ELECTRON SPIN POLARIZATION IN PHOTOSYNTHESIS AND THE MECHANISM OF ELECTRON TRANSFER IN PHOTOSYSTEM I

EXPERIMENTAL OBSERVATIONS

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ABSTRACT Transient electron paramagnetic resonance (EPR) methods are used to examine the spin populations of the light-induced radicals produced in spinach chloroplasts, photosystem I particles, and *Chlorella pyrenoidosa*. We observe both emission and enhanced absorption within the hyperfine structure of the EPR spectrum of $P700^+$, the photooxidized reaction-center chlorophyll radical (Signal I). By using flow gradients or magnetic fields to orient the chloroplasts in the Zeeman field, we are able to influence both the magnitude and sign of the spin polarization. Identification of the polarized radical and $P700^+$ is consistent with the effects of inhibitors, excitation light intensity and wavelength, redox potential, and fractionation of the membranes. The EPR signal of the polarized $P700^+$ radical displays a 30% narrower line width than $P700^+$ after spin relaxation. This suggests a magnetic interaction between $P700^+$ and its reduced (paramagnetic) acceptor, which leads to a collapse of the $P700^+$ hyperfine structure. Narrowing of the spectrum is evident only in the spectrum of polarized $P700^+$, because prompt electron transfer rapidly separates the radical pair. Evidence of cross-relaxation between the adjacent radicals suggests the existence of an exchange interaction.

The results indicate that polarization is produced by a radical pair mechanism between $P700^+$ and the reduced primary acceptor of photosystem I. The orientation dependence of the spin polarization of $P700^+$ is due to the g-tensor anisotropy of the acceptor radical to which it is exchange-coupled. The EPR spectrum of $P700^+$ is virtually isotropic once the adjacent acceptor radical has passed the photoionized electron to a later, more remote acceptor molecule. This interpretation implies that the acceptor radical has g-tensor anisotropy significantly greater than the width of the hyperfine field on $P700^+$ and that the acceptor is oriented with its smallest g-tensor axis along the normal to the thylakoid membranes. Both the ferredoxin-like iron-sulfur centers and the X^- species observed directly by EPR at low temperatures have g-tensor anisotropy large enough to produce the observed spin polarization; however, studies on oriented chloroplasts show that the bound ferredoxin centers do not have

this orientation of their g tensors. In contrast, X^- is aligned with its smallest g-tensor axis predominantly normal to the plane of the thylakoid membranes. This is the same orientation predicted for the acceptor radical based on analysis of the spin polarization of P700$^+$, and indicates that the species responsible for the anisotropy of the polarized P700$^+$ spectrum is probably X^-

The dark EPR Signal II is shown to possess anisotropic hyperfine structure (and possibly g-tensor anisotropy), which serves as a good indicator of the extent of membrane alignment.

INTRODUCTION

In the reaction centers of photosynthetic membranes the excitation produced by light absorption leads to a rapid transfer of electrons in an oxidation-reduction reaction. The electron donor and acceptor molecules are incorporated into a well-defined complex fixed in the membranes. For photosystem I of higher plants and algae the electron transfer results in optical absorption changes associated with the species P700, the electron donor, and P430, an early electron acceptor (1). Characteristic EPR signals associated with the unpaired electrons present in the donor and acceptor species are also observed (2).

The photoinduced changes in P700, easily monitored by optical absorption or EPR at room temperature under physiological conditions, have been interpreted in terms of the one-electron oxidation of a pair of chlorophyll A molecules located in the reaction center (3). The reduced acceptors are less readily seen, and their identity and sequence are the subject of active investigation. The optical changes designated P430 are reported only for detergent-treated membrane fragments (1). Furthermore, Ke et al. have recently presented evidence that P430 is not the primary electron acceptor (4). EPR signals, similar but not identical to those of known ferredoxin-like iron-sulfur proteins, are seen only at very low temperature, where much of the photochemistry has become irreversible. The proposal of a bound ferredoxin as the primary electron acceptor, based on EPR evidence of Malkin and Bearden (5), has been challenged in more recent studies. McIntosh and Bolton (6), Evans et al. (7,8), and Ke et al. (4) report evidence of a new, unidentified species, designated X, which exhibits properties characteristic of the primary acceptor when the bound ferredoxin has been reduced chemically. A light-induced EPR signal observed for X^- forms reversibly in parallel with P700$^+$. Even when no chemical reductant is present, a component of the EPR spectrum of P700$^+$ is formed reversibly at low temperatures (9–11), whereas the light-induced EPR signal of bound ferredoxin is irreversible (2).

In a previous publication we reported the observation of chemically induced dynamic electron polarization (CIDEP) associated with the initial charge separation in photosystem I at room temperature (12). At that time, it was believed that the so-called triplet mechanism (13) was responsible for the spin polarization. Our more recent results and interpretations indicate that polarization develops by a radical pair mechanism. This mechanism is established in systems of freely diffusing radicals (14). Systems that exhibit spin polarization are either born with or develop population dif-

ferences between their Zeeman levels, which are not in thermal equilibrium with their surroundings. According to the radical pair mechanism, unpaired electron spin density may develop via coherent mixing of singlet and triplet states on a weakly coupled radical pair. This mixing, driven by the difference in local magnetic fields and by spin exchange (J), is more rapid than incoherent relaxation with the lattice. The molecular state that is precursor to radical-pair formation can be determined from the sign (emission or enhanced absorption) of the polarization if the sign of J is known.

Spin polarization studies provide information on the identity of both radicals of the pair, even when only one of them exhibits measurable polarization. This is the case here, because the acceptor radical experiences prompt spin-lattice relaxation at room temperature. Spin exchange between the two partners causes the spectrum of each radical to reflect the properties of both. Furthermore, the dependence of the spectrum on membrane orientation provides information on the anisotropy of the magnetic interactions and the orientation of the radical species.

