Photosynth Res (2009) 102:403–414
DOI 10.1007/s11120-009-9407-5

REVIEW

Optically detected magnetic resonance (ODMR) of photoexcited triplet states

Donatella Carbonera

Received: 7 November 2008 / Accepted: 15 January 2009 / Published online: 24 February 2009

Abstract Optically Detected Magnetic Resonance (ODMR) is a double resonance technique which combines optical measurements (fluorescence, phosphorescence, absorption) with electron spin resonance spectroscopy. After the first triplet-state ODMR experiments in zero magnetic field reported in 1968 by Schmidt and van der Waals, the number of double resonance studies on excited triplet states grew rapidly. Photosynthesis has proven to be a fruitful field of application due to the intrinsic possibility of forming photo-induced pigment triplet states in many sites of the photosynthetic apparatus. The basic principles of this technique are described and examples of application in Photosynthesis are reported.

Keywords ODMR · Triplet state · FDMR · ADMR · T–S · Chlorophyll · Carotenoid

Abbreviations

ODMR	Optically detected magnetic resonance
FDMR	Fluorescence detected magnetic resonance
ADMR	Absorption detected magnetic resonance
Chl	Chlorophyll
MIF	Microwave-induced fluorescence
T–S	Triplet minus singlet

ODMR of triplet states

Optically Detected Magnetic Resonance (ODMR) is a double resonance technique which combines optical measurements (fluorescence, phosphorescence, absorption) with electron spin resonance spectroscopy. In the study biological samples, ODMR spectroscopy is mainly applied to triplet states, in the absence of external magnetic fields (the so-called zero field-ODMR or ZF-ODMR spectroscopy). Electronic triplet states can be seen as intrinsic molecular "spin probes" for a system, because they carry electronic paramagnetism and are endogenous probes of molecular structure and molecular interactions with the environment. As we will see, magnetic resonance transitions between the spin sublevels of a triplet state produce, under the right experimental conditions, changes in the optical properties of the system. Changes in phosphorescence (PDMR), fluorescence (FDMR), and absorption (ADMR) are often easily observed.

The first triplet state ODMR measurements of organic molecules were done in 1967, independently by Kwiram (1967), Schmidt et al. (1967), and Sharnhoff (1967), by adding a phosphorescence detecting system to a traditional X-band EPR spectrometer. The ODMR, in a high magnetic field while being suitable for the detection of well-oriented triplet states in single crystals, is far from ideal in the case of randomly oriented systems and thus, in particular, is not suitable for proteins which are usually not available in a single crystal form. The anisotropy of the interactions for randomly oriented systems leads to EPR spectra which are typically spread over 1,000 Gauss, with a considerable loss of sensitivity. In ODMR performed in zero-field, the main factor controlling the line-width is the inhomogenous broadening, caused by the differences in the local environment of the paramagnetic species, which leads to much higher resolution of the transition frequencies compared with EPR.

The first ZF-ODMR experiments have been reported in 1968 (Schmidt and van der Waals 1968) and the field of double resonance of the excited triplet states has grown

D. Carbonera (✉)
Dipartimento di Scienze Chimiche, Università di Padova, 35131 Padova, Italy
e-mail: donatella.carbonera@unipd.it

rapidly since then (Clarke 1982). A fruitful field of application has proven to be Photosynthesis. An important contribution to this field has been given by Hoff, especially in improving the ADMR technique (for a review, see Hoff 1996).

In this article, the physics of the triplet state will be first introduced, and then the basic principles of the ZF-ODMR spectroscopy will be discussed. At the end of the article, some representative examples of the applications in Photosynthesis will also be discussed.

Triplet state

A triplet state is an electronic state in which two electrons in different molecular orbitals have parallel spins, as shown in Fig. 1. The name "triplet" reflects the fact that there are three triplet sublevels. These sublevels are degenerate only for spherical molecular symmetry. For reduced symmetry, the three-fold degeneracy is removed, even in the absence of an applied magnetic field.

Electronic triplet states in molecules are rarely ground electronic states; instead, in chemical and biological systems, they are often present as states which can be populated thermally, by photo-excitation, or, in some cases, by chemical reactions, starting from singlet states. In Fig. 2, the Jablonski diagram, illustrating the relevant molecular electronic states and their population and decay routes, is shown. The ground state of a typical organic molecule is a singlet state, and absorption of light leads to excited singlet states. The unpairing of the electron spins is possible only if there is some degree of "spin-orbit coupling," in which the magnetic field arising from the orbital motion of the two electrons interacts with the spin magnetic moments. If this happens, then the molecule undergoes intersystem crossing (ISC) and becomes a triplet state. The atomic spin–orbit coupling is proportional to the nuclear charge. Often, in organic molecules, only light atoms (C, N, O) contribute to the molecular structure, thus the spin–orbit interaction is weak, and the ISC process is slow.

After an excited molecule crosses into a triplet state, it may remain there a long time because the de-excitation is spin-forbidden. The triplet state may decay to the ground singlet state with (phosphorescence) or without emission of radiation. The energy of the lowest triplet state, T_o, is usually lower than that of the first excited singlet state, S_1, due to the exchange interaction (the physical meaning is that, on average, the two electrons are farther apart in the triplet state than in the singlet state, hence the Coulombic repulsion is less intense and the state energy is lower).

Zero field splitting parameters

Together with the possibility of populating the triplet state by light absorption, the other element which is relevant for performing ODMR spectroscopy, is the paramagnetism associated with the triplet states. Those molecular triplet states, for which the spin–orbit coupling, i.e., the interaction between the spin angular momentum and the orbital angular momentum of the electron is not large, have an energy splitting of the three sublevels. Since there is a magnetic dipole moment associated with the spin angular momentum of each electron, the splitting is due to the magnetic dipole–dipole interaction between the two unpaired spins. The interaction is described by the spin hamiltonian H_{ss} which depends on the molecular symmetry and average distance vector **r** between the spins:

$$\hat{H}_{SS} = \frac{3}{4}\frac{g^2\beta^2\mu_0}{4\pi}\left(\frac{\hat{s}_1\hat{s}_2}{r^3} - \frac{(\hat{s}_1 r)(\hat{s}_2 r)}{r^5}\right)$$

in which g is the electronic g-value, β is the electronic Bohr magneton, $\hat{s_1}$ and $\hat{s_2}$ are the spin angular moments of the two electrons, and **r** is the distance between the two electrons.

By defining the total spin angular momentum $\hat{S} = \hat{s}_1 + \hat{s}_2$, and a dipolar interaction tensor D, whose matrix

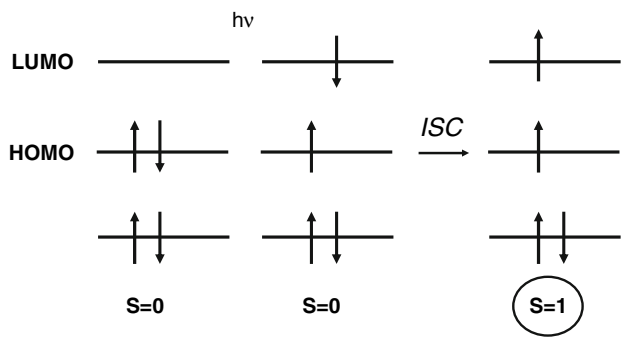

Fig. 1 Molecular orbitals of ground, excited singlet ($S = 0$), and triplet ($S = 1$) states. HOMO: highest-occupied molecular orbital; LUMO: lowest-unoccupied molecular orbital. Arrows indicate electrons with up/down spin. ISC: inter-system crossing

