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FDMR of chlorophyll triplets in integrated particles and isolated reaction centres of Photosystem II. Identification of P680 triplet

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Abstract

Fluorescence detected magnetic resonance (FDMR) of various chlorophyll *a* triplets in PS II large particles was studied with the aim of identifying the nature of the different components underlying the complex line shape of the signals. The latter are deconvoluted into sums of Gaussians in different experimental conditions, and the charge recombination triplet is unequivocally identified as the source of two lines with negative signs at 722.5 and 992 MHz ($|D| = 0.0286$ cm^{-1}, $|E| = 0.0043$ cm^{-1}). The origin of the other triplet signals from the integrated particles is discussed in terms of functional antenna components and products from alteration of the native environment of pigments. FDMR signals also arise from isolated reaction centre complexes and are all to be attributed to different triplet populations of the primary electron donor P680. The results are compared with those obtained by other authors on ADMR. The heterogeneity of P680 pigments in D_1D_2-cyt-b_{559} complexes is shown to arise during preparation procedures.

Key words: Fluorescence detected magnetic resonance; FDMR; Photosystem; Chlorophyll; Triplet; P680

1. Introduction

It is well known that magnetic resonance is suitable for studying the reaction centre and the antenna pigments of the photosynthetic apparatus in bacteria and in higher organisms. In particular, for RC pigments, optically detected magnetic resonance (ODMR) has been used extensively to study bacterial systems [1,2] and has recently been applied to the isolated RC complex of Photosystem II (PS II) of higher plants, the well known D_1D_2-cyt-b_{559} complex [3,4].

This complex is composed of D_1 and D_2 polypeptides, and of one or two molecules of cyt b_{559}, a redox component whose function has yet to be clarified [5]. D_1 and D_2 peptides are known to be the higher organism's counterpart of the M and L peptides contained in the purple bacteria reaction centre and are the locus of the pigments required for the primary steps of light induced electron transport: these pigments are the primary electron donor chlorophyll(s), P680; two molecules of pheophytin, one of which is the first electron acceptor and two (or four) more molecules of Chl *a*, whose function is not yet identified. Two further acceptor molecules, quinones Q_A and Q_B, are present in the complex but are lost during preparation procedures [6].

In the recent papers quoted above [3,4] the ODMR spectra were mainly discussed in terms of the triplet of P680, the primary donor of PS II, formed through the radical pair mechanism after charge separation and recombination. The highly inhomogeneous signal shape was attributed to conformational differences in the

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Abbreviations: PS I, Photosystem I; PS II, Photosystem II; BBY, Photosystem II enriched membranes, known as BBY particles; LCH II, light harvesting complex from Photosystem II; D_1D_2, isolated reaction centre of Photosystem II, known as D_1D_2-cyt-b_{559} complex; P680, primary electron donor to Photosystem II; RC, reaction centre; ODMR, optically detected magnetic resonance; ADMR, absorption detected magnetic resonance; FDMR, fluorescence detected magnetic resonance; ZFS, zero field splitting; ISC, intersystem crossing; Q_A, primary quinone acceptor of PS II; Q_B, secondary quinone acceptor of PS II; Chl *a*, chlorophyll *a*; MES, 4-morpholineethanesulphonic acid; DCM, β-dodecyl-maltoside; FeCy, potassium ferricyanide; MV, methylviologen.

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reaction centres trapped at the low temperature of the experiments. The ODMR version used was that of detecting differences in optical absorbance upon the application of microwave power, resonating with a pair of the levels of the triplet species (ADMR).

Pioneering work has been done on more integrated systems, such as PS II particles containing all the antenna complexes and the reaction centre (the so-called BBY particles), by using both the fluorescence (FDMR) [7,8] and the absorption detection (ADMR) method [9]. No definite conclusion has yet been reached about the identity of the triplets.

Recently we have shown that comparative analysis of the results obtained by the two above detection techniques may give additional information [10,11]. A recent FDMR study of the other reaction centre of plant chloroplasts, Photosystem I (PS I) [12] has also provided some new interesting information. Here we report new experiments on PS II particles and purified RC particles, using the fluorescence detection ODMR technique.

We thought it worthwhile to re-examine the FDMR spectra of BBY particles and also examine RC complexes by the same technique, in order to see if comparison between the two techniques, and between the isolated centre and the more integrated particles, could lead to better identification of functional P680 chromophores among the various triplet species detected within the very inhomogeneous ODMR spectral lines. The other possible sources of Chl *a* triplets are clearly antenna chlorophylls, RC accessory chlorophylls, and chlorophylls in unnatural environments created by preparation procedures.

The main result of this investigation shows that the FDMR signal shape depends on experimental settings such as light intensity, temperature and instrumental modulation frequency: these factors influence the composition of the steady state triplet population under continuous illumination, which is exploited to discriminate the various possible triplet species. The systematic recording of spectra of particles containing the RC together with antenna components, in different experimental conditions, clearly distinguishes the primary donor recombination triplet from other Chl *a* triplets which are formed at the same time in the steady state in anaerobic conditions.

Our experiments were carried out while bearing in mind the concept that, in large functional PS II particles, fluorescence within the 680–690 nm band is due to antenna components transferring efficiently to the RC, as is well known from the behaviour of the fluorescence on 'closing' the trap. Furthermore, we were guided by the conditions in which the triplet EPR spectrum with the characteristic AEEAAE pattern due to radical pair recombination is observed (see [13] for a recent review). The FDMR technique shows that this general model for energy transfer in PS II is also applicable at very low temperatures, as found for PS I [12] and that, in integrated particles, the recombination triplet is clearly distinguishable and gives rise to narrow single species FDMR lines. In isolated RC the various chlorophyll triplets detected all probably originate from P680, in agreement ADMR results. Comparison of ADMR results from BBY particle and isolated centre spectra is found to be useful in our discussion of the heterogeneity found in the RCs.

2. Materials and methods

Apparatus

Our FDMR apparatus is described in [10]. Microwave modulation was provided by a Wavetek Mod 164 square-wave genarator and, depending on the experiment, varied between 10 and 500 Hz. The emission was collected through an interference filter with a 680–690 nm band. The fluorescence emission spectra of our samples were recorded with a Jobin-Yvon monochromator in place of the interference filter. Maximum emission at 4 K was found at 683 nm for BBY particles and 685 nm for isolated RCs. Maximum photon flux was obtained by the use of a 250 W tungsten halogen lamp, a 10 cm diameter lens with a 10 cm focal length, and an 8 cm filter with 0.1 M $CuSO_4$. Flux was diminished to one-hundredth with a set of gray filters. The temperature of the experiment was set by adjusting the pressure downstream of the sample compartment of the liquid helium cryostat (Oxford Spectromag 4). Calibration was performed under experimental conditions.

