POLARIZED LIGHT SPECTROSCOPY ON ORIENTED SPINACH CHLOROPLASTS FLUORESCENCE EMISSION AT LOW TEMPERATURE

Gy. I. GARAB* and J. BRETON
Service de Biophysique, Département de Biologie
Centre d'Etudes Nucléaires de Saclay, BP 2, 91190 GIF-SUR-YVETTE, France

Received May 4, 1976

SUMMARY : The polarization spectrum of the fluorescence emission at -140°C has been studied in the spectral range 670-760 nm using magnetically oriented spinach chloroplasts. With edge viewing oriented membranes the measurement of the fluorescence polarization (FP), a value reflecting the orientation of the emitting oscillators with respect to the membrane plane, indicates the existence of at least five different fluorescing species. The highest FP is found in the long wavelength band, the smallest in the 675 nm region. A dip at 695 nm is observed in the FP spectrum. A determination of the depolarization of the fluorescence by energy transfer measured with face viewing oriented membranes shows a very high memory of the polarization of the excitation in the long wavelength band of the emission spectrum.

INTRODUCTION

Recent linear dichroism measurements on oriented chloroplasts have shown that the orientation of the photosynthetic pigments with respect to the membrane plane was higher than previously thought (1-3). Although there is still some uncertainty whether the short wavelength chlorophyll a absorbing oscillators in the red are at random or oriented near the magic angle (35°), it has been demonstrated that the long wavelength chlorophyll a Q_y absorbing dipoles are lying quite parallel to the membrane plane.

Using magnetically oriented chloroplasts or algae (2) the fluorescence polarization ratio (FP) has also been reported to be higher in the long wavelength than in the short wavelength region of the emission spectrum (4,5). However at room temperature the different fluorescing species are not spectrally resolved and the origin of the emission above 700 nm is unclear (6). In contrast the low temperature emission spectrum of chloroplasts from higher plants presents a more detailed structure characterized by the appearance of a new band at 695 nm and a very large enhancement

---

*On leave from Institute of Plant Physiology. Biological Research Center. Hungarian Academy of Sciences. Szeged, Hungary 6701
- Reprints are to be requested to J. Breton.

of the long wavelength emission. Consequently the measurement of the polarization of the fluorescence emission at low temperature should offer a new approach to the study of the orientation of chlorophyll in vivo. Although the information is limited to the chlorophyll forms that are fluorescing, such a measurement could also give new insight into the origin of the emission bands and the composite character of the emission spectrum which has been proposed by several authors (7-9).

Taking advantage of a new technique developped in this laboratory, namely the trapping at low temperature of magnetically oriented chloroplasts (10), we are investigating the polarization of the fluorescence emission spectrum at low temperature on such oriented samples.

In the present paper we report some preliminary results on the polarization at -140°C of the fluorescence emission of magnetically oriented spinach chloroplasts. Two types of oriented samples were used : in the first one, the fluorescence emitted along a direction parallel to the membrane planes is analysed. The ratio of the light emitted with a polarization parallel to the plane and perpendicular to it is connected to the anisotropic properties of the emission and is related to the orientation of the emission dipoles with respect to the photosynthetic membrane plane. In the second type of oriented samples the fluorescence is analysed along the normal at the membrane plane for polarizations parallel or perpendicular to the polarization of the excitation light. In this case the ratio of the two polarizations is related to the depolarization of the excitation by energy transfer and could give some information on the degree of relative order between adjacent molecules (4).

The results presented in this study are indicative of the presence of at least five different fluorescing species showing different orientations with respect to the membrane plane in the low temperature emission spectrum. The fluorescence emitted at 695 nm does not seem to originate from the PS II phototrap. Furthermore the longest wavelengths emission presents a very high memory of the polarization of the excitation.

MATERIAL AND METHODS

Freshly harvested leaves of greenhouse spinach were blended at low speed for 5 s. in a sucrose(.4M)-tris(20 mM, pH 7.8)-KCl (20 mM) buffer. The homogenate, after filtration through a nylon mesh (30 μ), was centrifuged at 1 000 x g for 1 minute. The pellet was resuspended in the isolation medium, and diluted with a glycerol-buffer (3-2, v/v) mixture. The chlorophyll concentration of the samples was 3-5 x 10^{-6}M.

