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FLUORESCENCE DETECTED MAGNETIC RESONANCE (FDMR) SPECTROSCOPY OF CHLOROPHYLL-PROTEINS FROM BARLEY

by

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Fluorescence detected magnetic resonance (FDMR) spectra and fluorescence emission spectra at 4.2 K of chlorophyll-proteins isolated and purified from barley thylakoids are presented. The FDMR spectra show the occurrence of chlorophyll *a* triplet states in all five chlorophyll-proteins studied, namely Chl_a-P1, Chl_a-P2, Chl_a-P3, $Chl_{a/b}$-P1 and $Chl_{a/b}$-P2.

The presence of more than one chlorophyll triplet each associated with a chlorophyll emitting at a specific wavelength gives rise to a characteristic wavelength dependence of the FDMR spectrum of chlorophyll-proteins. The zero field splitting parameters measured, combined with the observed fluorescence emission wavelengths suggest that three types of interactions of the Mg atom of chlorophyll *a* occur in these proteins: a type similar to that in the parallel dimer (Chl $a \cdot H_2O)_2$, seen at 721 nm for Chl_a-P1 leading to a positive FDMR signal; a type like that in Chl $a \cdot 2$ pyridine also giving a positive FDMR signal, seen in Chl_a-P2 and Chl_a-P3; and a third type similar to that in Chl $a \cdot 2H_2O$ leading to a negative FDMR signal, seen for Chl_a-P1 at 679 nm, and for $Chl_{a/b}$-P1 and $Chl_{a/b}$-P2.

The FDMR spectrum in the antenna of photosystem I (Chl_a-P1) can probably be ascribed to that of a trap formed by a pair of interacting chlorophyll *a* molecules, indicating that the organisation of chlorophyll in the antenna may not in principle be very different from that in the photosystem I reaction centre, and that it contains approximately plane-parallel chlorophyll *a* pairs. Chl_a-P2 and Chl_a-P3 do not show a long wavelength ($>$ 700 nm) emission, suggesting a much weaker interaction between chlorophyll molecules in these proteins compared to that in Chl_a-P1. For $Chl_{a/b}$-P1 and $Chl_{a/b}$-P2 the absence of a long wavelength emission and the observation of zero field splitting (ZFS) parameters similar to that of monomeric Chl $a \cdot 2H_2O$ both indicate the absence of strong interactions between chlorophyll *a* molecules in these proteins also, and it is suggested that chlorophyll *a* and chlorophyll *b* molecules occur in interacting pairs.

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1. INTRODUCTION

Fluorescence detected magnetic resonance (FDMR) spectroscopy is a recent offspring of optically detected magnetic resonance (ODMR) spectroscopy, performed in a zero magnetic field (8, 9, 26). Since a magnetic field is absent, there is no need to use oriented samples, such as single crystals, in order to obtain magnetic resonance spectra with satisfactory resolution ($\sim 10^{-2}$). In addition, optical detection of magnetic resonance transitions is well-known to be very sensitive as compared with detection of microwave-absorption by diodes or bolometers, such as in conventional high-field electron paramagnetic resonance (EPR) spectroscopy (11). This combination of properties makes FDMR spectroscopy an ideal technique for studying complex systems, such as the chlorophyll-proteins of a photosynthetic membrane which can be obtained only in limited amounts and in non-oriented form.

Triplet state FDMR spectroscopy makes use of the fact, that the three-fold degeneracy of the molecular triplet state is lifted by spin-spin interaction, even in the absence of an external magnetic field. For photosynthetic pigments, the resulting zero field splittings (ZFS) are $\sim 10^{-2}\text{cm}^{-1}$. The energies of these non-degenerate triplet spin levels are denoted X, Y and Z and are in the order $X > Y > Z$, representing eigenvalues of the spin-Hamiltonian (31) of the triplet state.

Thus, the distances between the spin levels (zero-field splittings) are given by X–Y, Y–Z and X–Z in energy units. Conventionally these splittings are expressed in the parameters D and E, related to X, Y and Z by (31):

$$D = -\tfrac{3}{2}\ Z$$

$$E = -\tfrac{1}{2}(X - Y)$$

The creation of a molecule in its lowest triplet state T_o is usually obtained after optical excitation into an excited singlet state S_n, followed by intersystem crossing $S_n \rightarrow T_o$. Resonance transitions between the spin-levels of T_o (X–Y, Y–Z and X–Z) can then in principle be detected as a change in the intensity of the phosphorescence of T_o, due to microwave absorption at any of the three resonance frequencies. Relaxation between T_o spin levels is sufficiently slowed down at low temperatures (typically $\leqslant 4.2$ K) so that they can be considered as isolated. This is a necessary condition for observing FDMR spectra.

