Secondary Pair Charge Recombination in Photosystem I under Strongly Reducing Conditions: Temperature Dependence and Suggested Mechanism

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ABSTRACT Photoinduced electron transfer in photosystem I (PS I) proceeds from the excited primary electron donor P700 (a chlorophyll *a* dimer) via the primary acceptor A_0 (chlorophyll *a*) and the secondary acceptor A_1 (phylloquinone) to three [4Fe-4S] clusters, F_X, F_A, and F_B. Prereduction of the iron-sulfur clusters blocks electron transfer beyond A_1. It has been shown previously that, under such conditions, the secondary pair $P700^+A_1^-$ decays by charge recombination with $t_{1/2} \approx 250$ ns at room temperature, forming the P700 triplet state (^{3}P700) with a yield exceeding 85%. This reaction is unusual, as the secondary pair in other photosynthetic reaction centers recombines much slower and forms directly the singlet ground state rather than the triplet state of the primary donor. Here we studied the temperature dependence of secondary pair recombination in PS I from the cyanobacterium *Synechococcus* sp. PCC6803, which had been illuminated in the presence of dithionite at pH 10 to reduce all three iron-sulfur clusters. The reaction $P700^+A_1^- \rightarrow {}^3$P700 was monitored by flash absorption spectroscopy. With decreasing temperature, the recombination slowed down and the yield of ^{3}P700 decreased. In the range between 303 K and 240 K, the recombination rates could be described by the Arrhenius law with an activation energy of ~170 meV. Below 240 K, the temperature dependence became much weaker, and recombination to the singlet ground state became the dominating process. To explain the fast activated recombination to the P700 triplet state, we suggest a mechanism involving efficient singlet to triplet spin evolution in the secondary pair, thermally activated repopulation of the more closely spaced primary pair $P700^+A_0^-$ in a triplet spin configuration, and subsequent fast recombination (intrinsic rate on the order of 10^9 s^{-1}) forming ^{3}P700.

INTRODUCTION

Photosystem I (PS I) is a membrane protein complex in oxygenic photosynthetic organisms. It performs light-driven transmembrane charge separation by transferring an electron from the singlet excited state of the primary electron donor (called P700) through a series of electron acceptors. The structure and function of PS I have been studied by biochemical and spectroscopic techniques (for reviews see Golbeck and Bryant, 1991; Brettel, 1997), and recently structural data to a resolution of 4 Å have been obtained from x-ray diffraction of PS I crystals (Krauss et al., 1996; Schubert et al., 1997). P700 is considered to be a dimer of chlorophyll *a*, the primary acceptor A_0 a monomeric chlorophyll *a*, and the secondary acceptor A_1 a phylloquinone (vitamin K_1). The acceptors F_X, F_A, and F_B are all [4Fe-4S] clusters. Electron transfer from P700 via A_0 to A_1 takes ~30 ps, further transfer to F_X ~200 ns, and the subsequent transfers to F_A and F_B (which have not yet been unambiguously resolved) presumably less than 500 ns. These electron transfer times, as well as all of the following ones quoted in the Introduction, refer to room temperature.

The forward electron transfer reactions compete with physiologically unproductive charge recombination reactions. The latter can hardly be observed under conditions of efficient forward electron transfer because, to achieve a high yield of charge separation, electron transfer from the *i*th acceptor A_i to A_{i+1} is much faster than the competing charge recombination in the pair $P700^+A_i^-$. Charge recombination occurs exclusively, however, when one of the forward electron steps is blocked. Thus, when the secondary acceptor A_1 is prereduced, inactivated, or removed, only the primary pair $P700^+A_0^-$ is formed and recombines with $t_{1/2} \approx 30$ ns (Sétif et al., 1985; Brettel and Sétif, 1987); this recombination forms two products: the singlet ground state of P700 (yield ~70%) and the P700 triplet state ^{3}P700 (~30%), which then decays by intersystem crossing to the singlet ground state within a few microseconds. The formation of ^{3}P700 can be explained (see Hoff, 1981, for a review) by dephasing of the unpaired electron spins of $P700^+$ and A_0^- (due to hyperfine interactions) during the lifetime of the primary pair; thus the spin state of the pair evolves between the singlet configuration (in which the pair is created from its singlet precursor ^{1}P700*) and the triplet spin configurations; charge recombination then forms either the singlet ground state or the triplet state of P700, depending on the spin configuration of the primary pair at the moment of recombination.

Received for publication 12 December 1997 and in final form 4 March 1998.

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0006-3495/98/06/3173/09 $2.00

With one exception (see below), recombination reactions between $P700^+$ and the acceptors following A_0 are much slower: $t_{1/2} \approx 10$ μs between $P700^+$ and A_1^- when F_X, F_A, and F_B are removed (Warren et al., 1993; Brettel and Golbeck, 1995), $t_{1/2} \approx 250$ μs between $P700^+$ and F_X^- and/or A_1^- when F_A and F_B are prereduced (Sauer et al., 1978; Brettel, 1989; Sétif and Brettel, 1993), $t_{1/2} \approx 1$ ms between $P700^+$ and F_X^- when F_A and F_B are removed (Parrett et al., 1989), and $t_{1/2} \approx 50$ ms between $P700^+$ and F_A^- and/or F_B^- when no extrinsic electron acceptors and donors are present (Hiyama and Ke, 1971), and there is no indication for the formation of $^3P700$ through these recombinations (see also Vassiliev et al., 1997, for a recent reexamination of the kinetics of charge recombination reactions in PS I). The slower recombination rates can be qualitatively explained by the larger distances between the partners, and the lack of triplet formation may simply be due to the fact that the free energy of these charge separated states is lower than that of $^3P700$. A remarkable exception is the charge recombination between $P700^+$ and A_1^- when all three iron-sulfur clusters (F_A, F_B, and F_X) are prereduced. Under these conditions, $P700^+A_1^-$ recombination proceeds with a half-time of only 250 ns and forms mainly the triplet state $^3P700$ (yield at least 85%) (Sétif and Bottin, 1989; Sétif and Brettel, 1990). To explain the high triplet yield, it has been proposed that prereduction of F_X, together with F_A and F_B, lifts the free energy of the pair $P700^+A_1^-$ to at least 30 meV above that of $^3P700$, possibly by electrostatic interaction between A_1^- and the reduced iron-sulfur clusters (Sétif and Brettel, 1990).

The recombination of $P700^+A_1^-$ with $t_{1/2} \approx 250$ ns in the presence of prereduced F_A, F_B, and F_X is not only much faster than the recombination of the same pair under all other conditions studied so far; it is also much faster than the recombination of the secondary pairs in other photosynthetic reaction centers: $t_{1/2} \approx 200$ μs in PS II (Renger and Wolff, 1976), 600 μs in *Rhodopseudomonas viridis* (Carithers and Parson, 1975), and 70 ms in *Rhodobacter sphaeroides* (Hsi and Bolton, 1974). This might indicate that the distance between primary donor and secondary acceptor is considerably shorter in PS I than in PS II and in purple bacteria. Alternatively, one may suggest that $P700^+A_1^-$ recombination in the presence of prereduced F_A, F_B, and F_X is not a direct one, but proceeds via repopulation of the more closely spaced primary pair $P700^+A_0^-$. In the latter case, the recombination should be a thermally activated reaction with an activation energy corresponding to the energy gap between $P700^+A_0^-$ and $P700^+A_1^-$. For this reason, we investigated the temperature dependence of the $P700^+A_1^-$ recombination rate after prereduction of F_A, F_B, and F_X.

