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THE DEPENDENCE OF REACTION CENTER AND ANTENNA TRIPLETS ON THE REDOX STATE OF PHOTOSYSTEM I

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The formation of chlorophyll triplet states during illumination of Photosystem I reaction center samples depends upon the redox state of *P*-700, X and ferredoxin Centers A and B. When the reaction centers are in the states $P\text{-}700^{+}A_1XFd_BFd_A^{-}$ and *P*-700 $A_1XFd_B^{-}Fd_A^{-}$ prior to illumination, we observe electron paramagnetic resonance (EPR) spectra from a triplet species which has zero-field splitting parameters ($|D|$ and $|E|$) larger than those of either the chlorophyll *a* or chlorophyll *b* monomer triplet, and a polarization which results from population of the triplet spin sublevels by an intersystem crossing mechanism. We interpret this triplet as arising from photo-excited chlorophyll antenna species associated with reaction centers in the states $P\text{-}700^{+}Fd_A^{-}$ and $P\text{-}700^{+}X^{-}$, respectively, which undergo de-excitation via intersystem crossing. When the reaction centers are in the states $P\text{-}700A_1XFd_B^{-}Fd_A^{-}$ and $P\text{-}700A_1X^{-}Fd_B^{-}Fd_A^{-}$ prior to illumination, we observe a triplet EPR signal with a polarization which results from population of the triplet spin sublevels by radical pair recombination, and which has a $|D|$ value similar to that of chlorophyll *a* monomer. We interpret this triplet (the radical pair-polarized triplet) as arising from $^3P\text{-}700$ which has been populated by the process $P\text{-}700^{+}A_1^{-} \rightarrow {}^3P\text{-}700A_1$. We observe both the radical pair-polarized triplet and the chlorophyll antenna triplet when the reaction centers are in the state *P*-700 $A_1XFd_B^{-}Fd_A^{-}$, presumably because the processes $P\text{-}700^{+}A_1^{-}X \rightarrow P\text{-}700^{+}A_1X^{-}$ and $P\text{-}700^{+}A_1^{-}X \rightarrow {}^3P\text{-}700\ A_1X$ have similar rate constants when Centers A and B are reduced, i.e., the forward electron transfer time from A_1^{-} to X is apparently much slower in the redox state *P*-700 $A_1XFd_B^{-}Fd_A^{-}$ than it is in state *P*-700 $A_1XFd_BFd_A$. The amplitude of the radical pair-polarized triplet EPR signal does not decrease in the presence of a 13.5-G-wide EPR signal centered at *g* 2.0 which was recorded in the dark prior to triplet measurements in samples previously frozen under intense illumination. This *g* 2.0 signal, which has been attributed to phototrapped A_1^{-} (Heathcote, P., Timofeev, K.N. and Evans, M.C.W. (1979) FEBS Lett. 101, 105–109), corresponds to as many as 12 spins per *P*-700 and can be photogenerated during freezing without causing any apparent attenuation of the radical pair-polarized triplet amplitude. We conclude that species other than A_1^{-} contribute to the *g* 2.0 signal.

Introduction

The transfer of electrons from *P*-700 to iron-sulfur Centers A and B during primary photochemistry in Photosystem I appears to involve two intermediate acceptors. MacIntosh et al. [1] and Evans et al. [2] observed EPR signals at liquid helium temperatures from photooxidized *P*-700 and a photoreduced acceptor, denoted X, during continuous illumination of samples in which Centers A and B had been chemically reduced prior to illumination. Evidence that X is not the only acceptor was obtained from studies of the kinetics of optical absorption changes by Sauer et al. [3], and of optical and EPR changes by Shuvalov et al. [4]. In both cases it was observed that photo-induced charge separation decays biphasically below

Abbreviations: PMS, phenazine methosulfate; Fd, ferredoxin.

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100 K in samples in which Centers A and B are reduced prior to measurement. The slower phase, which Shuvalov et al. [4] determined to have a halftime of 120 ms at 5 K, was attributed to the charge recombination between *P*-700 and X. The faster phase has a halftime of about 1 ms at 5 K and was attributed to charge recombination between *P*-700 and an additional acceptor, denoted A_1. The simplest interpretation of these data is that when X is not reduced prior to measurement, one observes the process

$$P\text{-}700\ A_1X \xrightarrow{h\nu} P\text{-}700^+A_1^-X \rightarrow P\text{-}700^+A_1X^- \quad (1)$$

