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ELECTRON PARAMAGNETIC RESONANCE SATURATION STUDIES OF *P*-700$^+$ REACTION CENTER CHLOROPHYLL IN PLANT PHOTOSYNTHESIS

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Summary

Electron paramagnetic resonance (EPR) power saturation and saturation recovery methods have been used to determine the spin lattice, T_1, and spin-spin, T_2, relaxation times of *P*-700$^+$ reaction-center chlorophyll in Photosystem I of plant chloroplasts for 10 K $\leqslant T \leqslant$ 100 K. T_1 was 200 μs at 100 K and increased to 900 μs at 10 K. T_2 was 40 ns at 40 K and increased to 100 ns at 10 K. T_1 for 40 K $\leqslant T \leqslant$ 100 K is inversely proportional to temperature, which is evidence of a direct-lattice relaxation process. At T = 20 K, T_1 deviates from the $1/T$ dependence, indicating a cross relaxation process with an unidentified paramagnetic species. The individual effects of ascorbate and ferricyanide on T_1 of *P*-700$^+$ were examined: T_1 of *P*-700$^+$ was not affected by adding 10 mM ascorbate to digitonin-treated chloroplast fragments (D144 fragments). The *P*-700$^+$ relaxation time in broken chloroplasts treated with 10 mM ferricyanide was 4-times shorter than in the untreated control at 40 K. Ferricyanide appears to be relaxing the *P*-700$^+$ indirectly to the lattice by a cross-relaxation process. The possibility of dipolar-spin broadening of *P*-700$^+$ due to either the iron-sulfur center A or plastocyanin was examined by determining the spin-packet linewidth for *P*-700$^+$ when center A and plastocyanin were in either the reduced or oxidized states. Neither reduced center A nor oxidized plastocyanin was capable of broadening the spin-packet linewidth of the *P*-700$^+$ signal. The absence of diplolar broadening indicates that both center A and plastocyanin are located at a distance at least 3.0 nm from the *P*-700$^+$ reaction center chlorophyll. This

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evidence supports previous hypotheses that the electron donor and acceptor to *P*-700 are situated on opposite sides of the chloroplast membrane. It is also shown that the ratio of photo-oxidized *P*-700 to photoreduced centers A and B at low temperature is 2 : 1 if *P*-700 is monitored at a nonsaturating microwave power.

Introduction

In chloroplast Photosystem I, the *P*-700 reaction-center chlorophyll and the electron acceptors and donors associated with it have received much attention in the past several years. The aim has been to identify and understand the mechanism of photochemical electron transfer in the *P*-700 reaction centers. The first evidence of a stable electron acceptor for *P*-700 was presented by Malkin and Bearden [1] when they identified a photo-induced rhombic electron paramagnetic resonance (EPR) signal ($g_z = 2.05$, $g_y = 1.94$, $g_x = 1.86$) which they ascribed to a bound iron-sulfur center now known as center A. They also observed an iron-sulfur signal, center B ($g_z = 2.05$, $g_y = 1.92$, $g_x = 1.89$), upon chemical reduction.

McIntosh et al. [2] identified by EPR an acceptor, now known as X, preceding centers A and B. Sauer et al. [3] have identified two acceptors, A_1 and A_2, preceding centers A and B by monitoring the flash-induced optical absorption changes of *P*-700$^+$ when centers A and B were both chemically reduced. Sauer et al. also suggest that A_2 and X are the same species. Shuvalov et al. [4] have observed the presence of A_1 by EPR; that is, an isotropic signal of g value 2.004 and linewidth of 1.0 mT.

On the donor side of *P*-700, a copper-containing protein, plastocyanin, in the oxidized state, was observed with EPR by Katoh [5] with $g_\parallel = 2.23$ and $g_\perp = 2.05$. Later evidence with optical flash spectroscopy by Haehnel [6] and Bouges-Bocquet [7] showed that plastocyanin was the primary donor to *P*-700$^+$.

