

Chlorophyll triplet states associated with Photosystem I and Photosystem II in thylakoids of the green alga *Chlamydomonas reinhardtii*

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Abstract

The analysis of FDMR spectra, recorded at multiple emission wavelengths, by a global decomposition technique, has allowed us to characterise the triplet populations associated with Photosystem I and Photosystem II of thylakoids in the green alga *Chlamydomonas reinhardtii*. Three triplet populations are observed at fluorescence emissions characteristic of Photosystem II, and their zero field splitting parameters have been determined. These are similar to the zero field parameters for the three Photosystem II triplets previously reported for spinach thylakoids, suggesting that they have a widespread occurrence in nature. None of these triplets have the zero field splitting parameters characteristic of the Photosystem II recombination triplet observed only under reducing conditions. Because these triplets are generated under non-reducing redox conditions, when the recombination triplet is undetectable, it is suggested that they may be involved in the photoinhibition of Photosystem II. At emission wavelengths characteristic of Photosystem I, three triplet populations are observed, two of which are attributed to the P₇₀₀ recombination triplet frozen in two different conformations, based on the microwave-induced fluorescence emission spectra and the triplet *minus* singlet difference spectra. The third triplet population detected at Photosystem I emission wavelengths, which was previously unresolved, is proposed to originate from the antenna chlorophyll of the core or the unusually blue-shifted outer antenna complexes of this organism.

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1. Introduction

Thylakoids are the site where the primary events of photosynthesis, associated with light absorbance and its conversion into chemical energy, take place. Two photosystems, namely Photosystem I (PS I) and Photosystem II (PS II), utilise

Abbreviations: PSII, Photosystem II; PSI, Photosystem I; RC, reaction centre; LHC, light harvesting complex; FDMR, fluorescence detected magnetic resonance; ADMR, Absorbance detected magnetic resonance; MIF, microwave-induced fluorescence spectrum

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visible light to transfer electrons from water to NADP⁺ against the electrochemical gradient. The photosystems are large multisubunit complexes which bind a large number of cofactors involved in electron transfer reactions and light harvesting. The crystallographic structure of PS II and PS I have been solved to medium–high resolution in recent years providing information with regard to the structural arrangement of redox and light harvesting cofactors [1–4]. Despite differences at the level of polypeptide composition, the two photosystems are organised in a similar fashion around a special pair (i.e. [5–7]), or cluster (i.e. [8–12]), of chlorophyll molecules which perform the photochemical charge-separation reactions (the reaction centres). The direct excitation of the RC is a rare event and excitation is efficiently transferred to the trap from the large antenna pigment

arrays. PS II has a complicated arrangement with the Chl *a*- β -carotene binding inner antenna complexes, CP43 and CP47, located on either side of the D1–D2 hetero-dimer, which binds the primary electron donor Chls, known as P₆₈₀, and the primary acceptor pheophytin ([13] for a review). In the case of PS I it is the gene products of the PsaA and PsaB subunits that act both as the inner antenna, co-ordinating about 100 Chl *a* and 30 β -Car molecules, and the reaction centre, binding the primary donor, P₇₀₀, as well as the electron acceptors A₀, A₁ and F_X. The terminal iron–sulphur clusters F_A and F_B are bound to the PsaC subunit ([14] for review). In higher plants and green algae the outer antenna complexes of PS I (LHCI pool) and PS II (LHCII pool) bind Chl *a*, Chl *b* and xanthophylls ([15] for a review).

In plant photosystems primary charge separation takes place from the lowest singlet excited state of the primary donors and energy equilibration in the antenna is considered to occur largely by a singlet resonance transfer (Forster) mechanism. Therefore the Chl triplet state, which in solvated chlorophyll has a yield of about 40% ([16] for a review), is of little direct importance in photosynthetic energy conversion. However, it has been suggested that Chl triplets have a primary role in light stress phenomena, which are known as photoinhibition, under aerobic conditions ([17] for a review). This is because of the interaction with molecular oxygen, which generates the highly oxidising singlet oxygen species. The Chl triplet may be populated in principle either by intersystem crossing (ISC) from the low lying singlet excited state or from the recombination of the charge separated radical pair state of the reaction centre. The rate of intersystem crossing in isolated protein–pigment complexes is of the same order of magnitude as that for the other singlet excited state de-excitation process [18]. Hence Chl *a* triplets have been detected in a variety of isolated Chl–protein systems (i.e. [19–23]). More recently Chl triplet states associated with Chl–protein complexes have been characterised in thylakoid membranes and also observed in leaves [24]. However in photosynthetic complexes Chl triplets are efficiently quenched by carotenoids (i.e. [24–30]). Although triplet–triplet energy transfer takes place *via* the Dexter mechanism which requires orbital overlap between the donor and the acceptor molecule, the yield of Chl triplet quenching by carotenoids has been estimated to be extremely efficient with yields in the range of 0.85–1 ([18,21,27]), depending on the system under investigation and the temperature at which the measurements were performed.

The Chl triplets formed by charge recombination at both PS I and PS II reaction centres are characterised by an anomalous Electron Paramagnetic Resonance (EPR) polarisation in high magnetic field compared to that produced by intersystem crossing because they are generated essentially from the pure singlet state of the radical pair precursor [31]. Thus spin polarised EPR spectra of reaction centre triplets were reported in PS I (³P₇₀₀) and the PS II reaction centre (³P₆₈₀), when the electron transfer acceptor(s) are reduced (i.e. [32–35]). However the PS II reaction centre triplet (the so-called ³P₆₈₀) attains a significant population only when the quinone acceptor Q_A is fully reduced [36], or when it is not present in its binding site, as in the case of the purified D1–D2–Cytb₅₅₉ complex [37,38].

As mentioned above, it is well known that the Chl triplet interacts with molecular oxygen to form singlet oxygen which produces photoinhibitory damage, mainly at the level of PSII. This has been attributed to the PS II recombination triplet [39,40], which has been suggested to form under conditions of saturating light intensity [41]. However the lifetime [42–44] and the zero field splitting parameters [43] of the PS II reaction centre triplet appear to be influenced by the redox state of the Q_A. Hence the physiological relevance of data obtained under strongly reducing conditions and in the isolated reaction centre complex needs to be re-evaluated. Moreover, studies on the relationship between the excited state quenching in PS II antenna and photoinhibition have provided strong evidence that light-induced damage in isolated thylakoids occurs via Chls which are energetically uncoupled from the main antenna matrix [45–51]. Also, it has been hypothesised that these chlorophylls, possibly associated with damaged or incompletely assembled PSII antenna complexes, may have a high triplet yield [45,47–50]. The presence of Chl triplet states associated with PS II of thylakoids, which do not belong to the reaction centre, has been recently described in isolated spinach thylakoids [26,43]. The lack of correlation between the lowering of the singlet excited state population in PSII antenna and photoinhibition has also been demonstrated in intact cells of *C. reinhardtii*, indicating that the energetically uncoupled Chls bound to damaged Chl–protein complexes might also be involved in photoinhibition *in vivo* [49]. Therefore, in order to investigate whether the triplet states previously observed in spinach are widespread in nature, we have searched for the presence of Chl triplet populations in *C. reinhardtii* thylakoids, which is considered as a model organism for green algae, under non-reducing conditions, using the ODMR technique.

At least three PSII triplet populations are shown to be present and are characterised with respect to their zero field splitting (EPR) parameters and microwave induced fluorescence emission dependence. In addition, the triplet state associated with the reaction centre of PS I is detected under non-reducing conditions. Moreover a third triplet population, which can be associated with the outer antenna of PS I is identified, based on its microwave-induced fluorescence emission spectrum.

2. Material and Methods

2.1. Purification of thylakoid membranes from *C. reinhardtii*

C. reinhardtii cells were grown in continuous light (100 μ E m^{−2} s^{−1}) in TAP medium in which acetate constitutes the main carbon source. The cells were harvested by centrifugation during the mid-logarithmic growth phase, and resuspended in minimum growth media and immediately frozen in liquid nitrogen for spectroscopic measurements. Thylakoids were prepared from freshly harvested cells that were broken by a Yeda Press at a pressure of 1000 PSI, (40 kg m^{−2}) at a concentration equivalent to 1 mg Chl mL^{−1}. The broken material was collected in a buffer containing 0.4 M Sorbitol, 20 mM NaCl, 10 mM MgCl₂, 2 mM CaCl₂, 100 mM Tricine–NaOH, pH 7.8 and centrifuged at 250 × g for 5 min to separate unbroken material. The supernatant was centrifuged at 1500 × g for 5 min, and the pellet subjected to osmotic shock by a solution containing 10 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 50 mM Tricine–NaOH, pH 7.8, to which two volumes of a 0.4 M Sorbitol, 10 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 50 mM Tricine–NaOH, pH 7.8 buffer were added prior to a second centrifuge run at 1500 × g for 5 min. The pellet containing the broken thylakoids

was suspended in 0.1 M Sorbitol, 20 mM NaCl, 5 mM MgCl_2 , 1 mM CaCl_2 , 50 mM Tricine–NaOH, pH 7.8. The chlorophyll concentration was estimated from the 80% acetone extract by the absorption coefficients given by McKinney [52]. All chemical additions were made in alcohol and the final V/V ratio never exceeded 1%. Anaerobiosis was obtained by means of the glucose/glucose-oxidase/catalase system. Extra pure degassed glycerol was added to the sample to a final 60:40 volumes ratio immediately before the sample was placed in the cryostat. Freshly prepared thylakoids were used in each experiment to avoid prolonged incubation of samples with cryoprotectant (glycerol) that leads to artefactual triplet production, probably due to the uncoupling of a small fraction of the protein-bound Chl [24].

2.2. Steady-state fluorescence spectroscopy

Steady state fluorescence emission spectra were detected in a home-built fluorimeter equipped with an EG&G OM4III (model 1460) intensified diode array detector mounted on a HR320 Jobin–Yvon spectrometer and adapted to fit a continuous helium flow cryostat (Oxford Instruments mod. Optistat^{CF}). The temperature of the sample was monitored by a thermocouple placed in the sample and controlled with an ITC-503 device (Oxford Instruments). The wavelength scale was calibrated using a neon spectral calibration source (Cathodeon). Excitation wavelength was selected by a Heath monochromator and two Corning CS 4-96 broad band pass filters and was fixed to 435 nm. The light path was 1 cm. In order to have an adequate signal to noise ratio, spectra were accumulated to around 10^6 counts at the emission maxima. All spectra were measured at a chlorophyll concentration of 4 $\mu\text{g/ml}$. Spectra were corrected for the detector sensitivity as previously described [53].