Several drops of the diluted suspension were used to fill 1 mm deep slots located on each sides of a quadratic block of brass; microscope cover-slips were used to hold the samples. This type of sample holder provides the possibility to study the fluorescence of four different samples in identical conditions and geometry.

The sample holder was then placed in a 12 kG electro-magnet, and the oriented

chloroplasts were trapped with progressive cooling as described elsewhere (10). The membranes being oriented perpendicularly to the magnetic field it was possible, using two adjacent slots of the sample holder, to trap the orientation of the membranes as depicted in fig. 1a. By a 90° rotation of the sample holder it was then possible to study the fluorescence of two different types of oriented membranes : one with membrane facing the direction of observation of the fluorescence (referred as face viewing oriented) fig. 1b, the other with the edges of the membranes aligned along this direction (called edge viewing oriented) fig. 1c. One of the slots was used for the glycerolic buffer alone in order to correct for the scattered light if it was necessary and the fourth slot for another sample or control (e.g. different concentrations).

The sample holder was secured in a partially unsilvered dewar in which the temperature was adjusted to -140° ± 2°C with a temperature regulated nitrogen stream and controlled with a thermocouple located in the center of the block. Because of the appearance of cracks, partially depolarizing the fluorescence, we did not lower the temperature further.

The polarization spectra of the fluorescence emission were performed in a laboratory built set-up (fig. 2). The excitation light (441.6 nm) provided by a He-Cd laser (Spectra Physics 185) was transmitted through an interference filter and a plastic sheet giving nearly circularly polarized light. For some experiments a polaroid sheet (HN 32) was set in the light beam providing vertically or horizontally polarized excitation. The usual power of excitation falling on the illuminated area (4-6 mm^2) of the sample was about 5 mW.

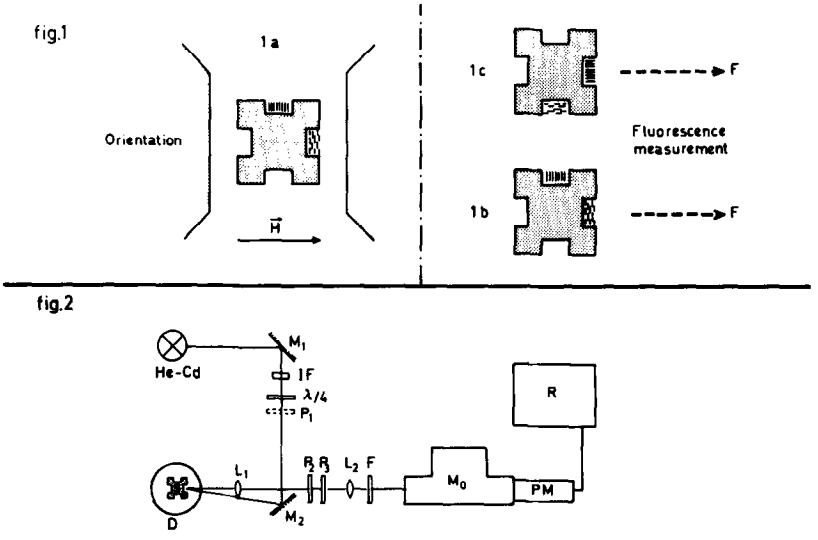

Fig. 1 Schematic top view of the sample holder during the trapping of the orientation (1a) and the measurement of the low temperature fluorescence emission for face viewing oriented (1b) or edge viewing oriented chloroplasts (1c). The small bars in the slots of the sample holder represent the oriented photosynthetic membranes. Details in text.

Fig. 2 Diagram of the set up for the determination of the fluorescence polarization ratio. He-Cd, laser for the excitation ; M_1-M_2, mirrors ; IF, interference filter ; $\frac{\lambda}{4}$, quaterwave plate ; P_1-P_2-P_3 sheet polarizers ; S, sample holder ; D, dewar ; L_1-L_2, lenses ; F, cut-off filter ; Mo monochromator ; PM, photomultiplier ; R, chart recorder.

