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# Trapping at Low Temperature of Oriented Chloroplasts: Application to the Study of Antenna Pigments and of the Trap of Photosystem-1

**A. Vermeglio, J. Breton, and P. Mathis**

*Service de Biophysique, Département de Biologie, Centre d'Etudes Nucléaires de Saclay, BP 2, 91190 Gif-Sur-Yvette, France*

A technique is described for the preparation of oriented samples from spinach chloroplasts whose linear dichroism is then studied by (flash) absorption spectroscopy. The chloroplasts are suspended in a glycerol-containing medium, oriented in a magnetic field, and slowly cooled in the magnet until the medium is rigid enough to avoid disorientation effects.

The absorption spectra in polarized light have been measured at $-50^{\circ}$ and $-170^{\circ}$C. They allow the orientation of chlorophyll *b* to be resolved, and the red transition moment is found to be tilted out of the membrane plane.

A study of the flash-induced absorption changes linked to Photosystem-1 activity reveals a progressive evolution of the difference spectra and of the linear dichroism with decreasing temperatures. At $-170^{\circ}$C, the difference spectrum of P700 in the red is well resolved. All transition moments are found to be largely parallel to the membrane plane.

The potential use of the technique for other experiments by differential absorption spectroscopy and by EPR techniques is discussed.

**Key words: chloroplasts, magnetic field orientation, membranes, photosystem-1**

## INTRODUCTION

One method of testing the molecular structure of photosynthetic membranes has been the measurement of the orientation of the pigments in situ. Early studies were usually performed by microscopy under polarized light (1–3). Later studies were conducted mainly on large collections of organites (chloroplasts, algae, bacteria, chromatophores) which were made anisotropic by various techniques: electric field (4), flow gradient (5, 6), air-drying (6–8), mechanical spreading of viscous suspensions (7, 8), sedimentation (9), and anisotropic photobleaching (10, 11). It has also been shown that some intact photosynthetic structures can be oriented by a magnetic field (8, 12–14). The advantage of this technique is to provide a high degree of orientation of materials kept under physiological conditions. It has been found useful for determining the orientation of pigments, by fluorescence polarization spectroscopy and by linear dichroism spectroscopy (12–18), and for determining the anisotropic properties of light-induced absorption changes in chloroplasts (19–21).

 

In this article we describe a procedure for the trapping, at low temperature, of chloroplasts oriented in a magnetic field at room temperature. This procedure should potentially allow for the study of the orientation of those species which are better detected at low temperature because of spectral or time resolution. The orientation is assayed and some new results are obtained on the linear dichroism of the antenna pigments and on the absorbance changes linked to Photosystem-1activity, which have been previously studied at physiological temperatures (10, 11, 20). In another report (22), we describe the application of the procedure to a study of the orientation of the Photosystem-2 primary donor, which has been observed at liquid nitrogen temperature (23).

## MATERIALS AND METHODS

Chloroplasts were prepared from young spinach leaves by a 5-sec gentle grinding in a blender, in a sucrose (0.4 M), Tris (20 mM, pH 8.0), KCl (20 mM) buffer. The slurry was filtered on a nylon cloth and centrifuged at 2,000 × g for 1 min. The pellet was carefully homogenized in a small volume of buffer. For the optical measurements, it was then diluted either with the buffer or with a glycerol-buffer mixture (2/1, v/v). The addition of glycerol was necessary in order to have a clear glass at low temperature, a prerequisite condition for optical measurements with polarized light.

The material was poured into a 1 mm optical path cuvette made of a copper frame and covered with plexiglass windows devoid of birefringence. The cuvette was inserted between the polar pieces of an electromagnet (H = 12,000 G), so that its optical path was perpendicular to the field. As it is known that chloroplast thylakoid membranes orient perpendicularly to a magnetic field (8, 14), the plane of the membranes is parallel to the optical path ("edge-viewing" orientation; see Fig. 1, left). In some control experiments, the cuvette was positioned in the magnet so that the membrane plane was perpendicular to the optical path ("face-viewing" orientation; see Fig. 1, right). The chloroplasts were

