Triplet states in photosystem I of spinach chloroplasts and subchlo-roplast particles

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ABSTRACT We report light-induced electron paramagnetic resonance triplet spectra from samples of chloroplasts or digitonin photosystem I particles that depend upon the dark redox state of the bound acceptors of photosystem I. If the reaction centers are prepared in the redox state P-700 A X Fd$_R$ - Fd$_A$, then upon illumination at 11K we observe a polarized chlorophyll triplet species which we interpret as arising from radical pair recombination between P-700$^+$ and A$. This chlorophyll triplet is apparently the analog of the P$_R$ state of photosynthetic bacteria [Parson, W. W. & Cogdell, R. J. (1975) Biochim. Biophys. Acta 416, 105-149]. If the reaction centers are prepared in the dark redox state P-700 A X Fd$_R$ - Fd$_A$, then upon illumination at 11K we observe a different triplet species of uncertain origin, possibly phoephtin or carotenoid. This species is closely associated with the photosystem I reaction center and it traps excitation when P-700 is oxidized.

Electron paramagnetic resonance (EPR) detection of triplet states has provided an effective probe of both the mechanism of the primary light reaction of bacterial photosynthesis and the structure and geometry of reaction center components (1). The first observation of a triplet state in photosynthetic bacteria by EPR methods by Dutton et al. (2) has led to a significant advance in our understanding of the initial charge separation process. They showed that intense, spin-polarized triplet EPR signals arise upon illumination at low temperatures under reducing conditions when normal photochemistry is inhibited. Further work in this area has revealed that this triplet state, designated P$_R$, forms on the primary electron donor, P-860, as the result of a charge recombination reaction between the photoreduced electron acceptor, I$, and the photooxidized primary donor P-860$(3). However, in green plant and algal preparations, only low-intensity EPR triplet signals, which most likely originate outside the reaction center, have been reported, and no unambiguous assignment of a triplet state to the primary photoreactions has been made (4-9). A component of delayed luminescence observed optically by Shuvalov et al. (10, 11) was attributed to triplet formation dependent upon the redox state of the reaction center of photosystem I.

Our choice of sample conditions for studying EPR triplet spectra was guided by the current model of the photosystem I reaction center, which may be represented as P-700 A X Fd$_R$ Fd$_A$. P-700 is the primary electron donor (12); Fd$_B$ and Fd$_A$ are iron sulfur centers (13, 14); and X is a species of unknown chemical composition that can be observed in its reduced state by EPR at liquid helium temperatures (15). In samples containing reduced Fd$_R$ and Fd$_A$, illumination at liquid helium temperature reduces X to X$, which then undergoes a back reaction with P-700$^+$ with a decay time of 190 msec (16). A is an acceptor species that participates as an intermediate in the light-induced charge separation between P-700 and X (16, 17). When X has been reduced prior to illumination (16, 17) or when X and the iron sulfur centers are chemically inactivated (18, 19), a back reaction attributed to P-700$^+$ and A$ occurs with a decay time of 1 msec at liquid helium temperatures in photosystem I subchloroplast particles. Under the same redox conditions in chloroplasts at liquid helium temperatures, two components with half-times of 122 msec and 1.7 msec contribute to the decay (20). Motivated by these new findings, we have designed experiments to detect triplet states by EPR in samples in which the normal photochemistry of photosystem I is blocked immediately beyond the initial electron acceptor.

MATERIALS AND METHODS

Chloroplasts were isolated from market spinach in 0.4 M sucrose/10 mM NaCl/10 mM Tris buffer, pH 8.0/10 μM EDTA and collected by centrifugation at 5000 × g for 5 min.

To prepare digitonin photosystem I subchloroplast particles, we resuspended chloroplasts to a chlorophyll concentration of 0.3-0.4 mg ml$^{-1}$ in 50 mM Tris buffer (pH 8.0) containing 10 mM Mg$^{2+}$ to assure clean fractionation of photosystem I from photosystem II (21). Digitonin was added to the chloroplast suspension as a 10% (wt/vol) solution to give 0.5% (wt/vol) digitonin. After incubation for 2 hr at 4°C, the detergent incubation mixture was centrifuged for 0.5 hr at 30,000 × g. The supernatant contained photosystem I particles with the characteristics: chlorophyll a/chlorophyll b = 5.6 and chlorophyll/P-700 = 175.

The supernatant was concentrated for EPR studies by precipitation with potassium sulfate as described by Nelson et al. (22).

Treatment with Dithionite. Chloroplasts or digitonin photosystem I pellets were degassed under reduced pressure and mixed with sodium dithionite in 100 mM glycine buffer (pH 10) under a nitrogen atmosphere to give 12 mM dithionite.