The fluorescence light was focused with a small angle on the analyser polaroid sheet (HN 32) secured in a hand rotating mount. A second polarizer accurately set at 45° and transmitting in equal amount F_V and F_H for an unpolarized light beam, provides a constant polarization of the light entering the monochromator (H-20 V. Jobin Yvon - 4 nm HBW). The scattered excitation light was blocked by a cut-off filter (OG 530-Schott). F_V and F_H were detected in 2 nm steps by a cooled photomultiplier (R 712-Hamamatsu) and recorded. Since the scattered light measured with the glycerolic buffer between 650 and 760 nm as well as the scattered light determined with samples at 650 nm was generally lower than 1 % of the fluorescence, usually no correction was necessary.

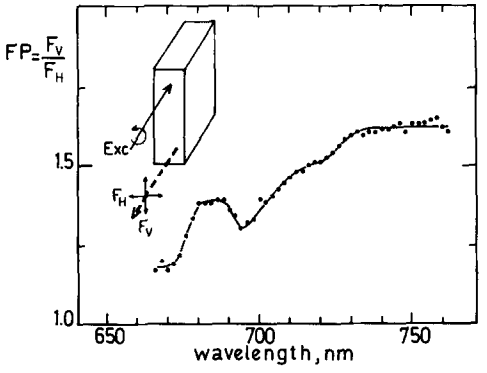

Fig. 3 Low temperature (-140°C) fluorescence polarization ratio spectrum of edge viewing oriented spinach chloroplasts excited with circularly polarized light.

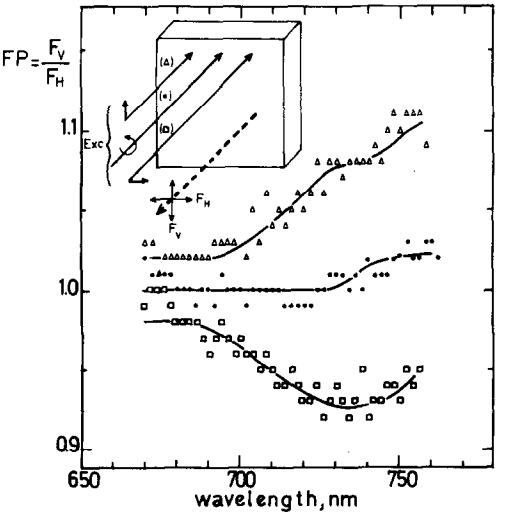

Fig. 4 Low temperature (-140°C) fluorescente polarization ratio spectra of face viewing oriented spinach chloroplasts excited with circularly (●), vertically (Δ) or horizontally (□) polarized light.

RESULTS

The -140°C FP spectrum of edge viewing oriented chloroplasts excited with circularly polarized light is depicted in fig. 3. The maximum values vary from 1.5 to 1.7 from samples to samples, but all the curves were similar. Fig. 4 illustrates the $\frac{F_V}{F_H}$ polarization ratio of face viewing oriented chloroplasts for three different polarizations of the excitation beam.

A small bleaching in all the fluorescence bands has been observed. This bleaching is still detectable with a 20-fold lower excitation energy (.25 mW). By repetitive scanning of the FP spectrum it has been checked hat this bleaching does not alter significantly the FP values. Fluorescence reabsorption effects have been studied by increasing the chlorophyll concentration by one order of magnitude. Some effects have been observed in the 680-690 nm region but they do not change the overall shape of the FP spectrum.

DISCUSSION

Edge viewing oriented chloroplasts

With this type of oriented chloroplasts, the FP values reflect the orientation of the emitting oscillators with respect to the membrane plane in a similar manner as the linear dichroism in the red absorption band reflects the orientation of the Qy absorbing oscillators. From theoretical and experimental evidence it has been concluded that these two oscillators are lying in the tetrapyrollic plane and are parallel to each other in chlorophyll monomeric species (11).