2.3. Optically detected magnetic resonance

2.3.1. Principle of the technique

The principle of the ODMR measurement has been extensively described in the literature (reviewed in [54,55]), therefore we limit ourselves to a brief summary of this principle. The technique monitors the changes in the singlet ground state population, as a result of irradiation with a resonant microwave field promoting transitions between the triplet sublevels manifold. The principle of the technique is illustrated in Scheme 1. The decay rates and the population of the triplet sublevels are generally anisotropic. Upon irradiation with a microwave field which promotes transitions between couples of sublevels within the manifold, the steady-state population of the triplet sublevels is modified. This in turn will lead to microwave-induced modification of the singlet ground state population which can be monitored as a change either in the steady-state absorption (ADMR) or in the emission (FDMR) of the chromophore. The ODMR technique presents the advantage of being extremely selective for molecular triplet states, which can be discriminated on the basis of

their zero field splitting (ZFS) parameters. Moreover, because the selective excitation is performed over the magnetic field, different triplet states can be examined based on their magnetic properties. This constitutes an advantage in the investigation of complex intact biological systems, such as thylakoid membranes, when selective optical excitation is hampered by spectral congestion due to overlapping absorption of several different Chl and carotenoid spectral forms associated with either PS I or PS II. Therefore, ODMR offers higher sensitivity compared to optical methods, as it is specific to triplet states, and presents the advantage of selectivity, since the magnetic resonance transitions can be monitored at relevant emission wavelengths, and in turn can be correlated with the presence of a triplet population associated with specific chlorophyll pools.

2.3.2. ODMR spectrometer

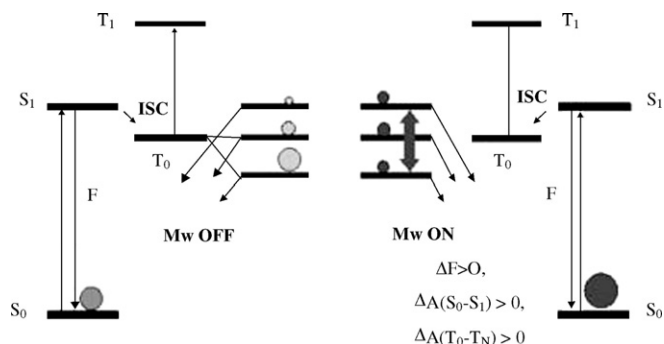
The microwave source of the ODMR apparatus consists of an HP8559b sweep oscillator equipped with an HP83522a plug-in device (0.01–2.4 GHz). The microwaves were amplified by a LogMetrics A210/L TWT amplifier in the range of 1–2 GHz and by a Sco-Nucleides 10-46-30 TWT amplifier in the 0.01–1 GHz range. The microwaves were irradiated to the sample through a low-Q resonator, consisting of a slow-wave helix with a pitch of about 2 mm. The microwaves were amplitude modulated by a square waveform signal generator (Wavetek, model 164), at a frequency between 20 Hz and 1.5 kHz. Optical excitation was provided by a tungsten lamp (Philips 250 W), supplied by a stabilised power supply (Oltronix B 32-20R). The light was focused by a 10 cm diameter lens with a 10 cm focal length, onto a flat Perspex cell (0.5×1.0 mm) placed in a liquid helium cryostat (Oxford instruments, model Spectromag 4), which can be pumped to reach the temperature of 1.8 K. In the FDMR experiments the light was filtered through an 8 cm wide 0.1 M CuSO_4 solution and a 630 nm cut-on filter, and the sample was oriented so that the normal to its plane was rotated by 40° , about the cryostat axis. The fluorescence emission was collected at 90° geometry through the appropriate combination cut-off and interference filters (FWHM 10–15 nm), with an OSI 5 K (Centronic) photodiode. The signal was detected with a phase sensitive lock-in amplifier (EG&G model 5210), interfaced to a PC by software written in the laboratory [23]. The same apparatus was adapted to perform the ADMR experiments: the beam was filtered through a 5 cm water path-length and a 575 nm cut-on filter (Coherent Ealing) then focused on the flat cell (1.5 mm width) placed perpendicular to the light beam. The transmitted light was analysed by a Jobin–Yvon HR250 monochromator, protected by a cut-on filter OG 550 (Schott), equipped with the Data-Link device controlled by a PC, and acquired by the same photodiode used for the fluorescence experiments. The photodiode voltage output is phase sensitive and was detected with an EG&G mod 5210 lock-in amplifier and presented, in this case, as the ratio of amplitude modulated and continuous components ($\Delta I/I$). For fluorescence detected resonance (FDMR) measurements the sample was diluted to a concentration of 100 $\mu\text{g/ml}$ Chl, which was reduced to 40 $\mu\text{g/ml}$ for microwave-induced absorption (Triplet minus Singlet (T–S)) experiments.

Complete reduction of Q_A inducing the population of the P_{680} recombination triplet was obtained by illumination at room temperature of sodium dithionite-incubated (20 mM) samples at a Chl concentration of 100 $\mu\text{g mL}^{-1}$ in a 1.5 mm path-width cuvette, with a 150 W tungsten lamp, filtered by a 5 cm water filter and a heat mirror (Ealing 35-6865). The intensity was 2000 $\mu\text{E m}^{-2} \text{s}^{-1}$. The samples were transferred to the pre-cooled cryostat within 30 s from the end of the room temperature illumination.

2.4. Deconvolution analysis of fluorescence emission and FDMR spectra

The $|D\rangle+|E\rangle$ and $|D\rangle-|E\rangle$ resonance lines of the FDMR spectra, recorded at multiple emission wavelengths, were simultaneously fitted using a global procedure based on a symmetrical Gaussian function. The use of Gaussian bandshapes is justified because at the temperatures at which the experiments are performed (1.8 K) the band-shape of the ODMR spectra is essentially determined by the inhomogeneous site distribution of the triplet sublevels energy [31].

The microwave frequency of the Gaussian maxima and bandwidths were fit parameters, and were constrained to remain constant for the different spectral series ($|D\rangle+|E\rangle$ or $|D\rangle-|E\rangle$). Amplitudes were allowed to change with



Scheme 1. Principle of ODMR spectroscopy. Upon irradiation with a resonant microwave (MW) field the steady-state population of the singlet ground state (S_0) is altered and can be monitored *via* increased or decreased absorption or emission of the chromophore. Legend to symbols— S_1 : first singlet excited state; T_1 : first triplet excited state; ISC: intersystem crossing; MW (on/off): resonant microwave field (on/off). The energy scale is arbitrary and serves to elucidate the triplet manifold resonance only.

corresponding bands in the $|D|+|E|$ and $|D|-|E|$ being coupled through a constant amplitude scaling factor. This scaling factor was determined by the fit procedure. To take into account the differences in the signal to noise levels between the spectra recorded at different emission wavelengths, spectra of the sample in a microwave region in which the radiation did not induce triplet sublevel transitions were recorded and the error estimated as the root mean square noise over the average output component, weighted for the number of scans. In this way a constant uncertainty was estimated for each point in the data set. The FDMR spectra were globally deconvoluted using software which minimises the sum of $\chi^2(\lambda_{em})$ by a combination of Simplex and Levenberg–Marquard non-linear least-square algorithms as previously described in detail [24,56].

3. Results and discussion

In the following we present a detailed characterisation of the chlorophyll triplet populations in freshly isolated thylakoid membranes from the green algae *C. reinhardtii* by means of the Optically Detected Magnetic Resonance (ODMR) technique. Since the fluorescence emission of PS II and PS I at low temperatures are readily discriminated, Chl triplet states associated with one or the other photosystem can be assigned on the basis of their microwave induced emission spectra (MIF). Fig. 1A shows the fluorescence emission spectrum of *C. reinhardtii* thylakoids at 4.5 K. The low temperature emission spectra of *C. reinhardtii* is significantly different from that generally observed in higher plants and other green algae [24,57,58], both in the blue wing which is due to PSII emission, and in the emission tail above 700 nm which is dominated by PS I emission. In order to characterise the terminal Chl *a* emission forms associated with each photosystem, we have further analysed the emission spectra by Gaussian deconvolution (Fig. 1B). A sharp emission peak at 689 nm is observed together with shoulders at 682 nm and 695 nm. The presence of at least three terminal emission forms in PS II is confirmed by the analysis of the second derivative (*data not shown*) and the Gaussian deconvolution of the emission spectra in the 4–50 K temperature range (*data not shown*). The bands peaking at 682 nm, 688 nm and 695 nm are consistent with terminal Chl *a* emitters as they are characterised by values of full width at half maximum

(FWHM) of about 130–140 cm^{-1} . From the analysis of the *chlorina f₂* barley mutant, which lacks most of the outer antenna complexes, Rijgersberg et al. [57] concluded that the 680–682 nm emission is associated with the LHCII emission while the longer emission forms originate from PS II core antenna. The fluorescence emission characteristics of PS II in *C. reinhardtii* are similar, in terms of sub-band positions and line widths to those previously observed for higher plants systems. However, the intensity of the three main terminal emitters is different. In particular the 688 nm sub-band is more prominent than that previously reported in spinach thylakoids [24,57], while the 695 nm band appears to be sharper and less intense. The fluorescence emission of the broad band attributed to PS I is strongly blue shifted to 715 nm compared to the maximum at 735–740 usually observed in higher plants [24,57] and other green algae [58]. However a shoulder at about 735 nm is still observable in the low temperature emission spectra. The presence of long-wavelength emission forms peaking at 713 nm in native LHCI complex from higher plants has been demonstrated by Jennings et al. [59]. Therefore it is likely that the 715 nm emission peak observed in *C. reinhardtii* thylakoids is originating from PS I outer antenna complexes, in which the most red-shifted Chl forms emitting at about 740 nm is present in a lower stoichiometric ratio compared to the 713 nm form observed *in vitro*.

