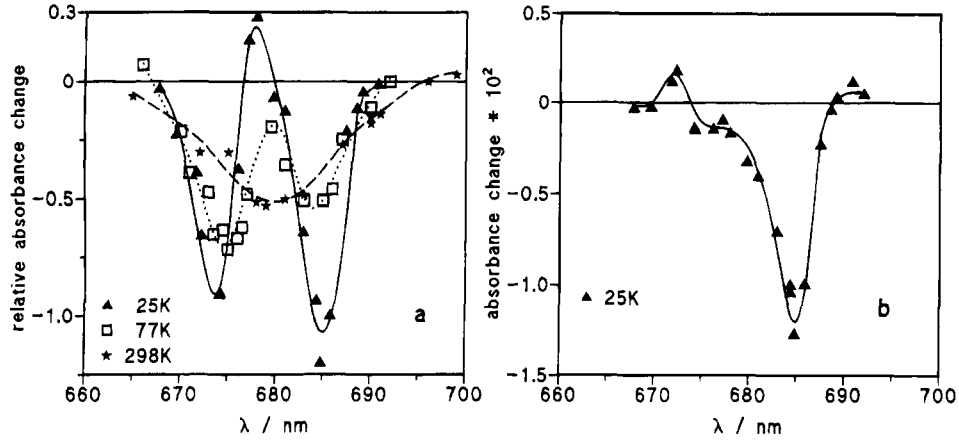

FIGURE 2: (a) Spectra of the flash-induced absorbance changes of PS II core complexes with singly reduced Q_A attributed to the formation of $P680^+Pheo^-$. The data of the 25 K spectrum were obtained from measurements like those depicted in Figure 1a,c. The absorbance change at 684 nm was arbitrarily set to 1. The difference spectra at 298 and 77 K were normalized to the 25 K spectrum assuming that the bleached area is temperature independent. The room temperature data are taken from Schlodder and Brettel (1988). (b) Spectrum of the flash-induced absorbance changes remaining after the decay of the primary pair in PS II core complexes with singly reduced Q_A at 25 K. The data were obtained from the same measurements as the data of the 25 K spectrum in panel a.

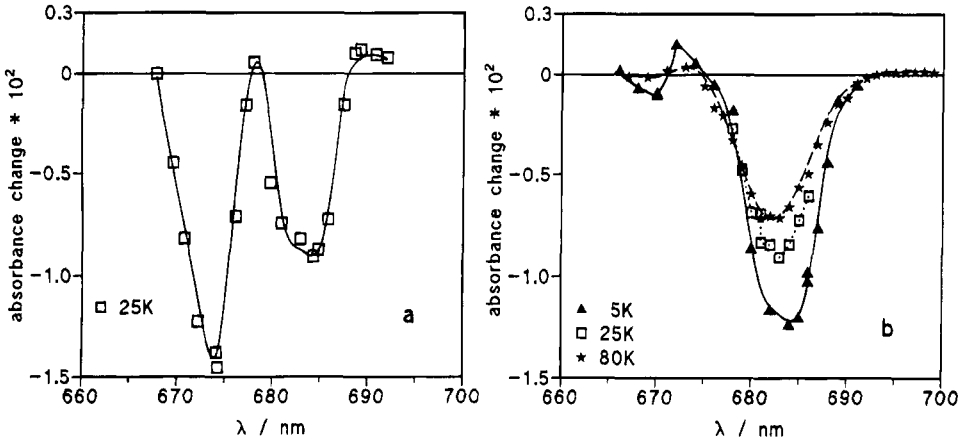

FIGURE 3: (a) Spectrum of the flash-induced absorbance changes in PS II core complexes with doubly reduced Q_A at 25 K attributed to the formation of $P680^+Pheo^-$. Conditions as in Figure 1, lower row. (b) Spectra of the amplitude of the millisecond phases ($t_{1/2}$ = 0.9 ms at 80 K, 1.3 ms at 25 K, and 1 and 5 ms at 5 K) measured in PS II core complexes with doubly reduced Q_A: [Chl] = 23 μM; d = 3.6 mm; excitation by 532-nm laser flashes (FWHM, 3 ns; repetition rate, 1 Hz). The average energy per pulse corresponds to approximately 60% saturation.

The signals obtained in the presence of doubly reduced Q_A have been fitted with one exponential ($t_{1/2}$ = 190 ns) plus a nondecaying component. The initial absorbance change shown in Figure 2a and 3a corresponds to the sum of the amplitudes of the three and two components, respectively. The main features of the difference spectra, namely, two distinct bleaching bands centered at 674 and 684 nm, are the same in samples with Q_A singly and doubly reduced (compare Figure 2a, triangles, with Figure 3a), indicating that the same state is formed by the flash, independent of the redox state of Q_A. We attribute the spectra to the formation by the flash, independent of the redox state of Q_A. We attribute the spectra to the formation of the primary radical pair $P680^+Pheo^-$. We note that the relative amplitudes of the two distinct bleaching bands and the extent of the absorbance increase at 678 nm depend on the reduction state of Q_A. These differences may be related to the negative charge on Q_A^- or to conformational perturbations caused by the double reduction treatment.

In Figure 2a, the corresponding difference spectra at 77 K (squares) and 298 K (stars are also shown. To derive the spectrum of the initial amplitude at 77 K, the signals were fitted using fixed half-lives of 16 and 140 ns and a straight line, while the data for the room temperature spectrum are taken from Schlodder and Brettel (1988). The three spectra were normalized to the same area under the curve. A comparison of the spectra in Figure 2a shows that there is a pronounced temperature effect on the shape of the spectra.

Formation and decay of the primary radical pair have also been studied at 20 K using PS II-enriched membrane fragments from spinach with an antenna size of 200−250 chlorophylls per reaction center. In Figure 6 of the accompanying paper (Van Mieghem et al., 1995), the time course of absorbance changes for samples with singly and doubly reduced Q_A at 674, 676, and 686 nm on the nanosecond time scale were presented. The kinetics were found to be similar to those described above for *Synechococcus* core complexes. At least two exponential decay components plus a constant were required for a satisfactory fit of the time course. In samples with Q_A singly reduced, the data were analyzed using half-lives of 18 and 90 ns. The contribution of both components was found to be approximately equal. In the doubly reduced sample the decay is significantly slower and can be described with two half-lives of 50 ns (≅30%) and 200 ns (≅70%). Slower components attributed to the decay of $^3P680$ are taken into

Table 1: Decay Kinetics of the Primary Radical Pair $P680^+Pheo^-$ and of the P680 Triplet State[a]

sample	state of Q_A	half-lives of $P680^+Pheo^-$ decay at ~20 K	half-lives of $^3P680$ decay at ~20 K
PS II core complexes from *Synechococcus*	singly reduced	20 and 140 ns (1:1)	1.5 and 15 μs (1:1)
	doubly reduced	190 ns	1.2 and 3.3 ms (6:1)
PS II-enriched membrane fragments	singly reduced	18 and 90 ns (1:1)	2.5 and 20 μs (3:2)
	doubly reduced	50 and 200 ns (1:2)	1.5 and 4.5 ms (3:2)

[a] The decay of the absorbance changes at all wavelengths was fitted with the half-lives listed in the table. In the case of biexponential fits the ratios of the amplitudes are given in parentheses. The amplitudes obtained by the fits have been used to derive the difference spectra at ~20 K depicted in Figures 2, 3, and 5.

account by a constant in samples with doubly reduced Q_A or by a third exponential plus a constant in samples with singly reduced Q_A. The kinetics of $^3P680$ decay resemble closely those obtained with PS II core complexes (Q_A singly reduced, 2.5 and 20 μs; Q_A doubly reduced, 1.5 and 4.5 ms). Table 1 summarizes the half-lives of the $P680^+Pheo^-$ and the $^3P680$ decay obtained for different redox states of Q_A and for different samples at around 20 K.

