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Identification of Electron-Transfer Reactions Involving the Acceptor A_1 of Photosystem I at Room Temperature

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ABSTRACT: The electron-transfer reactions of photosystem I (PS I) were investigated by flash-absorption spectroscopy at room temperature under highly reducing conditions in spinach and in the cyanobacterium *Synechocystis* 6803. Different conditions of background illumination were used in the presence of the reductant dithionite to generate two different redox states of the reaction center by photoaccumulating electrons on the PS I acceptors. When the iron–sulfur center Fe-S_X is prereduced by a weak illumination, the relaxation of flash-induced absorption changes exhibits two decay phases with $t_{1/2} \approx 750$ ns (in *Synechocystis*) and a few microseconds. These two phases can be ascribed respectively to the back-reaction between the oxidized primary donor P-700$^+$ and the reduced secondary acceptor A_1^- and to the decay of the P-700 triplet state, which is formed during this back-reaction with an efficiency larger than 90%. In contrast, at 10 K, the radical pair (P-700$^+$–A_1^-) decays directly to the ground state with $t_{1/2} \approx 20$–25 μs under the same conditions (Fe-S_X prereduced). Under illumination with a strong background light, the recombination kinetics of PS I are characteristic of the decay of the primary radical pair (P-700$^+$–A_0^-) ($t_{1/2} \approx 25$–30 ns) and of the decay of the P-700 triplet state which is formed during the back-reaction. This kinetic behavior is conserved when PS I reaction centers that have been submitted to strong illumination conditions are kept afterward in the dark. This indicates that the electron transfer is blocked at the level of A_0 although Fe-S_X is reoxidized during the dark adaptation. The results show that this block is probably due to the fact that A_1 stays reduced in the dark although no radical EPR signal is observable. To explain these observations, a process of A_1 double reduction is proposed that supports the identification of A_1 with a quinone molecule (vitamin K_1).

The reaction center of photosystem I (PS I)[1] in oxygen-evolving organisms is known to include the primary electron donor P-700 and five electron acceptors. One or possibly several of the iron–sulfur centers Fe-S_A, Fe-S_B, and Fe-S_X reduce soluble ferredoxin, which in turn is thought to be involved in cyclic electron flow around PS I as well as to engage the reduction of NADP$^+$. The primary acceptor A_0 is presumably made of chlorophyll molecule(s) and absorbs around 690–694 nm (Nuijs et al., 1986; Shuvalov et al., 1986; Wasielewski et al., 1987; Mathis et al., 1988). An acceptor named A_1 is generally thought to mediate electron transfer between the primary acceptor A_0 and the iron–sulfur center Fe-S_X (Bonnerjea & Evans, 1982; Gast et al., 1983).

In accordance with the presence of vitamin K_1 (phylloquinone) in PS I reaction centers (Interschick-Niebler & Lichtenthaller, 1981; Takahashi et al., 1985; Schoeder &

[1] Abbreviations: PS I, photosystem I; DPIP, dichlorophenolindophenol; chl, chlorophyll; EPR, electron paramagnetic resonance; CIDEP, chemically induced dynamic electron polarization.

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Lockau, 1986), recent experimental evidences strongly support the identification of A_1 with vitamin K_1 (Biggins & Mathis, 1988). In the latter study which involved extraction and readdition of vitamin K_1 in PS I reaction centers, the forward electron transfer from A_0^- as well as the photoreduction of $NADP^+$ appeared to be correlated with the presence of vitamin K_1. These data are consistent with the spectroscopic features of A_1 but are contradictory with other reports, concluding that reaction centers in which vitamin K_1 has been destroyed by UV irradiation are still fully photoactive (Palace et al., 1987; Ziegler et al., 1987). Evidence for the efficient photoreduction of the iron–sulfur centers at low temperature after depletion of vitamin K_1 also appears rather disconcerting with regard to the role of vitamin K_1 in PS I (Sêtif et al., 1987).

EPR and absorption features of the acceptor A_1 were obtained from photoaccumulation techniques under highly reducing conditions (Bonnerjea & Evans, 1982; Gast et al., 1983; Mansfield & Evans, 1986; Mansfield et al., 1987) as well as by the study of transient signals involving A_1 after a laser flash excitation of PS I reaction centers.

Transient type absorption experiments attempting to measure kinetics of forward electron transfer involving A_1 are still scarce: a 30-ps half-time has been measured for the reoxidation of A_0^- (Shuvalov et al., 1986), which presumably transfers an electron to A_1. Recent experiments indicate that A_1^- is reoxidized with a half-time of about 15 ns (Mathis and Sêtif, unpublished observation), in accordance with the upper limit of 100 ns found for the reduction of one of the iron–sulfur centers (Ke, 1972). The origin of another transient of 170 ns measured by pulsed EPR is not yet clear (Thurnauer et al., 1982).

The reoxidation of A_1^- in a recombination reaction with the oxidized primary donor $P\text{-}700^+$ has been identified by flash-absorption spectroscopy at low temperature ($t_{1/2} \approx 120$ μs) (Mathis & Conjeaud, 1979; Sêtif et al., 1984; Brettel et al., 1986). The EPR counterpart of this low-temperature recombination reaction exhibits polarization (CIDEP) effects which have been intensively studied [Petersen et al. (1987) and references cited therein] and the interpretation of which is still in debate (Hore et al., 1987). The same EPR polarization pattern has been also observed in room temperature studies (Furrer & Thurnauer, 1983; Manikowski et al., 1984), but the significance of this similarity is not yet clear.

In the presence of the fast physiological electron donor plastocyanin which allows to photoaccumulate rapidly electrons on the acceptor side of PS I, an absorption transient of 30 μs has been recently reported at room temperature. This transient was tentatively ascribed to a recombination reaction between $P\text{-}700^+$ and A_1^- (Bottin et al., 1987).

The present work reports the observation by flash-absorption spectroscopy of a back-reaction between $P\text{-}700^+$ and A_1^- with a half-time of about 750 ns. This decay phase is observed under highly reducing conditions in the presence of background illumination. It is also shown that this recombination reaction inside the secondary radical pair ($P\text{-}700^+ - A_1^-$) leads to efficient formation of the triplet state of the primary donor P-700. Evidence is also provided that A_1 can be doubly reduced by strong illumination under highly reducing conditions. This double reduction process gives additional support to the identification of A_1 with a quinone molecule.

Experimental Procedures

Biological Samples. PS I reaction centers were obtained from spinach and from the cyanobacterium *Synechocystis* 6803 as described respectively by Picaud et al. (1982) and Biggins and Mathis (1988). The chemical assay of these particles, using an extinction coefficient of 64 000 M^{-1} cm^{-1} for the red maximum of P-700 (Hiyama & Ke, 1972), gives chlorophyll to P-700 ratios of 110 and 120 for spinach and *Synechocystis* reaction centers, respectively. The same ratios are found in photochemical assays with a saturating laser flash using an absorption coefficient of 6500 M^{-1} cm^{-1} for $P\text{-}700^+$ at 820 nm (Mathis & Sêtif, 1981). Chlorophyll concentration was determined optically on 80% acetone extracts.

