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Chemically induced dynamic electron polarization in chloroplasts at room temperature: Evidence for triplet state participation in photosynthesis

(chlorophyll/radical pair/spin polarization/electron paramagnetic resonance)

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ABSTRACT **A transient electron paramagnetic resonance emission is observed after flash excitation of chloroplasts at room temperature. The spectrum of the emission signal is centered at $g = 2.0037$ and has a linewidth $\Delta H_{pp} = 4G$ (4×10^{-4} tesla). Inhibitor studies and chemical oxidation indicate that the signal is associated with Photosystem I, but the spectrum and kinetics indicate that it is neither P700 nor an iron-sulfur protein. The emission signal rises with the 2-μsec time response of the instrument, and decays during the actinic flash. The emission signal is produced on only the first of a pair of strong flashes separated by 100 μsec, indicating that the precursor has not been regenerated in that time.**

The results are discussed with reference to the two currently accepted mechanisms for chemically induced dynamic electron polarization: the radical pair and the photochemical triplet. For several reasons the photochemical triplet mechanism is the more attractive of the two. It is suggested that at room temperature the primary photochemistry of photosystem I proceeds via a triplet state of chlorophyll, and that the species giving rise to the emission signal is the primary electron acceptor of Photosystem I.

In the primary reactions of photosynthesis the first stable products resulting from photon absorption are one-electron oxidized and reduced species, but the metastable states preceding the redox reaction remain a source of controversy. The oxidized and reduced species contain unpaired electrons and, as such, often give rise to electron paramagnetic resonance (EPR) signals. The spectra, kinetic behavior, and response to inhibitors of these signals have been an invaluable aid in determining the reactions that occur in photosynthesis.

The first to be observed in these EPR signals in green plants is known as Signal I (1, 2). Signal I has been conclusively identified as arising from $P700^+$, an oxidized "special pair" of chlorophyll molecules which is in the reaction center of Photosystem I (3–5). Signal I is a structureless gaussian line, with $g = 2.0025$ and linewidth $\Delta H_{pp} = 7.5$ G (7.5×10^{-4} tesla).

At low temperature an additional EPR signal is observed which has been identified as a bound iron-sulfur protein (6). Largely through the work of Malkin and Bearden, this resonance has been assigned to the primary electron acceptor of Photosystem I (7). A component observed in optical studies and designated P430 may be identical to the bound iron-sulfur protein (8). However, recent experiments by Evans and Cammack (9) and McIntosh *et al.* (10) have suggested that the bound iron-sulfur protein is not the primary electron acceptor but rather a secondary acceptor.

Chemically induced dynamic electron polarization (CIDEP) is manifested as an emission or enhanced absorption of microwaves resulting from a nonBoltzmann population of spin states in a radical species. First observed for hydrogen atoms by Fessenden and Schuler (11) in 1963, the effect has since been detected in a variety of chemical systems in solution (12). There has been considerable theoretical interest in the phenomenon, centering around possible mechanisms by which spin polarization arises for radical products of chemical reactions (13, 14). These mechanisms are considered in detail in the *Discussion*.

Harbour and Tollin (15) observed CIDEP in a bacteriochlorophyll-quinone system *in vitro*. CIDEP effects have not been observed previously *in vivo* or in any artificial or natural membrane systems. Spin polarized triplet EPR signals have been observed by Leigh and Dutton (16) and by Uphaus *et al.* (17) in photosynthetic organisms in which photochemistry is blocked.

MATERIALS AND METHODS

All experiments were performed with a Varian E-3 EPR spectrometer modified for 1 MHz magnetic field modulation. The details of the modifications will be described elsewhere (G. Smith, R. Blankenship, and M. Klein, in preparation). The response time of our modified instrument is 2 μsec, in contrast to the previous value of 100 μsec (18). The output of the 1-MHz receiver is fed directly to a 1024 channel Biomation 802 transient recorder, with a minimum dwell time of 0.5 μsec per channel. After each cycle of the Biomation, the contents of the memory are transferred to a Nicolet NIC-80 computer for signal averaging, and subsequently plotted with an X-Y recorder.