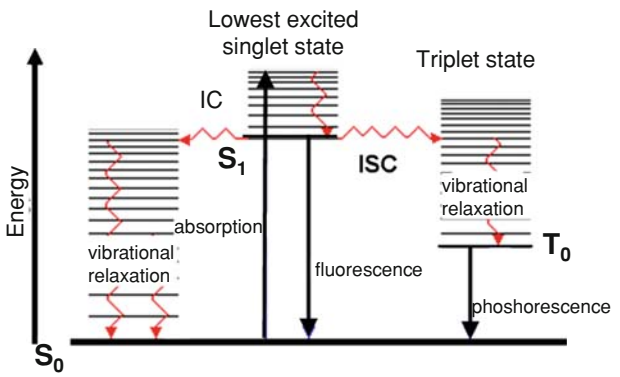

Fig. 2 Jablonski diagram showing the processes by which a molecule, on absorption of energy in the form of electromagnetic radiation, can return to the ground state

elements are averages over the spatial coordinates of the distance vector **r** between the two electrons, since they are not localized in space but are moving within the orbitals, we may write H_{ss} in the following form:

$$\hat{H}_{SS} = \hat{S}\underline{D}\hat{S} = -X\hat{S}_X^2 - Y\hat{S}_X^2 - Z\hat{S}_X^2$$

In fact the tensor may be made diagonal by a transformation in a proper coordinate system in the molecule. This special coordinate system is called the "principal axes system," giving the principal values *X, Y, Z,* which are the energies of the three triplet sublevels in zero field. Because $X + Y + Z = 0$ (the trace of the tensor is zero), the energies may be written in terms of two parameters, D and E, called zero field splitting (ZFS) parameters (see Fig. 3):

$$D = -\frac{3}{2}Z \qquad \text{by convention } |D| \geq 3|E|$$
$$E = -\frac{1}{2}(X - Y)$$

These parameters represent averages over the spatial coordinates of the distance vector of the two unpaired electrons:

$$D = \frac{3}{4}\frac{g^2\beta^2\mu_o}{4\pi}\left\langle\frac{r^2 - 3z^2}{r^5}\right\rangle$$
$$E = \frac{3}{4}\frac{g^2\beta^2\mu_o}{4\pi}\left\langle\frac{x^2 - y^2}{r^5}\right\rangle$$

D and E reflect the structural information, that is the spatial distribution of the electrons and, consequently, the symmetry and structure of the molecule carrying the triplet state. D is sensitive to the asymmetry (extension) of electronic distribution along the Z axis, while E reflects the distribution in the X,Y plane and thus is a measure of the deviation of the axial distribution about the Z-axis. The ZFS parameters are fingerprints of the molecular species and are also influenced by the electronic distortions introduced by the molecular environment.

The triplet states which are important in photosynthesis belong mainly to chlorophyll (Chl) and carotenoid (Car) molecules. In large aromatic molecules such as Chls, the electronic triplet state is a $^3(\pi\pi^*)$ state, and the electronic distribution is flattened along the Z axis and extended in the XY plane. D is positive (the z component of **r** is on the average much smaller than |**r**|) and, due to the symmetry in the plane, E is small. On the contrary for a rod-like molecule, such as a Car, D is negative because z is comparable to **r** (see Fig. 3).

The ZFS parameters of a triplet state give information about the molecular structure. Moreover, the local environment may induce a change in the D and/or E parameters, by producing a change in the electronic distribution. For instance, in the case of aromatic molecules, an increase of the polarizability of the local environment produces a more diffuse triplet state, with a consequent reduction of the D and E values. The ZFS parameters are also sensitive to electric fields.

Zero field transitions

Since there is a magnetic dipole transition moment between any pair of the triplet sublevels, it is possible to exchange energy with an electromagnetic field and induce a transfer of population from one level to another. All the three transitions of a triplet state are allowed and are induced by application of a resonant electromagnetic field, at frequencies corresponding to $2|E|$, $|D| + |E|$, $|D| - |E|$, energies (see Fig. 3). It is possible to determine the value

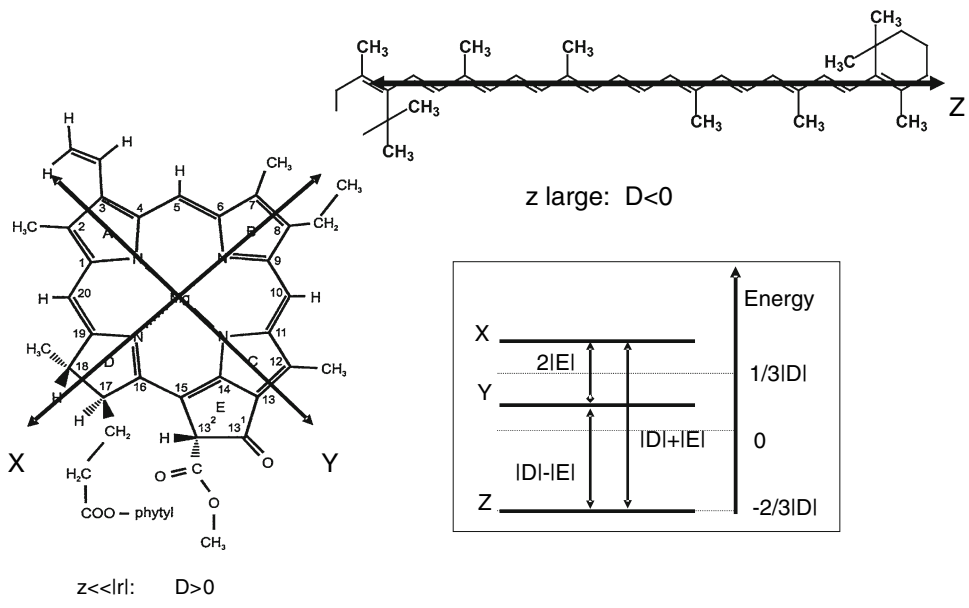

Fig. 3 Molecular structure of chlorophyll a and β-carotene with the directions of the zero-field splitting axes. For Chl a, the in-plane X–Y axes are shown, the Z axis is perpendicular to the porphyrin plane. For β-carotene, only the long Z axis is shown. In the box, the energy levels of the triplet spin sublevels are indicated together with the three possible transitions, corresponding to $(|D| \pm |E|)/h$ and $2|E|/h$ frequencies. D, E: zero field splitting parameters

of the ZFS parameters with high accuracy by ZF-ODMR spectroscopy. The transitions concerning organic molecules fall, in most cases, in the microwaves range (0.01–10 cm^{-1}).

The transitions from the singlet excited state to the three triplet sublevels (p_x, p_y, p_z) and the decay rates from the three triplet sublevels to the ground singlet state (k_x, k_y, k_z), have, in general, different probabilities, because they depend on the spin–orbit coupling which is an anisotropic process (see Fig. 4), and are determined by the molecular symmetry.

The sublevel populations (N_x, N_y, N_z), which are proportional to the ratio p_i/k_i ($i = x, y, z$), are often effectively equalized by spin–lattice relaxation (w), a process which contrasts the microwave-induced transition by reporting the spin populations at Boltzmann equilibrium. Due to this process, at temperatures as low as 5–10 K, it becomes often impossible to observe the transitions because of the little difference in populations at the Boltzmann equilibrium. However, at pumped liquid helium temperatures (1.2–2.1 K) the spin–lattice relaxation is blocked and the populations can achieve a state of spin alignment (polarization) which depends on the symmetry of each sublevel. The population difference between two levels (ΔN) allows to perform the magnetic spectroscopy, the intensity of the transition being proportional to ΔN. In principle, ESR spectroscopy of triplet states in zero field is possible but it is notably difficult experimentally because one must detect directly the absorption of the microwaves swept, but spurious microwaves reflections cannot be avoided. On the contrary, ZF-ODMR is very suitable for the scope, as it will be explained in the next section.