$t_{1/2}$ = 120 ms at 5 K

whereas when X is reduced prior to illumination, one observes the process

$$P\text{-}700\ A_1X^- \xrightarrow{h\nu} P\text{-}700^+A_1^-X^- \quad (2)$$

$t_{1/2}$ = 1 ms at 5 K

However, this hypothesis now appears to be questionable, partly because the mechanism of recovery by process 2 to the ground state *P*-700 A_1X^- has been shown to involve more than a single step. Frank et al. [5] reported evidence that the molecular triplet state of *P*-700 functions as an intermediate in the $P\text{-}700^+A_1^-$ back-reaction. They observed an EPR triplet from *P*-700, the polarization of which indicates that it has been populated during primary photochemistry from a radical pair precursor [5]. In addition, the kinetic data are suggestive that photoreduced A_1^- does not always transfer its electron to X when X is not reduced prior to photoexcitation. Sauer et al. [3] reported that the 1 ms phase was observed regardless of whether X was reduced prior to measurement, and Shuvalov et al. [4] observed the 1 ms phase in samples poised at −625 mV, a potential at which X is not reduced prior to measurement. Thus, under the conditions of these measurements, primary photochemistry would appear to be more complicated than the simple serial transfer of electrons from *P*-700 to A_1 and then to X depicted in process 1.

With these mechanistic questions in mind, we studied the relation between the state of reduction of Photosystem I acceptors and the amplitude of the radical pair-polarized EPR triplet signal from *P*-700. For these studies we used Photosystem I preparations isolated using Triton X-100 detergent. In these preparations we do not observe a triplet that is seen in chloroplasts and in digitonin Photosystem I subchloroplasts [5] and which Frank et al. [6] determined to arise from a carotenoid species. However, in addition to the radical pair-polarized triplet of *P*-700, in the Triton preparations we now observe an additional triplet species which appears to become populated when *P*-700 is oxidized prior to illumination; thus, it also depends on the redox state of Photosystem I. We observe the radical pair-polarized triplet in samples in which X was not reduced prior to illumination. We believe that it arises when A_1^- undergoes radical pair recombination with $P\text{-}700^+$ in preference to forward electron transfer to X. Our results therefore suggest that the processes

$$P\text{-}700^+A_1^-X \rightarrow P\text{-}700^+A_1X^-$$

and

$$P\text{-}700^+A_1^-X \rightarrow {}^3P\text{-}700\ A_1X$$

are in competition when Centers A and B are reduced prior to triplet measurements. We observe no correlation between the amplitude of the radical pair-polarized triplet and the amplitude of a *g* 2.0 signal present in the dark in samples which have been frozen under illumination prior to triplet measurements. This *g* 2.0 signal was attributed by Heathcote et al. [7] to A_1 which has been phototrapped in its reduced state. Our results suggest that the species giving rise to the *g* 2.0 signal is not the primary electron acceptor in Photosystem I.

Materials and Methods

Triton X-100 Photosystem I fraction

A subchloroplast Photosystem I fraction was isolated from spinach chloroplasts using Triton X-100 detergent in the presence of mono- or divalent cations as follows. Chloroplasts were isolated from market spinach in 0.4 M sucrose/0.1 M NaCl/0.05 M Tris (pH 8) buffer and centrifuged at 7000 × *g* for 5 min. The pellets were washed in 0.7 mM EDTA to remove any Mn^{2+} EPR signal and centrifuged for 10 min at 15 000 × *g*. The pellets were then resuspended to a chlorophyll concentration of 0.3–0.5 mg/ml in 0.05 M Tris (pH 8)/0.1 M NaCl or 0.01 M $MgSO_4$ and allowed to incubate 1 h at 4°C to assure clean frac-

tionation of Photosystem I from Photosystem II [8]. Triton X-100 was stirred into the chloroplast suspension to give 1% detergent, and the mixture was incubated in the dark at 4°C for 2–24 h. The supernatant of a 30 min spin at 30 000 × *g* contained Photosystem I complexes with the characteristics: chlorophyll *a*/chlorophyll *b* = 5–12, chlorophyll/*P*-700 = 175–200. This Photosystem I fraction was concentrated by dialysis against crystalline sucrose before it was used in EPR experiments or subjected to further purification.

Triton X-100 Photosystem I reaction centers

Photosystem I reaction centers were isolated from the Photosystem I fraction by the procedure of Golbeck et al. [9]. Concentrated Photosystem I fraction (25 ml) put through a 500 ml Sephacryl S-300 column yielded reaction centers with the characteristics chlorophyll *a*/chlorophyll *b* > 9, chlorophyll/*P*-700 = 35–65, that eluted with the column void volume. Photosystem I reaction centers were concentrated for EPR studies by dialysis against crystalline sucrose.

