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Applied Magnetic Resonance

FDMR of Carotenoid and Chlorophyll Triplets in Light-Harvesting Complex LHCII of Spinach

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Abstract. Fluorescence detected magnetic resonance (FDMR) of the light-harvesting complex LHCII of the spinach photosynthetic machinery revealed triplet contributions both from Carotenoids and Chlorophylls. All three carotenoids present in the complex (lutein, neoxanthin and violaxanthin) are evidenced as triplet states in the FDMR signals obtained as variation of the emission intensity of the Chlorophylls in the 680 nm region. The triplets show $|D|$ values of 0.0401, 0.0388 and 0.0382 cm^{-1}. A comparison with the results obtained by ADMR (Absorption Detected Magnetic Resonance) is made and discussed. An interesting concentration effect is discovered and discussed in terms of specific interactions between carotenoids and chlorophyll molecules. Signals are also obtained by microwave sweeping in the Chlorophyll regions and one triplet is detected ($|D| = 0.028–0.029$ cm^{-1}). The polarization of the carotenoid signals is discussed in terms of singlet-singlet and triplet-triplet energy transfer between carotenoids and chlorophylls, also with the help of double resonance experiments. Double resonance experiments involving both carotenoids and chlorophylls signals gave negative results. It is not possible as a consequence to assess that the chlorophyll whose triplets levels are scanned in the FDMR spectra are functionally connected to the carotenoids.

1. Introduction

It is well known that magnetic resonance is quite suited for studying the reaction centre (RC) and the antenna (AT) pigments of the photosynthetic apparatus in bacteria and in higher organisms. In particular, for AT pigments, optically detected magnetic resonance (ODMR) has been used quite extensively to study carotenoids in bacterial systems [1] and, following some older pioneering work [2, 3], has been recently applied to an isolated LHCII complex of higher plants [4] where a carotenoid triplet had been previously re-

vealed by conventional EPR [5]. Carotenoids are in fact present in the photosynthetic membrane both to extend the light harvesting spectrum and to protect Chlorophyll pigments from the harmful effects of photooxydation.

ODMR can be used specifically in different versions according to the method used to monitor the changes which occur under the microwave resonance. The version used in the LHCII case [4] was that of detecting differences in optical absorbance (ADMR). Here we report further experiments on the same complex by using a different detection method, that of monitoring differences in the fluorescence intensity of the sample (FDMR).

The investigation has more interest than that of a mere confirmation of a result by a different method. Carotenoids are in fact, as is well known, almost non fluorescing. For this reason any fluorescence change from the sample obtained by sweeping a microwave resonance known to be due to a carotenoid molecule would be in itself a direct evidence of an energy transfer interaction between carotenoids and chlorophyll molecules, one of the important functions carried out by the AT pigment-protein complexes.

In fact we have detected for the first time FDMR signals coming from carotenoid triplets in isolated pigment-protein complexes and we report in this paper on the informations obtained with this technique.

2. Materials and Methods

2.1. Apparatus

The microwave source was an HP-8350-B sweep oscillator with plug-in HP-83522A (0.01–2.4 GHz). The microwaves were amplified by a Log.Metrics-A210/L TWT amplifier in the range 1–2 GHz and by a SCO-Nucletudes 10-46-30 TWT amplifier in the range 0.01–1 GHz (the experiments were performed to a maximum output power of 2–3 W) and transmitted via a semi-rigid coaxial cable to the sample placed in a slow-wave helix (5 turns) with a pitch of about 2 mm.

The microwaves were amplitude modulated with a squared wave from a WAVETEK (model 164), at frequencies between 4 and 7000 Hz depending on the experiment, the demodulation being done by a EG & G 5210 lock-in amplifier.

Optical excitation was provided by a Tungsten-halogen 250 W Philips lamp supplied with a OLTRONIX B 32-20R power supply. The light was focused on a flat sample cell (0.5 × 1.0 mm) contained in a liquid-helium cryostat (Oxford Instruments, model Spectromag 4) which can be pumped down to 1.25 K. The cell was kept with the normal to its plane rotated 40° (about the cryostat axis) from the incident beam and the emission was collected at 90°

with a light pipe inserted between the inner window and the outer one of the cryostat.