As usual for macromolecular and biological samples at low temperature, the line shape for a single species transition was considered Gaussian (not Lorentzian) to take into account inhomogeneities deriving from the freezing of slightly different conformations of the species within its immediate environment. Gaussian deconvolution of the signals was performed by trial and error with the expression:

$$\Sigma a_i \exp\left\{-\left[(\nu - \nu_i)/\delta_i\right]^2\right\}$$

In the simulation of a series of BBY spectra taken on the same transition and on the same sample in different experimental conditions, only parameters a_i were allowed to vary. The position and width of the line were considered fixed for the species and not affected by photon flux, temperature (in the low-temperature domain imposed by the technique) or modulation frequency, the three main external parameters. EPR spectra were taken on a Bruker ER 200 D spectrometer equipped with an Oxford CF900 liquid helium cryostat, by modulating the light excitation (20 Hz) as described in [14].

Sample preparations

BBY particles were obtained from spinach leaves in the usual way [15] and the RC was prepared according to the methods described in [16]. Preparations were stored at 77 K in the dark until used. Room temperature absorption and emission maxima of the RC preparations were 675.5 and 683 nm respectively, indicating their good quality according to current standards [17,18]. Sample concentration was generally 100 μg/ml chlorophyll content or less. The medium for the BBY particles was 0.4 M sucrose, 10 mM NaCl, 50 mM MES (pH 6); for isolated RCs it was 50 mM Tris, 2 mM DCM (pH 7.2). Glycerol was always added to 66% v/v to the samples to avoid matrix cracking and heterogeneity. Oxygen was removed from the samples using a glucose/glucose oxidase system as described in [19]. Dithionite was added under nitrogen, when required for BBY acceptor reduction, together with 30 μM methylviologen (MV) and samples were incubated for some time to ensure double reduction of Q_A [20]. The same effects were practically obtained by incubating with 80 mM dithionite for a few minutes as with 10 mM dithionite for a few hours. In the latter case, observed effects were found to be proportional to incubation time. Degradation experiments were carried out on diluted samples of isolated centres (5 μg Chl *a*/ml) incubated at 0°C for 25 min after addition of 1% Triton X-100. 1 mM potassium ferricyanide (FeCy) was added in some RC experiments (as noted in the text) and incubated at 0°C for 20 min before recording the spectra.

3. Results

BBY particles

From the early stages of this investigation it was clear that the details of the FDMR spectra were quite reproducible when the samples were obtained from different batches of preparations or from preparations obtained with slightly varied procedures. Instead, it was evident that the spectra depended on other experimental conditions, such as temperature, reduction state of secondary acceptors, microwave amplitude modulation frequency and excitation light intensity.

The ODMR spectra of Chl *a* triplets consist of two regions of ΔI changes, one in the 710–780 MHz range (|D| − |E| transition) and the other in the 960–1010 MHz range (|D| + |E| transition). A third transition (2|E|) is located at a frequency equal to the difference between the former two; however, in chlorophyll systems, the intensity of this transition is low and differences among triplets ZFS are slight. Indeed, in BBY particles the 2E transition is covered by the analogous intense transition of carotenoid triplets in antenna complexes [10], and in isolated RCs it could be seen only under the highest light fluxes used.

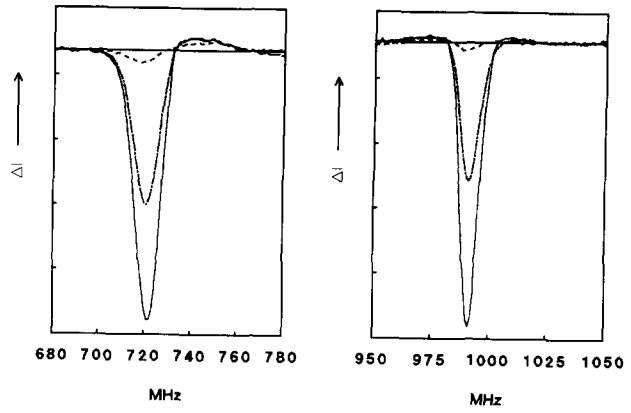

Fig. 1. FDMR spectra of dithionite treated BBY particles (D − E and D + E transitions). *T*: 1.8 K; mod. freq.: 17 Hz; microwave power: 20 mW; scan rate: 5 MHz/s; light flux: 60% of max.; scales are arbitrary but comparable. Dithionite incubation times: (— — —) 2 min; (·–·–·–) 90 min; (———) 150 min.

Fig. 1 shows typical FDMR spectra of BBY particles treated with dithionite. The addition of the reductant produces progressive increase, with incubation time, of a negative component peaking at 722.5 and 992.5 MHz. The maximum increase corresponds to a 30-fold increase with respect to the initial value. The very inhomogeneous nature of the signals and the selectivity of the reductant action clearly appears in Fig. 2, where spectra normalized at the negative maximum for fully reduced and untreated samples are compared.

Fig. 3 shows the effect of various experimental conditions on the |D| − |E| transition of dithionite untreated BBY particles. Increasing the temperature makes the signal weaker (it is no longer visible at 30 K), as shown by the DE line in Fig. 3a. The increase in

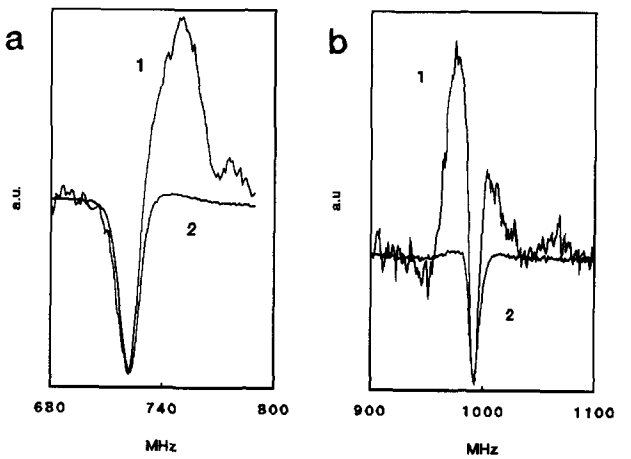

Fig. 2. Dithionite effect on the shape of FDMR spectra of BBY particles (a: D − E; b: D + E transitions). (1) untreated; (2) dithionite treated. (*T*: 1.8 K; mod. freq.: 17 Hz; microwave power: 20 mW; scan rate: 5 MHz/s; light flux: 40% of max.; spectra are normalized at the maximum of the negative peak).