In this paper we describe light-induced spin polarization in samples containing spinach chloroplasts, photosystem I particles, or whole cells of *Chlorella pyrenoidosa*, using time-resolved EPR. The detailed shape of the spectrum of spin polarization and its dependence on orientation of the photosynthetic membranes lead us to propose a mechanism significantly different from that presented in our previous paper. In particular, we now believe that the spin polarization observed is from P700$^+$ and that its spectrum reflects interaction with a counter-radical with EPR properties resembling a reduced iron-sulfur protein. This mechanism of development of spin polarization will be treated in detail in a later paper.[1]

METHODS

All transient experiments were performed with a modified Varian E-3 or E-9 spectrometer (Varian Associates, Palo Alto, Calif.) with 1 MHz magnetic field modulation (12,15). The Varian TE$_{102}$ microwave cavity was fitted with an optical transmission flange constructed from *P*-band waveguide to allow 100% light transmission. Typically, microwave power of 25 mW and modulation amplitude of 4 G or less were used. These provided good sensitivity without appreciable saturation or broadening of the signals. The 0–90% response time of the spectrometer was 2 μs. 500 kinetic traces were added at each field position with a Biomation 802 transient recorder (Biomation, Cupertino, Calif.) and an SDS Sigma 2 computer (Sigma Instruments, Inc., Braintree, Mass.).

A Q-switched ruby laser with a 50-ns pulse width served as a light source in some experiments. Energies to 0.15 J/pulse at a repetition rate of 2 s^{-1} were used. A Corning color filter (CS 2-58, Corning Glass Works, Corning, N. Y.) removed the stray flashlamp light, and a telescope or negative lens provided for beam expansion. The ruby laser oscillates at 694.3 nm. In other experiments utilizing a xenon flashlamp filtered to transmit above 300 nm, the response time of the measurements was limited to 10 μs by the flashlamp duration. Tests were performed with the spectrometer to measure the influence of the high-field modulation rate on the line shape of the radical spectra. The absence of any line shape dependence on modulation amplitude and

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[1]Friesner, R., G. C. Dismukes, and K. Sauer. 1977. In preparation.

No
flow

$\dfrac{dX''}{dH}$

Flow

⊢——⊢——⊢——⊢——⊣
0   200   400
      Time, μs

a) $\vec{V}$ $\nabla \vec{V}$ H_0

b) $\nabla \vec{V}$ H_0
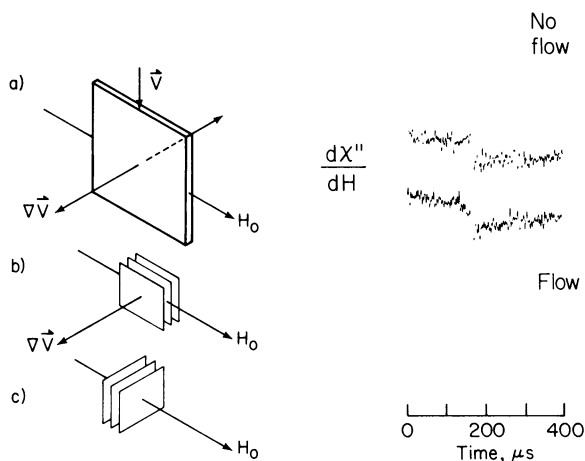
c) H_0

FIGURE 1   FIGURE 2

FIGURE 1 (a) EPR cell geometry. $\vec{V}$, flow direction; H_0, EPR spectrometer magnetic field axis; $\nabla \vec{V}$, direction of the velocity gradient and illumination. (b) Orientation of the thylakoid membranes of spinach chloroplasts relative to the EPR field when caused by the flow gradient. (c) Orientation of the thylakoid membranes caused by an intense magnetic field.

FIGURE 2 The effect of sample flow on the EPR signal from broken spinach chloroplasts. The average of 500 kinetic traces is shown. Flow rate was 0.5 ml/min through a cell of cross-section 0.025 cm × 1.0 cm; chlorophyll concentration, 8.3 mg/ml; room temperature; spectrometer conditions: microwave power, 25 mW; modulation amplitude, 3.8G; time constant, 0.5 μs; magnetic field strength, 3,385 G; pulsed ruby laser excitation: wavelength, 694 nm; duration 50 ns; repetition rate 2 s^{-1}.

the disappearance of the absorption signal upon 90° phase shift in the phase-sensitive detector indicate that fast passage distortion of the line shape was not important.

Broken spinach chloroplasts were prepared as previously described (16). All samples contained 100 μM EDTA, 25 $\mu g/ml$ ferredoxin, 1 mM NADP, 0.4 M sucrose, 0.05 M HEPES,[2] pH 7.5, and 0.01 M NaCl. Total chlorophyll concentrations of 5–6 mg/ml were used typically. Chloroplast fragments enriched in photosystem I were prepared by the digitonin procedure of Anderson and Boardman (D-144 particles) (17). The green alga *Chlorella pyrenoidosa* (original Emerson strain) was grown in Myers medium (18).

Measurements of electrochemical potential were made with a platinum electrode, with Ag/AgCl as a reference couple. The electrode was calibrated against a saturated solution of quinhydrone at pH 4.01 and 7.00, both at ambient temperature. Redox titrations were carried out on chloroplast suspensions buffered at pH 7.5, which were continuously flowed through the EPR cavity from the titration cell. Aliquots of a concentrated $K_3Fe(CN)_6$ solution were used for the oxidative titration, and a saturated ascorbate/ascorbic acid solution (pH 7.5) was used for back titration.

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[2] *Abbreviations used in this paper:* CIDEP, chemically induced dynamic electron polarization; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; HEPES, N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid; P700, Photosystem I reaction center chlorophyll P430, bound iron-sulfur proteins in Photosystem I.

Chloroplasts orient in a velocity gradient (19). We induced orientation by running the suspension through a flat quartz sample cell of cross-section 0.025 cm × 1.0 cm. The flow rate varies with the viscosity of the suspension but typically was 0.6 ml/min for suspensions containing 2 mg/ml of chlorophyll. Flow rates to 3 ml/min were available.

The geometry of the sample cell relative to the spectrometer's magnetic field axis is fixed by the design of the EPR cavity, as indicated in Fig. 1 a. This configuration results in the alignment of the short axis of the chloroplast along the velocity gradient, perpendicular to the magnetic field of the spectrometer. Spinach chloroplasts are shaped like oblate ellipsoids, within which the orientation of the normal to the thylakoid membranes is predominantly along the short axis of the chloroplast (20).