MATERIALS AND METHODS

Biological samples

PS I complexes from *Synechocystis* sp. PCC 6803 were prepared as described by Bottin and Sétif (1991). Strongly reducing conditions (to reduce F_A and F_B) were obtained by suspending the complexes in 0.2 M glycine buffer (pH 10) and adding excess sodium dithionite (25 mM). The final chlorophyll concentration in the samples was ~10 μM for transient absorption measurements in the red, and ~200 μM for measurements in the near IR. Samples for measurements at temperatures below 0°C contained 66% (v/v) glycerol.

Spectroscopic measurements

Flash-induced absorbance changes were measured at 691, 811, and 1064 nm (see Brettel, 1996, for some technical considerations related to the resolution of the usually very small absorbance changes encountered in plant photosynthesis research). The sample was contained in a plastic cuvette with a 1-cm path for the measuring light. For measurements at temperatures down to −25°C, the cuvette was placed in an argon-flushed box, and cooling was achieved by pumping an ethylene glycol/water mixture from a thermostat through the cuvette holder. Below −25°C, we used a helium flow cryostat. The temperature was measured with a thermocouple inserted into the sample. The samples were excited at 90° to the measuring beam by a frequency doubled (532 nm) Nd/YAG laser (YG501-10; Quantel, Les Ulis, France), providing pulses of 300 ps duration with an energy up to 20 mJ at a repetition rate of 2 Hz. The pulses were usually attenuated to give ~2 mJ/cm^2 at the window of the cuvette. As measuring light sources we used cw laser diodes (TOLD 9140, Toshiba, Tokyo, Japan, for 691 nm; SLD202U-3, Sony, Tokyo, Japan, for 811 nm) and a cw Nd/YAG laser (DPY101, Adlas, Lübeck, Germany) for 1064 nm. To diminish the actinic effect of the 691-nm measuring light, a shutter placed between laser diode and sample was opened for only 3 ms around the excitation flash. For measurements at 1064 nm, the measuring light beam was split before it passed the cuvette; the part not passing the sample was used as a reference for subtracting baseline oscillations (originating from the cw Nd/YAG laser) from the detected signal. The detection system consisted of a silicon photodiode (FND 100; EG&G, Quebec, Canada), an amplifier (10-20-1C; Nuclétudes, Les Ulis, France), and a digitizing oscilloscope (DSA 602A with plug-in amplifier 11A52; Tektronix, Beaverton, OR). The maximum electronic bandwidth was DC to ~100 MHz, but in many cases the 20-MHz low-pass filter of the 11A52 plug-in was used to improve the signal-to-noise ratio.

The transients were fitted to a multiexponential decay, using a Marquardt least-squares algorithm (program kindly provided by Dr. Pierre Sétif, CEA Saclay). The fitting was usually started 28 ns after the excitation flash (time 0), but the fit function was extrapolated back to time 0.

Prereduction of F_X

Prereduction of F_X (in addition to F_A and F_B) was achieved by illumination in the presence of dithionite at pH 10 (Sétif and Bottin, 1989). We performed extensive test measurements to establish optimal conditions for the prereduction treatment. We varied the intensity and duration of the illumination, the dithionite concentration, and the temperature of the sample, and we followed flash-induced absorbance changes at 1064 and 691 nm indicative of prereduction of F_X (transients rising with $t_{1/2} \approx 250$ ns, reflecting formation of $^3P700$) during and after the illumination. It turned out that after the illumination necessary to obtain a prominent 250-ns phase, there was already some reduction of A_1 (presumably to the quinol form; Sétif and Bottin, 1989), as indicated by the presence of an ~30-ns phase at 811 nm due to charge recombination in the primary pair $P700^+A_0^-$ in a fraction of the centers. During subsequent dark incubation, the latter fraction did not change markedly, whereas the amplitude of the 250-ns phase at 1064 and 691 nm decreased (half-life ~30 s), presumably because of reoxidation of F_X^- in the dark (see Sétif and Brettel, 1990). Hence we were not able to avoid contamination of our samples (supposed to have all three iron-sulfur clusters reduced and the other electron acceptors oxidized) by a fraction of centers with reduced A_1 and by another fraction with oxidized F_X. The following prereduction protocols turned out to yield samples with relatively low amounts of these contaminations:

1. For measurements at 1064 nm in the temperature range from +30 to −25°C, the sample was exposed for 40 s to white background illumination provided by a 800-W tungsten iodine lamp whose beam passed through 35 mm of water to remove infrared light. The illumination took place at room temperature in the argon-flushed box (see above), and experiments were started as soon as the sample reached the desired temperature.

2. For measurements at 1064 nm below −25°C, the sample was mounted on the sample holder of the cryostat and exposed to the same illumination at room temperature as in protocol 1. Then the sample was dipped into liquid nitrogen for rapid cooling and transferred to the precooled helium cryostat.

3. For measurements at 691 nm, the continuous measuring beam of the laser diode was used for illumination at room temperature for ~30 s, followed by cooling of the sample as in protocols 1 and 2. Laser flash-induced transients recorded at 691 nm during the preillumination served as a control for the reduction state of the sample.

We would like to mention a surprising observation made when we recorded flash-induced absorbance changes simultaneously at 1064 and 811 nm (the two beams were superimposed in front of the sample and separated behind the sample with dichroic mirrors) at different times during the illumination period of prereduction protocol 1 the build-up of the transient at 1064 nm rising with $t_{1/2} \approx 250$ ns and decaying with $t_{1/2} \approx 4$ μs (attributed to formation and decay of ^{3}P700; Sétif and Bottin, 1989; Sétif and Brettel, 1990) was slower than the build-up of an ~5-μs decay phase of the flash-induced absorbance change at 811 nm (attributed to the decay of ^{3}P700; Sétif and Bottin, 1989). This may indicate that the 5-μs decay at 811 nm observed relatively early during the prereduction treatment does not reflect the decay of ^{3}P700, but rather that of P700$^+$ (see Discussion), but a technical problem (imperfect superposition of the two measuring light beams within the sample, so that the average background intensity might have been weaker in the volume probed by the 1064-nm light than in the volume probed by the 811-nm light) cannot be ruled out completely at present. For the measurements presented in this paper, the illumination was always continued until the ~250-ns phase of ^{3}P700 formation as recorded at 1064 or 691 nm was fully developed.

