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Path of Electron Transfer in Photosystem 1: Direct Evidence of Forward Electron Transfer from A_1 to $Fe-S_X$[†]

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ABSTRACT: Pulsed EPR spectroscopy and selective removal of the iron–sulfur centers in photosystem 1 have been used to study forward electron transfer from the secondary electron acceptor A_1. At cryogenic temperatures where forward electron transfer is inhibited, we have observed a $g = 2.003$ electron spin–echo signal presenting a characteristic phase shift. This out-of-phase signal is attributed to the electron spin-polarized pair P_{700}^+/A_1^-, it decays with $t_{1/e} = 23$ μs, reflecting the recombination reaction. At room temperature the out-of-phase signal is also observed, but it decays with $t_{1/e} = 200$ ns in untreated photosystem 1, due to forward electron transfer from A_1^- to one of the iron–sulfur centers. This rate is unchanged in $Fe-S_{A/B}$-depleted PS1 but is lost when the iron–sulfur center $Fe-S_X$ is removed. In the preparations depleted of all iron–sulfur centers the out-of-phase signal decays with $t_{1/e} = 1.3$ μs, reflecting either the back reaction or the decay of polarization. These results demonstrate that the electron transfer pathway in photosystem 1 is $P_{700} \rightarrow A_1 \rightarrow Fe-S_X \rightarrow Fe-S_{A/B}$.

The photosystem 1 reaction center contains a primary electron donor, a dimer of chlorophyll *a* molecules (P_{700}),[1] and five electron acceptors: a chlorophyll molecule A_0, a phylloquinone A_1, and three [4Fe-4S] iron–sulfur (Fe-S) centers called $Fe-S_X$, $Fe-S_A$, and $Fe-S_B$ [for reviews see Evans and Nugent (1993) and Golbeck and Bryant (1991)]. In contrast with photosystem 2 and purple bacterial reaction centers, the electron transfer pathway of PS1 is not yet clearly characterized. Electron transfer from A_0 to A_1 is thought to occur within 30–200 ps [for a review see Sêtif (1992)]. Recently, electron transfer within the secondary electron acceptors and the kinetics of these reactions have been intensively studied by time-resolved absorption and EPR spectroscopy.

In the P_{700}^+/A_1^- pair induced by flash illumination the radical spins are correlated, giving them a non-Boltzmann distribution of populations of their magnetic sublevels in an external magnetic field. These radical pairs present potentially high-intensity EPR signals, called electron spin polarized (ESP) signals, with emissive and enhanced absorptive resonances [for reviews see Hoff (1984) and Snyder and Thurnauer (1993)].

Pioneering work by Thurnauer et al. (1979) used electron spin echo (ESE) pulsed EPR to study radical pair interactions at room temperature in PS1. They observed an unusual out-of-phase echo signal which decayed within a few hundred nanoseconds (Thurnauer & Norris, 1980). They tentatively attributed this signal to the disappearance of one of the spin pair components (A_1^-) during the microwave pulse sequence that generates the echo. If the interaction with A_1^- was lost between the two microwave pulses the Larmor frequency of P_{700}^+ could be changed, resulting in a phase shift of the echo. They concluded that the decay of this out-of-phase signal corresponded to the forward electron transfer from A_1^- and reported a lifetime of 200 ns (Thurnauer et al., 1979).

Using time-resolved continuous wave (cw) EPR, Stehlik and co-workers observed two consecutive ESP signals (Bock et al., 1989; Stehlik et al., 1991). The first EPR spectrum shows the characteristic emission/absorption/emission (e/a/e) ESP pattern attributed to P_{700}^+/A_1^- (Blankenship et al., 1975; Gast, 1982; Furrer & Thurnauer, 1983; Hore et al., 1987). The succeeding ESP signal, mostly emissive and covering the spectral region of the P_{700}^+ EPR signal, is proposed to be part of the spectrum due to the coupled radical pair $P_{700}^+/Fe-S^-$. From these data the authors deduced a kinetic rate of 260 ns for the forward electron transfer from A_1 (Bock et al., 1989).

A_1^- reoxidation at room temperature has also been studied by time-resolved absorption spectroscopy, and a kinetic phase of 250 ns is observed in cyanobacterial PS1 (Brettel, 1988). In spinach PS1 particles, this kinetic is biphasic with $t_{1/2} =$ 25 and 150 ns (Mathis & Sêtif, 1988; Sêtif & Brettel, 1993), the faster kinetic being attributed to altered electron transfer paths due to structural changes induced by harsh treatments during the preparation of the PS1 particles (Sêtif & Brettel, 1993).

In brief, a kinetic rate of about 200 ns (250 ns in cyanobacterial PS1) for A_1^- reoxidation at room temperature seems to be well established. However, because chemical prereduction of $Fe-S_A$ and $Fe-S_B$ inhibits the forward electron transfer, it is unclear if A_1^- transfers its electron to $Fe-S_X$ or directly to $Fe-S_A$ and/or $Fe-S_B$ without involving $Fe-S_X$ (Thurnauer et al., 1982; Sêtif et al., 1984; Brettel, 1989; Sêtif & Brettel, 1993).