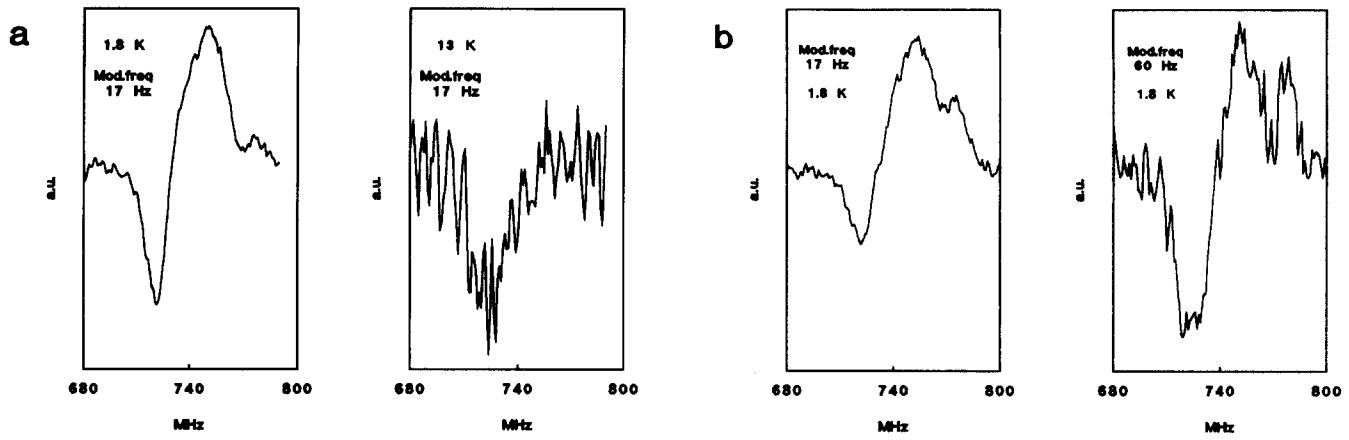

Fig. 3. Effect of temperature and modulation frequency on FDMR spectra of BBY particles (untreated). Only D – E transition shown. (a) temperature effect; (b) mod. frequency effect (microwave power: 20 mW; scan rate: 5 MHz/s; spectra are normalized at the maximum of the negative peak).

relative abundance of the negative signal peaking at 722 MHz is quite evident. The same effect is observed in the D + E transition at 992 MHz (not shown). The increase in microwave modulation frequency also has the effect of enhancing the relative intensity of the same negative component (Fig. 3b). A negative signal with its maximum at about 768 MHz is also enhanced. In the D + E transition only, the peak at 992 MHz is relatively altered.

The changes in the shape of the signals at different light fluxes are particularly evident in the absence of added reductant, as shown in Fig. 4. This dependence of the signal shape on light flux, which is to be expected in FDMR experiments (see Section 4), may be exploited to separate the signals into their single components, characterized by Gaussian shapes. Signals were recorded at various light intensities (a factor of about 100 from minimum to maximum) and the two spectral regions were simulated as sums of Gaussian components. Table 1 summarizes the results for both treated and untreated samples and Fig. 5 shows examples of fits. It is to be noted that the parameters of the Gaussians related to triplet T_1 are taken from the analogous fits of the spectra obtained in the presence of dithionite, where triplet T_1 is the dominant species (Fig. 1).

Some experiments were also carried out on very dilute samples (2–10 μg Chl/ml) in order to check that reabsorption did not corrupt the signals in any way.

RC preparations

The experiments described for BBY particles were repeated on RC preparations. A typical spectrum is shown in Fig. 6. It is immediately evident that at least two components are present, of which one (T_1) has negative maxima. However, the intensity ratio between the two components is almost unaffected by temperature and modulation frequency, and it is impossible to obtain T_1 as the dominant species by using reductant. Dithionite is not in fact expected to affect recombination triplet yield for RCs in which the stable quinone acceptor is lacking (see also [21]), although some increase is usually found. We did not in fact find any

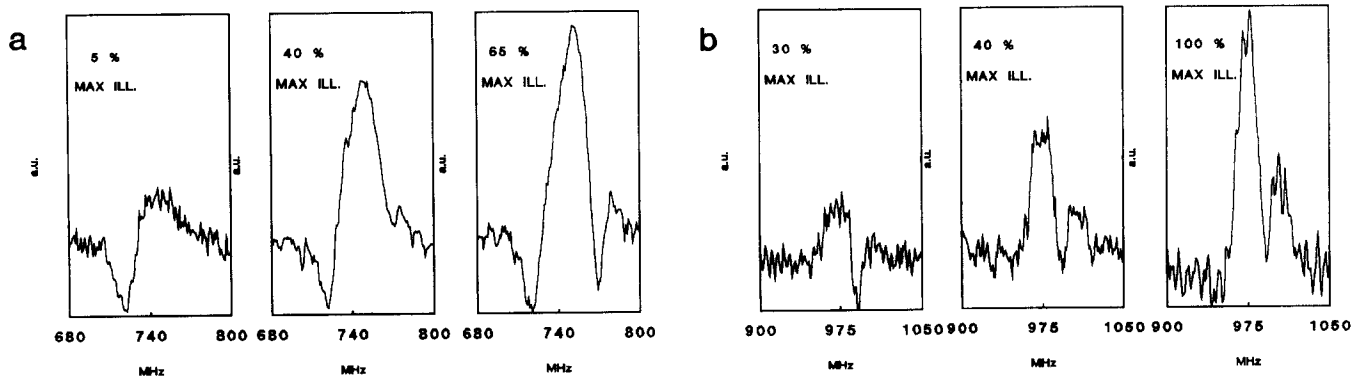

Fig. 4. Light flux effect on FDMR spectra of BBY particles (untreated). (a) D – E transition. (b) D + E transition. (T: 1.8 K; mod. freq.: 17 Hz; microwave power: 20 mW; scan rate: 5 MHz/s; spectra are in arbitrary units).