Treatment with Ferricyanide. Chloroplast or digitonin photosystem I pellets were mixed with K$_3$Fe(CN)$_6$ in 50 mM Tris buffer (pH 8) to give 1 mM ferricyanide.

The treated pellets were then combined with an equal volume of ethylene glycol, sealed in EPR tubes, and stored at 77K. Final chlorophyll concentration of the samples was about 2 mg ml$^{-1}$. Samples prepared in Condition 1 (described below) were illuminated by a tungsten lamp for approximately 3 min and frozen to dry ice/acetone temperature under illumination before they were stored at 77K.

EPR measurements were made with a Varian E-109 spectrometer at X-band with 100 kHz field modulation and equipped with an Air Products Helitron cryostat. The triplet state signals were detected by a light modulation technique by

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Abbreviation: EPR, electron paramagnetic resonance.
which the exciting light was chopped at 33.5 Hz. The output of the EPR system was fed directly to a PAR (Princeton Applied Research, Princeton, NJ) model 210 selective amplifier and then to a PAR model 220 lock-in amplifier that was referenced to the chopper.

Excitation was provided by an Oriel 1000-W xenon lamp filtered by 5 cm of water. Temperature was measured with a gold/chromel thermocouple. No change in the relative signs of the EPR signal occurred when the microwave power was reduced to 1 \( \mu \)W, when the chopper frequency was lowered to 11 Hz, or when the detector phase angle on the lock-in amplifier was altered.

RESULTS

Condition 1: Dithionite, pH 10, Frozen under Illumination. Chloroplasts or digitonin photosystem I particles that were frozen under illumination in the presence of dithionite gave the dark EPR spectrum shown in Fig. 1A. In the light-modulated triplet state spectra for chloroplasts (Fig. 2A) and digitonin photosystem I particles (Fig. 3A) prepared in condition 1, one major triplet, denoted triplet I, was observed. Triplet I has the spin polarization pattern \( aee \), where \( a \) denotes a signal in absorption and \( e \) denotes a signal in emission. The zero-field splitting parameters of triplet I are \( |D| = 0.0278 \pm 0.0009 \text{ cm}^{-1} \) and \( |E| = 0.0039 \pm 0.0009 \text{ cm}^{-1} \) (Table 1).

Condition 2: Dithionite, pH 10, Frozen Dark. Chloroplasts and digitonin photosystem I particles that were frozen dark in

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FIG. 1. EPR spectra of reduced chloroplasts observed in the dark at 11K. Conditions under which the samples were frozen were: (A) Dithionite, illumination while cooling; (B) dithionite, dark. EPR conditions for both spectra were: microwave power, 10 mW; sweep time, 2 min; time constant, 0.5 sec; modulation amplitude, 18 G; modulation frequency, 100 kHz; microwave frequency, 9.079 GHz; receiver gain, 2500.

FIG. 2. Light-modulated, triplet state spectra of chloroplasts. Conditions under which the samples were frozen were: (A) dithionite, illumination while cooling; (B) dithionite, dark; (C) ferricyanide. EPR conditions for all three spectra were: microwave power, 1 mW; sweep time, 1 hr; recorder time constant, 30 sec; modulation amplitude, 16 G; modulation frequency, 100 kHz; receiver gain, 80; microwave frequency, 9.075 GHz; light modulation frequency, 33.5 Hz.

the presence of dithionite gave the 11K dark EPR spectrum shown in Fig. 1B. Figs. 2B and 3B show the light-induced triplet signals obtained in condition 2 from chloroplasts and digitonin photosystem I particles, respectively. One major triplet state, denoted triplet II, was observed. Triplet II has the spin polarization pattern \( aee \) and zero-field splitting parameters \( |D| = 0.0383 \pm 0.0013 \text{ cm}^{-1} \) and \( |E| = 0.0040 \pm 0.0013 \text{ cm}^{-1} \).
Figure 3. Light-modulated triplet state spectra of digitonin photosystem I particles. Conditions under which the samples were frozen were: (A) dithionite, illumination while cooling; (B) dithionite, dark; (C) ferricyanide. EPR conditions for spectra A and B were: microwave power, 1 mW; sweep time, 1 hr; recorder time constant, 30 sec; modulation amplitude, 16 G; modulation frequency, 100 kHz; receiver gain, 20; microwave frequency, 9.075 GHz; light modulation frequency, 33.5 Hz. EPR conditions for spectrum C were the same as in A and B except for receiver gain, which was 80.