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[1] Abbreviations: A_0, chlorophyll electron acceptor in photosystem 1; A_1, phylloquinone electron acceptor in photosystem 1; EPR, electron paramagnetic resonance; ESE, electron spin echo; ESP, electron spin polarized; Fe-S, iron–sulfur center; $Fe-S_A$, $Fe-S_B$, and $Fe-S_X$, [4Fe-4S] centers of photosystem 1; PS1, photosystem 1; P_{700}, photosystem 1 primary electron donor.

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Iron–sulfur centers have small extinction coefficients and Fe–S_A, Fe–S_B, and Fe–S_X cannot be differentiated from each other by absorption spectroscopy. Low-temperature EPR can easily distinguish between these Fe–S centers. Unfortunately, although photoreduction of Fe–S_X^- is observed at low temperatures (Evans et al., 1975), forward electron transfer from A_1 is highly inefficient at cryogenic temperatures when measured by time-resolved absorption spectroscopy (Sétif et al., 1984). The dominant reaction at low temperature is the charge recombination between A_1^- and P_{700}^+.

In this paper, using time-resolved pulsed EPR at room temperature and at 4 K, we confirm that the out-of-phase ESP signal is characteristic of the radical pair P_{700}^+/A_1^- (Thurnauer & Norris, 1980). By selective depletion of Fe–S centers in spinach PS1, we provide direct evidence to show that the 200-ns decay of this signal at room temperature is due to the forward electron transfer from A_1 to Fe–S_X. When forward electron transfer is inhibited at either room temperature or low temperature, the lifetime of the out-of-phase signal is extended to the microsecond time range.

MATERIALS AND METHODS

Digitonin PS1 particles were prepared from spinach according to Boardman (1971) and contained about 200 chlorophylls/P_{700}. Comparative measurements were made on PS1 particles from *Synechocystis* PCC 6803 (Biggins & Mathis, 1988).

PS1 particles depleted of iron–sulfur centers were prepared according to Warren et al. (1990) using as starting material digitnonin PS1 particles from spinach. Selective depletion of Fe–S_A and Fe–S_B was checked by time-resolved absorption spectroscopy (kinetic phase change from 20 ms for P_{700}^+/Fe–S_A^- to 1 ms for P_{700}^+/Fe–S_X^- recombination was followed by monitoring P_{700}^+ absorption at 820 nm) and low-temperature cw EPR [for more details see Hanley et al. (1992)]. Depletion of all three Fe–S centers was confirmed by the disappearance of any millisecond-range absorption kinetic and low-temperature cw EPR signal from the iron–sulfur centers.

Room-Temperature Experiments. EPR samples were prepared in 0.5-mm internal diameter silica tubes. The PS1 particles (chlorophyll concentration of 4–5 mg/mL) were suspended in 50 mM Tris-HCl (pH 8) in the presence of 1 mM sodium ascorbate, 0.1 mM 2,6-dichlorophenolindophenol, and 0.1 mM methyl viologen. These conditions ensure full oxidation of the electron acceptors and reduction of the P700 prior to each laser flash. The iron–sulfur centers were prereduced by addition of 0.2% (w/v) sodium dithionite in 0.1 M potassium-glycine buffer, pH 10.0. This treatment extensively reduces the Fe–$S_{A/B}$ centers with little reduction of Fe–S_X before illumination.

Low-Temperature Experiments. EPR samples were prepared in 3-mm internal diameter silica tubes at a chlorophyll concentration of 2–4 mg/mL. Chemical prereduction of the Fe–S centers of PS1 was achieved in 0.2 M glycine–KOH (pH 10.0) and 10 mM sodium dithionite. After incubation for 30 min at room temperature in the dark, the sample was frozen in liquid nitrogen. This treatment extensively reduces Fe–$S_{A/B}$ centers with little reduction of Fe–S_X. During the ESE experiment, the sample was maintained at 4 K by an Oxford Instruments CF935 flowing gas cryostat.

The sample was excited by 532-nm laser pulses of 10-ns duration (doubled Nd-YAG laser, Spectra Physics DCR-11). The repetition rate (1–10 Hz) and flash energy (0.5–2 mJ/cm^2) were set to prevent sample degradation or photoaccumulation of reduced electron acceptors.

Spectra were recorded on a Bruker ESP380 X-band pulsed spectrometer with a variable Q, dielectric resonator (Bruker Model 1052 DLQ-H 8907). For a 16-ns pulse in a $s = {}^1/_2$, $g = 2.002$ system, the maximum microwave magnetic field generated by a 1-kW traveling wave tube amplifier was approximately 6 G within the 10-mm homogeneous region of the resonator.

The following microwave pulse echo sequence was used with quadratic detection (in- and out-of-phase channels): (*p*–τ–*2p*–τ–*echo*), where *p* corresponds to the first microwave pulse (90° impulsion), which probes the sample by tilting the total magnetization from the *z* axis (defined by the external magnetic field) to the *y* rotating axis (in-phase channel); τ corresponds to the time allowed for dephasing (fanning) of the individual magnetizations; *2p* is the second microwave pulse (180° impulsion) that flips the magnetizations which will rephase (refan) on the *y* axis after the time *t* to produce the echo (Norris et al., 1980).

At room temperature no dark signals are observed in PS1 particles when external electron donors and acceptors are present. In PS1 preparations where no reducing agent has been added, the P_{700}^+ signal can be photoaccumulated under continuous white-light illumination, and this signal can be used for the phasing of the detector. At low temperature the ESE signal from the chemically reduced Fe–S centers of PS1 is used for the setting of the in- and out-of-phase channels.