3.1. Chlorophyll triplet populations in non-reduced thylakoid membranes

In previous investigations on isolated spinach thylakoids [24,43] we found that while the majority of Chl triplet states have a lifetime in the order of 1–2 ms, a Chl triplet population having a shorter lifetime in the 100–150 μs range is also present, and is possibly associated with the PS II reaction centre recombination triplet under conditions where the primary quinone acceptor Q_A is semi-reduced [43,44]. Triplet states characterised by different lifetimes can be discriminated in an FDMR experiment because of phase-sensitive detection of the

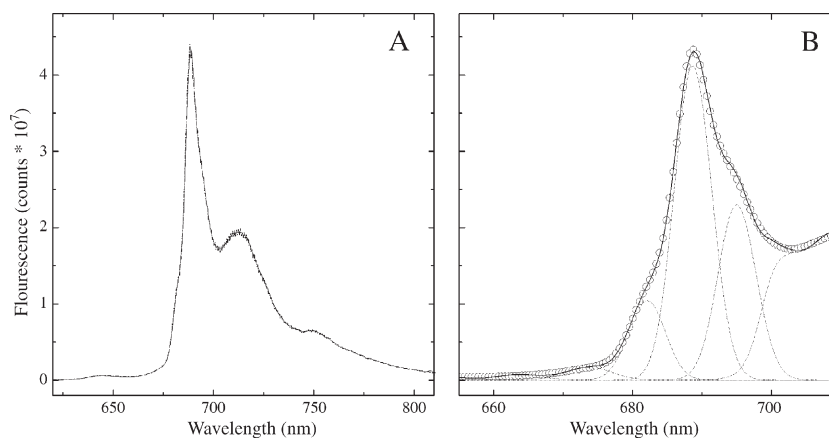


Fig. 1. (A) Fluorescence emission spectra of *C. reinhardtii* thylakoids at 4.5 K. Excitation wavelength 435 nm. Dashed-dotted line: experimental spectrum; solid line: smoothed spectrum. (B) Gaussian deconvolution of the fluorescence emission spectrum of *C. reinhardtii* thylakoids. Open circles: smoothed spectrum, dashed lines: Gaussian sub-bands; solid lines: fit function. Description of the experimental spectrum (peak, FWHM): band 1: 650 nm, 188 cm^{-1} ; band 2: 673 nm, 177 cm^{-1} ; band 3: 682 nm, 133 cm^{-1} ; band 4: 688.5 nm, 134 cm^{-1} ; band 5: 695 nm, 133 cm^{-1} .

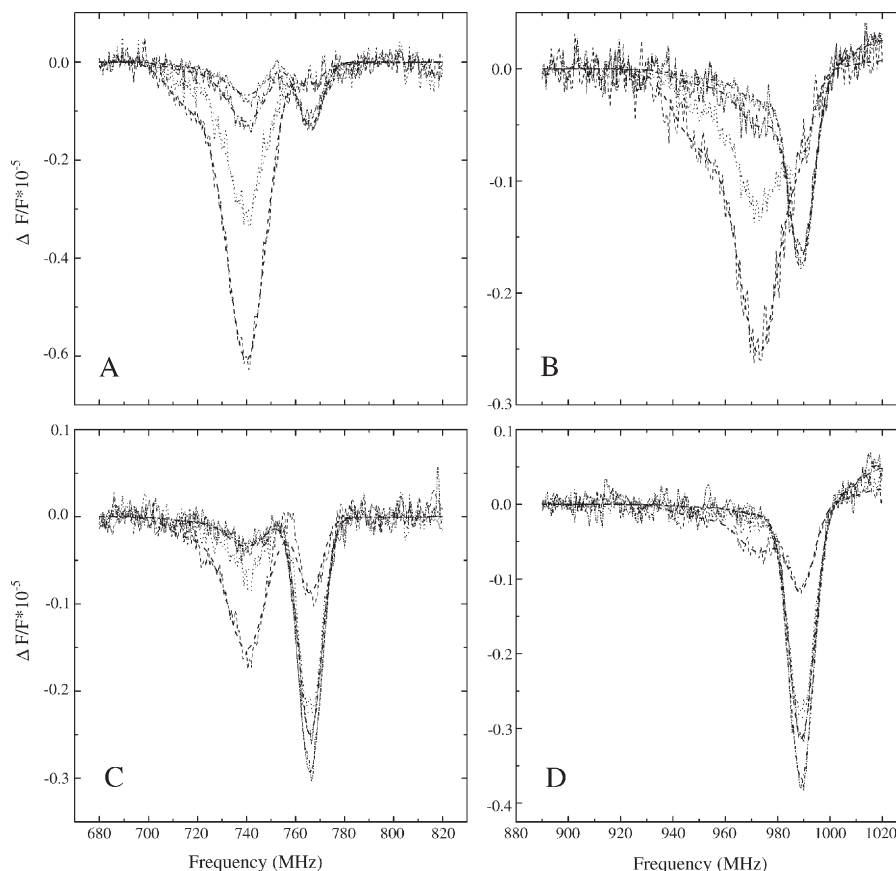


Fig. 2. Fluorescence detected magnetic resonance (FDMR) spectra of chlorophyll triplet states in *C. reinhardtii* thylakoids. $|D|-|E|$ transition: panels A and C; $|D|+|E|$ transition: panels B and D. In panels A and B the microwave/phase setting was optimised on the 740 MHz transition and spectra were acquired using the following conditions of Microwave Modulation (Hz) and Phase ($^{\circ}$)—dashed lines: 33 Hz, $\phi = -106^{\circ}$; dotted lines: 320 Hz, $\phi = -120^{\circ}$; dash-dot-dotted lines: 470 Hz, $\phi = -107^{\circ}$; dash-dot-dot-dotted lines: 1010 kHz, $\phi = -162^{\circ}$. In panels C and D, the microwave/phase setting was optimised on the 766 MHz transitions and spectra were acquired using the following conditions of Microwave Modulation (Hz) and Phase ($^{\circ}$)—dashed lines: 33 Hz, $\phi = 3^{\circ}$; dotted lines: 320 Hz, $\phi = 12^{\circ}$; dash-dot-dotted lines: 470 Hz, $\phi = 19^{\circ}$; dash-dot-dot-dotted lines: 1010 kHz, $\phi = 37^{\circ}$. In order to better distinguish the different spectra, the smoothed spectra (Solid lines) are also presented. Experimental conditions: emission wavelength: 690 nm; temperature: 1.7 K; microwave power: 600 mW.

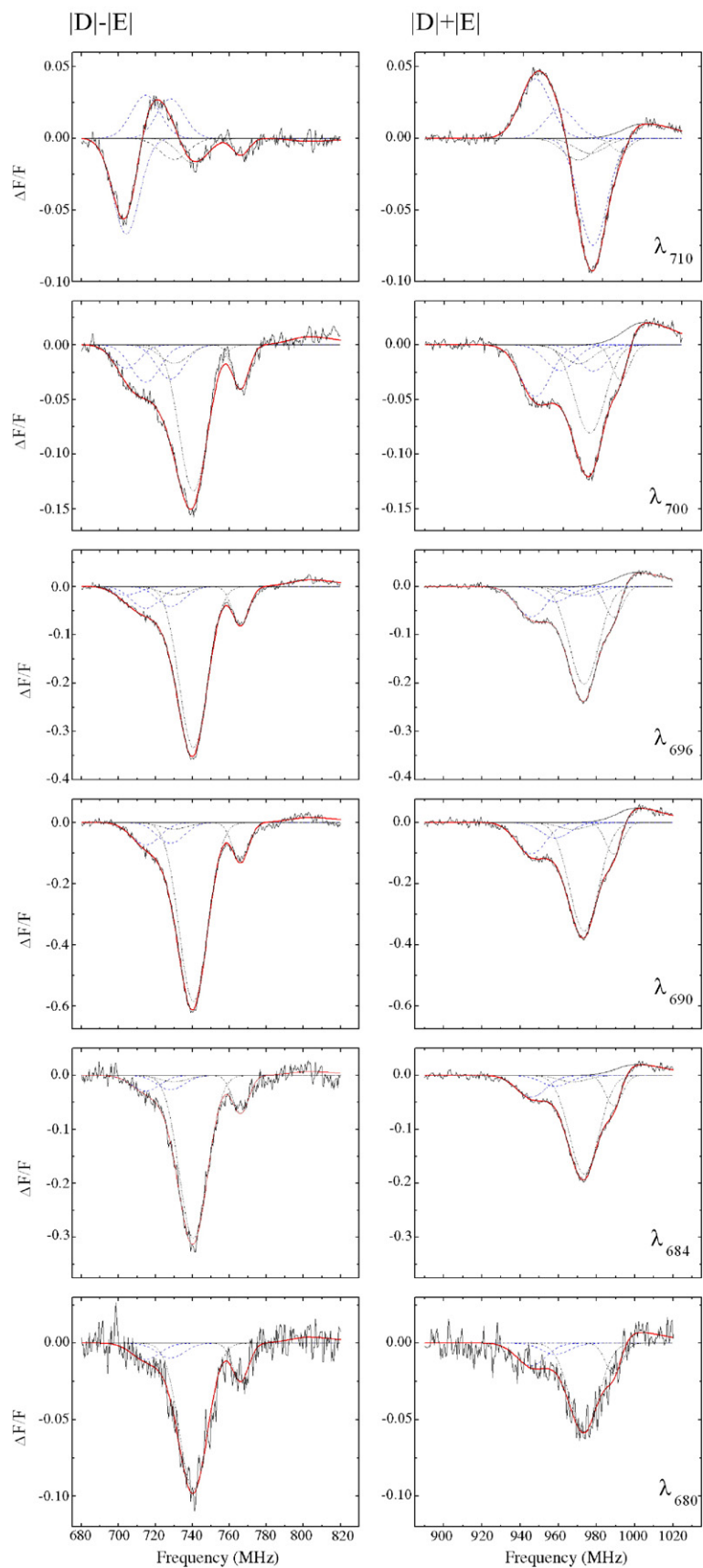
triplet sublevels resonance spectra. In Fig. 2 the FDMR spectra in the $|D|-|E|$ (680–820 MHz) and $|D|+|E|$ (890–1020 MHz) Chl *a* triplet resonance range, measured in *C. reinhardtii* thylakoids near the maximum of PS II emission ($\lambda = 690$ nm) at different microwave modulation frequencies, are presented. The $|2E|$ transitions were much weaker as expected for Chl triplet states and are not reported. In the short microwave region of both lines, the broadness of the FDMR spectra indicates that multiple, overlapping, contributions from different triplet populations are present. The presence of a population showing faster decay, and having resonance maxima at about 766 MHz and 989 MHz, is observed when either the detection phase is kept constant and the amplitude of the dominating triplet population (detected at low modulation frequencies) is reduced by the effect of phase suppression (Fig. 2A, B), or when the signal phasing is performed over this signal (Fig. 2C, D). In the