P-700$^+$ has been studied intensively by EPR and optical spectroscopy but little work has been done to determine T_1, the spin-lattice relaxation time, or T_2, the spin-spin relaxation time of this signal. T_1 is the time constant for transfer of energy from a paramagnetic spin to the surrounding lattice in the form of lattice vibration. T_2 is the time constant for relaxation of magnetization in the plane perpendicular to the external field for a population of spins; T_2 is inversely proportional to the linewidth of an EPR signal if it is homogeneously broadened or inversely proportional to the individual spin-packet linewidth in an inhomogeneously broadened signal [8]. Examples of inhomogeneous broadening mechanisms include hyperfine interaction, anisotropic broadening, dipole interactions between spins of different Larmor frequencies, and field inhomogeneities. Dipole interaction between spins of different environments can cause a shortening of T_2 if the spins are separated by a small distance [9,10]. Since T_2 relaxation is sensitive to interactions with other neighboring spins, T_1 and T_2 for *P*-700$^+$ have been determined as a function of temperature in order to estimate the *P*-700-center A distance and the *P*-700-plastocyanin distance in the chloroplast thylakoid membrane. In addition, the effects of sodium ascorbate and potassium ferricyanide on *P*-700$^+$ relaxation were mea-

sured since ascorbate and ferricyanide are used by many researchers to poise the chemical redox potential of chloroplast donors and acceptors.

Materials and Methods

Whole chloroplasts were isolated from spinach by disrupting leaves in a Waring blender in the following solution: 0.3 M sorbitol, 50 mM Tris buffer (pH 7.8), 10 mM NaCl, 1 mM EDTA and 1 mM diethylenetriamine pentaacetic acid. The slurry was squeezed through four layers of filtering silk and the filtrate centrifuged at $5000 \times g$ for 2 min. The precipitate, which contained whole chloroplasts, was resuspended in blending solution to give final chlorophyll concentrations of 4—5 mM. Photosystem I fragments were prepared following the procedure of Hauska et al. [11]. The $144\,000 \times g$ precipitate (D144) was resuspended in the same blending solution used for the whole chloroplasts with a final chlorophyll concentration of 4—5 mM. Chloroplast samples were placed in quartz EPR tubes and cooled in liquid nitrogen and then brought to the observation temperature in the EPR cavity by cold helium gas.

A modified x-band EPR spectrometer operating at 9.202 GHz with a TE_{011} mode cylindrical cavity was used for these experiments. First-derivative spectra and saturation-recovery signals were recorded using 80 Hz magnetic field modulation and phase-sensitive detection at that frequency. A 6 dB improvement in signal-to-noise over conventional EPR spectrometers was achieved by using a frequency-locked Gunn-diode microwave source (Varian VSX 9001, T2, Palo Alto, CA) and a double-balanced microwave mixer (RHG Electronics Lab Inc., model DM 8-12B, Deer Park, NY) as a homodyne detector.

The cavity was critically coupled in these experiments keeping the reflected power at a minimum to allow accurate determination of cavity power, and to protect the detection system from saturation during microwave pulsing. The microwave field intensity at the sample, $\langle H_1 \rangle_s$, was determined by the procedure outlined by Poole [12]. The filling factor (η) for a sample in a cylindrical tube of volume, V_s, extending along the axis of a cylindrical cavity of radius a and length d is:

$$\eta \approx \frac{6.16(V_s/V_c)}{1 + (0.82a/d)^2}$$

where V_c is the volume of the cavity. The relationship between $\langle H_1 \rangle_s$ and the filling factor is:

$$\langle H_1 \rangle_s = [2 \cdot 10^{-3} P_W Q_L \eta (V_w/V_s)]^{1/2}$$

where P_W represents the power in watts measured by a diode detector in the waveguide leading to the cavity, Q_L is the measured loaded Q value of the cavity (5000), and V_w is the volume per unit length of the waveguide. Substituting the measured values and using a critically-coupled cavity, $\langle H_1 \rangle_s$ as a function of measured power was:

$$\langle H_1 \rangle_s \simeq 2.6\, P_W^{1/2}.$$

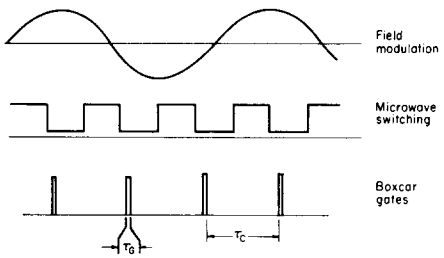


Fig. 1. The relationship between the field modulation, microwave switching, and boxcar gates used in the EPR saturation-recovery system. The boxcar gates are opened for a time τ_G during low microwave intensity to the cavity during signal recovery and closed for time τ_C between gates.

