LOW TEMPERATURE FLUORESCENCE POLARIZATION OF ORIENTED SPINACH CHLOROPLASTS

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The polarization spectra of the fluorescence emission at −140 °C was studied in the spectral range of 670—760 nm using spinach chloroplasts oriented by a magnetic field.

The fluorescence polarization (FP) spectra were determined in chloroplasts with their membrane planes oriented either parallel (edge viewing) or perpendicular (face wieving) to the direction of excitation and observation.

With edge viewing membranes the FP values indicated the existence of at least five different fluorescencing species. The highest FP was found in the long wavelength band (F 735), the lowest at around 675 nm. A dip at 695 nm was observed.

With face viewing membranes the FP spectra reflected a high degree of local order of the chlorophyll molecules emitting between 730—760 nm.

Introduction

Confirming the experimental data, it has been theoretically shown by Seely [1], that an optimal utilization of light energy in a photosynthetic unit can be achived only with a highly organized molecular array of photosynthetic pigments. This molecular architecture has to bring about an efficient pumping of absorbed light energy into the reaction center.

There are two major properties of chlorophyll *a in vivo*, which ensure efficient energy transfer: 1) It exists in different spectral forms. 2) There is mutual orientation of transition moment vectors.

The multiciplicity of chlorophyll forms has been unambigously shown (review paper 2), the number, the role, and nature of these forms, however, is not yet clear.

Recent linear dichroism (LD) measurements on oriented chloroplasts have shown that the red absorbing Q_y dipoles of chlorophyll *a* are not randomly oriented, but are lying almost parallel to the membrane plane [3—5]. Using magnetically oriented chloroplasts or algae the room temperature fluorescence polarization ratios (FP) — in accordance with the results obtained from LD measurements — have been reported to be higher in the longer, than in the shorter wavelength region of the emission spectrum.

Because of the large overlap of spectral bands the relative orientation of the chlorophyll forms, however, cannot be estimated on the basis of LD spectra. Inves-

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tigations on the polarization of room temperature fluorescence provide information only about the relative orientations of the fluorescing species of photosystem 2, emitting between 670 and 700 nm.

Compared to the absorption and room temperature fluorescence the emission spectra of chloroplasts at low temperature exhibit a much more detailed structure. Consequently, measurement of the polarization of fluorescence emission at low temperature should offer a new approach to the study of the orientation *in vivo*. Although the information is limited to those chlorophyll forms which are fluorescing, such measurements 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 [6—8].

Materials and Methods

Freshly harvested leaves of greenhouse spinach were blended at low speed for 5 s in a sucrose (0.4 M)-Tris (20 mM, pH7.8)-KCl (20 mM) buffer. The homogenate, after filtration through a nylon mesh (30 μ), was centrifuged at 1000xg for 1 minute. The pellet was resuspended in the isolation medium, and diluted with a glycerol-buffer (3—2, v/v) mixture.

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.

The sample holder was then placed in a 12 kG electro-magnet, and the oriented chloroplasts were trapped with progressive cooling as described elsewhere [9]. The membranes being oriented perpendicularly to the megnetic 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).

The sample holder was secured in a partially unsilvered dewar in which the temperature was adjusted to $-140° \pm 2°$ C with a temperature regulated nitrogen stream and controlled with a thermocouple located in the center of the block.

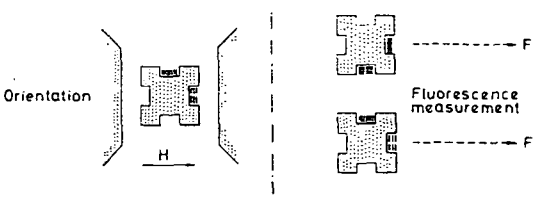

Fig. 1. Schematic top view of the sample holder during the trapping of the orientation (*1a*) and the measurement of the low temperature fluorescence emission for face viewing oriented (*1b*) or edge viewing oriented chloroplasts (*1c*). The small bars in the slots of the sample holder represent the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

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 mm²) of the sample was about 5 mW.

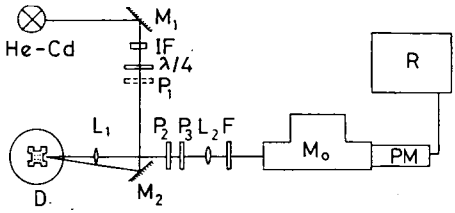

Fig. 2. Diagram of the set up for the determination of the fluorescence polarization ratio. He-Cd, laser for the excitation; M_1-M_2, mirrors; *IF*, interference filter; $\lambda/4$, quaterwave plate; $P_1-P_2-P_3$, sheet polarizers; *S*, sample holder; *D*, dewar; L_1-L_2, lenses; *F*, cut-off filter;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

Results and Discussion

Edge viewing oriented chloroplasts

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.

The wavelength dependence of the FP ratio indicates a rather low polarization at 670 nm. An increase in FP is observed from 670 up ot 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 shorter wavelength species being the less oriented one.

The dip observed at 695 nm is attributed to F 695 showing a low orientation. At 695 nm we measure a FP value of 1.3. However owing to the relatively small size of F 695 band and to the overlapping of this emission with fluorescence bands at higher and lower wavelengths which both show higher FP values it is probable that the true value for F 695 is small-

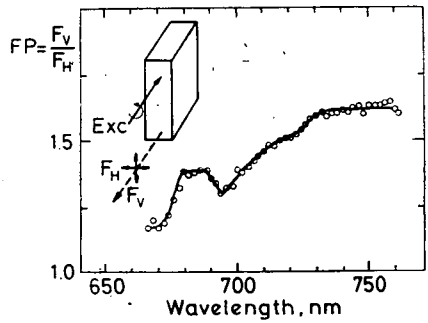

Fig. 3. Low temperature (−140 °C) fluorescence polarization ratio spectrum of edge viewing oriented spinach-chloroplasts excited with circularly polarized light

er 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 [10] whose red transition moment has been recently shown to be oriented in a planar configuration with respect to the photosynthetic membranes [11]. Similar conclusions have been reached from the enhancement of F 695 upon treatment of PS I particles with (DCMU 3/3,4-dichlorophenyl 1/-1,1-dimethylurea) [12].