In order to get the $P680^+Pheo^-$/P680Pheo spectra, the amplitudes obtained by the fits were corrected for particle flattening and signal loss due to the accmulation of reduced pheophytin [for details see Van Mieghem et al. (1995)]. Figure 5a shows the resulting spectra of the initial flash-induced absorbance changes for both types of samples (closed circles, Q_A singly reduced; open circles, Q_A doubly reduced). The very similar identical difference spectra give clear evidence that the primary radical pair is formed in PS II-enriched membrane fragments with approximately the same yield in the presence of singly and doubly reduced Q_A. Considering the correction factors necessary to construct the spectra from the data obtained with PS II-enriched membrane fragments, the spectra are also in good agreement with those of the PS II core complexes from *Synechococcus* at equivalent temperatures.

The P680 Triplet State. In the *Synechococcus* core preparations, the spectra of the absorbance changes remaining after the decay of the primary pair (see Figure 1) are shown in Figures 2b and 3b. In samples with Q_A singly reduced (Figure 2b) the amplitude of the slightly inclined straight line obtained by the fit (as described above for Figure 1a) is depicted versus wavelength. In the presence of doubly reduced Q_A, the longer lived absorbance changes have been followed on a millisecond time scale at three different temperatures. The kinetics of the $^3P680$ decay under conditions of doubly reduced Q_A are nearly monoexponential at 77 K with $t_{1/2} = 1.3$ ms. At 5 K, it can be decomposed into two exponential phases with half-lives of 1 ms (≅70%) and 5 ms (≅30%). The non-monoexponential decay of the triplet state at very low temperatures, with parts of it becoming faster and parts slowing down, is expected because interconversion between the three spin sublevels becomes slow (Searle et al., 1990). The spectra of the millisecond phases (Figure 3b) can be ascribed to $^3P680$ that is formed by charge recombination of the radical pair.

The spectra of Figure 3b are characterized by the main bleaching band, the absorbance increase above 693 nm, and two small bands: an absorbance increase around 673 nm and an absorbance decrease at 668 nm. The main bleaching band becomes slightly red shifted to 684 nm by lowering the temperature. At 5 K the full width at half-maximum (FWHM) is about 8 nm. The weak positive and negative bands on the short-wavelengths side are only observed at low temperature and are most pronounced at 5 K [Figure 3b; see also Durrant et al. (1990)].

The spectrum in Figure 2b from samples with singly reduced Q_A is globally similar to that in Figure 3b and is also attributed to $^3P680$. However, the main bleaching band at 685 nm is narrower (~5 nm) and is accompanied by a relative small absorbance decrease in the region between 674 and 679 nm.

To estimate the contributions from ^3Chl of decoupled antenna Chls to the $^3P680$/P680 spectrum of PS II core complexes with doubly reduced Q_A, a sample was illuminated for 15 min at 77 K in order to accumulate $Pheo^-$. After this preillumination the spectrum of the remaining millisecond phase has its maximum slightly blue shifted at 682 nm, and the bleached area corresponds to less than 15% of that measured before illumination (not shown). This value for the contribution of antenna Chl triplets is an upper limit, as primary charge separation was probably not blocked by $Pheo^-$ accmulation in all centers. Therefore, contributions from antenna Chl triplets are unlikely to be the reason of the broadening observed for the main bleaching band in PS II core complexes with doubly reduced Q_A.

In PS II-enriched membranes from spinach, the kinetics of the $^3P680$ decay and the absorbance difference spectra obtained from these kinetics [Figure 5b, closed circles, and Figure 8 in the accompanying paper (Van Mieghem et al., 1995)] resemble closely those obtained with PS II core complexes from *Synechococcus* in the presence of singly reduced Q_A. Under both redox conditions, the main bleaching band at about 685 nm has a FWHM of 4−5 nm. Both spectra include the positive band at about 674 nm and the feature around 678 nm.

The temperature dependence of the $^3P680$/P680 spectrum (see Figure 3b) indicates an increasing triplet yield with decreasing temperature. At 25 K, the $^3P680$ yield relative to the yield of $P680^+Pheo^-$ was calculated from the bleached area of the $P680^+Pheo^-$/P680Pheo spectrum and that of the corresponding triplet spectrum, derived from the same measurements. Considering that $Pheo^-$, Chl^+, and ^3Chl have a relative dipole strength of about 44%, 28%, and 25% compared to their neutral species in the Q_y region (Linschitz & Sarkanen, 1958; Fujita et al., 1978; Chauvet & Viovy, 1981) and that the dipole strength of Pheo is approximately 60% of that of Chl (Fujita et al., 1978), a factor of 1.4 between the areas is expected if the primary radical pair decays completely to the triplet state. The measured ratios at 25 K are 1.5 in the presence of singly reduced Q_A (calculated from the data in Figure 2a,b) and 2.1 in the presence of doubly reduced Q_A (calculated from the data from Figure 3a and the corresponding $^3P680$/P680 spectrum that is not shown); i.e., the triplet yields are 93% and 67% relative to primary radical pair yield.

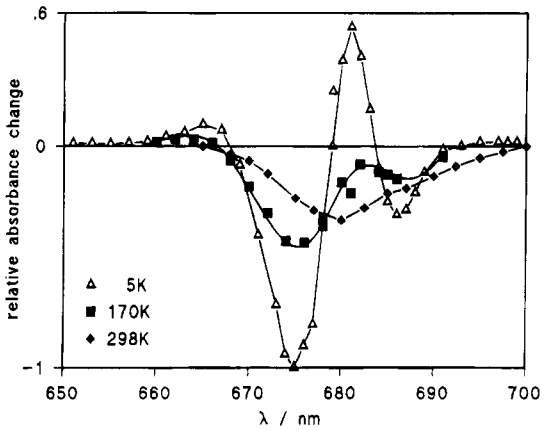

FIGURE 4: Difference spectrum of flash-induced absorbance changes measured in PS II core complexes in the presence of 5 mM $K_3[Fe(CN)_6]$ attributed to the formation of the secondary radical pair, $P680^+Q_A^-$. At 5 and 170 K the absorbance changes decayed with a half-life of 3 ms. The data of the 298 K spectrum were taken from Hillmann and Schlodder (1994). The maximum amplitude of the 5 K spectrum is arbitrarily set to 1. The difference spectra at 298 and 170 K were normalized to the 5 K spectrum assuming that the bleached area is temperature independent.

The absolute triplet yield can be calculated from the dipole strength corresponding to the bleached area of the $^3P680/P680$ spectrum when measured under saturating conditions. In the presence of doubly reduced Q_A the area under the curve corresponds to a dipole strength of about 13 D^2 at 5 K and 10 D^2 at 25 K (Figure 3b, triangles and squares). Assuming a dipole strength of 23 D^2 for the Q_y band of a Chl *a* molecule (Shipman et al., 1976) and taking into account that the ^{3}Chl itself contributes in this region with a dipole strength of about 6 D^2 (Linschitz & Sarkanen, 1958), it can be calculated that the triplet state is formed in 76% (5 K) and 59% (25 K) of the centers. The slight discrepancy between the two estimates of the triplet yield in samples with double-reduced Q_A at 25 K (67% versus 59%) may be explained by photoaccumulation of $Pheo^-$ in a fraction of centers during the measurement and by the yield of charge separation being slightly less than 100%. Both of these factors would diminish the absolute triplet yield per center without affecting the triplet yield relative to the primary pair yield.