Table 1
Decomposition into Gaussian components of FDMR lines of BBY particles treated and untreated with dithionite, detected at different light-fluxes

Triplets	Freq. (MHz)	Untreated contributions						Dithionite treated contributions		
		% max. ill.						% max ill.		
		2	3	6	9	30	80	5	40	80
T_1(D − E)	722.5	−0.2	−0.4	−1.2	−1.9	−4.6	−7.8	−6.2	−320	−322
T_1(D + E)	992.5	–	−0.2	−0.8	−1.5	−5.0	−9.9	−5.4	−280	−303
T_2(D − E)	739.5	0.1	0.2	0.9	1.5	6.4	10	–	9.6	16
T_2(D + E)	997.0	–	0.1	0.4	0.6	3.8	9.3	–	5.6	18
T_3(D − E)	753.0	0.1	0.2	0.6	1.1	9.5	16	–	9.6	22
T_3(D + E)	977.0	–	0.1	0.6	1.1	6.3	13	–	10	18
T_4(D − E)	768.0	–	–	−0.1	−0.9	−7.1	−14	–	–	–
T_4(D + E)	N.A.									
T_5(D − E)	772.0	–	0.1	0.2	0.9	6.4	11	–	–	5
T_5(D + E)	1016.0	–	–	0.1	0.1	0.5	1.3	–	–	–

Contributions are expressed in μV; δ_i values (defined in Section 2) are 13 MHz except for triplet T_1 where they are 9 MHz.

differences in the shape of the signals between treated and untreated samples, although about 10% enhancement was obtained in the former. It was also impossible to reach light flux saturation in the RC samples with the photon rate which was sufficient for BBY particles. Positive wide peaks appeared in the chloro-

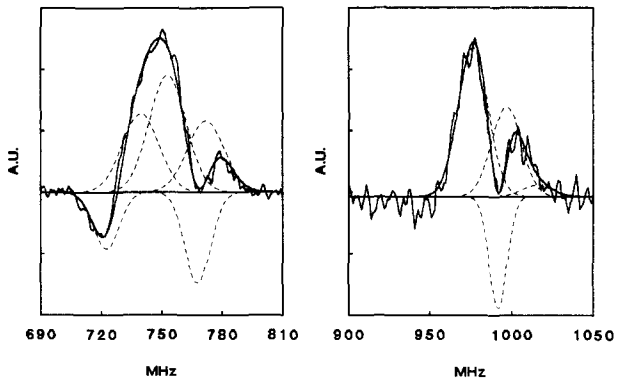

Fig. 5. Decomposition into Gaussian components of FDMR spectrum of BBY particles (untreated). The spectrum corresponds to data of Table 1 at 30% illumination.

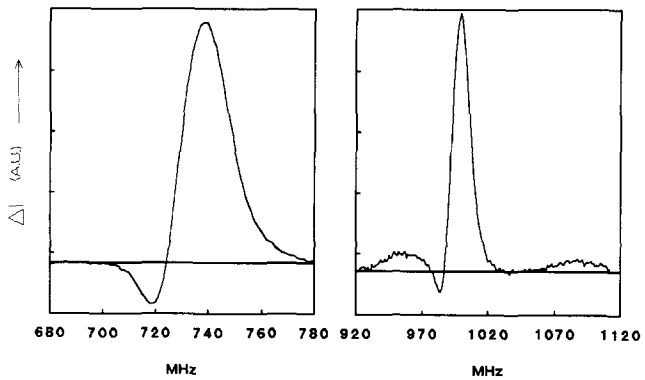

Fig. 6. FDMR spectra of D_1D_2-cyt b_{559}. Conc. μg/ml Chl (untreated). (microwave power: 20 mW; scan rate: 5 MHz/s; mod. freq.: 17 Hz; light flux: 20%; T: 1.8 K).

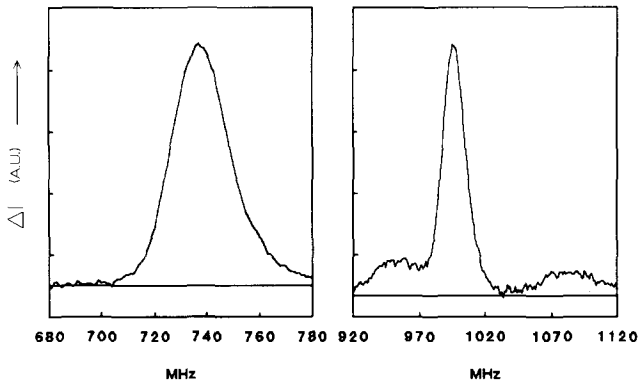

Fig. 7. FDMR spectra of D_1D_2-cyt b_{559}. Conc. 2 μg/ml Chl (untreated). (microwave power: 20 mW; scan rate: 5 MHz/s; mod. freq.: 17 Hz; light flux: 20%; T: 1.8 K).

phyll |D| + |E| region at 951 and 1084 MHz. These can be assigned to the |D| − |E| and |D| + |E| transitions of the triplet state of a pheophytin molecule of the RC. This observation was also made in the ADMR experiments [3,4] and will be discussed further elsewhere [22].

Experiments were also carried out on very dilute samples of RCs to check for reabsorption effects. In this case, signal shape was found to vary with dilution. In particular, the negative component was found to invert in sign with dilution, as shown in Fig. 7.

For the RCs, in the absence of a discriminating reductant effect due to the absence of the quinone acceptor, a few experiments with an exogenous acceptor were carried out. FeCy (1 mM) was added to some samples (10 μg Chl/ml), incubated for 25 min at 0°C. The signal decreased in intensity by a factor of about 60, with negligible shape variations (Fig. 8). As shown by Nugent et al. [23], FeCy acts as an electron acceptor, even at cryogenic temperatures, inducing a quantitative loss of the P680 recombination triplet.

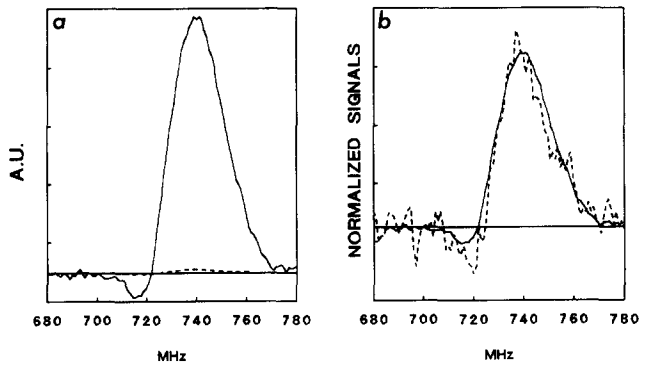

Fig. 8. Effect of FeCy on D−E FDMR transition of D_1D_2-cyt b_{559}. (———) untreated; (— — —) treated; (a) absolute spectra; (b) normalized spectra. Conc. 10 μg/ml Chl; other conditions as in Fig. 7.