The microwave saturation-recovery technique used in these experiments was a modification of the method devised by Isaacson [13]. This method uses a PIN diode to switch the microwave power in the cavity from a saturating level of 30 mW to a nonsaturating level of 10 μW or less within 1 μs. The EPR signal is field modulated at a frequency (ω_m) of 80 Hz in the cavity. A sample-and-hold (boxcar) gate detects the amplitude of the EPR signal during recovery and passes the 80 Hz component to a lock-in amplifier tuned to the modulation frequency of 80 Hz. The boxcar gate sweeps through the recovery signal in a period of 28 s, allowing the lock-in amplifier to signal-average each point of the recovery signal over several cycles of 80 Hz modulation. The microwave switching frequency (ω_s) was $250 \leqslant \omega_s \leqslant 2.5$ kHz. The relationship between ω_m, ω_s and the boxcar gates is shown as Fig. 1.

The recovery of the signal amplitude, $M(t)$, from saturation depends exponentially on T_1 according to the following equation:

$$M(t) = M_O(1 - e^{-t/T_1}),$$

where M_O is the maximum signal amplitude. A regression analysis program was used to fit the data to an exponential curve and give the time constant, T_1, of the saturation recovery signal.

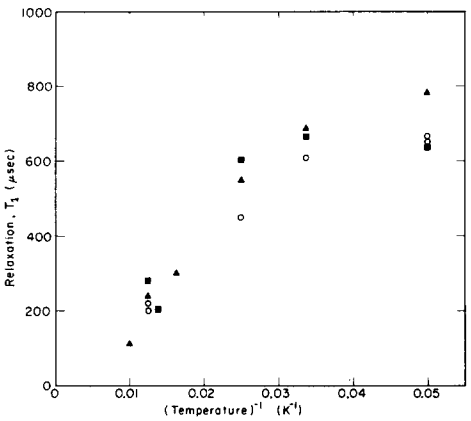
Spin-spin relaxation times for the P-700$^+$ were determined by the power-saturation method described by Beinert and Orme-Johnson [14] by plotting signal amplitude divided by the square root of power as a function of power. The inflection point of this saturation curve is the power at half-saturation ($P_{1/2}$) which is defined for first-derivative inhomogeneous signals as $P_{1/2} \equiv 4/\gamma^2 T_1 T_2$, where γ is the gyromagnetic ratio [15].

DCMU, (3-(3,4-dichlorophenyl)-1,1-diethylurea) was used as an electron transport inhibitor between Photosystem I and Photosystem II. Methyl viologen was used as an electron acceptor to reduce iron-sulfur center A.

Results

Saturation recovery

The spin-lattice relaxation times of P-700$^+$ in whole chloroplasts and D144 fragments were determined at temperatures from 10 K to 100 K. T_1 was 200 μs at 100 K and increased to 900 μs at 10 K. Fig. 2 shows T_1 as a function of



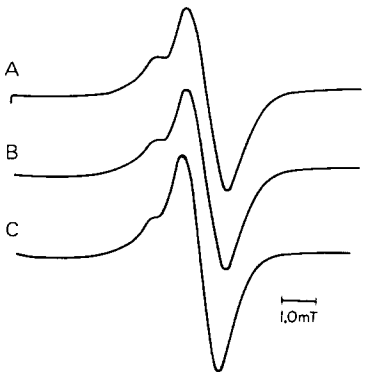


Fig. 2. Spin-lattice relaxation time of *P*-700$^+$ chlorophyll as a function of inverse temperature by the saturation-recovery method. Sample conditions: (▲) whole chloroplasts; (○) D144 fragments; (■) D144 fragments with 10 mM ascorbate.

Fig. 3. Chlorophyll EPR free-radical signal in broken chloroplasts at T = 40 K. Signal induced by: (a) 1 min red light at 40 K, (b) 10 mM ferricyanide, (c) 10 mM ferricyanide plus 1 min red light at 40 K. EPR conditions: power = 2 μW, H_m = 0.4 mT and field scan = 328.5 ± 5.0 mT.

inverse temperature. The spin-lattice times for *P*-700$^+$ in D144 fragments treated with 10 mM ascorbate for 4 h in the dark at 4°C were the same as D144 fragments without ascorbate added, over the temperature range examined.