Broad band xenon flashes 10 μsec long at half height were obtained from an ILC model L-391 programmable flash lamp system. For some experiments a Chromatix CMX-4 tunable dye laser was used. This unit produced 1-μsec flashes at 600 nm. Synchronization between start of the Biomation sweep and the flash was obtained with Tektronix 162 and 161 waveform and pulse generators.

Broken spinach chloroplasts were prepared as described (19) in 0.4 M sucrose, 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.5, and 0.01 M NaCl. Ten to 20 ml of a chloroplast suspension containing 1–3 mg of chlorophyll (Chl) per ml flowed continuously through the EPR flat cell at a rate of 0.6 ml/min, driven by an LKB

Abbreviations: CIDEP, chemically induced dynamic electron polarization; EPR, electron paramagnetic resonance; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol.

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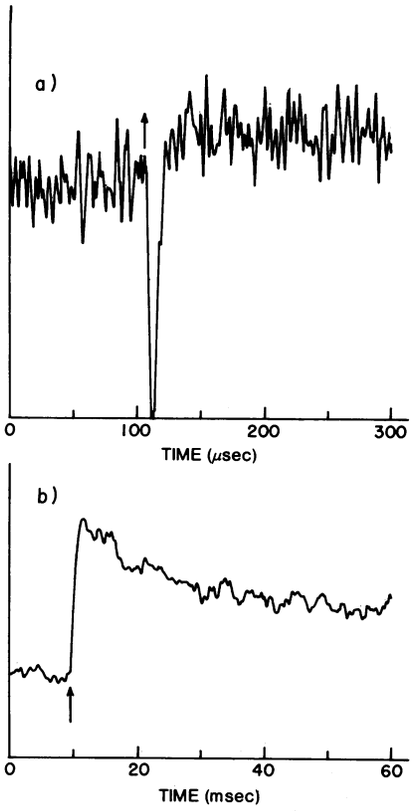

FIG. 1. Room temperature flash kinetic EPR experiment on spinach chloroplasts at 3392 G (0.3392 tesla). (a) 2-μsec time constant. (b) 1-msec time constant. Curve (a) is the average of 2000 events; curve (b) is the average of 500 events. Chloroplasts were prepared as described in *Materials and Methods*. Chlorophyll content, 2.0 mg of Chl per ml; microwave power, 25 mW; modulation amplitude, 3.8 G (3.8×10^{-4} tesla); flash duration, 10 μsec; repetition rate, 2 sec^{-1}.

12000 peristaltic pump. All samples contained 130 μM EDTA, 25 μg of ferredoxin per ml, and 1.3 mM NADP. All experiments were carried out at room temperature.

RESULTS

Fig. 1 shows the EPR response at 3392 G (0.3392 tesla) of spinach chloroplasts to a 10-μsec xenon light flash. This field position is near the low-field maximum in the derivative spectrum of Signal I. Trace (a) was obtained using the 2-μsec time response of the system, while trace (b) was obtained with an impressed time constant of 1 msec. In trace (a), a large spike is observed, opposite in sign to the normal Signal I observed with the slower response in trace (b). Signal I can be observed also in trace (a) as a change in baseline after the flash; there is little Signal I decay during the 200-μsec period shown. The amplitude of the negative going signal is approximately five times larger than the amplitude of Signal I. (We first observed this signal using 100 kHz field modulation and reported it without comment in a time-resolved trace in Fig. 3a of ref. 18.)

Point-by-point spectra of this rapid signal and of Signal I are shown in Fig. 2. The opposite sign of the derivative for the rapid transient indicates that it reflects emission, rather than absorption, as is seen for Signal I. The *g*-factor of the emission signal is 2.0037 ± 0.0004. This was obtained by reference to the standard Signal I value of 2.0025 (4). The linewidth, ΔH_{pp}, of the emission signal is 4 G (4×10^{-4} tesla),