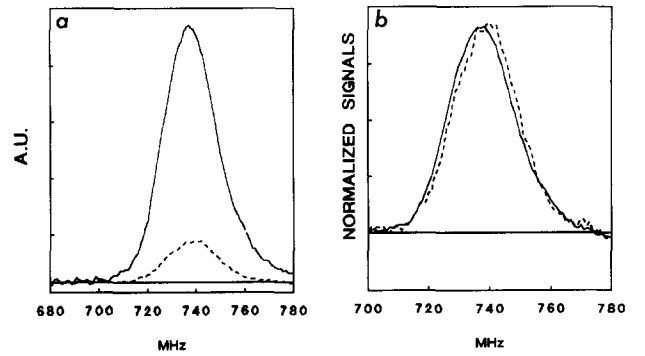

Fig. 9. Effect of detergent on D−E FDMR transition of D_1D_2-cyt b_{559}. (———) untreated; (— — —) treated; (a) absolute spectra; (b) normalized spectra. Conc. 5 μg/ml Chl; other conditions as in Fig. 7.

Experiments carried out to test the detergent effect (25 min incubation at 0°C with 1% Triton X-100), shown in Fig. 9 for the D − E transition, produced a sixfold decrease in the absolute signal and some change in the Chl *a* signal shape. The same effect was obtained in the D + E transition (not shown), while no decrease in absolute intensity was recorded for the presumed pheophytin signals (not shown, but see [22]).

4. Discussion

The P680 triplet in BBY particles

The P680 recombination triplet is unequivocally assigned by the effect of dithionite on the BBY particle spectra. In this condition, especially when secondary acceptor Q_A is doubly reduced to a neutral species Q_AH_2 [20,24], charge separation occurs but is limited to the primary pheophytin acceptor. From the latter only charge recombination occurs, giving rise to high triplet yields. We checked that this was actually so in our experimental conditions by running a conventional EPR spectrum in the X band. Only in samples incubated with dithionite were we able to see the typical high field AEEAAE polarized triplet spectrum of the primary donor.

The peculiarity of this triplet is also shown by temperature and frequency effects. Time-resolved EPR and transient absorption experiments [25–27] have clearly shown that the lifetime of the recombination triplet is of the order of 1 ms in the absence of oxygen. Not much is known about the lifetimes of chlorophyll triplets in antenna complexes. Some measurements on subchloroplast particles [28] indicate lifetimes similar to monoligated chlorophylls in vitro that are usually longer than 1 ms. From our experiments at higher modulation frequencies such as that shown in Fig. 3b, it is clear that the triplet with transitions centred at 722 and 992 MHz has slightly shorter lifetimes than those of the other positive components. We shall return later to the 768 MHz component, which is also enhanced at higher frequencies but which cannot be due to ^{3}P680 because it is not influenced by dithionite. Let us now consider the experiment at 13 K (Fig. 3a): the 722–992 MHz component decreases in intensity to a lesser extent than all other components. The decrease in overall signal intensity with temperature is an obvious consequence of the increased spin-lattice relaxation rates of the triplet levels, causing diminished polarization of the transitions. It could well be that the 'special triplet' has a spin-lattice relaxation rate less influenced by the rise in temperature in the range 213 K. Alternatively, on account of the different population mechanism (radical pair recombination as compared with ISC), a different change in precursor lifetime may give rise to the observed effect.

PS I, PS II and bacterial Photosystems are clearly recognized as non- or only weakly fluorescent traps embedded in a lake of fluorescent antenna pigments from which they draw their excitation energy. The FDMR of the trapped triplet state is mainly observed on the host fluorescence, a type of experiment which has been discussed earlier [29,30]. This situation is reflected in the well known Vredenberg-Duysens relation [31] which links fluorescence quantum yield to the fraction of open traps:

$$1/\phi = A + B(\text{fraction of open traps})$$

This is also the origin of the negative sign of the FDMR signal in bacterial cells and is to be expected in all cases where antennas are present and the trap is not or only weakly fluorescing. Consequently, if triplet P680 is recognized as a closed trap and the kinetics of populating and depopulating Chl *a* triplets in zero field is such as to produce a decrease in the overall triplet population under microwave resonance, the FDMR signal of this P680 triplet, monitored on the antenna fluorescence, should be negative (open trap population increases).

All ADMR experiments known to date on Chl *a* recombination triplets in higher plant Photosystems effectively show a decrease in the triplet population upon microwave resonance between a pair of levels, even in large PS II particles [9]. We conclude that the sign of the signal to be expected from ^{3}P680 which forms in BBY particles through the charge recombination process, may be opposite to that of any triplet formed by intersystem crossing in fluorescent antenna chlorophylls. This is consistent with the identification of the negative signals at 722 and 992 MHz in the BBY spectra as belonging to the ^{3}P680 species originating from charge separation and recombination, and monitored via antenna fluorescence, i.e., inverted in sign. The ZFS parameters of ^{3}P680 are calculated to be 0.0286 cm^{-1} for |D| and 0.0043 cm^{-1} for |E|, and the line width is characteristic of a single species showing conservation of the native structure of the pigment.

Triplets in D_1D_2

An immediate observation is the presence of negative peaks in the spectra of concentrated samples of D_1D_2. These peaks may be simulated with the same Gaussian parameters as those of triplet T_1 of the BBY spectra. The Gaussian deconvolution is less clear than in the case for BBY, because of the impossibility, mentioned in Section 3, of obtaining a spectrum with the negative peak alone. The frequency and width assigned to the negative peak influences deconvolution of the positive peaks. However, at least one component with negative peaks certainly exists and has frequencies and widths in the range of those found in BBY for triplet T_1.

The evidence at this point may suggest that the pair of lines assigned to ^{3}P680 in BBY is one of the components contributing significantly to the intensity of the FDMR spectrum of isolated RCs and that also in this case the signal sign is negative. Note that ADMR signals are also always positive in this case [3]. These facts may be taken as an indication that also in isolated RCs, the fluorescent species are accessory chlorophylls connected through energy transfer to P680. This interpretation is in agreement with recent work. The primary donor in D_1D_2 is equilibrated within very short times (less than a picosecond) with a pool of accessory chlorophylls, as has been shown by Durrant et al. [32]. This concept is also in line with recent hole-burning studies demonstrating Chl → P680 energy transfer in isolated RC complexes [33].

Although we believe that this mechanism of P680 excitation is certainly present in isolated centres, it cannot be important in determining the sign of FDMR signals belonging to T_1, which seems to be affected by a reabsorption artefact (Fig. 7).