For photosynthetic pigments, phosphorescence is too weak (22) to be suitable for observing zero field magnetic resonance. However, the fluorescence intensity arising from $S_1 \rightarrow S_o$ emission can be expressed as (3):

$$I_f = A\left[N - \sum_{i=1}^{3} n_i\right]$$

where A is an instrumental constant, N is the total number of pigment molecules and $\sum_i n_i$ represents the number of molecules in the spin levels of the lowest triplet state (T_o). It is assumed that the number of molecules in excited singlet states can be neglected under steady-state illumination of the sample.

It can be shown (3) that absorption of microwaves, resonant with any of the three zero field splittings of T_o, gives rise to a change of fluorescence ΔI_f, given by

$$\Delta I_f = A\,(k_i - k_j)(k_i + k_j)^{-1}\,(n_i^o - n_j^o)$$

where $i \neq j = 1, 2, 3$ and $n_{i,j}^o$ represents the steady state population of the T_o spin levels i or j under continuous illumination and in the absence of microwaves; $k_{i,j}$ are the decay rate constants of these spin-levels. This equation shows that resonances may be observed only when $n_i^o \neq n_j^o$, and $k_i \neq k_j$. The last condition almost always holds for chlorophylls. The transition at 2E (= Y–X) is often not observed since the top two spin-levels of T_o accidentally have almost equal steady state populations.

For the observation of triplet states, the sensitivity of the FDMR technique compares favourably with the standard EPR techniques. Due to high values of fluorescence and triplet

Abbreviations: Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *b*; Chl_a-P = chlorophyll *a*-protein; $\text{Chl}_{a/b}$-P = chlorophyll *a*/*b*-protein; EPR = electron paramagnetic resonance; FDMR = fluorescence detected magnetic resonance; I_f = fluorescence intensity; ODMR = optically detected magnetic resonance; SDS = sodium dodecyl sulfate; ZFS = zero field splitting.

yields of chlorophyll at low temperature a few micrograms of chlorophyll (*a* or *b*) in the chlorophyll-proteins are sufficient for observation. Under our experimental conditions resonance fluorescence changes of $\sim$ 0.1–1 % are observed, corresponding to a relative change of $\sum_i n_i$ of $\sim$ 10 %.

FDMR spectroscopy has been shown to be a suitable method (18, 19, 24, 25) to probe the environment of chlorophyll molecules in the triplet state under in vitro conditions. In the present communication we describe the application of this technique on purified chlorophyll-proteins which form integral parts of the photosynthetic membrane in higher plants. It is our aim to identify the way in which the chlorophylls are bound to their different apoproteins and to characterise chlorophyll-chlorophyll interactions from a comparison between FDMR parameters (ZFS values, signal sign) of chlorophyll in these proteins with those for various chlorophyll forms in vitro. The presence of chlorophyll in the triplet state arising from intersystem crossing can be expected in all chlorophyll-proteins at 4.2 K so long as triplet quenching processes are inhibited. A radical pair mechanism of chlorophyll triplet formation is possible in the chlorophyll-proteins containing active reaction centres (4, 14).

Recent advances in techniques for separation and isolation of chlorophyll-proteins from thylakoid membranes using detergent solubilisation followed by polyacrylamide gel electrophoresis have shown that virtually all chlorophyll is bound to specific proteins (21), and that there are in higher plants five different chlorophyll-proteins: Chl_a-P1, Chl_a-P2, Chl_a-P3, $Chl_{a/b}$-P1 and $Chl_{a/b}$-P2 (20). Polyacrylamide gel slices containing microgram quantities of individual chlorophyll-proteins form perfect objects for investigation by FDMR spectroscopy. Recently the groups of Hoff (13) and Clarke (6) have reported results obtained by FDMR on chloroplasts and a photosystem I fraction from chloroplasts; however these preparations still have a relatively complicated composition. A functionally undefined water-soluble chlorophyll *a/b*-protein from Atriplex hortensis has also been studied by Clarke, Leenstra and Hagar (7). The present study combines FDMR spectroscopy and low temperature fluorescence spectroscopy with the use of purified chlorophyll-proteins from wild-type and mutant barley (20, 30). The mutant *chlorina-f2* is unable to form chlorophyll *b* and therefore lacks in its thylakoids significant amounts of $Chl_{a/b}$-P1 and $Chl_{a/b}$-P2. Thus Chl_a-P1, Chl_a-P2 and Chl_a-P3 can be studied in this mutant without the interference of chlorophyll *a/b*-proteins (20). Conversely, the mutant *viridis-zb*^{63} is deficient in Chl_a-P1 and therefore allows specific studies of the chlorophyll-proteins associated with photosystem II (12, 30).