Assays

Chlorophyll was assayed by the method of Arnon [10]. *P*-700 was assayed by the ferricyanide-ascorbate absorbance difference at around 700 nm, assuming Ke's differential extinction coefficient of 64 $mM^{-1} \cdot cm^{-1}$ [11]. Carotenoids were assayed qualitatively by thin-layer chromatography as described by Barr and Crane [12].

EPR sample preparation

Samples of the Photosystem I fraction or Photosystem I reaction centers were prepared for EPR studies as previously described [5] and had final chlorophyll concentrations of about 0.5 or 0.1 mg/ml, respectively. The samples contained 1 mM phenazine methosulfate (PMS) or neutral red, as noted below. Samples which were illuminated before and during freezing were cooled to 156 K using a Varian liquid nitrogen temperature controller with a reproducible cooling cycle. Cooling from 273 to 156 K took about 30 s. Samples were illuminated with a Cary High Intensity tungsten lamp using intensities at or below 1000 $W \cdot m^{-2}$. The EPR samples were placed in a cold finger dewar located 40 cm from the lamp, and we did not place any lenses between the sample and the lamp. Photodamage was avoided by keeping the sample temperature below 0°C during illumination and by filtering the light beam through 20 cm water. We found that at room temperature up to 100% of the chlorophyll was irreversibly photobleached within 60 s of illumination by an unfiltered 1000 $W \cdot m^{-2}$ beam from a tungsten lamp.

EPR measurements

EPR measurements were made using a Varian E-109 spectrometer operating at X band. All measurements utilized 100 kHz field modulation and a TE-102 cavity that was fitted with an optical transmission flange. Light modulation experiments were performed at 33 Hz, as described previously [5] except that a Princeton Applied Research Model HR-8 lock-in amplifier was used for phase-sensitive detection. The light source was a Cary High Intensity tungsten lamp focused onto the optical flange to give 5000 $W \cdot m^{-2}$. A Bell Model 811 A digital gaussmeter was used to calibrate the magnetic field. An Air Products Helitran cryostat was used for liquid-helium temperature EPR measurements.

Results

The EPR spectra of Figs. 1–3 were obtained from four identical aliquots of Photosystem I reaction centers which were treated so as to produce four different redox states of the Photosystem I reaction center, as follows:

Sample (a): frozen in the dark and then illuminated at 10 K.

Sample (b): Frozen in the dark in the presence of dithionite and PMS.

Sample (c): same as sample b, but illuminated 60 s at 0°C and then frozen under illumination at low (50 $W \cdot m^{-2}$) light intensity.

Sample (d): same as sample c, but with higher (310 $W \cdot m^{-2}$) light intensity.

Figs. 1 and 2 show the EPR spectra recorded in the dark at 12 K of samples a–d. Illumination of sample a at 10 K resulted in the irreversible photooxidation of *P*-700 (Fig. 2a) and photoreduction of Center A (Fig. 1a), as reported by Bearden and Malkin [13]. In sample b, ferredoxin centers A and B are reduced by dithionite, but X is not (Fig. 1b), and there is not much signal in the *g* 2.0 region present in the dark (Fig. 2b). In sample c, component X has been phototrapped in its reduced state (Fig. 1c), and there is a

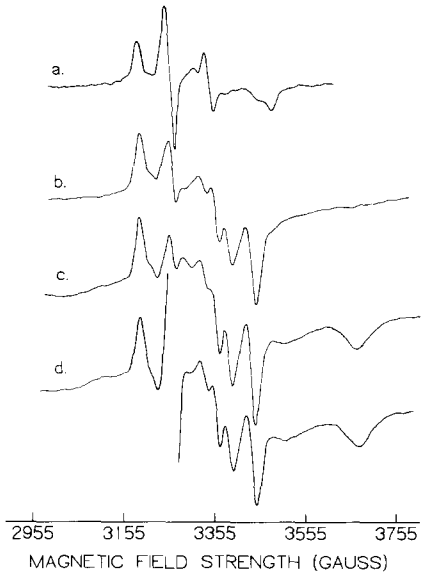

Fig. 1. EPR spectra of samples a–d recorded in the dark at 10 K. Photosystem I reaction centers were: (a) frozen in the dark in glycine buffer at pH 10 and then illuminated at 10 K; (b) frozen in the dark in the presence of 25 mM dithionite and 1 mM phenazine methosulfate at pH 10. (c) Sample is identical to sample b which was frozen under low intensity illumination. (d) Sample identical to sample b which was frozen under higher intensity of illumination. EPR spectrometer conditions: gain, 12 500 (Fig. 1a) and 10 000 (Fig. 1b–d); modulation amplitude, 20 G; microwave power, 20 mW; microwave frequency, 9.16 GHz; scan rate, 250 G · min^{-1}.