The Secondary Radical Pair $P680^+Q_A^-$. In order to complete the set of difference spectra at low temperature, the $P680^+Q_A^-/P680Q_A$ difference spectrum was also measured. At $T < 200$ K the secondary radical pair $P680^+Q_A^-$ decays mainly by charge recombination with a half-life of about 3 ms (not shown), a value similar to that reported in the literature (Mathis & Vermeglio, 1974; Malkin & Bearden, 1975; Reinman & Mathis, 1981). To prevent the electron donation of cyt *b*-559 to $P680^+$ that would lead to the accumulation of the state cyt *b*-$559^+P680PheoQ_A^-$, the cyt *b*−*559* was chemically oxidized by incubation of the sample with 5 mM $K_3[Fe(CN)_6]$ in the dark before being frozen.

Figure 4 shows the spectra of the 3-ms phase at 170 and 5 K obtained with the *Synechococcus* core preparation. The room temperature $P680^+Q_A^-/P680Q_A$ spectrum is taken from Hillmann and Schlodder (1994). The three spectra are normalized to the area under the curves. The spectra measured at low temperature are dominated by a strong bleaching band centered at 675 nm. At 5 K the width of the band is only 6 nm compared to about 16 nm of the 680-nm band at 298 K, while its amplitude at 5 K is approximately three times larger than at 298 K. It corresponds to a differential molar extinction coefficient of approximately 250 000 M^{-1} cm^{-1} under saturating conditions. In parallel, a positive band centered at 682 nm increases upon lowering the temperature. These spectral features suggest that an electrochromic band shift centered at 678 nm overlaps the bleaching due to the formation of $P680^+Q_A^-$ and becomes dominant at low temperature. The low-temperature spectra are further characterized by a small positive band between 660 and 670 nm and a more pronounced bleaching at 687 nm. Under saturating excitation, the bleaching area between 660 and 695 nm corresponds to a dipole strength of about 17 D^2, a value that is expected for the oxidation of one Chl *a* molecule (Chauvet & Viovy, 1981).

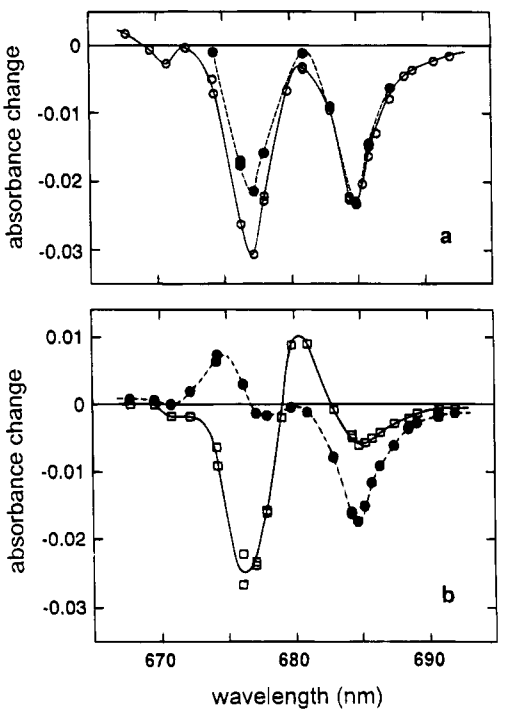

FIGURE 5: Absorbance difference spectra measured with PS II-enriched membrane fragments from spinach at 20 K. All data are corrected for particle flattening and light-induced signal loss [for details, see Van Mieghem et al. (1995)] and were normalized to [Chl] = 14.5 μM and d = 10 mm. The excitation flashes were about saturating. (a) Difference spectra of the absorbance changes attributed to the formation of $P680^+Pheo^-$. Open circles represent the spectrum obtained in samples with Q_A doubly reduced; closed circles, that in samples with Q_A singly reduced. (b) Open squares: $P680^+Q_A^-/P680Q_A$ spectrum obtained from the amplitudes of the 3-ms phase in a sample which had been incubated with 1 mM $K_3[Fe(CN)_6]$ for 1 h before cooling. Closed circles: $^3P680/P680$ spectrum measured in the presence of doubly reduced Q_A; the data are taken from Van Mieghem et al. (1995).

Formation and decay of the secondary radical pair have also been studied at 20 K using PS II-enriched membrane fragments from spinach. The decay kinetics of $P680^+Q_A^-$ and the $P680^+Q_A^-/P680Q_A$ difference spectrum (Figure 5b, open squares) are virtually identical to those described above for PS II core complexes from *Synechococcus*.

Comparative Study of the Primary Photochemistry in D1/D2/cyt b-559-Type Reaction Center Preparations from Synechococcus. The $P680^+Pheo^-/P680Pheo$ spectra shown above differ widely from that reported for the primary radical pair obtained from D1/D2/cyt *b*-559 preparations (Van Kan et al., 1990). Therefore, we repeated the measurements using

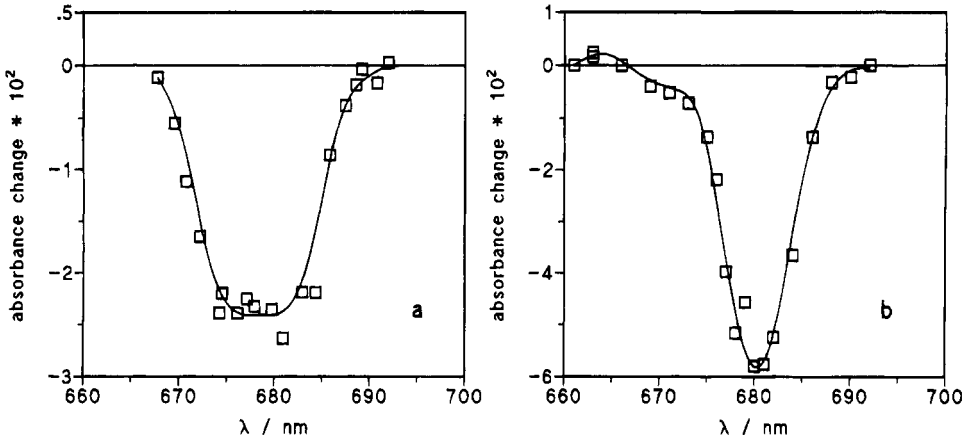

FIGURE 6: (a) Difference spectrum of the flash-induced absorbance changes in the Q_A-depleted D1/D2/cyt *b*-559-type preparation of *Synechococcus* at 77 K attributed to the formation of the primary radical pair. (b) Spectrum of the phase with a half-life of 1.3 ms at 77 K measured with the same preparation as in panel a. The amplitudes were normalized to [Chl] = 10 μM and d = 10 mm. The samples were excited (a) at 532 nm by 300-ps laser flashes with a pulse energy corresponding to approximately 40% saturation and (b) by saturating 15-μs xenon flashes filtered by a colored glass (CS 96-4 from Corning).

an equivalent preparation, which was isolated according to the method of van Leeuwen et al. (1991), starting from *Synechococcus* core complexes (see Materials and Methods).

