# Charge Recombination Reactions in Photosystem II. 1. Yields, Recombination Pathways, and Kinetics of the Primary Pair†

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ABSTRACT: Recombination reactions of the primary radical pair in photosystem II (PS II) have been studied in the nanosecond to millisecond time scales by flash absorption spectroscopy. Samples in which the first quinone acceptor ($Q_A$) was in the semiquinone form ($Q_A^-$) or in the doubly reduced state (presumably $Q_AH_2$) were used. The redox state of $Q_A$ and the long-lived triplet state of the primary electron donor chlorophyll ($^3$P680) were monitored by EPR. The following results were obtained at cryogenic temperatures (around 20 K). (1) The primary radical pair, $P680^+Pheo^-$, is formed with a high yield irrespective of the redox state of $Q_A$. (2) The decay of the primary pair is faster with $Q_A^-$ than with $Q_AH_2$ and could be described biexponentially with $t_{1/2} \approx 20$ ns (≈65%)/150 ns (≈35%) and $t_{1/2} \approx 60$ ns (≈35%)/250 ns (≈65%), respectively. The different kinetics may be due to electrostatic and/or magnetic effects of $Q_A^-$ on charge recombination or due to conformational changes caused by the double reduction treatment. (3) The yield of the triplet state $^3$P680 was high both with $Q_A^-$ and $Q_AH_2$. (4) The triplet decay was much faster with $Q_A^-$ [$t_{1/2} \approx 2$ μs (≈50%)/20 μs (≈50%)] than with $Q_AH_2$ [$t_{1/2} \approx 1$ ms (≈65%)/3 ms (≈35%)]. The short lifetime of the triplet with $Q_A^-$ explains why it was not detected earlier. The mechanism of triplet quenching in the presence of $Q_A^-$ is not understood; however it may represent a protective process in PS II. (5) Almost identical data were obtained for PS II-enriched membranes from spinach and PS II core preparations from *Synechococcus*. Room temperature optical studies were performed on the *Synechococcus* preparation. In samples containing sodium dithionite to form $Q_A^-$ in the dark, EPR controls showed that multiple excitation flashes given at room temperature led to a decrease of the $Q_A^-Fe^{2+}$ signal, indicating double reduction of $Q_A$. During the first few flashes, $Q_A^-$ was still present in the large majority of the centers. In this case, the yield of the primary pair at room temperature was around 50%, and its decay could be described monoexponentially with $t_{1/2} \approx 8$ ns (a slightly better fit was obtained with two exponentials: $t_{1/2} \approx 4$ ns (≈80%)/25 ns (≈20%). After 2000 flashes and subsequent dark adaptation for 20 min (in order to form the state P680 Pheo $Q_AH_2$), the yield of the primary pair was close to 100%, and its decay was slower [$t_{1/2} \approx 13$ ns or $t_{1/2} \approx 5$ ns (≈50%)/20 ns (≈50%). On the basis of these results and earlier work in the literature, we present a hypothesis providing a qualitative explanation for the photochemistry of PS II with regard to its dependence on temperature and the redox state of $Q_A$. This incorporates (a) an electrostatic effect of $Q_A^-$ which increases the standard free energy of $P680^+Pheo^-$ compared to centers containing $Q_A$ or $Q_AH_2$ or lacking $Q_A$, (b) exergonic primary charge separation at cryogenic temperatures, even in the presence of $Q_A^-$, and (c) an *effective* free energy for the excited state (equilibrated between P680 and the antenna chlorophylls) which decreases with increasing temperature, this decrease being more pronounced the larger the antenna system. Under physiological conditions, factors a and c may conspire to diminish charge separation in PS II whenever $Q_A^-$ is accumulated.

Photosystem II (PS II)[1] is a membrane-bound pigment−protein complex which catalyzes light-induced transfer of electrons from water to plastoquinone in oxygenic photosynthetic organisms (higher plants, algae, and cyanobacteria) [for a recent review, see Hansson and Wydrzynski (1990)]. The structure of the purple bacterial reaction center, which has been deduced from X-ray diffraction, is used as a structural model for PS II (Michel & Deisenhofer, 1988). The counterparts of the L and M polypeptides of the purple bacterial reaction centers are the D1 and D2 polypeptides in PS II. Spectroscopic studies indicate that the electron-transfer reactions in PS II and the cofactors involved are

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[1] Abbreviations: PS II, photosystem II; Chl, chlorophyll; P680 or P, primary electron donor of PS II; Pheo or Ph, pheophytin; $Q_A$ and $Q_B$, first and second quinone-type electron acceptor, respectively; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EPR, electron paramagnetic resonance; $t_{1/2}$, half-life; $\tau$, lifetime.

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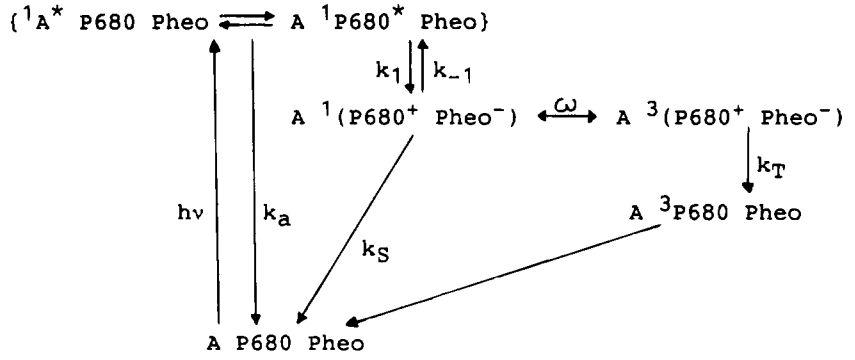
Scheme 1




similar to purple bacteria, with the exception of the components involved in the oxidation of water. In PS II, following light absorption by antenna pigments and excitation energy transfer to the reaction center, an electron is transferred within approximately 10 ps from the lowest excited singlet state of the primary electron donor, P680 (a special Chl *a* molecule), to the primary acceptor, a pheophytin *a* (Pheo). $Pheo^-$ transfers the electron within approximately 400 ps to the secondary acceptor, $Q_A$ (plastoquinone), and from there it moves within a few hundred microseconds to $Q_B$, another plastoquinone, which is, however, exchangeable with other members of the plastoquinone pool and functions as a two-electron gate. $P680^+$ is rereduced within 20−200 ns by a tyrosine, TyrZ, which subsequently (within 50 $\mu$s to 1 ms) oxidizes the Mn cluster, accumulating four oxidizing equivalents prior to $O_2$ evolution from water. The charge-accumulating states are known as S-states ($S_0$−$S_4$).

When electron transfer from $Pheo^-$ to $Q_A$ is blocked (by prereduction or extraction of $Q_A$), the resulting photoreactions may be represented by Scheme 1, which is based on the well-characterized situation occurring in the bacterial reaction center. In Scheme 1, A represents one of a variable number of antenna pigments. $k_a$ is the intrinsic decay rate of the quasiequilibrium between excited antenna pigments and excited P680; it represents all decay routes (including intersystem crossing to the triplet state of the pigment, which is omitted for simplicity in Scheme 1), except for primary charge separation (rate $k_1$). The primary pair, $P680^+$ $Pheo^-$, is initially created in a singlet configuration of the two unpaired electron spins but can evolve (due to hyperfine interactions) to a triplet spin configuration (and back again), as indicated by the symbol $\omega$ in Scheme 1. Charge recombination in the primary pair will produce either the singlet ground state of P680 (rate constant $k_S$) or the triplet state of P680 (rate constant $k_T$), depending on the spin configuration of the primary pair at the moment of recombination.

Prereduction of $Q_A$ to form $Q_A^-$ (changing PS II from the "open" to the "closed" state) can be achieved by addition of a reductant (e.g., dithionite) or by illumination under conditions in which the overall rate of electron input to PS II (from water) exceeds electron output (into the plastoquinone pool) [see Krause and Weis (1991) for a recent review]. It has been proposed that the presence of the semiquinone anion $Q_A^-$ in closed PS II may raise the energy of the primary pair by an electrostatic interaction, so that the driving force of primary charge separation is decreased compared to open PS II (Van Gorkom, 1985; Van Grondelle, 1985; Schatz & Holzwarth, 1986).

Studies by flash absorption spectroscopy of the photoreactions in closed PS II yielded rather different results for different samples (PS II prepared from different organisms, using different extraction methods, and having different antenna sizes) and even contradictory results for virtually identical samples. At room temperature, for example, according to Shuvalov et al. (1980), the primary pair is formed with a high yield and decays with a lifetime of approximately 4 ns in a closed PS II preparation from spinach (30−40 chlorophylls per P680). Schatz et al. (1987, 1988), studying a closed PS II preparation from the cyanobacterium *Synechococcus* sp. (approximately 80 chlorophylls per P680), reported that the primary pair was formed with a two to three times lower yield than in open centers and had a lifetime of only approximately 1.7 ns [they exclude the possibility of the presence of more than 15% of a longer lived primary pair (lifetime exceeding 2 ns)]. Schlodder and Brettel (1988), however, using a virtually identical sample, reported the observation of the primary pair with a yield of approximately 60% and a lifetime of 11 ns. A broad range of different yields and lifetimes of the primary pair was found in other studies [reviewed by Hansson and Wydrzynski (1990)].

A clear controversy arose from studies at cryogenic temperatures. Van Mieghem et al. (1989) monitored by EPR both the reduction state of $Q_A$ (in the dark) and the steady-state concentration during continuous illumination of the spin-polarized triplet state of P680 formed via charge recombination in the primary pair ($k_T$ in Scheme 1) in PS II membranes from spinach.[2] Surprisingly, virtually no triplet could be detected when $Q_A$ was singly reduced by dithionite (characterized by the presence of a large EPR signal attributed to the complex of $Q_A^-$ with the nearby $Fe^{2+}$). Only when stronger reducing conditions (chemical or photochemical) were applied could the spin-polarized triplet state of P680 be detected. In this case, however, the $Q_A^-Fe^{2+}$ signal was no longer present, indicating that $Q_A$ had become doubly reduced and presumably protonated (forming the quinol $Q_AH_2$) (Van Mieghem et al., 1989). The authors favored the explanation that primary charge separation depends on the reduction state of $Q_A$, namely, that it is inhibited in the presence of $Q_A^-$ due to repulsive electrostatic interaction with

[2] There has been some discussion whether the spin-polarized triplet state observed in EPR is located on P680 or on another Chl *a* molecule to which the triplet excitation is transferred from $^3$P680 (Rutherford, 1986; Van Mieghem, 1994). However, recent data from hole burning [Kwa (1993); see also review by Jankowiak and Small (1993)] and Fourier transform infrared spectroscopy (Noguchi et al., 1993) and the analysis of transient optical spectra in the accompanying paper (Hillmann et al., 1995) provide evidence that the triplet state stays on P680.

$Pheo^-$, whereas normal primary charge separation takes place in the presence of electrically neutral $Q_AH_2$, and only in the latter case would triplet formation via charge recombination be possible. The same electrostatic argument could explain the high primary pair and triplet yields observed at low temperature in D1/D2/cyt *b*-559 reaction center preparations which lack $Q_A$ (Takahashi et al., 1987; Van Kan et al., 1990).

In contrast to the proposal that the low triplet yield in the presence of $Q_A^-$ is due to a low yield of charge separation, Schlodder and Brettel (1990) reported that the yield of primary pair formation in the PS II core from *Synechococcus* increases with decreasing temperature to close to 100% (below 100 K), when the sample had been dark incubated with dithionite for only a few minutes at room temperature to reduce $Q_A$. A similar result was obtained even in the absence of dithionite, when the sample was illuminated at 200 K in order to trap PS II in the state $S_2P680PheoQ_A^-$ (Schlodder & Hillmann, 1992). However, EPR controls had not been done in these samples, leaving ambiguity concerning the intactness and the redox state of the quinone−iron complex.