C. We made this assignment as follows. The innermost signals were assigned to the \( Y^+ \) and \( Y^- \) transitions because of their characteristic shape. The other major features, labeled \( X^+ \) and \( X^- \), were used with the assignments of \( Y^+ \) and \( Y^- \) and the relation (23)

\[
H_{X^+} - H_{X^-} + H_{Y^+} - H_{Y^-} = H_{Z^+} - H_{Z^-}
\]

Table 1. Zero-field splitting parameters and electron spin polarization patterns of observed triplet state signals

| Triplet | \(|D|, \text{ cm}^{-1}\) | \(|E|, \text{ cm}^{-1}\) | Polarization pattern |
|---------|----------------|----------------|-----------------|
| I       | \(0.0278 \pm 0.0009\) | \(0.0039 \pm 0.0009\) | \(\text{ae ae}\) |
| II      | \(0.0383 \pm 0.0013\) | \(0.0040 \pm 0.0013\) | \(\text{ae ae}\) |

The errors represent the uncertainty in the parameters as deduced from the repeatability of the field position measurements. \(a\), Absorption; \(e\), emission.

To calculate the positions of the third transitions, \(Z^+\) and \(Z^-\). The calculated value agrees with small features that appear reproducibly in the spectra shown in Figs. 2 and 3.

Condition 3: Ferricyanide, pH 8. Figs. 2C and 3C show the light-induced triplet signals obtained in condition 3 for chloroplasts and digitonin photosystem I particles, respectively. Once again we observe triplet II as the major species, with the polarization pattern \(\text{ae ae}\) and zero-field splitting parameters \(|D| = 0.0383 \pm 0.0013 \text{ cm}^{-1}\) and \(|E| = 0.0040 \pm 0.0013 \text{ cm}^{-1}\) (Table 1).

DISCUSSION

The dark signals at \(g = 1.78\) and \(g = 1.89\) in Fig. 1A indicate that \(X^-\) and \(F_dB^-\) become reduced in samples frozen under illumination in the presence of dithionite (24). During subsequent light-modulation experiments in this redox state, we observe light-induced signals that accompany the process of charge separation and recombination (16, 17, 20) which we interpret as

\[
P_{-700} A^- X^- \text{Fd}B^- \text{FdA}^- = P_{-700} A^- X^- \text{Fd}B^- \text{FdA}^-, \quad T_{1/2} = 1 \text{ msec}.
\]

The \(\text{ae ae}\) polarization pattern of triplet I is characteristic of triplets formed via a charge recombination reaction and is best explained by the radical pair mechanism (1). According to this mechanism, the system is initially prepared in an excited singlet state. After one electron is transferred from a donor to an acceptor, a change in spin correlation occurs between the electron localized on the acceptor and the one remaining on the donor. This mixes the singlet state, \(S\), predominantly with the middle-energy high-field triplet spin sublevel, \(T_0\). If the effect of this process is to drive the population into the \(T_0\) level selectively. Hence, all \(T_0\) to \(T_{-1}\) transitions are in absorption and all \(T_0\) to \(T_{+1}\) transitions are in emission. Thus, the \(\text{ae ae}\) radical pair polarization pattern is observed in the triplet state EPR spectrum.

The radical pair mechanism explains the observation in the photosynthetic bacteria of the triplet state, \(P_{-860}\), which has the radical pair polarization pattern, \(3, 25, 25\). Also, the radical pair mechanism is known to be operating in photosystem I from recent studies of chemically induced dynamic electron polarization observed in green plant preparations (26–28), which were interpreted by Frieser et al. (29) to arise from a dynamic interaction between \(P_{-700}\), \(A^-\), and \(X\). We believe that triplet I, whose spectrum is shown in Figs. 2A and 3A, is the photosystem I analog of the bacterial \(P_{-860}\) state.

The zero-field splitting parameters for triplet I (see Table 1) are precisely those observed for monomeric chlorophyll a (25, 30). The bacterial \(P_{-860}\) state zero-field splitting parameters are about 20% smaller than the corresponding monomeric bacteriochlorophyll a values measured in vitro (1, 25). This is consistent with the idea that the triplet state, \(P_{-860}\), is delocalized over more than one molecule—i.e., the bacterial reaction center primary donor, \(P_{-860}\), is a bacteriochlorophyll dimer (1, 25).
In view of the fact that P-700 is thought to be a chlorophyll a dimer (1, 31), the observation of monomeric chlorophyll a $|D\rangle$ and $|E\rangle$ values for triplet I is puzzling.

If triplet I is not localized on P-700, it could be centered on a chlorophyll a monomer closely associated with the reaction center and on which the charge recombination reaction energy is trapped. Such a process would have to occur coherently so that the spin polarization is preserved. Alternatively, triplet I may remain on the acceptor A after charge recombination between P-700$^+$ and A$^-$ takes place. It has been suggested that A is a chlorophyll species (29). We are not aware, however, of any precedent for this kind of event.