Spectra of nonflowing chloroplasts were recorded with a fresh chloroplast suspension for each magnetic field position. Under these conditions, the chloroplasts are nearly isotropically oriented. In the absence of flow, the chloroplasts align partially (10–15%) in the 3.4 kG magnetic field used in the EPR spectrometer. This is casued by the anisotropic diamagnetic susceptibility of the chloroplast membranes and results in the short axis of the chloroplast aligning parallel to the field (21,22). Examination of the chloroplasts with an optical microscope at higher field strengths confirms this orientation.

Partial orientation of nonflowing chloroplasts is revealed by an anisotropy of the dark EPR Signal II for samples frozen in the presence or absence of the 3.4 kG field (*vide infra*). We examined samples oriented by intense magnetic fields of 10 or 13 kG and then frozen. These show complete alignment as evidenced by a saturation in fluorescence polarization (24) and in the anisotropy of EPR Signal II. For our sample geometry the orientation caused by the field is perpendicular to that produced by the flow. The orientation of thylakoid membranes relative to the EPR field is shown in Fig. 1 for both field and flow orientation. Field orientation during flow experiments is negligible, because the former is readily disrupted by the velocity gradient.

RESULTS

Spinach Chloroplasts

In a typical experiment we monitor the first derivative of the EPR absorption at a fixed field during a laser pulse. This is shown in the upper trace of Fig. 2 for broken spinach chloroplasts at a field position on the high-field side of the $P700^+$ spectrum. A large transient change in intensity occurs during the laser pulse, followed by a slower decaying signal afterwards. Both signals are field-dependent, indicating that they are not due to flash artifacts. Flow orientation of the photosynthetic membranes causes the brief pulse to invert for some field positions, as shown in the lower trace of Fig. 2. The 5-μs width of the short intense signal is limited by the spectrometer response time. The slow, smaller amplitude change is independent of sample flow at all field positions. The decay kinetics of the slow change is the same at all field positions and equals 30 ms, the same as found for $P700^+$ with both optical and EPR detection.

The dependence of both signals on magnetic field strength for flow-oriented and for nonoriented samples is plotted in Fig. 3. The amplitude of the rapid signal is taken as the difference between the peak amplitude and the amplitude after the pulse (see Fig. 2). The phases of the derivative signals indicate that the rapid component appears in net emission, while the slower component is in absorption. The *g* value (2.0025), line width (7.5 G, ΔH_{pp}), and isotropic character of the absorption signal are identical to those of spin-relaxed $P700^+$. Preferential excitation of photosystem I is expected at
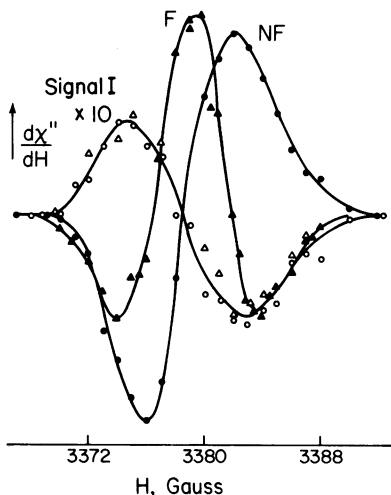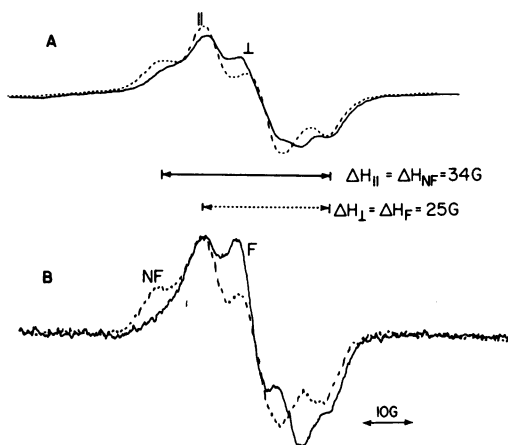
FIGURE 3

FIGURE 4

FIGURE 3 The first derivative of the EPR absorption is plotted as a function of magnetic field for the slow (Signal I) and fast (polarized) kinetic components of Fig. 2. Signal I: △, flowing sample and ○, nonflowing sample. Polarized signal: ▲, flowing sample and ●, nonflowing sample. The experimental conditions are the same as in Fig. 2, except that a 2 G modulation amplitude was employed and 700 flashes were averaged to obtain the flow spectra. The amplitudes are normalized to the same number of flashes and modulation amplitude. The amplitude of Signal I is multiplied by 10 for the purpose of display.

FIGURE 4 The effects of magnetic field orientation (A) and flow orientation (B) on the dark EPR signal in spinach chloroplasts. (A) chloroplasts oriented at 13 kG in 50% ethylene glycol, then frozen; spectrum recorded at 77°K. Scans are shown for both parallel and perpendicular orientations of the aligning field direction in the sample relative to the spectrometer field. Spectrometer conditions, 0.5 mW power, 2.0 G modulation amplitude, 100 kHz modulation frequency, 12.5 G/min scan, 1.0 s time constant, 1.5×10^5 gain. (B) chloroplasts under nonflowing (NF) and flowing (F) condition. Flow rate, 0.6 ml/min; EPR cell, 0.025 cm × 1 cm; temperature, 20°C; spectrometer conditions, 25 mW power, 4 G modulation amplitude, 1 MHz modulation frequency, 25 G/min scan, 1.0 s time constant, 30 dB attenuation, 5.0 gain.

the 694 nm laser wave length. Signals in emission or enhanced absorption are said to be spin-polarized. The line shape of the emission signal is a sensitive function of the orientation of the chloroplast with respect to the magnetic field. Without flow orientation we observe a symmetric emission signal displaying a g value identical to P700$^+$, but having a narrower line width of 5.5 G. For flow-oriented chloroplasts, an enhanced absorption peak appears on the high-field side. The amplitudes of the polarized signals cannot be compared to those of the slower kinetic signal, because the former are restricted by the instrument response time. The integral areas of the polarized signals for the oriented and unoriented samples were estimated by using a planimeter for the graphical integration. The areas are approximately equal. The low field cross-over point $(d\chi''/dH = 0)$ for the flow-oriented sample is shifted ca. 2.5G below that for nonoriented chloroplasts.