2. MATERIALS AND METHODS

2.1. Materials

Purified thylakoids were prepared from wild-type, *chlorina-f2* and *viridis-zb*^{63} barley leaves (Hordeum vulgare) according to the procedure described by Machold, Simpson and Møller (20). The thylakoids were solublized in 1 % sodium dodecyl sulfate using a SDS-chlorophyll ratio of 3.6:1. The dark-green supernatant obtained after ultracentrifugation was used immediately for SDS-polyacrylamide gel electrophoresis at 4 °C employing a Tris-sulfate buffer system (System IV in 20). After completion of electrophoresis the gels were inspected under visible and ultra-violet light and pieces of gel (1–2 mm wide and about 10 mm long) containing individual chlorophyll-proteins were cut out and immediately stored in stoppered vials in liquid N_2.

2.2. Methods

All measurements have been carried out at 4.2 K using an FDMR-spectrometer, as previously described (3), with minor modifications. Samples, consisting of thin slices of gel material containing a single chlorophyll-protein, were placed in a thin-walled Teflon cup, fitting snugly around the bottom end of a 4 mm diameter quartz light pipe, which was lowered into liquid helium. No precautions were taken to avoid contact of the sample with air during this operation, since the presence of oxygen turns out not to affect the triplet state properties of chlorophyll under our experimental conditions.

The sample is surrounded by a few turns of a non-resonant helical slow wave structure, con-

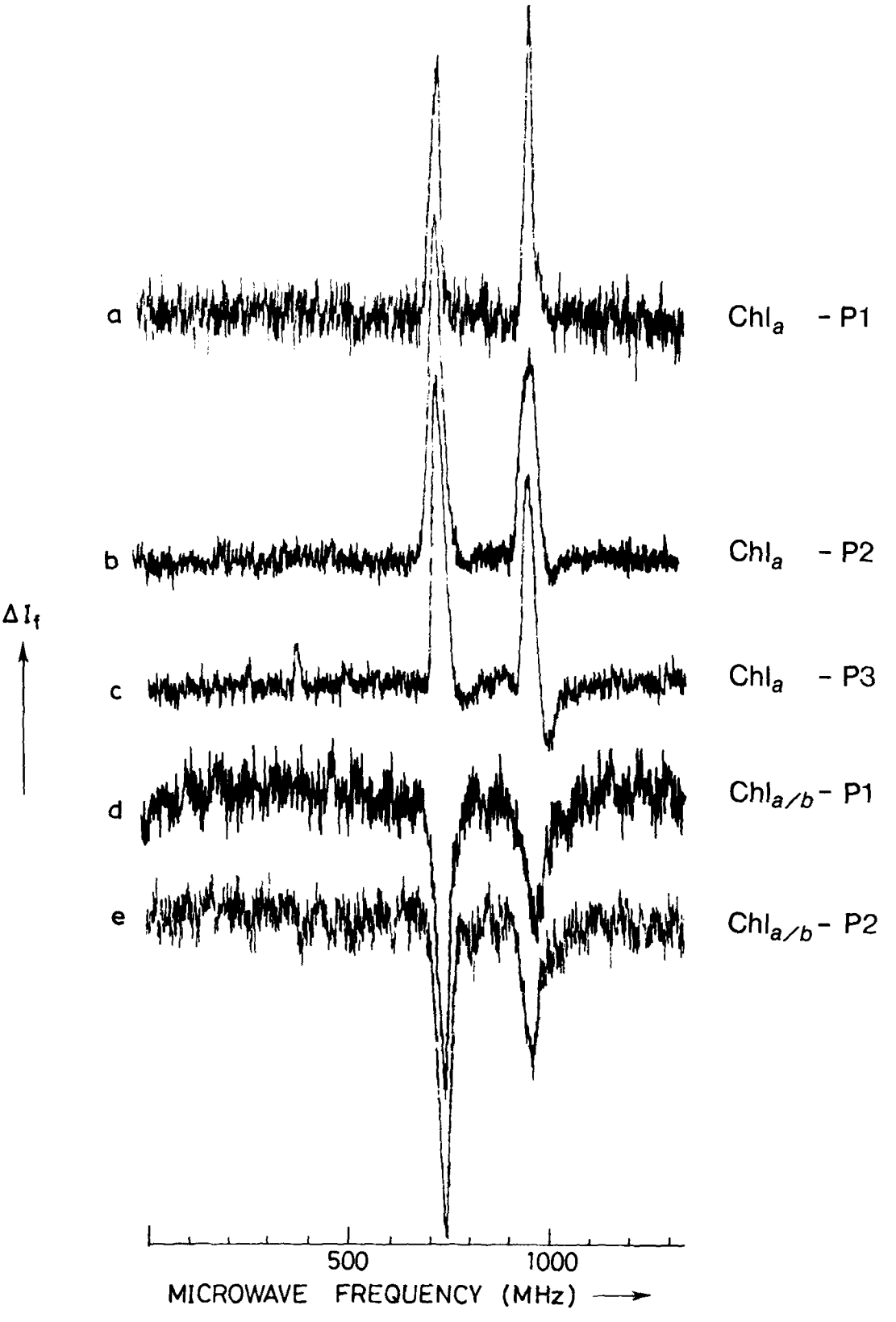
a
b
c
d
e
ΔI_f
Chl_a - P1
Chl_a - P2
Chl_a - P3
Chl_a/b - P1
Chl_a/b - P2
500
1000
MICROWAVE FREQUENCY (MHz)

Figure 1. FDMR spectra of chlorophyll-proteins obtained from wild-type and mutant barley as indicated in Table I.

