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EVIDENCE FOR A NEW EARLY ACCEPTOR IN PHOTOSYSTEM I OF PLANTS

AN ESR INVESTIGATION OF REACTION CENTER TRIPLET YIELD AND OF THE REDUCED INTERMEDIARY ACCEPTORS

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The yield of the triplet state of the primary electron donor of Photosystem I of photosynthesis (P^T-700) and the characteristic parameters (g value, line shape, saturation behavior) of the ESR signal of the photoaccumulated intermediary acceptor A have been measured for two types of Photosystem I subchloroplast particles: Triton particles (TSF 1, about 100 chlorophyll molecules per P-700) that contain the iron-sulfur acceptors F_X, F_B and F_A, and lithium dodecyl sulfate (LDS) particles (about 40 chlorophyll molecules per P-700) that lack these iron-sulfur acceptors. The results are: (i) In Triton particles the yield of P^T-700 upon illumination is independent of the redox state of A and of $F_{X,B,A}$ and is maximally about 5% of the active reaction centers at 5 K. The molecular sublevel decay rates are $k_x = 1100\ s^{-1} \pm 10\%$, $k_y = 1300\ s^{-1} \pm 10\%$ and $k_z = 83\ s^{-1} \pm 20\%$. In LDS particles the triplet yield decreases linearly with concentration of reduced intermediary acceptors, the maximal yield being about 4% at 5 K assuming full P-700 activity. (ii) In Triton particles the acceptor complex A consists of two acceptors A_0 and A_1, with A_0 preceding A_1. In LDS particles at temperatures below −30°C only A_0 is photoactive. (iii) The spin-polarized ESR signal found in the time-resolved ESR experiments with Triton particles is attributed to a polarized P-700-A_1^- spectrum. The decay kinetics are complex and are influenced by transient nutation effects, even at low microwave power. It is concluded that the lifetime at 5 K of P-700$A_0A_1^-$ must exceed 5 ms. We conclude that P^T-700 originates from charge recombination of P-700A_0^-, and that in Triton particles A_0 and A_1 are both photoaccumulated upon cooling at low redox potential in the light. Since the state P-700AF_X^- does not give rise to triplet formation the 5% triplet yield in Triton particles is probably due to centers with damaged electron transport.

Introduction

In recent years, ESR spectroscopy has contributed conserably to our knowledge of the acceptor side of PS I of plants. It is now generally accepted that three iron-sulfur proteins function as stable acceptors, whereas a chlorophyll monomer or dimer serves as transient acceptor (for reviews see Refs. 1 and 2). It was concluded that the primary reaction can be written as:

$$\text{P-700AF}_X\text{F}_B\text{F}_A \xrightarrow{h\nu} \text{P-700A}^- \text{F}_X\text{F}_B\text{F}_A \rightarrow \text{P-700A}(\text{F}_X\text{F}_B\text{F}_A)^- \quad (1)$$

Abbreviations: PS I, Photosystem I; LDS; lithium dodecyl sulfate; Chl, chlorophyll; Tricine, *N*-tris(hydroxymethyl)methylglycine

F_X, F_B and F_A represent the ferredoxin-type acceptors, A the intermediary acceptor and P-700 the primary donor. In higher plants and many algae the redox potentials of these acceptors obey the relation $E_m(F_X) < E_m(F_B) < E_m(F_A)$ [3]. The redox potential of the medium (E_h) can be poised such that F_A alone or the complex F_BF_A is reduced to F_A^- and F_B^- F_A^-; F_X can be reduced photochemically at low redox potentials. The state P-700AF_X^- F_B^- F_A^- is not stable. At about 10 K (the temperature at which the ESR measurements are carried out) it decays within 0.7 s [4] or 0.13 s [5] to the original state P-700AF_X F_B^- F_A^-.

When a fast electron donor is present, the intermediary acceptor can be photoaccumulated at low redox potentials under continuous illumination at 0°C followed by subsequent freezing in the light [6–8]:

$$\text{P-700A}F_X F_B^- F_A^- \xrightarrow{h\nu} \text{P-700A}F_X^- F_B^- F_A^- \xrightarrow{e} \text{P-700A}F_X^- F_B^- F_A^-$$

$$\xrightarrow{h\nu} \text{P-700A}^- F_X^- F_B^- F_A^- \xrightarrow{e} \text{P-700A}^- F_X^- F_B^- F_A^- \qquad (2)$$

The ESR spectrum attributed to A^-, however, is not well defined. There is considerable variance in reported g value (2.002–2.004) and line width (10–14 G) [5–8]. On the basis of the available ESR data the photoaccumulated intermediary acceptor appears to be a monomeric chlorophyll.

Flash excitation of PS I particles in the state P-700AF_X F_B^- F_A^- at 5 K results in a transient optical difference spectrum that decays biphasically with half-times of 130 and 1.3 ms [5]. The fast component was attributed to the back-reaction P-700A^- → P-700A. The observation of a fast transient ESR signal at 56 K with a half-time of 1.3 ms was taken to support this assignment. In contrast to Ref. 5, other workers observed under similar redox conditions but at different temperatures (10 and 300 K) spin-polarized doublet ESR signals with microwave and temperature-dependent decay kinetics [9–12]. The absorption difference spectrum of the reduced acceptor, constructed by subtracting the spectrum of P-700A from that of P-700A^- measured as the 1.3 ms component, was interpreted as originating from a Chl *a* dimer.

Under continuous illumination at 10 K of (sub)chloroplast particles poised in the state P-700AF_X^- F_B^- F_A^-, a triplet ESR signal was observed with a spin-polarization pattern characteristic of a triplet originating from radical pair recombination [13]:

$$\text{P-700A}F_X^- F_B^- F_A^- \xrightarrow{h\nu} \text{P-700A}^- F_X^- F_B^- F_A^-$$

$$\rightarrow \text{P}^T\text{-700A}F_X^- F_b^- F_A^- \qquad (3)$$

This reaction is analogous to that encountered in bacterial photosynthesis [1,2]. Obviously, when A^- is photoaccumulated, the reaction cannot proceed, and no triplet signal should be observed. This has been checked by Rutherford and Mullet [14], employing subchloroplast particles from which the ferredoxin-type acceptors had been removed by LDS treatment (LDS particles). Their results were suggestive of an inverse relation between triplet yield and the intensity of the photoaccumulated A^- ESR signal, but the experiments were carried out with optically very dense samples, making quantitative measurements difficult.