In the present work, we clarify the apparent contradictions with respect to the yield and lifetime of the primary pair and to triplet formation at low temperature in closed PS II by applying in parallel EPR and flash absorption spectroscopy on both PS II-enriched membranes from spinach and PS II core complexes from *Synechococcus*. In the course of this study, it was discovered that the low-temperature absorbance difference spectra for formation of the primary pair and the triplet state of P680 are much more structured and hence potentially more informative than those observed in a D1/D2/cyt *b*-559 reaction center preparation (Van Kan et al., 1990). The new spectral information and its implications will be treated in the accompanying paper (Hillmann et al., 1995). 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 Preparation.* PSII-enriched thylakoid membrane fragments were prepared from spinach according to Berthold et al. (1981) using the modifications described by Ford and Evans (1983) and stored in the dark at −80 °C until used. Samples with doubly reduced $Q_A$ were prepared as described by Van Mieghem et al. (1992) by incubation for 1.5 h in the presence of 40 mM sodium dithionite, 100 μM benzyl viologen, and 200 mM sodium formate at room temperature in the dark under argon. These samples were stored under argon and at −80 °C in the dark.

For (transient) absorbance measurements at low temperature, these samples were brought into a buffer containing 60% (v/v) glycerol, 20 mM MOPS (pH 7.0), 10 mM NaCl, 5 mM $MgCl_2$, 125 mM sucrose, and 20−40 mM dithionite. The sample with singly reduced $Q_A$ was prepared by mixing a sample from a concentrated stock solution, still at ambient redox potential, with the dithionite-containing buffer under argon, transferring it under argon into the cuvette, letting it incubate in the dark under argon for approximately 5 min, and then inserting it (sealed) in near darkness into a precooled optical cryostat (helium flow model from SMC). For the sample with doubly reduced $Q_A$, a sample from the already doubly reduced stock (see above) was used; in this case the sample was not incubated but immediately cooled down in the cryostat.

$O_2$-evolving PS II core complexes contaning approximately 40 chlorophylls per P680 were prepared from the cyanobacterium *Synechococcus* sp. according to Schatz and Witt (1984), subsequently purified by sucrose density centrifugation (Rögner et al., 1987) and by use of a Q-Sepharose column [for details, see the accompanying paper (Hillmann et al., 1995)], and stored at −25 °C in the dark. Double reduction of $Q_A$ in these samples was accomplished by 10-min illumination with white light from a 800-W tungsten lamp at room temperature in the presence of 40 mM dithionite, 100 μM benzyl viologen, and 200 mM formate, followed by dark adaptation for 25 min in order to allow reoxidation of accumulated $Pheo^-$ [see Van Mieghem et al. (1989)]. The extent of double reduction, using this method, was estimated by EPR to be 85% (see Results).

In order to get an indication of the extent of $Q_A$ double reduction during repetitive flash absorption measurements in the presence of dithionite at room temperature, we illuminated a PS II core sample from *Synechococcus* with saturating laser flashes (532 nm, 300 mJ per pulse) in the presence of 40 mM dithionite. After a certain number of flashes, the sample was kept in darkness for 30 min, and then it was frozen for low-temperature EPR measurements. As expected, the amplitude of the $Q_A^-Fe^{2+}$ signal decreased, and the amplitude of the spin-polarized triplet increased with the number of excitation flashes at room temperature, indicating double reduction of $Q_A$. The half-maximum effect was reached with approximately 500 flashes.

Sample preparation for low-temperature flash absorption measurements followed the same procedure as for PS II membranes from spinach, except for the buffer, which contained 20 mM MES/NaOH (pH 6.5), 10 mM $MgCl_2$, 20 mM $CaCl_2$, 0.025% dodecyl β- D-maltoside, and glycerol and dithionite as given above.

For flash absorption measurements at room temperature, glycerol was omitted from the buffer.

*Instruments.* EPR spectra were recorded using a Bruker 200 X-band spectrometer fitted with an Oxford Instruments cryostat and temperature control system. In order to record spectra during illumination, an 800-W tungsten projector was used. The light was filtered through 2 cm of water and three Calflex (Balzers) heat filters.

Flash absorption spectroscopy in the 680-nm region was carried out at 20 K in an optical cryostat cooled with helium gas. The sample was in a plastic cuvette with optical paths of 10 mm for the measuring light and 4 mm for the excitation light; the cuvette windows perpendicular to the excitation beam are diffusing in order to improve the homogeneity of the excitation. The temperature of the sample was measured by a temperature sensor close to the cuvette.

The sample was excited at 532 nm using a frequency-doubled Nd:YAG laser (Quantel) with a pulse width at half-maximum amplitude of 300 ps and pulse energies up to 20 mJ at a repetition rate of 5 Hz. The exciting beam was expanded and attenuated with neutral density filters. The resulting excitation energies per area at the position of the sample (in the cryostat) could not be measured directly. The values given in the Results section are only approximate, based on the pulse energies measured outside the cryostat and estimates for the losses due to the windows of the cryostat and the cuvette and for the beam cross-section at

the position of the sample. The relative energies per area (comparing different measurements), however, are precise within ±5%.

As measuring light source, CW laser diodes (Toshiba TOLD 9140 and 9215, emitting up to 20 mW) were used, fixed in a temperature-regulated holder and operated by a laser diode control system (LDC 400 from Profile). The emission wavelength of the laser diode was varied by changing its temperature and controlled using a monochromator. The measuring light was focused through the cuvette and through interference and cutoff filters (to reduce fluorescence contributions) on a photodiode (FND 100 from EG&G), the output of which was amplified (using an IV72A amplifier from the Hahn-Meitner Institut, Berlin) and measured with a transient digitizer (DSA 602A with plug-in 11A72 from Tektronix). The electronic bandwidth of the detection system was 500 Hz−100 MHz. In order to minimize actinic effects, the measuring light was pulsed, using the combination of a chopper and a shutter. The pulse duration thus obtained was 70 μs. In order to correct for the fact that the plateau of the pulses was not completely flat, the baseline was subtracted from the absorbance change measurements.

For measurements on time scales exceeding 60 μs, continuous measuring light was used, but it was attenuated by a factor of approximately $10^4$. The output of the photodiode in a 100-kΩ load resistance was amplified by a Tektronix AM 502 amplifier. The electronic bandwidth of the detection system was DC − 10 kHz.

Low-temperature flash absorption measurements were also performed at 820 nm. The setup for these measurements was the same as for the 680-nm region, except that the measuring light (from a laser diode SDL-5411-G1 from Spectra Diode Labs) was always continuous.

For room temperature flash absorption measurements at 820 nm, the cryostat was replaced by an open cuvette holder, and the electronic bandwidth of the detection system was extended to approximately 500 Hz−300 MHz.

Fitting of the absorbance change transients to a (multi)-exponential decay was performed on a personal computer, using a Marquardt least-squares algorithm (program kindly provided by Dr. Pierre Sétif). Time zero was chosen as the position of the maximum of the response of the detection system to scattered light from the excitation laser. Fitting was usually started later (see figure legends), but the decay phases were extrapolated back to time zero and the thus obtained extrapolated initial amplitudes were used for yield estimates.

*Correction for Light-Induced Signal Loss.* When repeated low-temperature flash absorption measurements were performed on the same sample, it was observed that the amplitude of the absorbance changes irreversibly decreased. This is explained by low quantum yield photoaccumulation of $Pheo^-$ due to the excitation light and the (red) measuring light [see Rutherford & Mathis (1983)]. The signal loss was estimated by measuring the absorbance change at some reference wavelengths several times during the course of the measurements. After the correction of a difference spectrum for the signal loss during the measurements, it was scaled to the very first measurement made. Despite the latter correction, there may remain some overall underestimation of the amplitude of the spectrum, due to exposure of the sample to light before the first measurement (e.g., during the alignment of the optics).

*Flattening Correction.* In order to correct the amplitude of the absorbance changes measured in the spinach PS II membranes for particle flattening [originally described by Duysens (1956); see, e.g., also Amesz (1964) and Pulles (1978)], we adapted the procedure described recently in Buser et al. (1992) to low temperature. This is briefly described below [for a detailed description, see Van Mieghem (1994)].

We first recorded the absorbance spectrum of the sample in a plexiglas cuvette (optical path, 2.7 mm) at 20 K, in the same buffer as that used in the time-resolved measurements, but without dithionite. This was done with a Cary 2300 UV-VIS-NIR spectrophotometer (Varian) equipped with the same cryostat as used in the time-resolved measurements. Spectra were recorded in the range 500−800 nm, with the following instrument settings: wavelength interval, 1 nm; spectral bandwidth, 1 nm; time constant, 0.5 s; scan rate, 1 nm/s. Furthermore, the beam size reduction (to 1/3) facility was used.

The spectrum was corrected for light scattering by the method of Latimer and Eubanks (1962): spectra were recorded in both the presence and absence of a diffusing plate behind the cuvette. The relative error in the scatter-corrected absorbance spectrum at 20 K is estimated to be approximately 10%, due to some lack of reproducibility in sample positioning.

Second, we determined the value of the wavelength-independent parameter $p$ [the projected area of all particles divided by the total illuminated area (Duysens, 1956)]. This was done at room temperature, based on the (scatter corrected) absorbance of a sample in buffer (containing glycerol) and a sample in ethanol in the peak at 435 nm [see Pulles (1978) for a detailed description]. To measure these spectra, the Cary spectrophotometer was equipped with its original cuvette holder, which allows good reproducibility of sample positioning. Correction for scattering again was done using the method of Latimer and Eubanks (1962). It was found that the value of $p$ in samples treated with dithionite and benzyl viologen (for measurements under conditions of doubly reduced $Q_A$) was greater (1.1 for a chlorophyll concentration of 5 μg/mL and an optical path of 10 mm) than that of samples which had not been treated in this way (0.9 for the same chlorophyll concentration and optical path). This means [see Duysens (1956)] that the particle size was smaller in the dithionite/benzyl viologen-treated samples. From the values of $p$ and the scattering-corrected absorbance spectrum, the flattening correction factors can be determined as a function of wavelength [see Buser et al. (1992)]. We did this for the low-temperature spectrum, using $p$ values adjusted to the concentration and optical path of the sample at low temperature. Two sets of correction factors were derived and depicted in Figure 1, one set for dithionite/benzyl viologen-treated samples (open symbols) and the other for samples not treated with these chemicals (closed symbols). The 10% error in the scatter-corrected low-temperature absorbance spectrum introduces an uncertainty in the flattening correction, which we estimate to be 15% at 676 nm for dithionite/benzyl viologen-treated samples and to be 30% at 676 nm for untreated samples [the relative error is largest at the absorbance maximum (676

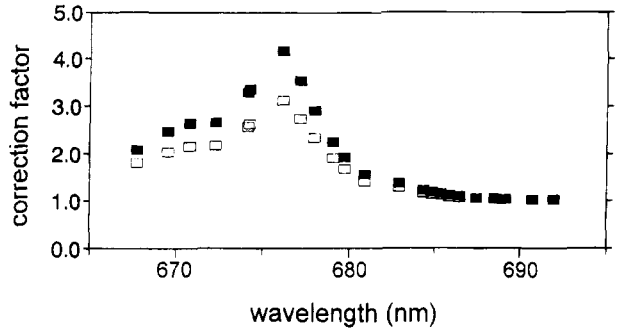



FIGURE 1: Flattening correction factors for PS II membranes from spinach at 20 K: open symbols, sample treated with dithionite and benzyl viologen in order to doubly reduce $Q_A$; closed symbols, untreated sample.

nm)]. An additional error may be caused by the assumption that the particles are identical and spherical.

It is of note that the flattening correction factors around the red absorption maximum for our PS II membranes are considerably larger than those determined for PS II membranes by Buser et al. (1992) and even slightly larger than those of spinach chloroplasts (Pulles et al., 1976). This presumably indicates aggregation of the PS II membranes used in the present study. Narrowing of the absorption bands at low temperature may also contribute to the unusually large flattening effect in our case (the lower correction factors cited above were obtained at room temperature).