The change in fluorescence intensity (ΔI_f, arbitrary units) is caused by resonance transitions between the chlorophyll triplet spin levels using microwave radiation of constant power and variable frequency. Fluorescence detection wavelengths: a: 721 nm; b–e: 686 nm; detection bandwidth in all cases 10 nm. Excitation source: Xe lamp with $CuSO_4$ and Schott BG 12 filters. Emission detection: RCA 31034 photomultiplier cooled to −40 °C. Microwave sweep rate: ~ 1000 MHz/s, power 40 mW, T = 4.2 K. The FDMR spectra a–e in this Figure correspond to 4.2 K fluorescence spectra a–e of Figure 2, i.e. Figures 1a and 2a, etc. refer to the same sample.

Table I

Distribution of chlorophyll-proteins in wild type barley and mutants.
The nomenclature system used for the chlorophyll-proteins is defined in (19). FDMR spectra of chlorophyll-proteins in heavy characters are shown in Figure 1.

	Photosystem I	Photosystem II	Light-harvesting
Wild-type	**Chl_a-P1**	Chl_a-P2[a]), Chl_a-P3	$Chl_{a/b}$-P1, $Chl_{a/b}$-P2
chlorina-f2	Chl_a-P1	Chl_a-P2[a]), Chl_a-P3	– –
viridis-zb^{63}	–	**Chl_a-P2[a]), Chl_a-P3**	**$Chl_{a/b}$-P1, $Chl_{a/b}$-P2**

[a]) Chl_a-P2 is located around photosystem II and may perform a light-focusing function.

Table II

Fluorescence emission and FDMR parameters of chlorophyll-proteins at 4.2 K.

Chlorophyll-protein	λ_F (nm)[a]	λ_D (nm)[b]	Microwave frequency (MHz)		Sign of FDMR signal	Zero field splitting parameters ($\times 10^{-4}$ cm^{-1})	
			D–E	D + E		D	E
Chl_a-P1	677 ± 1, 719 ± 1[c])	721	733 ± 3	958 ± 3	+	282 ± 1	38 ± 1
		679	754 ± 4	1000 ± 1	−	293 ± 1	40 ± 1
Chl_a-P2	676 ± 2, 683[d]), 687 ± 1	686	739 ± 6	968 ± 12	+	285 ± 3	38 ± 2
Chl_a-P3[e])	~ 670, 678[f]), 682	686	745 ± 2	975 ± 2	+	287 ± 1	38 ± 1
$Chl_{a/b}$-P1	683	686	755 ± 11	981 ± 8	−	290 ± 3	38 ± 1
$Chl_{a/b}$-P2	683 ± 1	686	769 ± 5	991 ± 8	−	292 ± 2	38 ± 1

[a]) Wavelengths of fluorescence emission maxima or shoulders. No difference was seen between wild-type and mutants, except where noted.
[b]) FDMR detection wavelength.
[c]) Seen in both wild-type and *chlorina-f2*.
[d]) Appeared in Chl_a-P2 from *viridis-zb*^{63} but not in *chlorina-f2*.
[e]) In Chl_a-P2 and Chl_a-P3 there is also an FDMR signal with negative sign (details not shown).
[f]) Not seen in *viridis-zb*^{63}.

Table III

Zero field splitting parameters for in vitro photosynthetic pigments at 4.2 K[a]).

Compound	Solvent[b])	λ_D (nm)[c])	Zero field splitting parameters ($\times 10^{-4}$ cm^{-1})	
			D	E
Chl *a*·H_2O	n−C_8	660	305 ± 3	38 ± 3
Chl *a*·2pyr	n−C_8/pyr	686	283 ± 5	40 ± 5
Chl *a*·$2H_2O$	n−C_8	687	291 ± 5	38 ± 4
(Chl *a*·H_2O)$_2$[d])	n−C_8	725	286 ± 5	31 ± 5
(Chl *a*·H_2O)$_n$[e])	n−C_8	750	275 ± 5	35 ± 6
(Chl *a*)$_2$	Tol	–	270 ± 4	40 ± 2
Chl *b*·Et_1	n−C_8/Et	644	332 ± 2	33 ± 2
Chl *b*·$2Et_1$·Et_2	n−C_8/Et	669	297 ± 2	40 ± 2
(Chl *b*·H_2O)$_2$	MP	700	281 ± 3	31 ± 3
Pheophytin *a*	n−C_8	668	350 ± 2	20 ± 1
		675	348 ± 2	25 ± 1
		683	353 ± 7	33 ± 1

a) Data taken from Kooyman et al. (18, 19) and van der Bent (2).
b) Solvents: n−C_8, n-octane; pyr, pyridine; Et, ethanol; MP, methylcyclohexane/pentane (1:1); Tol, toluene.
c) Fluorescence emission detection wavelength.
d) At low temperature an additional water ligand may be attached to each Mg atom in the dimer.
e) $n \geqslant 2$, but small (18, 19).

nected to a Hewlett-Packard variable frequency microwave source (10–1300 MHz), equipped with an Avantek UA 405 microwave amplifier, delivering maximally ~ 100 mW microwave power to the sample.