RESULTS

Monitoring of the reaction $P700^+A_1^- \rightarrow {}^3P700\ A_1$ by flash absorption spectroscopy and distinction from recombination to the P700 ground state are only possible at wavelengths at which the absorbance change due to ^{3}P700 is sufficiently large and clearly different from that due to $P700^+A_1^-$. As shown previously (Sétif and Bottin, 1989; Sétif and Brettel, 1990), the emission wavelength of the Nd/YAG laser (1064 nm) is particularly suitable because ^{3}P700 absorbs considerably more strongly than P700$^+$, whereas P700, A_1, and A_1^- hardly absorb at 1064 nm. Fig. 1 shows the flash-induced absorbance changes at 1064 nm at 20°C for a sample prereduced according to protocol 1 (see Materials and Methods). The instrument-limited rise of the absorbance is followed by a partial decay within ~10 ns (data points that are well above the *solid line* in Fig. 1), possibly arising from the decay of singlet excited antenna chlorophylls and charge recombination in the primary pair $P700^+A_0^-$ in a fraction of centers where A_1 was already prereduced (see Materials and Methods). We will refer to this fastest kinetic phase as the "fast phase." The subsequent slower rise of the absorbance ("intermediate phase"; $t_{1/2} = 212$ ns according to the fit shown as the *solid line* in Fig. 1) is attributed to the formation of ^{3}P700, which then decays on a microsecond time scale ("slow phase"; $t_{1/2} = 3.2$ μs

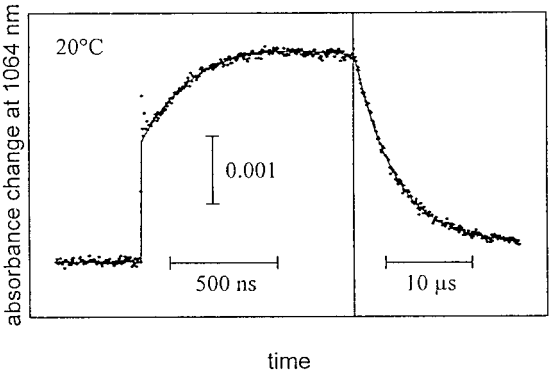

FIGURE 1 Flash-induced absorbance changes at 1064 nm for PS I complexes prereduced according to protocol 1. Temperature: 20°C; no glycerol present; electronic bandwidth: DC to 100 MHz; average of 384 transients. Parameters of the fit function (*solid line*; see Eq. 1): $a_2 = -1.99 \times 10^{-3}$; $k_2 = 3.26 \times 10^6\ s^{-1}$; $a_3 = 3.51 \times 10^{-3}$; $k_3 = 2.16 \times 10^5\ s^{-1}$; $a_4 = 0.23 \times 10^{-3}$. The "fast phase" (first exponential in Eq. 1) was omitted from the fit because the corresponding data (*initial spike above the solid line*) virtually do not contribute in the fit window (28 ns to 20 μs).

according to the fit). Measurements like the one depicted in Fig. 1 were performed at different temperatures. Fig. 2 shows three examples that were obtained in the presence of 66% (v/v) glycerol (to avoid freezing of the sample) and with a reduced electronic bandwidth (DC to 20 MHz, to improve the signal-to-noise ratio). It is evident from Fig. 2 that, with decreasing temperature, the intermediate phase becomes slower and its amplitude decreases.

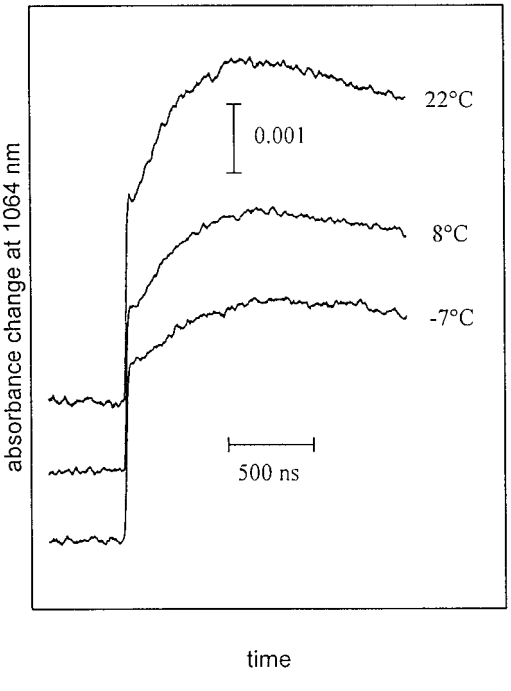

FIGURE 2 Flash-induced absorbance changes at 1064 nm for PS I complexes prereduced according to protocol 1, measured at different temperatures, as indicated. The sample contained 66% (v/v) glycerol; electronic bandwidth: DC to 20 MHz; 192 transients were averaged at each temperature.

We tried to monitor the reaction $P700^+A_1^- \rightarrow {}^3P700\ A_1$, also in the red spectral region. Based on spectra in the literature (Hoch, 1977; Den Blanken and Hoff, 1983; Vrieze et al., 1996), it appeared likely that the bleaching due to formation of ^{3}P700 exceeds that due to the formation of $P700^+$ in the region around 690 nm. By varying the wavelength of the measuring light (through variation of the temperature of the laser diode), we found that ^{3}P700 formation from the secondary pair could be monitored most easily at 691 nm. Fig. 3 shows a transient at 691 nm measured at 22°C. As at 1064 nm, one can distinguish three kinetic phases: the partial recovery of the initial bleaching (fastest phase; $t_{1/2} = 23$ ns according to the fit) may again be due to primary pair recombination in centers where A_1 was already prereduced. The subsequent intermediate phase (bleaching with $t_{1/2} = 223$ ns) should represent the reaction $P700^+A_1^- \rightarrow {}^3P700\ A_1$, followed by the decay of ^{3}P700 (slowest phase; $t_{1/2} = 2.4\ \mu$s). Measurements at 691 nm at different temperatures (Fig. 4) confirm qualitatively the result obtained at 1064 nm that, with decreasing temperature, formation of ^{3}P700 from the secondary pair slows down and decreases in amplitude.

For quantitative analysis, the transients measured at different temperatures were fitted to a three-exponential decay:

$$\Delta A(t) = a_1\exp(-k_1t) + a_2\exp(-k_2t) + a_3\exp(-k_3t) + a_4 \quad (1)$$

where the first, second, and third exponentials account for the fast, intermediate, and slow phases, respectively, and a_4 accounts for possible long-lived absorbance changes (not decaying on the time scale of the measurements). In all cases, the sign of the second exponential was opposite that of the first and third exponentials, which made it possible to perform reasonable fits to transients with a rather mediocre signal-to-noise ratio. Fig. 5 shows an Arrhenius plot of rate constant k_2, which we attribute to the decay of the secondary pair and the simultaneous formation of ^{3}P700. Triangles and crosses represent data at 1064 nm in the presence of 66% (v/v) glycerol and in the absence of glycerol, respectively. Circles refer to measurements at 691 nm (glycerol present).