The maximum FP is obtained in the 735—760 nm region where a plateau is observed. In this emission band 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) [9]. This high value indicates an orientation almost parallel to the membrane plane of the oscillators reponsible for the longest wavelength emission band.

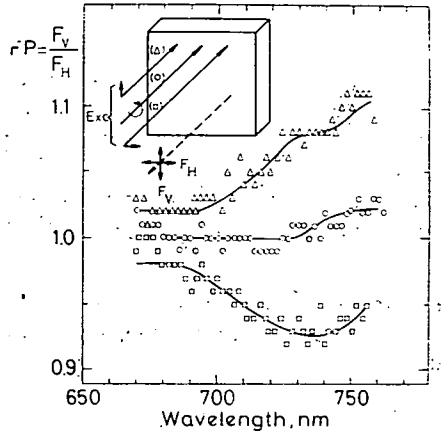

Fig. 4. Low termperature (−140 °C) fluorescence polarization ratio spectra of face viewing oriented spinach chloroplasts excited with circularly (○), vertically (△) or horizontally (□) polarized light

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 Q_y transition dipoles of chlorophyll *a* increases from the shorter towards the longer wavelenght. 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 [6—8].

Face viewing oriented chloroplasts

Fig. 4 illustrates the F_V/F_H polarization ratio of face viewing oriented chloroplasts for three different polarizations of the excitation beam.

In order to obtain valuable information on the extent of the depolarization of the fluorescence by energy transfer it has been demonstrated [13] 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 F_V/F_H increasing from 1.02 up to 1.1 when scanning the emission wavelength from 670 up to 760 nm. 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 F_V/F_H ratio when exciting with horizontally polarized light. 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, are to be attributed to (i) non perfect quarterwave characteristics of the plate we used for excitation and (ii) some incomplete orientation of the sample.

Using the more classical expression of the degree of fluorescence polarization definied for vertically polarized excitation as $p=F_V-F_H/F_V+F_H$ 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 [14]. The p value of 5% measured at 730—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 *in vivo* excistence of a long-wavelength fluorescencing 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 chlorlphyll *a*.

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References

[1] *Seely, G. R.:* J. Theor. Biol. **40**, 189 (1973).
[2] *Brown, J. S.:* Ann. Rev. Plant Physiol. **23**, 73 (1972).
[3] *Breton, J. and E. Roux:* Biochem. Biophys. Res. Comm. **45**, 557 (1971).
[4] *Geacintov, N. E., F. Van Nostrand, and J. F. Becker:* 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, (1972).
[5] *Breton, J., M. Michel-Villaz and G. Paillotin:* Biochim. Biophys. Acta **314**, 42. (1973).
[6] *Litvin, F. F. and V. A. Sineshchekov:* in Bioenergetics of Photosynthesis (Govindjee, ed.) pp. 620—661. Academic. Press, New York, (1975).
[7] *Mohanty, P., B. Z. Braun, Govindjee and J. P. Thornber:* Plant and Cell Physiol. **13**, 81 (1972).
[8] *Garab, Gy. I., G. Horváth, and Á. Faludi-Dániel:* Biochem. Biophys. Res. Comm. **56**, 1004 (1974).
[9] *Vermeglio, A., J. Breton, and P. Mathis:* J. Supramol. Struct. **5**, 109 (1976).
[10] *Kok, B.:* in Photosynthetic Mechanisms of Green Plants, 45—55. Nat. Acad. Sci. Nat. Res. Counc. Publ. 1145 Washington, DC (1963).
[11] *Mathis, P., J. Breton, A. Vermeglio, and M. Yates:* FEBS Letters **63**, 171 (1976)
[12] *Garab, Gy. I., S. Chernisheva, J. G. Kiss, and Á. Faludi-Dániel:* FEBS Letters **61**, 140. (1976).
[13] *Breton, J., J. F. Becker, and N. E. Geacintov:* Biochem. Biophys. Res. Comm. **53**, 1403 (1973).
[14] *Goedheer, J. C.:* in the Chlorphylls (Vernon, L. P. and Seely, G. R. eds. (pp. 147—184. Academic Press, New York. (1966).

ПОЛЯРИЗАЦИОННЫЕ СПЕКТРЫ ФЛУОРЕСЦЕНЦИИ ОРИЕНТИРОВАННЫХ ХЛОРОПЛАСТОВ ШПИНАТА ПРИ НИЗКОЙ ТЕМПЕРАТУРЕ

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Спектры поляризации флуоресценции испускаемой при −140 °С изучались в спектраьном диапазоне 670—760 нм, используя хлоропласты шпината ориентированные в магнитном поле.

Спектры поляризации определялись в хлоропластах в которых плоскости мембран ориентировались либо паралельно либо перпендикулярно к направлению возбуждения и наблюдения.

При паралельном наблюдении мембран обнаружились по крайней мере пять флуоресцирующих форм хлорофилла. Наивысшая поляризация обнаружилась в длинноволновой полосе (Ф735), а самая низкая при 675 нм. При 695 нм сушествует перегиб.

При перпендикулярном наблюдении мембран спектры поляризации указывают на высокий уровень упадрядоченности хлорофилла флуоресцирующего в области 730—670 нм.