Broken chloroplasts treated with 10 mM ferricyanide and frozen in the dark exhibit an EPR free-radical signal at 40 K (Fig. 3B) which is identical in *g*-value, linewidth and amplitude to the light-induced *P*-700$^+$ signal in untreated, broken chloroplasts (Fig. 3A). This indicates that ferricyanide at this concentration is capable of oxidzing *P*-700 only. If the ferricyanide-treated sample is then exposed to red light at low temperature, the free radical signal steadily increases. After 1 min of red light the free radical signal increases by 25% (Fig. 3C).

The spin-lattice relaxation time of the light-induced *P*-700$^+$ signal is 550 μs at 40 K. The saturation-recovery curve for this signal is shown in Fig. 4A. T_1 for the ferricyanide-induced signal is 150 μs (Fig. 4B). T_1 for the ferricyanide-plus-red-light signal is also 150 μs (Fig. 4C).

The effect of sodium dithionite on *P*-700 relaxation was examined, but the *P*-700 saturation recovery signals under these conditions were not observed because these signals were an order of magnitude smaller than the *P*-700 signals in the absence of dithionite.

Power saturation

Fig. 5A shows the power saturation curves for *P*-700$^+$ in dark-adapted whole chloroplasts at T = 15, 25 and 40 K under conditions where center A is reduced. An EPR spectrum under these conditions shows a fully reduced center A with lines at g = 2.05, 1.94 and 1.86 (Fig. 6A).

Fig. 5B shows the saturation curves for *P*-700$^+$ at the same temperatures under conditions where plastocyanin is oxidized but center A is not reduced.

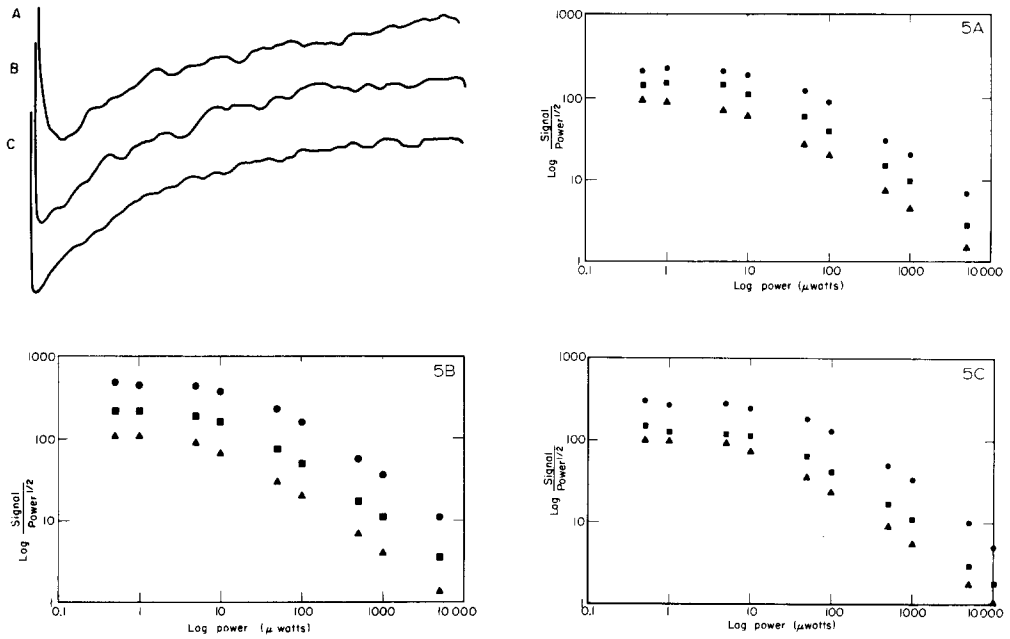


Fig. 4. (Top left) EPR saturation-recovery curves of the free radical signal in broken chloroplasts at 40 K. The signal is saturated with 30 mW power before the microwave power is switched to 10 μW to observe signal recovery from saturation in: (A) light-induced P-700$^+$ signal (2.0 ms time base); (B) P-700$^+$ signal induced by 10 mM ferricyanide (0.5 ms time base); (C) free radical signal induced by 10 mM ferricyanide and 1 min of red light (0.5 ms time base).

Fig. 5.EPR power saturation curves of P-700$^+$ chlorophyll plotted as signal amplitude/(power)$^{1/2}$ as a function of the log of power in (A) P-700$^+$ signal observed in dark-adapted whole chloroplasts illuminated at low temperature, (B) P-700$^+$ signal observed in broken chloroplasts with 250 μM DCMU, 5 μM methyl viologen and frozen in white light, and (C) P-700$^+$ signal observed in D144 fragments with 5 μM methyl viologen and frozen in white light. Temperature conditions: (▲) T = 15 K; (■) T = 25 K; (●) T = 40 K.