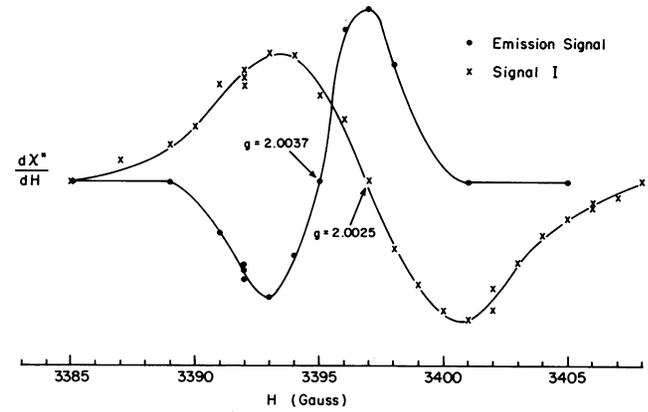

FIG. 2. Spectra of EPR signals in spinach chloroplasts at room temperature. Experiments similar to Fig. 1 were performed at various magnetic field values. ×, Signal I recorded with 3-msec time constant at 1.0 mg of Chl per ml; ●, emission signal recorded with 2-μsec time constant at 2.5 mg of Chl per ml. All other conditions are the same as in Fig. 1. The scale factor relating the amplitudes of the two curves is adventitious because of the different sample concentrations and instrument time constants.

while that of Signal I is 7.5 G (7.5×10^{-4} tesla). Identical spectra are obtained using either 4 G (4×10^{-4} tesla) or 2.5 G (2.5×10^{-4} tesla) magnetic field modulation amplitude, indicating that the spectra shown in Fig. 2 are not broadened by modulation effects. The different linewidths and *g* factors indicate that the two signals arise from different chemical species.

Because the emission signal rises and falls with the flash profile or the EPR instrument time constant, we need to rule out the possibility of flash artifacts. These are usually field-independent, unlike the spectrum shown in Fig. 2. Furthermore, they should not be sensitive to chemical inhibitors or kinetic limitations associated with the photosynthetic reactions.

When electron donation from Photosystem II is blocked by addition of the inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or by heating the chloroplasts at 50° for 5 min, little or no emission signal or Signal I transient is seen. With the repetitive-flash signal averaging technique that we use, the chain of electron carriers between the photosystems quickly becomes oxidized, and $P700^+$ does not become reduced between flashes. When an electron donor system is added, however, both transient signals reappear. Fig. 3 shows this behavior for DCMU-treated sample with an exogenous donor system (curves a and b, at instrument time constants of 2 μsec and 1 msec, respectively). When P700 is oxidized chemically by ferricyanide, no light-induced signals are observed (Fig. 3, curves c and d). The added electron donor system also restores the transient signals to heat-treated chloroplasts (Fig. 3, curves e and f). In every case we observe parallel behavior of the emission signal and Signal I. Because DCMU and heating are known to inactivate Photosystem II and not I, and ferricyanide is known to oxidize P700 chemically without affecting Photosystem II reactions, we conclude that the emission signal is associated with Photosystem I electron transport reactions.

Kinetic information on the rise and decay of the emission signal is obtained most reliably at a field position where Signal I does not appear. Fig. 4 shows the response at 3397 G (0.3397 tesla), which is the high-field maximum of the emission signal and the crossover point of Signal I (see Fig. 2). In response to a series of 1-μsec flashes from a dye laser, both