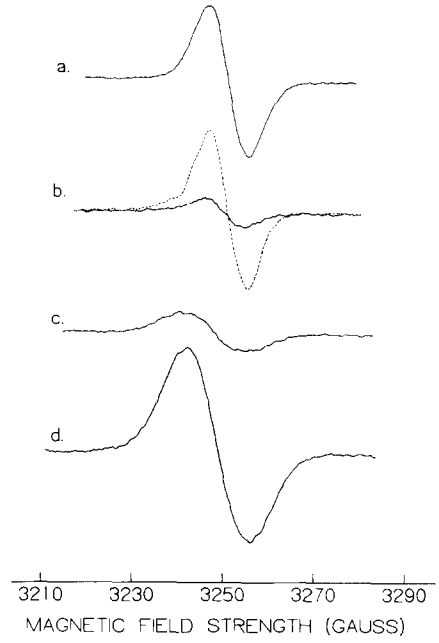

Fig. 2. EPR spectra in the g 2.0 region of samples a–d, which are as described in Fig. 1. (a) Spectrum recorded during illumination of sample a; (b, solid line), spectrum of sample b recorded in the dark before and after illumination; b, dotted line, spectrum of sample b recorded during illumination; (c and d), spectra of samples c and d, respectively, recorded in the dark. All spectra at 10 K. Spectrometer conditions: gain, 20 000; modulation amplitude, 4 G; microwave power, 10 μW; microwave frequency, 9.16 GHz; scan rate, 25 G · min^{-1}.

small signal centered at g 2.0 with a linewidth of 13.5 G (Fig. 2c). In sample d, component X has been phototrapped in its reduced state, and a much larger, 13.5-G wide signal is present at g 2.0 (Fig. 2d) than is present in sample c. We shall frequently refer to this photogenerated signal as 'the g 2.0 signal', to distinguish it from the photoinduced, 8-G-wide P-700$^+$ signal in samples like sample a, which we shall call 'the P-700$^+$ signal'.

Fig. 3 shows the EPR triplet spectra observed in samples a–d during 33 Hz light-modulation experiments. The phase of the lock-in amplifier was calibrated using the P^R triplet from a sample of photosynthetic bacteria in which quinones had been removed by sodium dodecyl sulfate treatment [14], and which was kindly provided by W.W. Parson and V.A. Shuvalov. The rise and decay times for the P^R state in this sample are known to be 10 ns and 120 μs [15], respectively, so this signal is a convenient zero-phase reference for 33 Hz experiments. The triplet spectra in Fig. 3 were recorded at zero phase with respect to the maximum amplitude of the P^R triplet. Figs. 3a and d show the two distinct triplet spectra which we observe in Photosystem I reaction centers; the spectra in Figs. 3b and c are convolutions of these two triplets.

Fig. 3d shows the radical pair-polarized triplet spectrum reported previously [5]. It has the polarization pattern AEEAAE and zero-field splitting parameters $|D| = 0.0278$ cm^{-1} and $|E| = 0.0038$ cm^{-1}. Fig. 3a shows the EPR spectrum of a triplet with a polarization pattern EEEAAA. This triplet is evidently populated by an intersystem crossing mechanism [5]. Its zero-field splitting parameters are $|D| = 0.0301$ cm^{-1} and $|E| = 0.0039$ cm^{-1}. The latter $|D|$ value is larger than that reported for chlorophyll a or chlorophyll b in vitro [16,17] or of the chlorophyll a triplet observed in spinach chloroplasts by Uphaus et