For the detection we used an OSI 5KM Centronic photo-diode after selection of the emission light with a band pass filter which includes the chlorophyll fluorescence maximum.

To run normal emission spectra a Jobin-Yvon monochromator was used in place of the interference filter.

The instrumental sensitivity of our apparatus is now $1 \cdot 10^{-6}$ ($\Delta I/I$), two orders of magnitude higher with respect to our previous set up [6].

2.2. Sample Preparation and Carotenoids Analysis

PSII membranes were obtained from spinach leaves in the usual way [7]. The membranes were then solubilized with 1% dodecyl-β-D-maltoside (DM), subjected to a separation in 0.1–1 M sucrose gradient (10 mM Hepes-NaOH, pH 7.5, and 0.06% DM) run at 39000 rpm in a SW 41 rotor for 24 h, at 4°C [8] and, finally, dialyzed against the medium. Sample concentration was 250 μg/ml as chlorophyll Glycerol to 66% was always added to the samples to avoid matrix cracking and heterogeneity.

The relative abundance of the different carotenoids in our sample was determined by HPLC using a reverse phase column. Elution was done by a linear gradient of 90% methanol to 100% ethyl acetate for 20 minutes at a flow rate of 1 ml/min. Detection was made at 430 nm. The ratios found were: Lutein: 1, Neoxanthin: 0.67, Violaxanthin: 0.21.

3. Results

Figure 1 shows the low-temperature absorption and emission spectra of LHCII. The main absorption peak at 677 nm is due to Chlorophyll-*a* (Chl-*a*) absorption; a shoulder is seen at 670 nm. The main emission band is at 683 nm and low intensity peaks are visible at lower energies with a prominent one at 740 nm.

Figure 2 shows the FDMR lines as detected for LHCII by sweeping the microwaves in the regions where ADMR signals were found [4] and monitoring the Chl-*a* fluorescence at 680 nm. (No differences were noted in experiments done by monitoring at 740 nm.) The line in the 1250–1370 MHz region is clearly inhomogeneously and asymmetrically broadened as one expects from the ADMR results which were clearly distinguishing two triplets. However the 1256 MHz triplet appears in FDMR with higher intensity and

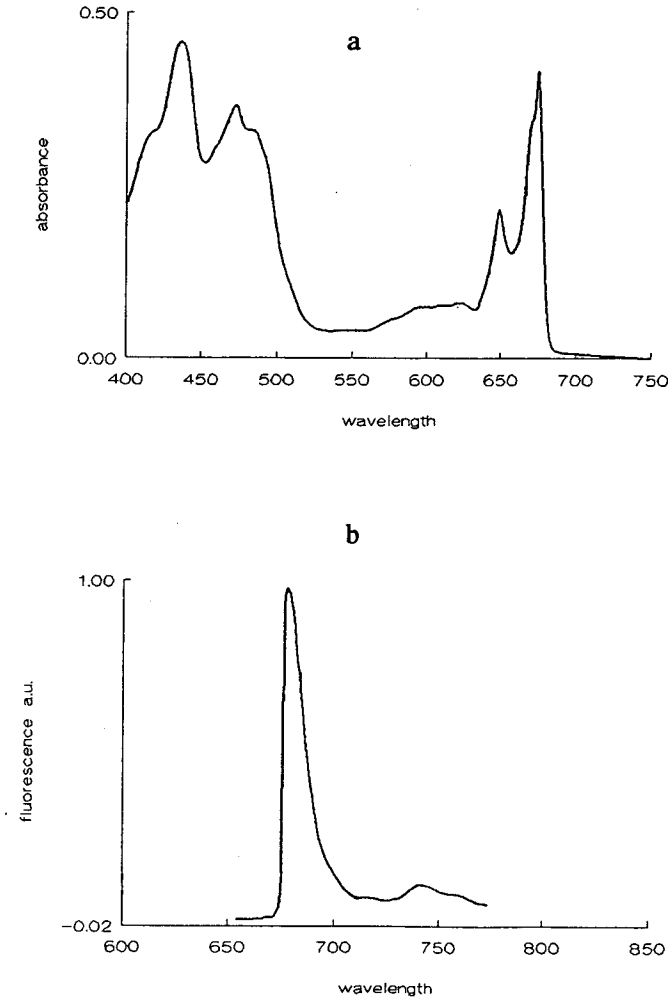

Fig. 1. Absorption and emission spectra at 4 K of LHCII: a) absorption; b) emission.