For kinetic measurements the delay between the actinic laser flash and the microwave pulse sequence is varied. At room temperature, the cavity ringing is quickly dumped and a τ of 80 ns can be used. In this condition the total echo sequence is 208 ns using a pulse sequence of *p*(16 ns)–τ(80 ns)–2*p*(32 ns)–τ–(80 ns). At low temperature, ringing is longer lived and the minimum value of τ is 112 ns. At low temperature, we used a pulse sequence of *p*(16 ns)–τ(112 ns)–*2p*(32 ns) for maximum time resolution. An echo sequence of *p*(96 ns)–τ(224 ns)–*2p*(192 ns) with longer microwave pulses of lower energy was also used when a better spectral resolution was required.

The time resolution of the experiment, i.e., the step interval of the time between the laser pulse and the first microwave pulse, can be as short as 8 ns, but in practice is usually restricted by the signal-to-noise ratio to about 40 ns. The signal is not detected until the end of the pulse sequence. However, the observed kinetics are not those occurring after a dead time equal to the pulse sequence length but reflect the kinetics occurring between the laser pulse and the first microwave pulse as the time between them is varied [see Norris et al. (1980) for a detailed analysis]. The behavior of the radical during the pulse sequence affects the ability to observe it. A radical will give an ESE signal only if the radical is present throughout the whole echo sequence. Signals with two different kinetic behaviors may be investigated:

(i) A signal formed instantaneously on the EPR time scale, which then decays. For a decaying radical the first point observed in a kinetic experiment does not represent the full intensity which would be observed for an instantaneous measurement since a fraction of the radicals will have decayed during the pulse sequence. The signal can only be detected if the signal intensity is sufficient for a detectable concentration of radicals to remain at the end of the pulse sequence. If for example the decay of this signal is as fast as 25 ns, it will essentially totally disappear during the time of a pulse sequence which is 350 ns long, and therefore this signal will be

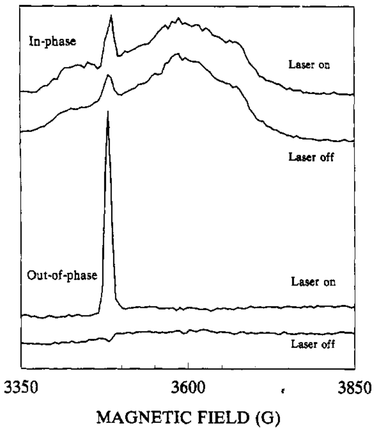

FIGURE 1: Field-swept spin–echo EPR spectra of prereduced PS1 particles at 4 K in the dark and following laser flash ilumination. Photosystem 1 particles were reduced with sodium dithionite as described in Materials and Methods to prereduce the iron–sulfur centers. Laser repetition rate of 10 Hz; microwave pulse sequence, $\pi/2$ pulse = 16 ns, τ = 112 ns; 0.5 mT/point, 10 sequences averaged/point; delay between laser pulse and microwave sequence t_0 = 1500 ns. Top to bottom: in phase, laser on; in phase, laser off; out of phase, laser on; out of phase, laser off.

undetectable. If the decay is longer than the pulse sequence, say in the microsecond time range, it will be readily detectable. Signals with intermediate decay rates with $t_{1/e}$ in the same range as the pulse train length will be detectable if the initial radical concentration is high enough for a detectable concentration to survive to the end of the pulse train. $t_{1/e}$ can then be calculated by fitting the decay observed.

(ii) A signal which presents a rise time in the nanosecond range. For this signal the first kinetic point observed corresponds to the amount of radical formed at the time of the first microwave pulse, and additional radicals formed during the echo sequence will not contribute to the signal intensity. The measured kinetics will again reflect changes between the laser pulse and first microwave pulse.

RESULTS

Low Temperatures. Figure 1 shows the field-swept ESE spectra obtained at 4 K with spinach digitonin PS1 particles in which the Fe–$S_{A/B}$ centers are prereduced. In the dark the in-phase channel shows the spectrum of the prereduced Fe–$S_{A/B}$ centers and a small g = 2.00 radical; this radical signal increases when the spectrum is recorded 1500 ns after a laser flash. In the out-of-phase channel there is no signal in the dark; a large light-induced signal at g = 2.003 with ΔH ptp = 1.2 mT is observed in the out-of-phase channel following the laser flash.

When the microwave pulse sequence is triggered immediately after the laser flash, a signal is first observed in-phase that rapidly converts to the out-of-phase signal. The in-phase kinetic trace (Figure 2B) is limited by the detection system so that the observed rise and decay of the in-phase signal corresponds to the instrument response time. The kinetic behavior is the same across the full width of the signal. No signals are observed off-resonance. The signals are only observed in samples in the appropriate redox state: if P700 is initially oxidized, or becomes oxidized as the result of irreversible electron transfer to Fe–S_A at low temperature, no signal is seen. The out-of-phase signal decays with a rate constant $t_{1/e}$ = 23 μs (Figure 2A). The field-swept spectrum of this out-of-phase signal is constant during its decay (Figure 3).