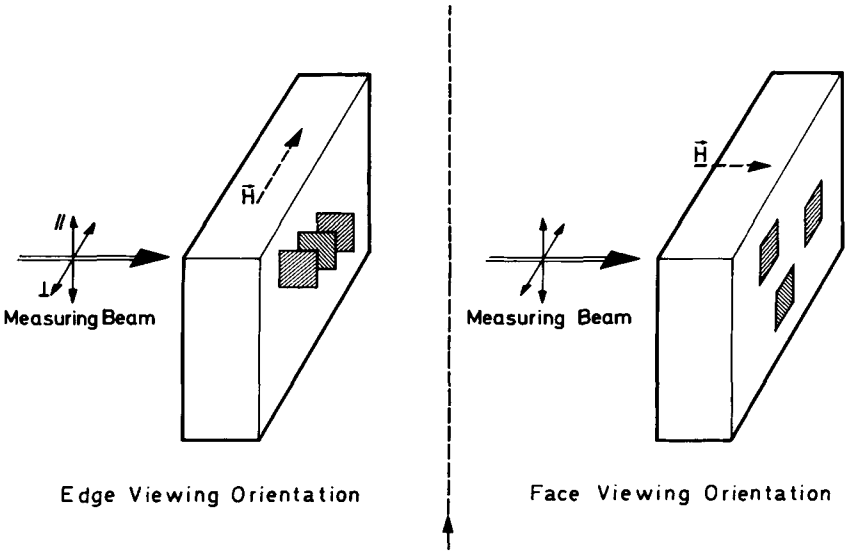


Fig. 1. A schematic representation of the geometrical arrangements used in this report. The squares represent the chloroplast thylakoid membranes.

oriented for 2 min in the field at room temperature. Then, with the field on, the cuvette was progressively cooled by liquid $N_2$, from the bottom. The temperature in the cuvette was about $-50°C$ after 30 sec, and $-70°C$ after 90 sec. After that time, the cuvette was rapidly transferred into a partly unsilvered Dewar flask, which was half filled with a mixture of ethanol dry ice or with liquid $N_2$, giving a final temperature in the cuvette of $-50°C$ or $-170°C$. The operations were all performed in complete darkness. The Dewar flask was mounted in a light-tight compartment, as previously used (24), which could fit all our absorption spectrophotometers.

The absorption spectra of the oriented samples were recorded with a Perkin-Elmer spectrophotometer (model 356), operating in the split-beam mode. We did not use any reference cuvette. A polarizer (Polaroid, type HN32) was inserted in each beam, before the sample compartment. The polarizers could be positioned so that the electric vectors of both beams were either vertical or horizontal. In the remaining part of this article, the absorbance ($\Delta A$) or the absorbance changes ($\Delta A$) for the two positions will be named $A_{/\!/}$ (or $A_\perp$) and $\Delta A_{/\!/}$ (or $\Delta A_\perp$), as with edge-viewing oriented membranes the electric vector is preferentially parallel (or perpendicular) to the chloroplast membrane plane.

The linear dichroism spectrum was measured with an apparatus built in the laboratory, designed similarly to the dichrograph described by Breeze and Ke (25). The measuring beam was modulated by a Morvue photoelastic modulator. The dichrograph could accommodate the cell for low-temperature measurements or an electromagnet providing a 12 kG field in which a usual $10 \times 10$ mm cell could be inserted.

Flash-induced absorption changes in polarized measuring light were measured in a single-beam apparatus, similar to that described by Breton et al. (20). The cuvette was perpendicular to the measuring beam. The sample was excited from the front side by flashes from a dye laser ($\lambda \sim 600$ nm, Electrophotonics model 23), at a repetition rate of 0.2 Hz. Each flash was of saturating intensity.

## RESULTS AND DISCUSSION

### Linear Dichroism of the Antenna Pigments

Absorption spectra of oriented chloroplasts have been measured at $-50°C$ and $-170°C$, with polarized light (Figs. 2, 3). The linear dichroism spectrum ($A_{/\!/} - A_\perp$) has been measured directly with the dichrograph on the same samples (inserts in Figs. 2 and 3). The difference spectra recorded with the dichrograph are corrected for baseline variations; this is not the case for the spectra recorded separately for each polarization in which crossing points in the 600–655 nm region are not significant. These measurements indicate that the membranes remain oriented at low temperature, after being oriented by a magnetic field at room temperature. At 678 nm, the wavelength of maximum absorption, the dichroic ratio $D\left(D = \frac{A_{/\!/}}{A_\perp}\right)$ has a value of 1.31 at $-50°C$ and 1.27 at $-170°C$ (these are representative values). The decrease at lower temperature is probably due to the appearance of some cracks in the sample. With the same preparation of chloroplasts, suspended in a buffer without glycerol, a value of 1.55 was measured at room temperature in the dichrograph, with the sample kept oriented by the magnetic field. Under the same conditions but with glycerol the dichroic ratio decreased to 1.37. Thus, the decreased orientation measured at low temperature is mainly due to the addition of glycerol, which is mandatory for obtaining a clear glass. In control experiments we found that the damaging effect of glycerol cannot be reversed by subsequent dilution with the buffer.