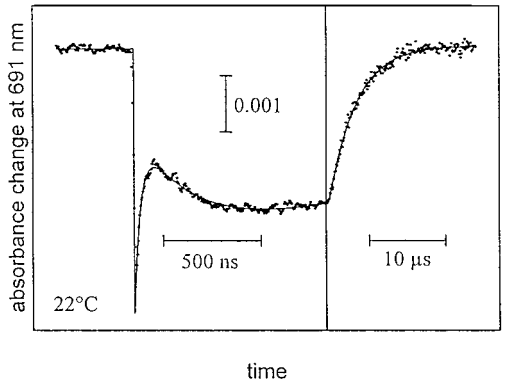

FIGURE 3 Flash-induced absorbance changes at 691 nm for PS I complexes prereduced according to protocol 3. Temperature: 22°C; the sample contained 66% (v/v) glycerol; electronic bandwidth: DC to 20 MHz; average of 64 transients. Parameters of the fit function (*solid line*; see Eq. 1): $a_1 = -3.26 \times 10^{-3}$; $k_1 = 3.08 \times 10^7\ s^{-1}$; $a_2 = 2.41 \times 10^{-3}$; $k_2 = 3.11 \times 10^6\ s^{-1}$; $a_3 = -3.93 \times 10^{-3}$; $k_3 = 2.91 \times 10^5\ s^{-1}$; $a_4 = 0.07 \times 10^{-3}$.

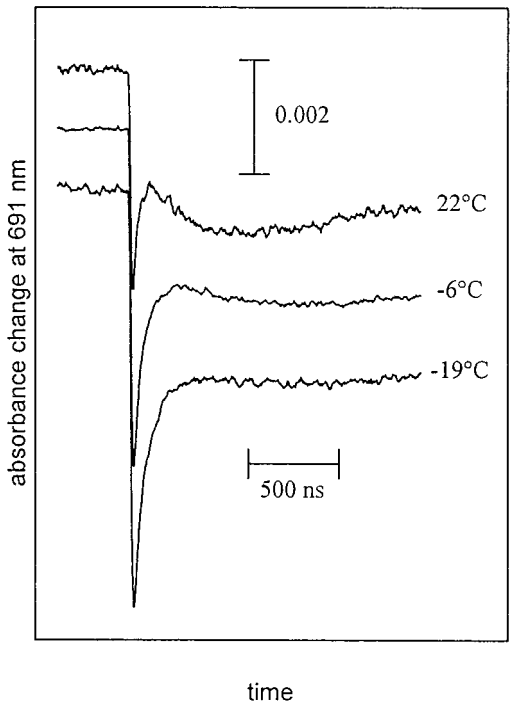

FIGURE 4 Flash-induced absorbance changes at 691 nm measured at different temperatures, as indicated. Other conditions as for Fig. 3.

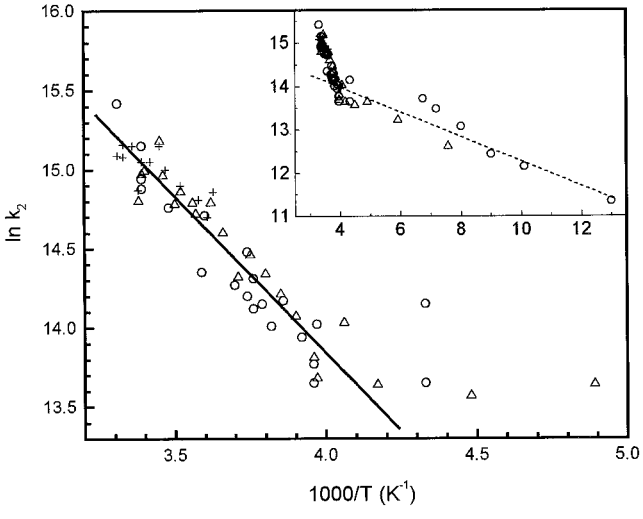

FIGURE 5 Temperature dependence of the rate of the intermediate phase (k_2), obtained by fitting transients like those shown in Figs. 1–4 to Eq. 1. +, △, Measured at 1064 nm in the absence and in the presence of glycerol, respectively; ○, measured at 691 nm in the presence of glycerol. The solid line was obtained by linear regression of the data between 303 and 240 K according to the Arrhenius law $\ln k = \ln k_0 - E_A/k_BT$, where $k_0 = 4.04 \times 10^9\ s^{-1}$ and $E_A = 171$ meV. The broken line in the inset represents the linear regression to the data below 240 K, where $k_0 = 3.69 \times 10^6\ s^{-1}$ and $E_A = 22$ meV.

Down to ~240 K, ln k_2 decreases approximately linearly with the inverse temperature; linear regression to the data between 303 and 240 K yielded a slope corresponding to an activation energy of 171 ± 8 meV. At lower temperatures, evaluation of k_2 became more and more difficult because of the decreasing amplitude of the intermediate phase. Nevertheless, it appears that the decrease in ln k_2 levels off at ~240 K. Below 120 K an intermediate phase became detectable again at 691 nm (but not at 1064 nm). Presumably this reflects a charge recombination of $P700^+A_1^-$ to the P700 ground state, which may be related to bleaching because the $P700^+$ minus P700 difference spectrum becomes positive around 690 nm at very low temperatures (Hoch, 1977, and references therein). Linear regression to the data below 240 K (*inset* of Fig. 5) yielded a slope corresponding to an activation energy of 22 ± 3 meV.

For the slow phase, the temperature dependence of rate constant k_3 is shown in Fig. 6. The data down to ~90 K are well desribed by the Arrhenius law with an activation energy of 40 ± 1 meV (*linear regression line* shown in Fig. 6). Only below 90 K does the temperature dependence of k_3 appear to level off. This behavior is in line with that observed previously for the decay of the triplet state of P700 (Mathis et al., 1978, in connection with Sétif et al., 1981). A very similar temperature dependence of the slowest kinetic phase was observed with a sample that had been illuminated in the presence of dithionite at pH 10 for a longer time to prereduce A_1 completely (data not shown; in this case, ^{3}P700 is formed via recombination of the primary pair).

DISCUSSION

In the present work we have investigated the temperature dependence of the charge recombination $P700^+A_1^- \rightarrow {}^3P700\ A_1$, which had been observed at room temperature after prereduction of all three iron-sulfur clusters (Sétif and Bottin, 1989; Sétif and Brettel, 1990). A major difficulty was that the treatments applied to prereduce cluster F_X led to some reduction of A_1 as well. Therefore, the transients presented above are contaminated by absorbance changes due to charge recombination in the primary pair $P700^+A_0^-$ (including formation of ^{3}P700 via this recombination) in some fraction of the centers. In another fraction, F_X was not prereduced, because the reduction treatment was not strong and prolonged enough to reduce F_X in all of the centers and because some reoxidation of F_X^- could occur during the delay between the end of the prereduction treatment and the flash absorption experiments. Despite these difficulties, our data demonstrate that the recombination $P700^+A_1^- \rightarrow {}^3P700\ A_1$ slows down with decreasing temperature. An activation energy of ~170 meV was estimated for the temperature range from 303 K to 240 K. The yield of ^{3}P700 formation from the secondary pair decreased with decreasing temperature. Because of the smaller amplitudes of the corresponding kinetic phase, the evaluation and interpretation of the data were more difficult and ambiguous at lower temperatures (see below).