## RESULTS

*EPR Measurements Relevant to Low-Temperature Flash Absorption Experiments.* PS II-enriched membranes from spinach with $Q_A$ in the doubly reduced state (Van Mieghem et al., 1989) were prepared by incubation with dithionite and benzyl viologen, as described in Materials and Methods. At different times during the incubation, the proportion of reaction centers with doubly reduced $Q_A$ was determined by EPR [see Van Mieghem et al. (1989)]. The formate-enhanced $Q_A^-Fe^{2+}$ signal (Vermaas & Rutherford, 1984) was maximal in the presence of dithionite only and decreased beyond detection after an incubation with dithionite and benzyl viologen for approximately 1.5 h at room temperature (see inset of Figure 2, traces a1 and b1). During this incubation, the amplitude of the light-inducible reaction center triplet signal, measured under continuous illumination by EPR [see Rutherford et al. (1981)], increased by a factor of approximately 10 (see inset of Figure 2, traces a2 and b2), in agreement with results in Van Mieghem et al. (1989, 1992).

PS II core complexes from *Synechococcus* were subjected to the same procedure. At the start of the incubation, the triplet EPR signal was very small and the $Q_A^-Fe^{2+}$ signal maximal (see inset of Figure 7, traces a1 and a2). After 3 h of incubation in the presence of benzyl viologen and dithionite, the triplet signal had increased considerably, but the $Q_A^-Fe^{2+}$ signal had only decreased to 50% of its initial size (not shown). Apparently, double reduction of $Q_A$ is more difficult in the *Synechococcus* preparation as compared to the spinach membranes. Double reduction of $Q_A$ can also be achieved by illumination at room temperature in the presence of dithionite (Van Mieghem et al., 1989). The $Q_A^-Fe^{2+}$ signal could indeed be further decreased in the *Synechococcus* sample by illumination at room temperature followed by dark adaptation for some 30 min (in order to allow for reoxidation of reduced pheophytin). The light-inducible triplet signal increased at the same time. These results are shown in the inset of Figure 7 (traces b1 and b2). From the size of the remaining $Q_A^-Fe^{2+}$ signal, we estimate that $Q_A$ was still singly reduced in approximately 15% of this sample.

*Synechococcus* samples with $Q_A$ singly reduced were also prepared in the absence of dithionite by illumination at 200 K, conditions under which only a single stable charge separation should take place forming the $S_2Q_A^-$ state (De Paula et al., 1985). The inset to Figure 8 shows the $S_2$ multiline EPR signal generated in this way (solid line; the broken line represents the control spectrum measured prior to the illumination at 200 K). Under different conditions of temperature and microwave power, we verified the formation of $Q_A^-Fe^{2+}$ by monitoring its signal at $g = 1.9$ (Rutherford & Zimmermann, 1984), and also we found very little formation of cyt *b*-559$^+$, Chl$^+$, and TyrD$^+$ by monitoring their respective signals [see Miller and Brudvig (1991) for a review]. The inset to Figure 8 shows, in addition to the $S_2$ multiline signal, a broad feature at around 400 mT ($g \approx 1.6$) which is attributed to the $Q_B^-Fe^{2+}Q_A^-$ state, generated by illumination in those centers in which $Q_B^-$ was present in the dark as seen in other cyanobacterial preparations (Hallahan et al., 1991).

*Low-Temperature Flash Absorption Measurements on PS II Membranes from Spinach.* Formation of the primary radical pair, P680$^+$Pheo$^-$, is expected to be accompanied by an absorbance increase at 820 nm (Takahashi et al., 1987). Figure 2 shows the transient absorbance changes at that wavelength in PS II membranes from spinach at 20 K induced by nonsaturating laser flashes (approximately 70 $\mu$J/cm$^2$). Traces a and b are from samples with $Q_A$ singly and doubly reduced, respectively, as checked by EPR (see above) prior to dilution of the samples for optical measurements. Both samples show a fast, instrument-limited absorbance increase with rather similar amplitudes. The subsequent decay of the signals in the nanosecond range is clearly faster in the sample with singly reduced $Q_A$ (trace a) than in the sample with doubly reduced $Q_A$ (trace b). The absorbance changes which remain at the end of the depicted time scale decay in the microsecond to millisecond range (see below). Fitting the decays with a single exponential for the nanosecond range [plus slower components determined from measurements on longer time scales (see below)] yielded half-lives of 62 and 188 ns for traces a and b, respectively, but the quality of these fits was rather poor. Better fits were obtained with two-exponential phases for the nanosecond range: trace a, $t_{1/2} = 36$ ns (15−50 ns) and $t_{1/2} = 201$ ns (100−300 ns) at an amplitude ratio of 2.5 (1−4); trace b, $t_{1/2} = 99$ ns (30−120 ns) and $t_{1/2} = 248$ ns (200−300 ns) at an amplitude ratio of 0.6 (0.2−1). The values in parentheses define the range of parameters which still yield an acceptable fit. These fits do not account for the "spike" seen in Figure 2 during the first 3 ns which is most likely due to the transient absorbance of singlet-excited chlorophylls (Huppert et al., 1976; Nuijs et al., 1986; Hansson et al., 1988). This spike will be omitted in all further analysis.

We attribute the transient absorbance changes depicted in Figure 2 to formation and subsequent recombination of the primary radical pair, P680$^+$Pheo$^-$ (see also Discussion). The rather similar extrapolated initial amplitudes indicate that the

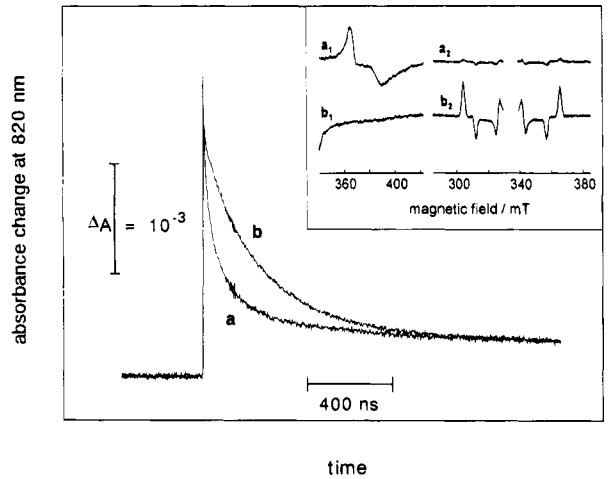



FIGURE 2: Absorbance changes at 820 nm in PS II membranes from spinach (122 $\mu$M Chl) at 20 K, induced by nonsaturating laser flashes (approximately 70 $\mu$J/cm$^2$) (average of 256 transients): trace a, sample treated with dithionite in order to obtain singly reduced $Q_A$; trace b, sample treated with dithionite and benzyl viologen in order to obtain doubly reduced $Q_A$ (see Materials and Methods for the procedures). Fitting in the time window from 15 to 1600 ns with two exponential decay phases yielded the following half-lives and relative amplitudes (extrapolated to time 0): trace a, $t_{1/2}$ = 36 ns (57.1%), 201 ns (22.7%), and 20.2% of the microsecond decay determined in Figure 3; trace b, $t_{1/2}$ = 99 ns (31.8%), 248 ns (53.3%), and 14.9% of the microsecond decay determined in Figure 3. The total extrapolated amplitudes of the fit functions are $\Delta A_{tot}$ = 2.06 × 10$^{-3}$ (trace a) and 2.23 × 10$^{-3}$ (trace b). Inset: EPR spectra of PS II membranes [2 mM Chl in a buffer containing 50 mM MOPS (pH 7.0), 300 mM sucrose, 1 mM EDTA, 10 mM NaCl, 5 mM $MgCl_2$, and 200 mM sodium formate] pretreated for single reduction of $Q_A$ (incubation with 40 mM dithionite for 8 min at room temperature; traces $a_1$ and $a_2$) and double reduction of $Q_A$ (incubation with 40 mM dithionite and 100 $\mu$M benzyl viologen for 1.5 h at room temperature; traces $b_1$ and $b_2$), respectively, measured under EPR conditions which allow monitoring of $Q_A^-Fe^{2+}$ (traces $a_1$ and $b_1$) and the light-induced spin-polarized triplet state of P680 (traces $a_2$ and $b_2$), respectively. Traces $a_1$ and $b_1$: dark spectra at 4.2 K; microwave power, 32 mW; microwave frequency, 9.44 GHz; modulation amplitude, 25 G. Traces $a_2$ and $b_2$: light minus dark difference spectra at 4.2 K; microwave power, 63 $\mu$W; other settings as for traces $a_1$ and $b_1$.

radical pair yield is similar for both reduction states of $Q_A$. The longer lived absorbance changes remaining at the end of the time scale of Figure 2 will be studied in more detail below.

In order to estimate the absolute radical pair yield, measurements like those shown in Figure 2 were performed at different excitation energies. For both samples, the signal amplitudes increased approximately three times for a 7-fold increase of the excitation energy (to approximately 500 $\mu$J/cm$^2$). There was no significant change in the kinetics up to this energy. Further increase of the excitation energy led to some increase of the contribution of long-lived ($t_{1/2}$ > 1 $\mu$s) absorbance changes, whereas the amplitude of the signal decaying in the nanosecond range was already saturated. The additional long-lived absorbance changes at "oversaturating" excitation energies may be due to triplet formation in antenna chlorophylls. The half-saturating energy for the signal decaying in the nanosecond range was approximately 100 $\mu$J/cm$^2$ both in the presence of singly and doubly reduced $Q_A$. Assuming an extinction coefficient of 12 400 M$^{-1}$ cm$^{-1}$ at 820 nm for the primary pair (Takahashi et al., 1987), the extrapolated initial absorbance changes at the virtually saturating excitation energy of approximately 500 $\mu$J/cm$^2$

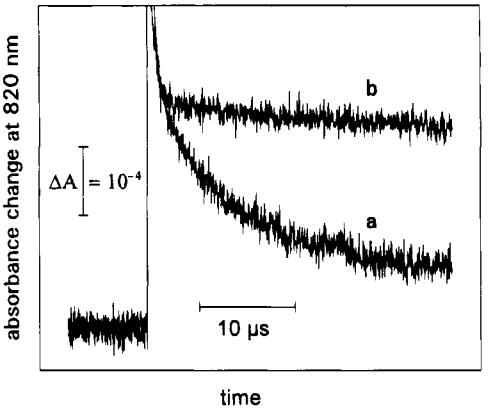



FIGURE 3: Continuation of the transients in Figure 2 on a microsecond time scale. Fitting yielded the following decay phases (in addition to the faster phases determined in Figure 2): trace a ($Q_A^-$), $t_{1/2}$ = 2.9 $\mu$s with $\Delta A$ = 2.28 × 10$^{-4}$ and $t_{1/2}$ = 27 $\mu$s with $\Delta A$ = 1.89 × 10$^{-4}$; trace b ($Q_AH_2$), $t_{1/2}$ = 187 $\mu$s with $\Delta A$ = 3.32 × 10$^{-4}$ (this decay is artificially induced by the AC coupling of the amplifier; the real half-life is approximately 1 ms, as measured with a DC-coupled detection system).

correspond to one primary pair per 240 and 220 chlorophylls in the presence of singly and doubly reduced $Q_A$, respectively. As the PS II membranes contain approximately 200–250 chlorophylls per P680 (Buser et al., 1992), it follows from this estimation that at low temperature the primary pair is formed in close to 100% of the centers, both in the presence of singly reduced and doubly reduced $Q_A$.