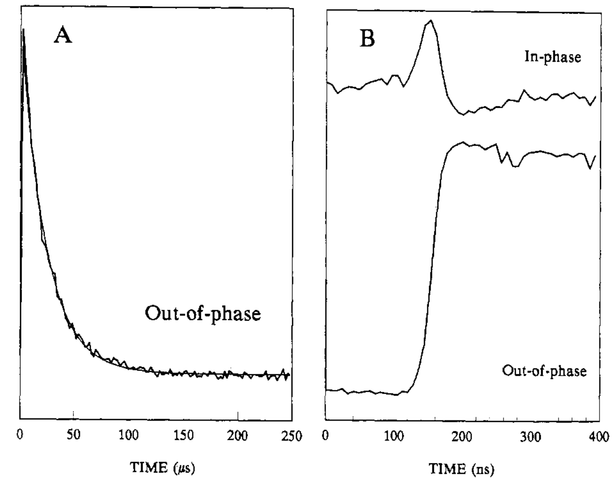

FIGURE 2: (a) Out-of-phase kinetic trace at 4 K of the decay of the laser flash induced signal at H_0 = 348.0 mT in photosystem 1 particles in which the iron–sulfur centers are prereduced with sodium dithionite. t_0 is incremented with 2-μs steps; 125 points with 10 pulse sequences/point were recorded. Fit: $t_{1/e}$ = 23 μs. (b) In-phase and out-of-phase kinetic trace observed under the same conditions, except that 8-ns increments of t_0 were used.

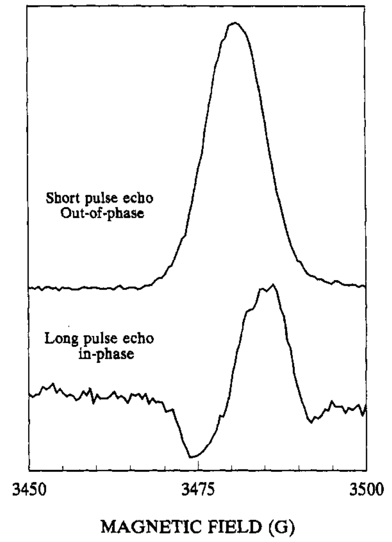

FIGURE 3: Field-swept spin–echo EPR spectra of the laser-induced signal (on–off, t_0 = 1 μs) in prereduced photosystem 1 at 4 K. Photosystem 1 particles were prereduced with sodium dithionite to reduce the iron–sulfur centers as described in Materials and Methods. Top spectrum: out-of-phase signal obtained with short pulse of high intensity ($\pi/2$ pulse = 16 ns); 0.05 mT/point; 20 added sequences/point. Bottom spectrum: in-phase signal obtained with longer pulses of lower intensity ($\pi/2$ pulse = 96 ns); 0.05 mT/point; 40 added sequences/point.

Using longer microwave pulses of lower intensity for greater spectral resolution (see Materials and Methods) results in a decrease of the out-of-phase signal in favor of an in-phase signal presenting an e/a/e electron spin polarization (ESP) pattern centered around g = 2.00 with essentially the same line width as the out-of-phase signal (Figure 3). This ESP spectrum is characteristic of the correlated spin radical pair P_{700}^{+}/A_{1}^{-} (Hore et al., 1987; Furrer & Thurnauer, 1983). Narrowing of the e/a/e signal seen with long pulses, and of the out-of-phase signal seen with short pulses, can be observed in a PS1 preparation from *Anabaena variabilis* containing selectively deuterated phylloquinone (Heathcote et al., manuscript in preparation).

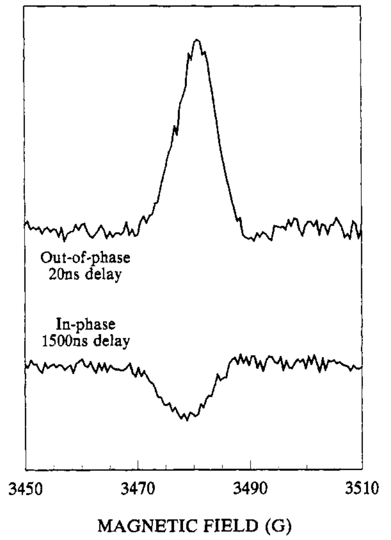

FIGURE 4: Field-swept spin–echo EPR spectra of the laser flash induced signal (on–off) obtained in intact PS1 particles at room temperature. Samples were prepared as described in Materials and Methods. Laser repetition rate of 2.4 Hz; microwave pulse sequence, $\pi/2$ pulse = 16 ns, τ = 80 ns; 0.1 mT/point, 10 added sequences/point. Top spectrum: out-of-phase signal, t_0 = 20 ns. Bottom spectrum: in-phase signal, t_0 = 1500 ns.

Kinetic traces at different field positions in the spectrum of the e/a/e ESP signal show identical rate constants for the decay of this signal of 23 μs.

Experiments carried out on other PS1 preparations, or when Fe–S$_A$/Fe–S$_B$ or all the Fe–S centers of PS1 particles have been removed, provided qualitatively the same results at 4 K, with only minor differences in the decay rate at 4 K.

