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A LIGHT-INDUCED SPIN-POLARIZED TRIPLET DETECTED BY EPR IN PHOTOSYSTEM II REACTION CENTERS

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Summary

A light-induced spin-polarized triplet state has been detected in a purified Photosystem II preparation by electron paramagnetic resonance spectroscopy at liquid helium temperature.

The electron spin polarization pattern is interpreted to indicate that the triplet originates from radical pair recombination between the oxidized primary donor chlorophyll, P-680$^+$, and the reduced intermediate pheophytin, I$^-$, as has been previously demonstrated in bacterial reaction centers. The dependence of the triplet signal on the redox state of I and the primary acceptor, Q, are consistent with the origin of the triplet signal from the triplet state of P-680.

Redox-poising experiments indicate the presence of an endogenous donor (or donors) which operates at 3—5 K and 200 K.

The zero field-splitting parameters of the triplet are very similar to those of monomeric chlorophyll *a*, however, this alone does not allow a distinction to be made between monomeric and dimeric structures for P-680.

Introduction

Primary photochemistry in Photosystem II (PS II) involves the light-driven transfer of an electron from the primary donor (P-680), a chlorophyll species [1], to the primary acceptor (Q), a special plastoquinone [2]. Recent studies suggest the involvement of a pheophytin molecule as an intermediate (I) in this process [3] as has been well established for the primary events in the bacterial

Abbreviations: BChl, bacteriochlorophyll; PS, photosystem; BPh, bacteriopheophytin; DCIP, dichlorophenolindophenol; Tricine, *N*-tris(hydroxymethyl)methylglycine; Chl, chlorophyll.

reaction center (for a review see Ref. 4). These reactions are similar to those which occur in the better-characterized purple photosynthetic bacterial reaction center [4].

In bacteria, EPR studies of signals attributed to a spin-polarized triplet state of the primary donor ($BChl_2$) [5] and to the reduced bacteriopheophytin intermediate (BPh) [6,7] have significantly contributed to the characterization of bacterial photochemistry. The triplet state of $BChl_2$ is formed when samples, in which the quinone-iron primary acceptor (QFe) is reduced, are illuminated at cryogenic temperatures [5]. Under these conditions charge separation occurs with the formation of the radical pair, $BChl_2^+$ BPh^- [8]. The short-lived radical pair decays via a spin-polarized triplet state of the primary donor which is relatively long lived and exhibits a characteristic EPR signal at liquid helium temperature.

$$\rightarrow BChl_2\ BPh\ Q^-Fe \xrightarrow{h\nu} \underbrace{BChl_2^+\ BPh^-\ Q^-Fe}_{\text{(radical pair)}} \rightarrow \underbrace{BChl_2^{\frac{1}{2}}\ BPh\ Q^-Fe}_{\text{(triplet)}}$$

The spin polarization of this triplet is quite distinct from that seen in triplets generated by excited state intersystem crossing and is characteristic of a chemical (i.e. radical pair) origin [8]. This triplet signal has been used to provide information concerning the structure and geometry of the primary donor [8—10].

An EPR signal from BPh^- has been observed in samples illuminated under strongly reducing conditions in the presence of efficient exogenous or endogenous electron donor to $BChl_2^+$ [6,7,11—13]. The trapped BPh^- frequently exhibits a split doublet EPR signal. The formation of the splitting is attributed to an interaction between BPh^- and the semiquinone-iron form of QFe [7,13].

With BPh trapped in its reduced state, the triplet is no longer observed under illumination [7,14]. This is strong evidence for formation of the triplet by recombination of $BChl_2^+$ BPh^-.

Recently a doublet signal, similar to that exhibited by BPh^- in bacteria, has been observed in PS II particles and has been attributed to I^- [19]. In this work we report the presence of a light-induced spin-polarized triplet in a purified PS II preparation. The I^- doublet signal can also be observed in this preparation. The interrelation between these signals has been used, along with redox-poising data, to identify the triplet as that of P-680 and to provide information on the mechanism of PS II photochemistry.