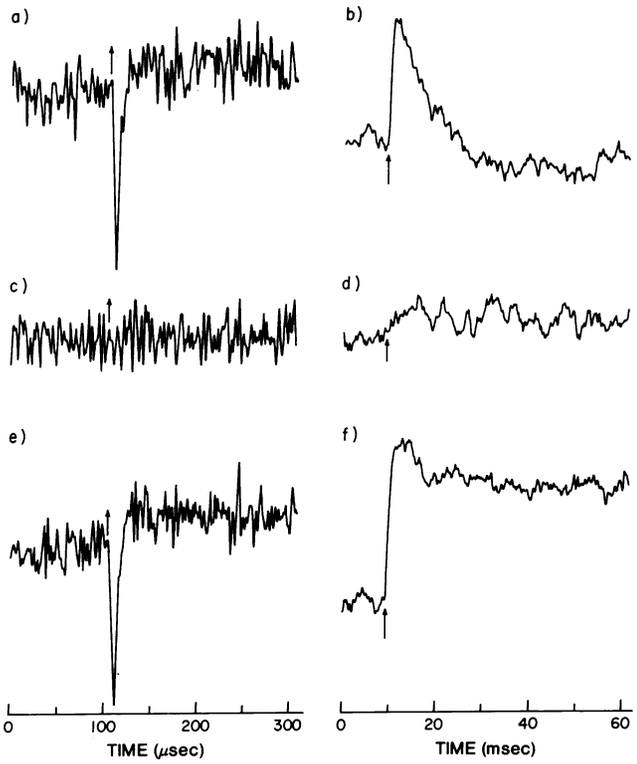

FIG. 3. Response of emission signal and Signal I to inhibitors. Curves (a) and (b), chloroplasts plus 10^{-5} M DCMU, 5 mM ascorbate, 30 μM 2,6-dichlorophenolindophenol (DCIP); curves (c) and (d), chloroplasts plus 6 mM $K_3Fe(CN)_6$; curves (e) and (f), chloroplasts heated for 5 min at 50°, plus 5 mM ascorbate and 30 μM DCIP. Curves (a, c, and e) were recorded with a 2-μsec time constant; curves (b, d, and f) were recorded with a 1-msec time constant. All other conditions as in Fig. 1. Chlorophyll content, 2.0 mg of Chl per ml.

the rise and decay of the emission were instrument-limited at 2 μsec. The rise time of Signal I also appeared to be instrument-limited.

Fig. 5 shows an experiment in which two flashes spaced 100 μsec apart were given to the system. Curve (a) shows the intensity profile of the flashes measured with a PIN photodiode. Curve (b) shows the results of an experiment where weak flashes were given to the system. Some emission signal is seen on both flashes. Curve (c) shows a similar experiment except that strong, nearly saturating flashes were used. The emission signal is seen only on the first flash of the pair, indicating that the precursor to the emission signal is not appreciably regenerated in 100 μsec. This is the expected result if reduced P700 is required for the emission signal to be observed. In our experiments with repetitive flashes the reduction of $P700^+$ is probably limited by the 30-msec turnover time of photosynthetic electron transport (20). Decay components with similar times occur for Signal I (Fig. 1) and $P700^+$ (21).

DISCUSSION

To facilitate discussion of the significance of these experiments, we present a summary of the origin of the CIDEP effect and the mechanisms that have been proposed to explain it. For a more complete treatment, the reader is referred to refs. 12–14, 22, and 23.

The polarization of electron spins results from the difference in population of the lower spin state, $|\beta\rangle$, and the upper spin state, $|\alpha\rangle$. This population difference, $N_\beta - N_\alpha$, is normally governed by the Boltzmann distribution and is calculated to be less than 0.1% for a thermally equilibrated system at room temperature. Since the observed EPR intensity is proportional to $N_\beta - N_\alpha$, it is apparent that any chemical reaction that provides even a slight preference for one or the other of the two spin states in its products will dramatically affect the intensities and even the signs of the observed signals. If state α is preferentially formed, the observed EPR signal will be an emission, while if state β is preferred, the signal will be an enhanced absorption. Because spin-lattice processes cause the return of the system to thermal equilibri-

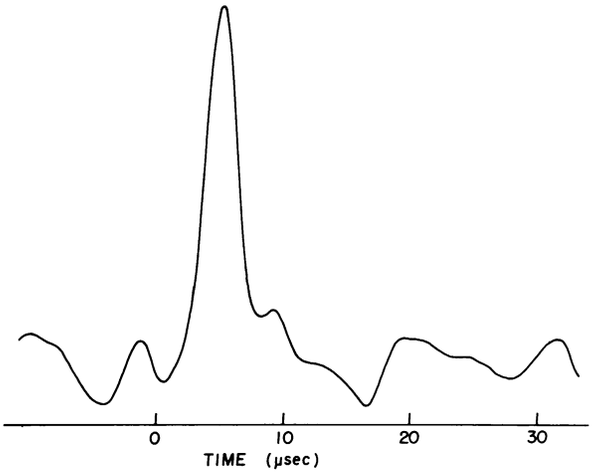

FIG. 4. Kinetics of emission signal recorded at 3397 G (0.3397 tesla) excited by a dye laser at 600 nm. The curve is the average of 2000 events. Microwave power, 25 mW; modulation amplitude, 3.8 G (3.8×10^{-4} tesla); chlorophyll content, 2.9 mg of Chl per ml. Laser flash duration, 1 μsec; repetition rate, 10 sec^{-1}. The large fluctuations in the baseline represent noise owing to the expanded time scale.