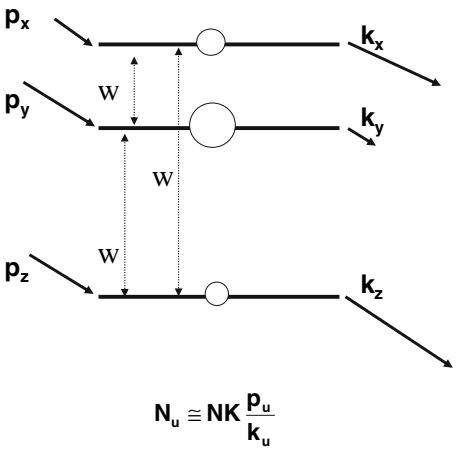

Fig. 4 Scheme of triplet sublevels with populating rates: p_x, p_y, p_z, and decay rates: k_x, k_y, k_z. The term, w, indicates the spin–lattice relaxation rate. Circles correspond to the population of the three sublevels, as determined by the ratio of the populating and decay rates (by neglecting the term, w). N_u represents the population of the u-level in steady state conditions of illumination. When $w = 0$, N_u depends on the ratio p_u/k_u and is proportional to the N (the whole population of the molecules in triplet and singlet states) and K (the rate constant for the triplet by the process: $S_0 \rightarrow S_1 \rightarrow T$)

The basic principle of ODMR spectroscopy

The principle of the ODMR technique may be easily illustrated by considering the example shown in Fig. 5, in which the triplet state of a molecule is populated under illumination. Under continuous illumination, a steady state population of the three sublevels will be generated. In the particular case of the example considered, the population rates are similar for all the sublevels ($p_x = p_y = p_z$) while the decay rates are: k_x,$k_y \gg k_z$. As a consequence the steady state populations of the three sublevels will be: $N_z \gg N_x$,N_y. Application of a resonant microwave field between a couple of sublevels will lead to a redistribution of their populations, because a new steady state will be generated by the presence of the microwave field. If, for instance, we switch on a microwave field of frequency corresponding to that of a transition between the **Y** and **Z** levels, the field will transfer population from the more populated and slowly decaying **Z** level to the much less populated, fast decaying **Y** level. This transferred population will quickly decay to the ground state leading to an increase of phosphorescence, if the triplet **Y** state decays with radiation. Moreover in the new steady state, established by the presence of the resonant microwave field, the singlet ground state population will be enhanced while the global triplet state population ($N_T = N_x + N_y + N_z$) will be decreased. This will induce an increase in fluorescence, an enhanced singlet ground state absorption, and a decrease in triplet–triplet absorption.

This is the basic principle of the ODMR technique. In fact, during an ODMR experiment, the sample is continuously illuminated at liquid helium temperature and irradiated by microwaves at a frequency v not far from one of the resonance frequencies. The frequency v is slowly scanned while the phosphorescence, the fluorescence or the absorption of the sample is being monitored. When v approaches the resonance frequency, the phosphorescence (PDMR), the fluorescence (FDMR) or the absorption (ADMR), will be enhanced or diminished depending on the relative values of the populating and decaying rates.

Once the ZFS parameters are known, it is possible to investigate the intensity dependence of the ODMR transitions on the detection wavelength. The sample is irradiated with microwaves at one resonance frequency, and the photo-detector output is monitored as a function of the probe beam wavelength. In this way, the microwave-induced phosphorescence (MIP), the microwave-induced fluorescence (MIF) or the microwave-induced absorption (MIA or triplet-minus-singlet = T − S) spectra can be

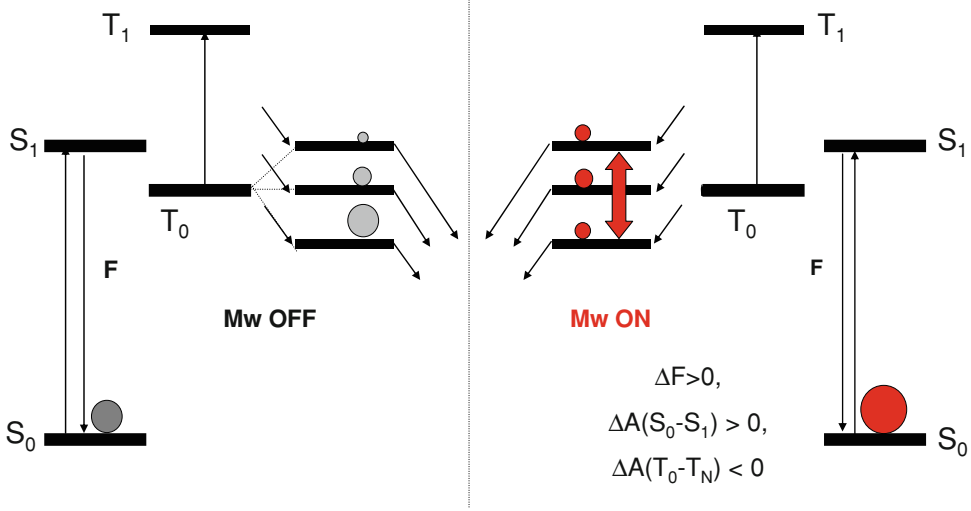

Fig. 5 Scheme illustrating the principle of ODMR spectroscopy. Change of the steady state populations of the singlet ground state and the triplet sublevels induced by the application of a resonant microwave (Mw) field, indicated by a thick arrow in the right panel. Details are found in the text

detected. Usually, the microwaves are ON–OFF-amplitude modulated, and the detector output is demodulated via a phase-sensitive lock-in amplifier. This allows to reach sensitivities as high as $\Delta I/I = 10^{-7}$.

In complex systems, the T–S spectrum of the molecule carrying the triplet state provides information also on the interactions involving the molecule itself and other molecules in the surroundings. In fact, the interactions of a particular molecule with the molecules in its environment may change when the electronic state of the molecule is converted from singlet to triplet. The T–S spectrum will reflect these changes in terms of spectral shifts and/or change of absorption intensity of the interacting molecules.

An important version of ADMR spectroscopy is Linear-dichroic ADMR spectroscopy, which allows to determine the direction of the magnetic transition moments with respect to the optical transition moments. This often gives precise structural information on complex molecular systems. The details of this version of ADMR technique may be found in Hoff (1989).

Basic instrumentation

The basic apparatus required for ODMR in zero field is not expensive; however, it is not commercial. The essential equipment is composed of the following:

a) an optical excitation source (lamp or continuous laser beam)
b) an optical detector (photomultiplier or photodiode)
c) a microwave sweep oscillator with a coaxial transmission line to couple the output with the helix inside the helium cryostat. The helix is a broad-band resonator which allows to sweep the microwave with almost constant power. The sample cell must be optically accessible either between turns in the helix or axially by means of a pipe. Microwaves are amplitude modulated to enhance the sensitivity, via demodulation by means of a lock-in amplifier (not used for slow-decaying triplet states).
d) a cryostat, with optical and microwave access, pumped to reach the temperature of superfluid liquid helium, under 2.1 K. At this temperature, the bubbles associated with the boiling of the liquid helium disappear, and scattering is completely suppressed.
e) a PC interfaced to the set-up for recording and handling of the data

Some differences in the assembly are necessary for FDMR and PDMR, in particular to collect the emission and to get rid of the scattering, due to the excitation. A 90° configuration between the excitation beam and the direction of the detection of the emission is suggested.

A simple basic scheme of the set-up, optimized for photosynthesis research, is shown in Fig. 6. Further details can be found in Hoff (1996).