The data reviewed above are difficult to fit into one model: (i) A proposed lifetime of about 1 ms for the pair P-700A^- contrasts with a lifetime of about 10 ns for the radical pair in bacterial photosynthesis. A radical pair with a lifetime of 1 ms is unlikely to produce a spin-polarized triplet upon recombination because of spin-lattice relaxation between the four radical pair energy levels. (ii) There is no agreement on the presence of a spin-polarized doublet ESR signal [5,8,12]. (iii) Data on the ESR line of photoaccumulated A^- do not agree with the ESR signal at g 2.00 measured after a flash [5,6] at redox potentials for which F_A and F_B are reduced. (iv) There is no agreement on the state of aggregation of A (monomer or dimer [5,12]). Moreover, many of the data have been obtained on different types of PS I subchloroplast particle preparations and are therefore difficult to compare. It seemed therefore highly desirable to make a comparative study of triplet yield, decay kinetics and the ESR spectrum of A^- in two well characterized preparations, viz., PS I subchloroplast particles with the ferredoxin acceptor complex intact (Triton particles) and particles lacking this complex (LDS particles).

Another object of the present study is to com-

pare the acceptor side of PS I with that of the photosystem of green photosynthetic bacteria. In a series of papers, Swarthoff et al. [15–17] have demonstrated that in this system two iron-sulfur centers function serially as stable electron acceptors and a BChl *a* functions as transient acceptor. Between the primary donor and this intermediate, another as yet unidentified acceptor must be present as evidenced by an increase in triplet yield upon photoaccumulation of $BChl^-$ *a*. In view of the similarity between the acceptor side of the green bacterial system and PS I a search for an early acceptor functioning between P-700 and A seemed warranted.

We shall present evidence that the ESR signal formerly ascribed to A^- in Triton particles is composed of two components and that in Triton particles an earlier acceptor than A which we call A_0 functions. In LDS particles the acceptor A_0 appears to function as the sole acceptor under our conditions of measurement. A will be used to denote the acceptor between P-700 and F_X in those cases where a distinction between A_0 and A_1 is either not possible or not practical. In the Triton particles poised in the state $P\text{-}700AF_XF_B^-F_A^-$ we found a flash-induced spin-polarized doublet signal at *g* 2.00 similar to that observed by McCracken et al. [12], with complex microwave-dependent decay kinetics that show transient nutation effects. The 1.3 ms component in the transient signal at *g* 2.00 measured by Shuvalov et al. [5] is shown to be most likely due to these nutation effects and not to represent a chemical reaction rate.

Materials and Methods

Reaction centers of the *Rhodopseudomonas sphaeroides* R-26 mutant were prepared according to Ref. 18. PS I particles of spinach chloroplasts containing about 100 Chl/P-700, so-called Triton particles, were prepared according to Ref. 19. PS I particles of spinach chloroplasts lacking the ferredoxin acceptors F_X, F_B and F_A (LDS particles) were prepared as follows, using a modification of the method of Ref. 14. A batch of Triton particles was dialyzed against 10 mM Tricine, pH 8, to remove the detergent Triton X-100. The particles were then diluted to 1 mg Chl/ml and incubated with LDS for 1 h at 4°C. Different LDS concentrations were used (0.75 –5%) in order to find the optimal concentration for the removal of the ferredoxin acceptors. The particles were then loaded on a linear sucrose gradient (0.1–1 M sucrose, 10 mM Tricine, 0.1% sodium cholate, pH 8) and centrifuged at $100\,000 \times g$ for 16 h. The LDS particles showed up as a dark green band about 2 cm from the bottom of the centrifuge tube. After removal of this band the LDS particles were dialyzed overnight against 10 mM Tricine and were concentrated to 2 mg Chl/ml by further centrifugation at $175\,000 \times g$ for 16 h. Protein composition of the LDS particles was analyzed by SDS gel electrophoresis [20] using a 10% polyacrylamide gel.

Chemical and/or photoreduction of the Triton and LDS preparations was carried out as follows: 2% of an ice-cold and freshly prepared 0.5 M sodium dithionite solution in 0.5 M glycine buffer, pH 9.5, was added to the anaerobic sample. To 500 µl sample, containing 60 vol% glycerol and 40 mM glycine buffer, pH 9.5, the following amounts were added: 250 µg glucose, 30 µg catalase (Sigma, from bovine liver; 21.000 U/mg), 60 µg glucose oxidase (Sigma, from *Aspergillus niger*, type VII; 203.400 U/g), 12 µg *N*-methylphenazonium methosulfate (as a donor to P-700) and 50 mg sucrose. In order to achieve low redox potentials, the sample was titrated before the addition of the dithionite solution to pH 10.2 using a micro pH electrode. Addition of dithionite lowered the pH to 10.0. The 4 mm outer diameter ESR quartz tubes were filled under nitrogen gas and frozen in a nitrogen flow cryostat. In order to accumulate the reduced acceptors F_X and/or A the samples were illuminated by white light for about 15 s and frozen in the light while rotating the sample. Illumination at 2 mW/cm^2 was enough to accumulate most of F_X^-. Variable amounts of A^- were accumulated by varying the light intensity between 2 and 350 mW/cm^2. A typical absorbance for the ESR samples was 4 mm^{-1} at 676 nm. For measurements of triplet yields, however, the ESR samples had an absorbance of 0.4 mm^{-1} at 676 nm and extra care was taken to avoid cracking of the sample during freezing.

The ESR spectrometer used for the experiments had a response time of 20 µs [21]. To obtain precise *g* values the microwave frequency was

measured with a HP 5342A frequency counter and the magnetic field inside the cavity was calibrated using α,α'-diphenyl-β-picrylhydrazyl as g-value marker (g 2.0037 $\pm$ 0.0002). For illumination of the samples inside the cavity a 1000 W projection lamp was used; the light was filtered by 5 cm water. Flash illumination was provided by a Lambda Physik dye laser (duration of the flash approx. 600 ns). To eliminate ESR background from the helium cryostat (Oxford flow cryostat type CA5279) the original quartz insert was replaced by a suprasyl quartz insert which was cleaned with a KOH/methanol mixture followed by rinsing with an EDTA solution. In the helium flow cryostat the indium seal and glass/metal transition was replaced by a glass/stainless-steel ground-glass joint. This improved the vacuum properties markedly and made it possible to clean the quartz insert without disassembling the cryostat.