Relatively small flow rates, 0.05 ml/min to 3.0 ml/min, through a cell of dimensions 0.025 cm × 1 cm achieved saturation of orientation.

Signal II Orientation

To confirm that the flow effect arises from the orientation of chloroplasts, we compared the EPR spectra of the dark Signal II (23) from flow-oriented chloroplasts with the analogous spectra from chloroplasts oriented and frozen in magnetic fields of 13 kG. Signal II can be used as a marker of the extent of orientation, because it displays partially resolved anisotropic hyperfine splitting and probably g tensor anisotropy that are sensitive to the orientation with respect to the EPR magnetic field. Signal II has been attributed to a membrane-bound semiquinone radical, most probably plastosemiquinone (24,25). We find that orientation of chloroplasts by a velocity gradient yields Signal II spectra with features identical to those of chloroplasts oriented and frozen in intense magnetic fields. The spectra are shown in Fig. 4, and the corresponding orientations of the thylakoid membranes induced by flow or magnetic fields are shown in Figs. 1 b and 1 c. The broadest hyperfine structure (34 G) occurs when the normal to the plane of the thylakoid membrane lies along the Zeeman field. This must be the direction of the largest component of the hyperfine tensors, on the average, and specifies the orientation of the radical. Nonflowing fluid samples show Signal II spectra slightly different from those of samples frozen in the absence of a magnetic field before the measurement. This suggests a partial alignment for nonflowing fluid samples, presumably by the weaker EPR magnetic field. We estimate orientation of 10–15% at 3.4 kG, in agreement with results from fluorescence polarization (21).

Babcock and Sauer observed changes in Signal II spectra with increasing ionic strength of the suspension medium (23). The spectral changes they observed are identical to those we see in oriented samples, which suggests that the increasing ionic strength may increase the degree of orientation by the EPR magnetic field.

Spinach Chloroplasts, Flashlamp Experiments

In an earlier paper, we reported spin-polarized EPR spectra for spinach chloroplasts using a 10-μs pulse width xenon flashlamp (12). The long pulse width excitation source is not favorable for observation of polarized signals with much more rapid kinetics of formation and decay, however. The earlier data on flow-oriented chloroplasts did not reveal a high-field enhanced absorption peak for the polarized spectrum, although the line shape asymmetry and 2 G downfield shift of the cross-over point relative to the relaxed P700$^+$ signal were observed.

We have performed additional experiments using the flashlamp at improved sensitivity, and we now find evidence for a high-field absorption peak on the otherwise predominantly emissive polarized spectrum of oriented chloroplasts. These data are shown in Fig. 5. A comparison of the data from Figs. 3 and 5 indicates that there is a considerable loss in amplitude for the polarized signal relative to Signal I when measured with the longer pulse width and instrument time constant. In contrast to those with laser excitation, the flashlamp experiments show an apparent decrease in polarized
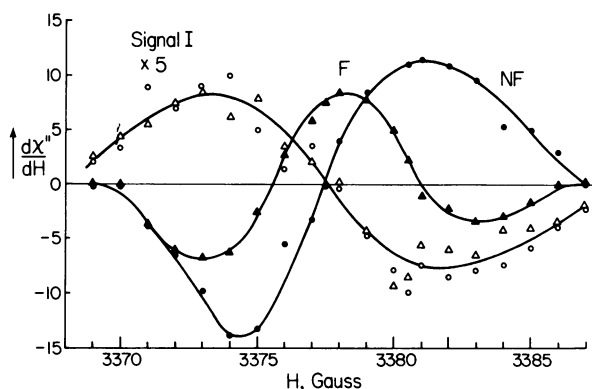
FIGURE 5 EPR spectra of the emission signal and Signal I in spinach chloroplasts under flow and no-flow conditions. Signal I: △, flowing sample and ○, nonflowing sample. Polarized signal: ▲, flowing sample and ●, nonflowing sample. Flow rate, 0.6 ml/min; microwave power, 25 mW; 4 G modulation amplitude; chlorophyll content, 2.5 mg/ml. Each point in the emission signal spectrum is the average of 200 events obtained with a 10-μs time constant. The amplitude of Signal I is multiplied by five for the purpose of display.

signal intensity for flowing samples relative to nonflowing samples. A 1.7-fold reduction of integral area is estimated from Fig. 5. There is also preferential loss in amplitude of the high-field enhanced absorption peak when the flashlamp and a longer time constant are used. Experiments performed with the laser pulse but with the EPR time constant increased from 2 to 10 μs give similar results, showing that the reduction is a consequence of the longer instrument response time. When the time constant is increased to 10 μs, and laser excitation is employed, a nonuniform decrease in signal amplitude is observed for the polarized signal: about a 3.6-fold decrease for the low-field and central peaks of the oriented sample, and about a five-fold decrease for the high-field peak. This indicates that relaxation decreases the polarized signals observed after the longer delay, and that relaxation is faster for the high-field unresolved hyperfine states.

To permit comparison of signal intensities between different experiments we nor-

TABLE I
RELATIVE INTEGRAL AREAS AND LINE WIDTHS OF THE
POLARIZED EPR SIGNALS

Sample	Conditions*	I_{NF}	I_F	I_{NF}/I_F	ΔH	ΔH_{NF}
Broken chloroplasts	Laser, 2 μs	13	13	1	7.5	5.6
Broken chloroplasts	Flashlamp, 10 μs	5	3	1.7	8	6.5
D-144 particles	Flashlamp, 10 μs	1.7	1.7	1	8	5.8
Chlorella pyrenoidosa	Flashlamp, 10 μs	2.5	2.3	1	8	6