To eliminate reduced center A and form a fully-oxidized plastocyanin signal, broken chloroplasts were incubated with 250 μM DCMU, 5 μM methyl viologen, and frozen in liquid nitrogen while under illumination by strong light. The EPR spectrum taken under these conditions shows as oxidized plastocyanin signal at g = 2.05 (Fig. 6B).

Fig. 5C shows the saturation curves for P-700$^+$ in the absence of reduced center A and oxidized plastocyanin. These conditions were brought about by treating D144 fragments with 5 μM methyl viologen and then freezing in the light. The EPR spectrum taken under these conditions (Fig. 6C) shows no reduced center A but a small oxidized plastocyanin signal due to approximately 20% of the total plastocyanin which remains in the fragments after detergent treatment.

The power saturation data of P-700$^+$ indicates that this signal saturates as an ideal inhomogeneous signal. T_2 for inhomogeneous signals cannot be derived from the linewidth of the signal because the linewidth represents an envelope of individual spin packets. However, the values of T_1 for P-700$^+$ determined by the saturation-recovery experiments and the values of $P_{1/2}$ from the power satu-

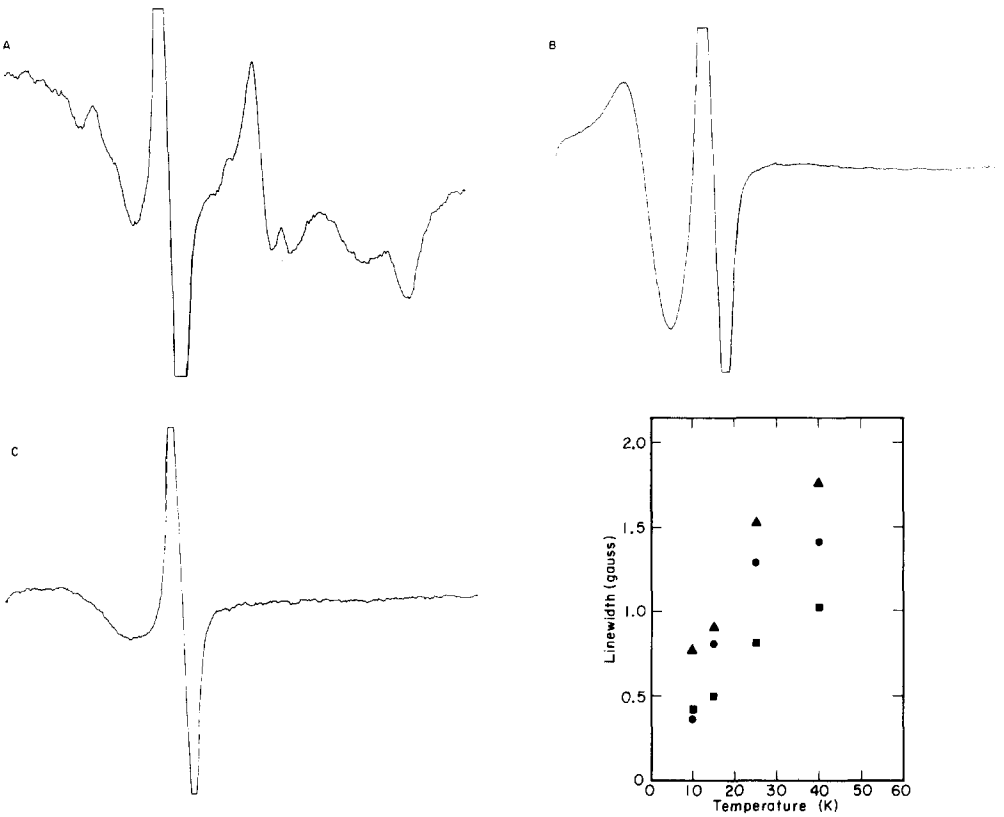


Fig. 6. Light-induced EPR signals in (A), dark-adapted whole chloroplasts, (B) broken chloroplasts with 250 μM DCMU, 5 μM methyl viologen, and frozen in white light, and (C) D144 fragments with 5 μM methyl viologen and frozen in white light. EPR conditions: field scan = 33.50 ± 25.0 mT; power = 15 mW; ω_m = 100 KHz; H_m = 1.0 mT; T = 15 K; gain = 100 for (A) and 25 for (B) and (C).