Triplets are generated by irradiating the sample with 400–450 nm light from a 900 Watt Osram XBO Xenon arc, via the bifurcated quartz light pipe. Emitted fluorescence, collected by the same light pipe passes either through a Balzer B40 interference filter or a Spex Minimate 0.25 m monochromator and is detected by a RCA 31034 photomultiplier with Peltier cooling.

FDMR spectra are recorded by monitoring the intensity of a selected fluorescence band, while sweeping the frequency of the microwave source. FDMR spectra are accumulated by a signal analyzer, in order to obtain satisfactory signal-to-noise ratio. Typically, 10–50,000 spectra are required, equivalent to a total sweeping time of 3–15 hours per spectrum. Surface-excited fluorescence spectra (4.2 K) were obtained employing the FDMR spectrometer with microwaves off and scanning the detection monochromator.

3. RESULTS

The FDMR spectrum of chlorophyll-proteins isolated from wild-type and mutant barley chloroplasts after SDS solubilisation and polyacrylamide gel electrophoresis have been measured in zero magnetic field at 4.2 K (Figure 1 a–e). In these spectra only the D–E and D + E resonances are observed and not the 2E resonance. All five different chlorophyll-proteins show a FDMR spectrum. In contrast to FDMR experiments on isolated photosystem I chlorophyll-proteins from the blue-green alga Synechococcus leopoliensis (28) the use of a phosphate buffer during gel electrophoresis was not found essential to obtain FDMR signals.

Table I presents a review of the occurrence and distribution of the chlorophyll-proteins studied. These proteins were also characterised by their fluorescence emission spectra at 4.2 K