Absorption Kinetic Measurements. Nanosecond absorption changes at 820 nm were measured as described by Van Best and Mathis (1978) with the modifications described by Brettel and Sêtif (1987). In brief, excitation laser pulses at 532 nm were of 30-ps duration, and the measuring light provided by a laser diode was detected by a rapid-response silicon photodiode (Lasermetrics 3117). The signal from the diode was first amplified (10-20-1C amplifier from Nuclêtudes: bandwidth 500 Hz–500 MHz) and then recorded by a Tektronix 7912 digitizer interfaced with a signal averager (Didac, Intertechnique). Most absorption experiments at 820 nm with a microsecond time resolution were done simultaneously with the same setup by separating in two parts the measuring light after the cuvette. The output signal of the rapid photodiode was amplified by using the 7A22 Tektronix plug-in amplifier (DC; 1 MHz). The rectangular sample cuvette (optical path: 10 mm in the direction of the measuring beam and 6 mm in the direction of the excitation beam) was placed in the center of a home-built electromagnet. Microsecond absorption experiments were also performed from 700 to 1000 nm with another apparatus which has been previously described (Bottin & Mathis, 1985). For all microsecond absorption experiments, the signals were recorded by using a Biomation 1010 or 2805 digitizer coupled to a Tracor 1710 signal averager. The same apparatus was used for wavelengths larger than 950 nm by replacing the silicon detector by a germanium detector (J16-P2 from Judson Infrared Inc.). Absorption changes with a time resolution of about 100 ns were measured from 1000 to 1300 nm by using a rapid germanium photodiode (J16-CER from Judson Infrared Inc.). The output signal of the photodiode was amplified by using the 7A13 Tektronix plug-in amplifier (5 MHz) before feeding a Tektronix 7912 digitizer (7A16A amplifier; 20 MHz).

Background illumination was provided by a 800-W tungsten–iodine lamp whose beam was filtered to remove infrared light (water cuvette + Calflex filters) and focused onto the sample. Illuminations with weak and strong light intensities were corresponding to light fluxes of about 30 and 250 mW/cm^2, respectively. These values are only indicative and are valid within 50%, as the intensities were varied from one experiment to another depending on the other conditions of the experiment (optical density of the sample and facility to photoreduce the acceptors, which depends itself on the pH of the sample and of the presence or absence of glycerol).

Low-temperature experiments at 820 nm were done with glycerol-containing samples placed inside an optical EPR cavity. The PS I particles were contained in a plexiglass tube which allows one to get a clear glass for absorption measurements. The measuring light was provided by a laser diode. The exciting light was provided by a YAG laser that was frequency-doubled ($\lambda = 532$ nm; duration 11 ns) and went almost antiparallel to the measuring light.

EPR Measurements. EPR spectroscopy was carried out at 10 K with an X-band Bruker ER200D spectrometer equipped with an Oxford ESR 900 helium cryostat. A standard cavity (TE 102 mode) or an optical transmission cavity were used. Continuous illumination was provided by an 800-W tung-

Table I: Half-Times of Decay in PS I Reaction Centers

initial state	final state	spinach reaction centers	*Synechocystis* reaction centers
	293 K		
$P\text{-}700^+\text{-}A_0^-$	P-700–A_0 and ^{3}P-700	25 ± 5 ns	30 ± 5 ns
$P\text{-}700^+\text{-}A_1^-$	^{3}P-700	≤1 μs	750 ± 200 ns
^{3}P-700	P-700	2.5 ± 0.5 μs	4.5 ± 0.7 μs
$P\text{-}700^+\text{-Fe-}S_X^-$	P-700–Fe-S_X	250 ± 20 μs	320 ± 40 μs
	10 K		
$P\text{-}700^+\text{-}A_1^-$ Fe-S_X	P-700–A_1 Fe-S_X	120 ± 10 μs	220 ± 30 μs
$P\text{-}700^+\text{-}A_1^-$ $Fe\text{-}S_X^-$	P-700–A_1 $Fe\text{-}S_X^-$	23 ± 5 μs	20 ± 5 μs

sten–iodine lamp whose beam was filtered to remove infrared light (water cuvette + Calflex filters) and concentrated onto the cavity window by using a plexiglass light pipe.

Results

The data reported in this paper were obtained with PS I reaction centers from spinach as well as from the cyanobacterium *Synechocystis* 6803. In every functional aspect that was studied, both organisms behave similarly, even though the decay half-times of the different electron-transfer steps slightly differ from one organism to another, as is shown in Table I.

The study of the recombination reactions involving the electron acceptors preceding the terminal iron–sulfur centers Fe-S_A and Fe-S_B necessitates the prereduction of these two acceptors by poising the reaction centers with a strong reductant. This prereduction can be achieved by the addition of sodium dithionite in excess at pH 10. Under such conditions, the charge separation induced by a flash leads to the photoreduction of the iron–sulfur center Fe-S_X, which is then reoxidized by recombination with $P\text{-}700^+$, with a half-time of about 250 μs in spinach (Sauer et al., 1978; Golbeck et al., 1978; Koike & Katoh, 1982). This kinetic behavior is shown again in the upper part of Figure 1, by measurement of the flash-induced absorption changes at 820 nm. In the absence of background illumination (thereafter called dark conditions), there is only a minor rapid decay ($t_{1/2} \leq 100$ ns), and on a longer time scale, the major kinetic component exhibits the characteristic half-time of 250 μs (320 μs in *Synechocystis*). Illumination by a strong background light has severe effects on the kinetic profile on both time scales (Figure 1, bottom traces). The initial size of the signal appears larger than in the dark, and an important fast component ($t_{1/2} \approx 25$–30 ns) is present which is followed by a 2.5-μs component (4.5 μs in *Synechocystis*). A very similar kinetic behavior has already been observed in PS I reaction centers in which electron transfer is blocked at the level of A_0, due to the lack or to the inactivation of secondary acceptors (Sêtif et al., 1985; Shuvalov et al., 1986; Brettel & Sêtif, 1987; Ikegami et al., 1987; Biggins & Mathis, 1988). By analogy with these studies, the rapid phase can be ascribed to a recombination reaction between $P\text{-}700^+$ and A_0^-, the increase in initial signal amplitude being due to the absorbance contribution of the reduced chlorophyll A_0^- at 820 nm (Nuijs et al., 1986; Mathis et al., 1988). This recombination reaction produces some triplet state of the primary donor P-700, which relaxes within a few microseconds in accordance with previous studies.

