ELECTRON SPIN RESONANCE IN ZERO MAGNETIC FIELD OF TRIPLET STATES OF CHLOROPLASTS AND SUBCHLOROPLAST PARTICLES

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1. Introduction

Recently, the new technique of electron spin resonance in zero magnetic field, as observed by Microwave Induced changes in Fluorescence (MIF) has been applied to the study of the light-induced triplet state in chloroplasts under reducing conditions [1]. Two resonances were detected at 723 and 952 MHz. The resonances were observed also in samples in which the acceptor of photosystem 2 was reduced with light in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and hydroxylamine (NH₂OH). This led to the conclusion that the observed triplet was linked to photosystem 2 (PS₂). The remarkable narrowness of the lines suggested that it was located on pigment molecules in identical surroundings, i.e., on the reaction center chlorophyll. In order to verify these conclusions, we have carried out MIF experiments on subchloroplast particles enriched in either photosystem 1 (PS₁ particles) or photosystem (PS₂ particles) by digitonin treatment. In contrast to the work on whole chloroplasts, the resonances described above could not be detected in the subchloroplast particles, even when the microwave power incident on the sample was greatly increased. Apparently, rupture of the photosynthetic complex of chloroplasts prevents the formation of this triplet. This means that in intact chloroplasts, it is formed by an excitation energy transfer from the PS₂ units with closed traps to PS₁ units, and is trapped on a fraction of antenna chlorophyll that after digitonin treatment is removed or is no longer available for energy transfer.

The application of stronger microwave fields had led to the discovery of a set of new MIF resonances at higher frequencies. The new resonances were present in chloroplasts, with or without reducing agents and unique differences were observed, between PS₁ and PS₂ particles. The signals can be attributed to two triplets with slightly different zero field splittings and different population and/or decay kinetics of the three triplet sublevels. The triplets are probably located on different fractions of antenna chlorophyll. MIF experiments monitoring the chlorophyll a fluorescence bands at 682, 692 and 735 nm of chloroplasts, PS₁ and PS₂ particles suggest that one of the triplets (triplet I) may be located on the F735 antenna chlorophyll of photosystem 1, whereas the other triplet (triplet II) is either located on the F692 antenna chlorophyll associated with photosystem 2 or on the F682 light harvesting protein pigment (LHPP).

In all preparations a third resonance at 232 MHz was observed, attributed to the 2E transition, common to triplet I and triplet II.

2. Materials and methods

2.1. Preparation and characteristics of the samples

Chloroplasts were prepared as described in ref. [2]. Fractionation into photosystem 1 and photosystem 2 enriched particles was carried out by digitonin treatment [3]. In order to remove solubilised chlorophyll the PS₁ and PS₂ fraction were passed over a Sephadex G-100 column and eluted with 50 mM phosphate-buffer (pH 7.2) containing 20 mM NaCl. Chlorophyll

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Table 1

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<thead>
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<th>Chloroplast</th>
<th>PS1 enriched particles</th>
<th>PS2 enriched particles</th>
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<tbody>
<tr>
<td>P-700: Chl a</td>
<td>1:390</td>
<td>1:135</td>
<td>1:500</td>
</tr>
<tr>
<td>Chl a: Chl b</td>
<td>2.2</td>
<td>3.5</td>
<td>1.6</td>
</tr>
<tr>
<td>F735: F685</td>
<td>1.1</td>
<td>4.8</td>
<td>0.75</td>
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a/b ratios (measured in 80% acetone [4]) and P-700 content (determined photochemically using an extinction coefficient of 64 mM⁻¹ cm⁻¹ at 700 nm [5]) of the preparations used are given in table 1.

Samples were taken from freshly prepared stock solutions with an A₇₀₀ 1 - 1.2 per mm at the red chlorophyll a maximum, diluted two-fold with glycerol and frozen in liquid nitrogen for our experiments. Reduction was carried out by adding excess solid dithionite under a nitrogen atmosphere prior to dilution with glycerol. Optical pathlength of the samples was 2 mm.