Advantages and limitations of ZF-ODMR

Compared to conventional EPR, ODMR is a quite sensitive technique. This is due to the fact that the energies of optical quanta (detected by ODMR) are much higher than the energies of microwaves quanta (detected by EPR), and thus enhances the sensitivity of the detector (the quantum-up conversion factor is about 10^5). Moreover, the lines are much narrower, because the anisotropy is not associated with an applied magnetic field. A unique feature of ODMR is the possibility to probe the resonance at different wavelengths, which may help to distinguish among different triplet states present in a sample, and which allows to correlate the optical

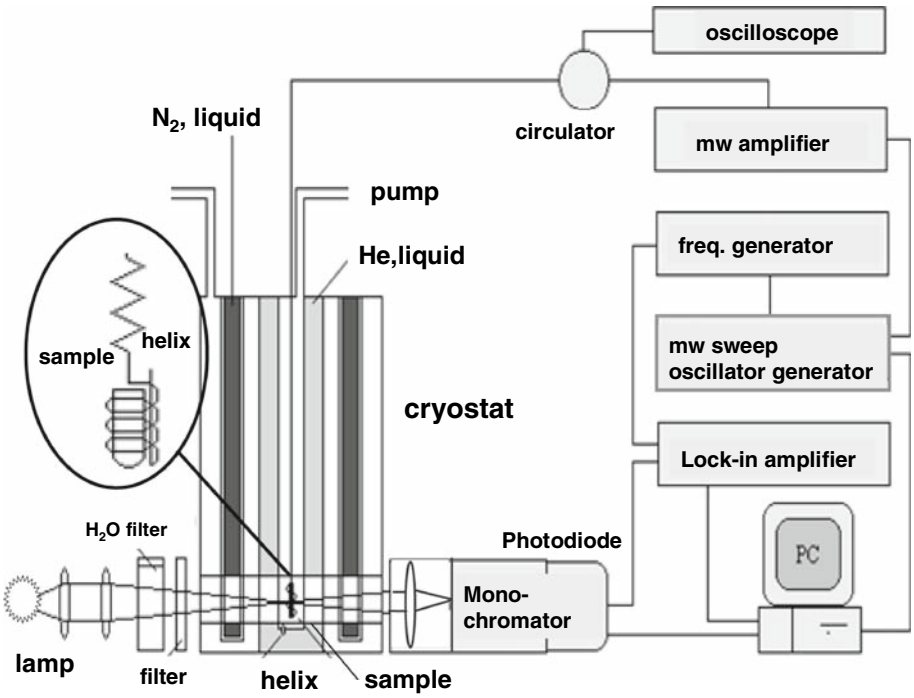

Fig. 6 Basic block scheme of the ODMR set-up. Only the absorption detection line is shown. Fluorescence detection is obtained with a 90° arrangement of the detection line with respect to the excitation line

and magnetic properties of the system. In particular, low temperature T–S spectra are much more accurate than those detected by conventional flash spectroscopy.

On the other hand, limitations arise from the need to operate at low temperature. The application at physiological temperatures is precluded except for crystals.

For triplet state transitions which do not saturate at low microwave power, the use of high power often induces microwave reflections and artifacts in the detection; however, saturation is required, for instance, to get the correct T–S spectra.

A limit of ODMR with respect to EPR is that it is restricted to triplet states, while radicals are not detectable.

ODMR in photosynthesis

All biological materials contain molecules such that their triplet states can be used as important probes of their molecular structure, of their structural environment, of their binding sites etc. All proteins contain as naturally occurring potential triplet probes their aromatic amino acids (phenylalanine, tryptophan, and tyrosine) which can be studied by ODMR (Maki 1995). Aromatic residues close to active sites of enzymes may be sensitive to substrate or dye binding. In other cases, the triplet states of endogenous cofactors, in biological systems, are potential tools for studying the dynamics and the function of the macromolecules (Clarke 1982).

The application of ODMR in photosynthesis research has been particularly fruitful since the first report by Gouterman et al. (1972) on the biologically important porphyrin molecule (for reviews, see: Hoff 1982, 1989, 1996; Giacometti et al. 2007).

Chlorophyll, bacteriochlorophyll, and carotenoid triplet states can be populated in the light-harvesting complexes of all the photosynthetic species, especially during light-induced stress conditions. These states have been studied by ODMR in many systems (for some examples, see: Carbonera et al. 1992a, b, 1996, 2001, 2002; Santabarbara et al. 2003, 2005, 2007; Santabarbara and Carbonera 2005; Bordignon et al. 2002; Van der Vos et al. 1991; Aust et al. 1990, 1991; Psencik et al. 1994; Ullrich et al. 1989; Lampoura et al. 2002; Di Valentin et al. 2008).

Moreover, triplet states, in photosynthetic membranes, may be generated on the primary donors (P) of photosynthetic reaction centers, by radical recombination, under conditions blocking the forward electron by removal, or chemical reduction, of the secondary acceptors:

$$Antenna \xrightarrow{light} Antenna^{*} PA_1A_2^{-} \longrightarrow AntennaP^{*}A_1A_2^{-} \xrightarrow{<3ps} {}^1\left[P^{+}A_1^{-}A_2^{-}\right] \overset{\omega}{\leftrightarrow} {}^3\left[P^{+}A_1^{-}A_2^{-}\right] \rightarrow {}^3PA_1A_2^{-}$$

The interactions between the primary donors and the other cofactors present in the reaction centers, have been largely investigated by ODMR, in different organisms (Aust et al. 1990; Angerhofer et al. 1994; Bordignon et al. 2002; Carbonera et al. 1994, 1997, 2002; Giacometti et al. 2007; Hoff 1982, 1989, 1996; Santabarbara et al. 2003, 2007; Ullrich et al. 1987; Witt et al. 2002, 2003; Krabben et al. 2000). Recently, site-directed mutants of photosynthetic bacteria and green algae have been studied by ODMR,

giving new insights about the effect of the protein environment on the electronic properties of the cofactors in close vicinity of the primary donor (Vrieze et al. 1996a, b; Witt et al. 2002, 2003; Krabben et al. 2000).

An important aspect of the application of ODMR in photosynthesis is that the technique can be used to study energy transfer and trapping processes in intact systems, such as large particles or even thylakoids, chloroplasts, and leaves, due to the high sensitivity and selectivity of the technique (Bordignon et al. 2002; Psencik et al. 1994; Santabarbara et al. 2003, 2007).

In the next sections some examples of application in photosynthesis, representative of the capabilities of the technique, are reported.

ODMR of carotenoid and chlorophyll triplet states

Carotenoid and Chlorophylls triplet states in photosynthesis are easily detected by ODMR. Carotenoids are present in all the photosynthetic systems where they have the dual functions of light-harvesting and photo-protection (Frank and Cogdell 1996). In fact, they absorb energy in the blue–green region and transfer the electronic excitation to chlorophylls with high efficiency. Moreover, their low-lying triplet state becomes populated by triplet–triplet energy transfer from chlorophyll triplets, thus preventing the formation of singlet oxygen and possible damage of the photosystems, as shown in the scheme reported in Fig. 7.

Carotenoid triplet formation has been detected in intact systems as well as in isolated light-harvesting complexes and reaction centers. Because the carotenoids are neither fluorescent nor phosphorescent species, it is not possible to perform (P)FDMR spectroscopy of carotenoids triplet states by monitoring their direct emission. However, FDMR spectra can be obtained by detection of the chlorophyll emission, while microwave resonant transitions are induced between a couple of spin sublevels of the carotenoid triplet state. The observed three FDMR transitions ($|D| + |E|$, $|D| - |E|$, and $2|E|$) corresponding to a change of emission upon resonant microwave field application are due to the energy-transfer processes connecting the pigments. According to the scheme of Fig. 7, it can be demonstrated, by solving the differential equations for the triplet state populations in the absence and in the presence of the resonant microwave field (Carbonera et al. 1992a, b; Van der Vos et al. 1991), that, for instance, a decrease in the carotenoid triplet population induced by a resonant microwave field causes, in turn, an increase in the Chl singlet population and therefore of the fluorescence intensity.

The same FDMR pattern of transitions ($2|E| \gg |D| + |E| > |D| - |E|$) has been reported for carotenoid triplet states occurring in several antenna complexes of plants, algae, and bacteria (Angerhofer et al. 1996; Aust et al. 1991; Ullrich et al. 1989; Van der Vos et al. 1991; Carbonera et al. 1992a, b).