Fig. 7. (Bottom right) Spin-packet linewidth of $P\text{-}700^+$ chlorophyll as a function of temperature calculated from saturation recovery and power saturation-recovery and power saturation data as described in text. Sample conditions: (●) dark-frozen whole chloroplasts; (■) broken chloroplasts with 250 μM DCMU, 5 μM methyl viologen, and frozen in white light; (▲) D144 fragments with 5 μM methyl viologen and frozen in white light.

ration experiments were used in the relation $P_{1/2} = 4/\gamma_2 T_1 T_2^1$ to give T_2 for the $P\text{-}700^+$ spin packets. T_2 was 40 ns at 40 K and increased to 100 ns at 10 K corresponding to spin-packet linewidths of 0.15 and 0.05 mT, respectively (Fig. 7).

Discussion

Bearden and Malkin [16] also used whole chloroplasts and D144 fragments in their experiments and therefore the experiments reported here are compatible with their work and support their assumption that ascorbate has no effect on $P\text{-}700$ signal intensity in whole chloroplasts and D144 fragments. The low power at which $P\text{-}700$ saturates in the present experiments indicates that conclusions drawn by Bearden and Malkin about the 1 : 1 ratio of photo-oxidized

P-700 to photoreduced center A are now in question. They measured the photoinduced *P*-700 signal intensity at a microwave power of 100 μW at an indicated temperature of 25 K. This power is approximately two orders of magnitude beyond the power saturation level of *P*-700 measured in this work at that temperature. If one measures the photo-oxidized *P*-700 signal intensity at a saturated power and compares its intensity to that of the photoreduced center A ratio will be artificially low. Preliminary experiments conducted in this laboratory using nonsaturating power levels indicate that the ratio of photo-oxidized *P*-700 to photoreduced centers A and B may be 2 : 1 with the assumption that centers A and B are photoreduced in equal quantities at low temperatures. This assumption is made here because no report is available on the quantification of the photoinduced center A to center B ratio. Centers A and B may have significantly different saturation characteristics and optimal signal conditions for the two centers may occur at different temperatures and powers. Hootkins (Hootkins, R., unpublished results) has observed that centers A and B in alga *D. parva* are photoinduced in approximately equal ratios at low temperature but center A saturates at a higher power than center B over the temperature range 10 K $\leqslant$ $T \leqslant$ 25 K.

The fact that Triton X and French Press particles were not examined in these experiments, as in the work of Williams-Smith [17], leaves open the possibility that the saturation properties of *P*-700 in Triton X and French Press particles with and without ascorbate may not be the same as in whole chloroplasts and D144 particles.

Broken chloroplasts treated with 10 mM ferricyanide and dark-frozen exhibit an EPR free-radical signal which is due to *P*-700$^+$. After illumination at low temperature, an additional free radical signal is created. Ke and Shaw [18] and Evans et al. [19] have both observed a similar effect by monitoring the optical absorption changes at 700 nm as a function of redox potential. They attributed the source of the signal in excess of *P*-700$^+$ to oxidized bulk chlorophyll.

Ferricyanide at a concentration of 10 mM shortens T_1 for *P*-700$^+$ by a factor of 4 at 40 K. Ferricyanide appears to be relaxing the *P*-700$^+$ indirectly to the lattice. Such a process, known as cross relaxation, has been described by Bloembergen et al. [20]. In this process, one paramagnetic center which is efficiently coupled to the lattice can relax a second paramagnetic center which has a longer T_1 if portions of the two EPR spectra overlap. Ferricyanide satisfies both of these conditions with respect to *P*-700$^+$.

A decrease in T_1 for the free radical signal in Photosystem I particles treated with 40 mM ferricyanide was also reported by Williams-Smith and coworkers [17]. However, the relative decrease in T_1 reported here is approximately 5-times greater than the relative decrease given by them.

The T_1 for *P*-700$^+$ for 40 K $\leqslant T \leqslant$ 100 K is inversely proportional to temperature as expected for a direct-lattice process. At T = 20 K however, T_1 deviates from a $1/T$ dependence. It is possible that the direct-lattice process is weakening for $T <$ 40 K due to a lack of lattice vibration modes available in this temperature region. Another relaxation process which can proceed at temperatures below which the direct process becomes inefficient is cross relaxation [21].