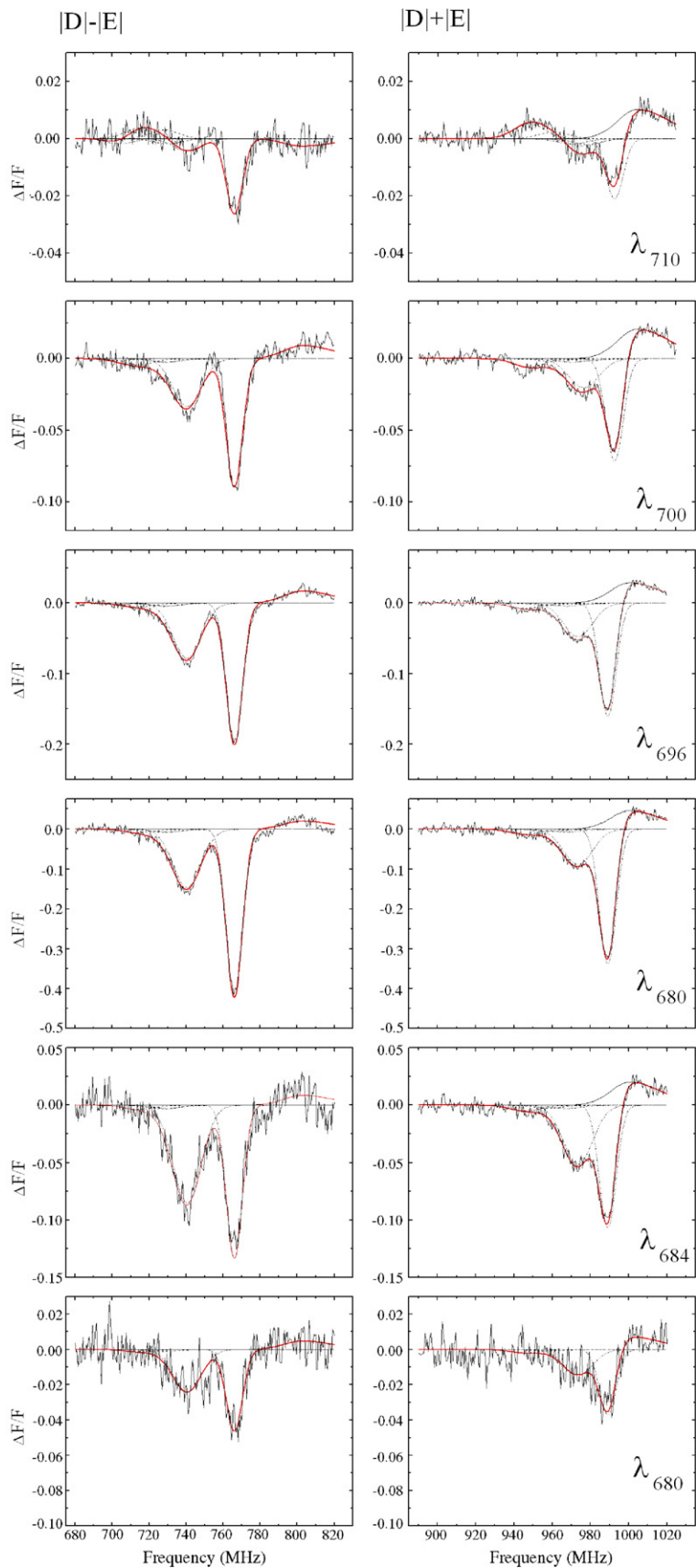
latter case the spectrum of the 766/989 MHz triplet population is resolved more clearly.

In order to untangle the different and overlapping contributions from Chl triplet populations present in thylakoids of *C. reinhardtii* we have analysed the collection of FDMR spectra recorded at multiple emission wavelengths and modulation frequencies of the amplitude of the microwave field by a global decomposition procedure based on Gaussian bands. This approach has been proved fruitful in the investigation of both Chl and Car triplet states in thylakoids purified from higher plants [24,30,43,56].

The FDMR spectra, recorded at emission wavelengths ranging from 680 nm to 700 nm, which are dominated by PS II emission together with their deconvolution in Gaussian bands are presented in Figs. 3 and 4. The FDMR spectra shown in Fig. 3 were collected at a phase angle of -106° and a

Fig. 3. Gaussian deconvolution of the FDMR spectra in the $|D|-|E|$ and $|D|+|E|$ chlorophyll resonance transition range detected at emission wavelengths ranging from 680 nm to 710 nm. Dashed lines: Gaussian components associated with PS I Chl triplet states; dotted lines: Gaussian components associated with PS II triplets states; Solid lines: experimental FDMR spectra; Thick solid lines: fit of the FDMR spectra. Experimental conditions: temperature: 1.7 K; microwave power: 600 mW; modulation frequency, 33 Hz; phase: -106° .





microwave field frequency modulation of 33 Hz, optimised on the maximum of the broad microwave signal peaking at 740 MHz. This corresponds to a triplet lifetime in the range of 1–2 ms, which is a typical range of values reported for the Chl *a* phosphorescence lifetime [16]. Similar lifetimes have been reported for reaction centre recombination triplets in higher plants (e.g. [18,24,39]) and Chl *a* triplet states in isolated Chl–protein antenna complexes (e.g. [20,21,27]).

In order to achieve a more comprehensive characterisation of the triplet population of an estimated lifetime in the 50–150 μ s range, we have recorded spectra at higher modulation frequency of the microwave field (320 Hz), optimised on the 766/989 MHz component ($\varphi=12^\circ$). The emission wavelength dependence of the FDMR spectra recorded under conditions optimised for the 50–150 μ s component are presented in Fig. 4.

The FDMR spectra presented in Fig. 3, detected at wavelengths dominated by PS II emission and at modulation frequency of 32 Hz, show two main structures in both the magnetic transitions investigated, sharp (FWHM ~ 10 MHz) peaks at 766 ± 0.5 MHz ($|D|-|E|$) and 989 ± 0.5 MHz ($|D|+|E|$) and rather broad (FWHM 17–20 MHz) and asymmetric bands peaking at about 735–740 MHz ($|D|-|E|$) and 970–975 MHz ($|D|+|E|$).

The sharp features with minima at 766/989 MHz suggests that this triplet originates from a single and rather homogeneous population of molecules. It is observed both at modulation frequencies of the microwave field of 32 Hz (Fig. 3) or 320 Hz (Fig. 4). However, in the short microwave region of both the $|D|-|E|$ and the $|D|+|E|$ resonance transitions detected at 32 Hz (detection optimised for triplet lifetimes in the ms timescale), the broadness, as well as the changes in symmetry as a function of the detection wavelength, indicates that overlapping contributions are present.

In Figs. 5 and 6 the FDMR spectra, detected in the wavelength region in which fluorescence is essentially emitted by PS I (720–780 nm) and collected at microwave modulations and phases selective for ms and μ s lifetimes respectively, are presented. The global deconvolution analysis of these spectra is also presented.

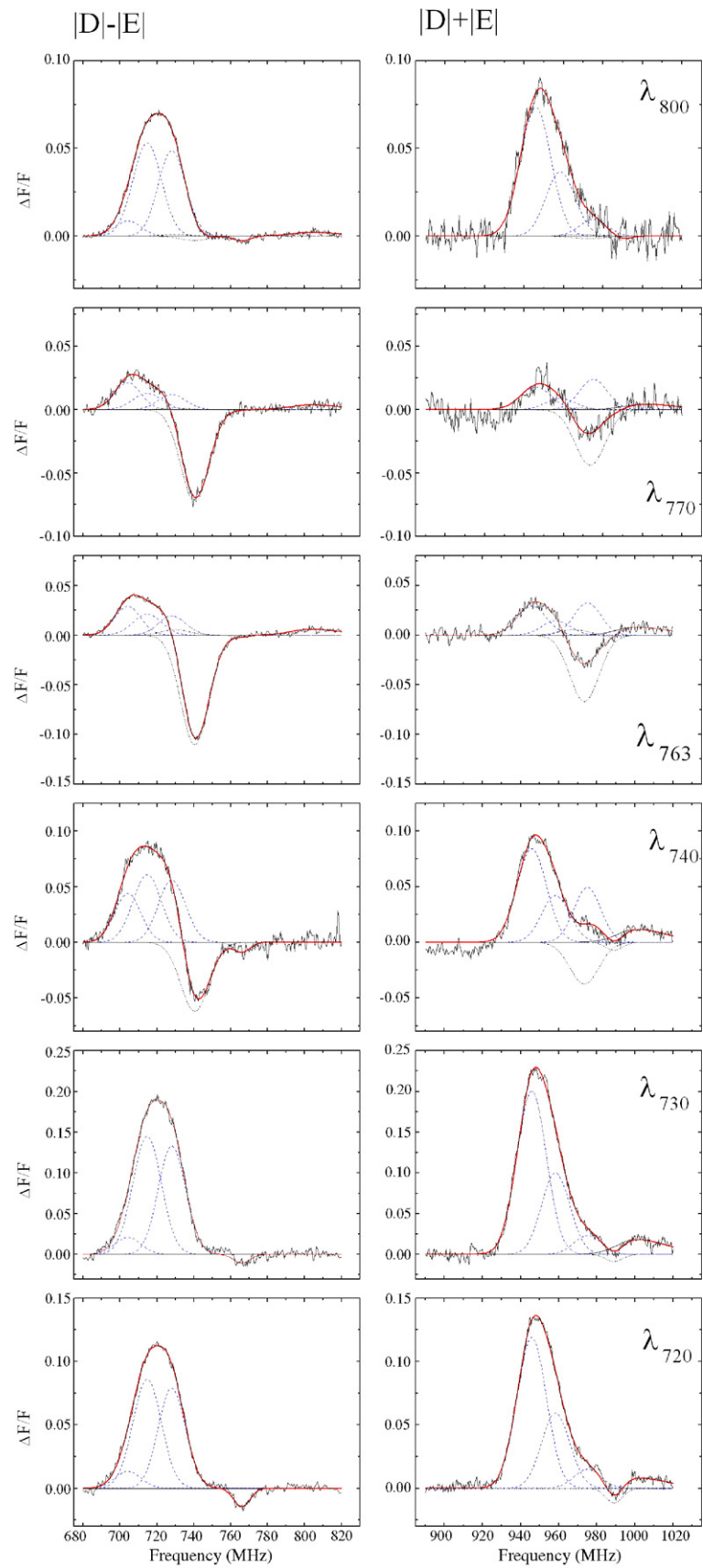
While in spinach thylakoids the FDMR spectra in the PS I fluorescence emission region do not change significantly with detection at the emission wavelengths ranging from 720 to nm [24], a variation of the broadness and symmetry of the transitions is observed in the FDMR spectra of *C. reinhardtii* thylakoids recorded in the 710–800 nm emission region. The spectra show a broad, asymmetric peak at about 735–740 MHz, suggesting that it originates from a contribution of at least two components. A shoulder on the low frequency side is also present, suggesting the presence of a third triplet population, which was also detected in spinach thylakoids although with a reduced amplitude. The spectra presented in Fig. 5 are in close agreement with those previously reported for isolated PSI-200

particles and thylakoids [24,60,61] and have been assigned to $^3P_{700}$ recombination triplets, originating from the reaction centre being frozen in at least two different conformational configurations [60,61]. The signals originating from PSI are almost completely suppressed at higher microwave modulation frequency (Fig. 6).