In samples with singly and with doubly reduced $Q_A$, the absorbance changes at 820 nm do not decay completely on the time scale depicted in Figure 2. This is not surprising for samples with doubly reduced $Q_A$ because the triplet state of P680 was detected by EPR (trace b2 in the inset of Figure 2). This triplet state lives for approximately 1–10 ms at liquid helium temperature (varying for the three sublevels) (Den Blanken et al., 1983; Rutherford et al., 1983, Searle et al., 1990) and absorbs at 820 nm (Takahashi et al., 1987). For samples with singly reduced $Q_A$, however, this triplet state has not been observed by EPR [see above and Van Mieghem et al. (1989)]. Flash absorbance measurements on a longer time scale revealed that the "long-lived" absorbance changes at 820 nm are in fact much shorter lived in samples with singly reduced $Q_A$ than in samples with doubly reduced $Q_A$. This is demonstrated in Figure 3 for PS II membranes from spinach. With singly reduced $Q_A$ (trace a), the signal decays largely on a microsecond time scale. With doubly reduced $Q_A$ (trace b), the absorbance change is stable on the time scale shown and finally decays within a few milliseconds (measured with DC coupling; not shown).

From these observations, it appeared necessary to determine the identity of the state which decays on a microsecond time scale when $Q_A$ is singly reduced. Therefore, the flash absorption measurements were extended to the red spectral region. Figure 4 shows kinetic traces on a nanosecond time scale at three characteristic wavelengths (traces a, $Q_A$ singly reduced; traces b, $Q_A$ doubly reduced) at virtually saturating excitation energy (approximately 500 $\mu$J/cm$^2$). The amplitudes of these signals are already corrected for the light-induced signal loss and for the flattening effect (see Materials and Methods).

The overall time course of the absorbance changes varies strongly with the detection wavelength, indicating that

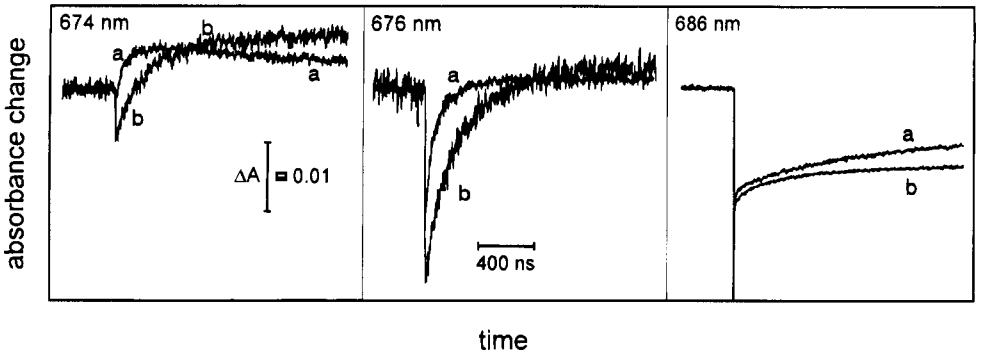



FIGURE 4: Absorbance changes at 674, 676, and 686 nm in PS II membranes from spinach at 20 K, induced by virtually saturating laser flashes (approximately 500 $\mu$J/cm$^2$). The labels a and b refer to samples with singly and doubly reduced $Q_A$, respectively (see Figure 2). The amplitudes were corrected for light-induced signal loss and flattening effect (see Materials and Methods) and normalized to a common Chl concentration of 14.5 $\mu$M.

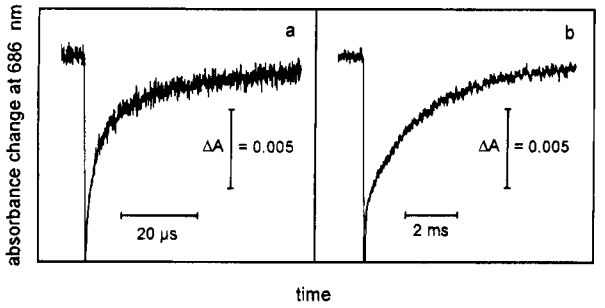



FIGURE 5: Absorbance changes at 686 nm in PS II membranes from spinach at 20 K recorded on a microsecond time scale for a sample with singly reduced $Q_A$ (trace a) and on a millisecond time scale for a sample with doubly reduced $Q_A$ (trace b). Other details as in Figure 4, except that trace b was measured with the setup for time scales exceeding 60 $\mu$s.

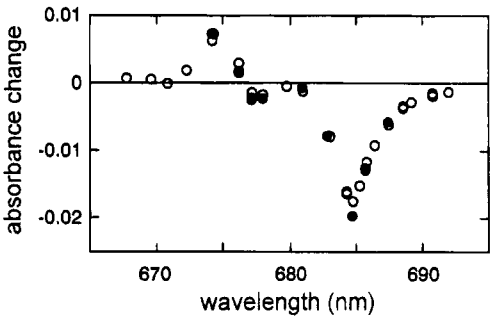



FIGURE 6: Spectra of the long-lived ($t_{1/2}$ > 1 $\mu$s) absorbance changes in PS II membranes from spinach at 20 K: closed symbols, sample with singly reduced $Q_A$; the amplitudes were obtained by fitting transients like trace a in Figure 5 in the time window from 850 ns to 55 $\mu$s; open symbols, sample with doubly reduced $Q_A$; the amplitudes represent the apparently nondecaying component of transients like those shown as traces b in Figure 4.

spectroscopically different states are involved. All these signals can be described by a transition from an initially formed state to a longer lived ($t_{1/2}$ > 1 $\mu$s) state. This transition is clearly faster in the sample with singly reduced $Q_A$ than in the sample with doubly reduced $Q_A$. For a given reduction state of $Q_A$, the kinetics of this transition agree rather well between the different wavelengths. They can be described by two phases with $t_{1/2} \approx 20$ and 100 ns at an amplitude ratio of approximately 1 for singly reduced $Q_A$ and $t_{1/2} \approx 50$ and 200 ns at an amplitude ratio of approximately 0.4 for doubly reduced $Q_A$, which is similar to the kinetics observed at 820 nm (see above). What varies between the different wavelengths are the signs and amplitudes of the initial and the longer lived ($t_{1/2}$ > 1 $\mu$s) absorbance changes. The initial absorbance changes are negative at all three wavelengths shown in Figure 4, while the longer lived absorbance changes are clearly positive at 674 nm (left panel) and weakly positive at 676 nm (middle panel). At 686 nm (right panel), the long-lived absorbance changes are negative and nearly as large as the initial ones (ignoring the negative "spike" during the first few nanoseconds), so that the transition between them is hardly visible. The pronounced negative "spike" at 686 nm may reflect stimulated emission due to singlet-excited chlorophylls and will not be analyzed further in the present work. The initial absorbance changes are ascribed to the formation of the primary pair, $P680^+Pheo^-$. Their amplitudes are somewhat smaller in the sample with singly reduced $Q_A$ than in the sample with doubly reduced $Q_A$, which may indicate some difference in the primary pair yields (see Discussion). Measurements like those in Figure 4 were performed at a series of wavelengths in the red spectral region. The absorbance difference spectra for the formation of the primary pair constructed from these measurements are presented and discussed in the accompanying paper (Hillmann et al., 1995).

With respect to the longer lived absorbance changes, an accelerated decay in the sample with singly reduced $Q_A$ compared to the sample with doubly reduced $Q_A$ is indicated already in Figure 4 (compare the slopes of traces a and b at the end of the time scale shown). Measurements on longer time scales in the red spectral region yielded decay kinetics rather similar to those observed at 820 nm (see Figure 3). This is shown in Figure 5 for signals at 686 nm. With singly reduced $Q_A$ (Figure 5a), the decay of the bleaching is nearly complete within 50 $\mu$s [a biexponential fit yielded half-lives of 1.8 and 14 $\mu$s at an amplitude ratio of 1.6 and a small longer lived bleaching (7% of the initial amplitude)]. With doubly reduced $Q_A$, however, most of the bleaching decays on a millisecond time scale (Figure 5b; a fit yielded half-lives of 1.0 and 3.1 ms at an amplitude ratio of 1.3).

Figure 6 shows difference spectra associated with the state which lives longer than approximately 1 $\mu$s and decays mainly in the microsecond range if $Q_A$ is singly reduced (closed circles) and mainly in the millisecond range if $Q_A$ is doubly reduced (open circles). These two spectra are virtually identical. For the sample with doubly reduced $Q_A$, it is straightforward to attribute the long-lived absorbance changes to the triplet state of P680 (i.e., the open circles in Figure 6 represent the triplet minus singlet spectrum). This assignment is based on the similarity with spectra in the

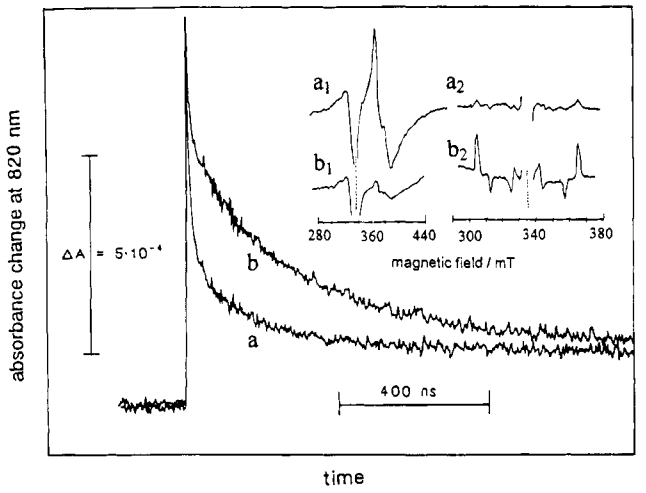



FIGURE 7: Absorbance changes at 820 nm and EPR spectra of PS II core complexes from *Synechococcus* analogous to Figure 2 (see Materials and Methods and Results for details of sample preparation). Measuring conditions deviating from Figure 2: absorbance changes, 10 $\mu$M Chl; excitation energy, approximately 500 $\mu$J/cm$^2$; EPR, 1.1 mM Chl; other conditions as in Figure 2. Fit results for the absorbance changes: trace a, $t_{1/2}$ = 17.8 ns (50.2%), 147 ns (27.2%), 2.7 $\mu$s (8.1%), and 38.5 $\mu$s (14.5%) (the latter two phases were determined from a measurement analogous to Figure 3, trace a); trace b, 63 ns (24.1%), 265 ns (55.3%), and 20.6% of a constant (decays within a few milliseconds, as determined with a DC-coupled detection system). The total extrapolated amplitudes of the fit functions are $\Delta A_{tot} = 5.9 \times 10^{-4}$ (trace a) and $7.95 \times 10^{-4}$ (trace b).

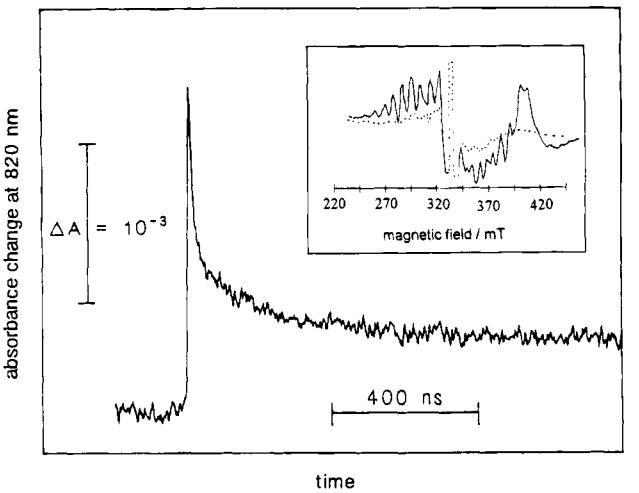



FIGURE 8: Absorbance changes at 820 nm in PS II core complexes from *Synechococcus* which had been illuminated at 200 K in the absence of external reductants in order to obtain singly reduced $Q_A$. Measurement was performed at 20 K and 24 $\mu$M Chl. Fit result (including a measurement on a microsecond time scale): $t_{1/2}$ = 15.1 ns (41.4%), 136 ns (28.7%), 2.0 $\mu$s (10.4%), 20.3 $\mu$s (6.6%), and 12.9% of a constant (decays within a few milliseconds, as determined with a DC-coupled detection system); $\Delta A_{tot} = 1.6 \times 10^{-3}$. Inset: EPR spectra of PS II complexes from *Synechococcus* (1.1 mM Chl; no formate present) measured under conditions which allow monitoring of the $S_2$ multiline signal. Conditions: temperature, 10 K; microwave power, 32 mW; microwave frequency, 9.44 GHz; modulation amplitude, 25 G. Lines: Solid, spectrum measured after illumination at 200 K; broken, control spectrum prior to illumination.

literature (Den Blanken et al., 1983; Van Kan et al., 1990) and on the arguments given above in the context of the measurements at 820 nm. The similarity of the two spectra in Figure 6 suggests that $^3$P680 is also formed in the sample with singly reduced $Q_A$ but is much shorter lived than in the sample with doubly reduced $Q_A$.