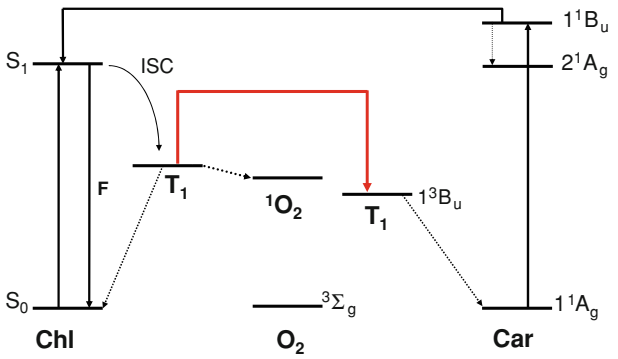

Fig. 7 Pathways of singlet and triplet energy transfer between chlorophyll and carotenoid molecules in photosynthesis. The Car triplet state can be populated from the Chl triplet excited state, via triplet–triplet energy transfer. Singlet oxygen production by the interaction of the excited Chl triplet state and ground-state oxygen is also shown. Because of singlet–singlet and triplet–triplet energy transfer, it is possible to monitor the ODMR spectra of non-fluorescing carotenoids on the Chl emission. Key: S_0, ground state; S_1, first singlet excited state; T_1, first triplet excited state; A, absorption; F, fluorescence; ISC, inter-system crossing

As an example, the ODMR spectra of carotenoids in peridinin–chlorophyll a–proteins (PCPs) will be discussed in some details in the next section.

Carotenoids are efficient quenchers of Chl triplets; however, it has been reported that small amounts of unquenched Chl triplet states are also formed in chloroplasts under illumination. It has been suggested that these Chl triplet states are related to the process of photo-inhibition (Santabarbara et al. 2003). ODMR is the most suitable technique to investigate the origin of the different triplet populations of Chl because of its high sensitivity and because it makes it possible to correlate the magnetic properties of the triplets under investigation to the optical properties of the system. A second example of application of ODMR, the study of the Chl triplet states populated in the thylakoid membranes of the green alga *Chlamydomonas reinhardtii*, will also be presented.

Peridinin–chlorophyll a–proteins (PCPs)

The peridinin–chlorophyll a–proteins are the peripheral light-harvesting complexes of the microalgae Dinoflagellates. The structure of the main form of PCP isolated from *Amphidinium carterae* has been determined by X-ray diffraction (Hofmann et al. 1996). Each monomer consists of two domains where the pigments occur in two clusters, each containing one Chl *a* and four carotenoid (peridinin) molecules related by a local twofold symmetry axis. The

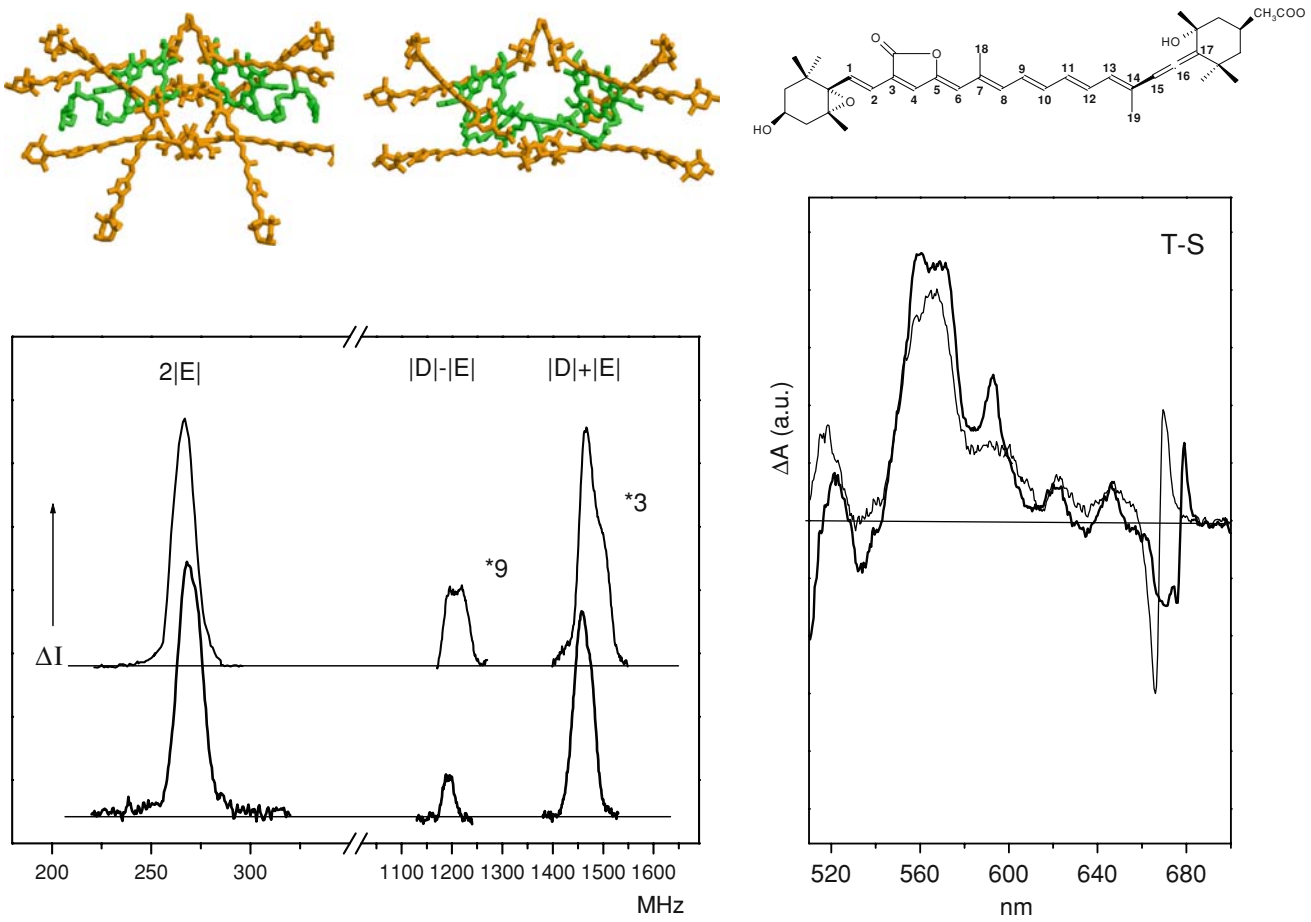

Fig. 8 Structures of the pigments associated with the monomeric basic unit of the PCP complexes from *A. carterae*. Left: main form PCP (MFPCP), right: high-salt PCP (HSPCP). Structure taken from coordinates 1PPR and 2C9E deposited in the Brookhaven Protein Data Bank. The molecular structure of peridinin is also reported. Left panel: FDMR spectra of peridinin triplet state in PCP (*top traces*) and high-salt PCP (*bottom traces*), at 1.8 K. Detection wavelength: 680 nm; modulation frequency: 323 Hz, Mw power 1 W. 10 scans. Right panel: T–S spectra of MFPCP (*thin line*) and HSPCP (*thick line*) detected at the 2|*E*| transition frequency. Spectra have been rescaled for better comparison. Line corresponding to $\Delta I/I = 0$ is shown. Modulation frequency: 323 Hz, microwave power: 1 W, $T = 1.8$ K. $\Delta I/I \cong \Delta A$. Figure based on the data from Di Valentin et al. 2008

closest distances between pigments, belonging to the two different clusters, are greater than those within a single cluster. Intra-cluster distances are in the range 4–11 Å, and the conjugated regions of all peridinins are in van der Waals contact with the chlorophyll molecule. More recently the X-ray structure of another form of PCP, the so-called high-salt PCP, has been resolved. In Fig. 8, the pigment arrangement in the two proteins is compared.

In these complexes, peridinin triplet states are formed by energy transfer from the Chl triplet states, as proved by the three FDMR transitions, assigned, on the basis of the ZFS parameters, to the peridinin triplet state, detected by monitoring the Chl a fluorescence changes (at about 680 nm). The FDMR spectra are shown for the two PCP proteins in Fig. 8 (Carbonera et al. 1996, Di Valentin et al. 2008, and references therein). No chlorophyll triplet states have been detected, demonstrating a 100% efficiency of triplet quenching by peridinins.