the 1318 MHz triplet with lower intensity than was deduced from the ADMR results. We have performed experiments at progressively lower concentrations of the sample and discovered marked changes in line shape with dilution. Simulations of the line-shape were made at each concentration by sums of two or three Gaussians having the same relative positions and line widths. Fig. 3 shows the simulations by using three Gaussians. The use of only two Gaussians is not sufficient for a global fit.

The Gaussian decomposition has been performed also on the 1090 MHz region line ($D - E$ transition); Fig. 4 shows the simulations with three Gaussians having positions obtained by subtracting from the frequency of the

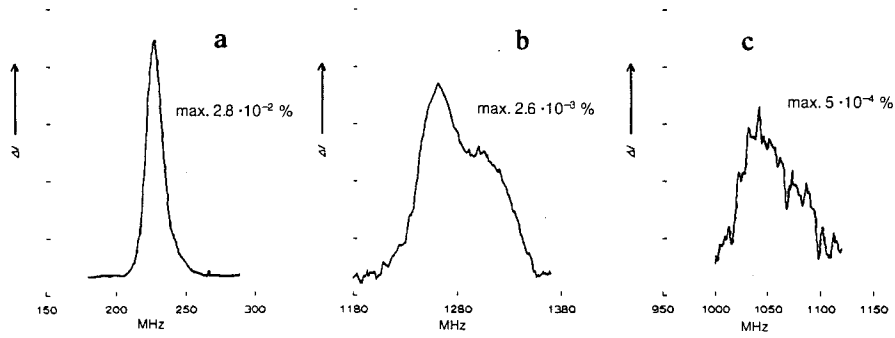

Fig. 2. Carotenoid regions of the FDMR spectrum at 1.8 K of LHCII. Mw modulation 315 Hz: a) $2E$ region, Mw power 1 W, sw.r. 0.3 MHz/s, t.c. 3 s; b) $D + E$ region, Mw power 1 W, sw.r. 0.3 MHz/s, t.c. 3 s; c) $2E$ region, Mw power 2 W, sw.r. 0.12 MHz/s, t.c. 10 s. Chl concentration 31 μg/ml.

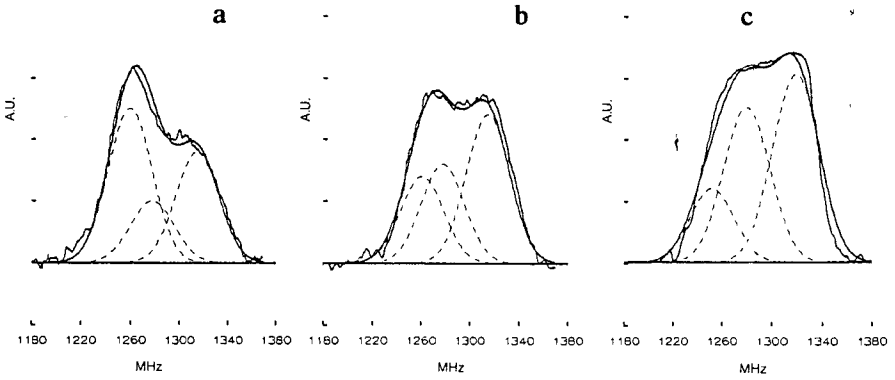

Fig. 3. Gaussian decomposition of carotenoid $D + E$ transitions at different concentrations: a) Chl conc. 31 μg/ml; b) Chl conc. 15.6 μg/ml; c) Chl. conc. 7.8 μg/ml. Detection conditions as in Fig. 2.