If the dark-adapted sample is first illuminated with a weaker background light, another kinetic behavior is prevailing (Figure 1, middle traces). The extent of submicrosecond decay has only slightly increased, as compared to the dark conditions, but a large 2.5-μs decay (4.5 μs in *Synechocystis*) precedes a remaining slower decay due to the recombination reaction

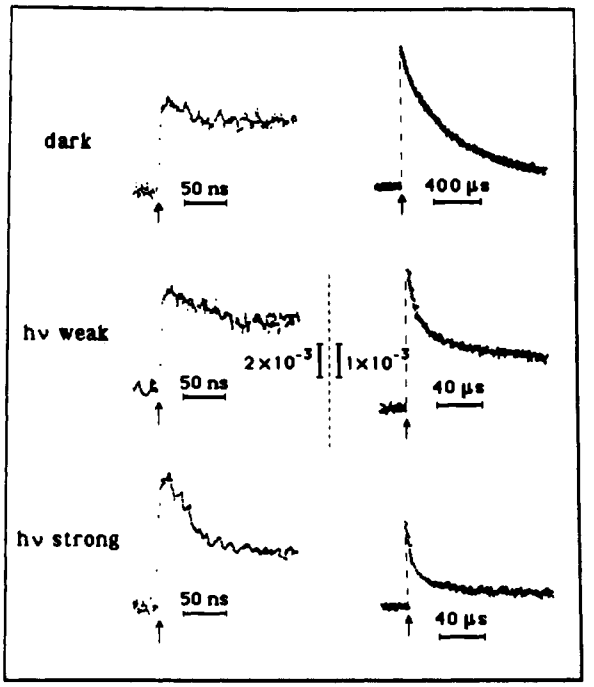

FIGURE 1: Kinetics of absorption changes measured at 820 nm and at 293 K in PS I reaction centers from *Synechocystis* and induced by a saturating laser flash (λ = 532 nm; duration 30 ps; frequency 1 Hz). The reaction centers are suspended in 0.2 M glycine–NaOH buffer, pH 10, in the presence of 10 mM sodium dithionite (absorption in the red maximum at 679 nm = 5.8 for an optical path of 10 mm). Dimensions of the cuvette: 10 mm in the direction of the measuring beam, 6 mm in the direction of the exciting beam. For the different conditions that have been studied (dark, *hν* weak, *hν* strong), kinetics with a 1-ns time resolution (left part; average of 10 experiments) and with a 1-μs time resolution (right part; average of 10 experiments) are recorded at the same time by dividing in two parts the measuring beam after the cuvette.

between $P\text{-}700^+$ and $Fe\text{-}S_X^-$. The percentage of this 2.5-μs decay at 820 nm can be controlled with the intensity of the background illumination, varying from 0 to about 60% of the whole change at 820 nm. Above that value, the kinetic profile described before for strong illumination conditions is rapidly developing. As a general rule, this percentage will be comprised in the following between 30 and 50% at 820 nm for weak illumination conditions.

After a period (from 1 to 30 min) of weak illumination, this kinetic profile evolves slowly in the dark so that the corresponding redox state of the PS I electron acceptors can be frozen at low temperature and characterized by EPR. This is shown in Figure 2 for a sample prepared in the presence of glycerol which exhibits the kinetic profile precedingly described under weak illumination conditions (not shown). Iron–sulfur centers Fe-S_A and Fe-S_B (signals at g = 1.89, 1.92, 1.94, and 2.05) together with a large fraction of center Fe-S_X (signal at g = 1.78, about 50% of the total amount) appear to be reduced, whereas no radical signal is present (as also measured under conditions of low microwave power, not shown). The same sample was studied by flash-absorption spectroscopy at 10 K inside the optical EPR cavity. It exhibits a major decay component with a half-time of about 23 μs together with two components of smaller magnitude ($t_{1/2}$ = 120 μs and about 1 ms). The amount of the 23-μs phase (46 ± 5% of the total decay) corresponds approximately to the percentage of reduced Fe-S_X (50 ± 10% of the maximum EPR signal of $Fe\text{-}S_X^-$) and to the percentage of the 2.5-μs phase that is observed at room temperature. This amount was estimated before freezing (43 ± 10% of the microsecond decay) and at the end of the experiment, after thawing the sample (40 ± 10% of the micro-

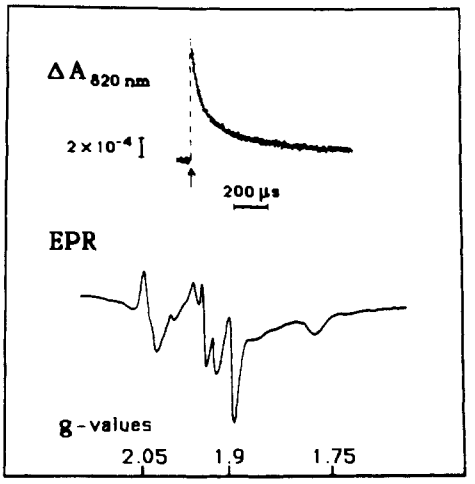

FIGURE 2: Upper part: Kinetics of absorption change induced at 820 nm and at 10 K in spinach PS I reaction centers by a laser flash (λ = 532 nm; duration 11 ns; frequency 1 Hz). Average of 100 experiments. The reaction centers (160 μg of chl/mL) are suspended in a mixture of 60% glycerol and 40% glycine–NaOH buffer (0.2 M, pH 10) with a final concentration of 15 mM sodium dithionite. The sample is contained in a plexiglass tube (internal diameter 3 mm) and the measurement is made inside an optical transmission EPR cavity (see Experimental Procedures). Lower part: EPR spectrum of the same sample. Instrument settings: temperature 10 K; microwave power 20 mW; modulation amplitude 10 G; average of 20 scans. The sample was first illuminated with background light for 10 min at room temperature so that it exhibited a kinetic behavior characteristic of the weak illumination conditions, and the temperature of the sample was then rapidly decreased. After completion of the low-temperature experiments, the sample was again studied at room temperature and it exhibited the same kinetic behavior as before freezing.