Results

Redox states and triplet yields in Triton and LDS particles

Fig. 1 shows the ESR spectra of Triton and LDS particles at different redox states of the acceptors at 10 K. The state $F_B^- F_A^-$, as indicated by the spectrum at g 2.05 and around g 1.9 in Fig. 1a, A, was formed by freezing the particles in the dark. The state $F_X^- F_B^- F_A^-$ (Fig. 1a, B) was accumulated by illumination at 0°C for 15 s and subsequently stabilized by freezing under low light conditions (about 2 mW/cm^2); the reduction of F_X is indicated by the ESR signal appearing at g 1.76. The state $A^- F_X^- F_B^- F_A^-$ (Fig. 1a, C), in which the presence of A^- is operationally defined by the 10–14 G wide ESR signal at g 2.00, was accumulated by illumination at 0°C for 15 s and subsequently frozen under high light conditions (about 350 mW/cm^2). This combination of chemi-

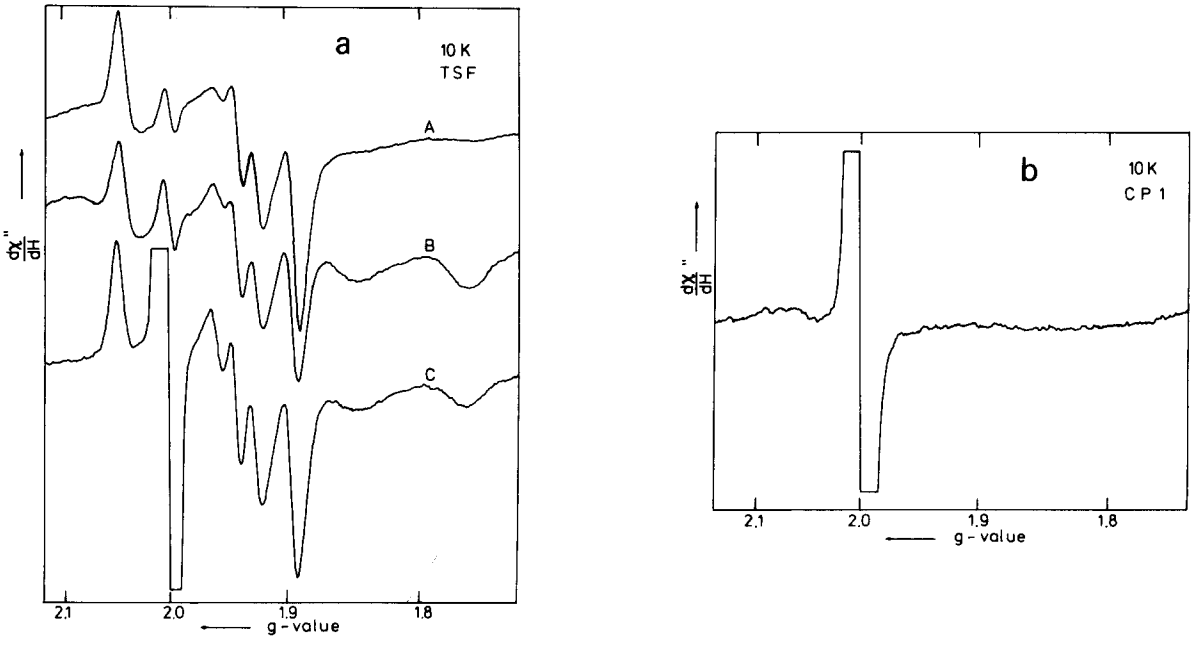
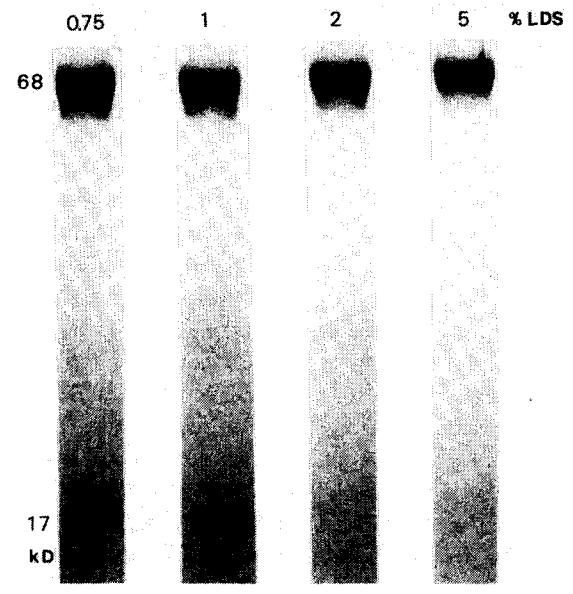
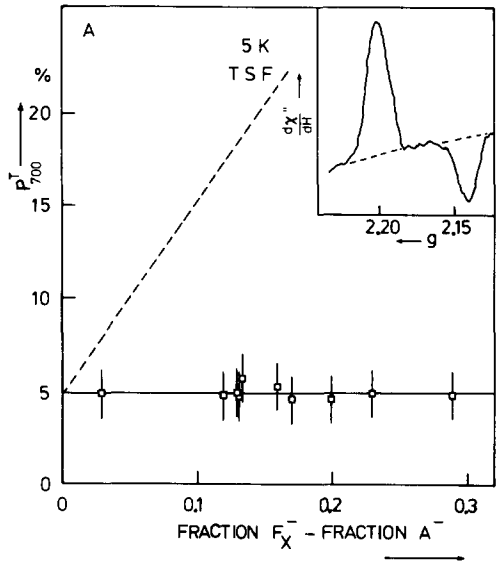


Fig. 1. (a) ESR spectra of the different redox states of Triton and LDS particles. Microwave power 10 mW, temperature 10 K, modulation amplitude 2.0 mT. (A) Triton particles reduced with dithionite at pH 10.0 in the presence of *N*-methylphenazonium methosulfate and frozen in the dark. (B) As a, A, but frozen under low-light conditions. (C) As a, A, but frozen under high-light conditions. (b) Spectrum of LDS particles reduced as in a, C.



cal reduction and accumulation of reduced states is analogous to the reduction method used for the acceptors of green bacteria [15–17]. Fig. 1B shows the ESR spectrum at 10 K of LDS particles which had been illuminated under maximum light condition at 0°C followed by subsequent freezing. It is seen that the ESR signals of F_X^- and $F_B^- F_A^-$ are absent. These particles were treated with 2% LDS; treatment with 5% LDS yielded identical spectra. Incubation with 0.75% LDS, however, resulted in the disappearance of $F_B^- F_A^-$ spectrum while traces of F_X^- were still present together with a broad ESR signal at g 2.1. Fig. 2 shows the results of polyacrylamide gel electrophoresis analysis of these particles at different LDS concentrations. It is

Fig. 2. SDS-polyacrylamide gel electrophoresis of Triton particles treated with different amounts of LDS during incubation as indicated.