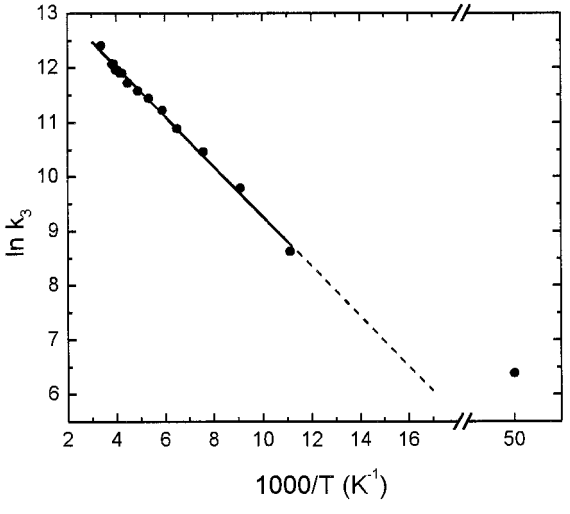

FIGURE 6 Temperature dependence of the rate of the slow phase (k_3) obtained by fitting transients at 1064 nm like those shown in Fig. 2, but measured on extended time scales. The line was obtained by linear regression as in Fig. 5, excluding the data point at the lowest temperature (20 K); parameters: $k_0 = 1.04 \times 10^6\ s^{-1}$ and $E_A = 40$ meV.

We were interested in the temperature dependence of the reaction $P700^+A_1^- \rightarrow {}^3P700\ A_1$ because it seemed conceivable that the high rate of this reaction ($t_{1/2} \approx 250$ ns at room temperature) is due to a recombination pathway involving thermal repopulation of the primary pair. Such an indirect recombination pathway, which could account for the observed activation energy, will be discussed in detail in the following. The alternative pathway, namely direct recombination of the secondary pair to form ^{3}P700, will be considered subsequently.

A kinetic scheme for secondary pair recombination, including repopulation of the primary pair, is shown in the right-hand panel of Fig. 7. The bold arrows indicate the pathway of ^{3}P700 formation, which we suggest to be dominant at room temperature under conditions of prereduced F_A, F_B, and F_X. Singlet-triplet spin evolution (indicated by the symbol ω) should occur nearly exclusively in the secondary pair (and not in the primary pair), because this process is much slower (on the order of 10 ns in comparable radical pairs; Werner et al., 1977, 1978) than forward electron transfer from A_0 to A_1 (on the order of 30 ps; see Brettel, 1997, for a review). Singlet-triplet spin evolution in the secondary pair $P700^+A_1^-$ is expected to be rather efficient because the magnetic interaction between the partners is very weak (exchange interaction $J \approx 1\ \mu$T, dipolar interaction $D \approx -170\ \mu$T (Zech et al., 1996); these values are comparable to those for the secondary pair $P^+Q_A^-$ in purple bacterial reaction centers reported in the same paper); hence the singlet level and the three triplet levels of the secondary pair are virtually isoenergetic, which is most favorable for singlet-triplet spin evolution (see Hoff, 1981, and Volk et al., 1995, for reviews). Thermal repopulation of the primary pair (in a triplet spin configuration) and subsequent charge recombination (rate constant k_T) would create

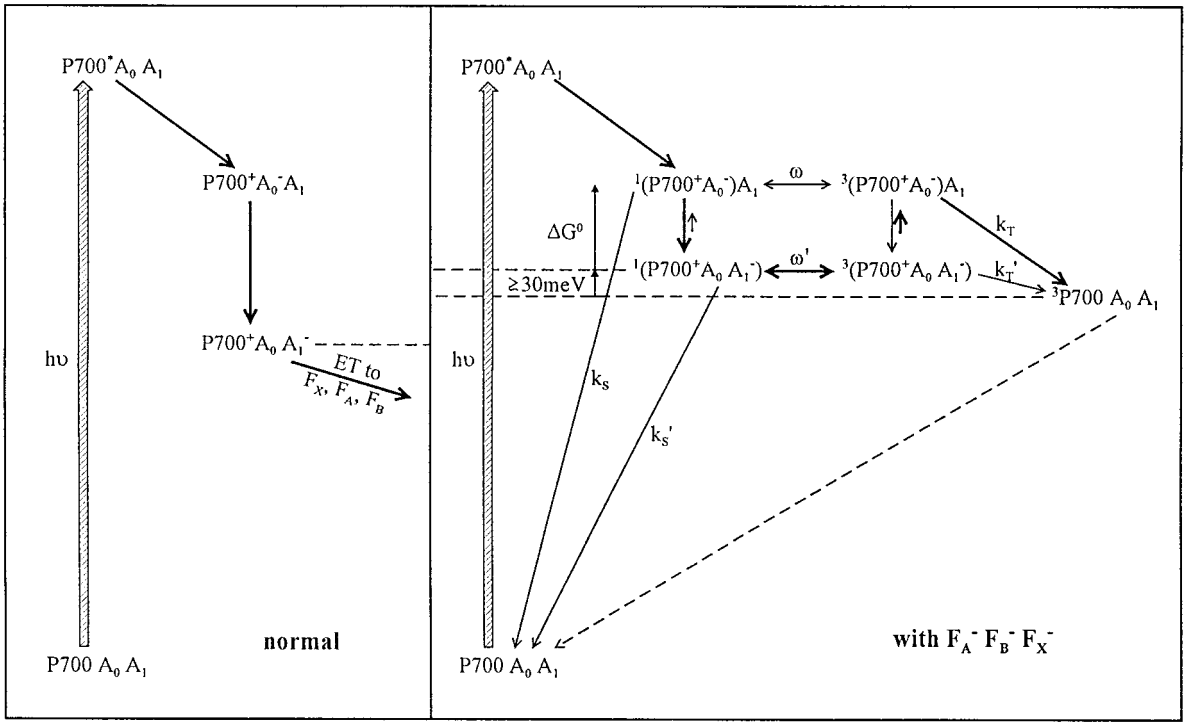

FIGURE 7 (*Left*) Reaction scheme for normal forward electron transfer (ET) in PS I. (*Right*) Reaction scheme for charge recombination of the secondary pair when F_A, F_B, and F_X are prereduced. Bold arrows indicate the reaction route for formation of ^{3}P700, which is suggested to dominate at room temperature. The vertical arrangement indicates the free energies of the different states qualitatively. Note that we assume that the secondary pair ($P700^+A_0A_1^-$) is energetically below ^{3}P700 under normal conditions, but above ^{3}P700 when F_A, F_B, and F_X are prereduced. Singlet-triplet spin evolution in the primary pair (ω) is expected to be less efficient than in the secondary pair (ω') because of a nonnegligible exchange interaction in the primary pair (singlet-triplet splitting $|2J| \approx 6$ mT; Brettel and Sétif, 1987). See text for further details.

the triplet state of P700. A high yield of ^{3}P700 is expected, if k_T exceeds k_S (recombination rate to the singlet ground state of P700), and if ^{3}P700 has a lower free energy than the secondary pair.