In order to satisfactorily describe the data, six Gaussian bands are required. The fit parameters are given in Table 1. The 714.7/945.9 and 727.9/958.3 MHz bands are attributed to P_{700} , following the interpretation given by Searle and Shaafsma [60] and Carbonera et al. [61] for isolated PSI particles. Although these bands dominate the FDMR spectra in the 720–740 nm emission window, they are also evident at lower emission wavelength (690–700 nm) with reduced amplitude and opposite signal polarity. The change in the signal polarisation has been attributed to energy transfer from antenna Chls emitting at shorter wavelength than P_{700} [60,61]. Similar observations were reported for spinach thylakoid membranes, but the relative amplitude of triplet components associated with PS I was somewhat less intense in higher plants thylakoids compared to *C. reinhardtii*. The contribution of $^3P_{700}$ components is more clearly observed in Fig. 7 in which the microwave induced fluorescence emission spectrum (MIF) obtained by a plot of the Gaussian band amplitudes versus the detection wavelengths are presented. It is interesting to note that the MIF spectra obtained for the 714.7/945.9 and 727.9/958.3 MHz triplet population attributed to $^3P_{700}$ shows a maximum at about 730 nm, which is about 10 nm blue shifted compared to that obtained in spinach thylakoids. This observation is in agreement with the blue shift of fluorescence emission of PS I in this organism as illustrated in Fig. 1. On the other hand the 704.1/975.1 MHz triplet population which describes the high frequency tail of the FDMR spectra and is dominant at wavelengths longer than 740 nm was observed only as a minor component in spinach thylakoids, and was characterised by a different set of ZFS and resonance frequencies. The difference in fluorescence emission characteristics of *C. reinhardtii* compared to higher plants, and other green algae (e.g. *Chlorella sorokinana*) are likely to originate from the different composition of the outer LHC I antenna pool in this organism. The difference observed in this study relative to the Chl triplet state attributed to PS I antenna might therefore arise from the fact that different terminal emitting Chls are present. The fact that the signal associated with the 704.1/975.1 MHz component is also more pronounced than in higher plant systems indicates a lower coupling and triplet quenching efficiency by the carotenoids in *C. reinhardtii* PS I terminal emitting chromophores.

In the PS II emission region three additional Gaussian bands are required at 730.0/967.6 MHz, 740.5/973.5 MHz and 766/989.1 MHz. We have previously reported similar values from Chl *a* triplet populations in spinach thylakoids (Table 2). The 730.0/967.6 MHz and the 740.5/973.5 MHz triplets have

Fig. 4. Gaussian deconvolution of the FDMR spectra in the $|D|-|E|$ and $|D|+|E|$ chlorophyll resonance transition range detected at emission wavelengths ranging from 680 nm to 710 nm. Dashed lines: Gaussian components associated with PS I Chl triplet states; dotted lines: Gaussian components associated with PS II triplet states; solid lines: experimental FDMR spectra; thick solid lines: fit of the FDMR spectra. Experimental conditions: temperature: 1.7 K; microwave power: 600 mW; modulation frequency, 320 Hz; phase: 12° .



millisecond lifetimes, while the 766/989 MHz triplet has a microsecond lifetime, as mentioned above. The sub-band associated with this triplet is narrow (FWHM 10.5 MHz) with respect to the others (FWHM \sim 17–20 MHz). This can be taken as an indication of a smaller inhomogeneous distribution. We have previously attributed this triplet population to the PS II recombination triplet in conditions of singly reduced Q_A , or to a Chl population tightly coupled, in the singlet state, to the reaction centre [43].

The MIF spectra of all three PS II triplets have a clear minimum at 690 nm (Fig. 7). The 766/989 MHz triplet has a sharper emission triplet-associated spectrum than the other two populations. Based on the assignment of the fluorescence emission bands presented in Fig. 1, and previous studies on antenna-deficient mutants [57], it is reasonable to assign all the three PS II triplet populations, which show a MIF peaking at 690 nm to terminal emitters located either in the inner antenna complexes or in the reaction centre complex of PS II.

However the assignment of a triplet state population to one particular terminal emitter is not always straightforward. Due to efficient singlet energy transfer from the external antenna complexes to the core complexes (which is demonstrated by the low yield of the external antenna complexes emission (682 nm band), even at 1.8 K) the triplet state populated in the outer antenna can, in principle, be monitored on inner antenna emission. We have previously discussed this situation either for Chl *a* [24] or carotenoids [54] triplet states. A triplet state would be observed on the photosystem terminal emitters even though they are initially populated on a shorter wavelength emitter (e.g. Chl^{F684}, Chl^{F688}), as long as the singlet transfer rate from the external antenna to the core is greater than that for transfer from the external antenna to its own terminal emitter (e.g. Chl^{F695}). In this case the MIF spectra would have a maximum at core emission wavelengths but the microwave induced fluorescence emission in the 680–685 nm region is expected to be relatively more intense than for triplets generated in the core complexes themselves.

From the comparison of the MIF spectra presented in Fig. 7 it can be seen that the 740.5/973.5 MHz triplet population has a somewhat broader emission spectra, with respect to both the 730.0/967.6 MHz and, particularly, the 766/989.1 MHz triplets. The increase in the relative fluorescence emission at shorter (680–685 nm) emission wavelengths suggests that the 740.5/973.5 MHz triplet may be generated in the external antenna, as discussed above, while the 730.0/967.6 MHz and the fast decaying 766/989 MHz triplets are probably generated in the core complexes. These assignments are consistent with those observed in spinach thylakoids. However, even though similar triplet populations are observed in thylakoids isolated from spinach and *C. reinhardtii*, the stoichiometric ratio is different. While the 740.5/973.5 MHz triplet population dominates the FDMR spectra in *C. reinhardtii* thylakoids (Fig. 4), the two

730.0/967.6 MHz and 766/989 MHz populations showed almost equal intensities in the FDMR recorded in spinach thylakoids and in leaves [24].

3.2. Chlorophyll triplet populations in reduced thylakoid membranes

None of the triplet populations observed in this present study under non-reducing conditions, can be assigned to the P_{680} recombination triplet ($|D|-|E|$: 720 MHz; $|D|+|E|$: 990 MHz).

We have previously suggested, based on an analysis of signal dependence as a function of the primary quinone acceptor Q_A reduction state, that the 766/989 MHz triplet population can be attributed to the PS II reaction centre under conditions in which Q_A is singly reduced [43]. In order to gain further evidence we performed ODMR experiments after pre-illumination treatments of *C. reinhardtii* thylakoids under reducing conditions, which are known to lead to full reduction of Q_A in PS II and photo-accumulation of the phyloquinone acceptor A_1 in PS I.

Fig. 8 shows the FDMR spectra detected near the maximal emission of PS II (λ_{690}) and PS I (λ_{730}) in *C. reinhardtii* thylakoid membranes incubated with 20 mM dithionite and illuminated with white light ($2000 \mu E m^{-2} s^{-1}$) at room temperature. The description in terms of Gaussian sub-bands is also presented. At PS II detection wavelengths the band shape of the FDMR spectra is strongly modified both in the $|D|-|E|$, 715 MHz and $|D|+|E|$, 991 MHz transitions. This is the signal which has been attributed to the P_{680} recombination triplet [6,62,63] and is strongly overlapped with the two shorter microwave PS II triplet populations observed in non-reducing conditions, and two bands attributed to the P_{700} triplet, monitored at longer emission wavelengths. At PS I emission wavelengths the FDMR band-shape is also partially modified. The 714.7/945.9 MHz and the 727.9/958.3 MHz triplets, arising from $^3P_{700}$ are the dominant triplet populations. The minor 704.1/975.1 MHz component, which is associated to LHC I antenna Chl(s), is hardly detectable after the photo-accumulation treatment. Moreover the 714.7/945.9 MHz $^3P_{700}$ triplet population exhibits a more pronounced increase in intensity than the 727.9/958.3 MHz one. The fast decaying 766/989 MHz triplet population is not detectable, even at optimised microwave frequency modulation and detection phase, in conditions which promote Q_A full reduction. This is in agreement with our previous studies in spinach thylakoids, which demonstrate an inverse correlation between the population of the 718.7/991.5 MHz triplet population originating from the P_{680} triplet under reducing conditions and the fast decaying triplet state.

The ADMR spectra detected at 685 nm and 701 nm absorption wavelengths are shown in Fig. 9. In these spectra only the contribution from the recombination triplets is

Fig. 5. Gaussian deconvolution of the FDMR spectra in the $|D|-|E|$ and $|D|+|E|$ chlorophyll resonance transition range detected at emission wavelengths ranging from 720 nm to 800 nm. Dashed lines: Gaussian components associated with PS I Chl triplet states; dotted lines: Gaussian components associated with PS I triplets states; solid lines: experimental FDMR spectra; thick solid lines: fit of the FDMR spectra. Experimental conditions: temperature: 1.7 K; microwave power: 600 mW; modulation frequency, 33 Hz; phase: -106° .