Materials and Methods

Chloroplasts were isolated as previously described [20] with the addition of 10 mM $MgCl_2$ in the grinding medium. Non-oxygen-evolving PS II particles were prepared by the isolation procedure described in Ref. 21. Chlorophyll determinations were done according to Ref. 22. P-700 content in the PS II preparation was one P-700 per 2500 chlorophylls as determined from oxidized minus reduced difference spectra [23]. PS II-mediated photoreduction of 0.02 mM dichlorophenolindophenol (DCIP) was measured in the presence of 100 μM diphenylcarbazide using a Hitachi spectrophotometer modified for side

illumination [20] (350 μmol DCIP reduced/mg Chl per h). PS II particles were concentrated for EPR studies by centrifugation at 150 000 × *g* for 1 h. PS II particles were resuspended in 20 mM Tricine/NaOH, pH 7.8, to a concentration of 1—2 mg Chl/ml for all experiments.

Oxidation-reduction potentiometry was carried out by the method described in Ref. 24. The following mediators were used (100 μM), 1,2-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, 1,4-naphthoquinone, duroquinone, anthraquinone-2-sulfonate, anthraquinone-1,5-disulfonate, methyl viologen and benzyl viologen.

The viologen dyes were omitted from the samples shown in Figs. 2 and 3 because of the large free radical signals which these dyes exhibit. The extent of the triplet signals in these samples (Figs. 2 and 3) was essentially the same as those poised at similar potentials in the presence of these mediators.

Cytochrome *b*-559 was assayed by spectrophotometry at room temperature and liquid nitrogen temperature using an Aminco-Chance DW2 spectrophotometer. EPR measurements were performed on a Varian E9 spectrometer with 100 kHz field modulation at liquid helium temperatures using an Oxford instruments cryostat and temperature-monitoring system. Illumination in the cryostat, at 200 K and at room temperature was provided by a 300 W projector.

Results and Discussion

Fig. 1 shows an EPR signal which is reversibly photoinduced at liquid helium temperature in a sample of PS II particles poised at −7 mV. The shape is remarkably similar to the spin-polarized triplet state of the primary donor in purple photosynthetic bacteria [5]. The signal consists both of absorption (A) and emission (E) peaks and has the same unusual polarization pattern as reported for the bacterial triplet [5], i.e., AEEAAE. This polarization pattern is indicative of the triplet's formation from a radical pair [8].

The large radical signal at a *g* value of approx. 2 present in Fig. 1 is partly due to an irreversible light-induced radical which is attributed to an oxidized donor functional at low temperature (see below for a discussion of this). A small unidentified radical signal is also present in the dark in all samples.

The amplitude of the triplet signal is maximized under conditions of low temperature (less than 6 K) and low power (less than 1 mW) and the signal is not detectable at power settings greater than 5 mW. A similar power dependency of the triplet in photosynthetic bacteria has been shown to be due to an increase in the decay rate of the triplet signal resulting from stimulated emission and absorption caused by the monitoring microwave field [25].

The ability to produce the triplet in the light should be related to the redox state of the primary reactants. Investigations of the conditions necessary for triplet formation have been carried out. Figs. 2A and 3A show that under conditions where I is trapped in the reduced state (i.e., by illumination while freezing or by illumination at 200 K [19]) the triplet signal is greatly diminished. This would be expected if the triplet is formed from the radical pair, $P\text{-}680^{+}I^{-}$, since charge separation is prevented by prereduction of I.

Fig. 2B and C show the changes occurring in the *g* 2 region after illumination while freezing. It can be seen that under conditions of low temperature and