Room Temperature. Figure 4 shows the field-swept ESE spectra of the light-induced signals, obtained at different times with respect to the laser flash, in untreated PS1 particles in the presence of external electron donors and acceptors (see Materials and Methods). These two signals have similar g values around g = 2.00 (Figure 4) but different phases. One is detected in the out-of-phase channel and is spectrally the same as that already observed at 4 K, and the other one is in-phase and emissively polarized. Their kinetic traces can therefore be obtained simultaneously in the two different channels, in- and out-of-phase.

The room-temperature ESE kinetic traces of untreated PS1 particles are shown in Figure 5 (top trace). In-phase, after an initial spike, a negative (emissive) signal is observed with a rise time of $t_{1/e}$ = 160 ± 50 ns and a decay with $t_{1/e}$ = 2000 ± 100ns. The out-of-phase signal decays with $t_{1/e}$ = 130 ± 50 ns. A minor component of this signal decays with a longer rate constant ($t_{1/e}$ = 1400 ± 100 ns). This contribution corresponds to less than 5% of the initial intensity of the out-of-phase signal. Within the error of the measurements, the rise of the emissive in-phase signal parallels the decay of the out-of-phase signal.

We have made comparative measurements on *Synechocystis* PS1 particles. These have a low signal-to-noise ratio due to a high chlorophyll/P$_{700}$ ratio and a low concentration of photosystem 1 reaction centers. However, they show kinetic decay rates similar to those obtained with spinach PS1 particles (300 ± 50 ns for the out-of-phase signal and 1500 ± 100 ns for in-phase signals, respectively). The rise of the in-phase signal again parallels the decay of the out-of-phase signal.

Very similar results to those obtained on untreated PS1 particles are also observed with Fe–Se$_{A/B}$-depleted PS1 particles (middle trace in Figure 5): a rise time $t_{1/e}$ = 170 ± 50 ns and decay time constant of $t_{1/e}$ = 2000 ± 100 ns are observed for the emissive in-phase signal, and a major decay time constant of $t_{1/e}$ = 190 ± 50 ns and an additional minor decay phase of $t_{1/e}$ = 1500 ± 100 ns, corresponding to less than 10% of the initial intensity, are observed for the out-of-phase signal. The intensity of the emissive in-phase signal relative to the out-of-phase signals is, however, significantly smaller in the Fe–S$_{A/B}$-depleted preparation than in untreated PS1 particles.

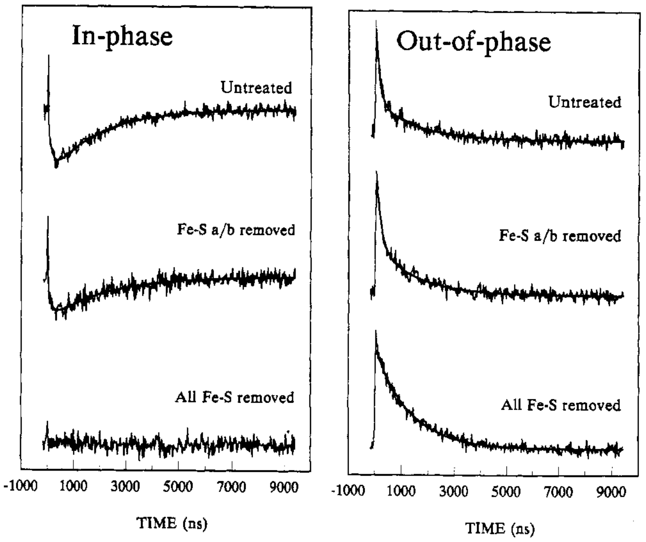

FIGURE 5: In-phase (left) and out-of-phase (right) kinetic traces at room temperature of the laser flash induced signals in photosystem 1. Samples were prepared as described in Materials and Methods. Laser repetition rate 2.4 Hz; H_0 = 348.05 mT; $\pi/2$ pulse = 16 ns, τ = 80 ns; t_0 was incremented with 32-ns steps, 300 points with 40 added sequences/point were recorded. In phase (left panel). Top: Intact, fit $t_{1/e}$ = 160 ± 50 ns (rise), 200 ± 100 ns (decay). Middle: Fe–S$_{A/B}$ removed, fit $t_{1/e}$ = 170 ± 50 ns (rise), 2000 ± 100 ns (decay) with a minor component (less than 10%) 1500 ± 100 ns. Bottom: All Fe–S centers removed. Out of phase (right panel). Top: intact, fit $t_{1/e}$ = 130 ± 50 ns with a minor component (less than 5%) 1400 ± 100 ns. Middle: Fe–S$_{A/B}$ removed, $t_{1/e}$ = 190 ± 50 ns with a minor component (less than 10%) 1500 ± 100 ns. Bottom: All Fe–S centers removed, $t_{1/e}$ = 1250 ± 50 ns.

The spectra of PS1 particles with all of the Fe–S centers removed (Figure 5, bottom trace), show no emissive in-phase signal. A small initial spike is detected in-phase, and the out-of-phase intensity decays monophasically with a rate constant $t_{1/e}$ = 1250 ± 50 ns.

Similar results are observed in intact photosystem 1 samples reduced with sodium dithionite in which the Fe–S$_{A/B}$ centers are prereduced. No emissive polarized signal is seen in the in phase channel, only the very fast initial spike decaying to the out-of-phase signal. The out-of-phase signal decays with $t_{1/e}$ = 500 ± 50 ns in the samples with the Fe–S$_{A/B}$ iron–sulfur centers prereduced.