The wavelength dependance of the FP ratio (fig. 3) indicates a rather low polarization at 670 nm. An increase in FP is observed from 670 up to 680 nm where FP stays constant up to 690 nm. From this observation we conclude that F 675 and F 685 originate from differently oriented species, with the shortest wavelength species being the less oriented one.

The dip observed at 695 nm is attributed to F 695 showing a low orientation. This dip cannot be explained by selective scattering because this effect tends to increase the FP values in this region (4). It cannot be either attributed to reabsorption of the fluorescence as (i) the maximum of this effect is expected around 680 nm at the maximum of the linear dichroism and (ii) only very small changes have been detected in this region when increasing the concentration by one order of magnitude. Variations in the magnitude of the dip at 695 nm are observed from samples to samples although the variations in the other regions of the FP spectrum are much smaller. Variations in F 695 intensity have already been reported (12). At 695 nm we measure a FP value of 1.3 (fig. 3). However owing to the relatively small size of the F 695 band and to the overlapping of this emission with fluorescence bands at higher and

lower vavelengths which both show higher FP values it is probable that the true value for F 695 is smaller than 1.3. A value even smaller than 1 for FP at 695 nm cannot be excluded. Such a low value of FP at 695 nm seems to indicate that F 695 does not originate from the PS II trap (12-13) whose red transition moment has been recently shown to be oriented in a planar configuration with respect to the photosynthetic membranes (14). Similar conclusions have been reached from the enhancement of F 695 upon treatment of PS I particles with DCMU [3(3,4-dichlorophenyl)-1,1]dimethylurea] (15).

The maximum FP is obtained in the 735-760 nm region where a plateau is observed (fig. 3). In this emission band, which has been related to PS I (6), the maximum value of FP that we have reproducibly obtained was 1.7, a value close to the dichroic ratio of P-700 measured at 703 nm under similar conditions (1.6) (10). This high value indicates an orientation almost parallel to the membrane plane of the oscillators reponsible for the longest wavelength emission band. It also shows that the influence in this region of the vibrational levels of the species fluorescing at shorter wavelength must be small, otherwise the FP between 735 and 760 nm should parallel its variations in the shorter wavelength region.

The increase in the FP ratio in the region 700-735 nm cannot be interpreted simply in terms of the overlapping of F 695 (small and narrow band) and F 735 (large band). It is necessary to introduce at least another band to account for the FP increase in this region. A small shoulder around 720 nm is indicative of such a band.

With the exception of F 695, there is good overall correlation between the FP values reported here and the linear dichroism data of oriented chloroplasts indicating that the degree of orientation relative to the membrane plane of the Qy transition dipoles of chlorophyll a increases from the shorter towards the longer wavelength. This similar behaviour observed both for the absorbing forms and the fluorescing forms strengthen models in which the various absorption and fluorescence bands can be correlated (7-9). Such a composite character of the emission spectrum at low temperature has to be confirmed by the study of the excitation spectra of the different fluorescing species using edge viewing oriented chloroplasts and polarized excitation light.

Face viewing oriented chloroplasts

In order to obtain valuable information on the extent of the depolarization of the fluorescence by energy transfer it has been demonstrated (16) that it is necessary to eliminate the effect of the orientational anisotropy described in the first section of this paper. This is possible by viewing the fluorescence along the normal to the plane of the photosynthetic membrane, which is a symmetry axis of the system.

Under these conditions and using vertically polarized exciting light we

found a ratio $\frac{F_V}{F_H}$ increasing from 1.02 up to 1.1 when scanning the emission wavelength from 670 up to 760 nm (fig. 4). This experiment deals with energy transfer depolarization and not with the orientational anisotropy of the chlorophyll within the membranes ; this is clear from the inversion of the $\frac{F_V}{F_H}$ ratio when exciting with horizontally polarized light (fig. 4). The non symmetry of the two curves with respect to the FP = 1 baseline, as well as the slight increase observed at the longest wavelengths with circularly polarized excitation (fig. 4), are to be attributed to (i) non perfect quarterwave characteristics of the plate we used for excitation and (ii) some uncomplete orientation of the sample (10).