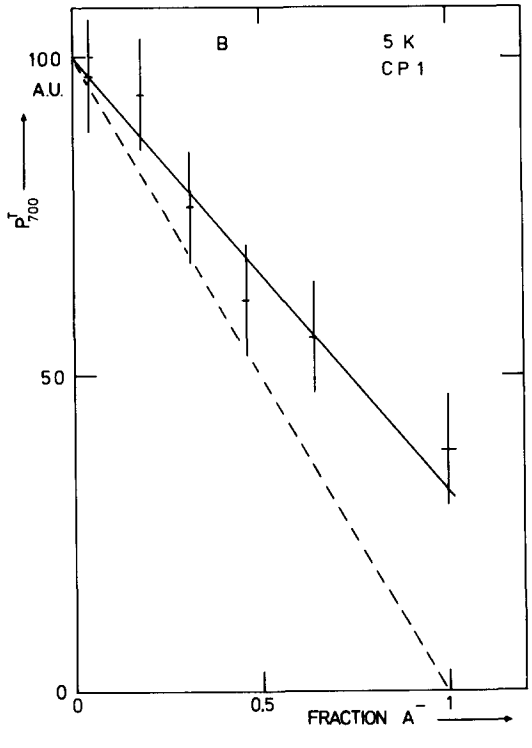


Fig. 3. (A) Dependence of the ESR triplet amplitude in Triton particles on the fraction of F_X^- minus the fraction of A^- (see text). The dashed line represents the theoretical dependence of P^T-700 if P^T-700 were to arise from the back-reaction of P^+-700A^- with a 100% yield. The amplitude of F_X^- was recorded at 10 K, 10 mW microwave power; the amplitude of A^- was recorded at 5 K, 2 μW microwave power. Sample concentration was 0.1 mg Chl/ml. Inset: low-field part of the polarized triplet of P-700 (0.8 mW, 5 K). (B) Dependence of the triplet amplitude (P^T-700) in LDS particles on the reduction of A. The dashed line shows the theoretical dependence of P^T-700 amplitude as in A, arbitrarily set equal to the measured amplitude of P^T-700 when no A is reduced.

seen that the LDS particles treated with 5% LDS exhibit the simplest protein composition and that the particles treated with 0.75% LDS still contain an additional protein band.

In order to express the dependence of the amplitude of the donor triplet signal on the redox state of the acceptors it is necessarily to express the ESR amplitude of F_X^-, A^- and P^T-700 in fractions of F_X, A and P^T-700 that are reduced. To find the fraction of F_X and A that is reduced we have assumed that in a sample which was illuminated for 1 min at full light intensity at 0°C all the A and all F_X was in the reduced state. Since in our Triton particles it was not possible to reduce F_X while keeping A totally oxidized, we have plotted in Fig. 3A the amount of P^T-700 against the fraction of F_X^- minus the fraction of A^-. The latter value represents the amount of reaction centers in the state $AF_X^- F_B^- F_A^-$ if we assume that there are three redox states possible in these particles: AF_X, AF_X^- and $A^- F_X^-$. The fraction of P-700 that is converted into the triplet state in Triton particles was calibrated using two different methods:

(1) In Triton particles preilluminated at 0°C in the absence of dithionite and *N*-methylphenazoniummethosulfate and frozen in the light all P-700 is irreversibly oxidized to P-700. In this sample no P^T-700 is observed. We assume that in this sample all P-700 is oxidized. The sample was then thawed so that P-700 became fully reduced again, frozen in the dark and illuminated at 5 K. Now, P-700 and F_A^- were generated irreversibly in the light. The amplitude of P-700 measured in the dark after illumination at 5 K was only slightly lower than the amplitude of the P-700 signal in the sample in which P-700 was fully oxidized. In the sample that was frozen in the dark only about 5% of total P-700 could not be irreversibly oxidized by illumination at 5 K. When the ESR spectrum was recorded under illumination at 5 K, in addition to the P-700 and F_A^- signal, a P^T-700 signal was observed. Apparently, in about 5% of the PS I reaction centers P-700 cannot reduce F_A and in these reaction centers the triplet state P^T-700 is formed by radical recombination. This amplitude served to calibrate the triplet signals in our other samples observed under various redox conditions.

(2) The fraction of P-700 that forms P^T-700 in a saturating flash of white light was compared at 5 K with the amplitude of P^T-860 in prereduced reaction centers of the *Rps. sphaeroides* R-26 mutant. Here it is assumed that in the bacterial reactions centers 100% P^T-860 is formed in a saturating flash at 5 K, that both triplets are solely populated through the T_0 spin state and that the small differences between the zero-field splitting parameters $|D|$ and $|E|$ of P^T-700 and P^T-860 and a possible difference in the spin-spin relaxation time, T_2, that may influence the amplitude of the differential ESR triplet spectrum can be neglected. To correct for the different decay rates of P^T-700 and P^T-860 the triplet amplitudes were extrapolated to $t = 0$. The result was that about 3% of P-700 formed P^T-700. This value agrees well with the 5% P^T-700 found using the first method.

Using the methods mentioned above, we have monitored the dependence of the amount of P^T-700 (in all cases the amplitude of the peak at either the lowest or highest field position was measured under continuous illumination) on the redox state of A or F_X for Triton and LDS particles at 5 K (Fig. 3). These measurements were all performed on very dilute samples (0.1 mg Chl/ml). At higher concentrations such as those used in Ref. 14, the effects were highly irreproducible, probably because of strong local heating under illumination at 5 K and a stronger dependence of the effective optical path length on cracks that arise during freezing. The inset in Fig. 3A shows part of the triplet spectrum of P^T-700 in Triton particles. The polarization pattern clearly shows that it arises from radical pair recombination [21,22]. We regard this as proof that we observed the triplet formed by radical recombination, and not a triplet formed by intersystem crossing on antenna chlorophylls. Kinetic experiments on P^T-700 yielded for the sublevels decay rates $k_z = 83\ s^{-1} \pm 20\%$, $K_y = 1300\ s^{-1} \pm 10\%$ and $k_x = 1100\ s^{-1} \pm 10\%$. For P^T-700 in LDS particles we found similar decay rates. These values were extracted from the kinetic data by extrapolating the decay rates of the different peaks of the triplet to zero microwave power [21]. The average decay time $\bar{k} = 1/3(k_x + k_y + k_z)$ then becomes $\bar{k} = 800\ s^{-1} \pm 20\%$. These decay rates are in agreement with those found by Mathis (personal communication).