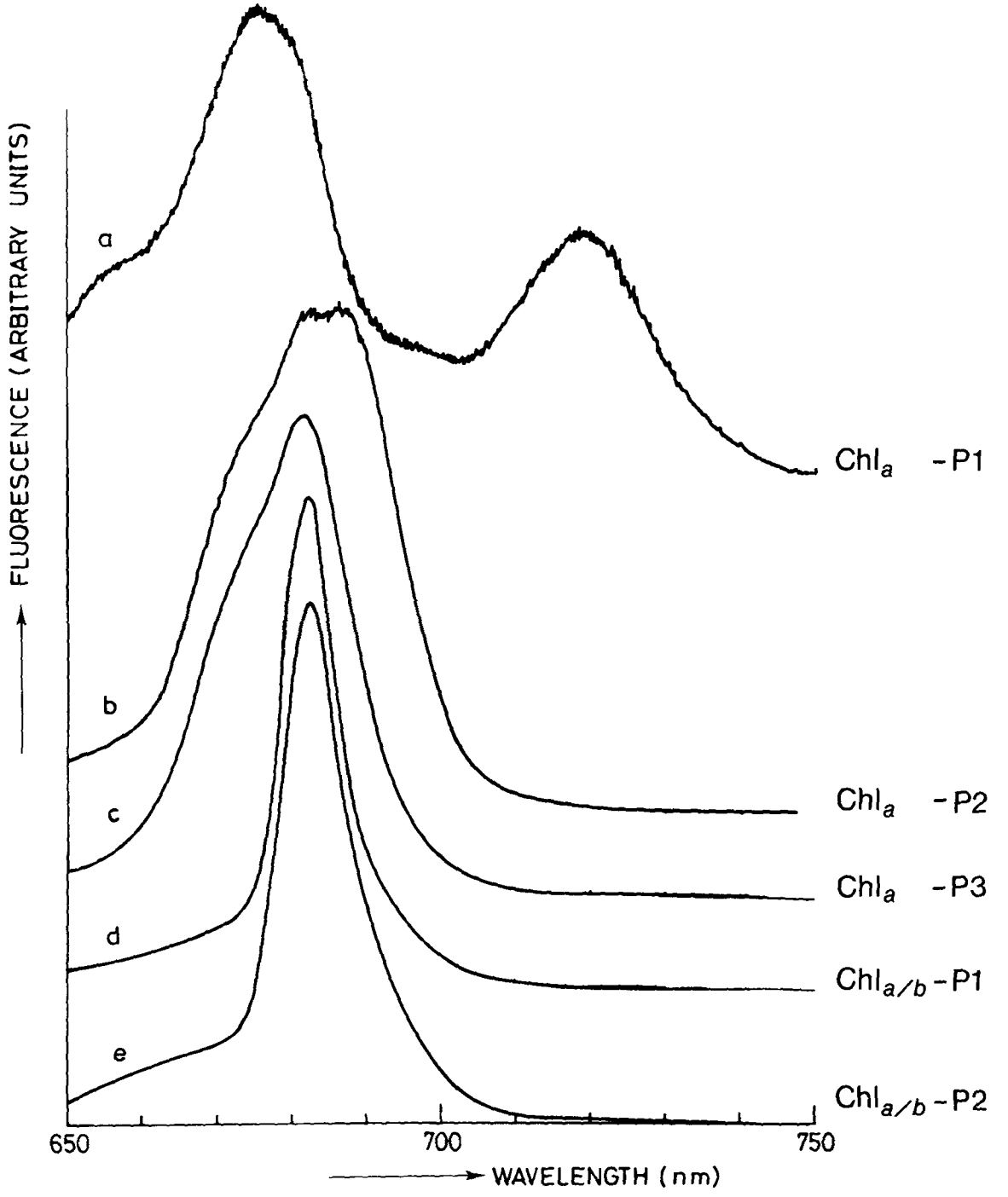

Figure 2. Fluorescence emission spectra of chlorophyll-proteins from barley obtained as indicated in Table I. The emission spectra were measured in the FDMR spectrometer under the same conditions as for the FDMR spectra, by the use of front surface excitation and with a Spex monochromator (bandwith 5 nm) placed between sample and photomultiplier. Spectra a–e refer to the same sample as in Figure 1.

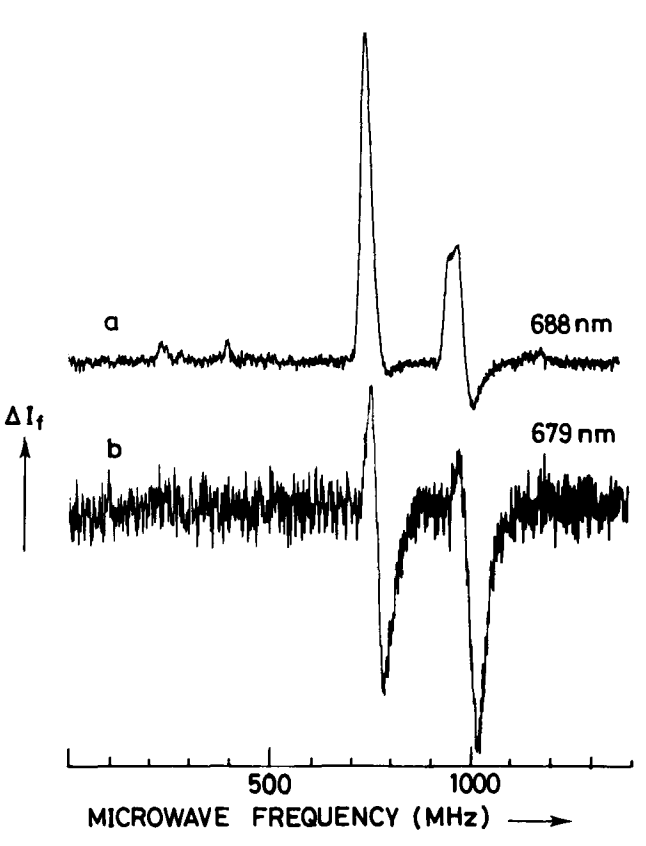

Figure 3. The FDMR spectrum of Chl_a-P2 protein from barley mutant *viridis-zb*^{63} measured in the 688 nm (a) and 679 (b) fluorescence emission.

Other experimental conditions as in Figure 1.

(Figure 2, Table II). All three proteins containing only Chl *a* show a FDMR spectrum consisting of a positive signal (increase in fluorescence on resonance with microwaves) but with slightly different ZFS values (D varies between 282 and 287 $\times$ 10^{-4} cm^{-1} whilst E = 38 $\times$ 10^{-4} cm^{-1}). These values correspond closely to those for monomeric Chl *a* biligated with pyridine and to those of the chlorophyll dimer (Chl *a*·H_2O)$_2$ (Table III). The emission wavelength at which this triplet state is detected in Chl_a-P1 is far to the red of that seen for Chl *a*·2pyr and corresponds closely with the emission maximum (725 nm) for (Chl *a*·H_2O)$_2$.

The ZFS values for the positive FDMR signal in Chl_a-P2 show a relatively large variation and this is probably attributable to the presence of at least two signals of varying relative proportions close together. This heterogeneity is not very apparent in Figure 1b but is more clearly seen by the splitting of the D + E peak in Figure 3a.