The paramagnetic center reponsible for cross relaxation $P\text{-}700^+$ has not yet been identified.

The spin-packet linewidth of $P\text{-}700^+$ determined from the power saturation and saturation recovery data (see Results) shows that the $P\text{-}700^+$ spin packets are approximately 0.05 mT wide at 10 K and gradually broaden to approximately 0.15 mT at 40 K. Over this temperature range, the spin-packet linewidth of $P\text{-}700^+$ follows the same temperature dependence regardless of the redox state of plastocyanin or center A. This indicates that dipole broadening does not exist between $P\text{-}700^+$ and reduced center A or between $P\text{-}700^+$ and oxidized plastocyanin. Because $P\text{-}700^+$ line broadening was not detected, a minimum distance of separation was calculated for the P-700-center A pair and the P-700-plastocyanin pair using the magnetic dipole field equation

$$H_D = \gamma\mu(1 - 3\cos^2\theta)/r^3.$$

H_D represents the dipole magnetic field at $P\text{-}700^+$ due to an electron spin dipole on either oxidized plastocyanin or on reduced center A. The distance between interacting spins is r, μ is the magnetic moment of center A or plastocyanin, and θ is the angle between the direction of the external magnetic field and the vector connecting the two paramagnetic spins.

Since $P\text{-}700^+$ line broadening was not observed at 10 K, at which the $P\text{-}700^+$ spin-packet linewidth is 0.05 mT, the dipole field, H_D, must be equal to or smaller than that value. The values chosen for θ were $0 \leqslant \theta \leqslant \pi$, representing a random orientation of two spins relative to each other. Given the above information, it was found that both the P-700-center A pair and the P-700-plastocyanin pair are at least 3.0 nm apart.

A minimum separation distance of 3.0 nm for the P-700-center A pair is reasonable by reference to other work involving reaction center components in plant and bacterial photosynthetic systems. Visser et al. [22] examined the kinetics of the reversible photooxidized P-700 signal at low temperature by monitoring optical absorption changes in the 670—720 nm region. They used a tunneling model to calculate the distance between P-700 and center A of approx. 4.0 nm. Bowman et al. [23] recently detected a decrease in spin-lattice relaxation for the reaction center bacteriochlorophyll (P-860) in bacteria attributable to cross relaxation with the primary stable electron acceptor, an iron-ubiquinon complex. They estimated the distance of separation between the P-860-(iron-ubiquinone) pair to be 2.4 nm. In bacteria it is known that one intermediate electron acceptor, a pheophytin anion, is located between the P-860 and the iron-ubiquinone molecules [24,25]. Therefore, the P-860-(iron-ubiquinone) pair could be closer together than the P-700-center A pair in chloroplasts since there are two intermediate electron acceptors, A_1 and A_2, located between P-700 and center A.

The estimated minimum distance between P-700 and plastocyanin also appears reasonable because Tiede and coworkers [26] failed to observe a magnetic-dipole interaction between P-860 and cytochrome c-553 in the bacteria *Chromatium vinosum*. Cytochrome c-553 is the primary electron donor to P-860 in *C. vinosum* and is capable of reducing P-860 at temperatures down to 4 K [27]. The lack of dipole interaction has led these researchers to set a minimum separation between the P-860-c-553 pair of at least 2.5 nm.

Finally, the distances between electron carriers reported here support the concept proposed by Trebst [28] and by Junge and Witt [29] that the electron donor and acceptor to *P*-700 are situated on opposite sides of the chloroplast thylakoid membrane. They cite research which shows the accessibility of soluble ferredoxin and plastocyanin to antibodies, artificial electron donors, and chemical labels. They conclude that soluble ferredoxin is located on the outer surface of the membrane and that plastocyanin is located on the inner surface. Since bound iron-sulfur center A donates electrons directly to soluble ferredoxin [1,30], center A must also be located on the outer surface of the thylakoid membrane close to the soluble ferredoxin. If plastocyanin, *P*-700 and center A are located along a line, the overall distance between plastocyanin and center A, as reported here, would be at least 6.0 nm. Since the thylakoid membrane width is approx. 7.5 nm [31], a distance of 6.0 nm would be sufficient to place plastocyanin and center A at opposite sides of the membrane as proposed by Trebst, Junge and Witt.

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