DISCUSSION

While the spectral characteristics of the out-of-phase reported here are identical to earlier data from Thurnauer and Norris (1980), its kinetic behavior is shown to depend on the experimental conditions. Thurnauer and Norris suggested that the phase shift occurred because forward electron transfer during the pulse sequence resulted in only one of the original radical pair surviving to the second microwave pulse, so that the interaction between them was "switched off" during the pulse sequence. This mechanism does not explain the phase shift results described here, as the out-of-phase signal is seen at 4 K when little forward electron transfer from A$_1^-$ is expected and the lifetime of the radical pair is long compared to the microwave pulse sequence. The first few data points in each kinetic trace obtained using high-power microwave pulses show a "spike" of an in-phase signal rapidly converting to the out-of-phase signal. This signal is only seen in photosystem 1 samples in an appropriate redox state and in the spectral region of the out-of-phase signal. It is not, therefore, a laser artifact and is probably related to the $P700^+/A_1^-$ radical pair. Whether the signal remains in-phase or shifts out-of-phase appears to depend on the pulse energy. The spectrum of the signal observed using relatively low-power pulses is that of the classical photosystem 1 ESP signal. A discussion of the original of the phase shift will be presented elsewhere (Moënne-Loccoz et al., manuscript in preparation). For the present discussion only the assignment of the out-of-phase signal to the radical pair P_{700}^+/A_1^- is relevant.

Our low-temperature ESE experiments on PS1 particles show the following results from comparison of the well-characterized e/a/e/ ESP signal of P_{700}^+/A_1^-, observed using long, relatively low-intensity microwave pulses, and the out-of-phase signal, observed using short, high-intensity pulses: (i) They span the same spectral region. (ii) As the pulse length is changed, the relative signal intensities in the in-phase and out-of-phase channels change in parallel. The relative intensity of the in-phase signal observed with long pulses and of the out-of-phase signal seen with short pulses is similar between different samples. (iii) They decay with the same rate constant. (iv) The line width of both is decreased when phylloquinone is biosynthetically deuterated (Heathcote et al., manuscript in preparation). These results show that it is the same radical pair that gives rise to these two signals. The out-of-phase signal can therefore be assigned to the radical pair P_{700}^+/A_1^- as proposed by Thurnauer and Norris (1980).

The observed decay of the out-of-phase signal at low temperature may be due either to the relaxation of spin polarization or charge recombination between the $P700^+$ and A_1^- radicals. The 23-μs time constant that we observed at low temperature for this decay is consistent with the time-resolved absorption measurements of $P700^+/A_1^-$ recombination (Sétif et al., 1984) and can be assigned to the back reaction within the radical pair P_{700}^+/A_1^-.

When the kinetics of intact PS1 particles are investigated at room temperature in the presence of external electron donors and acceptors, the out-of-phase signal characteristic of the pair P_{700}^+/A_1^-, decays with a rate of approximately 200 ns. (The measured rates vary between 130 and 190 ns in spinach and 300 ns in *Synechocystis*. Literature values which seem to refer to this decay vary between 150 and 300 ns. For convenience we will refer to it as the "200-ns" rate.) This is in good agreement with rates observed by cw EPR (Block et al., 1989), the original pulsed EPR work of Thurnauer and Norris (1979), and the optical work of Setif and co-workers [reviewed by Setif (1992)]. All of these measurements indicate forward electron transfer from A_1^- at room temperature occurs with a kinetic rate of about 200 ns. The decay of the out-of-phase signal is paralleled by the rise of the in-phase signal, which can therefore be assigned to the radical pair consisting of the $P700^+$ radical and the electron acceptor taking the electron from A_1^- (Block et al., 1989). Removal of the Fe–$S_{A/B}$ centers from the preparation has no effect on the kinetic properties. Removal of Fe–S_X in addition results in the disappearance of the in-phase signal and a 10-fold slowing in the decay of the out-of-phase signal. These results indicate that the electron is transferred from A_1^- to Fe–S_X.

Minor contributions (less than 10%) of a slow-decaying out-of-phase signal in Fe–$S_{A/B}$-depleted PS1 particles probably indicate the presence of a small fraction of photosystems where Fe–S_X has been damaged or removed during the Fe–S_A/Fe–S_B extraction. A similar slow decay which is observed in untreated PS1 particles (less than 5%) may corresponds to a minority of PS1 reaction centers inefficient in forward electron transfer.

The emissive in-phase signal that we observe is very similar to PS1 ESP signals studied by time-resolved cw EPR (Blankenship et al., 1975; Dismukes & Sauer, 1978; Bock et al., 1989). The 2-μs decay rate of this signal has been clearly assigned to relaxation of P_{700}^+ spin polarization by Bock et al. (1989), who observed a 2-μs decay rate of this P_{700}^+/Fe–S^- ESP signal with transient cw EPR using low microwave power and a faster relaxation with higher microwave energy (Bock et al., 1989).

Two interpretations of this signal have been proposed (Bock et al., 1989): (i) This signal is the ESP signal of P_{700}^+ within the radical pair P_{700}^+/Fe–S^-, and no contributions from the Fe–S radical are observed because of its large *g* anisotropy. (ii) This signal is the residual spin polarization of P_{700}^+ (induced during the state P_{700}^+/A_1^-) after A_1^- reoxidation.

