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ZERO FIELD RESONANCE AND SPIN ALIGNMENT OF THE TRIPLET STATE OF CHLOROPLASTS AT 2°K

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Summary

Microwave induced transitions in zero magnetic field have been observed in the photoinduced triplet of chloroplasts treated with dithionite by monitoring changes in the intensity of the 735 nm fluorescence band at 2°K. Similar results were obtained with chloroplasts treated with hydroxylamine plus 3-(3,4-dichlorophenyl)-1,1-dimethylurea and preillumination. The zero field parameters are $D = 0.02794 \pm 0.00007$ cm^{-1}, $E = 0.00382 \pm 0.00007$ cm^{-1}, i.e. equal to those of monomeric chlorophyll *a* to within the experimental error. The photoinduced triplet appears to be linked to Photosystem II. This indicates that the low temperature 735 nm fluorescence band of chloroplasts is at least partly due to Photosystem II.

The discovery of a photoinduced triplet state in photosynthetic bacteria and chloroplasts [1,2] under reducing conditions has generated much debate whether this triplet state is a precursor of the primary charge separation. The low temperature ESR spectra of these triplets are spin polarized, i.e. some of the ESR lines are emissive rather than absorptive.

Here we report electron resonance experiments in zero field on chloroplasts in the presence of reductant by the Microwave Induced Fluorescence method developed in this laboratory [3]. Analogous experiments have been carried out by R.H. Clarke (private communication) on photosynthetic bacteria.The results of his and our work concur in showing that the microwave induced fluorescence technique is not restricted to in vitro molecular problems but may be used with success on photosynthetic systems of bacteria and plants.

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

From the present experiments it follows that the photoinduced triplet in reduced chloroplasts is linked to Photosystem II and our results provide an explanation for the spin polarization of the triplet signal observed by Uphaus et al. [2]. Our results imply that an essential difference exists in the structure of the chlorophyll complex of the primary donor of Photosystem II and of photosynthetic bacteria.

In our experiments the sample is continuously illuminated and a steady-state distribution is established over the ground state and lower excited states. Then, one of the transitions between the spin components of the triplet state is saturated with resonant microwaves; this equalizes the populations of two of the components and, indirectly, causes a slight displacement in the entire steady-state distribution. In the microwave induced fluorescence technique this perturbation is detected via a small change in the fluorescence intensity [3].

Chloroplasts were prepared from spinach as described in [4]. Reduction of the chloroplasts was carried out by adding dithionite (10^{-1} M for 30 min under N_2 atmosphere) or by adding 10^{-2} M hydroxylamine plus 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea, and illumination prior to freezing. The microwave induced fluorescence apparatus has been described [3].

Fluorescence was excited by broad banded blue light, centered at 400 nm; detection took place via a cut-off filter and a monochromator or narrow band interference filters. A fluorescence spectrum obtained at 2°K of chloroplasts treated with hydroxylamine and DCMU, is displayed in Fig. 1. Spectra of chloroplasts with dithionite, or without reductant were similar in shape. Microwave induced fluorescence experiments were carried out monitoring the fluorescence at the 682, 692 or 735 nm band positions. Only at the 735 nm fluorescence peak zero field transitions were observed. Fig. 2 shows the microwave induced fluorescence resonance for chloroplasts treated with dithionite or hydroxylamine and DCMU. In agreement with ESR data [1,2] we observed two transitions at 723.0 and 952.0 MHz (± 2 MHz) having a width of 25 and 20 MHz, respectively. The intensities of the resonances are about 0.02% of the 735 nm fluorescence peak. When identifying the resonances with the $D-E$ and $D+E$ transitions within the triplet one obtains $D = 0.02794 \pm 0.00007$ and $E = 0.00382 \pm 0.00007$ cm^{-1} for the zero field parameters. This compares to $D = 0.0274 \pm 0.0005$ and $E = 0.0032 \pm 0.0005$ cm^{-1} for chlorophyll *a* in a glass [5]. The remarkable narrowness of the lines compared to ZFR lines in glassy solution suggests that the triplet resides on molecules in identical surroundings, e.g. in reaction centers rather than on bulk chlorophyll.

In principle one might expect a third resonance at a frequency of $2E/h$. We have not been able to find a signal at 229 MHz, even with a double resonance technique in which two microwave transitions are irradiated simultaneously.