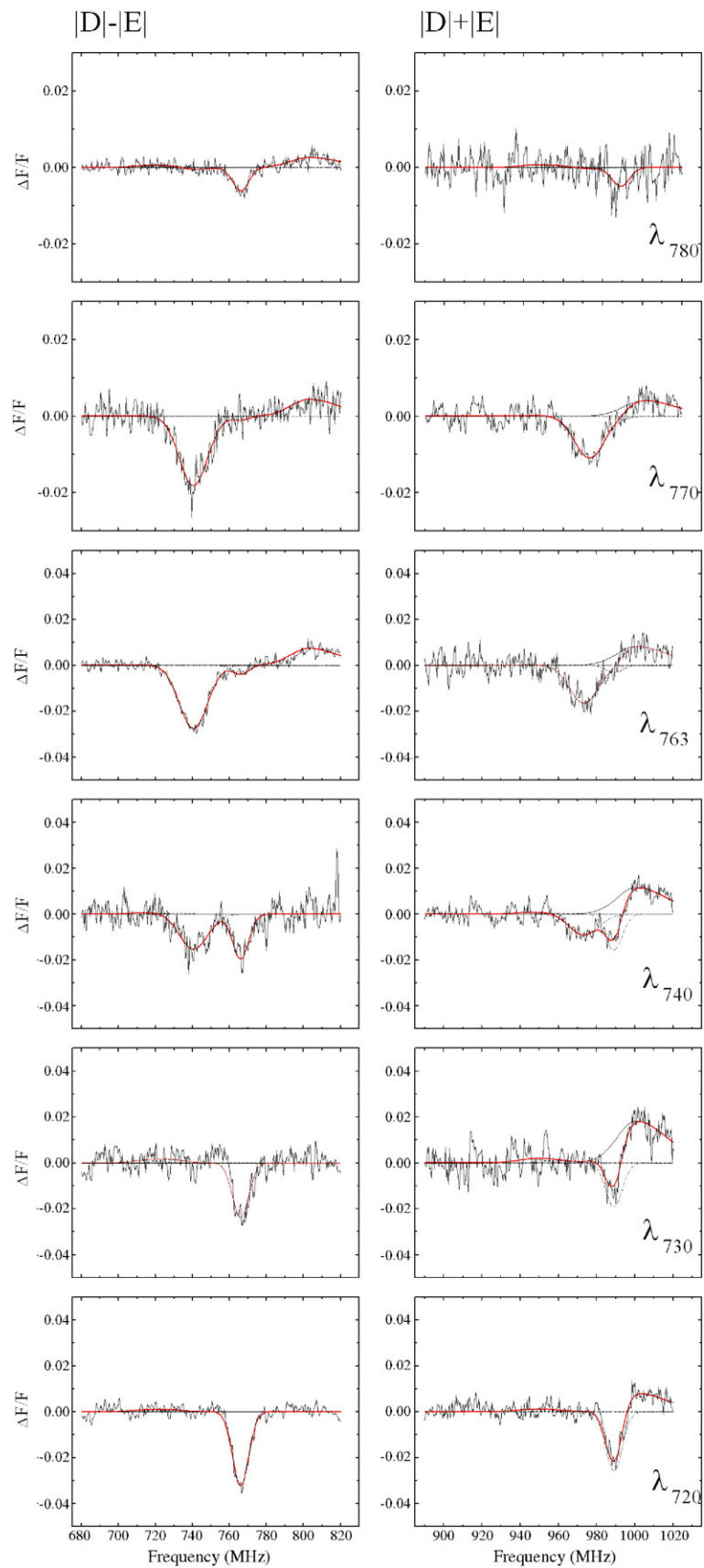


Table 1
Parameters of Global Gaussian Decomposition of FDMR Spectra of Thylakoids

Triplet	$ D - E $ (MHz)	$ D + E $ (MHz)	FWHM (MHz)	$ D $ (cm^{-1})	$ E $ (cm^{-1})	Assignment
T_{PSI}^1	714.7	945.9	18.2	0.0277	0.0039	$^3P_{700}$
T_{PSI}^2	727.9	958.3	17.5	0.0281	0.0038	$^3P_{700}$
T_{PSI}^3	704.1	975.1	18.5	0.0280	0.0045	PSI Core or outer antenna
T_{PSII}^1	730.0	967.6	18.0	0.0287	0.0039	PSII Core antenna
T_{PSII}^2	740.5	973.5	18.0	0.0286	0.0039	PSII Core antenna/ PSII Outer antenna
T_{PSII}^3	766.0	989.1	10.3	0.0293	0.0037	PSII Core antenna
T_{PSII}^4	720.5	991.0	14.7	0.0285	0.0045	$^3P_{680}$

This table reported the value of maxima and the width of Gaussian functions employed in the global deconvolution analysis of FDMR spectra of thylakoids monitored at multiple emission wavelengths. Also indicated are the Zero Field Splitting parameters $|D|$ and $|E|$. Errors are $\pm 0.0001 \text{ cm}^{-1}$.

observed. At 700 nm the ADMR spectra shows contributions from the $^3P_{700}$ triplet only. The description in Gaussian bands is in agreement with the analysis of the FDMR spectra obtained under reducing conditions (Fig. 8, Table 1). Two components with resonance maxima at 714.7/945.9 MHz and 727.9/958.3 MHz are observed. On the other hand, the ADMR detected at 685 nm shows a more complex structure as contributions by both the PS I and PS II reaction centre recombination triplets are observed. While in the $|D|-|E|$ transition the signal arising from PS II and PS I recombination triplets are strongly overlapping, in the $|D|+|E|$ transition the contribution from these triplet states can be easily discriminated. The PS II recombination triplet has a maximal resonance frequency at 992 MHz, while the PS I recombination triplet has maxima at 946 MHz and 958 MHz.

Therefore we have acquired microwave-induced Triplet minus Singlet (T-S) spectra, employing selective magnetic pump frequencies of 992, 945 and 960 MHz in the $|D|+|E|$ transition. The T-S spectrum of the PS II recombination triplet has a main singlet bleaching located at 685 nm, and a weak positive feature near 675 nm (Fig. 10). Also shown in Fig. 10 is the description of the T-S spectrum in terms of Gaussian bands. The details of the fit parameters are listed in Table 2. This spectrum shows strong similarities to the one previously obtained in spinach thylakoids [24], and the one published by Carbonera et al. [61,62] in grana membrane fractions. However the spectra recorded in thylakoids are red shifted by about 0.5 nm compared to the isolated PS II. It is clear that the recombination triplet resides on the long wavelength absorbing chlorophyll form of the PS II reaction centre complex. Moreover the spectrum presented in Fig. 10 differs from those obtained with the isolated reaction centre (D1–D2–Cyt_{b559}) complex (e.g. [6,39,62]). The maximum bleaching in the T-S spectra recorded in the isolated reaction centre is located at 680.5 nm.

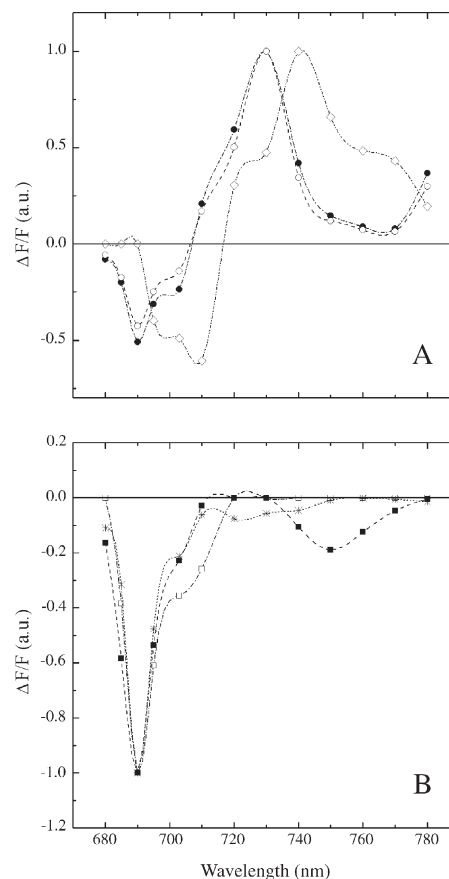


Fig. 7. Microwave-induced fluorescence emission spectra of Chl triplet states in *C. reinhardtii* thylakoids. (A) Triplet states associated with PS I. Open circles: 714.7/945.9 MHz component ($^3P_{700}$); full circles: 727.3/958.3 MHz component ($^3P_{700}$); open diamond: 704.1/975.1 MHz (T_{PSI}^3) component. (B) Triplet states associated with PS II. Open squares: 730.0/967.6 MHz component (T_{PSII}^1); full squares: 740.5/973.5 MHz component (T_{PSII}^2); stars: 766/989.1 MHz component (T_{PSII}^3).

A long wavelength structure near 684 nm is also observed. However the positive structure near 675 nm, present in our spectrum, is also present in those of the isolated reaction centre complex. In the isolated complex, an electronic transition located at 683–684 nm is usually detected [7,64–66] which appears to be red-shifted to 685 nm in intact thylakoids. We therefore interpret the difference observed between the intact thylakoids and that of the isolated D1–D2–Cyt_{b559} complex in terms of a perturbation of pigment arrangement occurring during reaction centre isolation, which leads to a blue-shift of the optical properties of the Chl which carries the triplet state at cryogenic temperatures. The minor structure present in the T-S spectrum of the reaction centre complex near 684 nm probably reflects the 685 nm band seen in our spectrum. The absence of major changes in the T-S spectrum outside the band peaking at 685 nm indicates that the Chl₆₈₅ may be considered to be

Fig. 6. Gaussian deconvolution of the FDMR spectra in the $|D|-|E|$ and $|D|+|E|$ chlorophyll resonance transition range detected at emission wavelengths ranging from 720 nm to 800 nm. Dashed lines: Gaussian components associated with PS I Chl triplet states; dotted lines: Gaussian components associated with PS II triplets states; solid lines: experimental FDMR spectra; thick solid lines: fit of the FDMR spectra. Experimental conditions: temperature: 1.7 K; microwave power: 600 mW; modulation frequency, 320 Hz; phase: -12° .