At 77 K the radical pair, $P680^+Pheo^-$, formed in this Q_A-depleted PS II preparation recombines with half-lives of 20 and 140 ns forming mainly the state $^3P680$, which decays as in the samples with Q_A doubly reduced with a half-life of 1.3 ms. In order to obtain the $P680^+Pheo^-/P680Pheo$ spectrum, the sum of the amplitudes of the three decay components was depicted in Figure 6a as function of the wavelength. The spectrum resembles closely the reported difference spectra for the primary radical pair in D1/D2/cyt *b*-559 complexes from spinach but is considerably different from the primary radical pair spectra measured here with the more intact preparations. Instead of the two distinct bleaching bands, there is only one broad band (FWHM $\cong$ 13 nm) centered at 680 nm. The amplitude of the 1.3-ms phase attributed to the decay of $^3P680$ at 77 K is shown as a function of the wavelength in Figure 6b (note that the absolute amplitudes in panels a and b of Figure 6 are not comparable because of different excitation energies; see legend of Figure 6). The $^3P680/P680$ spectrum is also modified compared to the spectrum obtained with PS II core complexes in the presence of doubly reduced Q_A at the same temperature (see Figure 3b). The main bleaching band is shifted to the blue and centered at 680 nm.

DISCUSSION

The yield, recombination pathways, and kinetics of the primary radical pair have been discussed in detail in the accompanying paper (Van Mieghem et al., 1995). In the present work the kinetic data taken at low temperature over an extended spectral range confirm the conclusions drawn in the accompanying paper: (1) the primary pair yield and the $^3P680$ yield at low temperature are high irrespective of whether Q_A is singly or doubly reduced; (2) $^3P680$ decays more than 100 times faster when Q_A is singly reduced than when Q_A is doubly reduced or absent. This rules out the proposal that the charge separation in PS II at low temperature is prevented in the presence of singly reduced Q_A due to the electrostatic repulsion between Q_A^- and $Pheo^-$ [Van Mieghem et al., 1989; see also Schatz et al. (1988) and Vass and Styring (1992)].

The main purpose of this work was to analyze the absorbance difference spectra of the transient states of primary photochemistry in PS II: $P680^+Pheo^-$, $P680^+Q_A^-$, and $^3P680$. So far, spectral features of PS II have been almost exclusively studied using D1/D2/cyt *b*-559 reaction center preparations (Tetenkin et al., 1989; Durrant et al., 1990; Braun et al., 1990; Van Kan et al., 1990; Otte et al., 1992; Van der Vos et al., 1993; Kwa et al., 1994b). These preparations lack the secondary acceptor Q_A, and it is not yet clear to what extent other properties are modified compared to more intact PS II preparations. We found that the spectra obtained with O_2-evolving PS II core complexes from *Synechococcus* and PS II-enriched membrane fragments from spinach are strikingly different from those reported for D1/D2/cyt *b*-559 preparations. The interpretation of the spectra and in particular the assignment of the absorbance bands in the Q_y region to the pigments involved will be discussed below.

$^3P680/P680$ Spectrum. $^3P680/P680$ spectra were obtained for PS II core complexes from *Synechococcus* and for PS II-enriched membrane fragments from spinach with Q_A singly or doubly reduced (see Figures 2b, 3b, and 5b and Figure 8 of the accompanying paper). They all have in common a strong bleaching centered at approximately 685 nm (at $T \leq$ 25 K) and a smaller positive band at about 673 nm. The location of the main bleaching band at 685 nm is probably due to a red shift induced upon lowering the temperature (see Figure 3b). The spectrum that has been measured at 5 K using *Synechococcus* cores with Q_A double reduced exhibits additionally a small negative band at 668 nm. Of note is that in this sample the main bleaching band is broader than in the other three spectra. A feature at approximately 678 nm is only observable in the spectra which exhibit the narrower main bleaching band. Since the double reduction treatment in *Synechococcus* required a more severe treatment than used for the PS II membranes from spinach, it might be assumed that antenna Chl triplets contribute to these spectra. As only a low yield of such triplets was observed at 77 K in a sample, where reduced pheophytin was accumulated by preillumination, this assumption seems unlikely. Instead, it seems more likely that the centers were modified by this treatment, resulting in a broadening due to

a less homogeneous environment for P680 as found for D1/D2/cyt *b*-559 complexes (see below).

For comparison, the ^{3}P680/P680 spectrum was recorded in a D1/D2/cyt *b*-559-type preparation from *Synechococcus*. The spectrum (Figure 6b) is similar to those reported earlier from comparable preparations from plants, showing a bleaching maximum at 680.5 nm and a width of the main bleaching band of 5−8 nm (Takahashi et al., 1987; Durrant et al., 1990; Van Kan et al., 1990, 1992; Otte et al., 1992; Schlodder & Hillmann, 1992; Van der Vos et al., 1993; Kwa et al., 1994b). The small positive (673 nm) and negative (668 nm) bands were not found at 77 K (see Figure 6b) in accordance with results of Van Kan et al. (1992) but have been reported to be present in D1/D2/cyt *b*-559 preparations at temperatures below 10 K (Van Kan et al., 1990, 1992; Otte et al., 1992; Van der Vos et al., 1993; Kwa et al., 1994b). The temperature dependence of the ^{3}P680/P680 spectrum in the PS II core from *Synechococcus* with Q_A double reduced (see Figure 3b) shows also that the spectral features on the short wavelength side of the strong bleaching band are more pronounced at the lowest temperature.

ADMR experiments (Van der Vos et al., 1993) and site-selection spectroscopy (Kwa et al., 1994b) revealed a spectral heterogeneity of P680 in D1/D2/cyt *b*-559 preparations at low temperatures. At least two forms of the primary donor were distinguished absorbing around 680.5 and 684 nm (Otte et al., 1992; Kwa et al., 1994b). From our results, we conclude that the primary donor absorbs at 685 nm at low temperatures in intact PS II preparations and that the absorbing band is shifted 4−5 nm to the blue in D1/D2/cyt *b*−559 preparations due to the detergent treatment needed for removing the core antenna. The isolation procedure might cause changes in the pigment−pigment and pigment−protein interactions of the reaction center. The observed heterogeneity with parts of P680 absorbing at 684 nm in these preparations might originate form a residual fraction of centers which are more comparable to intact PS II.

As a first approximation, the ^{3}P680/P680 difference spectrum may be explained by the bleaching of the Q_y band of a monomeric Chl *a* upon triplet formation, if the red shift of the Q_y band to ≥680 nm is attributed to interactions with the surrounding protein. However, the spectral features on the short wavelength side of the strong bleaching band, especially the positive band at 673 nm, are not observed in the triplet-minus-singlet spectrum of monomeric chlorophyll *a* in organic solvents or in detergent micelles [e.g., Triton X-100 (Kwa et al., 1994a) or β-DM (Hillmann, unpublished results)]. These features can be attributed to the loss of the excitonic coupling with the neighboring pigment(s) upon the formation of the triplet state of P680. The fact that these features become more intense at low temperature, accompanied by a slight red shift of the main bleaching band, may in part be due to the narrowing of the absorbance bands with decreasing temperature. Furthermore, it may indicate an increase of the coupling strength at low temperature. One explanation might be that the average distance between the pigments decreases slightly because of a contraction of the protein, when the temperature is lowered. Summarizing, the ^{3}P680/P680 difference spectrum contains information not only on the Chl *a* molecule on which the triplet state islocated but also on possible excitonic coupling(s) between this Chl *a* and its neighboring pigment(s).