Using the more classical expression of the degree of fluorescence polarization defined for vertically polarized excitation as $p = \frac{F_V - F_H}{F_V + F_H}$ we obtain a variation of p from 1 up to 5 % when the wavelength of the emission increases from 670 up to 760 nm. The degree of polarization of the fluorescence of chlorophyll a in viscous solutions at room temperature is 6-7 % when excited around 442 nm (17) and is not much modified at lower temperature (6). The p value of 5 % measured at 750-760 nm with face viewing oriented chloroplasts is very close to the value obtained with isolated, non transfering, non rotating chlorophyll a molecules. This can be explained assuming the existence in vivo of a long-wavelength fluorescing chlorophyll a species that could not transfer its energy and which keeps the memory of the polarization of the 442 nm excitation ; this species might be the chlorophyll a form absorbing in the longest wavelength part of the spectrum. Another non conflicting hypothesis would be to consider a very high degree of local order (transition moments parallel to each others) in the long wavelength forms of chlorophyll. In both cases this high polarization strengthens the attribution of the fluorescence observed between 730 and 760 nm to some defined, long wavelength form of chlorophyll a. However more detailed conclusions could be obtained by measuring the degree of fluorescence polarization using face viewing oriented chloroplasts at low temperature with well defined excitation wavelengths in the red band of chlorophyll a.

ACKNOWLEDGEMENTS

We wish to express our thanks to Dr N.E. GEACINTOV for his interest in this work and for revision of the manuscript.

REFERENCES

1. Breton, J. and Roux, E. (1971) Bioc . Biophys. Res. Comm. 45, 557-563
2. Geacintov, N.E., Van Nostrand, F. and Becker, J.F. (1972) Proc. IInd Int. Congr. Photosynthesis Res., Stresa (Forti, G., Avron, M. and Melandri, A., eds) Vol. 1, pp. 283-290, Dr W.Jung N.V. Publishers, The Hague.

3. Breton, J., Michel-Villaz, M. and Paillotin, G. (1973) Biochim. Biophys. Acta, 314, 42-56
4. Becker, J.F.,Breton J., Geacintov, N.E. and Trentacosti, F. (1976) Biochim. Biophys. Acta, (in press)
5. Breton, J. (1975) Proc. Third Int. Congr. Photosynthesis, Rehovot (Avron, M. ed.) Vol. 1, pp. 229-234. Elsevier, Amsterdam
6. Goedheer, J.C. (1972) Ann. Rev. Plant. Physiol. 23, 87-112
7. Litvin, F.F. and Sineshchekov, V.A. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.) pp. 620-661. Academic Press, New-York
8. Mohanty, P., Braun, B.Z., Govindjee and Thornber, J.P. (1972) Plant and Cell Physiol. 13, 81-91
9. Garab Gy. I., Horvath, G. and Faludi-Daniel, A. (1974) Biochem. Biophys. Res. Comm. 56, 1004-1009
10. Vermeglio, A., Breton, J. and Mathis, P. (1976) J. Supramol. Struct. (in press)
11. Gouterman, M. and Stryer, L. (1962) J. Chem. Phys. 37, 2260-2266
12. Cho, F. and Govindjee (1970) Biochim. Biophys. Acta, 205, 371-378
13. Kok, B. (1963) in Photosynthetic Mechanisms of Green Plants, 45-55. Nat. Acad. Sci. Nat. Res. Counc. Publ. 1145 Washington, DC
14. Mathis, P., Breton, J., Vermeglio, A. and Yates, M. (1976) FEBS Letters 63, 171-173
15. Garab, Gy.I., Chernisheva, S., Kiss, J.G. and Faludi-Daniel, A. (1976) FEBS Letters 61, 140-143
16. Breton, J., Becker, J.F. and Geacintov, N.E. (1973) Biochem. Biophys. Res. Comm. 54, 1403-1409
17. Goedheer, J.C. (1966), in the Chlorophylls (Vernon, L.P. and Seely, G.R. eds.) pp. 147-184. Academic Press, New-York