If the second hypothesis is correct, the emissive signal intensity should be directly related to the intensity of the previous ESP signal. We observe a decrease of the in-phase/out-of-phase intensity ratio in Fe–$S_{A/B}$-depleted PS1 particles compared to the ratio in intact particles. A slight modification in P_{700}^+/Fe–S_X^- spin–spin interactions, related to the removal of Fe–$S_{A/B}$, would be likely to change the intensity of the ESP signal of the radical pair P_{700}^+/Fe–S^-. Our experiments therefore support the first interpretation. However, it would be expected that the electron would be transferred from Fe–S_X to Fe–$S_{A/B}$ in less the 2 μs, so the origin of the signal may be more complex than these interpretation suggest. These results clearly show that the immediate electron acceptor from A_1^- is Fe–S_X and confirm the identification of the emissive signal as arising from the electron spin-polarized signal of $P700^+$ within the $P700^+$/Fe–S_X^- radical pair.

When there is no forward electron transfer from the radical pair P_{700}^+/A_1^-, the out-of-phase signal decay is slowed down. In PS1 particles where all of the Fe–Se centers have been removed, the out-of-phase signal decays with a $t_{1/e} = 1.3$ μs. The decay of the P_{700}^+/A_1^- out-of-phase signal may due be either to relaxation of spin polarization or to charge recombination between these two radicals. In this type of preparation, the radical pair P_{700}^+/A_1^- is thought to recombine with a rate of 3 μs (Warren et al., 1993). In intact PS1 particles in the presence of sodium dithionite, when the Fe–$S_{A/B}$ centers are extensively chemically reduced, our measurements show no emissive in-phase signal from P_{700}^+/Fe–S_X^-, confirming the inhibitory effect of this treatment on the forward electron transfer from A_1. We observed a relatively fast decay of the P_{700}^+/A_1^- signal ($t_{1/e} = 500$ ns) in these samples. P_{700}^+/A_1^- is reported to recombine with a kinetic rate of 750 ns in samples prepared under similar conditions (Setif & Bottin, 1989). It therefore seem likely that the observed decays of the out-of-phase signal reflect the back reaction between $P700^+$ and A_1^- in both reduced intact samples and iron–sulfur-depeleted samples. The faster decay rate may reflect coulombic repulsion between the charges on A_1^- and the reduced iron–sulfur centers or perhaps structural changes occurring on reduction. The effect of changes in redox state of the iron–sulfur centers is extensively discussed by Warren et al. (1993).

Thurnauer et al. (1982) reported a decrease in the intensity of the out-of-phase signal when the preparation was reduced to -550 mV in the presence of low-potential mediators at pH 11. This procedure was used to reduce Fe–$S_{A/B}$. They interpreted the loss of the out-of-phase signal as being due to an increase in the lifetime of the radical pair. We observe an increase in the lifetime of the out-of-phase signal in samples with Fe–$S_{A/B}$ reduced. We attribute this to an increase in the lifetime of the $P700^+/A_1^-$ radical pair caused by the inhibition of forward electron transfer. Although the observation of the behavior of the out-of-phase signal is different in the two experiments, it is likely that this is a reflection of the different experimental parameters of the pulse experiment; the conclusion with regard to the electron transfer process is the same. However, Botton and Setif (1991) have shown that incubation for less than 5 min under the conditions used by Thurnauer et al. results in double reduction of A_1; Snyder et al. (1991) have shown that double reduction of A_1 results in loss of the spin-polarized signal. It is possible, therefore, that the loss of signal observed by Thurnauer et al. (1982) was the result of double reduction of A_1. No mediators were used in our experiments with dithionite reduction, and under these conditions double reduction of A_1 is negligible.

Our ESE experiments show no evidence of a biphasic reoxidation of A_1^- with $t_{1/2}$ = 150 and 25 ns observed by time-resolved absorption spectroscopy (Sétif & Brettel, 1993). This is probably due to limitations in the time response of our equipment. Because of the length of the microwave pulse echo sequence, we could not detect a 25-ns decay rate of the P_{700}^+/A_1^- radical pair signal (see Materials and Methods). Sétif and Brettel (1993) also observed that the 25-ns half-time phase is not inhibited by the prereduction of Fe–$S_{A/B}$ and report the formation of 40% ± 10% of P_{700}^+/Fe–S_X^- in digitonin PS1 particles through the 25-ns phase. We observed no in-phase signal indicative of the formation of $P700^+$/Fe–S_X in spinach particles in which Fe–$S_{A/B}$ were prereduced. We would expect to detect a signal reflecting 40% of the centers, although we would not resolve the kinetics of its appearance. The decay time of the out-of-phase signal suggests that significant electron transfer to the iron–sulfur centers does not occur in this experiment. Setif and Brettel suggest that the P_{700}^+/Fe–S_X^- radical pair is formed in the 25-ns phase as a result of structural change. The failure to detect this reaction may therefore reflect variations in the properties of the preparation or the experimental conditions.