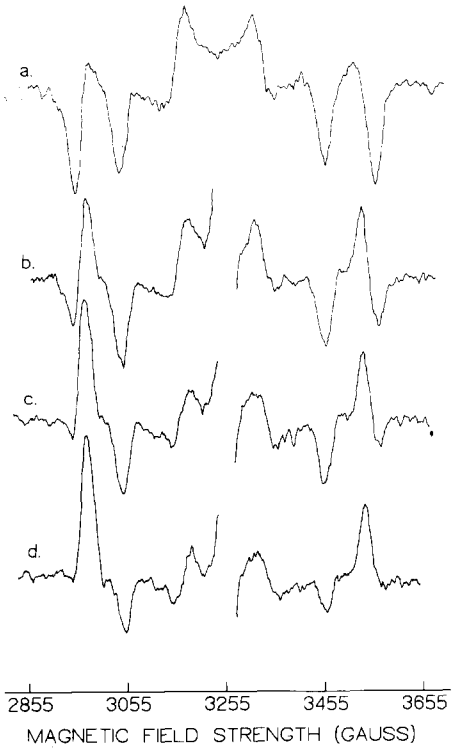

Fig. 3. Light-modulated EPR triplets observed in samples a–d at zero degrees phase with respect to the maximum of the bacterial P^R triplet amplitude, as described in the text. All spectra were recorded at 10 K. Spectrometer conditions: 33 Hz light modulation; gain, 200; modulation amplitude, 40 G; microwave power; 100 μW; microwave frequency, 9.16 GHz; scan rate, 63 G · min^{-1}.

al. [16]. However, it seems likely that this triplet arises from a chlorophyll antenna species, and so we shall refer to it as the antenna triplet for clarity. Table I gives the zero-field splitting parameters of the radical pair-polarized and antenna triplets as well as those reported elsewhere [5,16,17] for chlorophyll and pheophytin triplets.

The results presented in Figs. 1–3 typify those observed in measurements on eight similar sets of samples of Photosystem I reaction centers. The results from five sets of the Photosystem I fraction were essentially the same. We never observed the carotenoid triplet which has been observed in whole chloroplasts and in digitonin Photosystem I subchloroplasts [5], despite the fact that we found substantial amounts of carotenoid in the Photosystem I fraction by TLC analysis.

TABLE I

ZERO–FIELD SPLITTING PARAMETERS REPORTED FOR CHLOROPHYLL AND PHEOPHYTIN TRIPLET STATES IN VIVO AND IN VITRO

Species	Zero-field splitting parameters	
	$\|D\|$ (cm^{-1})	$\|E\|$ (cm^{-1})
Radical pair triplet [a]	0.0278	0.0038
Antenna triplet [a]	0.0301	0.0039
In vitro [b]		
Chlorophyll *a* monomer	0.0275	0.0036
Chlorophyll *b* monomer	0.0287	0.0037
Pheophytin *a*	0.0339	0.0033
In vivo		
Spinach chloroplasts [b]	0.0284	0.0039
Spinach chloroplasts [c]	0.0278	0.0039

[a] Error limits 0.0002 cm^{-1}.
[b] From Ref. 16.
[c] From Ref. 5.

The *g* 2.0 signal in Fig. 2d has the same characteristics as the signal that Heathcote et al. [7] observed in Triton X-100 reaction centers which had been frozen under intense illumination. They attributed this signal to A_1 which had been phototrapped in its reduced state. Fig. 4 shows the results of experiments intended to correlate the number of spins represented by the *g* 2.0 signal with the total reaction center concentration and with the amplitude of light-inducible radical pair-polarized triplet. Fig. 4 shows the results of three separate trials. In each trial, a set of EPR samples was prepared from identical aliquots of Photosystem I reaction centers or the Photosystem I fraction in 0.1 M glycine buffer at pH 10. Several aliquots in each set were treated with dithionite and PMS and were then illuminated before and during freezing, each with a different light intensity, in order to vary the amplitude of the phototrapped *g* 2.0 signal. (We found that the *g* 2.0 signals photogenerated in sets of samples containing PMS and dithionite had the same amplitudes as those in otherwise identical sets of samples which did not contain PMS.) Each set of samples included a sample which did not contain dithionite or PMS, so that *P*-700 could be irreversibly photooxidized at low temperatures, e.g., sample a above. The number of $P\text{-}700^+$ spins in the