2.2. The MIF method

The technique of detection of electron spin resonance in zero magnetic field by monitoring microwave induced changes in fluorescence was first described by van Dorp et al. [6,7]. In this technique, a sample at low temperature (in our experiments, 2°K) is continuously irradiated and a steady-state distribution of molecules over the ground state S₀ and the lower excited states (S₁, T₀) is established. Then, one of the transitions between the spin components (Tₓ, Tᵧ, T₀) of the triplet T₀ is saturated with resonant microwaves; this equalizes the population of two of the components and, indirectly, causes a slight displacement in the entire steady-state distribution. This perturbation is detected via a small change in fluorescence intensity which is proportional to the change in the population of S₀. The MIF set-up was essentially as described earlier [1,7]. Microwaves were generated by a Hewlett-Packard HP 8690B sweep oscillator (output, 20 mW) and amplified between 500 and 1000 MHz by a solid-state amplifier to a level of 1 W and between 1000 and 2000 MHz by a travelling wave tube amplifier (Varian VZL-6941-A1) to a level of 8 W.

Chlorophyll a fluorescence was monitored at the 682, 692 and 735 nm band positions using Balzer B-400 684 nm and Schott AL 692 and AL 730 nm interference filters. The excitation light source was an Oriel HBO 1000 W high-pressure mercury arc filtered by a solution of CuSO₄ (100 g/l) in H₂O and Corning 4-76 and Schott BG 25 filters.

3. Results

In chloroplasts treated with dithionite, but not in chloroplasts without reducing agents, the earlier described [1] resonances at 723 and 952 MHz were observed. However, in the PS1 and PS2 particles these resonances were absent, also for microwave power 50-fold higher than previously used. In contrast, the higher power levels allowed the observation of new resonances. Investigation of the range between 1000 and 2000 MHz revealed the spectra displayed in fig.1. Broad peaks are observed at 1047 and 1279 MHz with some structure. Chemical reduction of the primary acceptors by dithionite had only little influence on the spectra. No new resonances were observed in the 500-1000 MHz range, but at 232 MHz a third resonance was detected.

We have attempted to assign the new signals to a particular photosystem by carrying out MIF experiments on the 682, 692 and 735 nm fluorescence bands. The 735 nm band is associated mainly with PS1, whereas the 682 nm and 692 nm bands are associated mainly with PS2 [8]. Figure 1 summarises the results obtained at these fluorescence bands for the three preparations investigated. Apart from some differences in fine structure, the most notable change in the spectra is the appearance of a strong resonance
Fig. 1. Zero field resonance Microwave Induced changes in Fluorescence (MIF) spectra of untreated chloroplasts, photosystem I and photosystem 2 particles at 2°K. Arrows indicate increase in fluorescence. Spectra are single scans, scanning rate 0.6 MHz/s, time constant 30 s. Photomultiplier current was kept constant for the various fluorescence intensities.
Table 2

Characteristics of triplets I and II

<table>
<thead>
<tr>
<th>Transition frequency MHz</th>
<th>D (× 10^-4 cm⁻¹)</th>
<th>E (× 10^-4 cm⁻¹)</th>
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<tbody>
<tr>
<td>Triplet I</td>
<td>232.5 ± 0.5</td>
<td>1047 ± 5</td>
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<tr>
<td></td>
<td></td>
<td>388 ± 1.5</td>
</tr>
<tr>
<td>Triplet II</td>
<td>232.5 ± 0.5</td>
<td>995 ± 5</td>
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at 995 MHz of opposite polarity, observed at the 692 and 682 nm band positions for chloroplasts and PS2 particles but not (or only very little) for the PS1 particles. Discrimination between the 692 and 682 positions is rather poor, because of appreciable overlaps of the interference filters used (bands centered at 684 and 693 nm, bandwidth 14 and 20 nm respectively). We assign the 1047 and 1279 MHz lines to the D-E and the D+E transitions of one triplet (triplet I) respectively and the 232 MHz line to the 2E transition; its asymmetric shape is probably due to the fine structure on the high frequency lines. The 995 MHz line must be due to a second triplet (triplet II) with somewhat different zero-field splitting parameters and population characteristics. The intensity of the 232 MHz line and the line shape, varies little for the three preparations and various wavelengths. This suggests, that it is common to the two triplets. D- and E-values of triplet I and II calculated on this basis are given in table 2.