Study of the dependence on excitation energy of the absorbance changes in the red spectral region yielded a similar saturation behavior as at 820 nm (see above). The half-saturating excitation energy was approximately 100 $\mu$J/cm$^2$. The shapes of the kinetic traces as shown in Figures 4 and 5 are independent of the degree of saturation up to an excitation energy of approximately 500 $\mu$J/cm$^2$.

*Low-Temperature Flash Absorption Measurements on PS II Core Complexes from Synechococcus.* Measurements like those described above were also performed on PS II core complexes from *Synechococcus*, where the reduction state of $Q_A$ had been verified by EPR (see above). The results were similar to those obtained with PS II membranes from spinach, except that higher excitation energies were required to saturate the absorbance changes. The absorbance changes at 820 nm shown in Figure 7 (trace a, $Q_A$ singly reduced by dithionite; trace b, $Q_A$ doubly reduced by incubation with dithionite and benzyl viologen and subsequent illumination at room temperature) were measured at an excitation energy of approximately 500 $\mu$J/cm$^2$, which was approximately 25% saturating. Using the extinction coefficient of the primary pair given above and the extrapolated initial amplitudes of the fit curves described in the legend of Figure 7, it follows that under saturating excitation one primary pair would be formed per approximately 50 and 40 chlorophylls in the presence of singly and doubly reduced $Q_A$, respectively. This corresponds to approximately 0.8 and 1 primary pair per PS II. Flash absorption measurements in the red spectral region on PS II core complexes from *Synechococcus* yielded kinetic data and absorbance difference spectra similar to those from PS II membranes from spinach shown in Figures 4−6. These data are presented in the accompanying paper (Hillmann et al., 1995).

As outlined above, single reduction of $Q_A$ can also be achieved in the absence of any external reductant by illumination at 200 K. Figure 8 shows the 820-nm absorbance changes measured at 20 K in PS II core complexes from *Synechococcus* which had been illuminated at 200 K in the optical cryostat. The kinetics are very similar to those observed in the presence of dithionite (Figure 7, trace a), except for a somewhat larger contribution of long-lived absorbance changes in Figure 8. This may be due to a fraction of centers where $Q_A$ was not reduced by the illumination at 200 K and which show reversible formation of $P680^+Q_A^-$ at 20 K [half-life of recombination approximately 3 ms (Hillmann et al., 1994, and references therein)].

*Flash Absorption Measurements at 820 nm at Room Temperature.* In an attempt to compare the yield and decay kinetics of the primary pair, $P680^+Pheo^-$, between PS II with singly reduced $Q_A$ and PS II with doubly reduced $Q_A$ at room temperature, we performed the following experiment: To a sample of PS II core complexes from *Synechococcus*, dithionite was added in the dark, and the cuvette was closed to minimize entrance of oxygen. After approximately 3 min in the dark, flash absorption measurements at 820 nm were performed under continued repetitive excitation at a rate of 5 Hz. The measured transients were stored individually for each of the first 64 excitation flashes and subsequently in groups of 64 averaged transients. After approximately 2000 flashes, the excitation was stopped, and the sample was kept

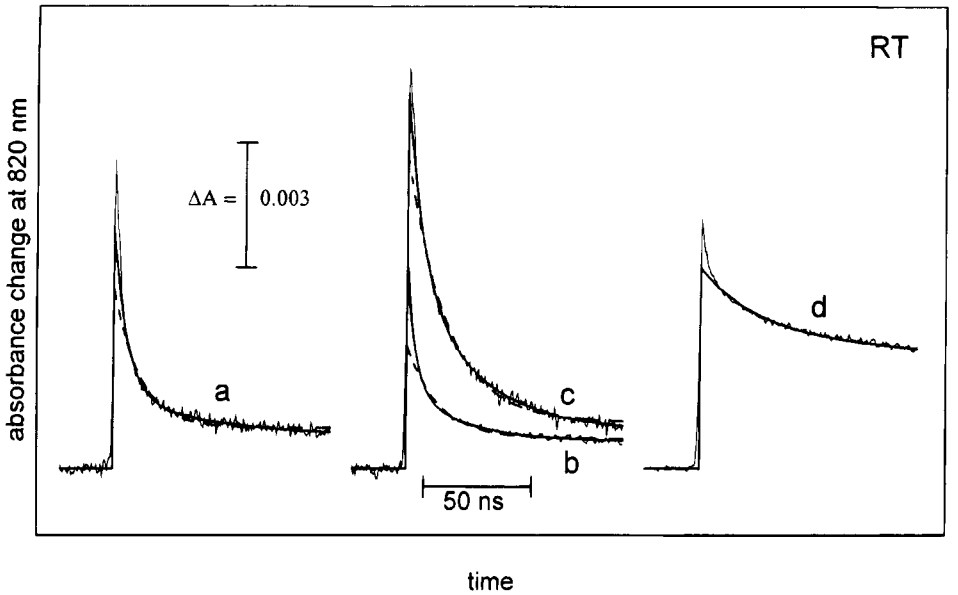



FIGURE 9: Absorbance changes at 820 nm in PS II core complexes from *Synechococcus* (33 $\mu$M Chl) at room temperature. The excitation energy is approximately 2 mJ/cm$^2$. The thin, noisy lines represent measured transients; the thicker broken and solid lines were obtained by fitting with a constant plus one and two exponential decay phases, respectively. Time window for the fits: 5−100 ns for traces a−c and 5−850 ns for trace d. Trace a: Average of the transients due to the first eight flashes given to a sample which had been incubated with 30 mM dithionite for 3 min in the dark; monoexponential fit, $t_{1/2}$ = 8.3 ns (77.6%), constant 22.4%, and total extrapolated amplitude $\Delta A_{tot}$ = 4.40 × $10^{-3}$; biexponential fit, $t_{1/2}$ = 4.1 ns (67.6%) and 26.6 ns (18.6%), constant 13.8%, and $\Delta A_{tot}$ = 5.85 × $10^{-3}$. Trace b: Average of the transients due to flash number 701−764 given to the sample from trace a; monoexponential fit, $t_{1/2}$ = 10.2 ns (75.5%), constant 24.5%, and $\Delta A_{tot}$ = 2.97 × $10^{-3}$; biexponential fit, $t_{1/2}$ = 2.9 ns (59.0%) and 18.3 ns (27.2%), constant 13.8%, and $\Delta A_{tot}$ = 4.69 × $10^{-3}$. Trace c: Average of the transients due to the first eight flashes given to the sample from trace a after it had received 2000 flashes and had been dark-adapted for 20 min; monoexponential fit, $t_{1/2}$ = 12.8 ns (85.3%), constant 14.7%, and $\Delta A_{tot}$ = 7.66 × $10^{-3}$; biexponential fit, $t_{1/2}$ = 5.1 ns (47.3%) and 21.1 ns (43.0%) constant 9.7%, and $\Delta A_{tot}$ = 9.18 × $10^{-3}$. Trace d: Average of 64 transients measured in the presence of electron acceptors (2 mM potassium ferricyanide and 200 $\mu$M phenyl *p*-benzoquinone); biexponential fit, $t_{1/2}$ = 19.4 ns (34.2%) and 185 ns (23.4%), constant 42.4%, and $\Delta A_{tot}$ = 4.91 × $10^{-3}$.

in the dark for 20 min before a new series of flash absorption measurements were started.

Trace a of Figure 9 shows the averaged signal due to the first eight excitation flashes (the individual signals did not vary significantly, so that averaging appeared justified in order to improve the signal to noise ratio). This signal should be representative of PS II with mostly singly reduced $Q_A$, as EPR measurements showed (1) that short dark incubation with dithionite of PS II core complexes from *Synechococcus* yields essentially singly reduced $Q_A$ (see above) and (2) that approximately 500 flashes are required to achieve double reduction of 50% of the $Q_A$ present (for experimental details, see Materials and Methods). The general shape of trace a in Figure 9 is similar to the 820-nm signals at 20 K (Figure 7), but the absorbance change decays much faster at room temperature. The signal was fitted starting 5 ns after the excitation flash in order to minimize contribution from species with a lifetime below 2 ns (mainly singlet-excited chlorophylls) which cannot be resolved correctly with the present setup. Fitting with a single exponential (plus a constant) yields a half-life of 8.3 ns (broken line in Figure 9, trace a). Allowing for two exponential phases (plus a constant), one obtains half-lives of 4.1 and 26.6 ns at an amplitude ratio of 3.6:1 (solid line in Figure 9, trace a).

When the flash absorption measurements were continued under repetitive excitation, a decrease of the amplitude of the absorbance changes was observed. An example is given as trace b in Figure 9, which is the average of 64 signals measured after approximately 700 preceding excitation flashes. The decrease of the signal amplitude during repetitive excitation can be explained by progressive accumulation of reduced pheophytin due to competition between charge recombination in the primary pair and electron donation to $P680^+$ by TyrZ[3] so that primary charge separation is no longer possible in part of the centers. The decay kinetics of the remaining absorbance changes in trace b of Figure 9 is not much different from trace a (see legend of Figure 9 for the results of the fits).

Trace c in Figure 9 is the average of the first eight transients measured after 2000 flashes, followed by dark adaptation for approximately 20 min. According to EPR measurements (see Materials and Methods for details), this signal should be representative of PS II with mostly doubly reduced $Q_A$. This signal shows a clearly larger amplitude and a clearly slower decay of the absorbance changes compared to trace a in Figure 9 (mostly singly reduced $Q_A$). A fit with one exponential (plus a constant) yields a half-life of 12.8 ns (broken line in Figure 9, trace c), and a fit with two exponentials (plus a constant) yields half-lives of 5.1 and 21.1 ns at an amplitude ratio of 1.1:1 (solid line). Continuation of flash absorption measurements on this sample led again to a marked decrease of the signal amplitude (not shown), presumably due to accumulation of reduced pheophytin. The number of excitation flashes (at 5 Hz) necessary for a certain relative decrease of the signal

[3] As incubation with dithionite should reduce and destroy the Mn cluster of the oxygen-evolving complex, it is likely that electron transfer from TyrZ to $P680^+$ was slowed down to the microsecond range, as observed usually after inactivation of the oxygen-evolving complex (Conjeaud & Mathis, 1980). This explains the relatively low yield of $Pheo^-$ accumulation and implies that the kinetics attributed to the recombination of the primary pair was not significantly affected by the parallel electron transfer from TyrZ to $P680^+$.

amplitude was approximately five times lower than during the first series of flash absorption measurements.

As a reference for the estimation of the primary pair yields of traces a–c in Figure 9 (see Discussion), we measured absorbance changes under otherwise identical conditions also in the presence of artificial electron acceptors, i.e., under conditions where the excitation flash should induce formation of the pair $P680^+Q_A^-$ and subsequent multiphasic rereduction of $P680^+$ with half-lives of 20 ns and slower (Brettel et al., 1984). The measured signal and the fit function (for parameters, see legend of Figure 9) are depicted as trace d in Figure 9. The ratios between the extrapolated initial amplitudes of the prereduced samples and the corresponding amplitude in the oxidized sample (solid line in trace d of Figure 9) were obtained (see legend of Figure 9). Using extinction coefficients of 7000 $M^{-1}$ $cm^{-1}$ and 12 400 $M^{-1}$ $cm^{-1}$ for $P680^+Q_A^-$ and $P680^+Pheo^-$, respectively (Takahashi et al., 1987), these ratios would correspond to primary pair yields (relative to the secondary pair yield in open centers which is taken as 100%) of 51% (monoexponential fit) and 67% (biexponential fit) for trace a ($Q_A$ singly reduced), 34% and 54% for trace b (partial accumulation of $Pheo^-$), and 88% and 106% for trace c ($Q_A$ doubly reduced).