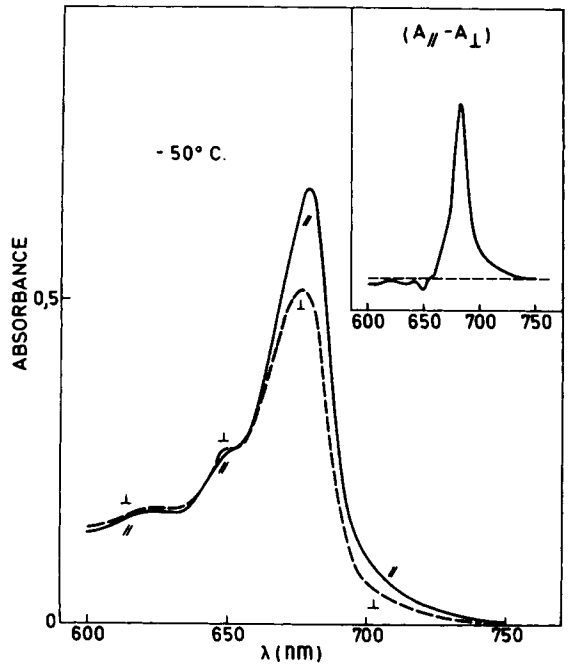


Fig. 2. Absorption spectra, recorded with the double-beam spectrophotometer, of a suspension of oriented chloroplasts at −50°C. A polarizer, with indicated direction, was inserted before the cuvette (optical path: 1 mm). The insert represents the spectrum of linear dichroism, measured on the same sample with the dichrograph. Vertical scale: arbitrary units.

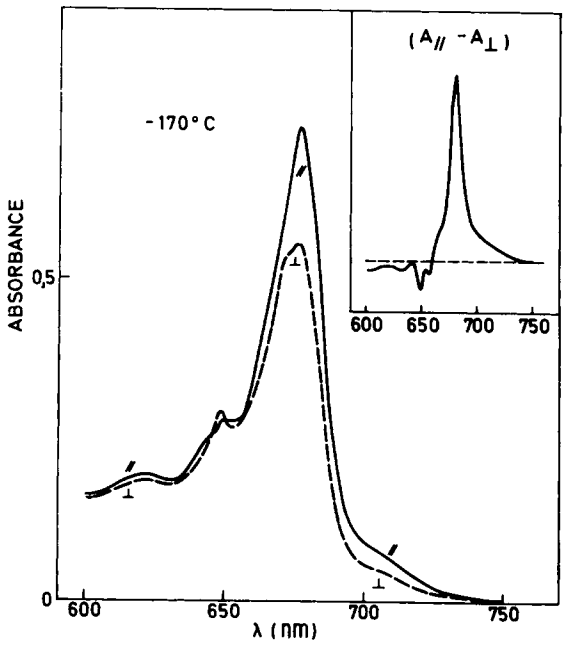


Fig. 3. Absorption spectra and linear dichroism spectrum, measured as in Fig. 1, at −170°C.

The spectra presented in Figs. 2 and 3 indicate that, as already observed at room temperature (7, 8), the Qy oscillator of chlorophyll *a* forms, absorbing at long wavelength, has a rather high degree of orientation in the plane of the membrane. A new result is obtained concerning chlorophyll *b*, whose absorption peak at 648 nm is well resolved at −170°C. A negative value is obtained for $(A_{//} - A_{\perp})$, which indicates that the corresponding transition moments are preferentially oriented perpendicular to the membrane plane. From a comparison of the linear dichroism of chloroplasts from wild-type barley and chlorophyll *b* less mutants, Demeter (personal communication) came to the same conclusion. In a previous report (7), based on spectra recorded at room temperature in conditions where the peak of chlorophyll *b* could not be precisely sorted out, a different conclusion had been attained because the positive shoulder at 652 nm in the linear dichroism spectrum had been attributed to chlorophyll *b*.