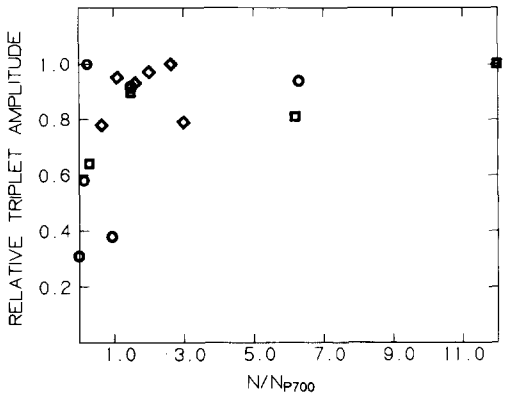

Fig. 4. Plot of the ratio $N/N_{P\text{-}700}$ vs. the amplitude of the radical pair-polarized triplet, where N is the number of spins represented by the g 2.0 signals (e.g., Fig. 2d) which are photo-generated by freezing aliquots of reaction centers in the presence of dithionite and phenazine methosulfate during illumination of varying intensity, and $N_{P\text{-}700}$ is the number of spins represented by the $P\text{-}700^+$ signal (e.g., Fig. 2a) in an identical reaction center aliquot which does not contain dithionite or phenazine methosulfate but has been illuminated at 77 K. ○, ◇ and □ represent three different sets of measurements. See Results section for details. Conditions for triplet measurements are as in Fig. 3, except that 11 Hz light modulation frequency was used. The g 2.0 EPR signal measurements were made at 77 K and 10 μW microwave power.

latter sample was assumed to be equimolar with the total reaction center concentration. The $P\text{-}700^+$ signal in the latter sample, and the g 2.0 signals in the other samples in the set were recorded in the dark at 100 K at non-saturating microwave powers. Under these conditions, the integrated areas of the signals are a direct measure of the number of spins, N, giving rise to the g 2.0 signal and of the number of spins giving rise to the $P\text{-}700^+$ signal, $N_{P\text{-}700}$. The ratios $N/N_{P\text{-}700}$ relate the moles of the g 2.0 species to the moles of P-700. EPR triplet spectra were then recorded using a 11 Hz chopper, a frequency at which the antenna triplet makes a minimal contribution to the observed triplet spectra. The amplitude of the low-field z peak of the radical pair-polarized triplet was used in Fig. 4 to represent the triplet amplitude. Fig. 4 shows that up to 12 spins per reaction center may be photogenerated in the g 2.0 signal without having any apparent effect on the amplitude of light-inducible radical pair-polarized triplet.

Discussion

On the zero-field splitting parameters of the radical pair-polarized and antenna triplets

In bacterial systems, the $|D|$ value of the P^R triplet is 20% smaller than that of bacteriochlorophyll monomer [18]. This effect is well accounted for by the argument that it results from a delocalization of the P^R triplet over two bacteriochlorophyll molecules in the reaction center special pair [17,18]. The evidence presented here and elsewhere [5,16] suggests that such a simple relation does not hold between observed zero-field splitting parameters and the aggregation state of chlorophylls in vivo or in vitro. Table I allows comparison of the zero-field splitting parameters of the radical pair-polarized and antenna triplets with those previously reported [16,17] for chlorophyll and pheophytin triplets. Although P-700 is also thought to be a dimer [19], the radical pair-polarized triplet $|D|$ value is not significantly different from that of chlorophyll a monomer. This is consistent with the observation of Uphaus et al. [16] that the $|D|$ values of chlorophyll a dimer and chlorophyll b oligomer were within 3% of their respective monomeric values. The question of the correlation between the zero-field splitting parameters and chlorophyll environment is also raised by the chlorophyll antenna $|D|$ value, which is significantly larger than that of either chlorophyll a or chlorophyll b monomer, and smaller than that of pheophytin. We cannot exclude the possibility that the antenna triplet arises from chlorophyll b or pheophytin on the basis of its $|D|$ value. However, it is now generally thought that chlorophyll b and pheophytin do not play a role in Photosystem I, so that it is likely that the antenna triplet arises from chlorophyll a.

On the redox dependence of the antenna triplet

Figs. 3a–d show that the amplitudes of both the radical pair-polarized triplet and of the antenna triplet depend on the state of reduction of Photosystem I acceptors at cryogenic temperatures.

The antenna triplet is not observed in samples in which X has been reduced prior to illumination (Figs. 3c and d); it is observed together with the radical pair-polarized triplet in samples in which centers A and B are reduced but X is not reduced prior to illumination (Fig. 3b). These results are consistent with the assignment of the antenna triplet to an excited