Table 2
Parameters of the Gaussian decomposition of T–S spectrum of PS II Reaction Centre triplet

Bands	Peak (nm)	FWHM (nm)	Height (a.u.)
Band 1	652.9	8.34	0.0427
Band 2	662.3	8.73	0.0587
Band 3	674.2	6.40	0.2474
Band 4	679.5	4.60	−0.285
Band 5	684.7	4.78	−0.948
Band 6	689.4	6.66	−0.183
Band 7	696.0	5.10	−0.0815

This table reported the value of maxima and the width of Gaussian functions employed in the deconvolution analysis of T–S spectrum of PS II reaction centre triplet presented in Fig. 10.

predominately monomeric. This is in agreement with the measured ZFS $|D|$ 0.0285 cm^{-1} and $|E|$ 0.00451 cm^{-1} (Table 1) which are typical for a monomeric Chl *a* in the penta-coordinated state [67,68]. Electron paramagnetic resonance spectroscopy on orientated reaction centres and PSII enriched membrane and single crystals [38,69,70] have shown that the triplet states reside on a Chl which is tilted by about 30° with respect to the axis perpendicular to the membrane plane. It is interesting to note that Noguchi et al. [72,73] have shown that

the triplet states reside on a Chl monomer which is closely associated, but distinct, from the primary donor cation P_{680}^{+} . Moreover photon-echo spectroscopic studies on the isolated reaction centre have provided evidence for primary charge separation originating from the accessory chlorophyll bound to the D1 subunit, rather than from the pair of Chl *a* parallel to the photosystem symmetry axis [71]. It is therefore likely that the triplet state resides on the Chl from which the primary charge separation is initiated [71,10,11], and it is the electron hole which is transferred to the parallel Chl dimer. Although the electronic transition of this chlorophyll appears to be red-shifted with respect to the bulk absorption of PS II antenna, careful examination of the T–S spectra indicates the presence of a long wavelength transitions which extends up to 695 nm and are generally not observed in the isolated D1–D2–Cytb₅₅₉ complex (Table II). The presence of long wavelength Chl forms has been recently reported in isolated PS II core purified by improved biochemical procedures [74]. The long wavelength absorption forms might result from weak excitonic interaction bands, which are perturbed upon triplet state population.

In Fig. 11 the microwave induced T–S spectrum, obtained with a pump frequency of 945 and 960 MHz in the $|D|+|E|$ transition, are presented. The T–S spectra recorded at the two

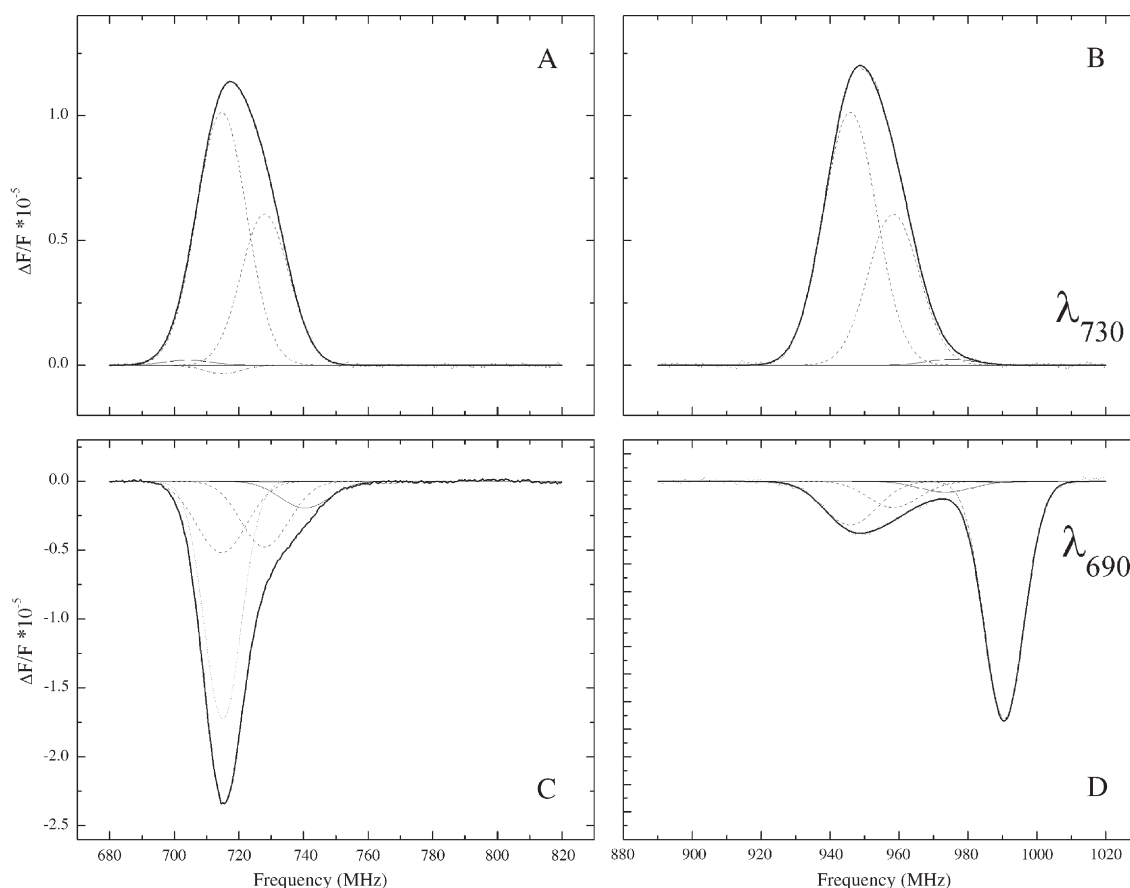


Fig. 8. FDMR spectra of the $|D|-|E|$ (A, C) and $|D|+|E|$ (B, D) Chl transitions of *C. reinhardtii* thylakoids reduced by 20 mM sodium–dithionite and preilluminated for 4 min at room temperature. Dashed lines: Gaussian components associated with PS I Chl triplet states; dotted lines: Gaussian components associated with PS II triplets states; dash-dotted: PSII recombination triplet ($^3P_{680}$); solid lines: experimental FDMR spectra, thick solid lines: fit of the FDMR spectra. Experimental conditions: temperature: 1.7 K; microwave power: 600 mW; modulation frequency, 33 Hz; phase: -106° . Panels A, B emission wavelength: 730 nm; panels C and D emission wavelength 690 nm.

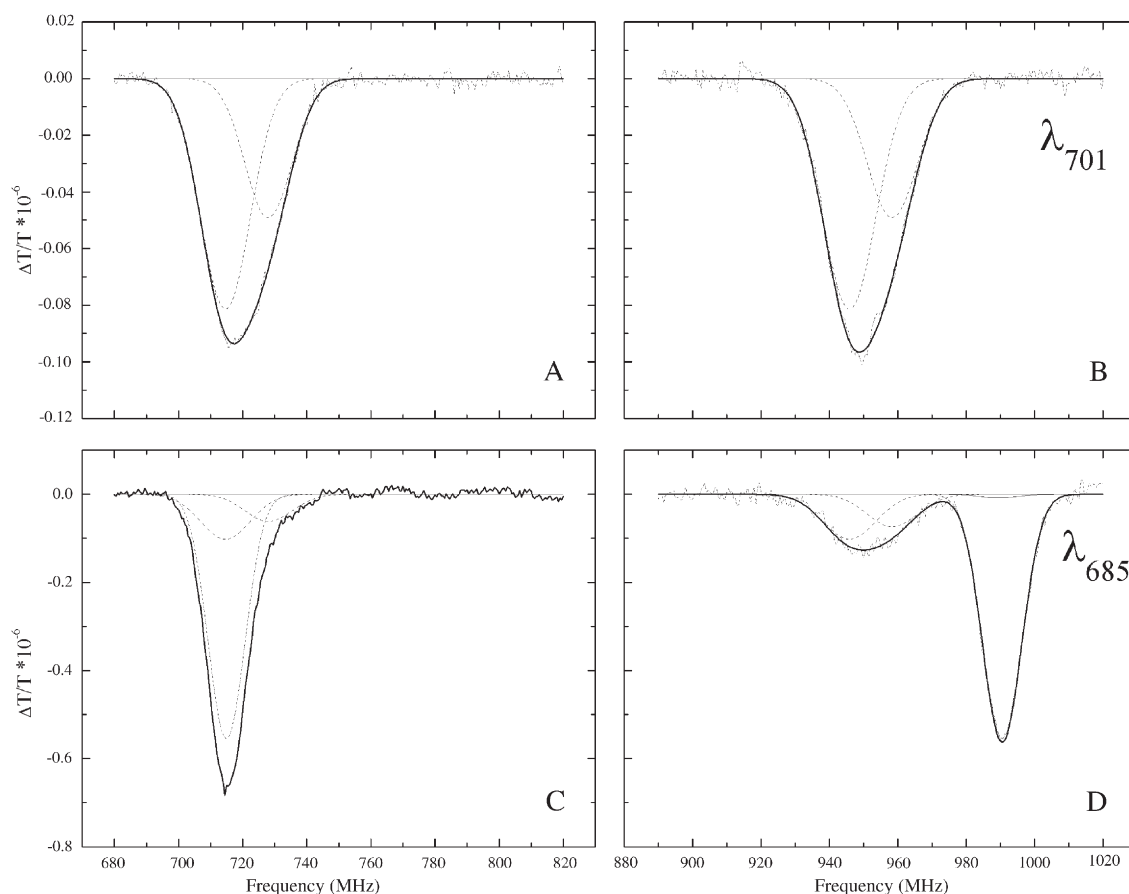


Fig. 9. Absorbance detected magnetic resonance spectra of the $|D|-|E|$ (A, C) and $|D|+|E|$ (B, D) Chl transitions of *C. reinhardtii* thylakoids reduced by 20 mM Na–Dithionite and preilluminated for 240 s at room temperature. Dashed lines: Gaussian components associated with PS I Chl triplet states; dotted lines: Gaussian components associated with PS II triplets states; dash-dotted lines: PSII recombination triplet ($^3P_{680}$); solid lines: experimental FDMR spectra; thick solid lines: fit of the FDMR spectra. Experimental conditions: temperature: 1.7 K; microwave power: 683 mW; modulation frequency, 33 Hz; phase: -106° . Panels A, B absorption wavelength: 701 nm; panels C and D absorption wavelength 685 nm.

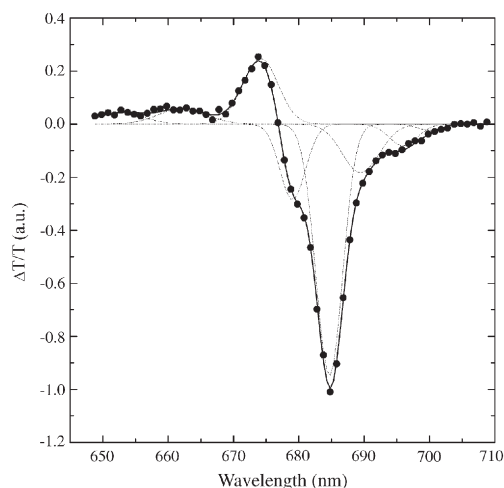


Fig. 10. Microwave induced Triplet minus Singlet (T–S) spectra of PS II reaction centre recombination triplet ($^3P_{680}$) in the Chl Q_y absorption region, detected in *C. reinhardtii* thylakoids reduced by 20 mM Na–Dithionite and preilluminated for 240 s at room temperature. The description in terms of Gaussian sub-bands of the T–S spectra the $^3P_{680}$ are also presented. The microwave resonance frequency was set near the maximum in the $|D|+|E|$ transition at 992 MHz. Full circles: experimental spectrum; solid lines: fit function; dashed lines: Gaussian sub-bands. Optical resolution: 0.5 nm, scan rate: 0.1 nm/s. All the other conditions as in the legend of Fig. 8.