4. Discussion

The absence in subchloroplast particles of the 723 and 952 MHz resonances previously observed in chloroplasts [1] indicates that the triplet giving rise to these transitions is not located on reaction center chlorophyll. Apparently, in intact chloroplasts in the presence of reducing agents it is formed by trapping of an excitation in antenna chlorophyll a monomer. Our new MIF data do not support the conclusion that the primary donor of photosystem 2 is a chlorophyll a monomer.

The new resonances observed with high microwave power in chloroplasts and subchloroplast particles must be due to, at least, two different triplets. The resonance of 990 MHz with negative polarity (upward peak in fig.1) is practically absent in preparations devoid of photosystem 2 and when MIF is detected on the 735 nm band. The high frequency resonances with positive polarity vary little for the various preparations and wavelengths.

The two triplets giving rise to the 995, 1047 and 1279 MHz lines have presumably, in common, the 232 MHz transition. In order to assign the two triplets to particular antenna pigment fractions, we use the assignments suggested recently by Butler and Kitajima [9]. In this model, F735 originates in antenna chlorophylls of PS1 (also see [8]), F682 to the light harvesting pigment protein complex (LHPP) and F692 to the antenna chlorophyll of the PS2 complex. Our particles labelled PS1 contain an enrichment in the F735 nm antenna chlorophyll fraction and the photosystem 1 reaction center, and the PS2 particles contain an enrichment of the F692 and F682 containing fractions. It may be noted that the PS2 particles still contain some F735. This is either due to incomplete removal of PSI material or to an intrinsic contribution of the PS2 complex at this wavelength, enhanced by selfabsorption. With regard to the location of triplet II (which gives rise to the 995 MHz line) we can explain the data of fig.1 in two ways: either triplet II is located on the LHPP fraction or it resides on the F692 antenna pigment. It is not formed on photosystem 1 F735 component.

The available evidence with regard to the location
of triplet I is less clear. The variations in the 1047 and 1279 MHz lines are small. The high MIF intensity at 739 nm in chloroplasts suggests that it may be due to F735. However, the lines appear almost as strongly in the PS2 as in the PS1 particles. The lines cannot be due to triplet formation on solubilised chlorophyll since this is removed by the Sephadex treatment. Triplet I is either located in PS1 only or on both PS1 and PS2. Experiments with higher spectral resolution using higher purified subchloroplast particles are needed to pin down the precise location of triplet I.

With regard to the population of the three sublevels of triplet I and II, we can derive tentative conclusions from the polarity of the observed lines. In fig. 2, one solution for triplet I and two possible solutions for triplet II are shown. It must be kept in mind that the difference in the populations (schematically represented by the size of the circles) are much exaggerated; they are probably of the order of a few percent at most [10].

It is seen that the scheme for triplet I gives an increase in fluorescence for all combinations of two out of three sublevels, whereas the schemes for triplet II give an increase for the $2E$ transition, a decrease for the $D-E$ transition and no change for the $D+E$ transition in accord with our observations. It should be noted that one can reverse the relative values of decay rates and populations in a concerted way and obtain the same stick spectra. An investigation of the kinetics of the system is necessary to remove this ambiguity. This would also constitute to the identification of the molecular species giving rise to triplet I and triplet II. The high $D$-values seem to point to a non-chlorophyll component, possible pheophytin. Recently, weak MIF resonances observed in reduced algae, at frequencies of 1040 and 1160 MHz, were attributed to pheophytin derived from photoreduced chlorophyll [11]. The weak phosphorescence recently measured in vivo by Krasnovsky et al. [12] may be due to either one, or a combination of the triplets I and II and the triplet observed only in reduced material.

Although much has yet to be cleared up, we feel that the MIF technique is a powerful new tool to study not only the intricacies of the primary reactions [10] but also to learn more about the configuration of the entire photosynthetic apparatus.

Acknowledgements

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Fig. 2. Schematic representation of the triplet I and triplet II systems (see discussion). Length of arrows represents decay rates, the site of the circles represents the populations of the sublevels. The triplet I representation is one of two possible configurations, as is each of the triplet II configurations. The difference in population is much exaggerated. Energy levels are not drawn to scale.

References