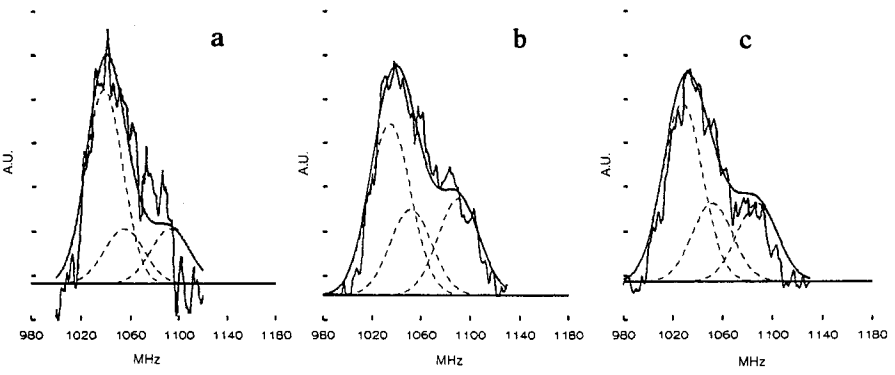

Fig. 4. Gaussian decomposition of carotenoid $D - E$ transitions at different concentrations. Conditions as in Fig. 3.

Table 1. Relative intensities of $D+E$ and $D-E$ transitions of carotenoid triplets in LHCII from Gaussian decomposistions. Full with at 1/e: 44 MHz for each component. (a) Chl conc. 31 μg/ml; (b) Chl conc. 15.6 μg/ml; (c) Chl conc. 7.8 μg/ml.

$D+E$ (MHz)	Relative Intensities (a)	(b)	(c)	$D-E$ (MHz)	Relative Intensities (a)	(b)	(c)
1260	0.47	0.26	0.18	1035	0.64	0.48	0.51
1278	0.19	0.30	0.36	1053	0.18	0.24	0.24
1315	0.37	0.44	0.46	1090	0.18	0.27	0.24

corresponding Gaussian in the $D+E$ transition that of the center of the very intense and narrow $2E$ transition (228 MHz), the linewidth is the same as before. Table 1 summarizes the results of the Gaussian decompositions for both lines at three different dilutions.

Double resonance experiments were performed by pumping on the $D+E$ and monitoring the $D-E$ regions. Increase in the $D-E$ intensity was observed, an example is shown in Fig. 5.

Emission spectra were scanned while sitting on the different carotenoids lines, and in different positions within the lines. Our spectral resolution was not sufficient to detect differences in the emission spectrum of the sample.

Other signals are observed in the microwave frequency regions where chlorophyll triplet lines are expected. Fig. 6 shows the lines observed in the two regions (the $2E$ transition is too weak to be observed). Good signals are ob-

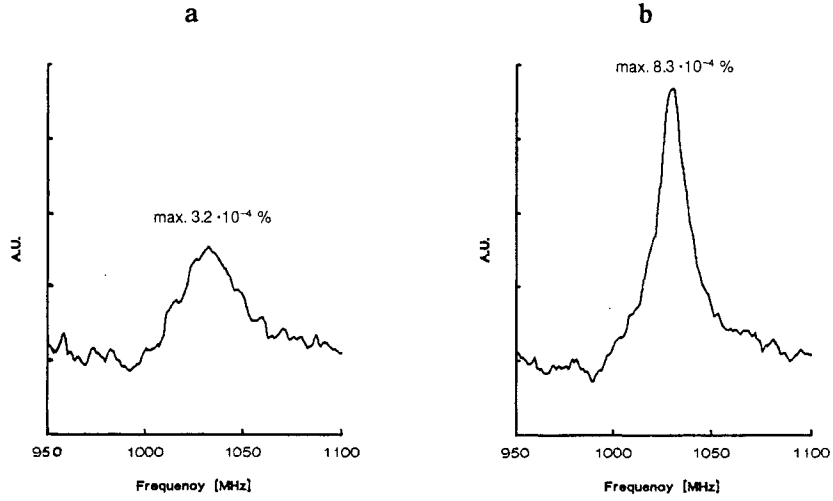

Fig. 5. Double resonance experiment on $D-E$ transition: a) no CW pumping; b) CW on $D+E$ at 1260 MHz, power 1 W, other conditions as in a).