From Fig. 3A (continuous line) it is clear that

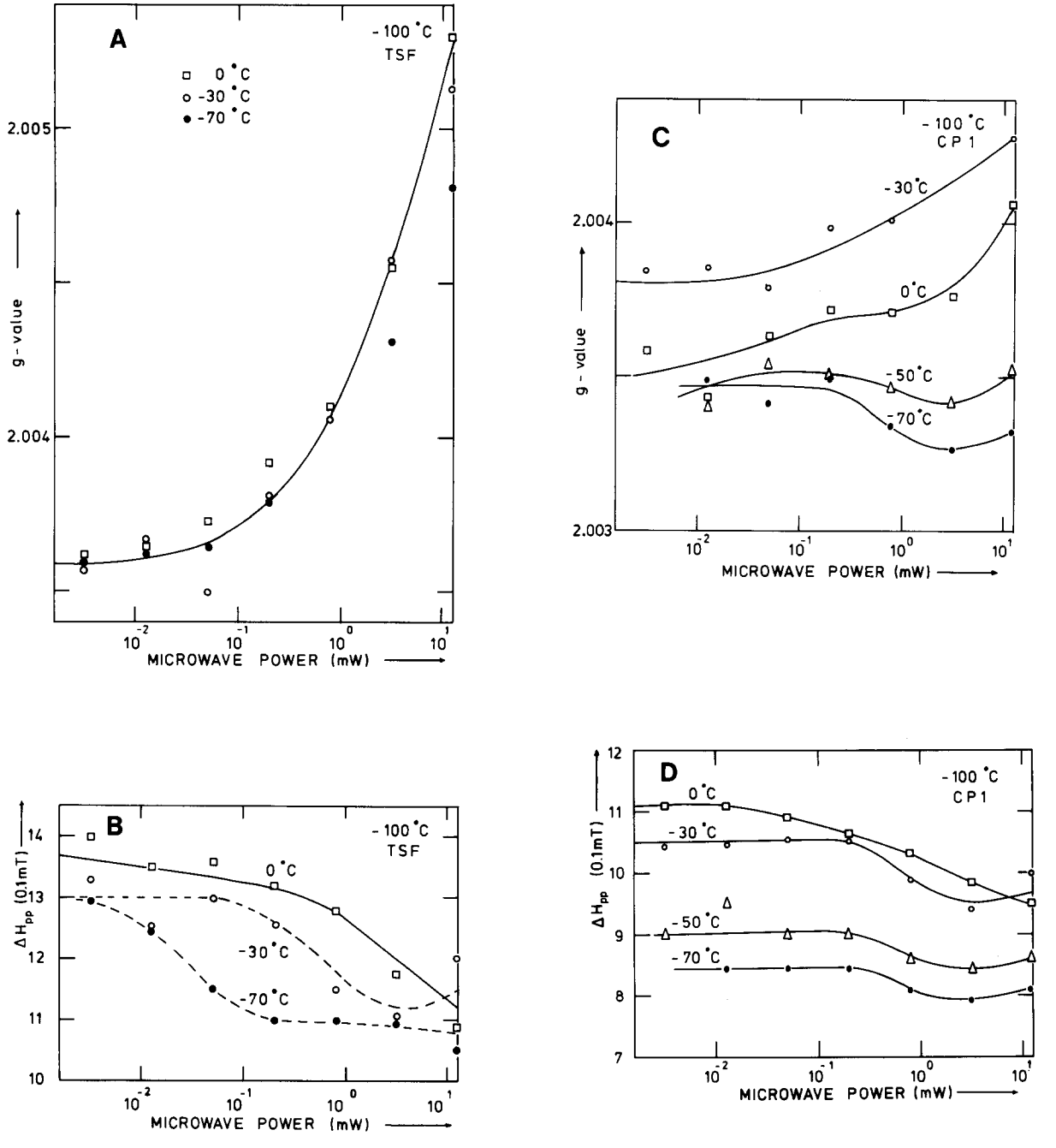


Fig. 4. (A, B) Dependence of g value (A) and line width (B) of the spectra of A^- in Triton particles on the microwave power. Temperatures indicated are those at which the samples were illuminated. The continuous line shows the result of the simulation of these effects using two radicals with different g value and line width (see text), and saturation behavior as in Fig. 5. Measuring temperature 170 K, modulation amplitude 0.25 mT. Other conditions as in Fig. 1a, C. (C, D) As in A and B for LDS particles treated with 5% LDS.

the amount of P^T-700 does not depend on the redox state of F_X. The dashed line in Fig. 3A represents the theoretical dependence of P^T-700 amplitude on the redox state of F_X if P^T-700 were to originate from the reaction P-700 $A^- \rightarrow P^T$-700 A [14]. The maximum triplet yield per mg Chl in LDS particles was twice that of Triton particles. Fig. 3B shows the dependence of the P^T-700 amplitude in these particles as a function of the reduction of A. Since we have no data on the activity of the LDS particles in terms of the quantum yield of P-700 production at low temperature, we cannot express the triplet yield in triplets per active P-700 as was possible in Triton particles. In Fig. 3B we have therefore plotted the P^T-700 amplitude in arbitrary units. It can be seen that in these particles the amount of P^T-700 is linearly dependent on the amount of A^-. Apparently, in LDS particles A is indeed part of the radical pair whose recombination generates P^T-700 while in Triton particles A^- is not a member of this radical pair. This contradiction suggests that A^- as defined by the ESR signal at g 2.00 does not represent the same entity in Triton and in LDS particles, i.e., the ESR spectrum at g 2.00 of Triton particles that are photoreduced at low redox potential either represents another radical than the intermediary A^- of photoreduced LDS particles, or is a composite of at least two radical species representing two intermediary acceptors acting in series. We have checked this hypothesis by carefully measuring the g value and line width (ΔH_{pp}) of A^- in Triton and LDS particles as a function of microwave power.