In order to simulate the ^{3}P680/P680 spectrum obtained in samples that we consider to be more intact (Figures 2b and 5b), we assumed as a first approach that only two identical pigments are excitonically coupled and, on the basis of EPR results, that the triplet state is localized on only one Chl at low temperatures. In this simplified model, the main band in the ^{3}P680/P680 spectrum at 685 nm with a FWHM of 5 nm is attributed to the bleaching of the lower Q_y exciton band of the two interacting pigments. It was shown by LD−triplet-minus-singlet spectroscopy using a D1/D2/cyt *b*-559 preparation that the small negative band at 669 nm and the main bleaching band are polarized perpendicularly to each other (Kwa et al., 1994b). CD spectra, measured at different temperatures (Tetenkin et al., 1989; Braun et al., 1990; Otte et al., 1992) show opposite rotational strength below 670 nm and around 680 nm. These observations suggest that the absorbance decrease around 668 nm in the ^{3}P680/P680 spectra results from the bleaching of the higher energy Q_y exciton band. On the basis of these results it has been proposed that P680 might consist of two weakly coupled chlorophylls with a coupling energy of about 170 cm^{-1}, and the angle between the two unperturbed Q_y transitions has been estimated to be 15−21° (Kwa et al., 1994b). Following this suggestion in the simulation (see Figure 7a), the two exciton bands of the interacting pigments are located at 669 and 685 nm with nearly all of the dipole strength (corresponding to that of two chlorophylls) in the lower energy transition. Upon triplet formation, the exciton interaction is lost, resulting in two noninteracting Chls, one in the triplet state and one in the singlet ground state. It is assumed that the exciton bands are bleached upon triplet formation (dashed line in Figure 7a) and a positive absorption band (dotted line) with the oscillator strength of one chlorophyll appears due to the absorption of the Chl which is not carrying the triplet state. This band has been located at 685 nm to account for the intensity of the bleaching observed around 685 nm being apparently similar to that associated with triplet formation on a monomeric Chl *a* molecule. The absorption of the Chl triplet state, which is weak and nearly constant in this wavelength region, has been neglected. The agreement of the simulation shown in Figure 7a (solid line) with the measured ^{3}P680/P680 spectrum (see Figures 2b and 5b) is rather poor. Slight shifting of the positive band does not lead to marked improvements in this simulation [in contrast to the situation in D1/D2/cyt *b*-559 preparation; see Kwa et al. (1994b)]. Improved agreement might be achieved if excitonic interactions to a third (or more) pigment(s) is taken into account. However, at this point we prefer to explore further simulations in which only two pigments are involved.

An acceptable simulation with only two interacting Chl *a* molecules can be obtained in the following way (see Figure 7b). Two excitonic bands of approximately equal oscillator strength are located at 678 and 685 nm. The positive band at 675 nm (due to the absorption of the Chl which is not carrying the triplet state) cancels much of the bleaching of the higher energy excitonic band. The superposition (solid line) gives rise to a positive band at 673 nm and the spectral feature around 678 nm in accordance with the ^{3}P680/P680 spectra obtained with the more intact samples. This simulation could be accounted for by two different models. (1) In the case of identical monomers absorbing at the same wavelength, the 676- and 685-nm bands with equal intensity would indicate an excitonic splitting of about 100 cm^{-1} and

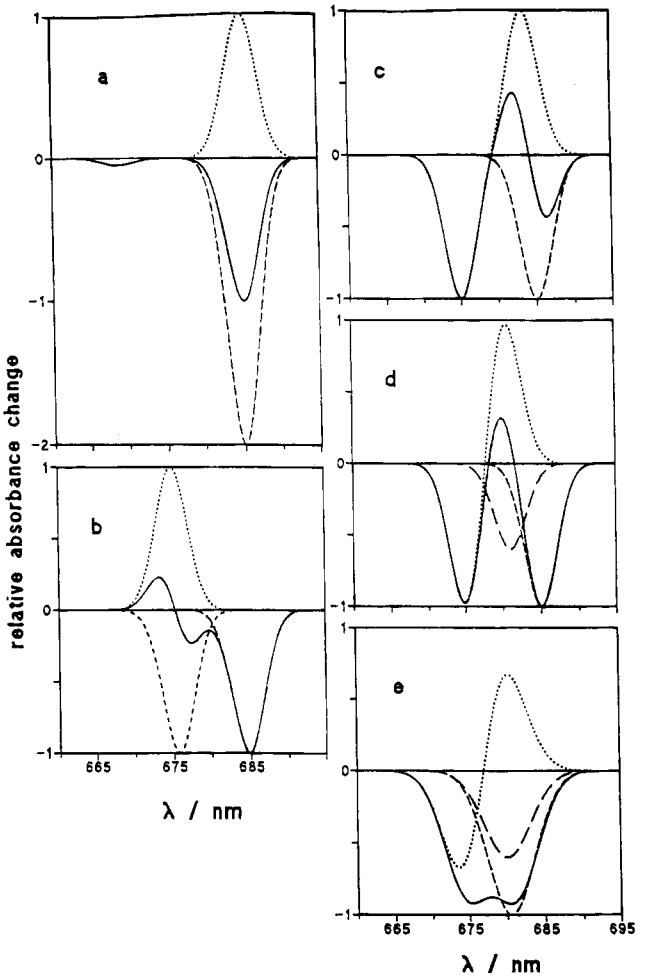

FIGURE 7: (a) Simulation of the ^{3}P680/P680 spectrum (see Figures 2b and 5b) based on the assumption that P680 is a dimer of two weakly coupled Chl *a* molecules with identical Q_y transition energies. ^{3}P680 formation leads to the bleaching of the two exciton bands described by two Gaussian bands (dashed line) centered at 685 and 669 nm with FWHM of 5 nm and relative amplitudes of −2 and −0.05. The appearing Chl monomer band (dotted line) is positioned at 685 nm (FWHM of 5 nm and relative amplitude of 1). The ^{3}P680/P680 spectrum resulting from the simulation (solid line) is the sum of the components. Note that the dashed and solid lines are superimposed around 670 nm. For further details see text. (b) Simulation of the ^{3}P680/P680 spectrum (see Figures 2b and 5b) based on the assumption that P680 absorbing at 685 nm is one Chl *a* molecule weakly coupled to another pigment absorbing at 675 nm. ^{3}P680 is assumed to be localized on the 685-nm Chl. Upon triplet formation this band bleaches (dashed line) whereas the 675-nm band is slightly shifted due to the breaking of exciton coupling described by the disappearance of a band at 676 nm (dashed line) and its appearance at 675 nm (dotted line). The Gaussian bands have a FWHM of 5 nm and identical amplitudes of −1 and 1, respectively. The ^{3}P680/P680 spectrum resulting from the simulation (solid line) is the sum of the components. Note that the broken and the solid lines are superimposed around 685 nm. For further details see text. (c) Simulation of the $P680^+Q_A^-$/$P680Q_A$ difference spectrum (see Figures 4 and 5b) with two components. The bleaching of P680 is taken into account by one Gaussian band (dashed line) at 685 nm, FWHM = 5 nm, and a relative amplitude of −1, which corresponds to the main bleaching band observed in the triplet spectrum. The second component (dotted line) results from the electrochromic red shift of an absorbance band with a relative amplitude of −1 and FWHM of 5 nm, corresponding to the dipole strength of one Chl. The shift occurs from 675 to 683.4 nm. Note that the dotted and the solid lines are superimposed around 675 nm. (d) Simulation of the $P680^+Pheo^-$/P680Pheo spectrum obtained with PS II core complexes in the presence of reduced Q_A (see Figures 2a, 3a, and 5a). The $P680^+$/P680 bleaching band (dashed line) is the same as in (c), while the $Pheo^-$/Pheo band is centered at 681 nm with a FWHM of 5 nm and a relative amplitude of −0.6 (broken line). The contribution of the electrochromic band shift is slightly varied compared to (c). An absorbance band with a relative amplitude of −1 and a FWHM of 5 nm is assumed to be red shifted from 675 to 680.5 nm (dotted line). Note that the dotted and solid lines around 675 nm and the broken and solid lines around 685 nm are superimposed. (e) Simulation of the spectrum of the $P680^+Pheo^-$/P680Pheo spectrum obtained with the D1/D2/cyt *b*-559-type reaction center preparations (see Figure 6a). The $P680^+$/P680 bleaching band (relative amplitude, −1; FWHM = 7 nm) is positioned at 680.5 nm (dashed line). The $Pheo^-$/Pheo band is centered at 680 nm with a FWHM of 7 nm and a relative amplitude of −0.6 (broken line). The dotted line describes the electrochromic shift of an absorption band with a FWHM of 7 nm and a relative amplitude of −1 from 675 to 679 nm.