antenna species which undergoes intersystem crossing when *P*-700 is in its oxidized state during the illumination period. The antenna triplet has its maximum amplitude in sample a, in which presumably the entire population of reaction centers has undergone irreversible charge separation to the state $P\text{-}700^+Fd_A^-$, as indicated by the spectra in Figs. 1a and 2a. Antenna chlorophyll molecules excited during illumination at low temperature cannot transfer their excitation to reaction centers in this state, so they undergo intersystem crossing to the triplet state. In sample b, illumination results in charge separation to the state $P\text{-}700^+X^-$ and we observe the EPR signals from $P\text{-}700^+$ (Fig. 2b) and X^- (data not shown), as reported by Evans et al. [2]. Figs. 2a and b show that the amplitude of the light-induced $P\text{-}700^+$ EPR signal in sample b is equal to that observed in sample a, so that both signals represent the same number of spins. Because the $P\text{-}700^+$ in sample a arises from an irreversible light-induced electron transfer to center A in all of the reaction centers, it appears that all of the reaction centers in sample b can achieve the state $P\text{-}700^+X^-$ during illumination. The 120 ms [4] lifetime of the state $P\text{-}700^+X^-$ is slow compared to the 33 Hz chopper period, so there will be a significant steady-state population of reaction centers in the state $P\text{-}700^+X^-$ during experiments using 33 Hz phase-sensitive detection. This steady-state population of reaction centers in which *P*-700 is oxidized during the illumination period will give rise to the antenna triplet.

In samples c and d, X is reduced prior to measurement. Illumination results in charge separation to the state $P\text{-}700^+A_1^-$, which has a lifetime more than an order of magnitude shorter than the 33 Hz illumination period. In this case, no significant steady-state population of reaction centers in the state $P\text{-}700^+$ is achieved, and the antenna triplet is not observed.

On the redox dependence of the radical pair-polarized triplet

In sample b, we observe a convolution of the radical pair-polarized triplet with the antenna triplet. We that believe this is evidence, in addition to the kinetic evidence [3,4] discussed in the Introduction, that the processes

$$P\text{-}700^+A_1^-X \rightarrow {}^3P\text{-}700\ A_1X$$

and

$$P\text{-}700^+A_1^-X \rightarrow P\text{-}700^+A_1X^-$$

have similar rate constants when centers A and B are reduced, for the following reasons. The observation that the photo-induced $P\text{-}700^+$ EPR signal has the same amplitude in sample b as it has in sample a is suggestive that the reaction centers are fully converted to the state $P\text{-}700^+X^-$ during continuous illumination of sample b. The presence of the radical pair-polarized triplet EPR signal is suggestive that the reaction centers also populate the state ${}^3P\text{-}700$. One explanation of this observation is that the state ${}^3P\text{-}700$ is populated as an intermediate in the $P\text{-}700^+X^-$ back-reaction. However, this does not seem likely, because the latter process cannot give rise to a polarized triplet on *P*-700 if the spin states of the $P\text{-}700^+X^-$ radical pair become thermally relaxed before charge recombination populates ${}^3P\text{-}700$. The X^- EPR signal is not saturated at 100 mW at 10 K, indicating that its spin-lattice relaxation time is not longer than a few microseconds. The spin-lattice relaxation time of $P\text{-}700^+$ was reported to be 800 μs at 10 K [20]. Spin-lattice relaxation will destroy any polarization on the $P\text{-}700^+X^-$ radical pair within the 120 ms lifetime. Therefore, it seems likely that the radical pair-polarized triplet arises from $P\text{-}700^+A_1^-$ radical pair recombination rather than from $P\text{-}700^+X^-$ pair recombination. If so, then the observation of the radical pair-polarized triplet in sample b suggests that every time a reaction center is excited, it has some probability that A_1^- will not transfer its electron to X but will instead undergo radical pair recombination to ${}^3P\text{-}700$. The lifetime of the state $P\text{-}700^+X^-$ (120 ms) is much longer than the lifetime of the state ${}^3P\text{-}700$ (1 ms), so that the reaction centers are in the former state most of the time during continuous illumination. This is why we observe the full extent of *P*-700 photooxidation during steady-state EPR measurements. However, because the reaction centers return periodically to the ground state, they can also give rise to the radical pair-polarized triplet if there is a competition between forward electron transfer from A_1^- to X and back reaction between $P\text{-}700^+$ and A_1^- which populates ${}^3P\text{-}700$. This can happen only if the two rate constants are similar.

In the case of samples c and d, we observe that the reduction of the species giving rise ot the *g* 2.0 EPR signal shown in Fig. 2d has no apparent effect on the amplitude of the radical pair-polarized triplet. This suggests that the *g* 2.0 radical is not the primary Photosystem I acceptor in its reduced state. If this radical