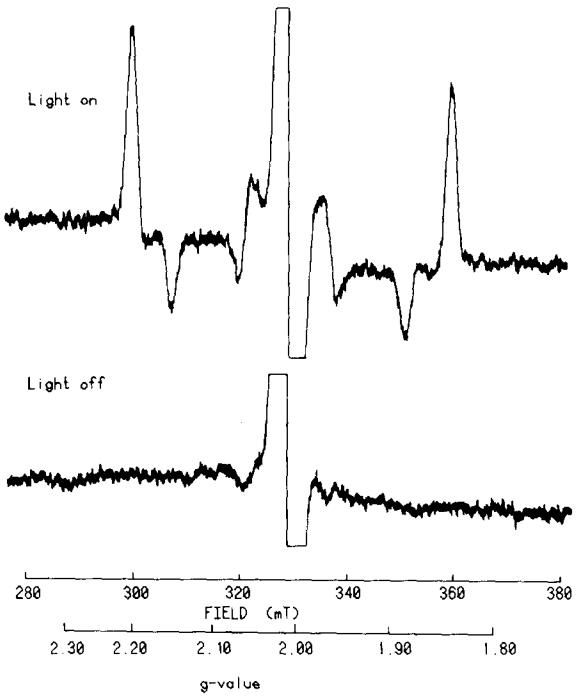


Fig. 1. The effect of illumination at 3—5 K on the EPR spectrum of PS II particles. The sample was poised at an E_h of —7 mV. EPR conditions were as follows: modulation amplitude 1.0 mT, microwave power 10 μW, frequency 9.26 GHz, instrument gain $2 \cdot 10^4$.

high power (Fig. 2B) a signal, split by approx. 5.5 mT, is present. This signal is attributed to reduced I, a pheophytin which is thought to be interacting with the semiquinone-iron primary acceptor. A similar signal has been reported in PS II particles (TSF-2a) prepared from spinach [19]. Fig. 2C shows that the split signal is replaced by a singlet close to g 2.00 under conditions of higher temperature and lower power.

Illumination at 200 K (Fig. 3 B and C) induces larger changes in the g 2 region (note the smaller instrument gain) and the split signal cannot be observed. It is possible that 200 K illumination photoinduces a signal from an oxidized donor which swamps out and/or interacts with the split signal. This oxidized donor is absent in the sample frozen under illumination either because a different donor is functional under these conditions or more likely because it is quickly rereduced by dithionite.

These results are in contrast to those reported earlier in which the split signal was observed after 200 K illumination but not after illumination at room temperature [19]. This discrepancy may be due to differences in the endogenous donors found in the two PS II preparations.

In Figs. 2 and 3 it is also demonstrated that the trapping of I^- is reversed by thawing, dark adaptation at room temperature and refreezing in the dark. The reactions occurring in Figs. 2 and 3 may be summarized by the following scheme:

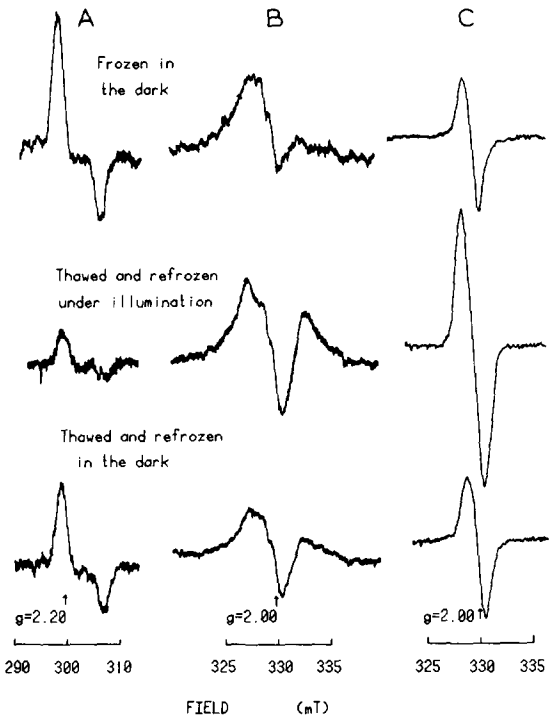
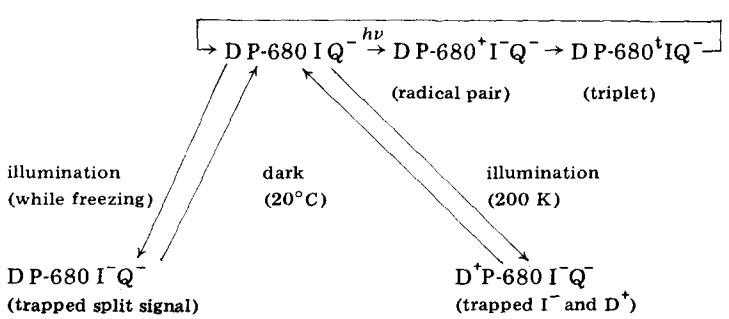