## DISCUSSION

*Formation of the Primary Pair at 20 K.* In the present work, low-temperature photochemistry of PS II was studied by flash absorption spectroscopy in samples for which the redox state of the secondary acceptor $Q_A$ had been monitored by EPR.

For samples with doubly reduced $Q_A$, the transient absorbance changes can be attributed to formation of the primary pair, $P680^+Pheo^-$, and subsequent charge recombination forming the long-lived triplet state of P680. This assignment is suggested by the appearance of a relatively large light-induced triplet EPR signal which shows a spin-polarization pattern characteristic of triplet formation from a singlet radical pair precursor [traces $b_2$ in the insets of Figures 2 and 7 and Van Mieghem et al. (1989)]. Time-resolved EPR measurements of this triplet state at 4.2 K yielded a rise time of a few hundred nanoseconds (F. J. E. Van Mieghem, C. H. Bock, A. W. Rutherford, and D. Stehlik, unpublished results; Van Mieghem, 1994), in line with our assignment of the optical data. Absorbance changes with kinetics at low temperature similar to those described here were observed in D1/D2/cyt *b*-559 preparations (which lack $Q_A$) and were assigned in the same way (Van Kan et al., 1990). The assignment to a process in the reaction center is also consistent with the saturation behavior of the absorbance changes (see Results), which is comparable to that of room temperature photochemistry in "open" PS II with similar antenna sizes (Schlodder et al., 1984; Brettel & Witt, 1983; Brettel, 1984). Triplet formation in functionally disconnected antenna chlorophylls and subsequent triplet transfer to carotenoids, which are the most obvious alternative candidates for absorbance changes at 820 nm and in the red spectral region, are expected to saturate at higher excitation energies because of a smaller effective absorption cross section. Rereduction of $P680^+$ by secondary electron donors can also be excluded as a significant contribution to the observed absorbance changes as in this case $Pheo^-$ should have accumulated rapidly during repetitive excitation.

For PS II with singly reduced $Q_A$, it had been suggested that the yield of primary charge separation at low temperature might be low, since, under these conditions, the spin-polarized triplet signal is not observed by standard EPR [Van Mieghem et al. (1989) and traces $a_2$ in the insets of Figures 2 and 7]. On the other hand, primary pair formation at low temperature had been demonstrated for PS II core complexes from *Synechococcus* incubated with dithionite (Schlodder & Brettel, 1990) and very recently also for a sample illuminated at 200 K in the absence of external reductants (Schlodder & Hillmann, 1992). As, however, the reduction state of $Q_A$ had not been controlled directly for these samples, it could be argued that in the first case $Q_A$ may have in fact been doubly reduced, especially since in plant PS II, the detergent treatment used to make core preparations results in damage to $Q_A$ in a variable fraction of centers [see Van Mieghem et al. (1989, 1992)].

In the present work, the redox states were monitored by EPR. The flash-induced absorbance changes measured at various wavelengths do have similar amplitudes in samples with singly and doubly reduced $Q_A$. Additionally, the absorbance changes taken a few nanoseconds after the flash saturate at about the same excitation energy density irrespective of the reduction state of $Q_A$. This holds both for PS II core complexes from *Synechococcus* and for PS II membranes from spinach. These results provide strong evidence that the primary pair is formed with similar yields in the presence of singly reduced $Q_A$ and in the presence of doubly reduced $Q_A$. The absolute primary pair yields for saturating excitation were estimated to be in the order of 80–100% (see Results), based on an extinction coefficient at 820 nm of 12 400 $M^{-1}$ $cm^{-1}$ for the primary pair. The latter value is derived from extinction coefficients of $P680^+$ and pheophytin $a^-$ obtained under conditions different from those used here (Mathis & Sétif, 1981; Fujita et al., 1978), which may affect the estimates of the absolute yield.

From the experimental data (see Figures 2, 4, and 7), there appears to be a trend that the initial absorbance changes and hence the primary pair yields are slightly smaller in the presence of singly reduced $Q_A$ than in the presence of doubly reduced $Q_A$. In view of the uncertainties in the determination of the initial amplitudes attributed to the primary pair (uncertainties of the fit functions and their extrapolation to time zero, correction for light-induced signal loss, and flattening correction), however, the data do not allow us to affirm that these differences are significant. One might speculate that primary charge separation at low temperature is slowed down by the presence of $Q_A^-$ to such an extent that the competing intrinsic decay of the singlet excited states leads to some reduction of the primary pair yield. Hence, it would be interesting to resolve the rise kinetics of the primary pair at low temperature in the presence of singly and doubly reduced $Q_A$. This is not possible with our current instrument.

*Decay of the Primary Radical Pair at 20 K.* The decay of the primary pair state (and the concomitant rise of $^3P680$) as followed by flash absorption spectroscopy is clearly faster in samples with singly reduced $Q_A$ than in samples with doubly reduced $Q_A$ (see Figures 2, 4, and 7). The primary pair decay is expected to depend on the rate constants $k_S$ and $k_T$ (see Scheme 1) of charge recombination to the singlet and triplet state of P680, respectively, and on the kinetics of singlet–triplet conversion within the radical pair [see Hoff (1986), Lersch and Michel-Beyerle (1989), and Angerhofer

(1991) for recent reviews on these processes in different photosynthetic reaction centers]. At least one of these processes must vary considerably between samples with singly and with doubly reduced $Q_A$. The back-reaction via the excited singlet state ($k_{-1}$) can be neglected at low temperature ($T < 30$ K) (B. Hillmann, I. Moya, and E. Schlodder, unpublished results).

A possible explanation for the kinetic effect of the $Q_A$ reduction state might be the electrostatic influence of the negative charge of $Q_A^-$. It has been proposed that, in the doubly reduced state, the negative charges on $Q_A$ are neutralized due to protonation (i.e., formation of $Q_AH_2$) (Van Mieghem et al., 1989). An electrostatic influence could affect charge recombination rates to the singlet and/or triplet state of P680, making the radical pair decay faster in samples with singly reduced $Q_A$ compared to doubly reduced $Q_A$ [see Van Mieghem et al. (1992) and Liu et al. (1993) for indications at room temperature]. According to current electron-transfer theories [see Marcus and Sutin (1985) for a review], the recombination to the singlet ground state of P680 (rate constant $k_S$) should be slowed down rather than accelerated due to the electrostatic effect of $Q_A^-$, because this recombination is most likely in the Marcus' "inverted region" (free energy gap exceeds the reorganization energy). Recombination to the triplet state of P680 ($k_T$), however, may be in the "normal region" and may well be accelerated by the electric field. In addition, conformational effects associated with the $Q_A$ redox state might influence the charge recombination rates. There are indeed some indications that conformational changes occur upon double reduction of $Q_A$ (Van Mieghem et al., 1989; Van Mieghem, 1994).

With respect to singlet−triplet conversion in the primary radical pair, this process may be favored by magnetic interactions with the semiquinone−iron complex (Werner et al., 1978). Such effects are expected to increase the yield of $^3$P680 but may also change the lifetime of the primary pair (provided that the rate constants of charge recombination to the singlet and to the triplet state of P680 are different). Magnetic interactions between the semiquinone−iron complex and the primary radical pair state have been proposed in purple bacteria to explain changes observed in the yield and properties of the reaction center triplet in relation to the state of the quinone−iron complex [Schenck et al. (1982), Vidal et al. (1986), Hore et al. (1988), and references therein]. In summary, then, an electrostatic effect of $Q_A^-$ is not the only possible mechanism to account for the observed difference in primary pair decay kinetics between samples with singly reduced and with doubly reduced $Q_A$.

The quantitative analysis of the flash absorption data at 20 K at different wavelengths revealed that—at the present signal to noise ratios—the transition from the absorbance changes at a few nanoseconds after excitation to the long-lived absorbance changes ($t_{1/2} > 1$ μs) can be sufficiently well described with two exponential decay components. The corresponding half-lives are approximately 20 and 150 ns (ratio of amplitudes approximately 2:1) in the presence of singly reduced $Q_A$ and approximately 60 and 250 ns (ratio of amplitudes approximately 1:2) in the presence of doubly reduced $Q_A$. With a better signal to noise ratio it may turn out that the kinetics contain more components than found in our fits or are governed by a distribution of lifetimes.

The reason why the kinetics in the nanosecond range contain more than one exponential phase is not obvious. The two kinetic phases found in our fits cannot be distinguished spectroscopically at the present signal to noise ratios, so that they appear to reflect the same reaction, namely, recombination of the primary pair and concomitant formation of $^3$P680. According to the EPR results, the "contamination" of $Q_A^-$ samples with $Q_AH_2$ (and vice versa) was too small to account for the relative amplitudes of the two phases in the biexponential fits. As more likely possible explanations for the non-monophasic primary pair decay, we consider the following: (1) a heterogeneity of the samples at a level different from the reduction state of $Q_A$, e.g., frozen conformational substates of the protein complex or an inherent heterogeneity of the hyperfine-induced singlet−triplet mixing in the primary pair due to the statistical orientation of the nuclear spins (Volk et al., 1993); (2) a relaxation of the primary pair state on a tens of nanoseconds time scale to a conformation with lower energy and altered recombination kinetics [see Schlodder and Brettel (1988) for a similar suggestion to account for observations at room temperature].

*Yield and Decay of $^3$P680 at 20 K.* In line with the literature (Van Mieghem et al., 1989; Den Blanken et al., 1983; Rutherford et al., 1983), we have attributed the long-lived ($t_{1/2} \simeq 1-4$ ms) absorbance changes in samples with doubly reduced $Q_A$ to the triplet state of P680 which is detectable by standard EPR. The difference spectrum of these absorbance changes (Figure 6, open circles) resembles published spectra attributed to formation of the triplet state of P680 (Den Blanken et al., 1983; Van Kan et al., 1990; Otte et al., 1992; Van der Vos et al., 1993), but there are also significant differences which will be discussed in the accompanying paper (Hillmann et al., 1995). In samples with singly reduced $Q_A$, absorbance changes decaying with half-lives of approximately 2 and 20 μs have a virtually identical spectrum and very similar amplitudes (Figure 6, closed circles) and show also a similar saturation behavior. This result strongly suggests that $^3$P680 is formed with similar yield in the presence of singly reduced $Q_A$ as in the presence of doubly reduced $Q_A$ but is much shorter lived in the former case (see below for a discussion of the triplet lifetimes). Thus the observation by EPR of little or no P680 triplet signal under continuous illumination in samples with $Q_A$ singly reduced is not due to a low yield of triplet formation as suggested before (Van Mieghem et al., 1989) but due to a shortened triplet lifetime. It should also be mentioned that, according to simulations of the kinetics of singlet−triplet conversion in comparable radical pairs (Werner et al., 1978; Haberkorn & Michel-Beyerle, 1979), this process should essentially be complete already after 10−20 ns, so that the shortened lifetime of the primary pair in the presence of singly reduced $Q_A$ should still allow for efficient triplet formation.