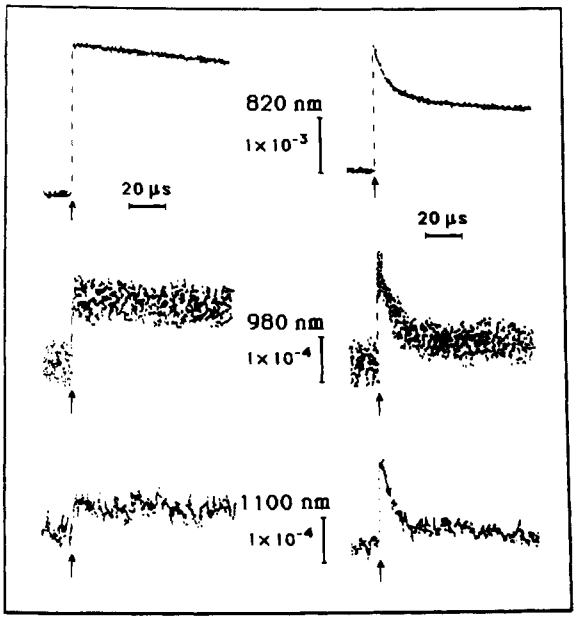

FIGURE 3: Kinetics of absorption changes measured at 820, 980, and 1100 nm and at 293 K in PS I reaction centers from *Synechocystis* and induced by a saturating ruby laser flash (λ = 694.3 nm; duration 6 ns; frequency 0.15 Hz). Left part: Dark conditions. Right part: Conditions of illumination with a weak background light. The reaction centers are suspended in glycine–NaOH buffer (0.2 M, pH 10) in the presence of 10 mM sodium dithionite (absorption in the red maximum at 679 nm = 3.3 for an optical path of 10 mm). Dimensions of the cuvette: 10 mm in both directions (measuring and exciting beams). A silicon photodiode was used at 820 nm (average of four experiments) whereas a germanium photodiode was used at 980 nm and at 1100 nm (average of eight experiments at both wavelengths). Under the conditions of background illumination that were used, the kinetic behavior was stable (for a period of 1 h), thus allowing us to change the detector during the experiment.

second decay). The two slower phases of absorption decay at low temperature (120 μs and 1 ms) have been characterized at 10 K: they correspond respectively to the back-reaction between P-700$^+$ and A_1^- in reaction centers where Fe-S_X is not reduced (Sêtif et al., 1984) and to the P-700 triplet-state decay (Sêtif et al., 1981). The spectrum of a kinetic phase with $t_{1/2} \approx 20$ μs obtained under similar conditions has been previously measured and unambiguously corresponds to a recombination reaction involving P-700$^+$ (Sêtif & Mathis, 1986). This recombination was assumed to involve A_1^- as the partner of P-700$^+$ in reaction centers where center Fe-S_X is prereduced. The present observations confirm this assignment and open the possibility that the 2.5-μs phase measured in spinach reaction centers (4.5 μs in *Synechocystis*) which is observed at room temperature under conditions of weak illumination is due to the recombination reaction between P-700$^+$ and A_1^-.

However, the kinetic similarity between microsecond phases of absorption decay at 293 K under weak or strong background illumination suggests another hypothesis, namely, that the triplet state of P-700 is observed under both weak and strong illumination conditions. A convenient wavelength range to discriminate between these two possibilities lies from 950 to 1300 nm, where the absorption coefficients of ^{3}P-700 are much larger than those of P-700$^+$ (Mathis & Sêtif, 1981; Sêtif et al., 1981). Figure 3 shows the flash-induced absorption changes at 820 nm as well as at 980 and 1100 nm measured with a microsecond time resolution in the dark and under weak illumination conditions in *Synechocystis* reaction centers. For the two latter wavelengths, the initial signal amplitude appears larger under illumination than in the dark. It strongly suggests that the triplet state of P-700 is responsible for the kinetic decay of a few microseconds. Other wavelengths were studied

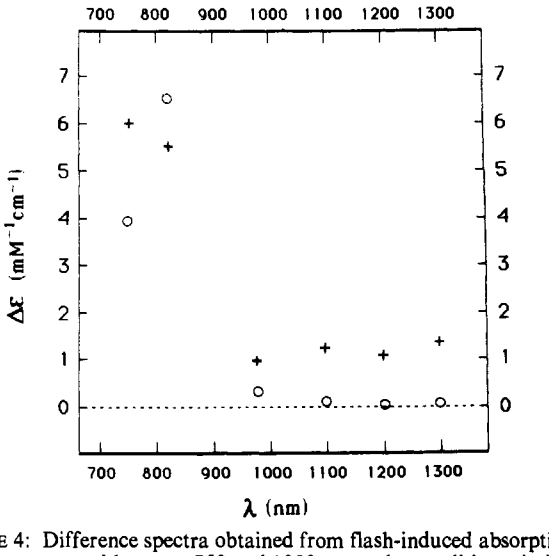

FIGURE 4: Difference spectra obtained from flash-induced absorption changes measured between 750 and 1300 nm under conditions similar to conditions described in Figure 3 (weak background illumination, reaction centers from *Synechocystis*, 293 K). At each wavelength, the absorption decay could be deconvoluted into two exponential phases with $t_{1/2} \approx 4.5$ μs (crosses) and 320 μs (open circles). The flash-induced kinetics were also measured at 703 nm (not shown) and can be deconvoluted into the same two exponential phases. The two spectra were normalized by assuming an extinction coefficient of 64 000 M^{-1} cm^{-1} for both phases at 703 nm. The photodetector was a silicon photodiode at 703, 750, and 820 nm and a germanium photodiode from 980 to 1300 nm.

in the red (703 nm, not shown) and infrared regions, and the spectrum of this fast phase together with the spectrum of the slower component corresponding to the recombination reaction

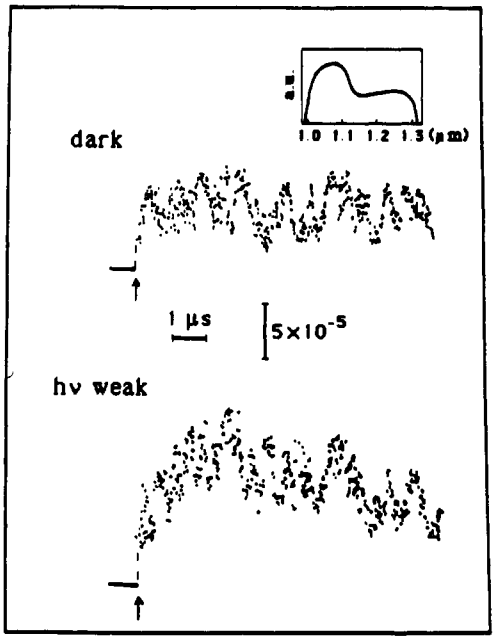

FIGURE 5: Kinetics of absorption changes measured at 293 K between 1000 and 1300 nm in PS I reaction centers from *Synechocystis* and induced by a saturating ruby laser flash (λ = 694.3 nm; duration 6 ns; frequency 0.15 Hz). The reaction centers are suspended in glycine–NaOH buffer (0.2 M, pH 10) in the presence of 10 mM sodium dithionite (absorption in the red maximum at 679 nm = 2.15 for an optical path of 10 mm). Dimensions of the cuvette: 10 mm in both directions (measuring and exciting beams). Upper trace: Dark conditions. Lower trace: Conditions of illumination with a weak background light. Average of 60 experiments under both conditions. The wavelength range of the experiments was imposed by a high-pass filter transmitting light above 1000 nm, by the absorption of the measuring light by the sample, and by the response profile of the germanium photodetector. The inset shows the response profile of the setup for an absorption change identical at every wavelength, taking into account these three parameters together with the emission profile of the tungsten–iodine lamp.