I_{NF} and I_F are normalized integral areas for the polarized signals in nonflowing (NF) and flowing (F) samples. See the text for the normalization procedure. ΔH, peak-to-peak line width for the relaxed P700$^+$ signal; ΔH_{NF}, peak-to-peak line width for the polarized signal in nonflowing samples.
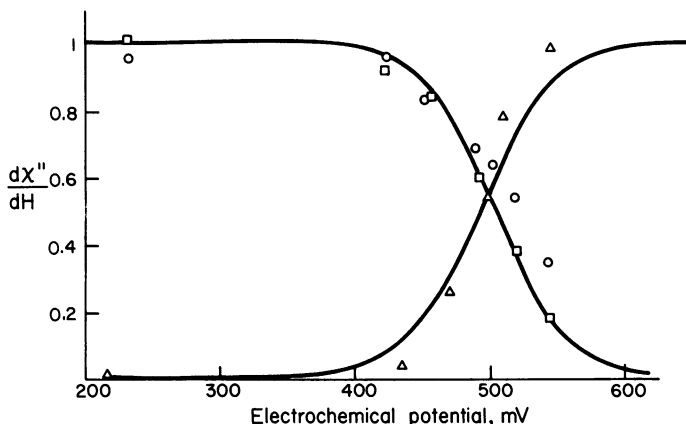*Light source and overall instrument response time.

FIGURE 6 Potentiometric titration of the light-induced EPR Signal I (o) and emission signal (□) from broken spinach chloroplasts. The data are an average of an oxidative and a reductive titration in which the 10-μs flashlamp was the light source. Aliquots of a concentrated solution of $K_3Fe(CN)_6$ were used to oxidize P700, followed by back titration with a solution of ascorbate/ascorbic acid (pH = 7.5). The chemically oxidized (steady-state) amplitude (△) was also followed. The curves represent the theoretically expected behavior for a one-electron transfer process. The midpotentials could only be estimated because the data are incomplete above 545 mV, but suggest a common midpoint of E_m = 500–525 mV. The experiment was done at room temperature and pH = 7.5. Potentials are reported relative to the normal hydrogen electrode.

malize the integral areas for the polarized signals by dividing by the area under the relaxed P700$^+$ spectrum. This internal normalization is justified because we observe a direct correlation between the two signals for different sample concentrations and instrument conditions. The normalized areas for the signals in Figs. 3 and 5 are tabulated in Table I.

Redox Titration

We investigated the relation between the spin-polarized and the relaxed EPR signals in spinach chloroplasts by measuring their dependence on the steady-state redox level of the chloroplast suspensions. The data are given in Fig. 6; they include an oxidative titration with $K_3Fe(CN)_6$ and a reductive titration with ascorbate. Data are absent above 545 mV, so only estimates of midpotentials are possible. Both the light-induced polarized signal and the relaxed P700$^+$ signal show a midpoint potential of ca. 500–525 mV. This agrees with the results reported by Knaff and Malkin (26) for P700 using optical and EPR methods, but is greater than the value of +375 mV found by Evans et al. (27). The redox behavior appears to be that of a one-electron transfer process.

Photosystem I Particles

Spin polarization is observed in subchloroplast preparations which contain predominantly photosystem I. The spin-polarized and relaxed P700$^+$ spectra for D-144 particles are shown in Fig. 7. No change in the EPR spectrum of either kinetic component is observed when the flow is varied from zero to ca. 3 ml/min. The polarized spectrum
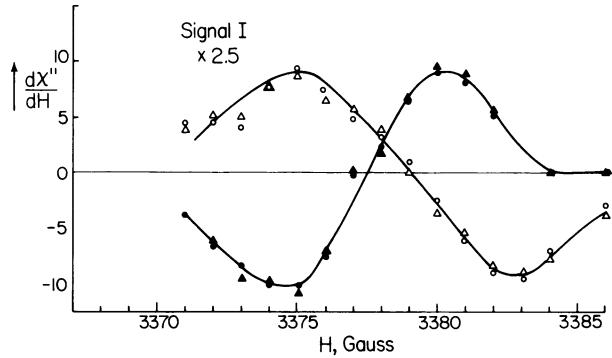
FIGURE 7 EPR spectra of the emission signal and signal I in photosystem I particles (D-144) under flow and no-flow conditions. Each point represents the average of 300 events. Flow rate, 0.6 ml/min; 25 mW microwave power; 2.5 G modulation amplitude; 1 MHz modulation frequency; chlorophyll content, 0.83 mg/ml. The solution contained 2×10^{-3}M NADP, 70 μg/ml ferredoxin, 2×10^{-4}M EDTA, 1.4×10^{-7}M ferredoxin-NADP reductase, 1×10^{-2}M ascorbate, and 4×10^{-5} M DCIP,[2] in 0.05M HEPES, 0.4 M sucrose, and 0.01 M NaCl at pH 7.5. The scale for Signal I is 2.5 times that for the emission signal.

shows spectral features intermediate between the flow and no-flow spectra of chloroplasts (Fig. 5). Although no high-field absorption peak is present, a slightly asymmetric line shape and 1.5 G downfield shift in the cross-over point relative to the relaxed P700$^+$ spectrum are observed. There is a sharp decrease in the magnitude of spin polarization for photosystem I particles compared to chloroplasts, as shown in Table I. Flow orientation of the small membrane fragments of detergent-solubilized photosystem I particles is not expected under our experimental conditions. Also, magnetic field orientation of these particles is negligible up to 100 kG (22).
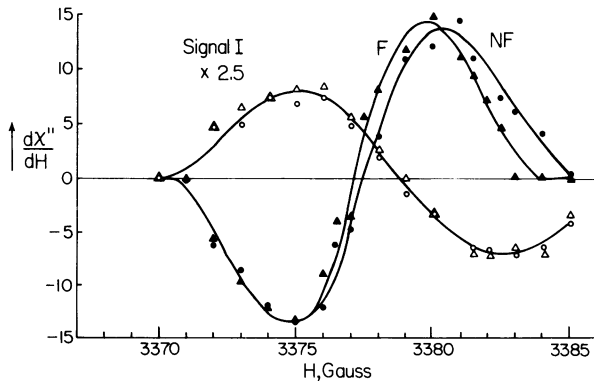

FIGURE 8 EPR spectra of the emission signal and Signal I in whole cells of *Chlorella pyrenoidosa*. Each point represents the average of 400 events. Flow rate, 0.6 ml/min; 25 mW microwave power; 4 G modulation amplitude; 1 MHz modulation frequency; time constant, 10 μs. The scale for Signal I is 2.5 times that for the emission signal.