Reduction with NH_2OH plus DCMU and light only reduces Photosystem II, leaving Photosystem I unchanged [6]. Control microwave induced fluorescence experiments with chloroplasts without reductant gave a negative result. Since chloroplasts treated with dithionite gave the same signal as after treatment with NH_2OH plus DCMU and light we conclude that the photoinduced triplet in reduced chloroplasts is due to Photosystem II reaction

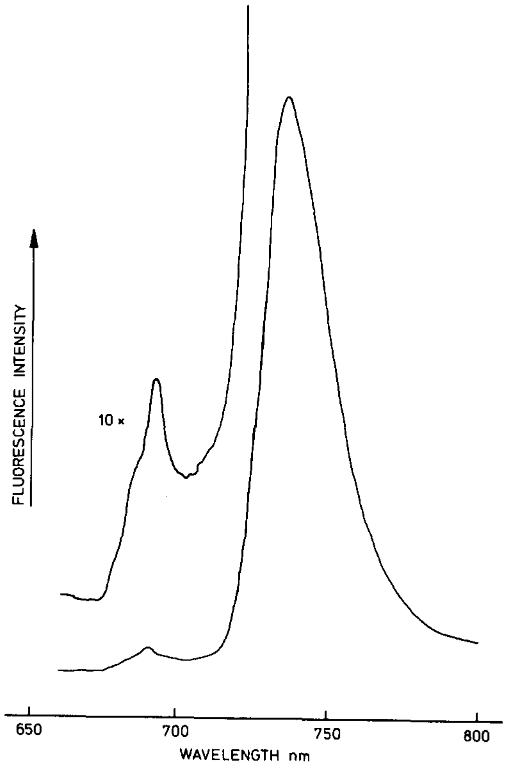

Fig. 1. Fluorescence emission spectrum at 2°K of chloroplasts treated with 10 mM NH_2OH plus 10 μM DCMU and preillumination before freezing. Chlorophyll concentration 0.2 mg/ml, pathlength 2 mm. Excitation: Philips SP 1000 high pressure mercury arc filtered by 10 cm of a solution of $CuSO_4$ (100 g/l) in H_2O, and Corning CS 4-76 and Schottky BG 25 filters, the light falling perpendicular on the sample. Detection: Schottky RG 630 cut-off filter followed by a ¼ m Ebert monochromator and a EMI type 9658 A photomultiplier. No corrections for self absorption and photomultiplier sensitivity have been applied.

centers. That the ZFR peaks were only observed at the 735 nm fluorescence peak, but not at the 682 or the 692 bands is an unexpected result, since the 735 band (which appears only at low temperatures) is considered to be due to Photosystem I [7,8]. Our results indicate that at least part of the 735 nm fluorescence is excited via Photosystem II.

The kinetics of the population and depopulation of the triplet sublevels has not yet been measured quantitatively, owing to the weakness of the signals. From the change of the signal with modulation frequency and the fact that the signal corresponds to a decrease in fluorescence intensity, it was concluded that there are two fast and one slowly decaying level. This, coupled to the fact that only two resonances are observable, leads to a steady-state population roughly as in Fig. 3. Hence in a magnetic field, the populations are expected to be distributed as shown in Fig. 3 for the three principal derictions of the magnetic field. The predicted ESR spectrum shown at the bottom of Fig. 3 agrees remarkably well with the spectrum published by Uphaus et al. [2]. Schemes similar to that of Fig. 3 can account for the ESR

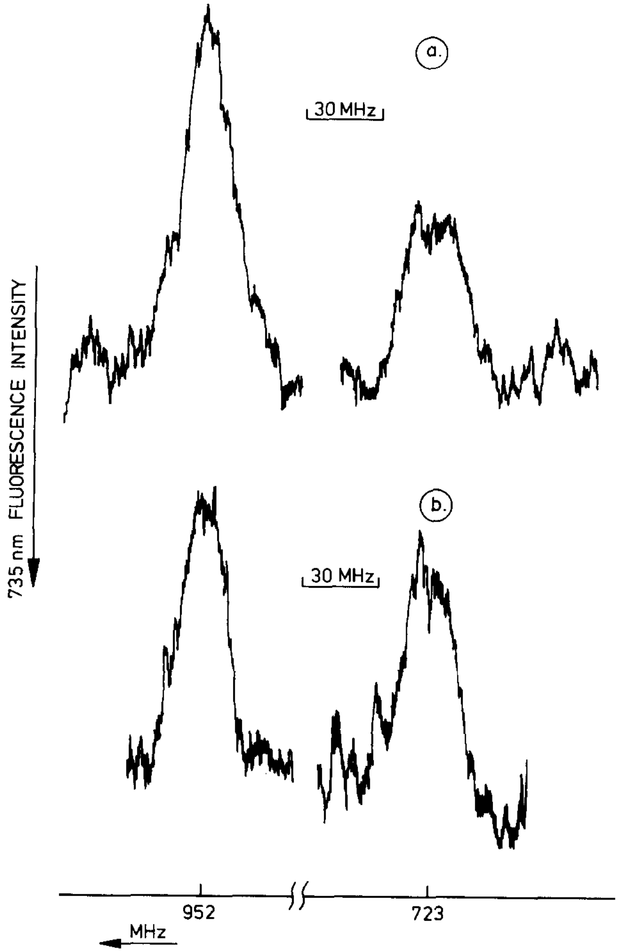

Fig. 2. Zero field resonance microwave induced fluorescence spectra of chloroplasts at 2°K (a) treated with 10^{-1} M dithionite for 30 min under N_2 atmosphere, (b) treated with hydroxylamine (10^{-2} M) and DCMU (10 μM) followed by illumination prior to freezing. Excitation as in Fig. 1. Detection: RG 630 cut-off and Balzer B-40 738 nm interference filter. Microwaves were generated by a Hewlett Packard HP 8690 B sweep oscilator, power level 20 mW, amplitude modulation 237 Hz, scanning rate 7.5 MHz/min, time contant 30 s. All spectra are single scans; maximum peakheight about 0.02% of the 735 nm fluorescence intensity.