After the three transitions have been determined, the change in the absorption, induced by the microwaves, at different wavelengths can be probed by setting the microwave field at a frequency corresponding to one of the transitions and sweeping the detection wavelength. In this way, the T–S spectra are detected. For the two PCP complexes, the well-resolved T–S spectra detected at the maximum of the 2|*E*| transitions, are shown in Fig. 8. The positive bands in the range 450–550 nm, correspond to triplet–triplet absorption bands of the peridinin, while the negative bands correspond to singlet–singlet absorption bleaching. It is interesting to note that there are negative and positive bands also in the region between 650 and 700 nm. These bands are in the spectral region where only Chl *a* molecules in these proteins absorb. Therefore they correspond to the Chl *a* band shifts induced by the change of the electronic state of the peridinin molecule. The intensity of the Chl *a* bleaching in the T–S spectra of the

carotenoids is proportional to the triplet–triplet transfer efficiency (Angerhofer et al. 1996). It is clearly seen that in the high-salt PCP complex, two spectrally distinct forms of Chl *a* interact with the carotenoids carrying the triplet states.

Chlorophyll triplet states detection in thylakoids

Illumination of thylakoids of both plant and algae at low temperatures leads to the formation of several chlorophyll and carotenoid triplet populations which can be detected by ODMR (Santabarbara et al. 2002, 2005, 2007; Santabarbara and Carbonera 2005). The technique is unique in allowing the assignment of specific triplet populations in an intact environment, such as large membrane preparations. The analysis of the FDMR spectra of thylakoids has revealed the presence of carotenoid as well as chlorophyll triplet states, belonging to Photosystems I and II. In Fig. 9, the spectra relative to the $|D| + |E|$ and $|D| - |E|$ transitions of the chlorophyll triplet states in thylakoids of *Chlamydomonas r.* detected at multiple wavelengths are reported. The $2|E|$ transitions were too week to be detected, as usually observed for Chl triplet states (data from Santabarbara et al. 2007). On the basis of the decomposition of the spectra by Gaussian components, the MIF spectra associated to each triplet population can be reconstructed (not shown). Thus it is possible to assign the triplet components to specific regions of the photosynthetic membrane (see Table 1). The Chl triplets, which are formed under non-reducing redox conditions when the recombination triplet state of the primary donors are undetectable, have been suggested to be involved in the photo-inhibitory damage of Photosystem II (Santabarbara et al. 2003, 2005, 2007; Santabarbara and Carbonera 2005).

After reduction of the secondary acceptors by sodium dithionite, followed by few minutes of illumination at room temperature, the recombination triplet states, ^{T}P680 and ^{T}P700, belonging to the two reaction centers, appear as new components in the FDMR spectra of thylakoids (see Fig. 10, based on data from Santabarbara et al. 2007). Vredenberg and Duysens quantified, in 1963, the process of fluorescence yield of the antenna depending on the fraction of open/closed reaction centers. Their scheme explains why it is possible to observe the FDMR spectra of the recombination triplet states localized in the reaction centers via emission of the antenna chlorophylls, the primary donor being connected to the antenna chlorophylls via singlet–singlet energy transfer.

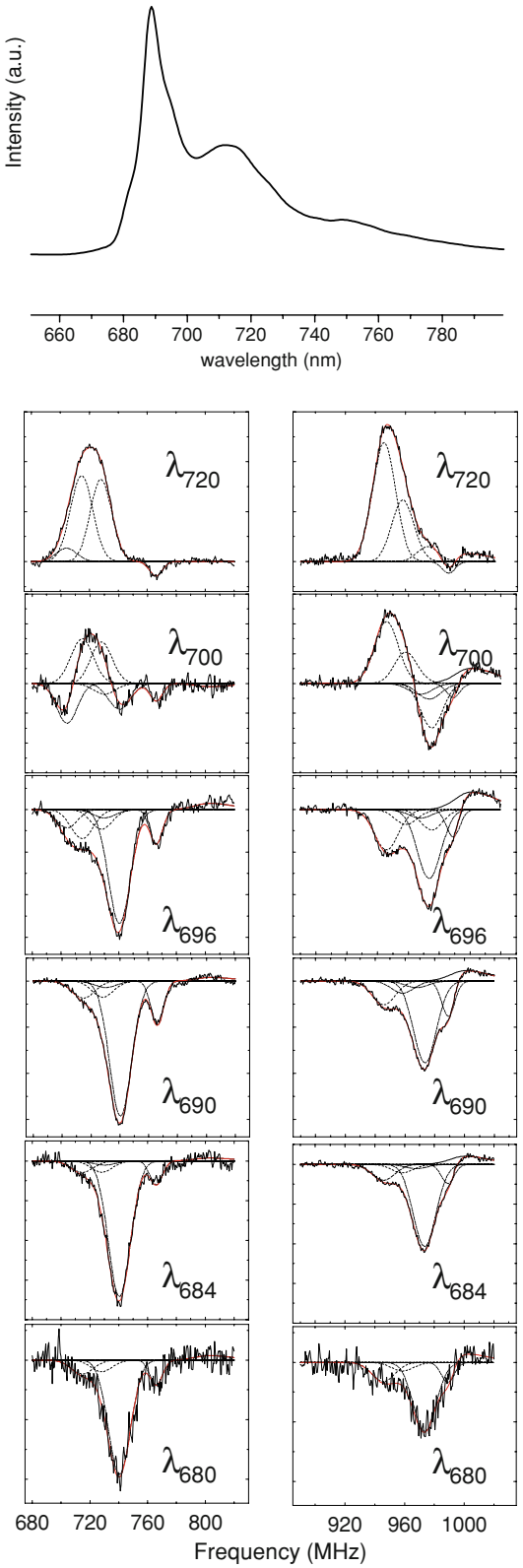

Fig. 9 (*Top*) Fluorescence emission spectra of *C. reinhardtii* thylakoids at 4.5 K. Excitation wavelength 435 nm. (*Bottom*) Gaussian deconvolution of the FDMR spectra of *C. reinhardtii* thylakoids, in the $|D| - |E|$ and $|D| + |E|$ chlorophyll resonance transition range, detected at different emission wavelengths as indicated. Experimental conditions: temperature: 1.7 K; microwave power: 600 mW; modulation frequency, 33 Hz. Figure based on the data from Santabarbara et al. 2007 ▶

Table 1 Parameters of global Gaussian decomposition of FDMR spectra of thylakoids of *Chlamidomonas r.* monitored at multiple emission wavelengths

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	$^{3}P700$
$^{T}PSI^{2}$	727.9	958.3	17.5	0.0281	0.0038	$^{3}P700$
$^{T}PSI^{3}$	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	$^{3}P680$

From Santabarbara et al. 2007

By selecting the frequency of the microwaves at 992 MHz, corresponding to the specific resonance of $^{T}P680$, the associated T–S spectrum of $^{T}P680$ in thylakoids can be detected. It shows its main negative peak at 685 nm, red-shifted with respect to earlier reports on isolated reaction centers (Van der Vos et al. 1992; Carbonera et al. 1994). A weak signal, of opposite sign, at approximately 675 nm is also present. The 685-nm peak indicates that, at cryogenic temperatures, the triplet is indeed located not in P680, but on the long-wavelength absorbing chlorophyll present in the reaction center of Photosystem II. From the absence of a clear structure in the 680-nm absorption region, this long-wavelength absorbing state does not appear to be strongly coupled to P680, though it must be associated with one of the "inner core" pigments recently identified in the Photosystem II crystallographic structure.