Fig. 2. The effect of illumination at room temperature upon the triplet and radical EPR signals. The sample was poised at −430 mV. The same sample was used for all the spectra shown. Thawing and refreezing of the sample were carried out under anaerobic conditions. Illumination at room temperature was for 30 s before the sample was frozen, still under illumination. Dark adaptation at room temperature was allowed for appox. 2 min. (A) the g 2.2 feature of the light-induced triplet spectrum recorded under illumination after each treatment. EPR conditions were as in Fig. 1. (B) the changes that occur in the radical region after each treatment with EPR conditions as follows, temperature 3.5 K, modulation amplitude 0.2 mT, microwave power 100 mW, frequency 9.26 GHz, instrument gain $2 \cdot 10^3$. (C) Same as (B) except the temperature was 16 K and the microwave power was 1 mW.



The operation of the donor, D, is analogous to the donor in some photosynthetic bacteria (i.e. *Chromatium vinosum*). During illumination at liquid helium temperature the donation from D to P-680$^+$ does not occur since the reaction is very slow in comparison to the decay of the radical pair [7,15]. At 20°C and

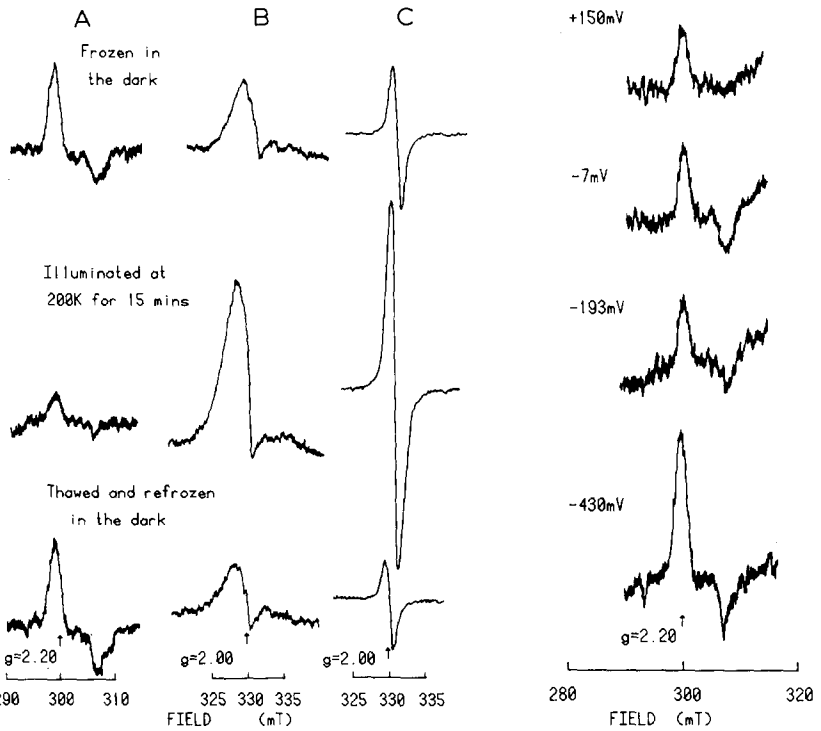


Fig. 3. Effect of 200 K illumination upon the triplet and radical EPR signals. The sample was poised at −430 mV. 200 K illumination was performed in an ethanol/solid CO_2 bath in an unsilvered dewar for 15 min. Thawing and refreezing were carried out as in Fig. 2 and EPR conditions were as in Fig. 2 except (B) and (C) were at a gain of $1.3 \cdot 10^3$.