At low temperature, it is known [17] that the percentage of fluorescence yield originating directly from P680 is low but, in any case, it is the change of this fluorescence we certainly observe by FDMR, as the triplet gives rise to a normal positive peak under resonant microwave irradiation in diluted samples. This peak may be inverted in concentrated samples through large fluorescence reabsorption owing to a singlet–singlet transition, abundantly overlapping in the red. This mechanism appears clearly if we compare our results with those obtained using ADMR by Van der Vos and others [3]. With the help of microwave induced absorption spectra, they were able to analyse the ADMR signals in terms of five triplet species, according to the values reported in Table 1 of [3]. We calculated the D − E and D + E transition frequencies corresponding to the ZFS values reported for the five species and estimated the spectral line width from the associated Fig. 7 of [3]. We used the five Gaussians thus obtained to simulate the spectra of our Figs. 5 and 6. The results are reported in Fig. 10, and Table 2 lists the details of the simulations together with the relevant data of Van der Vos et al. It is quite clear that the ADMR analysis is not only fully compatible with the FDMR results but also gives us further information. Table 2 shows that the spectrum of the diluted sample can be simulated simply by inverting the sign of the component with |D| = 0.0285 cm^{-1} in the Gaussian

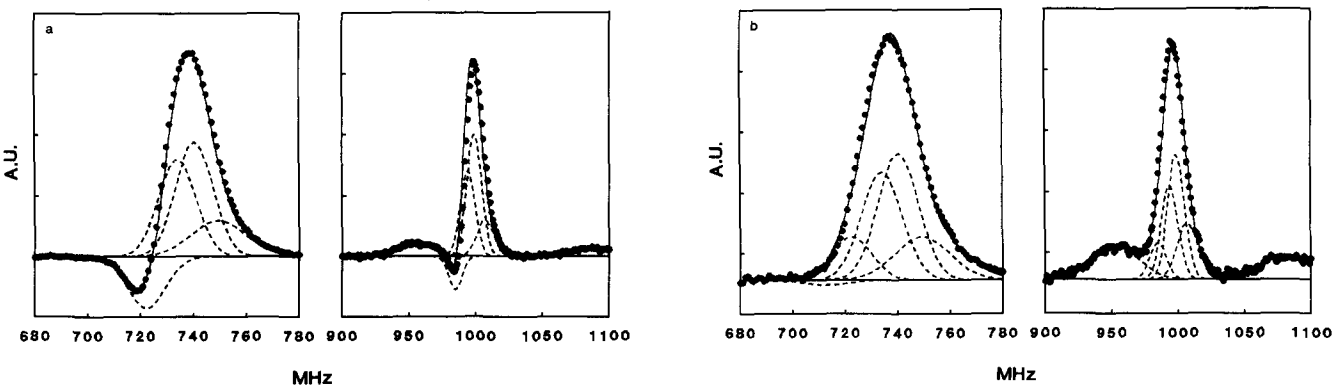

Fig. 10. Fits of spectra shown in Figs. 6 and 7 (see Table 2). (a) Fig. 6 (concentrated samples); (b) Fig. 7 (diluted samples).

Table 2
Decomposition into Gaussian components of FDMR lines of isolated RCs and comparison with ADMR (see text); ADMR data taken from [3]

ADMR			FDMR		
Centre (nm ± 0.1)	|D| value (cm^{-1} ± 0.0001)	Comp. (% ± 2.5)	|D| value (cm^{-1} ± 0.0001)	Comp. dil. (% ± 2.5)	Comp. conc. (% ± 3.0)
685.6	0.0281	5	0.0281	−1	0
683.3	0.0284	25	0.0285	13	−15
680.4	0.0288	30	0.0288	31	32
678.9	0.0290	35	0.0290	39	40
677.2	0.0293	5	0.0293	16	13

deconvolution. In the case of BBY particles, owing to the greater percentage of fluorescence originating from energy donor molecules residing in the antennas, the intrinsic negative sign is clearly apparent and is shown to persist down to the greatest dilutions.

In spite of the misleading appearance of the concentrated samples, we must conclude that the different sign of the ^{3}P680 FDMR signals, which in the integrated particles originates from donor fluorescence, cannot be appreciated in D_1D_2 complexes, if present, being masked by the overlap of two effects with opposite sign (donor fluorescence and the direct fluorescence of P680 itself). As stated above, in the absence of ADMR data, we could not have analysed the spectra in terms of five different species, but we accept the fit which is compatible with the global analysis of all the experiments. We also agree with the conclusions of [3] that the observed triplets all originate from P680 and not from other possible sources such as accessory or exogenous chlorophylls. The low similar effect of both exogenous acceptor (FeCy) and detergent on all triplets (Figs. 8 and 9) support this interpretation of the data.

We must now comment on the different values of the five contributions to the FDMR spectra compared with the ADMR spectra, due to the different fluorescence properties of the species absorbing at different wavelengths. We find that the percentage of the FDMR signal is larger than that resulting from ADMR analysis for those species with larger D values and absorbing toward the shortest wavelengths, and correspondingly smaller for species with smaller D values absorbing at longer wavelengths. The detergent action we observed