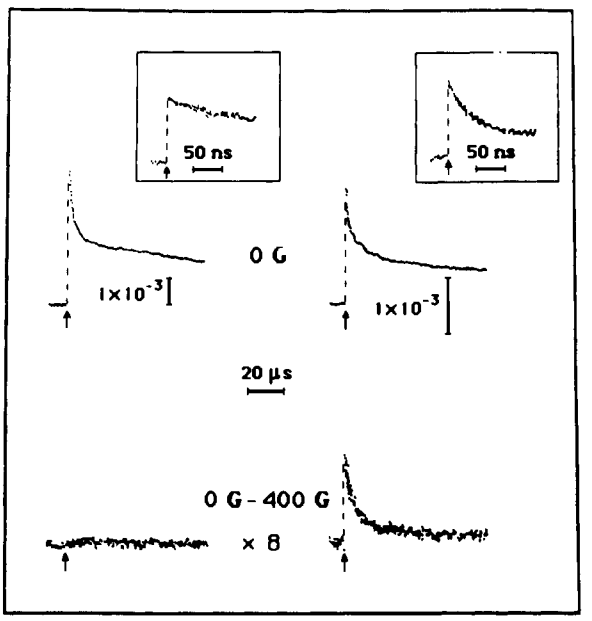

FIGURE 6: Kinetics of absorption changes measured at 820 nm and at 293 K in spinach PS I reaction centers and induced by a saturating laser flash (λ = 532 nm; duration 30 ps; frequency 1 Hz). The reaction centers are suspended in glycine–NaOH buffer (0.2 M, pH 10) in the presence of 10 mM sodium dithionite (absorption in the red maximum at 678 nm = 6.2 for an optical path of 10 mm). Dimensions of the cuvette: 10 mm in the direction of the measuring beam, 6 mm in the direction of the exciting beam. Conditions of background illumination: The left and right parts correspond to weak and strong lights, respectively. Insets: Kinetics with a 1-ns time resolution (average of 16 experiments). Middle traces: Kinetics with a 1-μs time resolution in the absence of magnetic field B (average of 500 experiments at 5 Hz). Lower traces: Differences between signals for B = 0 G and B = 400 G. The signals are expanded by a factor of 8 as compared to the middle traces.

between P-700$^+$ and Fe-S_X^- is shown in Figure 4. After normalization of the two red maxima at 703 nm, and assuming an extinction coefficient of 64 000 M^{-1} cm^{-1} for the oxidation of P-700, the spectral features of the fast and slow components correspond closely to those of the differences (^{3}P-700–P-700) and (P-700$^+$–P-700), respectively (Mathis & Sétif, 1981; Sétif et al., 1981). The differences between these two spectra can be briefly summarized: (a) the ratio ($\Delta A_{820}/\Delta A_{750}$) is about 1.6 in the case of P-700$^+$ and about 0.95 in the case of ^{3}P-700. (b) In the region 950–1300 nm, the absorption coefficients of ^{3}P-700 lies around 1000–1300 M^{-1} cm^{-1} whereas the coefficients of P-700$^+$ are comprised between 100 and 350 M^{-1} cm^{-1}. By taking into account the spectrum of ^{3}P-700 that was previously determined (Sétif et al., 1981) and by comparing the magnitudes of the absorption changes in the dark and under weak illumination (Figures 3 and 4), it can be estimated that, in *Synechocystis* reaction centers that exhibit a fast relaxation of a few microseconds, the population of the P-700 triplet state is larger than 60% at 2 μs after the flash.

Figure 5 displays flash-induced absorption changes measured with a very large wavelength bandwidth (from 1000 to 1300 nm; the response profile of the setup is shown in the inset) with a time resolution of less than 100 ns. In the dark, the flash-induced signal appears stepwise within the noise level and can be attributed to the fast photooxidation of P-700. Under weak illumination conditions, a distinct signal rise can be detected with a half-time of 750 ± 200 ns. In accordance with the above interpretation, this signal rise can be ascribed to the formation of the P-700 triplet state. This rate of formation is considerably slower than the rate of formation of ^{3}P-700 from the primary radical pair (P-700$^+$–A_0^-) which decays completely within 100 ns at room temperature. Thus it appears that under conditions of weak illumination the P-700 triplet state is probably formed through a recombination reaction between P-700$^+$ and A_1^-. The experiments depicted in Figures 3–5 repeated with spinach PS I reaction centers gave similar results. However, the rise time of the P-700 triplet state is more difficult to measure in that case. This is presumably due to the fact that the triplet state decay is faster in these reaction centers ($t_{1/2} \approx 2.5$ μs instead of 4.5 μs in *Synechocystis*) and competes more efficiently with the triplet formation. Thus, due to the noise level of our experiments, only an upper limit ($t_{1/2} \leq 1$ μs) can be estimated for the rise time of P-700 triplet formation in spinach reaction centers.

When formed from the primary radical pair, the yield of the primary donor triplet-state formation has been shown to depend, at room temperature, on the presence of a magnetic field in different kinds of reaction centers. This effect has been extensively studied experimentally in bacterial reaction centers and theoretically interpreted within the framework of the radical pair mechanism [reviewed by Hoff (1981 and 1986) and Boxer et al. (1983)]. Such magnetic field effects have been also observed in PS I reaction centers devoid of functional secondary acceptors (Brettel & Sétif, 1987). A similar observation can be made in PS I reaction centers having a full complement of electron acceptors under strong illumination conditions (Figure 6, right part) for a magnetic field of 400 G (Figure 6, bottom trace). By contrast, no magnetic field effect on the yield of triplet-state formation is detectable under weak illumination conditions (Figure 6, bottom trace of left