were the fully reduced primary acceptor, then charge separation could not occur and we would not observe the radical pair-polarized triplet. Fig. 4 shows that up to 12 spins per *P*-700 may be photo-generated and contribute to the *g* 2.0 signal without having any apparent effect on the amplitude of the radical pair-polarized triplet. Thus, the results presented in Fig. 4 show that there are species other than A_1^- contributing amplitude to the *g* 2.0 signal. We suggest that photooxidized antenna chlorophyll contributes to this amplitude. However, it is also possible that the *g* 2.0 signal contains a contribution from phototrapped A_1^-. The observation of the radical pair-polarized triplet in samples in which A_1 was known to be fully reduced would be evidence for the existence of an earlier acceptor than A_1. Unfortunately, the EPR spectrum of A_1^- is apparently very similar to that of the other photogenerated species which contribute to the *g* 2.0 signal, and so it may be difficult to establish the state of reduction of A_1 on the basis of these EPR data. Recently, Rutherford and Mullet [21] reported studies of the EPR triplets in Photosystem I reaction centers in which X and Centers A and B had been denatured by treatment with lithium dodecyl sulfate. They observed the radical pair-polarized triplet in samples which contained dithionite and neutral red and were frozen in the dark. After these samples had been thawed and refrozen during illumantion, the *g* 2.0 signal attributed to A_1^- was present and the radical pair-polarized triplet amplitude was smaller, suggesting that charge separation is not occurring when the *g* 2.0 radical is phototrapped. After the latter samples had been thawed and refrozen in the dark, the *g* 2.0 signal was smaller, and the original amplitude of the radical pair-polarized triplet was restored. Our results (data not shown) with Photosystem I reaction centers that have been treated with urea and ferricyanide by the method of Golbeck et al. [9] to remove iron from X and Centers A and B are in agreement with the results of Rutherford and Mullet [21]. These observations can be understood if A_1 or an even earlier acceptor is easier to phototrap in the reduced state in preparations in which X and Centers A and B are not present. When these secondary acceptors are active, they must be reduced before earlier acceptors can be photoreduced, and the latter must then be reduced in close proximity to three or four negatively charged sites.

Conclusion

The observation of the antenna triplet and of the full extent of $P\text{-}700^+$ steady-state photooxidation in sample b suggest that the reaction centers are able to be completely converted to the state $P\text{-}700^+X^-$ during continuous illumination. The observation of the radical pair-polarized triplet, on the other hand, indicates that the reaction centers also undergo charge separation followed by radical pair recombination between $P\text{-}700^+$ and A_1^-, which populates the radical pair-polarized triplet. This suggests to us that when Centers A and B are reduced, the forward electron transfer to X and radical pair recombination between $P\text{-}700^+$ and A_1^- are in competition, and that the reaction kinetics may be represented by the scheme:

$$P\text{-}700\ AXFd_B^-Fd_A^- \xrightarrow{h\nu} P\text{-}700^+A^-XFd_B^-Fd_A^- \xrightarrow{k_f} P\text{-}700^+AX^-Fd_B^-Fd_A^-$$
$$\nwarrow \quad \swarrow \ k_b$$
$$^3P\text{-}700\ AXFd_B^-Fd_A^-$$

where $k_f \cong k_b$. Our results are thus in agreement with the results of Sauer et al. [3] and of Shuvalov et al. [4] who observed the 1 ms phase associated with $P\text{-}700^+A_1^-$ recombination in samples in which X was not reduced prior to measurement. Similar observations were recently reported in Photosystem I by Setif et al. [22] and in green bacteria by Swarthoff et al. [23]. There is indeed evidence that this situation exists also at room temperature. In room-temperature kinetics measurements, Shuvalov et al. [24] observed a biphasic decay of optical changes attributed to $P\text{-}700^+A_1^-$ radical pair formation under conditions where Centers A and B were reduced prior to measurement. They assigned a 10 ns component at 694 nm to the recovery of changes in the spectrum of A_1 due to radical pair recombination to a triplet state, presumably ^3P-700. They also observed a 3 μs component which they attributed to the ^3P-700 lifetime. However, they noted that the amplitude and decay rate of the 10 ns component were the same regardless of whether X was reduced by exogenous donors and background illumination during the kinetics measurements. This result again suggests a similarity between the two rate constants for the processes of $P\text{-}700^+A_1^-$ pair recombination and forward electron transfer from A_1^- to X. If we take this argument seriously, then it would appear that when Centers A and B are reduced, the forward electron transfer time from

A_1^- to X is on the order of 10 ns, which is 50-times slower than the 200 ps forward transfer time when Centers A and B are not reduced prior to illumination [24]. Thus, our results suggest that the reduction of Centers A and B affects the kinetics of primary events in Photosystem I. If so, then the kinetics involving *P*-700, A_1 and X occurring during primary photochemistry under physiological conditions will need to be studied in some other way, such as by selectively removing Centers A and B from the reaction center. So far, however, any treatment which removes Centers A and B also removes X [9,21].

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