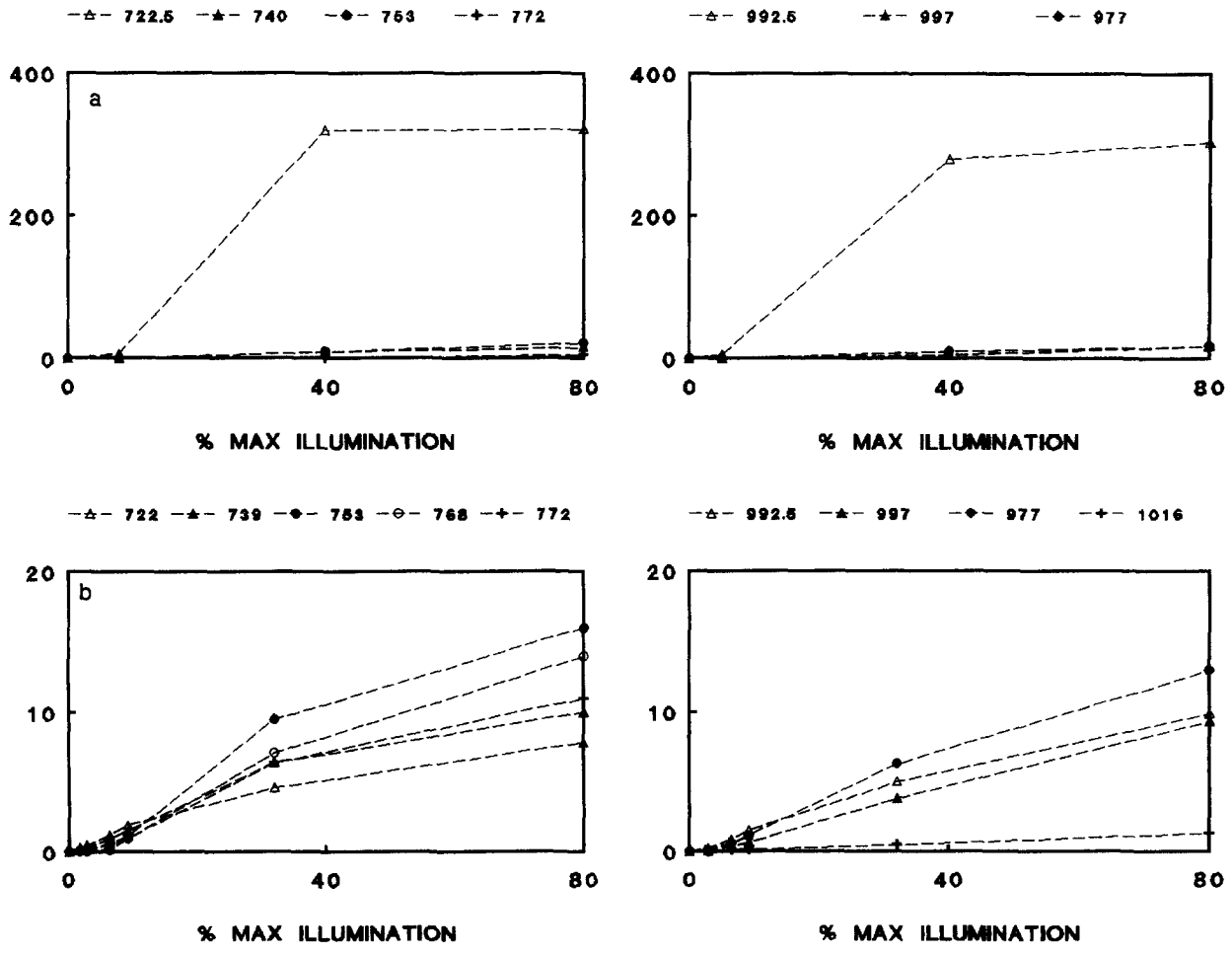

Fig. 11. Contributions (μV) of Gaussian components vs. light flux (see Table 1). (a) Dithionite treated BBY particles; (b) untreated.

is also in agreement with the ADMR scenario. There is an overall decrease in the intensity of the signal which shows impairment of the electron transferability of the centres affected and a consequent decrease in the recombination triplet yield. The slight changes in shape occurring upon detergent action also show a tendency toward a triplet composition richer in high D values and high fluorescence species (Fig. 9).

To conclude this section, we point out what seems to emerge as an ODMR feature characterizing 'functional' isolated PS II reaction centres. It is well known that the optical properties of D_1D_2 are quite sensitive to agents such as temperature and detergents [17,18,34] and our results clearly show that detergent action during preparation produces changes in some of the centres, giving rise to various kinds of spectroscopic objects. The major species are all to be associated to forms of P680, because there is almost no discriminating effect from exogenous acceptors, as shown by the FeCy effect, nor from detergent action. Comparison with the BBY is most revealing. In that case P680 gives rise to a triplet with a very narrow signal, characteristic of a single species, showing conservation of the native structure of the pigment. In the isolated RCs, the signals are much more heterogeneous, showing an effect of preparation on the micro-environment or binding of pigments. It is safe to conclude that some centres are less affected than others and that those remaining in the 'native' state are to be identified with those giving rise to triplets having smaller ZFS parameters, and whose ground state absorbs at longer wavelengths (680–685 nm) and shows low fluorescence yield.

Antenna triplets

The dependence of FDMR transition amplitudes on excitation light intensity can be quite complex [35]. A quadratic phase is expected at low light fluxes, followed by a linear phase and finally by saturation.

With our light fluxes the quadratic regime which is characteristic of FDMR signals at low light is observed as can be seen from Fig. 11 obtained from Table 1. At high light levels, the saturation regime is already approached and is especially apparent in the case of dithionite-treated BBY particles.

Since the light flux behaviour of the signal intensity for the different triplet species is a consequence of the ratios between photon flux and the rate constants of the various processes involved, it should be possible to gather quantitative information on these processes by using FDMR. In this work, low light flux behaviour is only exploited in the deconvolution of the spectrum into Gaussian components; the latter had to fit the shape of the signal changing with light flux. In any case, qualitative evidence of high yield of energy funnelling from the antenna is contained in the fact that, when charge separation is efficient, saturation occurs even at low light fluxes in ^{3}P680. When no dithionite is present, at low temperature, Q_A is in the form Q_A^-, charge separation occurs at a lower speed, and ^{3}P680 saturates at a low population level; other triplets can then easily form, with comparable yields.

In Table 1 five triplets are identified. Triplet T_1 has been dealt with in the previous sections. Signals from the other triplets are probably to be attributed to species deriving from the antenna system or to preparation artefacts.

The assignment of the pair of transitions (D − E and D + E) to individual triplets in Table 1 was made bearing in mind that, for chlorophylls, D + E and D − E polarizations are often found to be equal. This seemingly turns out to be the case for T_1, T_2 and T_3 triplets. Instead triplet T_5 shows different polarization, but the increase in both 1016 and 772 MHz components during photodegradation (see later) seems to indicate pairing. Some doubts remain about the T_4 species, whose D + E transition may be masked by the 992 MHz component. At least one triplet with similar ZFS characteristics and with the same sign has been found in isolated LCH II preparations [10]. It may be suggested that this triplet is formed in the external principal antenna system of PS II, mainly in conditions of saturation of the P680 trap when, in the absence of dithionite, Q_A is photoreduced to Q_A^-, charge separation does not occur, and ^{3}P680 has a low yield.

Triplets T_2 and T_3 have resonance frequencies and sign similar to those found in isolated CP43 and CP47 complexes [36] respectively. It is therefore probable that these triplets belong to proximal antenna chlorophylls. However the lines in isolated antenna complexes are broader, possibly on account of the detergent treatments necessary for their preparation. The growth in the BBY spectra of the component labeled T_5 in Table 1 was found to increase progressively with increasing light flux even when T_1 starts decreasing after saturation. Some sort of photodegradation seems to be occurring, paralleled at very high light fluxes (data not shown) also by a decrease in all other triplet species.

It has already been noted that the use of detergents in preparation procedures, even the mild ones used in recent practice, may affect properties and stoichiometry of pigments which come within their range of interaction. It is therefore possible that chlorophylls affected in such a way acquire magnetic resonance parameters which are determined more by the interaction with the detergent than by their own native environment; they are also expected to give high fluorescence yield. It is reasonable that molecules exposed to the surface of the complexes are affected in this way, and this leaves some doubt about the functionality of the antenna triplets observed. We are planning further FDMR experiments on PS II preparations with differ-

ent antenna sizes, and with selected wavelength detection and excitation, to gather more information on the origin of these triplets and their degree of intactness.