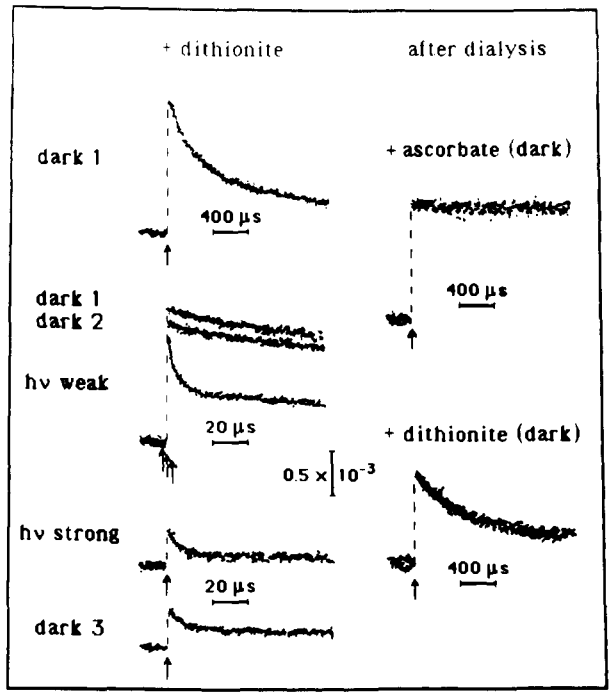

FIGURE 7: Kinetics of absorption changes measured at 820 nm and at 293 K in spinach PS I reaction centers and induced by a saturating ruby laser flash (λ = 694.3 nm; duration 6 ns; frequency 0.15 Hz). Each trace was the average of eight experiments. The same sample was used in a square cuvette (10 mm) throughout the experiment, and kinetic traces were obtained in the following order: Reaction centers were first suspended in glycine–NaOH buffer (0.2 M, pH 10) in the presence of 10 mM sodium dithionite (absorption in the red maximum at 678 nm = 1.06 for an optical path of 10 mm). The following letters correspond to the chronological order of the experiments: (a) Dark 1; shown on two different time scales. (b) *hν* weak; background illumination with a weak light. (c) Dark 2; 1 h of dark adaptation after the weak illumination treatment. The three traces, dark 1 (faster time scale), *hν* weak, and dark 2, share a common base line. (d) *hν* strong; background illumination with a strong light. (e) Dark 3:3 h of dark adaptation after the strong illumination treatment. The reaction centers were then dialyzed during 14 h against Tris buffer (50 mM, pH 8). They were diluted by the dialysis treatment (absorption in the red maximum at 678 nm = 0.37). (f) After dialysis; addition of 10 mM sodium ascorbate and 20 µM DPIP in the dark. (g) Addition of 2 M glycine–NaOH buffer, pH 10, to a final concentration of 0.2 M and 10 mM sodium dithionite in the dark. The two right traces (after dialysis) were multiplied by a factor of 2.85 (upper trace) and 3.15 (lower trace) to normalize the sample absorptions for comparison to the left traces.

part). This makes another difference in the properties of the P-700 triplet state, according to its formation from either the primary radical pair ($P\text{-}700^+\text{–}A_0^-$) or the secondary radical pair ($P\text{-}700^+\text{–}A_1^-$).

From the flash-induced kinetics at 820 nm, it can be derived that the effect of a weak illumination is reversible: a period of dark adaptation after a weak light treatment (Figure 7, *hν* weak) restores almost completely (Figure 7, dark 2) the signal first observed in the dark (Figure 7, dark 1). This restoration of flash-induced kinetics has been correlated with the reoxidation of $Fe\text{-}S_X^-$ in darkness described in a preceding study (Sétif & Mathis, 1984). By contrast, a period of strong illumination induces kinetic changes (Figure 7, *hν* strong) as described before that are not reversed by a period of dark adaptation (Figure 7, dark 3): after 2 h in the dark, the flash-induced absorption changes at 820 nm are unchanged compared to strong illumination conditions. A large fast transient is still present ($t_{1/2} \approx 25$ ns, not shown) whereas the same amount of P-700 triplet state is formed. An identical kinetic behavior is observed if the strong light treatment is

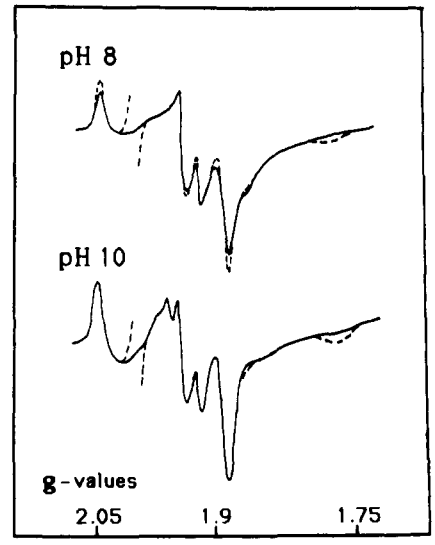

FIGURE 8: EPR spectra of PS I reaction centers from *Synechocystis* (0.5 mg of chlorophyll/mL) which have been submitted at room temperature to strong light illumination followed by dark adaptation during 1 h. The samples are suspended respectively in Tris-HCl buffer (50 mM, pH 8) and glycine–NaOH buffer (0.2 M, pH 10) for the upper and lower traces with the addition of 20 mM sodium dithionite in both cases. Solid curves: Dark conditions. Dashed curves: Under illumination. Instrument settings: Standard cavity (TE 102 mode); temperature 10 K; microwave power 20 mW; modulation amplitude 10 G.

performed at pH 8 in the presence of dithionite, which produces a redox potential in the medium which is about 120 mV higher than at pH 10. The EPR spectra of PS I reaction centers from *Synechocystis* which have been submitted to prolonged dark adaptation after a strong light treatment are shown in Figure 8 (solid curves). These spectra indicate the absence of any radical signal in both samples. Less than 10% of centers $Fe\text{-}S_X$ are still reduced in the pH 10 sample, while there is no more $Fe\text{–}S_X^-$ signal at pH 8. Moreover, a small fraction of centers $Fe\text{-}S_B^-$ is reoxidized at pH 8. Illumination of these samples at 10 K induces the photoreduction of iron–sulfur centers (Figure 8, dashed curves). At pH 8, the fraction of reoxidized $Fe\text{-}S_B$ centers is irreversibly photoreduced while a small amount of $Fe\text{-}S_X$ centers is reversibly reduced under illumination. The fraction of reversible photoreduction of $Fe\text{-}S_X$ is larger in the pH 10 sample and corresponds to about 15% of the total amount of $Fe\text{-}S_X$. A large radical signal due essentially to $P\text{-}700^+$ is also observed under illumination.

PS I reaction centers from spinach and *Synechocystis*, which are depleted of vitamin K_1, have been obtained in previous studies after solvent extraction (Sétif et al., 1987; Itoh et al., 1987; Biggins & Mathis, 1988). The behavior of these reaction centers is very similar to that of reaction centers that have been treated with a continuous strong light under highly reducing conditions. In both cases, the electron transfer appears to be essentially blocked at the level of the primary acceptor A_0 at room temperature (Ikegami et al., 1987; Biggins & Mathis, 1988), whereas the low-temperature photoreduction of iron–sulfur centers is not dramatically affected by the extraction procedure (Sétif et al., 1987). Therefore, the strong light treatment appears to induce the same effects on electron-transfer processes in PS I as the solvent extraction of vitamin K_1. These observations strongly suggest that A_1 has been inactivated by the strong light treatment.