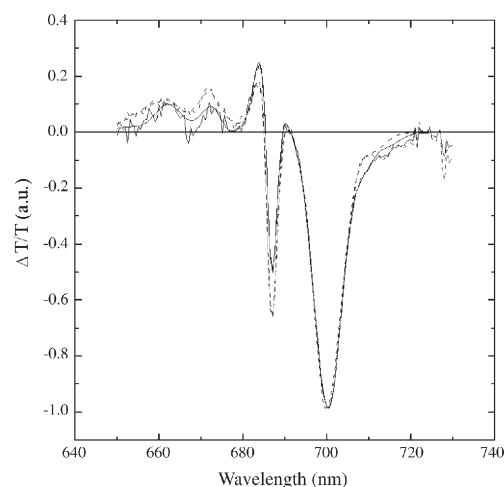


Fig. 11. Triplet minus Singlet (T–S) spectra of PS I reaction centre recombination triplet ($^3P_{700}$) in the Chl Q_y absorption region, detected in *C. reinhardtii* thylakoids reduced by 20 mM Na–Dithionite and preilluminated for 240 s at room temperature. The microwave resonance frequency was set near the maxima in the $|D|+|E|$ transition derived from the deconvolution of the ADMR spectra at 940 MHz (dashed lines) and 955 MHz (dashed dotted lines). The smoothed spectra are also presented for clarity. All the other conditions as in the legend of Fig. 10.

microwave resonance frequencies are very similar. These provide further confirmation to the assignment of the 714.7/945.9 MHz and the 727.9/958.3 MHz triplet state to $^3\text{P}_{700}$ conformers. The T–S spectra of PSI recombination triplets which have a broad singlet bleaching peaking at 700 nm also exhibit sharp and intense derivative-shaped features with minima at 683 nm and maxima at 686 nm (Fig. 11). These bands can not originate from an electrochromic effect on the primary donor, because the triplet state is electrically neutral. Broader and less intense contributions are observed in the 660–680 nm region as well. The crystallographic structure of PS I shows distances of about 5–7 Å within the four central chlorophylls [4]. Thus they are close enough to establish quite strong (greater than 100 cm^{-1}) excitonic interactions between the excited singlet states. Excitonic coupling strengths in the order of $80\text{--}130\text{ cm}^{-1}$ have been recently calculated based on the analysis of the T–S spectra, as well as the $\text{P}_{700}^+ \text{ minus } \text{P}_{700}$ difference spectra, in PS I particles purified in the cyanobacterium *Synechococcus elongatus* [12]. These interactions give rise to excitonic band splitting and redistribution of dipole oscillator strengths which are perturbed upon triplet population formation and lead to a redistribution of oscillator strength as well as band shifts, which are observed for the T–S spectra recorded at both microwave resonance frequencies. It should be noted that such complex structures are not observed in the case of the PS II reaction centre T–S spectrum, indicating that the excitonic coupling within the PS II reaction centre Chls is much weaker than in the reaction centre of PS I.

4. Additional discussion

The triplet states of Chl do not play an important role in terms of the photochemical reactions which take place from the first singlet excited state. However, the physiological importance of Chl triplet states reside in their involvement in photo-oxidative processes, through the generation of the highly reactive oxygen singlet species that are capable of damaging most biological compounds (e.g. protein, lipids and the chromophores themselves). Common to the most popularly proposed mechanisms for PS II photoinhibition is the involvement of a state generated from the forward electron transfer processes or from charge recombination reactions, like the reaction centre triplet, as a result of a malfunction of the acceptor side. The recombination triplet ($^3\text{P}_{680}$) is populated with high efficiency in the isolated reaction centre of PS II and it leads both to singlet oxygen production [39,40] and damage to the reaction centre itself. Based on this evidence it has been suggested that $^3\text{P}_{680}$ is the principal species involved in PS II photoinhibition [39–41]. This suggestion however is not waterproof as the long living recombination triplet observed in the isolated reaction centre is not readily formed in intact PSII, being detectable only under strongly reducing conditions which are unlikely to occur naturally. Of course this triplet readily forms in the isolated in the isolated reaction centre of PS II, due to the lack of binding of both the Q_A and the Q_B quinones, and therefore mimics the strongly reducing condi-

tions which are needed to detect a long living (ms) reaction centre Chl triplet in thylakoids and intact PS II preparations [24,35,36,39–43]. However, this is not of physiological relevance. Moreover the recombination triplet hypothesis is not supported by earlier data in the literature in which the rate of photodamage exhibits only a slight dependence on the excited state level (i.e. singlet quenching) in PS II antenna, both in thylakoids isolated from several species [45–51] as well as in whole algae of *C. reinhardtii* [49]. Neither it is supported by evidence from the high-resolution action spectra of photo-inhibition in visible light [47,49] where it was suggested that either damaged, or incorrectly assembled Chl-binding complexes could be involved in photo-inhibition. Furthermore the action spectra of photo-inhibition show striking similarities with phosphorescence excitation spectra, which is a direct measurement of the absorption of the molecules giving rise to triplet state formation in thylakoids and green algae, [75]. Both the photoinhibition action spectra and the phosphorescence excitation spectra are 3–4 nm blue shifted compared to the photon absorption spectra of PS II in the thylakoid membranes [47,49]. The action spectra measured both with the wild-type and with the *Chlorina f₂* mutant of barley indicate the involvement of inner and outer antenna complexes in which singlet energy coupling amongst the bound Chl cofactors is perturbed [47,49]. Since quenching of the Chl triplet state by carotenoids requires orbital overlap between the donor and the acceptor molecule, while singlet energy transfer can take place even at distances of tens of Å, it is reasonable to expect that pigments which are weakly coupled in the singlet state will also be uncoupled in the triplet state, and hence have an higher probability of interacting with oxygen and thus producing strongly reactive species. With this aspect in mind, over recent years, we have searched for other triplet populations which are more readily formed in PS II i.e. not under strongly reducing conditions. In fact, using ODMR techniques we initially found three such populations in isolated spinach thylakoids [24] and have now extended these studies to include thylakoids prepared from *Chlamydomonas*. The relative population intensity of the three PS II associated triplet states, varies between *C. reinhardtii* and spinach. Nevertheless, the formation of triplet states in PS II distinct from the recombination triplet, is widespread in nature and, under our measuring conditions, are readily populated. The lifetime of the triplet states detected, excluding the fast decaying 766/989.1 MHz population, are in the ms timescale, a value close to those reported for unquenched Chl in organic solvents (see ref. [43] for a discussion). Therefore the triplets observed in the present study are not quenched by carotenoids. This may indicate that these triplets form on complexes in which Chl–Car coupling is weak or absent possibly due to incorrect pigment–protein assembly, as suggested by the photoinhibition action spectra [47,49]. The observation that triplet states characterised by similar ZFS and magnetic resonance transition frequencies are observed both in higher plants and green algae, also point towards the idea that the proposed molecular mechanism of visible-light induced photo-damage involving weakly coupled Chl–protein complexes is

common in evolutionary divergent phyla, rather than being species-specific. Moreover, the three triplet populations associated with PS II are also observed in whole cells of *C. reinhardtii* (data not shown) and were previously monitored in spinach leaves [24], excluding the possibility that they might originate from a purification artefact.

Of course, it may be argued that the triplet populations which we detect at cryogenic temperatures may not be populated at physiologically relevant temperatures. At the present stage we have no clear answer to this point. However, some relevant comments can be made. First, the rate of intersystem crossing, in the absence of quenching phenomena, is not strongly temperature dependent. Second, quenching of Chl triplet states by the carotenes, occurs *via* the Dexter mechanism, which requires orbital overlap, and is therefore strictly dependent on the geometrical arrangement of the donor (Chl triplet) and acceptor (Car triplet) molecules. Considering the high packing density of Chl–protein complexes large changes in pigment conformations due to the lowering of the temperature, which could affect the Chl–Car coupling are unlikely. It is worth noting that all existing crystallographic models [1–4] are also obtained by X-ray diffraction data collected at very low temperature (10–40 K). Thus Chl triplets formed under the very low temperatures required for ODMR measurements are also expected to be formed at higher temperatures at which the ODMR detection is impaired. However, these Chl triplets have not been detected and are probably not detectable by optical methods at room temperature due to the weakness of the signal, which is estimated to originate from only 1–3 Chl per PS II [50]. Consistent with this suggestion is the fact that microwave-induced T–S spectra associated with the three PS II triplet observed in non-reducing conditions can not be recorded even at 1.8 K.

The presence of a short lifetime (~ 150 μ s) recombination triplet was reported originally by time-resolved optical spectroscopy [42] and more recently by ODMR [24,43] and time-resolved EPR [44] techniques, at low temperatures (<20 K). The fast decaying triplet (766/989 MHz) is probably located on a Chl population close to the reaction centre and could represent the reaction centre recombination triplet populated under conditions of singly reduced Q_A . This triplet species can obviously also be involved in photo-inhibitory phenomena. Differently from case of Chl triplet populated in antenna complexes in which singlet–singlet and triplet–triplet energy transfer are impaired, the steady-state level of a triplet state populated by charge recombination in the reaction centre is expected to be proportional to the single excited state level in the bulk of the coupled antenna. Therefore, singlet excited state quenching phenomena, such as photochemical and non-photochemical quenching, should lead to proportional lowering of the recombination triplet population in the reaction centre. As mentioned above, the protective effect of singlet excited state quenching in PS II antenna on photo-inhibition is weak, and can be quantified in 20–25% of the expected [45,47,49,50]. This residual protection conferred by singlet excited state quenching can in principle be explained by the involvement of the fast-decaying triplet state populated in the reaction centre of PS II as

a singlet oxygen sensitizer. However, because of its shorter lifetime, it is expected to have a reduced probability of interaction with molecular oxygen. Therefore, this value of $\sim 25\%$ traces an upper limit for the reaction centre triplet states contribution to photo-inhibition.

Acknowledgements

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