an approximately orthogonal orientation of the Chl Q_y transitions. (2) Alternatively, the two interacting Chl *a* molecules may have different Q_y transition energies (corresponding to absorption bands at about 676 and 685 nm) due to different environment shifts. In this case, the additional excitonic interaction could be less than 100 cm^{-1}. If the exciton interaction is equal to or smaller than the difference between the Q_y transition energies of the two Chls, the two excitonic transitions would be very similar to those of the uncoupled Chls. If the triplet state is localized on the 685 nm Chl, this band would bleach upon triplet formation, whereas the 676 nm band would be slightly shifted due to the breaking of exciton coupling. This shift (in our simulation this is a shift from 676 to 675 nm) would give rise to an absorbance increase at 673 nm and an absorbance decrease around 678 nm in the ^{3}P680/P680 spectrum. Model 2 corresponds to a situation in which P680 is essentially a monomeric chlorophyll [albeit weakly coupled to its neighboring pigment(s)] and is in accordance with the observation that most properties of P680 are in favor of a monomeric Chl *a* molecule (see the introduction). Furthermore, it would explain the only rather small linear dichroism effects observed around 670 nm in D1/D2/cyt *b*-559 preparations (Kwa et al., 1994b).

The exciton interaction energy calculated from the simulation shown in Figure 7b is equal to or smaller than 100 cm^{-1}. This value is much lower than that for the bacterial special pair in accordance with other values reported in the literature (Tetenkin et al., 1989; Braun et al., 1990; Van Kan et al., 1990; Kwa et al., 1994b). By comparison, the coupling between the BChls of the special pair in the bacterial reaction center of *Rps. viridis* is about 950 cm^{-1} (Knapp et al., 1985). In a putative Chl special pair, with a structure the same as the special BChl pair in the bacterial reaction center (Michel & Deisenhofer, 1988; Deisenhofer et al., 1985) and assuming a dipole strength of the Q_y band of 51 D^2 (BChl *b*) and of 23 D^2 (Chl *a*), an excitonic coupling of 430 cm^{-1} would be expected. Therefore, a geometry of P680 identical to that of the special pair in the bacterial reaction center can be excluded. As a further comparison, if we take the structure of the bacterial reaction center and substitute BChl *vs* Chl and BPheo *vs* Pheo (dipole strength of the Pheo Q_y band = 14 D^2), we can calculate the predictable interactions between the pigments. The interaction between the Chl, which is the counterpart of the M-side BChl of the special pair and the nearest monomeric Chl (counterpart of the monomeric L-side BChl), is calculated to be 105 cm^{-1}, while the interaction between Pheo (L-side) and the nearest monomeric Chl is calculated to be 90 cm^{-1}. These calculations show that

interactions among other pigments in the reaction center are comparable to those obtained in the simulations done above.

An additional argument that the primary donor must be quite differently organized in PS II compared to the bacterial reaction center comes from EPR measurements with oriented samples. It was shown that the plane of the Chl bearing the triplet state is PS II is tilted 30° out of the membrane plane (Rutherford, 1985; Van Mieghem et al., 1991), while in the special pair of the bacterial reaction center, both BChls are nearly perpendicular to the membrane plane. On the other hand, a high homology of the amino acid sequences of the L and M and the D1 and D2 proteins exists. In particular, the two histidines ligating the BChls of the special pair in the bacterial reaction center are conserved in PS II (Trebst, 1986; Michel & Deisenhofer, 1988), suggesting that Chl counterparts to these BChls are present in PS II. One proposal made by Van Mieghem et al. (1991) was that P680 is a Chl which is an analogue to one of the monomeric BChls in the bacterial reaction center, which are tilted about 30° out of the membrane plane. In this proposal, it was considered that excitonic coupling occurred between the Chl monomer and one or both of the chlorophylls which are structurally analogous to the constitutents of the bacterial special pair. A weakness of this model is that the histidines His L153 and His M180, which are ligands to the accessory BChls in the bacterial rection center, are missing in D1 and D2 (Trebst, 1986; Michel & Deisenhofer, 1988), and therefore the structural analogy in this region must be questioned although, however, histidine ligation is not obligatory in Chl-containing proteins (Kühlbrandt et al., 1994).

An alternative model, also proposed by Van Mieghem et al. (1991), involved a rearrangement of the two special pair Chls so that one of the Chls is tilted 30° out of the membrane plane and carries the triplet state at low temperatures, while the ring plane of the second Chl is oriented perpendicular to the membrane as in the bacterial reaction center. This changed orientation may lead to a weaker exciton coupling caused by different angles between the Q_y transition moments or between the Q_y transitions and the line connecting the Mg atoms. A larger distance between the two Chls forming this "dimer" might also be responsible for a weaker coupling. This model was recently favored by Noguchi et al. (1993), who suggested, on the basis of FTIR measurements, that this different conformation might be forced by additional hydrogen bond formation between the chlorophyll carbonyl groups with the surrounding protein. In this model the charge separation between P680 and Pheo occurs over a distance similar to that between the special pair and BPheo in the bacterial center. As a consequence, according to the distance dependence of electron-transfer rates (Moser et al., 1993), an intermediate, functionally equivalent to monomeric BChl, would be required in this model too in order to explain the similar electron-transfer kinetics. A weakness of this model, as pointed out by Van Mieghem et al. (1991), is that tilting of a chlorophyll molecule from a position parallel to the transmembrane α-helices to a position closer to being parallel to the membrane plane would require significant perturbation of the protein structure. These models were put forward to maintain as far as possible the structural analogy to the bacterial reaction center. It is clear, however, that a variety of structural models, which allow greater variations between the arrangement of the pigments in both reaction centers, could explain the current spectroscopic data of PS II.

Comparison of $P680^+Q_A^-/P680Q_A$ and $^3P680/P680$ Spectra. The low-temperature difference spectrum associated with the formation of the secondary radical pair, $P680^+Q_A^-$, is characterized by a strong, narrow bleaching band, centered at 675 nm, a strong absorbance increase at 680.5 nm, and a smaller bleaching band at 687 nm (see Figure 4). Additionally, a small absorbance increase at 667 nm was observed. The low-temperature spectra of $P680^+Q_A^-/P680Q_A$ reported previously had their maximum bleaching at 680 nm (Ke & Dolan, 1980; Ke et al., 1982) and did not show the sharp features observed in this work. However, this could have been due to a lower spectral resolution in the previous work.