A similar scheme has been proposed by Shopes and Wraight (1987) to explain the temperature dependence of secondary pair recombination in *Rhodopseudomonas viridis*, but there the free energy of the triplet state of the primary electron donor is well above that of the secondary pair; therefore activated recombination of the latter was assumed to occur predominantly via repopulation of the primary pair in the singlet spin configuration and recombination to the singlet ground state of the primary donor (Shopes and Wraight, 1987). The opposite situation in PS I under the strongly reducing conditions applied in the present study is presumably due to an upshift of the free energy of the secondary pair due to the prereduction of F_A, F_B, and F_X (Sétif and Brettel, 1990); at moderate ambient potential (all iron-sulfur clusters oxidized), the secondary pair is most likely well below ^{3}P700 in free energy, and therefore, under normal conditions, the triplet recombination pathway is inefficient and cannot compete with forward electron transfer ($t_{1/2} \approx 200$ ns) from A_1^- to the iron-sulfur clusters (*left-hand panel* of Fig. 7; distinction between singlet and triplet radical pairs was omitted because the rate of forward electron transfer should be independent of the spin configuration). A possible competition between forward electron transfer from A_1^- and triplet recombination has previously been discussed by Vos and van Gorkom (1990).

For an approximate quantitative description of the rate of ^{3}P700 formation according to the scheme in the right-hand panel of Fig. 7, we neglect all decay pathways of the secondary pair other than recombination via the primary pair to form ^{3}P700. As equilibration between the primary and the secondary pair can be assumed to be much faster than the overall decay of these radical pairs, the effective rate k_{eff} for the formation of ^{3}P700 can be expressed as

$$k_{eff} = [1 + \exp(\Delta G^0/k_B T)]^{-1} \alpha_T k_T \qquad (2)$$

where $\Delta G°$ is the free energy difference between the primary and secondary pair, k_B is the Boltzmann constant, and α_T is the fraction of the radical pairs in triplet radical pair states. According to simulations of singlet-triplet spin evolution in comparable radical pairs (exchange interaction neglected), α_T is expected to rise from zero (immediately after creation of the pair from a singlet precursor) to ~0.7 within ~10 ns (Werner et al., 1977, 1978); because of relaxation processes, α_T should finally reach the equilibrium value of 3/4 (the singlet state and the three triplet states are virtually degenerate in an only weakly coupled radical pair like $P700^+A_1^-$). For the sake of simplicity, we neglect

the variation in α_T with time and use an estimated average value of 0.7 for α_T throughout the lifetime of $P700^+A_1^-$. To a first approximation, $\Delta G°$ may be identified with the activation energy E_A of the reaction $P700^+A_1^- \rightarrow {}^3P700\ A_1$, because the primary pair would be the transition state in the suggested mechanism (this approximation neglects a possible entropic contribution to $\Delta G°$ and any temperature dependence of k_T). With $\Delta G° = E_A = 171$ meV and $k_{eff} = 3 \times 10^6\ s^{-1}$ (corresponding to $t_{1/2} = 231$ ns) at 298 K, Eq. 2 yields $k_T = 3.3 \times 10^9\ s^{-1}$. For comparison, estimates of the triplet recombination rate constant of the pair P^+H^- in purple bacterial reaction centers (H denotes the bacteriopheophytin at the active branch) range from $4 \times 10^8\ s^{-1}$ to $2.5 \times 10^9\ s^{-1}$ (reviewed by Volk et al., 1995). Given the rather rough approximations made in our estimation, it is not certain that k_T in PS I is really faster than the corresponding rate in purple bacterial reaction centers.

Having estimated k_T to be on the order of $10^9\ s^{-1}$, the much slower decay of the primary pair $P700^+A_0^-$ when electron transfer to A_1 is blocked ($t_{1/2} \approx 30$ ns) should be dominated by the singlet recombination rate constant k_S (see Volk et al., 1995, and references therein for a quantitative evaluation of the similar situation for the pair P^+H^- in purple bacterial reaction centers). Hence k_S should be on the order of $2 \times 10^7\ s^{-1}$, i.e., $k_S \ll k_T$, in line with a requirement for a high $^3P700$ yield during secondary pair recombination, according to the scheme in the right-hand panel of Fig. 7.

The recombination pathway proposed in Fig. 7 has implications for the energetics of PS I. With a free energy of 1.29 eV for $^3P700$ (Shuvalov, 1976), a free energy difference of at least 30 meV between $P700^+A_1^-$ (when all three iron-sulfur clusters are reduced) and $^3P700$ (Sétif and Brettel, 1990), and assuming that $\Delta G° \approx 150$ meV, the free energy of the primary pair should be at least 1.47 eV, i.e., at most 300 meV below P700* (free energy ~1.77 eV, corresponding to the energy of a 700-nm photon). This is compatible with a recent estimate of 250 meV for the free energy gap between P700* and the primary pair obtained from the yield of delayed fluorescence under conditions in which A_1 and all three iron-sulfur clusters were presumably reduced (Kleinherenbrink et al., 1994).

Summarizing these considerations, a recombination pathway via repopulation of the primary pair (*bold arrows* in Fig. 7) can account for the present experimental data above ~240 K and is largely consistent with data from the literature. Of course, this consistency does not disprove the alternative pathway, namely direct recombination of the secondary pair to the triplet state of P700 (rate constant k'_T in Fig. 7). If the latter pathway were dominating, the observed activation energy would have to be inherent to the electron transfer process and the high reaction rate at room temperature would have to be due to a rather short distance between P700 and A_1.

The position of A_1 within the PS I complex could not yet be resolved from the x-ray diffraction data at a resolution of 4 Å (Krauss et al., 1996; Schubert et al., 1997). The distance between P700 and A_1, however, has been deduced from pulsed EPR measurements; the published result of 25.4 ± 0.3 Å (Zech et al., 1996; Bittl and Zech, 1997) or 25.3 ± 0.3 Å (Dzuba et al., 1997) should represent the distance between the centers of spin density of $P700^+$ and A_1^-. The distance relevant for an estimation of the electron transfer rate, however, is not that between the centers, but the shortest distance between edge atoms of the reactants. Taking into account the geometry of the chlorophyll dimer P700 (Krauss et al., 1996; Schubert et al., 1997), the orientation of the carbonyl bonds of the phylloquinone A_1 as parallel to the vector joining P700 and A_1 (Van der Est et al., 1997), and the orientation of the latter vector as tilted by 27° from the normal to the membrane plane (MacMillan et al., 1997; Bittl et al., 1997), the edge-to-edge distance between P700 and A_1 cannot be shorter than 15 Å. This lower bound can be obtained under the extreme assumption that the spin of $P700^+$ is centered on the dimer half, which is more distant from A_1, but that the other half contributes nevertheless to the electronic coupling, which mediates electron transfer from A_1^- to $P700^+$; for a position of A_1 proposed recently (Bittl et al., 1997; Schubert et al., 1997), the edge-to-edge distance would be as large as 21 ± 2 Å (Schubert, 1997). Using empirical data on the distance dependence of electron transfer in purple bacterial reaction centers (Moser et al., 1992), the optimal electron transfer rate over an edge-to-edge distance of 15 Å would be $10^6\ s^{-1}$, i.e., slower than the observed rate of $3 \times 10^6\ s^{-1}$ at room temperature. Furthermore, the optimal rate could only be achieved if the standard free energy of reaction would match the reorganization energy, and in this case the electron transfer should be activationless (Moser at al., 1992). For a reaction with a considerable activation energy, as in our case, the mismatch between free energy and reorganization energy should decrease the electron transfer rate at room temperature to far below the optimal rate estimated above. Hence the observed rate at room temperature of the reaction $P700^+A_1^- \rightarrow {}^3P700$ under conditions of prereduced F_A, F_B, and F_X appears to be too fast to be explained by direct charge recombination over the edge-to-edge distance of at least 15 Å between P700 and A_1. This leaves the activated recombination via the primary pair, as suggested in Fig. 7, as a plausible pathway for temperatures above ~240 K. As pointed out previously (Brettel, 1997), a primary pair recombination rate k_T on the order of $10^9\ s^{-1}$, as required for this pathway (see above), would be compatible with present data on the distance between P700 and A_0.