Although pheophytin *a* is considered to be associated with the photosystem II reaction centre (16), and Chl_a-P3 to contain this reaction centre (20), the FDMR spectrum of Chl_a-P3 shows no evidence for a triplet state of pheophytin *a* under the conditions used (see Table III). The remaining FDMR spectra reported in Table II all have a negative sign (decrease in fluorescence on resonance) and show D values $\geqslant$ 290 $\times$ 10^{-4} cm^{-1}. As expected, the emission maxima and ZFS parameters of the two $Chl_{a/b}$-proteins are similar, and the triplet state observed at 686 nm is that of Chl *a* and not of Chl *b*. At shorter wavelengths however, a FDMR spectrum with ZFS values similar to that for Chl *b* in vitro is observed (29). The ZFS values for $Chl_{a/b}$-P1 and $Chl_{a/b}$-P2 at 686 nm correspond closely to that for Chl *a*·$2H_2O$ (Table III). The 677 nm emission of Chl_a-P1 (Figure 2) is seen in the protein prepared from the *chlorina-f2* mutant as well as in the wild-type so that it cannot be ascribed to contamination by $Chl_{a/b}$-P2*** (20).

The significance of the difference in sign of the FDMR spectra for Chl_a-P and $Chl_{a/b}$-P at the same wavelength (Figure 1) is not clear (5). It is possible that it is simply related to the state of ligation of the chlorophyll molecule, and the nature of the ligands, which can affect the depopulation rates of the three triplet spin-levels (2).

In Figure 3 the FDMR spectrum of Chl_a-P2 recorded in 688 nm emission shows that a high signal to noise ratio (about 20:1) can be obtained for chlorophyll-proteins. The difference between the spectra in Figure 3a (for 688 nm) and 3b (for 679 nm) illustrates the strong wavelength dependence of the FDMR spectrum for this protein. This may allow the use of the fluorescence microwave double resonance technique (see for example ref. 1) to resolve the emission from separate types of chlorophyll *a* molecules in Chl_a-P2. The wavelength dependence of FDMR spectra of chlorophyll-proteins appears to be a general phenomenon and is also seen for Chl_a-P1 (Table II).

Figure 3a demonstrates the presence of three distinct chlorophyll triplet states in a single

chlorophyll-protein, two with a positive FDMR signal having ZFS values of D = 282, E = 37 and D = 286, E = 40 (in units 10^{-4} cm^{-1}), and a third signal with a negative sign. In Figure 3b the latter is dominant and this signal can be assigned to a triplet state present in the chlorophyll form emitting at 676 nm (see also Figure 2 and Table II). It is quite possible that the other two signals may be similarly correlated each with a chlorophyll form emitting at a specific wavelength.

From Figures 1b and 1c it is seen that Chl_a-P2 and Chl_a-P3 show similar FDMR spectra, although the broader D + E peak in Figure 1b suggests the heterogeneity of Chl_a-P2 already mentioned. The fluorescence emission spectrum of Chl_a-P2 at 4.2 K is however, distinct from that of Chl_a-P3 having a 687 nm emitting component (Figures 2b and 2c). Therefore these two proteins can be regarded as similar, but nevertheless spectrally distinguishable.

4. DISCUSSION

The ability to detect chlorophyll triplet states using the FDMR technique at 4.2 K in zero magnetic field in welldefined chlorophyll-proteins provides a new and useful means for the study of the structure and function of these building blocks of the photosynthetic apparatus. Already from the experiments reported here we are able to draw several relevant conclusions about the way in which certain chlorophyll molecules interact with protein and with other chlorophyll molecules.

In Chl_a-P1 derived from photosystem I, the reaction centre (*P*700) can be expected to remain in the oxidized form under the conditions of the FDMR experiment – high intensity illumination at 4.2 K – so that the formation of a chlorophyll triplet at the reaction centre via a radical pair mechanism (4, 14) probably can be ruled out (5). The chlorophyll triplet state observed must therefore be located in the photosystem I antenna chlorophyll. The ZFS parameters for the 721 nm emission of Chl_a-P1 are rather close to those of monomeric Chl *a*·2pyr and also but to a less extent to those of the (Chl $a \cdot H_2O)_2$ dimer (see Table III). The observed Chl_a-P1 ZFS values are not however compatible with those of the (Chl $a)_2$ dimer or the higher aggregate (Chl $a \cdot H_2O)_n$ (Table III). As the wavelength of the emission is far to the red of that of a monomeric form of chlorophyll we must tentatively conclude that the trap in the antenna where the triplet state is located is comprised of an approximately plane parallel chlorophyll *a* dimer held together by the protein. That the ZFS parameters do not exactly match those of (Chl $a \cdot H_2O)_2$ is to be expected as the chlorophylls in the chlorophyll-protein are most probably interacting not with water molecules but rather with protein side-chains containing either oxygen or perhaps nitrogen electron-donating groups. This finding is of interest, in view of the reported coordination of Mg in bacteriochlorophyll to histidine residues of the protein in the bacteriochlorophyll-protein complex isolated from the green bacteria P. aestuarii 2K (23). Apparently, therefore the organisation of the chlorophyll molecules in the antenna does not differ in principle from that of the reaction centre, in contrast to the hypothesis of KATZ, NORRIS and SHIPMAN (15).