The direct comparison of the low-temperature spectra of $^3P680/P680$ and $P680^+/P680$ shows that the main bleaching bands are centered at 675 and 684 nm, respectively. This result leads to the question of whether the positive charge and the triplet state are localized on the same pigment. The migration of the triplet state formed by charge recombination from P680 to another Chl has been discussed before (Rutherford, 1986). It would be the latter Chl which would correspond to the 30° oriented Chl as measured by EPR of the triplet state (Rutherford, 1985; Van Mieghem et al., 1991). No evidence for such triplet transfer was obtained either from kinetic analysis of the absorbance changes in the Q_y region reported here or from kinetic EPR studies of oriented PS II (F. J. E. Van Mieghem, C. H. Bock, A. W. Rutherford, and D. Stehlik, unpublished results; Van Mieghem, 1994). These results, however, do not rule out the possibility that the rate of the triplet transfer might be faster than primary pair decay. On the other hand, hole-burning experiments indicate that the triplet state is localized on P680 itself (Jankowiak & Small, 1993; Kwa, 1994b). In addition, results from FTIR studies are consistent with the triplet state being located on P680 (Noguchi et al., 1993). In the following discussion, we provide an explanation for the different positions of the bleaching maxima found for the $^3P680/P680$ and $P680^+/P680$ spectra in which triplet and positive charge is assumed to be located on the same pigment [an alternative explanation for the spectra involving triplet transfer was additionally discussed by Van Mieghem (1994)].

The temperature dependence of the $P680^+Q_A^-/P680Q_A$ spectrum (see Figure 4) suggests that an electrochromic band shift becomes dominant when the temperature is lowered. The observation that absorption changes due to electrochromic band shifts increase upon lowering the temperature can be explained for the most part by the sharpening of absorption bands at cryogenic temperatures. The red shift of an absorbance band located at about 675 nm, which is induced by the electric field of $P680^+$ and Q_A^-, probably gives rise to the absorbance decrease at 675 nm and the absorbance increase at 681 nm. In a simplified approach toward simulating the $P680^+Q_A^-/P680Q_A$ spectrum the following assumptions were made: (a) The positive charge is located on the same pigment as that of the triplet state; i.e., the formation of $P680^+$ in itself leads to a bleaching at 685 nm. A Gaussian band at 685 nm with FWHM of 5 nm and a dipole strength of one Chl *a* molecule (dashed line in Figure 7c) accounts for this bleaching. Smaller spectral features due to the loss of weak excitonic coupling with neighboring pigment(s) as observed in the $^3P680/P680$ spectrum are neglected for the sake of simplicity. (b) The

absorbance of the Chl cation is neglected. (c) An electrochromic red shift occurs. The dotted line in Figure 7c describes the red shift of a Gaussian band with a dipole strength of one Chl *a* from 675 to 683 nm. The result of this simulation (solid line in Figure 7c) describes the main features of the experimental $P680^+Q_A^-/P680Q_A$ spectrum (Figure 4) quite well except for the weak positive band around 665 nm. One possible explanation for this additional band is that the radical cation $P680^+$ absorbs more strongly than the ground state of P680 in this wavelength region.

The assumption of an electrochromic band shift of several nanometers is not unreasonable according to calculations for the electrochromic effect of a charge on the special BChl pair on the BChl monomer in the bacterial reaction center (Eccles et al., 1988). It is assumed that the strong red shift of the 675-nm band is caused by the electric field of $P680^+$ (spectral shifts due to the formation of Q_A^- are discussed below). It may be assumed that the pigment shifted by the charge on $P680^+$ is that which is closest to it, i.e., presumably that which is excitonically coupled to the pigment bearing the charge after oxidation of P680. The simulation of the triplet spectrum shown in Figure 7b is consistent with this assumption. However, we cannot rule out alternative assignments for the shifted 675-nm absorption band. (1) The band can be ascribed to a pigment other than that which is excitonically coupled in P680. (2) The band shift can result from a smaller shift of an absorbance band with an oscillator strength of more than one Chl, i.e., a weaker electrochromic effect on several pigments including possibly Chls from the core antenna, absorbing around 680 nm. However, to avoid oxidation of Chls by the highly oxidizing species $P680^+$ under physiological conditions, the distance between the primary donor and Chls with usual redox potentials should be rather large (Van Gorkom & Schelvis, 1993), and it is therefore less likely that the shifted pigment(s) is (are) antenna Chls. Comparing absorbance difference spectra of charge-separated states measured in chromatophores and reaction centers of bacterial organisms, there is—according to our knowledge—also no indication of electrochromic band shifts of antenna pigments.

It has been reported that the formation of Q_A^- is accompanied by a blue shift of a band centered at 685 nm (Van Gorkom, 1974) giving rise to a bleaching around 690 nm and an absorbance increase around 680 nm, both corresponding to a differential extinction coefficient of approximately 5000 M^{-1} cm^{-1} at room temperature. If such a blue shift is taken into account in the simulation of the $P680^+Q_A^-/P680Q_A$ spectrum (not shown), an acceptable simulation can be achieved by shifting the 675-nm band slightly more to the red; i.e., the dominating red shift is still required to obtain the main bleaching at 675 nm instead of 685 nm as in the $^3P680/P680$ spectrum.

Interestingly, the electrochromic band shifts observed in bacterial reaction centers are in opposite direction to that seen in PS II. The positive charge on the primary donor induces a blue shift (on the absorbance band of the BChl monomer), and the negative charge on the first quinone acceptor induces a red shift (on the neighboring BPheo). As the magnitude and sign of electrochromic shifts depend on the arrangement of the involved pigments, these differences indicate also a different arrangement of the pigments in both reaction centers.

Comparison of the $P680^+Q_A^-/P680Q_A$ and $P680^+Pheo^-/P680Pheo$ Spectra. The low-temperature absorbance difference spectra of the primary radical pair obtained with PS II core complexes from *Synechococcus* and PS II-enriched membrane fragments from spinach show two distinct bleaching band at 674 and 684 nm. In the middle between these bands, the absorbance change is positive (Figures 2a and 3a) or close to zero (Figure 5a). The behavior of the spectra with temperature (see Figure 2a) is similar to that described above for the secondary radical pair (Figure 4) and might also be explained by the presence of an electrochromic band shift which becomes more pronounced at lower temperatures.

A comparison between the absorbance difference spectra of the primary and the secondary radical pair should yield information about the position of the Q_y absorption band of the pheophytin, which has been the subject of controversy (Nuijs et al., 1986; Braun et al., 1990; Van Kan et al., 1990; Van der Vos et al., 1993). Both spectra obviously differ in the region above 680 nm, where the $P680^+Pheo^-/P680Pheo$ spectrum seems to have an additional bleaching superimposed on the $P680^+Q_A^-/P680Q_A$ spectrum. At first glance, the bleaching at about 685 nm may be attributed to the formation of $Pheo^-$.