Chlorella pyrenoidosa

A spin-polarized EPR signal is present in the green alga *Chlorella pyrenoidosa*. The formation and decay of polarization are again faster than can be resolved with the 2-μs EPR instrumentation. The magnetic field dependence of the polarized signal is illustrated in Fig. 8, along with the relaxed P700$^+$ spectrum which accompanies it. A considerably smaller flow-induced effect is observed for the polarized spectrum in *Chlorella* than for spinach chloroplasts. Since *Chlorella* are nearly spherical cells, they are not expected to orient readily in a flow gradient. The spectrum recorded without flow shows a 1.5 G shift in the cross-over point, downfield from relaxed P700$^+$ and similar to the position for photosystem I particles.

Bacteria

Using the xenon flashlamp and 1 MHz field modulation (limiting response time, 10 μs), we found no polarized EPR absorption or emission at room temperature for whole cells or chromatophores of the photosynthetic bacteria *Rhodospirillum rubrum* or for whole cells of *Rhodopseudomonas spheroides*.

DISCUSSION

In an earlier paper we reported our initial observations of an EPR emission signal in chloroplasts (12). At that time we believed that the signal was due to the acceptor radical and that polarized emission occurred because of radical formation from the triplet state of P700, by the so-called triplet mechanism of CIDEP. The new evidence presented here forces us to reject this interpretation.

The results of the redox titration experiment, the observation of the polarized signal in photosystem I particles and with far red light (694 nm) are in agreement with our earlier interpretation that the polarized radical is involved in electron transfer in photosystem I. However, these observations can be explained if the polarized signal arises from either P700$^+$ or the photo-reduced acceptor radical. We now believe that the *g* value and line shape of the polarized signal can be understood best in terms of polarization of the P700$^+$ radical. The complex line shape changes that we observe upon orientation can be accounted for by a radical pair mechanism of electron transfer. In this view the transfer of an electron from the chlorophyll donor, P700, to an adjacent acceptor molecule produces two radicals sufficiently close to one another that orbital overlap and, hence, spin exchange coupling occurs. This weakly coupled radical pair is born with the same spin configuration as that of the excited molecular state of P700 from which transfer occurred, i.e., spin singlet; hence, there will be no initial polarization. However, in a weakly coupled radical pair coherent mixing of the singlet and triplet states is produced by the difference in local magnetic fields on the two radicals (hyperfine and spin-orbit fields). Mixing of this type produces spin polarization in freely diffusing radicals in solution (14,28). We believe that this process is also significant for membrane-bound radicals where rapid electron transfer takes the place of bulk radical diffusion in the dynamics of radical pair formation and separation.[1]

Our results indicate that the counter-radical of P700$^+$ is oriented in the membrane

and possesses a distinctly anisotropic g tensor. Anisotropic g tensors are characteristic of the two leading candidates for the electron acceptor: membrane-bound ferredoxin and X^- (6-8). The coupling between the paired radicals results in a highly anisotropic spin-polarized P700$^+$ signal, because exchange coupling between the two radicals causes the magnetic anisotropy of the acceptor radical to be observable in the P700$^+$ spectrum. In this manner the predominantly isotropic unresolved hyperfine structure of P700$^+$ can exhibit anisotropic features. Anisotropic features are not observed after spin relaxation, evidently because rapid electron transfer separates the paired radicals faster than relaxation occurs. The participation of the triplet state of P700 via a triplet mechanism of radical formation can be rejected, because this mechanism cannot account for transitions of both emission and enhanced absorption among the hyperfine states of P700$^+$ (13).

Consideration of the qualitative features of the radical pair mechanism developed by Adrian (14), or given in the next paper,[1] yields insights into the identity and orientation of the radicals and the spin multiplicity of the precursor state of P700. Radicals born from a pure spin singlet or triplet molecular state will populate either Zeeman level of the separated doublet radical with equal probability and, therefore, yield no spin polarization. If separation occurs so that a small orbital overlap still exists, then mixing of the resulting singlet and triplet radical pair states can produce unequal population of the Zeeman levels. The local magnetic fields on P700$^+$ and the acceptor radical produce this mixing at a frequency given by

$$\omega = \frac{1}{2}(g_1 - \hat{g}_2)\frac{\beta_0 H}{\hbar} + \frac{1}{2\hbar}\sum_i a_i m_i \qquad (1)$$

The first term is due to a difference in g values and the last term is the hyperfine field on radical 1 (here P700$^+$). g_1 and $\hat{g}_2$ are isotropic and anisotropic g tensors, respectively; β_0 is the Bohr magneton; $\hbar$ is Planck's constant divided by 2π; H is the magnetic field strength; a_i is the hyperfine coupling constant for nucleus i on P700$^+$, and m_i is the corresponding nuclear magnetic quantum number.

The expression given in Eq. 1 is suitable when the dominant mixing occurs between the singlet level and the triplet level for which $<S_z> = 0$. This occurs when the anisotropy of the g and hyperfine tensors is small and when the singlet-triplet splitting (exchange energy) is much less than the Zeeman energy. We ignore any effect of a hyperfine field on radical 2 for simplicity, because it does not alter the following arguments. The $(g_1 - \hat{g}_2)$ or Δg term in Eq. 1 populates the upper spin states of the radical with the larger g factor and the lower spin states of the radical with the smaller g factor. This produces on the average, emission from radical 1 and absorption from radical 2. Because the sign of the hyperfine term changes in the center of the P700$^+$ spectrum, the polarization changes from emission to absorption at the field value where the two terms of Eq. 1 become equal and of opposite sign in going from the low to the high-field hyperfine states.

This mixed emission/absorption pattern for P700$^+$ becomes apparent for the oriented membranes, where the difference in g values becomes less than the hyperfine

field of P700$^+$. Using this model we can learn something about the orientation and estimate the magnitude of the g tensor anisotropy of the acceptor radical.