Fig. 4. Effect of redox poising at different values of E_h upon the extent of the triplet signal. The figure shows the g 2.2 feature of the light-induced triplet in samples poised at the E_h values shown. EPR conditions were as in Fig. 1.

200 K donation from D to P-680$^+$ occurs and competes with the back reaction thus trapping I^-.

Fig. 4 shows the result of preliminary redox-poising experiments. It can be seen that the triplet signal is present in samples in which the primary acceptor, Q, is oxidized in the dark (i.e., +150 mV and −7 mV). This indicates that a secondary donor, D, is functioning under these conditions. If no donor were present no triplet would be formed at potentials where Q is oxidized before illumination.

$$\mathrm{D\,P\text{-}680\,I\,Q} \xrightarrow{h\nu} \mathrm{D^+P\text{-}680\,I\,Q^-} \xrightarrow{h\nu} \mathrm{D^+P\text{-}680^+I^-Q^-} \rightarrow \mathrm{D^+P\text{-}680^tI\,Q^-}$$

It is unlikely that D is cytochrome *b*-559 since the donor operating at low temperature is functional at +150 mV, an E_h where cytochrome *b*-559, which is in its low-potential form in this preparation (not shown), is oxidized. Also no optical or EPR changes [26,27] attributable to cytochrome *b*-559 photooxidation at low temperature could be observed in this preparation. Under redox conditions where D would be expected to operate, an irreversible signal

at g 2 is photoinduced which is tentatively attributed to D^+ (not shown). Since subtraction facilities were not available it is not possible to determine whether the donor, D, operating at 5 K is the same as that operating at 200 K.

Redox titrations of the primary acceptor in the PS II preparation obtained by measuring changes in fluorescence yield (Rutherford, A.W., Paterson, D.R. and Mullet, J.E., unpublished results) gave results very similar to those obtained in chloroplasts [28]. The redox titration curve revealed two steps ($E_{m(7.8)}$ values of approx. −40 mV and −275 mV) which have been previously assigned to two populations of PS II centers differing with respect to the midpoint potentials of Q [29]. In Fig. 4 the increase in the extent of the triplet occurring at −430 mV probably reflects the reduction of PS II particles which contain the low-potential Q.

Recently a light-induced spin-polarized triplet state of P-700, the primary donor of Photosystem I (PS I) has been observed by EPR in chloroplasts and PS I particles in which the primary acceptor, X, is reduced [30]. This signal has the same electron spin polarization pattern and zero field-splitting parameters (see below) as the signal reported here (Table I). Several lines of evidence allow the PS II triplet to be distinguished from that of PS I. Firstly the PS I triplet is small and it is unlikely that the PS I contamination in the PS II preparation (i.e., approx. one P-700 per 2500 Chl) could account for the signal observed here. Secondly the conditions which are necessary for the formation of the PS I triplet (i.e., photochemical reduction of X by illuminating while freezing) actually result in the disappearance of the triplet in the PS II preparation. The disappearance of the triplet in PS II is matched by the appearance of the I^- doublet. This correlation is taken as evidence that the triplet originates from the decay of the $P\text{-}680^+I^-$ radical pair. Thirdly, the disappearance of the triplet during 200 K illumination, conditions which are known to trap I^- in the reduced state in PS II [19], would not be expected, if the triplet observed were associated with the PS I reaction center since no secondary donors are known to function in PS I at this temperature. Fourthly, the redox-poising experiments (Fig. 4) show the presence of the triplet in PS II at all values of E_h tested. Any PS I present in the preparation would not have X in the reduced form and light induction of the triplet would not occur. It is conceivable that PS I reaction centers without the primary acceptor and bound iron sulfur centers (like those obtained by SDS treatment [31]) would show a reaction center triplet

TABLE I

A COMPARISON OF THE ZERO-FIELD-SPLITTING PARAMETERS OF THE P-680 TRIPLET WITH THOSE OF P-700, $BChl_2$, MONOMERIC Chl AND BChl

Source of triplet	$\|D\|$ (cm^{-1})	$\|E\|$ (cm^{-1})	Polarization pattern	
P-680	0.0290	0.0040	AEE	AAE
P-700 *	0.0278 ± 0.0009	0.0039 ± 0.0009	AEE	AAE
$BChl_2$ **	0.0187	0.0034	AEE	AAE
Monomeric Chl *a* **	0.0275	0.0036	EEE	AAA
Monomeric BChl *a* **	0.0221	0.0053	EEE	AAA

* Values from Ref. 28.
** Values from Ref. 8.

under these redox conditions. However, in such particles no redox potential dependence, like that shown in Fig. 4, would be expected.