The ESR spectrum of A^- in Triton and LDS particles

Fig. 4 shows the results of the measurements on A^- of Triton and LDS particles. In Fig. 4A and B the results obtained with Triton particles are presented for different temperatures of accumulation of A^- (−70 to 0°C). The ESR spectra were measured at −100°C, since at lower temperatures microwave power-induced line broadening obscured the effects. It is seen that in the Triton particles the g value shifts (Fig. 4A) and the line width narrows upon increasing microwave power (Fig. 4B). The g value shift is practically insensitive to the accumulation temperature; the line narrowing is only slightly dependent on the accumulation temperature. These two effects – g value shift and line narrowing – indicate that the g 2.00 signal assigned to the acceptor A^- consists of more than one radical with different g value, line width and saturation behavior. This is supported by the fact that the amplitude of the ESR signal at g 2.00 indicated the generation of two to three unpaired electrons per P-700. The spectra of A^- in Triton particles accumulated at 0°C could be fitted very well over the whole range of microwave power by two Gaussians with g_1 2.0017 ± 0.0006, $\Delta H_{pp,1}$ = 1.15 mT ± 0.1, and g_2 = 2.0054 ± 0.0006, $\Delta H_{pp,2}$ = 1.08 mT ± 0.1. We shall denote the radicals that are presented by these two Gaussians that form the spectra of A^- as A_0^- and A_1^-. The continuous lines in Fig. 4A and B show the curves of the g-value shift and line narrowing as a function of microwave power simulated simultaneously with one pair of Gaussian curves of equal area in the nonsaturating region. The saturation behavior of the two radicals is shown in Fig. 5.

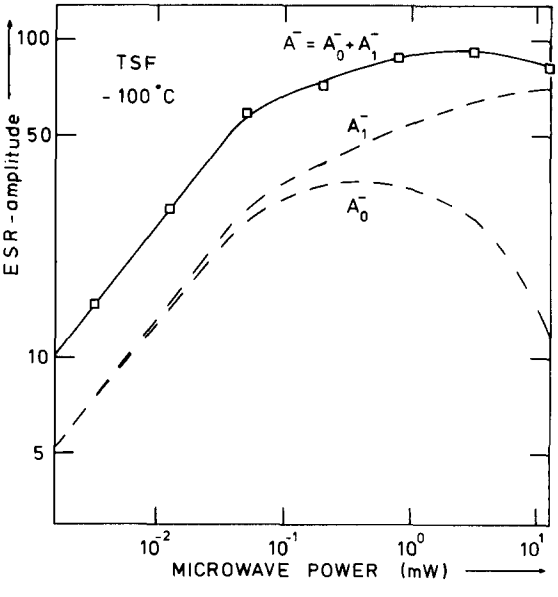


Fig. 5. Saturation behavior of the two radicals that constitute the spectrum of A^- derived from simulating the 0°C results from Fig. 4A. The data points show the amplitude of the spectrum of A^- measured at −100°C. The dashed curves represent the assumed individual saturation curves of A_0^- and A_1^- used for the simulation in Fig. 4A and B, the continuous line is the sum of the amplitudes of A_0^- and A_1^-.

Fig. 4C and D shows the results of a similar set of experiments for LDS particles. It is seen that when A^- is accumulated at 0°C the same effects (g-value shift and line narrowing) are still present, though much less pronounced than in Triton particles. When A^- is accumulated at lower temperatures the g-value shift and line narrowing become much smaller and the line width at low microwave power is appreciably narrower than for samples with A^- accumulated at 0°C. The amplitude of the spectrum of the acceptor was half as much as that in Triton particles. The above results were obtained for LDS particles that were treated with 5% LDS at 4°C. Experiments with LDS particles treated with 2% LDS showed a g-value shift and line width narrowing identical with those in Triton particles when A^- was accumulated at 0°C. Accumulation of A^- at −70°C in the 2% LDS particles resulted in extents of g-value shift and line narrowing intermediate between those of the Triton particles and the 5% LDS particles. If it is assumed that in LDS particles the earlier acceptor A_0 is retained upon treatment with LDS, A_0^- is represented by the Gaussian with low g value and A_1^- by the Gaussian with high g value.

We conclude that at 0°C there are two acceptors active in LDS particles (as in Trion particles), but that at low temperature only one of them retains photoactivity. The experiments with different LDS concentration in the incubation show that the amount of the second acceptor A_1 in the reaction center decreases with increasing LDS concentration.

Electron spin polarization in TSF particles

In order to obtain more information about the properties of the acceptors of PS I we studied the spectrum and the kinetics of the spin-polarized spectrum at g 2.00.

After a laser flash at 600 nm (pulse width 600 ns) at 5 K a 150 ms component was observed in Triton particles prepared in the state $AF_XF_B^-F_A^-$. The ESR spectrum of this component was identical to that of P-700. Since the 150 ms component was also found at g 1.76, we attribute this component to the back-reaction $P\text{-}700F_X^- \rightarrow P\text{-}700F_X$ [5]. At shorter times, faster kinetics were observed around g 2.00 that showed complex microwave power-dependent decay rates. The points in Fig. 6A represent the ESR amplitude 100 μs after a laser flash at 5 K. The spectrum is identical to the electron spin-polarized spectrum reported in Ref.

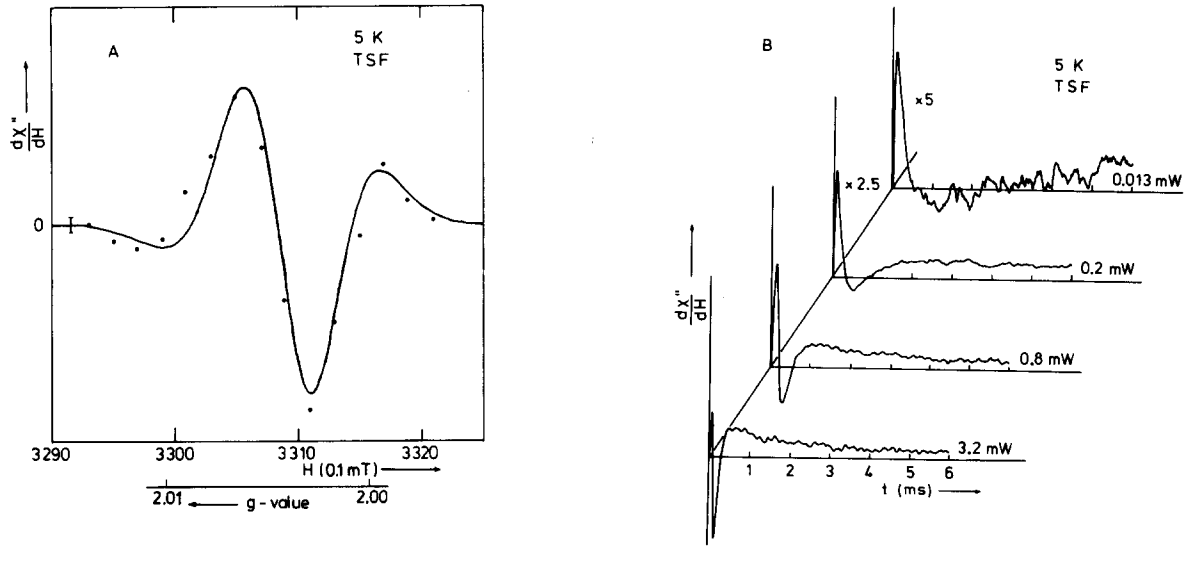


Fig. 6. (A) Spectrum for Triton particles in the state $F_B^-F_A^-$ taken 100 μs after a light flash at 5 K. Conditions: microwave power 20 μW, modulation amplitude 0.2 mT. The continuous line shows the simulation of this spectrum (see Discussion). (B) Kinetic behavior of the polarization effects of A measured at 0.3304 T for different microwave powers.