The yield of $^3$P680 at 20 K has been estimated from the measurements at 820 nm (Figures 2 and 7) by comparing the amplitude of the long-lived ($t_{1/2} > 1$ μs) absorbance changes with the extrapolated initial amplitude attributed to the primary pair. Using an extinction coefficient of 12 400 $M^{-1}$ $cm^{-1}$ for the primary pair (see above) and 3800 $M^{-1}$ $cm^{-1}$ for $^3$P680 [taken from the triplet state of chlorophyll *a* in cyclohexanol at room temperature (Mathis & Sétif, 1981)], we obtain the following ratios between triplet yield and primary pair yield: 0.66 and 0.49 for PS II membranes from spinach with $Q_A$ singly and doubly reduced, respectively, and 0.74 and 0.67 correspondingly for PS II core complexes

from *Synechococcus*. The yield estimation can only be an approximate one as the extinction coefficients used were determined under rather different conditions (temperature and environment of the molecules) and because the uncertainties in the fit functions and their extrapolation to time zero may introduce some error. Nevertheless, it is noteworthy that in both materials the relative triplet yield appears to be somewhat higher with singly reduced $Q_A$ than with doubly reduced $Q_A$; this would be in line with an acceleration of the recombination of the primary pair to the triplet state of P680 (rate constant $k_T$) due to the electrostatic effect of $Q_A^-$. The data also show a trend to somewhat higher triplet yields in the PS II core complexes from *Synechococcus* than in PS II membranes from spinach, for which we have no explanation. Contributions from antenna chlorophyll triplets are unlikely to be the origin, as only a very low yield of such triplets was observed in a *Synechococcus* sample where primary charge separation was blocked by prereduction of Pheo (measured at 77 K; Hillmann et al., 1995). From the saturation behavior of the absorbance changes at 20 K in both spinach and *Synechococcus* samples, we estimate that the long-lived absorbance changes used to determine the $^3$P680 yields (Figures 2 and 7) contain at most 10% contamination by antenna chlorophyll triplets.

The lifetime of $^3$P680 in samples with singly reduced $Q_A$ appears to be shorter by more than 2 orders of magnitude than the lifetime in samples with doubly reduced $Q_A$, the latter being close to the lifetime of the triplet state of monomeric chlorophyll *a* in vitro at cryogenic temperatures (Clarke & Hofeld, 1974). A significantly shorter lifetime of the reaction center triplet state with singly reduced $Q_A$ compared to doubly reduced $Q_A$ has been found before in purple bacteria (Vidal et al., 1986; Shuvalov & Parson, 1981; Chidsey et al., 1985). However, this difference was absent at cryogenic temperatures (Shuvalov & Parson, 1981), and the effect at higher temperatures was not as dramatic as that observed here in PS II at low temperature. Quenching by carotenoids is probably not the reason for the short lifetime of $^3$P680 in the presence of singly reduced $Q_A$: preliminary measurements in the green spectral region showed that the amplitudes of the absorbance changes attributable to carotenoid triplets are too small and that their rise time is too fast to match with the decay of $^3$P680 (Van Mieghem, 1994). Looking for other explanations, one may suggest that a magnetic interaction with the semiquinone−iron complex could be the origin of the accelerated decay of the triplet state. However, this would be surprising in view of the large distance of the latter from the primary donor site. The distance problem might be overcome, if the triplet excitation would first be transferred to a molecule which is closer to $Q_A$, obvious candidates being the two pheophytins in the reaction center of PS II. The subsequent decay of the pheophytin triplet to the singlet ground state would have to be strongly accelerated by the magnetic interaction with the semiquinone−iron complex. This interaction would be missing in the presence of the diamagnetic doubly reduced $Q_A$. As another speculative mechanism accelerating the decay of the putative pheophytin triplet state, we consider a two-step electron-transfer process consisting of (1) oxidation of the semiquinone by the pheophytin triplet state and (2) "normal" forward transfer from reduced pheophytin to $Q_A$. Step 1 may become blocked if $Q_A$ is doubly reduced and protonated (the redox potential of the couple $Q_AH_2/Q_AH_2^+$ is expected to be much higher than that of the couple $Q_A^-/Q_A$) or if $Q_A$ is absent. For both mechanisms (magnetic interaction and two-step electron transfer), the maximal transient concentration of the pheophytin triplet may be low and hence hardly detectable, either because of its rapid decay or because of an unfavorable equilibrium between the triplet state of P680 and the pheophytin triplet state. According to *in vitro* data [Krasnovsky (1982) and references therein], the energy of the triplet state of pheophytin *a* should be close to or slightly higher than that of the (chlorophyll *a*) triplet state of P680. In the latter case, the decay of $^3$P680 via the mechanisms discussed above should be an activated reaction. Preliminary data at 685 nm (not shown) indicate in fact that the decay of $^3$P680 accelerates (and that the triplet yield decreases) with increasing temperature.[4] A pheophytin triplet with higher energy than $^3$P680 could not function as a real intermediate at very low temperature but may participate as a virtual state, similar to the intermediate in electron transfer via superexchange [Bixon et al. (1989) and references therein].

It is worth mentioning that the decay of the reaction center triplet state in purple bacteria can be accelerated by an external electric field (Franzen, 1992). This observation may be relevant for the triplet lifetimes in PS II because the electric field at the P680 site may vary with the reduction state of $Q_A$. Clearly, further work (e.g., studies of the dependence of the triplet kinetics on temperature and the presence of a magnetic or an electric field) is required for an understanding of the unusually short lifetime and also of the non-monoexponential decay of the P680 triplet state in the presence of singly reduced $Q_A$.

The rapid triplet quenching which is observed in $Q_A^-$-containing PS II, if present at room temperature, may represent a protective mechanism with physiological relevance. An unusually short-lived chlorophyll triplet would be less likely to react with oxygen to form harmful singlet oxygen. The double reduction of $Q_A$, which is shown here to result in the loss of triplet quenching, has already been suggested to be relevant to some kinds of photoinhibition (Van Mieghem et al., 1989; Vass et al., 1992), the processes by which PS II is damaged in plants which are exposed to high light intensities. To understand this process, research must focus on the origin of the triplet quenching effect.

*Yield and Lifetime of the Primary Pair at Room Temperature.* The results presented in Figure 9 demonstrate that the yield and the lifetime of the primary pair in the PS II core from *Synechococcus* at room temperature are clearly larger when $Q_A$ is doubly reduced (trace c) than when $Q_A$ is singly reduced (trace a). This result is in line with conclusions from recent fluorescence (Van Mieghem et al., 1992) and flash absorption measurements (Liu et al., 1993) on PS II preparations from spinach. The change in the yield of the primary pair may be explained by an increase of the free energy of the primary pair due to the electrostatic interaction with $Q_A^-$ (compared to the neutral $Q_AH_2$) which would shift the quasiequilibrium between the excited state and primary

[4] It was recently concluded from flash absorption measurements at 77 K that virtually no $^3$P680 is formed under conditions of singly reduced $Q_A$ (Schlodder & Hillmann, 1992), in apparent contradiction with the present data at 20 K. It is possible that $^3$P680 escaped detection in the measurements at 77 K because of its lower yield and because its lifetime is already in the submicrosecond region at that temperature.

pair into the direction of the excited state (concomitant with a decrease of the rate constant of charge separation, $k_1$, and a slight increase of the rate constant of repopulation of the excited state, $k_{-1}$) (Van Gorkom, 1985; Schatz et al., 1987, 1988; Schlodder & Brettel, 1988; Van Mieghem et al., 1989; see also below). The change in the lifetime of the primary pair at room temperature may have the same origin, as the same shift of the equilibrium should favor the decay of the primary pair via repopulation of singlet-excited chlorophylls and their subsequent decay to the singlet ground state or the triplet state. In addition, the mechanisms discussed above for low temperature may also contribute to the shortening of the lifetime of the primary pair in the presence of $Q_A^-$ at room temperature.

In the kinetic analysis of the signals in Figure 9, we omitted the fastest component ($t_{1/2} < 2$ ns) which cannot be resolved correctly with our setup. As the fluorescence decay in closed PS II core particles from *Synechococcus* shows major components with lifetimes of 0.22 and 1.3 ns (Schatz et al., 1987) and as singlet-excited Chl *a* absorbs at 820 nm [$\epsilon \simeq 4800\ M^{-1}\ cm^{-1}$ (Hansson et al., 1988)], we assume that the fastest component observed at 820 nm corresponds to the decay of singlet-excited Chl *a*. The subsequent absorbance decay on the nanosecond time scale was fitted by both one (broken lines in traces a–c in Figure 9) and two (solid lines) exponential phases. For singly reduced $Q_A$, the monoexponential fit ($t_{1/2} = 8.3$ ns, corresponding to a lifetime $\tau = 12.0$ ns) agrees well with $\tau = 11 \pm 3$ ns obtained previously (Schlodder & Brettel, 1988) for a very similar sample [the similarity of the two estimates of the primary pair lifetime indicates that the estimate of Schlodder and Brettel (1988) was not greatly affected by the slower charge recombination ($\tau = 18.5$ ns for a monoexponential fit) occurring in centers where $Q_AH_2$ had accumulated due to the greater number of excitation flashes used]. The better fit obtained with two exponential phases ($t_{1/2} = 4.1$ and 26.6 ns at an amplitude ratio of 3.6) is similar to recently published data for PS II core complexes from spinach in the presence of dithionite (Liu et al., 1993). For doubly reduced $Q_A$, our biexponential fit ($t_{1/2} = 5.1$ and 21.1 ns at an amplitude ratio of 1.1) resembles that for PS II core complexes from spinach (after preillumination in the presence of dithionite) as well (Liu et al., 1993).

We would like to stress that the parameters obtained by our biexponential fits are rather ambiguous (fits of nearly equal quality can be obtained with considerably different parameters). To obtain more reliable fits, a better signal to noise ratio and a higher time resolution would be required. Nevertheless, the mediocre quality of the monoexponential fits indicates that the primary pair decay deviates from a simple first-order reaction. For possible origins of this behavior, we refer the reader to the above discussion of the kinetics at low temperature.

The absorbance changes remaining at the end of the time scale depicted in Figure 9 may represent triplet states of Chl *a* in the antenna and/or in the reaction center. Whether and to what extent $^3$P680 contributes cannot be decided from the present measurements.

The question whether (and with what yield) the primary pair is formed in closed PS II at room temperature is rather controversial in the literature (see the introduction). As noted by Hansson et al. (1988), there is a trend toward an increase of the primary pair yield with decreasing antenna size (see also below). As a second factor responsible for the reported variation of the yield and lifetime of the primary pair, Van Mieghem et al. (1989) proposed that the reduction state of $Q_A$ ($Q_A^-$ or $Q_AH_2$) and the intactness of the quinone–iron complex may have varied between different studies and different samples. The present results show that the quinone iron complex in the PS II core preparation from *Synechococcus* is intact, unlike some preparations from higher plants (Van Mieghem et al., 1992), but confirm that $Q_A$ can become doubly reduced during repetitive flash absorption measurements in the presence of dithionite and that this is accompanied by an increase of the yield and the lifetime of the primary pair. However, different extents of double reduction of $Q_A$ cannot be the main origin of the discrepancy (see the introduction) between the negligible (<15%) yield of the long-lived ($\tau > 2$ ns) primary pair reported by Schatz et al. (1987) and the (60 ± 15)% yield reported by Schlodder and Brettel (1988) for the PS II core from *Synechococcus* incubated with dithionite, because the results obtained with mostly singly reduced $Q_A$ in the present study confirm a yield in the order of 50%. Presumably, the decay of the long-lived primary pair state was not resolved by Schatz et al. (1987) because of the use of a time window which was too short. Transients were detected only up to 1.4 ns after excitation; at this time only approximately 50% of the initial absorbance change at 680 nm had decayed.