The break in the Arrhenius plot of the intermediate phase (*inset* of Fig. 5) indicates that a recombination pathway with a much smaller activation energy dominates below ~240 K. The concomitant decrease in the yield of $^3P700$ formation from the secondary pair suggests that direct (nearly activationless) recombination to the singlet ground state of P700 (k'_S in Fig. 7) competes with the activated triplet recombination pathway. Direct recombination to the P700 triplet state (rate constant k'_T) may also contribute to the nearly activationless decay of the secondary pair, but below ~120

K, only recombination to the singlet ground state was observed. The singlet recombination of the secondary pair has been reported previously to proceed with $t_{1/2} \approx 20$ μs at 10 K when all three iron-sulfur clusters were reduced (Sétif and Mathis, 1986). This is close to our observation for the intermediate phase at 691 nm ($t_{1/2} \approx 15$ μs at 20 K; not shown). Unfortunately, the quality of our kinetic data (small amplitudes of the intermediate phase; heterogeneity of the samples with respect to the reduction state of the electron acceptors) did not allow a more detailed analysis of the recombination pathways below ~240 K.

With respect to the mechanism that enables recombination of the secondary pair to form the triplet state of P700 under strongly reducing conditions, it has been proposed (Sétif and Brettel, 1990) that Coulombic interaction with F_X^- lifts the free energy of the secondary pair $P700^+A_1^-$ to above that of ^{3}P700, but other modifications (e.g., structural changes or reduction of other components within the PS I complex) may also be involved. Interestingly, we observed a tendency that an ~5-μs decay phase at 811 nm (where $P700^+$ and ^{3}P700 absorb similarly) appeared earlier during the illumination in the presence of dithionite at room temperature than the transient at 1064 nm (rising with $t_{1/2} \approx$ 250 ns and decaying with $t_{1/2} \approx 4$ μs), which reflects formation and decay of ^{3}P700 (see Materials and Methods). This might be explained by two consecutive modifications of the PS I complex: the first modification would accelerate the recombination of the secondary pair to $t_{1/2} \approx 5$ μs with negligible or low triplet yield, and the second modification would enable the fast, activated recombination forming ^{3}P700. Further work is necessary to characterize in detail the modifications of the PS I complex during the reduction treatment, which finally makes possible recombination of the secondary pair to form the triplet state of P700.

We thank Dr. Hervé Bottin for providing the PS I complexes and Dr. Pierre Sétif for useful discussions and critical reading of the manuscript.

REFERENCES

Bittl, R., and S. G. Zech. 1997. Pulsed EPR study of spin-coupled radical pairs in photosynthetic reaction centers: measurement of the distance between $P_{700}^{\bullet+}$ and $A_1^{\bullet-}$ in photosystem I and between $P_{865}^{\bullet+}$ and $Q_A^{\bullet-}$ in bacterial reaction centers. *J. Phys. Chem. B.* 101:1429–1436.

Bittl, R., S. G. Zech, P. Fromme, H. T. Witt, and W. Lubitz. 1997. Pulsed EPR structure analysis of photosystem I single crystals: localization of the phylloquinone acceptor. *Biochemistry.* 40:12001–12004.

Bottin, H., and P. Sétif. 1991. Inhibition of electron transfer from A_0 to A_1 in photosystem I after treatment in darkness at low redox potential. *Biochim. Biophys. Acta.* 1057:331–336.

Brettel, K. 1989. New assignment for the 250 μs kinetics in photosystem I: $P\text{-}700^+$ recombines with A_1^- (not F_x^-). *Biochim. Biophys. Acta.* 976: 246–249.

Brettel, K. 1996. Flash absorption spectroscopy. Principle and applications in plant photosynthesis research. *Bull. Soc. R. Sci. Liège.* 65:262–268.

Brettel, K. 1997. Electron transfer and arrangement of the redox cofactors in photosystem I. *Biochim. Biophys. Acta.* 1318:322–373.

Brettel, K., and J. H. Golbeck. 1995. Spectral and kinetic characterization of electron acceptor A_1 in a photosystem I core devoid of iron-sulfur centers F_X, F_B and F_A. *Photosynth. Res.* 45:183–193.

Brettel, K., and P. Sétif. 1987. Magnetic field effects on primary reactions in photosystem I. *Biochim. Biophys. Acta.* 893:109–114.

Carithers, R. P., and W. W. Parson. 1975. Delayed fluorescence from *Rhodopseudomonas viridis* following single flashes. *Biochim. Biophys. Acta.* 387:194–211.

Den Blanken, H. J., and A. J. Hoff. 1983. High-resolution absorbance-difference spectra of the triplet state of the primary donor P-700 in photosystem I subchloroplast particles measured with absorbance-detected magnetic resonance at 1.2 K. *Biochim. Biophys. Acta.* 724: 52–61.

Dzuba, S. A., H. Hara, A. Kawamori, M. Iwaki, S. Itoh, and Yu. D. Tsvetkov. 1997. Electron spin echo of spin-polarised radical pairs in intact and quinone-reconstituted plant photosystem I reaction centres. *Chem. Phys. Lett.* 264:238–244.

Golbeck, J. H., and D. A. Bryant. 1991. Photosystem I. *In* Current Topics in Bioenergetics, Vol. 16. C. P. Lee, editor. Academic Press, San Diego. 83–177.

Hiyama, T., and B. Ke. 1971. A further study of P430: a possible primary electron acceptor of photosystem I. *Arch. Biochem. Biophys.* 147: 99–108.

Hoch, G. E. 1977. P-700. *In* Photosynthesis I. A. Trebst and M. Avron, editors. Springer Verlag, Berlin, Heidelberg, New York. 136–148.

Hoff, A. J. 1981. Magnetic field effects on photosynthetic reactions. *Q. Rev. Biophys.* 14:599–665.