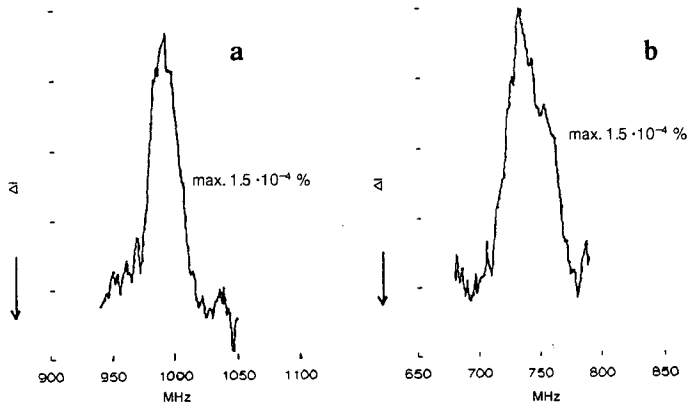

Fig. 6. Chlorophyll regions of the FDMR spectrum at 1.8 K of LHCII. Mw modulation frequency 17 Hz; Mw power 25 mW; sw.r. 0.12 MHz/s; t.c. 10 s.

tained down to much lower modulation frequencies than necessary for the carotenoids signals. The sign of the chlorophyll signals is inverted with respect to that of the carotenoids. This means that chlorophyll triplet overall population increases under microwaves resonance.

Double resonance experiments by pumping on the carotenoids lines and monitoring the detected chlorophyll resonances and vice versa have been attempted with negative results.

4. Discussion

LHCII is a multigenic population of pigment-protein complexes with a composition which can vary with species and with growth conditions of the plants [9]. The chlorophyll content of at least one of the LHCII complexes is sufficiently well known, after the recent investigations on two-dimensional crystals by Kuhlbrandt and co-workers [10] which have given also some hint on the relative position of the Chlorophyll molecules within a complex. The basic LHCII unit, investigated by Kuhlbrandt, contains 15 chlorophyll molecules per complex of which seven are Chl-*b* and eight Chl-*a*.

Three types of carotenoid molecules are known to be present in LHCII: Lutein (**1**), Neoxanthine (**2**) and Violaxanthine (**3**) [11]. Carotenoid to chlorophyll relative abundance is about 1 : 2 while the ratios of carotenoid abundance are claimed to be on the average 1 : 0.57 : 0.25 [5]. They are in fact very similar to those measured in our samples.

Practically nothing is known about the location of the carotenoids in the complexes and on the specificity of their interaction with the chlorophylls. We will try to extract some informations about these aspects from our data.

4.1. The Sign of the Carotenoid Signals

First of all we shall comment on the fact that all three regions of the carotenoid FDMR spectra correspond to an increase of chlorophyll fluorescence when resonant microwaves are applied to each pair of triplet levels. The connection with the chlorophyll fluorescence is depicted in Fig. 7, a scheme illustrating energy transfer from carotenoid to chlorophyll via the singlet states and from chlorophyll to carotenoid via the triplet states. A steady state analysis (see Appendix) based on this scheme predicts that the fluorescence intensity change (ΔI) should be opposite to the triplet concentration change (ΔC_t). The latter has been shown to be negative for the carotenoid signals obtained in the ADMR experiments [4] and the positive FDMR signals are consequently in agreement with them. From the three signals having all the same sign and from the further observed fact that the intensity of the three lines (at the same rf power) is in the order $2E \gg D+E \gg D-E$ we extract the steady state population and decay pattern illustrated in Fig. 8a. This is in agreement with the double resonance experiment shown in Fig. 5. Under saturation excitation of the $D+E$ transition at 1260 MHz, the $D-E$ transition becomes almost twice more intense. The proposed scheme is to be preferred to the one proposed in [4] which would not be compatible with the $2E$ transition being more intense than the $D+E$. The labeling of the three levels is done with z being the long axis of the carotenoid molecule, consistently with previous discussions [5, 12]. The x and y labels remain unspecified.