In conclusion, due to the conditions under which the triplet signal is observed, the triplet signal's interrelationship with the split I^- signal and the results of preliminary redox-poising experiments which indicate an increase in the extent of the triplet as Q goes reduced, the triplet signal reported here is ascribed to a triplet state generated by radical pair recombination in the reaction center of PS II. It seems likely that the triplet state itself is localized on P-680, however, other possibilities do exist. It has been suggested (Wraight, C.A., personal communication) that the triplet state, having been formed by radical pair recombination, could migrate out of the reaction center and onto an antenna pigment. Under these circumstances the unusual electron spin polarization pattern would be conserved (Norris, J.R., personal communication). However, such a migration process would not be expected for two reasons: firstly, the transfer of energy from the reaction center to the antenna would be an energetically up-hill process, since the reaction center is accepted to be long-wavelength energy sink and secondly, in photosynthetic bacteria, migration of the reaction center triplet to the antenna does not occur.

Little information is available concerning the structure of P-680. The narrow linewidth of the EPR signal ascribed to $P\text{-}680^+$ has been taken as an indication that P-680 was a dimer [32,33] or a trimer of chlorophyll [33]. However, recent in vitro work has shown that liganded monomeric chlorophyll can exhibit EPR signals with unusually narrow linewidths and that this is dependent upon environmental factors [34]. From this evidence, together with in vitro optical and redox data it was suggested that P-680 was a monomer [34].

In bacteria the zero field-splitting parameters measured from the triplet EPR spectra when compared to in vitro monomeric bacteriochlorophyll provided evidence that the primary donor was a special pair of bacterial chlorophyll molecules [8,16]. Measurement of the zero field-splitting parameters in the P-680 triplet and comparison with the zero field-splitting parameters reported for monomeric chlorophyll should provide similar evidence concerning the structure of P-680. Table I shows that the zero field-splitting parameters for P-680 are very similar to those reported for monomeric chlorophyll. This in vivo evidence is consistent with the assignment of P-680 as a monomeric chlorophyll. However, the similarity of the $|D|$ zero-field-splitting parameter to that of monomeric chlorophyll cannot alone be used as evidence that P-680 is monomeric since a $|D|$ zero-field-splitting parameter similar to that of the monomer, could be obtained from a special pair if the two molecules were oriented in a particular way (i.e., if all the axes of the two molecules of the special pair are parallel) [35].

Conclusion

The acceptor side of PS II bears a remarkable resemblance to that of purple photosynthetic bacteria. The primary acceptor in both cases is a specialized quinone molecule while the intermediate is a pheophytin. The split I^- signal reported here and earlier [19] suggests that the primary acceptor is associated with a ferrous iron atom in a manner analogous to that in bacteria. The characteristics of the spin-polarized triplet reported here further extend this analogy.

The polarization pattern of the triplet and the dependence on the redox state of I/I^- of its formation strongly support a radical pair configuration for the early charge transfer states similar to that observed in bacteria.

Although the evidence from the $|D|$ zero-field-splitting parameter does not alone provide evidence for the assignment of P-680 as a monomer, it does agree with the previous suggestion of this possibility [32] and it does indicate that P-680 is different from the bacterial primary donor. Differences between the donor side of PS II and that of bacteria are not unexpected since P-680 has to provide the oxidizing power to extract electrons from water (greater than 900 mV) and thus must have an E_m approx. 500 mV more oxidizing than that of the primary donor of photosynthetic bacteria.

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