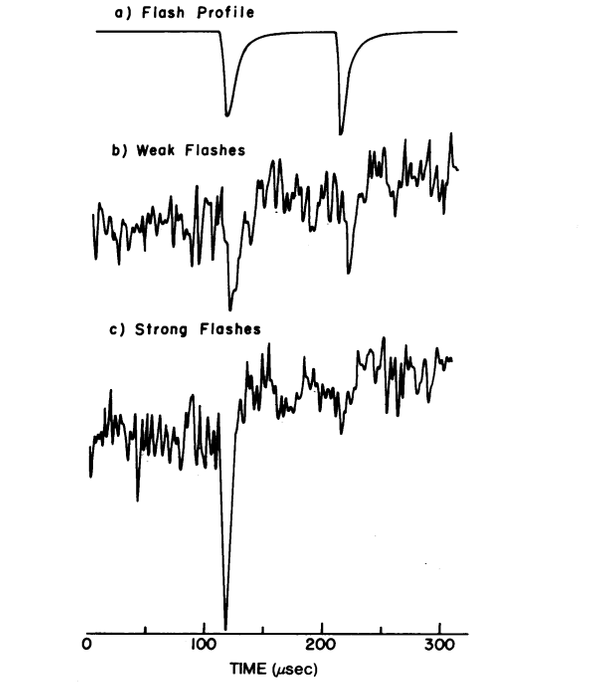

FIG. 5. Response of emission signal at 3393 G (0.3393 tesla) to paired 10-μsec flashes separated by 100 μsec. All conditions are the same as in Fig. 1 except flash rate 0.8 sec^{-1}. Chlorophyll content, 1.8 mg of Chl per ml.

um in a time T_1, the spin lattice relaxation time of the species, the emission or enhanced absorption will decay quickly to the equilibrium absorption signal. Hence, a time-resolved EPR spectrometer is needed to document the polarization effects.

Two different mechanisms have been invoked to explain various experimental CIDEP observations: the radical pair and the photochemical triplet mechanisms. The two mechanisms differ primarily in terms of the origin of the polarization. In the radical pair theory separation occurs first, followed by diffusive recombination of the separated radicals into a closely associated radical pair. Mixing of singlet and triplet states of the radical pair results in the subsequent growth of spin polarization. The photochemical triplet mechanism involves intersystem crossing from an excited singlet to a triplet state of a photosensitive species. Owing to unequal rates of crossing to the three triplet sublevels, the triplet state can become spin polarized. Subsequent rapid electron transfer leaves the resulting radical ions spin polarized. Recent evidence indicates that neither mechanism can explain all of the experimental findings (22, 23). Certain systems appear to operate via a radical pair mechanism, while others utilize the photochemical triplet pathway. The subject of CIDEP mechanisms remains controversial and requires further experimental testing of the various theories.

It is not possible, on the basis of the experiments presented in this paper, to choose one of the two CIDEP mechanisms as unambiguously correct in this system. However, the photochemical triplet mechanism appears to be more appropriate to the photosynthetic primary process for several reasons.

(*i*) The radical pair mechanism is dependent on the radicals being free to diffuse in solution. This is not an obligate feature of the photochemical triplet mechanism, even though all of the studies where it has been invoked have been in liquid solution. In chloroplast membranes the radical species are most likely immobile and restricted to a fixed geometry. The consequences of rigid geometry have not been considered in theoretical studies. The requirement of the radical pair mechanism for diffusive recombination would seem to be absent in chloroplast membranes, however.

(*ii*) Triplet states are well established as intermediates in photochemical studies of chlorophyll *in vitro* (24).