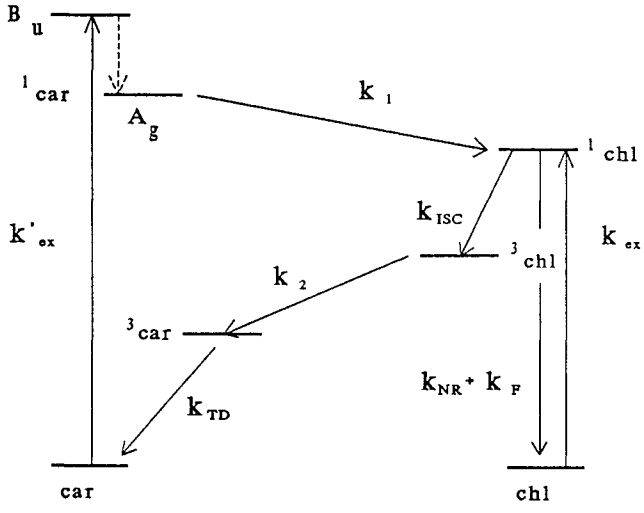

Fig. 7. Energy transfer scheme of chlorophyll-carotenoid connections.

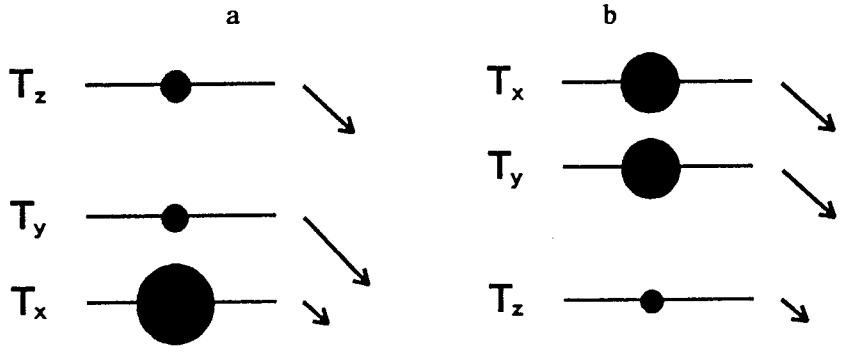

Fig. 8. Qualitative polarization pattern of triplet states in LHCII: a) carotenoids; b) chlorophylls.

4.2. The Identity of the Carotenoid Triplets and their ZFS

The Zero Field Splitting (ZFS) values for the three triplets are reported in Table 2. They agree with those obtained from the EPR and ADMR experiments [4, 5], if one considers that those data were interpreted on the basis of one or two triplets only.

As for the assignment to the specific xantophylls known to be present in the complex, the differences in the conjugation pattern of the three molecules is too small to be used as a key. The three D values are in agreement with carotenoids having 8–9 conjugated double bonds if we interpolate within one of the theoretical correlation diagrams of D vs. the number of such bonds [1, 5]. The correlation is in fact based on bacterial systems carotenoids whose number of double bonds span a much larger range of values (9–13). In the region 8–9 we expect D values between $|0.040|$ and $|0.038|$ cm^{-1} as found for our systems. In such a small region however inversions in the trend may well occur due to structural effects modulating the triplet wave function.

Table 2. Absolute values of ZFS (cm^{-1}) of carotenoid triplets in LHCII complexes.

trip.	FDMR [this work]		ADMR [4]		EPR [5]	
	D	E	D	E	D	E
I	0.0401	0.0038	0.0400	0.0038	0.0395	0.0037
II	0.0388	0.0038	0.0385	0.0038		
III	0.0382	0.0038				

4.3. The Concentration Effect

We now discuss one of the most interesting features of our experiments, the changes occurring on dilution in the line shapes of the $D+E$ and $D-E$ regions.

The relative intensity of each carotenoid FDMR line is made up by the product of a triplet relative concentration term (C_i) and of a triplet relative polarization term (P_{ij}), the index i denoting the carotenoid and the index j the transition.