12. We note that the polarized spectrum of Fig. 6A is very similar to that found in nonreduced reaction centers of the bacterium *Rps. sphaeroides* strain 2.4.1 (AUT-s particles [23]). Fig. 6B shows the kinetics of the polarized signal for different microwave powers. The kinetics show oscillations that are dependent on microwave power (note that at high microwave power three 'components' are seen). Our conclusion is that these oscillations arise from a combination of spin polarization, field modulation and transient nutation effects in inhomogeneously broadened lines and do not represent chemical decay. Even at extremely low microwave power (20 nW) oscillations were still present that lasted about 3.5 ms. The fact that even at this low power the decay of the ESR signal still shows a slow oscillation indicates that the decay does not approach the limit in which the decay curve is a simple exponential with half-time equal to $0.7T_1$ [24]. Hence, at 5 K T_1 must be appreciably slower than 5 ms. This lower limit for the spin-lattice relaxation time is compatible with the T_1 of 0.8 ms at 20 K found by Rose and Bearden [25] using the saturation recovery method and the T_1 of 4 ms obtained in our laboratory using electron spin echo spectroscopy at 5 K (Mushlin, R.A., unpublished results).

Discussion

Previous work in this laboratory on green bacteria showed that the amount of triplet state of the primary donor, P^T-840, formed in the light increased upon reduction of the acceptor X_1. This led to the conclusion that there exists at least one acceptor earlier than X_1. Because of the similarity between the acceptor side of PS I and that of the green bacterial photosystem, it is of interest to check whether similar behavior is exhibited for PS I. In LDS particles that lack the acceptor F_X,F_B and F_A [14] the amplitude of the ESR signal of P^T-700 appeared to decrease upon reduction of A, although it was not shown that P^T-700 decreased proportionally to the level of reduction of A [14]. It was concluded that P-700A^- is the radical pair that generates P^T-700. Similar experiments were as yet not performed on PS I particles with intact acceptor side (Triton particles). If A is indeed the earliest acceptor (one of the geminate radicals

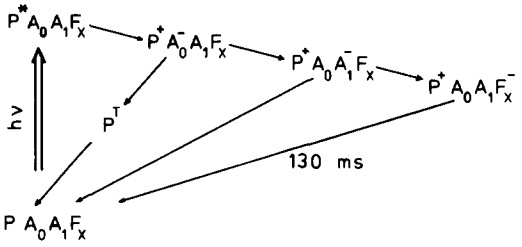


Fig. 7. Schematic representation of the photoreaction in Triton particles. P is the primary donor, F_X represents the most primary iron-sulfur center; A_0 and A_1 are the two acceptors that give rise to the ESR spectrum at g 2.00.

giving rise to P^T-700) one would expect in Triton particles an increase in P^T-700 on reduction of F_X and a decrease in P^T-700 on subsequent reduction of AF_X^- to $A^-F_X^-$.

Our results show that Triton and LDS particles behave differently with respect to the formation of P^T-700. In Triton particles, the triplet does not increase in amplitude when F_X is reduced while A remains oxidized (Fig. 3A). These results strongly suggest that P^T-700 does not originate from P-700A^-. In support of this it can be seen in Fig. 4 that the g value and line width of the so-called A^- spectrum in Triton particles (Fig. 4A and B) is microwave dependent. These effects are explained using a simulation of two Gaussians of equal area with different g value, line width and saturation behavior (continuous lines in Fig. 4A and B). The ESR characteristics of these two radicals are g_1 2.0017; $\Delta H_{pp} = 1.15$ mT and g_2 2.0054; $\Delta H_{pp} =$ 1.08 mT. This indicates that A^- consists of two radicals of different g value, line width and T_1 : A_0 and A_1. A^- represents the g 2.00 signal that consists of $A_0^- + A_1^-$. Our conclusion is that the triplet state P-700 originates from the recombination P-700$A_0^- \rightarrow$ P-700A_0. Fig. 7 shows a scheme of the primary reaction in PS I in which the above conclusions have been incorporated. On the basis of the linear relation between the triplet yield and reduction of A in LDS particles found in Fig. 3B, one would expect that in these particles the spectrum of A^- accumulated in the light consists of only one of these two radicals. Presumably, this species represents the earlier intermediary acceptor, which we designated by A_0^-. In Fig. 4B it is shown that the microwave power dependence of

the g value and the line width of the g 2.00 signal in LDS particles treated with 5% LDS are indeed much less pronounced than for Triton particles. This is especially clear for accumulation at $-70°C$. Apparently, at this temperature, illumination at low redox potential produces only one of the two radicals with an ESR spectrum around g 2.00 that are found in Triton particles after similar treatment. At higher temperature of accumulation also in LDS particles two radicals are produced. From the simulation from Fig. 4A using Triton particles it was shown that the g value of A_0 is 2.0017 with $\Delta H_{pp} = 1.15$ mT. In LDS particles, however, we find that the radical accumulated at $-70°C$ (A_0^-) has a g value of 2.0033 with $\Delta H_{pp} = 0.8$ mT. These values deviate considerably from the values found for A_0^- and A_1^- in Triton particles. If our contention that predominantly a single radical species is produced in LDS particles at $-70°C$ is correct, then the question arises as to why the ESR parameters are different from that of A_0^- in Triton particles. In addition, the g values and line widths of A_0^- and A_1^- in Triton particles are not typical for chlorophyllous anions, whereas the parameters for the radical in LDS particles are close to the g value and line width of either a chlorophyll or a pheophytin anion [26]. The above discrepancies might be explained by the presence of reduced ferrodoxins in the proximity of $A_0^- + A_1^-$ in Triton particles, giving rise to a g-value shift and line broadening arising from magnetic interactions [27]. Pure exchange interaction between A_0^- and A_1^- would give rise to a split ESR line of asymmetric shape [28]. In view of the different saturation behavior of A_0^- and A_1^- such an interaction is probably not the sole cause of the apparent g-value shift but a contribution to the observed signal shape in Triton particles is not excluded. Experiments conducted at higher microwave frequency might sort out these effects. The kinetics of optical changes in the submicrosecond time range have so far given no clear evidence for the existence of more than one chlorophyll acceptor. However, these studies have been hampered by the relative large changes due to P-700 photooxidation and possibly antenna reactions [30,31].