The right part of Figure 7 shows that restoration of forward electron transfer from A_0^- and efficient photoreduction of the iron–sulfur centers at room temperature are restored after a prolonged dialysis has eliminated the strong reductant di-

thionite. In the presence of ascorbate, the dominant absorption change then represents the back-reaction between the species P-700$^+$ and P-430$^-$ (Ke, 1973). Recombination between P-700$^+$ and Fe-S$_X^-$ with a half-time around 250 μs (Sauer et al., 1978) becomes the major process upon readdition of dithionite at pH 10 after 30 s of illumination with a very weak light. This reactivation of electron transfer after extensive dialysis indicates that A_1 has not been irreversibly damaged by the light treatment. Moreover, it shows that A_1 stays reduced during dark incubation in the presence of dithionite and becomes oxidized when this reductant is eliminated. Instead of a dialysis step, the addition of ferricyanide to the suspension medium can restore as well forward electron transfer (not shown).

When reduced by a strong light treatment, A_1 is unable to transfer an electron to Fe-S$_X$ which is reoxidized by dark adaptation. During dark incubation at pH 8 (Figure 8, upper part), iron–sulfur center Fe-S$_B$ becomes partly reoxidized while no significant change in the room temperature kinetic behavior of the reaction centers can be detected. If the system can be considered to be in redox equilibrium with the suspension medium, this observation implies that the process of A_1 reoxidation occurs above the redox potential of Fe-S$_B$, which is about −590 mV (Ke et al., 1973; Evans et al., 1974).

Discussion

Three different kinetic profiles have been described under Results. These profiles correspond to absorption changes obtained with PS I reaction centers poised at a low redox potential and submitted to different conditions of background illumination (dark, and weak and strong illumination). They can be associated with different redox states of the electron acceptors. It is quite obvious that the transition between one condition and another is progressive, and mixing of the states corresponding to these kinetic profiles can be observed. However, the intensity of the background illumination can be adjusted so as to produce states that are clearly distinguishable. Under the so-called high illumination conditions, a fast decay with $t_{1/2} \approx 25$–30 ns corresponds to the recombination reaction between P-700$^+$ and A_0^-. The yield of the P-700 triplet state that is formed through this reaction is affected by the presence of a magnetic field. Under weak illumination conditions, these two features are absent. Moreover, the rise time of the P-700 triplet state is much longer (≈750 ns) than when it is formed from the primary radical pair.

Another essential difference between the two conditions (weak and strong background illumination) consists in the reversibility properties of the kinetic behaviors when the reaction centers are dark-adapted after illumination. The changes in the kinetics of absorption decay induced by a light treatment are reversible in the dark in the case of a weak light but not in the case of a strong light. However, in both cases, the iron–sulfur center Fe-S$_X$ becomes reoxidized in the dark in accordance with its redox potential around −705 mV (Chamorovsky & Cammack, 1982). In the case of the weak light pretreatment, the slow process of Fe-S$_X^-$ reoxidation ($t_{1/2} \approx 10$–15 min) in the dark has been correlated with the appearance of the flash-induced reduction of Fe-S$_X$ (Sétif & Mathis, 1984).

As shown by the EPR characterization of the sample, the weak illumination induces the reduction of iron–sulfur center Fe-S$_X$ in a large fraction of reaction centers. In reaction centers where Fe-S$_X$ is prereduced, it is expected that the relaxation of flash-induced absorption changes corresponds to a recombination reaction between P-700$^+$ and A_1^-. This is confirmed by the results obtained both at room temperature and at 10 K, where half-times of about 750 ns and 20–23 μs are found at these two temperatures for this back-reaction. The room temperature value disagrees with the results of a recent study in which the pair (P-700$^+$–A_1^-) has been proposed to recombine with a half-time of about 30 μs at room temperature (Bottin et al., 1987). The prereduction of Fe-S$_X$ which is necessary to observe the 30-μs decay phase was obtained in the latter case by using the fast physiological donor plastocyanin in double- and triple-flash experiments in the absence of background illumination. When background illumination was used to promote the prereduction of Fe-S$_X$, no 30-μs phase and only a 4-μs phase could be observed with a microsecond time resolution. The present data agree with these observations as the 30-μs phase appears to be absent under background illumination. This absence can be ascribed neither to the presence or absence of glycerol nor to the pH (between 7.5 and 10) nor to the organism [this work and Bottin et al. (1987)]. The 30-μs phase was observed in the presence of glycerol under conditions where Fe-S$_A$ stays oxidized, and where Fe-S$_X$ stays reduced during 300 ms. By contrast, the three iron–sulfur centers Fe-S$_A$, Fe-S$_B$, and Fe-S$_X$ must stay in a reduced state for the 750-ns decay phase to be observed. So we hypothesize that the redox states of Fe-S$_A$ and Fe-S$_X$ are responsible for the difference between the two kinetic behaviors. This difference might be due to a direct electrostatic effect of the charge carried out by Fe-S$_A$ or to an indirect effect of the redox state of Fe-S$_X$ which could modify the protein environment of A_1^- with a half-time larger than 300 ms. Recent data indicate that the 30-μs decay phase is not due to a direct recombination reaction but more probably reflects a transition between two different states of the reaction center (H. Bottin and P. Sétif, unpublished observation). However, the nature of the different states of PS I reaction centers, differing by their kinetic behavior and containing a reduced center Fe-S$_X$ (oxidoreduction state of Fe-S$_A$, protonation, or conformational state of the protein), as well as the number of these states is not yet known. A better understanding of this issue certainly requires further investigation, as the redox state of the PS I acceptors may have some regulatory function on the electron flow inside the PS I reaction center.

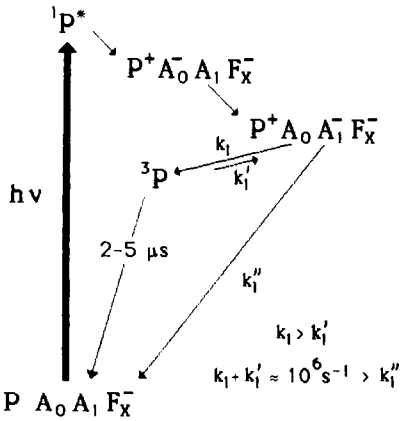

FIGURE 9: Electron-transfer pathways in PS I at room temperature for the different states involving the primary electron donor P-700 and the electron acceptors A_0 and A_1 in PS I reaction centers where the iron–sulfur centers Fe-S$_A$, Fe-S$_B$, and Fe-S$_X$ are prereduced.