Hsi, E. S. P., and J. R. Bolton. 1974. Flash photolysis-electron spin resonance study of the effect of *o*-phenanthroline and temperature on the decay time of the ESR signal B1 in reaction-center preparations and chromatophores of mutant and wild strains of *Rhosopseudomonas sphaeroides* and *Rhodopspirillum rubrum*. *Biochim. Biophys. Acta.* 347: 126–133.

Kleinherenbrink, F. A. M., G. Hastings, B. P. Wittmershaus, and R. E. Blankenship. 1994. Delayed fluorescence from Fe-S type photosynthetic reaction centers at low redox potential. *Biochemistry.* 33:3096–3105.

Krauss, N., W.-D. Schubert, O. Klukas, P. Fromme, H. T. Witt, and W. Saenger. 1996. Photosystem I at 4 Å resolution represents the first structural model of a joint photosynthetic reaction centre and core antenna system. *Nature Struct. Biol.* 3:965–973.

MacMillan, F., J. Hanley, L. van der Weerd, M. Knüppling, S. Un, and A. W. Rutherford. 1997. Orientation of the phylloquinone electron acceptor anion radical in photosystem I. *Biochemistry.* 36:9297–9303.

Mathis, P., K. Sauer, and R. Remy. 1978. Rapidly reversible flash-induced electron transfer in a P-700 chlorophyll-protein complex isolated with SDS. *FEBS Lett.* 88:275–278.

Moser, C. C., J. M. Keske, K. Warncke, R. S. Farid, and P. L. Dutton. 1992. Nature of biological electron transfer. *Nature.* 355:796–802.

Parrett, K. G., T. Mehari, P. G. Warren, and J. H. Golbeck. 1989. Purification and properties of the intact P-700 and F_X-containing photosystem I core protein. *Biochim. Biophys. Acta.* 973:324–332.

Renger, G., and C. Wolff. 1976. The existence of a high photochemical turnover rate at the reaction centers of system II in Tris-washed chloroplasts. *Biochim. Biophys. Acta.* 423:610–614.

Sauer, K., P. Mathis, S. Acker, and J. A. van Best. 1978. Electron acceptors associated with P-700 in Triton solublilized photosystem I particles from spinach chloroplasts. *Biochim. Biophys. Acta.* 503:120–134.

Schubert, W. D. 1997. Röntgenkristallographische Untersuchungen zur Struktur des cyanobakteriellen Photosystem I der oxygenen Photosynthese aus *Synechococcus elongatus* bei 4 Å Auflösung. Ph.D. thesis. Freie Universität, Berlin.

Schubert, W.-D., O. Klukas, N. Krauss, W. Saenger, P. Fromme, and H. T. Witt. 1997. Photosystem I of *Synechococcus elongatus* at 4 Å resolution: comprehensive structure analysis. *J. Mol. Biol.* 272:741–769.

Sétif, P., and H. Bottin. 1989. Identification of electron-transfer reactions involving the acceptor A_1 of photosystem I at room temperature. *Biochemistry.* 28:2689–2697.

Sétif, P., H. Bottin, and P. Mathis. 1985. Absorption studies of primary reactions in photosystem I. Yield and rate of formation of the P-700 triplet state. *Biochim. Biophys. Acta.* 808:112–122.

Sétif, P., and K. Brettel. 1990. Photosystem I photochemistry under highly reducing conditions. Study of the P700 triplet state formation from the secondary radical pair ($P700^+\text{-}A_1^-$). *Biochim. Biophys. Acta.* 1020: 232–238.

Sétif, P., and K. Brettel. 1993. Forward electron transfer from phylloquinone A_1 to iron sulfur centers in spinach photosystem I. *Biochemistry.* 32:7846–7854.

Sétif, P., G. Hervo, and P. Mathis. 1981. Flash-induced absorption changes in photosystem I. Radical pair or triplet state formation? *Biochim. Biophys. Acta.* 638:257–267.

Sétif, P., and P. Mathis. 1986. Photosystem I photochemistry: a new kinetic phase at low temperature. *Photosynth. Res.* 9:47–54.

Shopes, R. J., and C. A. Wraight. 1987. Charge recombination from the $P^+Q_A^-$ state in reaction centers from *Rhodopseudomonas viridis. Biochim. Biophys. Acta.* 893:409–425.

Shuvalov, V. A. 1976. The study of the primary photoprocesses in photosystem I of chloroplasts. Recombination luminescence, chlorophyll triplet state and triplet-triplet annihilation. *Biochim. Biophys. Acta.* 430: 113–121.

Van der Est, A., T. Prisner, R. Bittl, P. Fromme, W. Lubitz, K. Möbius, and D. Stehlik. 1997. Time resolved X-, K-, and W-band EPR of the radical pair state $P_{700}^{\bullet+}A_1^{\bullet-}$ of photosystem I in comparison with $P_{865}^{\bullet+}Q_A^{\bullet-}$ in bacterial reaction centers. *J. Phys. Chem. B.* 101:1437–1443.

Vassiliev, I. R., Y.-S. Jung, M. D. Mamedov, A. Y. Semenov, and J. H. Golbeck. 1997. Near-IR absorbance changes and electrogenic reactions in the microsecond-to-second time domain in photosystem I. *Biophys. J.* 72:301–315.

Volk, M., A. Ogrodnik, and M. E. Michel-Beyerle. 1995. The recombination dynamics of the radical pair P^+H^- in external magnetic and electric fields. *In* Anoxygenic Photosynthetic Bacteria. R. E. Blankenship, M. T. Madigan, and C. E. Bauer, editors. Kluwer Academic Publishers, Dordrecht, the Netherlands. 595–626.

Vos, M. H., and H. F. van Gorkom. 1990. Thermodynamical and structural information on photosynthetic systems obtained from electroluminescence kinetics. *Biophys. J.* 58:1547–1555.

Vrieze, J., P. Gast, and A. J. Hoff. 1996. Structure of the reaction center of photosystem I of plants. An investigation with linear-dichroic absorbance-detected magnetic resonance. *J. Phys. Chem.* 100:9960–9967.

Warren, P. V., J. H. Golbeck, and J. T. Warden. 1993. Charge recombination between $P700^+$ and A_1^- occurs directly to the ground state of P700 in a photosystem I core devoid of F_X, F_B, and F_A. *Biochemistry.* 32:849–857.

Werner, H.-J., Z. Schulten, and K. Schulten. 1977. Theory of the magnetic field modulated geminate recombination of radical ion pairs in polar solvents: application to the pyrene-*N,N*-dimethylaniline system. *J. Chem. Phys.* 67:646–663.

Werner, H.-J., K. Schulten, and A. Weller. 1978. Electron transfer and spin exchange contributing to the magnetic field dependence of the primary photochemical reaction of bacterial photosynthesis. *Biochim. Biophys. Acta.* 502:255–268.

Zech, S. G., W. Lubitz, and R. Bittl. 1996. Pulsed EPR experiments on radical pairs in photosynthesis: comparison of the donor-acceptor distances in photosystem I and bacterial reaction centers. *Ber. Bunsenges. Phys. Chem.* 100:2041–2044.