McLean and Sauer [29] recently reported observations qualitatively similar to ours for particles similar to our Triton preparation. However, they found that the maximal amplitude of their photoaccumulated ESR signal at g 2.00 (A^-) amounted to 12 spins per P-700, in contrast to our maximal value of 2–3 spins per P-700. This, and our near stoichiometry (i.e., the g-value shift and line narrowing as a function of microwave power can only be explained with about equal concentrations of A_0^- and A_1^-) suggest that at least for our preparations the two radical species in Triton particles represent two intermediate electron acceptors and not one acceptor plus reduced antenna chlorophyll. If the latter were to be the case, it would be hard to understand why the triplet yield in Triton particles is independent of the amplitude of $A_0^- + A_1^-$ and why in LDS particles the purported antenna chlorophyll cannot be photoreduced.

Heathcote and Evans [7] observed for the ESR spectrum of A^- in digitonin particles a spectrum with complex line shape. They attributed the line shape of the spectrum to partly resolved hyperfine interaction. However, this spectrum may also be explained with two radical signals having different line width and g value. Baltimore and Malkin [8] found a light-induced signal at g 2.0025 with $\Delta H_{pp} = 1.0$ mT in LDS particles that was almost completely reversible in the dark. Their conclusion was that P-700A^- was formed in the light. From an analysis of the line shape, A^- was found to have a g value of 2.0025 with $\Delta H_{pp} = 1.2$ mT. This result deviates from our findings and those of Ref. 14. However, since the preparation procedure of the LDS particles in Ref. 8 was more refined than ours and that of Ref. 14, this contrasting result may imply that is is possible to prepare PS I particles that only lack the ferredoxin acceptors and still contain A_1 that is active at low temperature. It would be of interest to monitor in such particles the decay time of the g 2.0025 signal and the dependence of P^T-700 on the redox states of the acceptors.

Shuvalov et al. [5] found in Triton particles at 50 K a flash-induced radical signal at g 2.00 which decayed in 1.3 ms. They attributed this signal to the radical pair P-700A^-. In contrast, we found in Triton particles with prereduced acceptor F_B and F_A a flash-induced spin-polarized signal at g 2.00 (Fig. 6A) which is similar to that reported in Ref. 12 and which lasted for at least 3 ms at 5 K. The

spectra we observed at 50 K were difficult to interpret owing to faster spin-lattice relaxation at that high temperature, but strong polarization effects were still present. The fact that Shuvalov et al. [5] did not observe these effects, which are most clearly notable in the low- and high-field wings of the spectrum, might be attributed to overmodulation (a 6 G wide field modulation was used) and inadequate spectrometer time constant (200 μs). In view of the fact that decay rates as measured with ESR for polarized signals are dependent on microwave power, the agreement between the decay half-time measured as decay of the ESR signal and as the decay of the optical absorption difference spectrum must be regarded as coincidental. This conclusion is strengthened by a recent paper by Setif et al. [32] in which a careful study has been made of the optical absorption difference spectra and decay kinetics after flash excitation of LDS particles. Their results corroborate our findings that the 1 ms component found at cryogenic temperature is not due to the back-reaction P^+-$700A^-$ → P-700A but is due to the triplet state of P-700 that is generated by radical pair recombination. This assignment agrees with the average decay rate of P^T-700 ($\bar{k}$) at 5 K which we and Mathis and co-workers (personal communication) found to be 1.3 ms for Triton and LDS particles. The complex kinetics of the spin-polarization effects around g 2.00 are explained by us as arising, to a large extent, from transient nutation effects [24]. When the effects are taken into account, T_1 of the radicals involved has a lower limit of several milliseconds.

Following a suggestion of Pedersen [33], we have attempted to simulate the spin-polarized ESR spectrum around g 2.00 using the concepts of static polarization (polarization of the geminate radical pair) and dynamic polarization (polarization that is formed by transfer of polarization from one of the radical pair partners to another acceptor or donor):

$$(P^+A^-)_{RP} \rightarrow P^+_{static}A^-_{static}$$

$$(P^+A_0^-)_{RP}A_1 \rightarrow P^+_{static}A_0A^-_{1dynamic}$$

The result of the simulation is shown in Fig. 6A (continuous line). As can be seen, this simulation model, which we have also succesfully used to explain the polarization effects found in bacterial reaction centers [23], gives a very satisfactory fit to the experimental spectrum. In the simulation we used a statically polarized ESR spectrum with g 2.0025, ΔH_{pp} = 0.65 mT and a dynamically polarized ESR spectrum with g 2.0053, ΔH_{pp} = 0.9 mT, while the intermediary acceptor was centered at g 2.0016. The g value of the intermediary acceptor found with this simulation agrees with the g value for the intermediary acceptor A_0 found by simulating the microwave saturation effects on the line shape of A^- (Fig. 4). The static-dynamic polarization model as applied by us does not immediately explain the orientation effects on the polarized signal [12]. To include such effects, anisotropic electron transfer has to be assumed. Therefore, although the fit with the static-dynamic polarization model is at least as good as that obtained with the two-site model [9,10], further experiments, e.g., on oriented Triton particles or on LDS particles as prepared by Baltimore and Malkin [8], have to be done before the static-dynamic model can be accepted as an alternative explanation of the spin-polarization data.

Summarizing our results we come to the following conclusions:

The acceptors of PS I are: A_0, A_1, F_X, F_B and F_A.

The g value and line width of A_0^- and A_1^- are g_1 2.0017 ± 0.0006; $\Delta H_{pp,1}$ = 1.15 mT ± 0.1 and g_2 2.0054 ± 0.0006; $\Delta H_{pp,2}$ = 1.08 mT ± 0.1, respectively.

Strong illumination at 0°C in the presence of *N*-methylphenazonium methosulfate leads to accumulation of A_0^- plus A_1^-, in equal amounts for Triton and LDS particles treated with 2% LDS.

A_1 in LDS particles is not photoactive at low temperatures; the amount of A_1 present in these particles depends on the LDS concentration used during the preparation.

P^T-700 originates from P^+-$700A_0^-$.

P^T-700 in Triton particles is insensitive to any redox state of the acceptors and most likely arises from particles with damaged electron transport.

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