In the Results section, the yield of the long-lived primary pair in the PS II core from *Synechococcus* with singly reduced $Q_A$ was estimated from the extrapolated initial amplitudes of the absorbance changes at 820 nm to be 51% (monoexponential fit) or 67% (biexponential fit) of the secondary pair yield with oxidized $Q_A$. This estimation neglects the contribution of singlet-excited chlorophylls which are in quasiequilibrium with the primary pair (see Scheme 1). An upper bound for their possible contribution to the absorbance changes at 820 nm is obtained as follows:

The observed decay rate ($k_{obs}$) of the equilibrium state between the excited state (E) and primary radical pair (RP) must be larger than that of its decay via the excited state alone:

$$k_{obs} > k_a[E]/([E]+[RP]) \quad \text{or} \quad [RP]/[E] > k_a/k_{obs} - 1$$

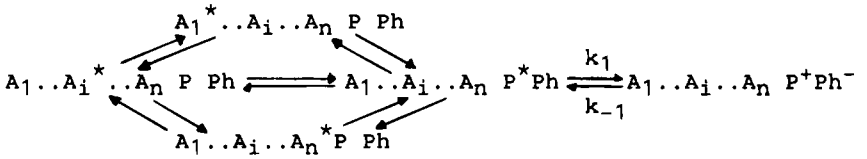
This limits the absorbance ratio of the radical pair and excited state in equilibrium:

$$A_{RP}/A_E = (\epsilon_{RP}/\epsilon_E)[RP]/[E] > (\epsilon_{RP}/\epsilon_E)(k_a/k_{obs} - 1)$$

Using the extinction coefficients for the primary radical pair ($\epsilon_{RP}$) and singlet-excited Chl *a* ($\epsilon_E$) given above and an intrinsic decay rate of the excited state ($k_a$) of at least 0.3 $ns^{-1}$ (Roloefs & Holzwarth, 1990), the contribution of singlet-excited chlorophylls should not exceed 33%, 13%, and 3% for phases with $t_{1/2} = 4.1$, 8.3, and 26.6 ns, respectively. After correction for this contribution, the yield of the long-lived ($t_{1/2} \geq 4$ ns) primary pair in the PS II core from *Synechococcus* with singly reduced $Q_A$ (relative to the secondary pair yield in open PS II) at room temperature is at least 44% (monoexponential fit) or 49% (biexponential fit).

The same approach for the sample with doubly reduced $Q_A$ gives a primary pair yield of at least 81% (monoexponential fit) or 85% (biexponential fit).

Scheme 2




Finally, we would like to comment on the question of primary pair relaxation in closed PS II. Similar to a proposal by Woodbury and Parson (1984) for purple bacteria, Schlodder and Brettel (1988) suggested that the primary pair state generated initially in closed PS II relaxes (with a rate constant in the order of 1 $ns^{-1}$) to an energetically more favorable conformation, which may involve a reorganization of the surrounding protein. Because of the lower free energy of the relaxed primary pair state, the concentration of excited states in quasiequilibrium with it would be rather low, and this would explain why the fluorescence decay (Schatz et al., 1987) does not show a prominent component with $\tau >$ 2 ns, corresponding to the decay of the long-lived primary pair state. The observed $\tau = 1.3$ ns fluorescence component would then reflect the decay of the excited states in quasiequilibrium with the initial (unrelaxed) primary pair state. Opposing this idea, Roelofs and Holzwarth (1990) demonstrated that the fluorescence kinetics observed with intact cells of the green alga *Scenedesmus obliquus* with closed PS II are not compatible with a long-lived ($\tau \geq 3$ ns) relaxed primary pair state at a yield exceeding 15%. This is not surprising, however, and cannot be used as an argument against a relaxed primary pair in the PS II core from *Synechococcus*, as the antenna size of the alga cells is much larger than that of the PS II core preparation from *Synechococcus*. Hence, one expects a much lower primary pair yield and also a shorter lifetime than in the PS II core. Interestingly, in a very recent report, Vass et al. (1993) provide an analysis of the fluorescence decay in isolated PS II membranes from spinach with doubly reduced $Q_A$, which accepts the possibility of the formation of a relaxed primary pair state. We consider it as likely that such a relaxation takes place whenever the primary pair exists for a sufficiently long time, as is the case in the PS II core from *Synechococcus* even in the presence of singly reduced $Q_A$.

*Hypothesis on the Origin of the Variation of the Primary Pair Yield with Temperature, Antenna Size, and Reduction State of $Q_A$.* In the following, we propose a qualitative explanation for the observation that the yield of primary pair formation in closed PS II decreases with increasing temperature and becomes dependent on the reduction state of $Q_A$ and the antenna size.

We consider the simplified reaction, Scheme 2, for excitation energy transfer and primary charge separation. In Scheme 2, $A_i$ denotes the $i$th antenna pigment, and P and Ph are used for P680 and pheophytin, respectively. We use $\Delta G^\circ_{*i} = G^\circ_{P*} - G^\circ_{Ai*}$ and $\Delta G^\circ_{cs} = G^\circ_{P+Ph-} - G^\circ_{P*}$ as the standard free energy changes for energy transfer from excited $A_i$ to P680 and for charge separation, respectively, and $K_{cs} = k_1/k_{-1} = \exp(-\Delta G^\circ_{cs}/k_BT)$ as the equilibrium constant of charge separation. Excitation energy transfer between the antenna pigments and to P680 appears to be sufficiently fast so that, with completion of charge separation, a quasiequilibrium between the primary pair and all excited states is established. This quasiequilibrium determines the primary pair concentration (Van Gorkom, 1985; Van Grondelle, 1985; Schatz et al., 1987, 1988; Schlodder & Brettel, 1988; Trissl, 1993).

We define an effective equilibrium constant $K_{eff}$ which is relevant for the primary pair yield:

$$K_{eff} = \frac{[A_1...A_i...A_n\ P^+Ph^-]_{eq}}{[A_1...A_i...A_n\ P^*Ph]_{eq} + \sum_i [A_1...A_i^*...A_n\ P\ Ph]_{eq}}$$

One obtains

$$K_{eff} = K_{cs}/N \tag{1}$$

with

$$N = 1 + \sum_i \exp(\Delta G^\circ_{*i}/k_BT) \tag{2}$$

[see also Trissl (1993)]. On the basis of $K_{eff}$, we define an effective free energy change for charge separation ($\Delta G^\circ_{eff}$) and an effective free energy of the equilibrated excited states ($G^\circ_{*eff}$):

$$\Delta G^\circ_{eff} = -k_BT \ln K_{eff} = G^\circ_{P+Ph-} - G^\circ_{*eff}$$

We obtain

$$\Delta G^\circ_{eff} = \Delta G^\circ_{cs} + k_BT \ln N \tag{3}$$

and

$$G^\circ_{*eff} = G^\circ_{P*} - k_BT \ln N \tag{4}$$

In general, $N$ is a function of temperature. For the following qualitative arguments, we assume that the free energies of the excited antenna Chl *a* molecules are exactly the same as that of excited P680; i.e., $\Delta G^\circ_{*i} = 0$ for all Chl a, and we neglect the contribution to $N$ of the other antenna pigments because their excited states are energetically far above P680*. With these approximations, $N$ becomes independent of temperature and equals the total number of Chl *a* molecules (including P680, which is counted as one molecule). As another crude approximation, we assume that the free energies of the individual states ($G^\circ_{Ai*}$, $G^\circ_{P*}$, and $G^\circ_{P+Ph-}$) are independent of temperature.

Figure 10 summarizes schematically the resulting free energies as a function of temperature. The free energy levels of excited P680 and of the primary pair in open centers (presence of $Q_A$) are indicated respectively by the upper and lower solid horizontal line. The presence of $Q_A^-$ is assumed to increase the free energy of the primary pair due to electrostatic interaction [broken horizontal line; the increase may be in the order of 50 meV (Schlodder & Brettel, 1988; Schatz et al., 1988)], whereas in the presence of doubly reduced and protonated $Q_A$ ($Q_AH_2$) or in the absence of $Q_A$, the free energy of the primary pair is assumed to be the same as in open centers (lower horizontal line). The three

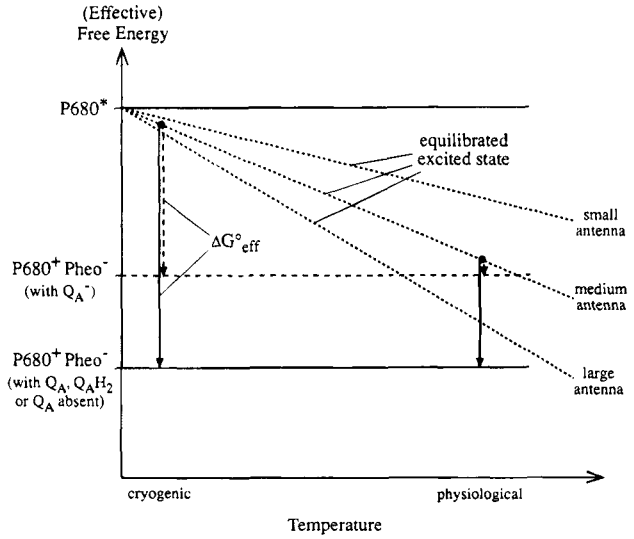



FIGURE 10: Schematic representation of the energetics of primary charge separation in PS II as a function of temperature, as proposed and explained in the Discussion.

decreasing dotted lines represent the effective free energies of the equilibrated excited state ($G°_{*eff}$) for three different antenna sizes; the larger the antenna, the more pronounced the decrease of $G°_{*eff}$ with increasing temperature (cf. eq 4). The effective free energy change for charge separation ($\Delta G°_{eff}$) is indicated by vertical arrows (with broken lines for centers with $Q_A^-$ and solid lines for centers with $Q_A$, $Q_AH_2$, or $Q_A$ absent). On the basis of the high primary pair yields observed in the present study, $\Delta G°_{eff}$ at cryogenic temperatures is taken as negative, even in the presence of $Q_A^-$. With increasing temperature, $\Delta G°_{eff}$ increases. At physiological temperatures and for a certain antenna size, $\Delta G°_{eff}$ in the presence of $Q_A^-$ may be rather small (i.e., $K_{eff} \approx 1$), but with $Q_A$, $Q_AH_2$, or $Q_A$ absent, $\Delta G°_{eff}$ may still be well negative (i.e., $K_{eff} \gg 1$). Such a situation is indicated by the right-most vertical arrows in Figure 10 and may be realized in the PS II core from *Synechococcus* where we observed long-lived primary pair yields at room temperature in the order of 50% and close to 100% in the presence of singly and doubly reduced $Q_A$, respectively. For PS II with larger antenna (e.g., lowest dotted line in Figure 10), $\Delta G°_{eff}$ at room temperature would be positive ($K_{eff} < 1$) in the presence of $Q_A^-$, implying a low primary pair yield, while $\Delta G°_{eff}$ may still be negative ($K_{eff} > 1$) in the presence of $Q_AH_2$, allowing for a higher yield of primary charge separation. For small effective antenna sizes, Figure 10 predicts $\Delta G°_{eff} < 0$ and $K_{eff} > 1$ and hence a high primary pair yield even in the presence of $Q_A^-$.

It should be stressed that eqs 1–4 and Figure 10 are not suitable for a quantitative prediction of primary pair yields because of the crude approximations outlined above and because reaction pathways, which were not considered in Scheme 2, influence the primary pair yield as well (decay of excited antenna pigments to the ground or triplet state, singlet–triplet conversion and charge recombination in the primary pair, relaxation of the initially primary pair state to a conformation with lower free energy; of course, these processes would also be essential to model the fluorescence properties of PS II). Nevertheless, the highly simplified picture presented in Figure 10 accounts well for the trends observed so far in the dependence of the primary pair yield on temperature, antenna size, and redox state of $Q_A$. To prove the predictions of Figure 10 further, the complete temperature dependence of the primary pair yield should be measured for PS II preparations with different antenna sizes and with $Q_A$ singly and doubly reduced. To avoid confusion which may arise from Figure 4 in the work of Schatz et al. (1988), we would like to stress that the effective free energy change $\Delta G°_{eff}$ was introduced by us only to describe the quasiequilibrium between excited states and the primary pair state and should not be used as the free energy gap for estimating the rate of primary charge separation by electron-transfer theory; rather $\Delta G°_{cs}$ should be used in this context, and it turns out from the present study that $\Delta G°_{cs}$ in PS II is negative even in the presence of $Q_A^-$, as is the case in purple bacteria [e.g., Woodbury and Parson (1984)].

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