If the frequency of the microwaves is set at 940 MHz, the T–S spectrum of P700 can be selectively recorded (see Fig. 10). It presents several bands, which can be interpreted in terms of the interactions between P700 and the other Chl molecules close to it, in the reaction centers. These interactions give rise to excitonic band splittings and redistribution of dipole oscillator strengths, which are perturbed upon $^{T}P700$ formation. This leads to a change of the intensity of the absorption bands and band shifts, which are observed in the T–S spectrum. It should be noted that such complex structure of the T–S spectrum is not

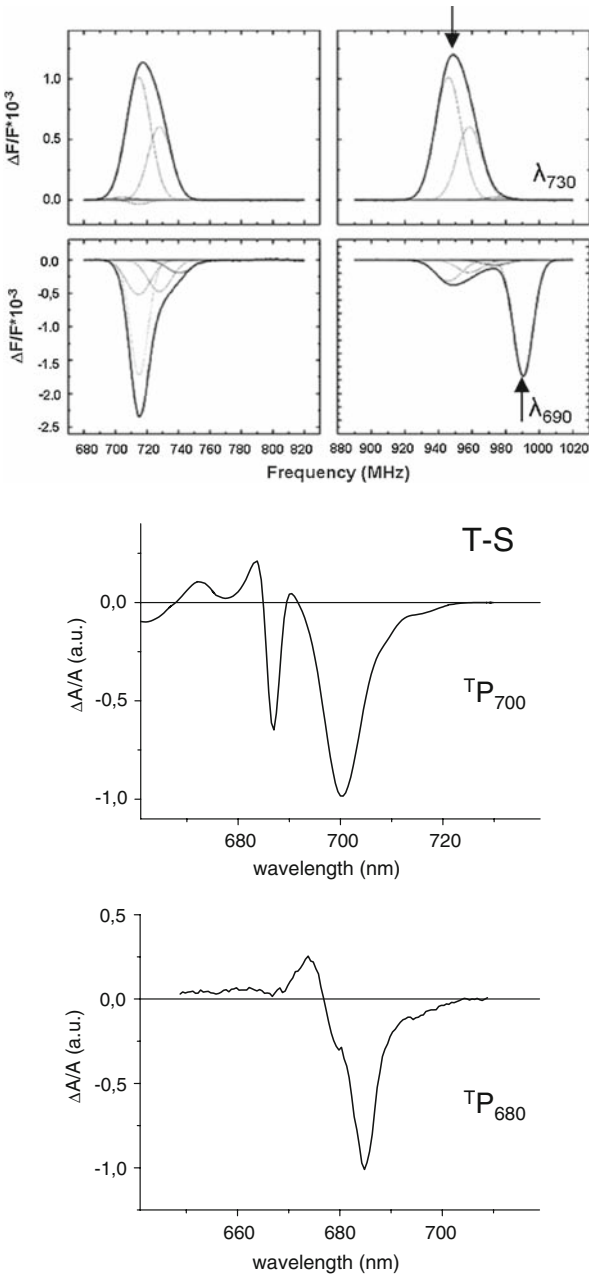

Fig. 10 (*Top*) FDMR of Chl triplets of thylakoids from *C. reinhardtii* ▶ reduced by 20 mM sodium-dithionite and preilluminated for 4 min at room temperature. Experimental conditions: temperature: 1.7 K; microwave power: 600 mW; modulation frequency: 33 Hz, emission wavelength as indicated. (*Bottom*) T–S spectra of $^{T}P700$ and $^{T}P680$ in the Chl Qy absorption region, detected in *C. reinhardtii* thylakoids reduced by 20 mM sodium-dithionite and preilluminated for 4 min at room temperature. The microwave resonance frequency was set near the maxima in the $|D| + |E|$ transitions, at 940 MHz for $^{T}P700$ and 992 MHz for $^{T}P680$, as indicated by the arrows in the FDMR spectrum above. Experimental conditions: temperature 1.8 K, microwave power: 600 mW; modulation frequency: 33 Hz, optical resolution: 0.5 nm, scan rate: 0.1 nm/s. Figure based on the data from Santabarbara et al. 2007

observed in the case of the P680, indicating that the excitonic coupling within the PSII reaction centre is much weaker than in the reaction center of PS I.

This study on thylakoids describes well the potentiality of ODMR technique, whose sensitivity and selectivity allow to determine the spectroscopic properties of specific pigments and cofactors in an intact molecular environment, which is not altered by the biochemical manipulation and isolation procedures of the protein complexes.

Conclusions and prospects

ODMR is a powerful tool for investigating triplet states in photosynthesis. Although triplet states are not directly involved in the main path of photosynthesis, they can be seen as molecular probes to get information on the structure and interactions of specific pigments in the protein complexes.

The accurate measure of ZFS parameters by ODMR will acquire more importance in the future, when reliable calculations of ZFS parameters by means of modern computational methods will be available, and the effect of the protein environments on the ZFS parameters will be estimated.

The possibility to apply mutagenesis techniques to photosynthetic protein complexes has thrown open further possibilities for future applications of ODMR in photosynthesis, in particular, for the study the interactions of the triplet probes with the protein environment, both in reaction centers and in light-harvesting complexes.

Further developments of ODMR methods could include CD (circular-dichroism), Raman, and photoacoustic detection of the magnetic resonance.

References

Angerhofer A, Friso G, Giacometti GM, Carbonera D, Giacometti G (1994) Optically detected magnetic resonance study on the origin of the pheophytin triplet state in D1/D2-cytochrome b-559 complexes. Biochim Biophys Acta 1188:35–45. doi:10.1016/0005-2728(94)90019-1

Angerhofer A, Bornhauser F, Gall A, Cogdell RJ (1996) Optical and optically detected magnetic resonance investigation on purple photosynthetic bacterial antenna complexes. Chem Phys 194:259–274. doi:10.1016/0301-0104(95)00022-G

Aust V, Angerhofer A, Parot PH, Violette CA, Frank HA (1990) Temperature-dependent ADMR on borohydride-treated reaction centers of *Rhodobacter sphaeroides* R26. Chem Phys Lett 1731:439–442. doi:10.1016/0009-2614(90)87231-F

Aust V, Angerhofer A, Ullrich J, von Schütz JU, Wolf HC, Cogdell RJ (1991) ADMR of carotenoid triplet states in bacterial photosynthetic antenna and reaction center complexes. Chem Phys Lett 181:213–221. doi:10.1016/0009-2614(91)90357-F

Bordignon E, Scarzello M, Agostini G, Giacometti G, Vianelli A, Vannini C, Carbonera D (2002) Optically detected magnetic resonance of intact membranes from *Chloroflexus aurantiacus*. Evidence for exciton interaction between the RC and the B808-866 complex. Photosynth Res 71:45–57. doi:10.1023/A:1014947412940

Carbonera D, Giacometti G, Agostini G, Angerhofer A, Aust V (1992a) ODMR of carotenoid and chlorophyll triplets in CP43 and CP47 complexes of spinach. Chem Phys Lett 194:275–281. doi:10.1016/0009-2614(92)86051-I

Carbonera D, Giacometti G, Agostini G (1992b) FDMR of carotenoid and chlorophyll triplets in light-harvesting complex LHCII of spinach. Appl Magn Reson 3:361–368

Carbonera D, Giacometti G, Agostini G (1994) A well resolved triplet minus singlet spectrum of P680 from PSII particles. FEBS Lett 343:200–204. doi:10.1016/0014-5793(94)80555-5

Carbonera D, Giacometti G, Segre U (1996) Carotenoid interactions in peridinin chlorophyll-a proteins from Dinoflagellates: evidence for optical excitons and triplet migration. J Chem Soc, Faraday Trans 92:989–993. doi:10.1039/ft9969200989

Carbonera D, Collareta P, Giacometti G (1997) The P700 triplet state in an intact environment detected by ODMR. A well resolved triplet minus triplet spectrum. Biochim Biophys Acta 1322:115–128. doi:10.1016/S0005-2728(97)00068-6

Carbonera D, Bordignon E, Giacometti G, Agostini G, Vianelli A (2001) Fluorescence and absorption detected magnetic resonance of chlorosomes from green bacteria *Chlorobium tepidum* and *Chloroflexus aurantiacus*. A comparative study. J Phys Chem B 105:246–255. doi:10.1021/jp001778+