In intact PS II preparations, $Pheo^-$ accumulation at room temperature leads to a main bleaching at 685 nm and a positive band at 676 nm (Klimov et al., 1977). For comparison, we performed $Pheo^-$ accumulation at 77 K in PS II core complexes from *Synechococcus* and found a similar difference spectrum (not shown) that exhibits the main bleaching band at 685 nm with a FWHM of about 5 nm and an absorbance increase around 677 nm. If we superimpose this difference spectrum on the $P680^+Q_A^-/P680Q_A$ spectrum, the resulting simulation describes the main features of the $P680^+Pheo^-/P680Pheo$ spectrum, (not shown); however, the resulting bleaching at 684 nm is too large relative to the bleaching at 674 nm and the position of the absorbance increase is too far to the red.

Following the approach used by Ganago et al. (1982) to explain their room temperature LD measurements of $Pheo^-$, we deconvoluted the 77 K spectrum of photoaccumulated $Pheo^-$ into a bleaching at 681 nm due to the disappearance of the absorption of Pheo and a blue shift of an absorption band from 685 to about 680 nm due to the electric field of $Pheo^-$. The position of the shifted band indicates that the shift is induced on P680 itself. This has already been proposed in the literature on the basis of room temperature experiments (Ganago et al., 1982; Schlodder & Brettel, 1988; Breton, 1990). Therefore, we performed the following simulation of the $P680^+Pheo^-/P680Pheo$ spectrum using again somewhat simplifying assumptions: (a) A Gaussian Band at 685 nm with FWHM of 5 nm and a dipole strength of one Chl *a* molecule (dashed line in Figure 7d) accounts for the bleaching due to the oxidation of P680, identical to that used in the simulation of the $P680^+Q_A^-/P680Q_A$ spectrum. (b) The bleaching due to the reduction of the pheophytin is described by an additional Gaussian band centered at 681 nm and a dipole strength corresponding to approximately 60% of that of Chl *a* (Fujita et al., 1978) (broken line in Figure 7d). Assuming that it is the absorption band of P680 itself, which is shifted by $Pheo^-$ (see above), a spectral shift caused by $Pheo^-$ is not taken into account in the simulation of the $P680^+Pheo^-/P680Pheo$ spectrum. (c) The absorption of the Chl cation, the Pheo anion, and smaller

spectral features due to the loss of weak excitonic coupling with neighboring pigment(s) are neglected. $Pheo^-$ has a strong absorption band, but it is blue shifted by about 30 nm from the ground state Q_y absorption band. Contribution from this anion band might therefore be expected at about 650 nm, i.e., outside of the spectral range dealt with here. (d) As in the simulation of the $P680^+Q_A^-/P680Q_A$ spectrum, an electrochromic red shift of an absorption band at 675 nm with the dipole strength of one Chl is included to account for the bleaching at 675 nm and for the absorbance increase at about 679 nm. Thereby, it is assumed that the absorbance increase, which is much less pronounced than in the $P680^+Q_A^-/P680Q_A$ spectrum (compare Figure 2a or 3a with Figure 4 and Figure 5a with Figure 5b), is for the most part compensated by the bleaching upon Pheo reduction. The dotted line in Figure 7d describes the red shift of a Gaussian band with a dipole strength of one Chl *a* from 675 to 680.5 nm; i.e., to achieve a satisfying simulation, the 675-nm band is shifted less (5.5 nm instead of 8 nm) to the red than in the $P680^+Q_A^-/P680Q_A$ simulation. The result of this simulation (solid line in Figure 7d) resembles closely the experimental $P680^+Pheo^-$/P680Pheo spectrum (Figures 2a, 3a, and 5a).

The $P680^+Pheo^-$/P680Pheo spectrum measured with D1/D2/cyt *b*-559-type preparations [see Figure 6a and Van Kan et al. (1990)] is quite different from those we measured with more intact PS II preparations (see Figures 2a, 3a, and 5a). There is only one broad bleaching band with FWHM of about 13 nm. As pointed out by Van Kan et al. (1990), it seems as if an additional bleaching band around 675 nm is simply superimposed on the $^3P680/P680$ spectrum. In contrast to these authors we do not attribute this band to the pheophytin reduction. The assignment of an absorbance band at 676 nm to the pheophytin requires further assumptions in order to explain CD data of $Pheo^-$ in reaction centers (Otte et al., 1992), and it is also contradictory to results of spectral hole-burning experiments (Jankowiak & Small, 1993).

On the basis of our results obtained with the intact PS II preparations, we attribute the bleaching at 675 nm in the D1/D2/cyt *b*-559-type preparation also to an electrochromic red shift caused by $P680^+$. The strikingly different spectrum in the D1/D2/cyt *b*-559-type preparation (compared to that of intact PS II at the same temperature) can be explained by the assumption that the 685-nm band of P680 is shifted to shorter wavelengths (680.5 nm) due to the isolation procedure used for the D1/D2/cyt *b*-559-type preparations. Such a blue shift has already been observed in the triplet difference spectra. To simulate the $P680^+Pheo^-$/P680Pheo spectrum measured in the D1/D2/cyt *b*-559-type preparation, it was additionally assumed that the absorbance bands are inhomogeneously broadened (alternatively, different discrete spectral forms of P680 may be assumed) and that the pigment absorbing at 675 nm is shifted 4 nm to the red. The simulation using this interpretation (shown in Figure 7e) describes the main features of the experimental $P680^+Pheo^-$/P680Pheo spectrum in the D1/D2/cyt *b*-559-type preparation (Figure 6a) quite well. In agreement with this simulation is the observation that the absorbance difference spectrum obtained by photoaccumulation of $Pheo^-$ at 77 K in the D1/D2/cyt *b*-559-type preparation leads to a main bleaching at 682 nm and a positive band at 675 nm (not shown). According to the deconvolution of the difference spectrum induced by photoaccumulation of $Pheo^-$ (Ganago et al., 1982; Breton, 1990) described above, the shift to shorter wavelength can be mainly ascribed to the shift of the P680 absorption band from 685 nm in more intact PS II preparations to about 680 nm in D1/D2/cyt *b*-559 preparations.

Pronounced differences between the $P680^+Pheo^-/P680Pheo$ spectra measured with D1/D2/cyt *b*-559-type preparations and with more intact PS II preparations were found in this work at low temperature. At room temperature the spectra are rather similar in the different preparations [compare, e.g., Schlodder and Brettel (1988) and Durrant et al. (1990)] presumably due to broadening of the absorption bands which obscures the differences visible at low temperatures. Summarizing, the simulations of the $P680^+Pheo^-/P680Pheo$, $^3P680/P680$, and $P680^+Q_A^-/P680Q_A$ spectra at cryogenic temperature lead to a consistent assignment of the absorption bands of P680 and of Pheo if it is assumed that a strong red shift of an absorption band at 675 nm is induced by the electric field of $P680^+$ in the charge-separated states. To prove this interpretation, the analysis of the linear dichroism of the time-resolved difference spectra is in progress.

We assume that the red shift which is strong at low temperature but weakens with increasing temperature may still contribute significantly to the difference spectra of $P680^+Q_A^-/P680Q_A$ and $P680^+Pheo^-/P680Pheo$ at room temperature; i.e., the bleaching observed at 680 nm upon $P680^+$ formation at room temperature is a combination of the red shift and the bleaching of P680 itself. To clarify this point, the measurement of the $^3P680/P680$ spectrum at higher temperatures in intact PS II preparations is required.

ADDED IN PROOF

After submission of the manuscript, we became aware of a recent ADMR study on PS II membranes from spinach by Carbonera et al. (1994). They obtained a low-temperature difference spectrum for the formation of $^3P680$ which resembles closely the spectrum presented here (Figure 5b, closed circles).

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