The ground state principal values of the g tensor for ferredoxin center A are g_z = 2.05, g_y = 1.95, and g_x = 1.87, while center B has g_z = 2.05, g_y = 1.93, and g_x = 1.89 (29). The corresponding values for X^- are 2.09, 1.90, and 1.78 (30). The average values are 1.95 for the ferredoxins and 1.92 for X^-. The average values are suitable estimates for the unoriented samples. Either value is sufficiently smaller than that for P700$^+$, 2.0025, to produce a Δg term overwhelmingly larger than the hyperfine contribution to the polarization (the unresolved hyperfine envelope of P700$^+$ on a g scale is 0.0049 or 8 G). This gives very nearly a symmetric emission signal centered on the relaxed P700$^+$ spectrum, which is what we observe for nonflowing samples. If g_z and g_y of the acceptor radical are in the direction of the plane of the thylakoid membrane, a flow-oriented sample would possess an average g value of 2.00 for either X^- or the bound ferredoxins. For this orientation we expect a decrease in the Δg term involved in generating polarization. The hyperfine state of P700$^+$ then becomes important, and the appearance of a high-field absorption peak is expected.[3] We observe this behavior when the sample is oriented by flow. Eq. 1 indicates that a decrease in the Δg term upon orientation causes a reduction in the rate of mixing, which should decrease the magnitude of spin polarization. This would be true only if the extent of mixing had not yet reached saturation, which inevitably occurs as complete polarization is approached. The data in Table I taken with the shortest time constant indicate that oriented and unoriented chloroplasts have the same integrated polarization intensity, and so suggest that nearly the maximum spin polarization is produced. We observed saturation of polarization in model calculations[1] employing an oriented X^- radical coupled to P700$^+$. The integral areas of the polarized signals observed at longer time constants are more severely influenced by relaxation effects, which we have not considered in our calculations.

The symmetric signals that we observed for samples that are not flow oriented show a consistent narrowing of about 2–2.5 G compared to the 7.5 G width of the relaxed P700$^+$ spectrum. The data are given in Table I. The narrowing cannot be explained by a simple model involving averaging of the hyperfine structure by an electron spin exchange or electron spin dipole-dipole interaction between P700$^+$ and X^-. This may be deduced from the Anderson treatment of exchange narrowing of dipolar broadened spectral lines (31). Under certain conditions the hyperfine structure on P700$^+$ may be narrowed, at least in principal, by an interaction between the unpaired electron on the photoreduced acceptor molecule and the nuclear spins on P700$^+$ that contribute to the EPR hyperfine structure. To meet this condition and still maintain a weak exchange interaction ($|J| < g\beta H$, as implied by the observation of spin polarization), the acceptor would have to be located in an unfavorable position for overlap with the P700$^+$ molecular orbital bearing the unpaired spin, yet situated so that a hyperfine interaction with some of the protons on P700$^+$ is comparable to the hyperfine inter-

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[3]See note added in proof.

actions within P700$^+$. These restrictions may be met if the acceptor is located in a nodal plane of one of the chlorophyll A molecules of P700$^+$.

Other causes for the narrowing of the polarized P700$^+$ spectrum can be postulated. For example, the narrowing could arise from delocalization of the unpaired electron on P700$^+$ over three or four chlorophyll molecules, which would cause a further reduction in line width of $(3/2)^{1/2}$ or $(4/2)^{1/2}$. This would produce the necessary narrowing to explain our results. It requires that the spin delocalization decreases with time to that expected for a uniform distribution within a dimer, in order to account for the broader spectrum after relaxation. Still another possibility is that the spin density distribution in P700$^+$ immediately after photo-oxidation is not uniform over a pair of identical chlorophyll A molecules, but becomes uniform as the P700$^+$ dimer relaxes to its equilibrium geometrical arrangement, which may be quite different from that of reduced P700.

We cannot distinguish between X^- and reduced ferredoxin as the counter-radical solely on the basis of the magnitude of the spin polarization they might produce, because of the inaccuracy with which the g tensors are known currently. Also, both ferredoxin and X^- have thermally accessible excited spin states that are occupied at room temperature where our measurements are made. A completely accurate comparison of the two would require a knowledge of the g tensors for the excited states. The best approach to determining which molecule functions as the earliest electron acceptor is to look for polarization at low temperatures, where both X^- and ferredoxin may be observed by EPR. These experiments are in progress. We can also look for an orientation dependence in the EPR spectra of X^- or reduced ferredoxin in samples of oriented chloroplasts. We have observed an orientation dependence in the EPR spectrum of X^- in which the smallest g tensor axis lies predominantly along the normal to the thylakoid membrane. By contrast, reduced ferredoxin center A shows no orientation dependence and center B is oriented with its smallest g tensor axis in the membrane plane (30). Only the X^- orientation is the same as predicted for the electron acceptor on the basis of spin polarization in P700$^+$, and is strong evidence implicating X^- as the species responsible for the anisotropy of the polarized P700$^+$ spectrum.

McIntosh and Bolton recently reported spin polarization in spinach chloroplasts and in whole cells of *Anacystis nidulans* and *Scenedesmus obliquus* (32). These authors attribute the two emission signals that they observe at 200°K or below to photosystem II radicals on the basis of a decrease in signal upon treatment with red light in the presence of the inhibitors 3-(3,4-dichlorophenyl)-1,1-dimethylurea[2] and hydroxylamine. Because this will also result in photooxidation of P700, their experiment is ambiguous and permits the interpretation that P700$^+$ is responsible for the emission signal. The first-derivative EPR signals that they report do not integrate to zero, as is necessary for a physically reasonable signal. This indicates that their spectra are distorted by rapid passage effects or microwave power saturation at the low temperatures. This may explain their apparent observation of two radicals. Alternatively, the two peaks that they saw split by ca. 5 G in deuterated algae may be due to the radical pair,

P700$^+$X$^-$, in which spin exchange or dipolar coupling has split the P700$^+$ spectrum. In any case, severe distortions appear to be present. Also, these authors propose that the triplet mechanism is responsible for the polarization on the basis that molecular diffusion is impossible in a membrane-bound system. We have shown that electron transfer reactions are quite capable of producing large spin polarization via a radical pair mechanism in membrane-bound systems (30).[1] Our evidence indicates that it is the excited singlet state of P700 which is the molecular state involved in charge separation.[1] If it is accepted that the radical pair mechanism is responsible for spin polarization, as is clear from our experiments, then the appearance of both primary donor and acceptor radicals in total emission is clearly impossible when ST_0 mixing is dominant. ST_+ mixing can lead to emission for both donor and acceptor radicals and can be dominant only for $J \sim g\beta H$. Accordingly, we believe the signal reported by McIntosh and Bolton is probably due solely to polarized P700$^+$.