Our experiments show that, at room temperature, the P-700 triplet state is formed as a result of the back-reaction between the primary donor and the secondary acceptor A_1. This appears to be a unique feature of PS I reaction centers, as the formation of the triplet state of the primary donor has only been shown to occur from the primary radical pair in other kinds of reaction centers. The energy levels and the kinetic

pathways of electron transfer that govern the formation of the state ^{3}P-700 from the state (P-700$^+$–A_1^-) are schematically displayed in Figure 9. A kinetic model of the P-700 triplet-state formation from the radical pair (P-700$^+$–A_1^-) and of its decay can be made on the basis of the experimental half-times and of the absorption changes at different wavelengths which have been measured more precisely in *Synechocystis* reaction centers. The data can be satisfactorily fitted by assuming that the radical pair (P-700$^+$–A_1^-) decays essentially to the triplet state of P-700 [$(k_1 + k_1')/k_1'' \geq 0.9$] and that the triplet state lies lower in energy than the radical pair ($k_1 > k_1'$). Consequently, assuming a redox potential of +0.49 V for the oxidation of P-700 (Sētif & Mathis, 1980) and that the triplet state of P-700 lies 1.27 eV above the ground state (Shuvalov, 1976), the redox potential for the reduction of A_1 should be lower than −0.79 V, under conditions where Fe-S_X is prereduced. This is in agreement with the postulated role of A_1, which reduces Fe-S_X, the redox potential of which is around −705 mV. The process of P-700 triplet formation from the radical pair (P-700$^+$–A_1^-) appears to be temperature dependent, as this radical pair decays essentially directly to the ground state at low temperature.

The absence of detectable magnetic field effect on the yield of P-700 triplet formation from the secondary radical pair can receive two simple explanations. First, such an effect indeed requires that the decay rate from the triplet sublevels of the radical pair is not too large compared to the decay rate from the singlet sublevel, which is probably not the case here. Second, the iron–sulfur center Fe-S_X^- could be close enough to A_1^- and act as a fast relaxer. This relaxer would annihilate the spin-coherent motion of the two unpaired electron spins on P-700$^+$ and A_1^- which is needed for a magnetic field effect to occur. Studying the magnetic field dependence of the rate of ^{3}P-700 formation under these conditions should allow one to distinguish between these two possibilities.

There is an obvious interest in the study of electron-transfer processes in PS I reaction centers from different organisms. The results presented here are very similar in spinach and cyanobacterial reaction centers. This gives additional support to the idea that the essential features of a given type of reaction centers are conserved throughout evolution whatever organism is considered. Another interest in these parallel studies comes from the fact that although most absorption studies of PS I have been made with higher plants, recent studies are often made with cyanobacterial reaction centers. Additionally, many recent time-resolved EPR studies of PS I were done with cyanobacterial reaction centers, taking advantage of isotope labeling (Furrer & Thurnauer, 1983; Manikowski et al., 1984; Gast et al., 1987; Petersen et al., 1987). Two of these studies (Furrer & Thurnauer, 1983; Manikowski et al., 1984) report a polarization pattern of the EPR signals at room temperature that is very similar to the pattern observed at low temperature. At low temperature, the experiments clearly indicate that the EPR signals can be attributed essentially to the superposition of the two polarized radicals P-700$^+$ and A_1^- [McCracken & Sauer, 1983; Petersen et al. (1987) and references cited therein]. The present evidence for a recombination reaction between P-700$^+$ and A_1^- at room temperature with a half-time of about 750 ns appears consistent with the EPR transients observed at room temperature and supports the idea that these data should be interpreted in the same way as the low-temperature EPR data.

During a period of dark adaptation following a period of strong illumination under highly reducing conditions, the electron transfer remains blocked at the level of A_0 while no A_1^- radical is present and while the iron–sulfur center Fe-S_X is slowly reoxidized. In the meanwhile, reaction centers that have been submitted to the same strong light pretreatment followed by prolonged dark adaptation are still capable of photoreducing the iron–sulfur centers at low temperature.

A similar behavior has been encountered in previous studies on reaction centers that have been depleted of vitamin K_1 by solvent extraction (Sētif et al., 1987). The absorption properties and the rates of NADP$^+$ photoreduction of these reaction centers have been investigated at room temperature (Biggins & Mathis, 1988). Together with reconstitution experiments involving the readdition of vitamin K_1, these experiments support the identification of A_1 with vitamin K_1, acting as an intermediate acceptor between the primary acceptor A_0 and the terminal iron–sulfur centers. In the same depleted reaction centers, a bypass of electron transfer from A_0 to the iron–sulfur centers was put forward to explain the efficiency of the photoreduction of iron–sulfur centers at low temperature.

In the present experiments, the similarity in behavior supports a similar interpretation, with the difference that A_1, although not functional, is present and in a reduced state as it is supported by the restoration of forward electron transfer at room temperature, after the elimination of dithionite. The dark reoxidation of Fe-S_X^- in reaction centers where the electron is still blocked at the level of A_0 shows that the reduced A_1 can no longer transfer an electron to Fe-S_X. Dark incubation of a pH 8 sample indicates that the redox potential for the reoxidation of A_1 may be higher than −590 mV. These data, together with the absence of the EPR signal of A_1^- in blocked reaction centers, can be simply explained by a double reduction of A_1. This interpretation raises some questions about the radical A_1 which can be observed after photoaccumulation at 200 K (Bonnerjea & Evans, 1982). In this work, photoaccumulation is preceded by a period of illumination at room temperature under highly reducing conditions followed by a short dark period before freezing. According to the preceding interpretation, the same room temperature treatment leads to the double reduction of A_1 when the intensity of the background illumination is sufficiently high. In reaction centers containing A_1 in a doubly reduced form, the following illumination at 200 K could then lead to the formation of a radical distinct from A_1. Such a behavior could explain some recent experimental evidence that the photoaccumulated radical A_1 is not vitamin K_1 (B. A. Barry and G. T. Babcock, personal communication).

The double reduction hypothesis fits with the idea that quinone molecules can be doubly reduced to the quinol form, thus leading in the present case to the state A_1H_2. This also explains the higher redox potential necessary for the reoxidation of reduced A_1. This could appear surprising with regard to the fact that the environment of A_1 has to be aprotic to ensure the low redox potential for a one-electron reduction which has been estimated above to be lower than −790 mV. However, this double reduction process may be very slow, and the fully reduced state could be progressively accumulated with a low efficiency. The sequence of events leading to this double reduction may also involve some changes in the protein matrix under these exceptional conditions of very low redox potential. The possibility exists that, after photoaccumulation of the state (A_1^- Fe-S_X^-), the second electron necessary for the double reduction of A_1 is provided by the center Fe-S_X^- according to the mechanism:

$$A_1^- \text{ Fe-}S_X^- + 2H^+ \rightarrow A_1H_2 \text{ Fe-}S_X$$

Titration of the reoxidation of the reduced A_1 via the measurement of flash-induced absorption changes at room tem-

perature should help in testing the hypothesis of quinol formation. At the moment this is the best hypothesis we can propose, as our results argue for the identification of A_1 with a chemical species able to undergo a double reduction process.

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