We can assume, for simplicity, that P_{ij} are properties of the carotenoid, negligibly dependent on the chlorophyll interactions. The C_i, on the other hand, must mainly be determined by the chlorophyll interactions because the triplet is populated via the chlorophyll triplet state (Fig. 6). On dilution (or by the action of detergents which have been shown to produce similar effects), aggregation among the complexes in solution is made less effective [13] and this has consequences on the fluorescence yield. We must now accept that only some of the chlorophyll molecules of a complex, or only some of the complexes of the heterogeneous LHCII population, are affected in this way by aggregation and that these molecules and/or complexes are connected to different carotenoids in specific ways.

Another source of apparent C_i variations could be a singlet-triplet fluorescence quenching, dependent on aggregation in a selective way for each carotenoid. In this case the carotenoid triplet population would not depend greatly on dilution, but the intensity of the lines would be affected by the differences in fluorescence yield. In any case the C_i' are to be considered parameters determining the intensity of the line at constant polarization.

By a trial and error global analysis of the line shape at the different sample concentrations we arrive to a set of C_i for each concentration and a set of P_{ij}. The values are reported in Table 3; they reproduce neatly the intensity composition of the lines as shown.

Table 3. P and C parameters describing concentration behaviour of relative intensities (exp. in parenthesis) in carotenoids transitions. (a) Chl conc. 31 μg/ml; (b) Chl conc. 15.6 μg/ml; (c) Chl conc. 7.8 μg/ml.

				(a)			(b)			(c)	
trip.	$P(D+E)$	$P(D-E)$	C	$I(D+E)$	$I(D-E)$	C	$I(D+E)$	$I(D-E)$	C	$I(D+E)$	$I(D-E)$
I	0.18	0.44	0.64	0.44 (0.47)	0.74 (0.64)	0.37	0.24 (0.26)	0.52 (0.48)	0.35	0.20 (0.18)	0.47 (0.51)
II	0.30	0.22	0.16	0.19 (0.19)	0.10 (0.18)	0.29	0.31 (0.30)	0.20 (0.24)	0.37	0.35 (0.36)	0.25 (0.24)
III	0.51	0.34	0.20	0.34 (0.37)	0.17 (0.18)	0.24	0.45 (0.44)	0.27 (0.27)	0.28	0.45 (0.46)	0.28 (0.24)

The simplest way of figuring out a carotenoid-chlorophyll interaction pattern giving rise to the observed effects is to envisage a minimum of Car-Chl complexes as there are different carotenoids in the system. Each one of these complexes involves chlorophylls which behave differently when the complexes aggregate.

Different interactions between carotenoids and chlorophyll were detected also in the ADMR work [4]. From the ADMR data a speculative model hypothesis of the interaction was attempted which could be easily reconciled with the finding that three carotenoids rather than two contribute to the spectra.

On the other hand, the polarization differences among carotenoids and between lines should be reflected by differences in decay rate constants and/or population rate constants. Differences contained within such limits that the qualitative polarization pattern of Fig. 7a is not affected, are clearly sufficient. Data up to now available [5] are very scanty and quite uncertain being largely based on line-shape simulations of conventional EPR spectra. Time resolved measurements on the ODMR lines in the microseconds range and new ADMR spectra at different sample concentrations would provide further insight in this problem. Until these are available we feel it would be uncautious to try further speculations on possible models of interaction.

4.4. The Chlorophyll Signals

The frequencies of the $D+E$ and $D-E$ lines attributed to chlorophyll triplets are 990 and 745 MHz respectively. The sign is inverted with respect to the carotenoid signals. The corresponding ZFS values are $D=|0.0292|$ and $E=|0.041|\,\mathrm{cm}^{-1}$. The $2E$ transition is not detectable.

Chlorophyll FDMR signals in a preparation similar to the present one were already obtained [3] in experiments performed without microwaves modulation. The observations and the measured values are in perfect agreement with the present ones. The previous authors were not able to detect the carotenoid signals probably because of their use of a DC detection technique.