It is interesting that the other two chlorophyll *a*-proteins, Chl_a-P2 and Chl_a-P3 do not show a long wavelength emission comparable to that seen in Chl_a-P1. A possible explanation for this is that the chlorophyll molecules in these two proteins are on average further apart and that interaction between chlorophylls is less strong than in Chl_a-P1. The shorter lifetime for fluorescence from photosystem I at room temperature as compared to photosystem II (27) would then be consistent with a more efficient singlet energy transfer to the photosystem I reaction centre (assuming energy transfer and not trapping to be rate limiting) and a closer average spacing of chlorophyll *a* molecules in photosystem I. In this connection it is of interest that leaves of the barley mutant *viridis-zb*^{63} have a long wavelength emission band even though this mutant lacks photosystem I (12). After SDS polyacrylamide gel electrophoresis none of the chlorophyll-proteins isolated from this mutant retained this emission. Recently it has been observed that the light harvesting chlorophyll *a*/*b*-protein 2 isolated and purified by Triton solubilization at 4 °C had a major emission band at 735 nm (10). The long wavelength emission of the mutant in situ could thus derive from the chlorophyll *a*/*b* proteins.

The SDS solubilized chlorophyll-proteins con-

taining both chlorophylls *a* and *b* ($Chl_{a/b}$-P1 $Chl_{a/b}$-P2) did not show a long wavelength emission band. This seems to exclude appreciable Chl *a* pair interaction. The work of KNOX and VAN METTER on a similar chlorophyll *a*/*b* protein from spinach (17) led to the conclusion that the chlorophyll *b* molecules were clustered together in the centre of the protein and that the chlorophyll *a* molecules were placed at the periphery at such a distance from each other that only weak chlorophyll *a*-chlorophyll *a* interaction is seen. We would like to adapt this model to include relatively strong pairing between chlorophyll *a* and chlorophyll *b* molecules, as our work on FDMR reported here and on triplet spin level kinetics of $Chl_{a/b}$-P1 and -P2 to be reported in a subsequent publication suggest that triplet energy transfer is possible at low temperature from Chl *b* to Chl *a*, a phenomenon known to require short range interaction. A similar suggestion has been made by CLARKE et al. (6). Thus we propose that chlorophyll *a* and *b* in these proteins occur in pairs, with the interaction between pairs being relatively weak (29).

The ZFS parameters found for the chlorophyll-proteins and reported in Table II can give us an indication of the nature of the liganding groups binding to the centre Mg atom in the axial positions in these proteins. For Chl_a-P1 at 721 nm, and for Chl_a-P2 and -P3 the experimental ZFS parameters approximately agree with those seen in vitro for (Chl $a \cdot H_2O)_2$ and Chl $a \cdot$ 2 pyridine, respectively. This indicates biligation of the chlorophyll and further suggests the imidazole group of histidine as a possible candidate in Chl_a-P2 and -P3 and possibly also in Chl_a-P1, if one considers the differences between the ZFS parameters of Chl_a-P1 and that for (Chl $a \cdot H_2O)_2$. For $Chl_{a/b}$-P1 and $Chl_{a/b}$-P2 the agreement of the observed ZFS parameters with those of Chl $a \cdot 2H_2O$ is very good (Tables II and III), so that in these chlorophyll-proteins the chlorophyll again appears to be biligated, but in this case with water or perhaps oxygen-containing protein side groups.

A feature of the emission spectrum for Chl_a-P1 is the presence of the 677 nm band in addition to the long wavelength fluorescence maximum. This shorter wavelength emission could be from chlorophyll molecules which transfer their singlet energy relatively inefficiently to the dimer-like trap referred to above and which emits at 719 nm. The cause for this inefficiency could be: an increase in inter-chlorophyll distances, a change in relative orientation of the chlorophyll molecules or a decrease in spectral overlap. The observation that the ZFS parameters of the FDMR signal in the 679 nm emission are different to those in the 721 nm emission, both with regard to the D and E values and the sign, suggests in fact the last of the three possibilities, i.e., a change in the bonding of the chlorophyll which leads to shifts in absorbance and emission spectra and so possibly to a reduction in resonance energy transfer.

This communication shows that FDMR spectra together with fluorescence emission spectra of chlorophyll-proteins are able to provide important characteristics of these proteins which should allow us to probe the interaction of chlorophyll with protein side chains and with other chlorophyll molecules to gain an understanding of the way in which the chlorophyll is organised. In addition, these characteristics can be employed to monitor the integrity of the chlorophyll-protein during procedures designed to prepare purified reaction centres from higher plants.

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