spectra of the triplets of chlorophyll *a* and *b* and bacteriochlorophyll in glassy solution. However, as noted by Norris [5], the distribution of emissive and absorptive character over the ESR spectrum of the photosynthetic bacterium Rhodospirillum rubrum cannot be explained in the same way. in fact the spin polarization of this spectrum cannot be obtained by any population mechanism along the usual spin-orbit coupling scheme within a single molecule. This together with the fact that the zero field parameters of the chloroplast triplet are, within the limits of error, identical to those of monomeric chlorophyll *a* in vitro as measured from the triplet ESR spectra by Norris et al. [5] suggests that the unpaired electrons giving rise to the triplet state in the

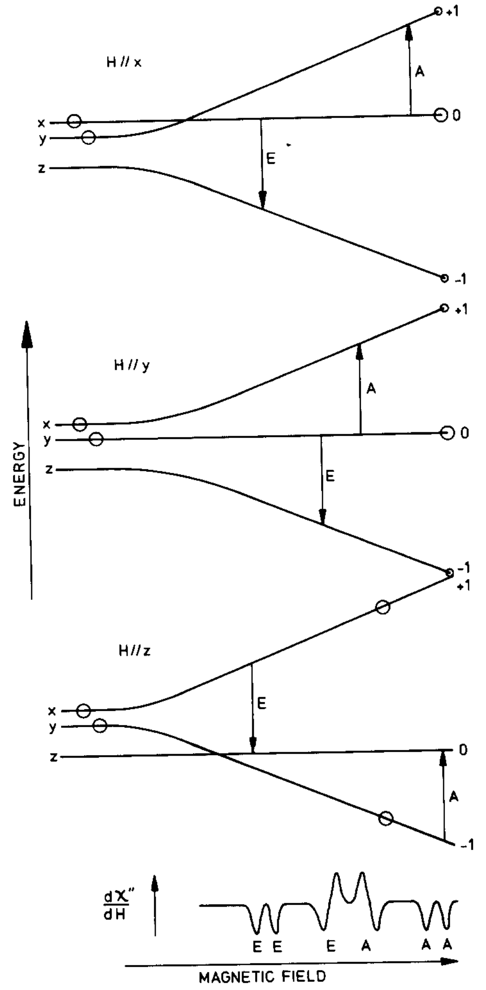

Fig. 3. Schmatic representation of the triplet sublevels and their population in zero and high magnetic field at direction of the field parallel to the molecular x, y or z axis. ESR transitions are indicated by arrows. The corresponding ESR spectrum is schematically drawn at the bottom of the figure. A and E stand for absorptive and emissive lines respectively. The size of the circles represents the steady-state population.

reaction center of System II are not delocalized over two chlorophyll molecules, as is the case with the unpaired spin of the oxidized primary donor in the bacterial and Sytem I reaction center [9,10,11] and possibly also in the System II reaction center [12]. This would then rule out a structure analogous to the $BChl^{-} \cdot BChl^{+}$ complex proposed by Feher et al. [10] and Norris et al. [5].

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References

1 Dutton, P.L. and Leigh, J.S. (1973) Biochim. Biophys. Acta 292, 654—664
2 Uphaus, R.A., Norris, J.R. and Katz, J.J. (1974) Biochem. Biophys. Res. Commun. 61, 1057—1063
3 Van Dorp, W.G., Schaafsma, T.J., Soma, M. and van der Waals, J.H. (1973) Chem. Phys. Lett. 21, 221—225
4 Visser, J.W.M., Amesz, J. and van Gelder, B.F. (1974) Biochim. Biophys. Acta 333, 279—287
5 Norris, J.R., Uphaus, R.A. and Katz, J.J. (1975) Chem. Phys. Lett. 31, 157—161
6 Bennoun, P. (1970) Biochim. Biophys. Acta 216, 357—363
7 Butler, W.J. (1961) Arch. Biochem. Biophys. 93, 413—422
8 Lozier, R.H. and Butler, W.L. (1972) FEBS Lett. 26, 161—164
9 Feher, G., Hoff, A.J., Isaacson, R.A. and McElroy, J.D. (1973) Biophys. Soc. Abstr. 13, 61
10 Feher, G., Hoff, A.J., Isaacson, R.A. and Ackerson, L.C. (1975) Ann. N.Y. Acad. Sci. 244, 239—259
11 Norris, J.R., Druyan, M.E. and Katz, J.J. (1973) J. Am. Chem. Soc. 95, 1680—1684
12 Van Gorkum, H.J., Tamminga, J.J., Haverman, J. and van der Linden, I.K. (1974) Biochim. Biophys. Acta 347, 417—438