In our experiments chlorophyll signals can be observed with modulation frequencies up to 300–400 Hz. At higher modulation frequencies the $D-E$ carotenoid line can be observed without the perturbing presence of the $D+E$ chlorophyll line. This must be a consequence of the different lifetimes of the two types of triplet states.

The chlorophyll triplets detected by low frequency modulated FDMR must have lifetimes in the millisecond range, while the carotenoids triplets, as is known [5], have lifetimes in the microseconds range.

The chlorophyll ZFS and the polarization of the triplet levels (Fig. 7b), as deduced from the sign and from the absence of the $2E$ transition, are in agreement with the features shown by Chl-*a* monomers *in vitro* [3].

The preceding discussion points toward the fact that the observed signals derive from Chl-*a* molecules not connected by energy transfer to the carotenoids. For chlorophyll molecules connected to carotenoids as described by the scheme of Fig. 6, we would expect in fact very short triplet lifetimes, and a very small stationary concentration. Also a strong interaction with carotenoid molecules should affect the D values as it occurs *in vitro* when perturbations, e.g. dimerization, are present.

It is possible to envisage the existence of chlorophyll molecules in LHCII complexes not directly connected to carotenoids since the stoichiometry is quite in favour of the chlorophylls. It is however also possible that the chlorophyll signals are due to isolated chlorophyll molecules, separated from the complexes during the preparation procedures and trapped in detergent micelles not eliminated by the dialysis. This latter explanation would also justify the absence of double resonance effects from carotenoid to chlorophyll signals and vice versa.

5. Conclusions

This paper has shown that FDMR can demonstrate directly the existence of energy transfer connections between carotenoids and chlorophyll molecules in photosynthetic antenna complexes. The technique is also able to distinguish all different carotenoids existing in LHCII complexes and to point out specific differences in Chl-Car interactions.

It appears that ODMR is indeed among the few experimental handles to be utilized in the search of much needed informations on the function of the numerous isolated pigment-protein complexes of the Antenna system of higher plants photosynthetic apparatus.

Time resolved ODMR experiments in the microseconds range are needed to help in further clarifying some of the questions which have been raised in this paper.

Appendix

The scheme of Fig. 6 supposes that both carotenoid and chlorophyll are light absorbing species while only chlorophyll is emitting. The scheme is simplified in such that the chlorophyll triplet decay to the ground state is neglected with respect to transfer to the carotenoid triplet and the carotenoid

ISC from singlet to triplet is also neglected with respect to transfer to the chlorophyll singlet. These simplifications have no effect on the form of the result.

The kinetic expressions of interest are those related to the singlet states:

$$\frac{d[C_1]}{dt} = k'_{ex} \cdot [C_0] - k_1 \cdot [C_1][Ch_0] ,$$

$$\frac{d[Ch_1]}{dt} = k_{ex} \cdot [Ch_0] - (k_f + k_{nr} + k_{isc}) \cdot [Ch_1] + k_1 \cdot [C_1][Ch_0] ,$$

the last term on the right side of both equations expressing the "bimolecular" process of singlet energy transfer

$$C_1 + Ch_0 \xrightarrow{k_1} C_0 + Ch_1 .$$

In the approximation that neglects the concentration of the fastest decaying states (C_1, Ch_1 and Ch_t), the mass balance equations are simply:

$$[Ch_0] = NCh,$$

$$[C_0] = NC - [C_t],$$

where NCh and NC are the chlorophyll and carotenoid concentration independently of electronic state.

From the steady state conditions we obtain

$$[Ch_1] = \frac{k_{ex} NCh + k'_{ex}(NC - [C_t])}{k_{nr} + k_f + k_{isc}}$$

and

$$\Delta[Ch_1] = -\frac{k'_{ex}}{k_{nr} + k_f + k_{isc}} \Delta[C_t] ,$$

and finally for the fluorescence intensity:

$$\Delta I = k_f \cdot \Delta[Ch_1] = - k'_{ex} \cdot \varphi \cdot \Delta[C_t]$$

with

$$\varphi = \frac{k_f}{k_{nr} + k_f + k_{isc}} .$$

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