If triplet I is localized on P-700, then we must explain the fact that the zero-field splitting parameters correspond to monomeric chlorophyll a values. Clarke et al. (32, 33) have proposed a simple exciton model which they use to calculate the angle between the chlorophyll planes of the bacterial dimer, P-860, based on a comparison between monomeric bacteriochlorophyll zero-field splittings measured in vitro and those obtained for several species in vivo. Following this reasoning, our results could be explained by a plane-parallel P-700 dimer structure where the monomeric magnetic axes are all parallel. This possibility was suggested as the model for P-700 by Fong (34). However, this interpretation is not fully consistent with the P-700 circular dichroism spectrum obtained by Philipson et al. (35). Further experimental and theoretical investigations will be needed to corroborate structural models based on observed triplet state parameters with those obtained from optical measurements.

The signal at $g = 1.89$ in Fig. 1B indicates that Fd$A$ and Fd$B$ are reduced (24), but the absence of a signal at $g = 1.78$ shows that X is not reduced when samples are frozen dark in the presence of dithionite. Subsequent illumination at liquid helium temperatures of samples in this redox state causes the rapid transfer of an electron from P-700 to X. Because the donor system to P-700$^+$ does not function at low temperatures (20) and the charge recombination time between P-700$^+$ and X$^+$ is 130 msec (16), illumination produces the redox state P-700$^+$ A X$^-$$\rightarrow$ Fd$B$ Fd$A$ during light modulation experiments.

The eae aea polarization pattern seen in triplet II is one among many patterns that can arise from a molecular inter-system crossing mechanism populating the lowest triplet state of an aromatic molecule. Such a mechanism has been studied in great detail for aromatic hydrocarbons (36–38) and chlorophylls (23, 39, 40). The eae aea polarization pattern indicates that the most populated triplet spin sublevel is the middle-energy zero-field level (39). The population is driven into this level as the result of spin-orbit and vibronic coupling between the singlet and triplet manifolds of states of an isolated molecule (41, 42). In contrast to the radical pair mechanism, no charge transfer-recombination process need be operating for the triplet to be formed. Consequently, spin polarization patterns distinct from the radical pair mechanism pattern are observed.

Thus, we believe that triplet II forms while the photosystem I reaction center is in the charge separated P-700$^+$ A X$^-$$\rightarrow$ Fd$B$ Fd$A$ state. Because the back reaction time between X$^-$ and P-700$^+$ is sufficiently slow (see above), the P-700 trap remains closed to excitation during the light-modulation time period. The excess excitation energy incident on the sample is funnelled into a different trap where molecular inter-system crossing takes place to form triplet II.

The zero-field splitting parameters of triplet II (see Table 1) are substantially larger than those of either chlorophyll or phoephophytin monomers (25, 43). Similar triplet state parameters were obtained by Hoff et al. (9), using optical detection of magnetic resonance techniques on reduced chloroplasts or digitonin particles prepared without intense illumination while freezing (our condition 2). They suggested that the observed signals arose from a non-chlorophyll species, possibly phoephophytin. We feel that it is also possible that the signals arise from carotenoids. Numerous optical experiments on green plants and bacteria have revealed that carotenoid triplet states serve as sinks for excess energy (44–48). However, triplet state EPR spectra of these systems are sparse, presumably due to the difficulty of photoexciting carotenoids directly into their triplet states (49). Chlorophyll sensitization greatly enhances the carotenoid triplet population (49).

Our studies of chloroplasts and digitonin photosystem I particles treated with ferricyanide confirm the hypothesis that triplet II appears when the P-700 traps are closed. In the presence of ferricyanide we have the redox state P-700$^+$ A X Fd$B$ Fd$A$ in the dark. One can see from Figs. 2 B and C and 3 B and C that the same triplet state spectrum, triplet II, arises either when all P-700 traps are closed by chemical oxidation (condition 3) or when X is not reduced (condition 2). This result provides evidence that triplet II is not localized on one of the main components active in the primary charge transfer events of photosystem I, but serves as an energy sink for excess excitation that never reaches P-700.

Our observation of the radical pair polarization of triplet I is consistent with the observation of a radical pair mechanism polarizing the EPR signal from P-700$^+$ and giving rise to chemically induced dynamic electron polarization during normal forward photochemistry at room temperature in chloroplasts (27–29). Furthermore, the observation of triplet I provides independent corroborations that an electron carrier A mediates in the charge transfer from P-700 to X (16, 17).

Our conclusion that triplets I and II are associated with P-700 in the photosystem I reaction center is supported by (i) the light-indcued appearance of triplet I only when X is reduced prior to the EPR experiment; (ii) the disappearance of triplet I and the appearance of triplet II when P-700 is oxidized; and (iii) the observation of these events in a photosystem I–enriched digitonin fraction.

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