Setif and Brettel (1993) suggest that while the 25-ns phase reflects A_1^- to Fe–S_X electron transfer, the 200-ns phase reflects reduction of Fe–$S_{A/B}$. They cannot determine whether Fe–$S_{A/B}$ is reduced directly by A_1^- or through Fe–S_X, but they favor direct transfer from A_1 to Fe–$S_{A/B}$, with reduction of Fe–S_X occurring only in damaged centers. Our results show that this intepretation is not correct, as the 200-ns phase is unaffected by the removal of Fe–$S_{A/B}$ but is eliminated when Fe–S_X is depleted. Warden (1990) presented evidence from optical measurements that the iron–sulfur centers are reduced in less than 5 ns. Such a rapid process would not be detected by our technique. However, we find no evidence for centers in the state $P700^+$/Fe–S^- at early times, while our results clearly demonstrate the presence of $P700^+/A_1^-$ at longer times with a 200-ns decay associated with reduction of Fe–S_X.

In summary, we have shown that the 200-ns kinetic rate of reoxidation of A_1^- in PS1 particles specifically involves the iron–sulfur center Fe–S_X and confirm that $P_{700} \rightarrow A_1 \rightarrow$ Fe–S_X is the forward electron transfer pathway functioning in photosystem 1 under physiological conditions.

NOTE ADDED IN PROOF

After this paper was submitted for publication, a paper describing very similar experiments using optical spectroscopic analysis was published (Luneberg et al., 1994).

REFERENCES

Biggins, J., & Mathis, P. (1988) *Biochemistry 27*, 1494–1500.

Blankenship, R., McGuire, A., & Sauer, K. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4943–4947.

Block, C. H., Van der Est, A. J., Brettel, K., & Stehlik, D. (1989) *FEBS Lett. 247*, 91–96.

Boardman, N. K. (1971) *Methods Enzymol. 23*, 268–276.

Bottin, H., & Setif, P. (1991) *Biochim. Biophys. Acta. 1057*, 331–336.

Brettel, K. (1988) *FEBS Lett. 239*, 93–98.

Brettel, K. (1989) *Biochim. Biophys. Acta 976*, 246–249.

Dismukes, G. C., & Sauer, K. (1978) *Biochim. Biophys. Acta 504*, 431–445.

Evans, M. C. W., Sihra, C. K., Bolton, J. R., & Cammack, R. (1975) *Nature 259*, 668–670.

Evans, M. C. W., & Nugent, J. (1993) in *The Photosynthetic Reaction Center* (Deisenhofer, J., & Norris, J. R., Eds.) Vol. 1, pp 391–352, Academic Press, San Diego, CA.

Furrer, R., & Thurnauer, M. C. (1983) *FEBS Lett. 153*, 399–403.

Gast, P. (1982) Ph.D. Thesis, University of Leiden, Leiden, The Netherlands.

Golbeck, J. H., & Bryant, D. A. (1991) *Curr. Top. Bioenerg. 16*, 83–117.

Hanley, J. A., Kear, J., Bredenkamp, G., Li, G., Heathcote, P., & Evans, M. C. W. (1992) *Biochim. Biophys. Acta 1009*, 152–156.

Hoff, A. J. (1984) *Q. Rev. Biophys. 17*, 153–282.

Hore, P. J., Hunter, D. A., McKie, C. D., & Hoff, A. J. (1987) *J. Chem. Phys. Lett. 137*, 495–500.

Luneberg, J., Fromme, P., Jekow, P., & Schlodder, E. (1994) *FEBS Letts. 338*, 197–202.

Mathis, P., & Sétif, P. (1988) *FEBS Lett. 237*, 65–68.

Norris, J. R., Thurnauer, M. C., & Bowman, M. K. (1980) in *Advances in Biological and Medical Physics* (Lawrence, J. H., Gofman, J. W., & Hays, T. L., Eds.) pp 365–416, Academic Press, New York.

Sétif, P. (1992) in *The Photosystems: Structure, Function and Molecular Biology* (Barber, J., Ed.) pp 471–499, Elsevier, Amsterdam.

Setif, P., & Bottin, H. (1989) *Biochemistry 28*, 2689–2697.

Sétif, P., & Brettel, K. (1993) *Biochemistry 32*, 7846–7854.

Sétif, P., Mathis, P., & Vänngard, T. (1984) *Biochim. Biophys. Acta 767*, 404–414.

Sieckman, I., Vanderest, A., Bottin, H., Setif, P., & Stehlik, D. (1991) *FEBS Lett. 284*, 98–102.

Snyder, S. W., & Thurnauer, M. C. (1993) in *The Photosynthetic Reaction Center* (Deisenhofer, J., & Norris, J. R., Eds.) Vol. 3, pp 285–330, Academic Press, San Diego, CA.

Snyder, S. W., Rustandi, R. R., Biggins, J., Norris, J. R., & Thurnauer, M. C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9895–9896.

Thurnauer, M. C., & Norris, J. R. (1980) *Chem. Phys. Lett. 284*, 98–102.

Thurnauer, M. C., Bowman, M. K., & Norris, J. R. (1979) *FEBS Lett. 100*, 390–312.

Thurnauer, M. C., Rutherford, A. W., & Norris, J. R. (1982) *Biochim. Biophys. Acta 682*, 332–338.

Warden, J. T. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. II, pp 653, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Warren, P. V., Parret, K. G., Warden, J. T., & Golbeck, J. H. (1990) *Biochemistry 29*, 6545–6550.

Warren, P. V., Golbeck, J. H., & Warden, J. T. (1993) *Biochemistry 32*, 845–857.