(*iii*) Spin polarized triplet EPR spectra have been observed for chlorophylls *in vitro* (17, 25–27) and also for photosynthetic systems in which the normal photochemistry is blocked (16, 17). This shows that photo-induced spin polarization of the chlorophyll triplet state does occur under certain conditions. Polarization of the triplet level is a necessary, but not a sufficient, condition for the photochemical triplet mechanism.

These points constitute the basis of a preference for the photochemical triplet mechanism, but are not a proof of it. A definite assignment requires further experimental evidence. The two mechanisms differ in their predictions of the relative signs of the polarization of the two radicals. The photochemical triplet mechanism predicts that the two radicals will have the same sign of polarization (12, 22). We have not been able to observe polarization in the supposed counter radical, Signal I. This could arise if Signal I is not the counter radical, or if the spin lattice relaxation time of Signal I is rapid enough to thermalize the spin system before our instrument can respond. No T_1 value for Signal I has been reported. A particularly revealing experiment in this regard would be the temperature dependence of the polarization. Lower temperatures may slow the T_1 of Signal I sufficiently to allow observation of the sign of its polarization, as was seen by Wong *et al.* (23) in the 1,4-napthoquinone system. The sign of polarization of Signal I could therefore distinguish between the two mechanisms.

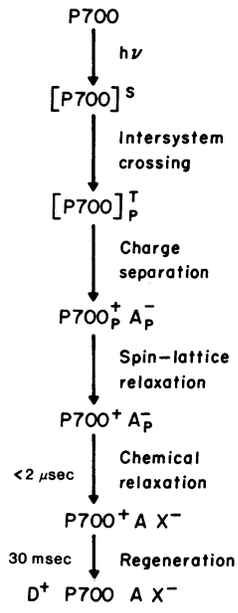

FIG. 6. Possible mechanism for primary photochemistry of Photosystem I assuming the photochemical triplet mechanism for CIDEP. S and T refer to singlet and triplet states of P700; subscript P denotes a spin polarized species. D and X are secondary electron donors and acceptors, respectively.

In the CIDEP studies reported so far, the emissive radical decays into absorption in a time T_1, the spin lattice relaxation time of the doublet species. The signals reported in this paper do not appear to do so, when measured at the crossover of Signal I, the position of least interference. Within the rather large noise envelope, the emission signal appears to decay directly to zero amplitude. This is the expected behavior if the dominant relaxation mechanism of the radical is by chemical reaction.

A possible scheme for the primary photochemistry of Photosystem I, assuming the photochemical triplet mechanism, is shown in Fig. 6. Absorption of the photon results in excitation of P700 to the first excited singlet state. This is followed by intersystem crossing to the triplet state, with polarization arising from unequal rates of intersystem crossing to the three triplet sublevels. Charge separation then occurs rapidly with retention of the polarization. Spin lattice relaxation restores thermal equilibrium quickly for the $P700^+$ species, but the polarized spins of the acceptor relax primarily by an electron transfer (chemical) process. Both P700 and the acceptor must return to their neutral states before the process can occur again. Our results show that the precursor to the emission signal is regenerated in the interval between 100 μsec and 500 msec after a flash.

Regardless of the CIDEP mechanism involved, we conclude that the species giving rise to the observed emission signal is the counter radical produced by P700 oxidation. As such, it would correspond to the primary electron acceptor of Photosystem I, a species which is currently controversial. The emission signal is not due to the iron-sulfur protein of Malkin and Bearden (6), which has a very different EPR spectrum and is observable only at low temperature. The spectrum of the emission signal indicates an organic radical,

possibly a quinone or a flavin. Its relation to P430 is difficult to know because detergent-treated preparations were used for the P430 kinetic studies (28).

The quantum yield of formation of the emission signal is difficult to measure because its magnitude depends on both the population difference of the two spin states and the absolute number of spins. Preliminary studies do show that the emission signal and Signal I begin to saturate at about the same flash intensity.

In conclusion, we have observed an EPR transient that has some of the characteristics of the primary acceptor of Photosystem I. The spin polarization of the species suggests that it is formed from triplet chlorophyll as a precursor in the electron transfer reactions of photosynthesis under physiological conditions.

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