The absence of a flow effect on the line shape of the polarized P700$^+$ signal for *Chlorella* and photosystem I particles is consistent with the expectation that a hydrodynamic velocity gradient cannot orient spherical bodies or exceptionally small fragments. Also, the small degree of magnetic field orientation which occurs at 3.4 kG for chloroplasts is negligible for *Chlorella* and absent for photosystem I particles (21,22).

The polarized signals in nonflowing samples of spinach chloroplasts, *Chlorella*, and photosystem I particles are all in total emission, as expected if g value differences dominate in the production of polarization. Table I shows that the integrated polarization intensity can vary considerably depending on the organism or the extent of fractionation of the chloroplast. This is not unreasonable, since the separation of the radicals may also be influenced by these changes. The emission spectra in *Chlorella* and photosystem I particles are not centered about the relaxed P700$^+$ spectrum, but appear 1–1.5 G to lower field. This is expected if both a g value difference and a hyperfine field of similar magnitude contribute to the polarization. The average g value difference between P700$^+$ and X^- at room temperature may be in this range when the g tensors for the excited states of X^- are considered. Presently, these are unknown. The emission signal in chloroplasts is centered on the relaxed P700$^+$ signal in nonflowing samples. If partial orientation of the nonflowing chloroplasts occurs in the EPR magnetic field, then an increase in the average g value difference is expected. This could easily account for the symmetric emission spectrum of nonflowing chloroplasts.

The difference between the polarized P700$^+$ signal in Fig. 3, observed with the 40-ns laser pulse, and that in Fig. 5, observed with the 10-μs xenon flashlamp and 10-μs time constant, is due to a decrease in overall instrument response from 2 to 10 μs, rather than to an effect of wavelength. This is proved by our observation that spectra obtained with the laser and with an imposed EPR time constant of 10 μs are the same as those obtained with the flashlamp. At the longer time constant we observe a decrease in integrated polarization intensity with a preferential decrease in intensity of the high-field hyperfine transitions. In addition to nuclear spin-independent electron spin-lattice relaxation, cross-relaxation between the paired radicals is possible. The

latter relaxation process is important when the radicals have near-degenerate spin transitions. Both reduced ferredoxin and X^- show EPR spectra which overlap with $P700^+$ and have their predominant absorption to higher fields. The high-field hyperfine states of $P700^+$ may therefore relax more readily via direct and Raman-assisted cross-relaxation processes. Cross-relaxation rates which decrease with increasing disparity in transition frequency are well documented (33, 34).

We did not observe spin polarization in whole cells and chromatophores of the photosynthetic bacteria *R. rubrum* and *R. spheroides* at room temperature. Recently, Hoff et al. (36) reported spin polarization at $100°K$ in isolated reaction centers of *R. spheroides* depleted of iron. Evidently removal of iron and reduction of temperature slows spin relaxation to a point where the polarization can be observed in the tens of microseconds time region. Also, the magnitude of the exchange coupling is crucial in determining whether spin polarization will be observed. Thus if $|J| \gtrsim g\beta H$ and the radical pair lifetime $\tau > |J|^{-1}$, polarization is quenched (14).

CONCLUSION

Spin polarization studies have significance for the primary process of photosystem I of chloroplasts. (*a*) A unique measure of the interaction between primary photoproducts at room temperature is possible from observations of the populations of the electron spin energy levels of the generated radicals before relaxation. (*b*) The dynamics of charge separation involves electron transfer in which the radical pair, $P700^+X^-$, forms in less than 2 μs, and probably much less than this. Subsequent electron transfer to a more remote acceptor molecule, possibly bound ferredoxin, also occurs in less than 2 μs, as suggested by the absence of a radical pair EPR spectrum. (*c*) Electron transfer occurs from the first excited singlet state of the reaction-center chlorophyll. This argues against a triplet state precursor. (*d*) After spin relaxation, $P700^+$ has predominantly isotropic hyperfine and g tensors, consistent with the spin density being distributed equally between two chlorophyll molecules. (*e*) A 30% narrower line width for $P700^+$ is observed before spin relaxation. Several explanations for this narrowing are discussed. (*f*) An early acceptor has a distinctly anisotropic g tensor and is oriented in a definite spatial relation with respect to $P700^+$. The smallest g tensor axis lies in the direction of the normal to the thylakoid membrane. The X^- radical is the leading candidate on the basis of its g tensor anisotropy and its observed orientation within the thylakoid membrane. (9) The radical responsible for dark Signal II in spinach chloroplasts possesses anisotropic hyperfine structure (and probably g anisotropy). It is aligned so that the axes of the hyperfine tensors have their largest values, on the average, normal to the plane of the thylakoid membrane.

NOTE ADDED IN PROOF

Calculations in which the detailed orientation averaging is performed confirm that X^- can cause the anisotropy of the polarized $P700^+$ spectrum.[1] However, the absolute amplitude of the polarized signal calculated for oriented chloroplasts is too small by a factor of 10. This is resolved if an earlier acceptor molecule A_1 is postulated. A_1 must have isotropic magnetic properties and also forms a radical pair with $P700^+$. This model leads to a term dependent on the hyperfine state of $P700^+$ and a second term dependent on Δg between $P700$ and X^-. This suggests that spin polarization of $P700^+$ is developed within both radical pairs, $P700^+A_1^-X$ and $P700^+A_1X^-$, which form by successive electron transfer. These calculations are supported by recent optical experiments reported by K. Sauer, S. Acker, P. Mathis, and J. A. Van Best (1977. *In* Bioenergetics of Membranes, L. Packer, A. Trebst and G. Papageorgiou, editors. Elsevier/North Holland Biomedical Press, Amsterdam, The Netherlands), who found evidence for two acceptors before P430. One of these is presumably X, while the other may correspond to A_1.

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