Carbonera D, Burzomato V, Bordignon E, Giacometti G, Agostini G, Heathcote P, Leech HK (2002) Fluorescence and absorption detected magnetic resonance of membranes from the green sulfur bacterium *Chlorobium limicola*. Full assignment of detected triplet states. J Phys Chem B 106:7560–7568. doi:10.1021/jp020181m

Clarke RH (1982) Triplet state ODMR spectroscopy. John Wiley and Sons, New York

Di Valentin M, Ceola S, Salvadori E, Agostini G, Giacometti GM, Carbonera D (2008) Spectroscopic properties of the peridinins involved in chlorophyll triplet quenching in high-salt peridinin-chlorophyll a-protein from *Amphidinium carterae* as revealed by optically detected magnetic resonance, pulse EPR and pulse ENDOR spectroscopies. Biochim Biophys Acta 1777:1355–1363

Frank HA, Cogdell RJ (1996) Carotenoids in photosynthesis. Photochem Photobiol 63:257–264. doi:10.1111/j.1751-1097.1996.tb03022.x

Giacometti G, Agostini G, Santabarbara S, Carbonera D (2007) ODMR spectroscopy of molecular functions in photosynthetic membrane proteins. Appl Magn Reson 31:179–191

Gouterman M, Yamanashi BS, Kwiram AL (1972) Zero-field splitting of the triplet state in zinc etioporphyrin. J Chem Phys 56:4073–4078. doi:10.1063/1.1677817

Hoff AJ (1982) ODMR spectroscopy in photosynthesis II. In: Clarke RH (ed) Triplet state ODMR spectroscopy. John Wiley and Sons, New York, pp 367–425

Hoff AJ (1989) Optically-detected magnetic resonance of triplet states. In: Hoff AJ (ed) Advanced EPR application in biology and biochemistry. Elsevier, Amsterdam, pp 633–684

Hoff AJ (1996) Optically detected magnetic resonance (ODMR) of triplet states. In: Amesz J, Hoff AJ (eds) Advances in photosynthesis, vol 3 Biophysical techniques in photosynthesis. Kluwer Academic Publishers, Amsterdam, pp 277–298

Hofmann E, Wrench PM, Sharples FP, Hiller RG, Welte W, Diederichs K (1996) Structural basis of light harvesting by carotenoids: peridinin-chlorophyll-protein from *Amphidinium*

carterae. Science 272:1788–1791. doi:10.1126/science.272.5269.1788

Krabben L, Schoddler E, Jordan R, Carbonera D, Giacometti G (2000) Influence of the axial ligands on the spectral properties of P700 of Photosystem I: a study of site directed mutants. Biochemistry 39:13012–13025. doi:10.1021/bi001200q

Kwiram AL (1967) Optical detection of paramagnetic resonance in phosphorescent triplet states. Chem Phys Lett 1:272–275. doi:10.1016/0009-2614(67)80017-4

Lampoura SS, Barzda V, Owen GM, Hoff AJ, van Amerongen H (2002) Aggregation of LHCII leads to a redistribution of the triplets over the central xanthophylls in LHCII. Biochemistry 41:9139–9144. doi:10.1021/bi025724x

Maki AH (1995) Optically detected magnetic resonance of photoexcited triplet states in biochemical spectroscopy. Methods Enzymol 246:611–638

Psencik J, Searle GFW, Hála J, Schaafsma TJ (1994) Fluorescence-detected magnetic-resonance (FDMR) of green sulfur photosynthetic bacteria *Chlorobium* sp. Photosynth Res 402:1–10. doi:10.1007/BF00019040

Santabarbara S, Carbonera D (2005) Carotenoid triplet states associated with the long-wavelength-emitting chlorophyll forms of Photosystem I in isolated thylakoid membranes. J Phys Chem B 109:986–991. doi:10.1021/jp047077k

Santabarbara S, Bordignon E, Jennings RC, Carbonera D (2002) Chlorophyll triplet states associated with Photosystem II of thylakoids. Biochemistry 41:8184–8194. doi:10.1021/bi0201163

Santabarbara S, Jennings RC, Carbonera D (2003) Analysis of Photosystem II triplet states in thylakoids by fluorescence detected magnetic resonance (FDMR) in relation to the redox state of the primary quinone acceptor Q_A. Chem Phys 294:337–342. doi:10.1016/S0301-0104(03)00279-9

Santabarbara S, Agostini G, Heathcote P, Carbonera D (2005) A fluorescence detected magnetic resonance investigation of the carotenoid triplet states associated with Photosystem II of isolated spinach thylakoid membranes. Photosynth Res 86:283–296. doi:10.1007/s11120-005-2840-1

Santabarbara S, Agostini G, Casazza AP, Syme CD, Heathcote P, Böhles F, Evans MCW, Jennings RC, Carbonera D (2007) Chlorophyll triplet states associated with Photosystem I and Photosystem II in thylakoids of the green alga *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1767:88–105. doi:10.1016/j.bbabio.2006.10.007

Schmidt J, van der Waals JH (1968) Optical detection of zero-field transitions in phosphorescent triplet states. Chem Phys Lett 2:640–642. doi:10.1016/0009-2614(63)80039-1

Schmidt J, Hesselmann IAM, De Groot MS, Van der Waals JH (1967) Optical detection of electron resonance transitions in phosphorescent quinoxaline. Chem Phys Lett 10:434–436. doi:10.1016/0009-2614(67)85066-8

Sharnoff M (1967) ESR-produced modulation of triplet phosphorescence. J Chem Phys 46:3263. doi:10.1063/1.1841199

Ullrich J, von Schütz JU, Wolf HC (1987) Zero-field absorption ODMR of reaction centers of *Rhodobacter sphaeroides* at temperatures between 4.2 and 75 K. Chem Phys Lett 140:416–420. doi:10.1016/0009-2614(87)80758-3

Ullrich J, Speer R, Greis J, von Schütz JU, Wolf HC (1989) Carotenoid triplet states in pigment–protein complexes from photosynthetic bacteria: absorption-detected magnetic resonance from 4.3–225 K. Chem Phys Lett 155:363–370. doi:10.1016/0009-2614(89)87170-2

Van der Vos R, Carbonera D, Hoff AJ (1991) Microwave and optical spectroscopy of carotenoid triplets in light-harvesting complex LHCII of spinach by absorbance-detected magnetic resonance. Appl Magn Reson 2:179–202

Van der Vos R, van Leeuwen PJ, Braun P, Hoff AJ (1992) Analysis of the optical absorbance spectra of D1-D2- cytochrome b-559 complexes by absorbance-detected magnetic resonance. Structural properties of P680. Biochim Biophys Acta 1140:184–196. doi:10.1016/0005-2728(92)90008-P

Vrieze J, Williams JC, Allen JP, Hoff AJ (1996a) An LD-ADMR study on reaction centers of the LH(L131) and LH(M160) hydrogen-bonding mutants of *Rhodobacter sphaeroides*. Biochim Biophys Acta 1276:221–228. doi:10.1016/0005-2728(96)00083-7

Vrieze J, Schenck CC, Hoff AJ (1996b) The triplet state of the primary donor in reaction centers of the HL(L173) and HL(M202) heterodimer mutants of *Rhodobacter sphaeroides*. Biochim Biophys Acta 1276:229–238. doi:10.1016/0005-2728(96)00082-5

Witt H, Schlodder E, Teutloff C, Niklas J, Bordignon E, Carbonera D, Kohler S, Lbahn A, Lubitz W (2002) Hydrogen bonding to P700: site-directed mutagenesis of threonine A739 of Photosystem I in *Chlamydomonas reinhardtii*. Biochemistry 41:8557–8569. doi:10.1021/bi025822i

Witt H, Bordignon E, Carbonera D, Dekker JP, Karapetyan N, Teutloff C, Webber A, Lubitz W, Schlodder E (2003) Species-specific differences of the spectroscopic properties of P700—analysis of the influence of non-conserved amino acid residues by site-directed mutagenesis of Photosystem I from *Chlamydomonas reinhardtii*. J Biol Chem 278:46760–46771. doi:10.1074/jbc.M304776200