5. Conclusions

We have shown in this paper that the triplet localized in 'active' P680 may be unequivocally assigned to specific FDMR transitions and that it is formed together with many other triplet species during continuous illumination in integrated PS II particles. We have also shown that in these particles P680 is a species with low fluorescence, as the sign of its FDMR signals is the opposite of that expected from the relative population levels and the decay rate constants of the triplet level system. In this way, energy transfer at low temperature from fluorescing antenna chlorophylls to P680 is clearly seen in the FDMR experiment.

We also studied triplet formation in isolated RCs and concluded that, although some ^{3}P680 may form through energy transfer from accessory chlorophylls to weakly fluorescent P680, the phenomenon is masked by the much lower number of donor molecules with respect to the system containing the complete antennas. Instead, it is quite apparent that the isolation procedures for D_1D_2-cyt b_{559} complexes induce changes in the P680 environment, giving rise to various kinds of spectroscopic objects. The species most affected seem to become also the most fluorescent. The triplet components found in isolated RCs with ZFS parameters very close to those of integrated BBY particles also belong to the less fluorescent P680s and are to be assigned to the more 'intact' complexes.

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7. References

[1] Den Blanken, H.J., Van der Zwet, G.P. and Hoff, A.J. (1982) Chem. Phys. Lett. 85, 335–338 (and refs. therein).
[2] Angerhofer, A., von Schuetz, J.U. and Wolf H.C. (1985) Z. Naturforsch. 40c, 379–387 (and refs. therein).
[3] Van der Vos, R., Van Leeuwen, P.J., Braun, P. and Hoff, A.J. (1992) Biochim. Biophys. Acta 1140, 184.
[4] Bernlochner, D., Angerhofer, A. and Robert, B. (1993) Zeitschr. Phys. Chem., in press.
[5] Barber, J., Gounaris, K. and Chapman, D.J. (1987) in Cytochrome Systems (Papa, S., Chance, B., and Ernster, L., eds.), pp. 657–666, Plenum Press, New York.
[6] Barber, J., Chapman, D.J. and Telfer, A., (1987) FEBS Lett. 220, 67–73.
[7] Hoff, A.J., Govindjee and Romijn, J.C. (1977) FEBS Lett. 73, 191–196.
[8] Searle, G.F.W., Koehorst, R.B.M., Schaafsma, T.J., Moeller, B.L. and Von Wettstein, D. (1981) Carlsberg Res. Commun. 46, 183.
[9] Den Blanken, H.J., Hoff, A.J., Jongenelis, A.P.J.M. and Diner, B.A. (1983) FEBS Lett. 157, 21–27.
[10] Carbonera, D., Giacometti, G. and Agostini, G. (1992) Appl. Magn. Res. 3, 859–872.
[11] Carbonera, D. and Giacometti, G. (1992) Rend. Fis. Acc. Lincei 3, 361–368.
[12] Searle, G.F.W. and Schaafsma, T.J. (1992) Photosynth. Res. 32, 193–206.
[13] Miller, A.F. and Brudvig, G.W. (1991) Biochim. Biophys. Acta 1056, 1–18.
[14] Carbonera, D., Giacometti, G., Agostini, G. and Toffoletti, A. (1989) Gazz. Chim. Ital. 119, 225–228.
[15] Berthold, D.A., Babcock, G.T. and Yokum, C.F. (1981) FEBS Lett. 139, 231–234.
[16] Chapman, D.J., Gounaris, K. and Barber, J. (1990) in Methods in Plant Biochemistry Vol. V (Rogers, L.J., ed.), pp.171–193, Academic Press, London.
[17] Roelofs, T.A., Kwa, S.L.S., Van Grondelle, R., Dekker, J.P. and Holzwarth, A.R. (1993) Biochim. Biophys. Acta 1143, 147–157.
[18] Braun, P., Greenberg, P.M. and Scherz, A. (1990) Biochemistry 29, 10376–10387.
[19] Crystall, B., Booth, P.Y., Klug, D.R., Barber, J. and Porter, G. (1989) FEBS Lett. 260, 138–140.
[20] Van Mieghem, F.J.E., Nitschke, W., Mathis P. and Rutherford, A.W. (1992) Biochim. Biophys. Acta, 977, 207–214.
[21] Okamura, M.Y., Satoh, K., Isaacson, R.A. and Feher, G. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), vol. I, pp. 379–381, Kluwer, Dordrecht.
[22] Friso, G. (1994) Thesis, University of Padova.
[23] Nugent, J.H.A., Telfer, A., Demetriou, C. and Barber, J. (1989) FEBS Lett. 255, 53–58.
[24] Vass, I. and Styring, S. (1993) Biochemistry 32, 3334–3341.
[25] Searle, G.F.W., Telfer, A., Barber, J. and Schaafsma, T.J. (1990) Biochim. Biophys. Acta 1016, 235–243.
[26] Durrant, J.R., Giorgi, L.B., Barber, J., Klug, D.R. and Porter, G. (1990) Biochim. Biophys. Acta 1017, 167–175.
[27] Telfer, A., Durrant, J.R. and Barber, J. (1990) Biochim. Biophys. Acta 1018, 168–172.
[28] Clarke, R.H., Hotchandani, S., Jagannathan, S.P. and Leblanc, R.M. (1982) Photochem. Photobiol. 36, 575–579.
[29] Hoff, A.J. (1982) in Triplet State ODMR Spectroscopy (Clarke, R.H. ed.), Chap. 9, pp. 394–395, Wiley, New York.
[30] Hala, J., Searle, G.F.W., Schaafsma, T.J., Van Hoek, A., Pancoska, P., Blaha, K. and Vacek, K. (1986) Photochem. Photobiol. 44, 527–534.
[31] Vredenberg, W.J. and Duysens, L.N.M. (1963) Nature 197, 355–357.
[32] Durrant, J.R., Hastings, G., Joseph, D.M., Barber, J., Porter, J. and Klug, D.R. (1992) Proc. Natl. Acad. Sci. USA 89, 11632–11636.
[33] Small, G.J., Jankowiak, R., Seibert, M., Yocum, C.F. and Tang, D. (1990) in Reaction Centers of Photosynthetic Bacteria (Michel-Beyerle, M.E., ed.), pp. 101–111, Springer, Berlin.
[34] Booth, P.J., Crystall, B., Ahmad, I., Barber, J., Porter, G. and Klug, D.R. (1991) Biochemistry 30, 7573–7586.
[35] Hoff, A.J. (1989) in Advanced EPR - Applications in Biology and Biochemistry (Hoff, A.J., ed.), Chap. 18, pp. 639–641, Elsevier, Amsterdam.
[36] Carbonera, D., Giacometti, G., Agostini, G., Angerhofer, A. and Aust, V. (1992) Chem. Phys. Lett. 194, 275–281.