### Absorption Changes Related to Photosystem-1 Activity

**Measurements at −50°C.** Absorption changes induced by saturating flashes were measured with oriented samples in polarized measuring light (Fig. 1, edge-viewing configuration). A typical result is presented in Fig. 4 (at 703 nm). The absorption decrease for a vertically polarized measuring beam ($\Delta A_{//}$) is greater than for an horizontally polarized beam ($\Delta A_{\perp}$). With nonoriented samples (and an unpolarized measuring beam), the spectrum of the flash-induced absorption changes is reported in Fig. 5. A major decrease is observed at 703 nm (P700), as well as smaller peaks at 690 and 685 nm. This spectrum can be attributed to Photosystem-1 only, as the measurements are performed by a repetitive flash technique (0.2 Hz), and Photosystem-2 reactions can be considered to be irreversible at −50° (24). At 820 nm we observed the absorption increase due to oxidized P700 (23; see also Ref. 26). At 520 nm we observed the absorption change attributed to a shift in the absorption band of carotenoids (27, 28).

With oriented samples, typical values of the dichroic ratio for a few selected wavelengths are presented in Table I. We have routinely controlled the orientation by the magnetic field of the same chloroplasts suspended in sucrose buffer at room temperature. For P700, an average value D = 2.0 has been obtained at 702 nm (compare with Ref. 20, in which chloroplasts presented a higher orientation, D = 2.3). Possible artifacts in the

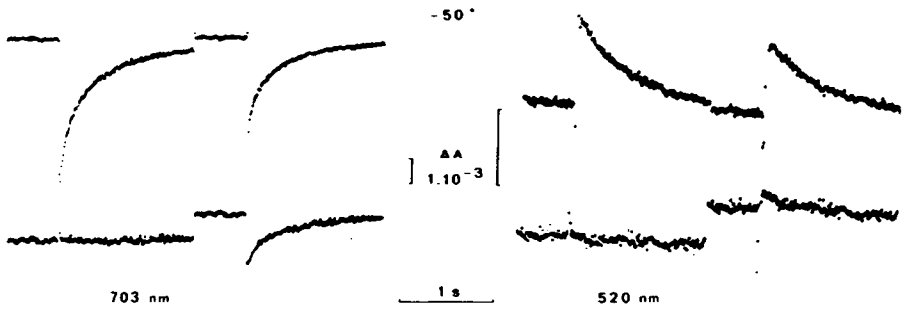


Fig. 4. Absorption changes obtained by repetitive laser flash excitation of a suspension of oriented chloroplasts, at −50°C. Number of averaged flashes: 40 at 703 nm, 160 at 520 nm. At each wavelength are presented the absorbance changes for the two positions of the polarizer (// and ⊥, edge-viewing configuration), their difference (bottom traces, on the right), and their difference in the face-viewing configuration (bottom traces, on the left). Chlorophyll concentration: 500 $\mu g/ml^{-1}$.

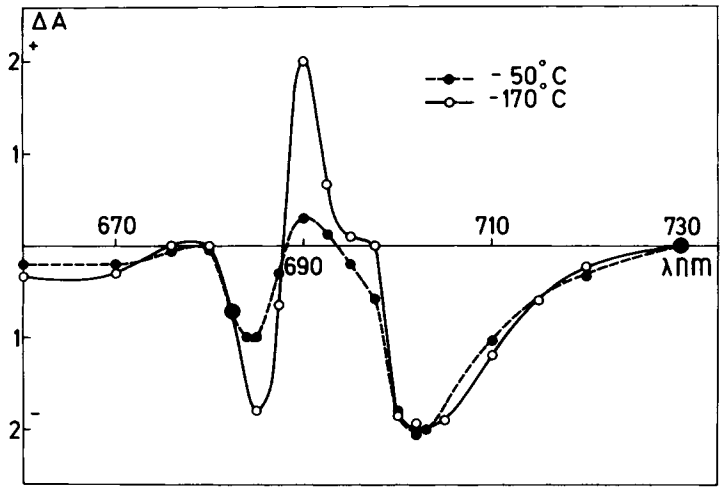


Fig. 5. Difference spectra of absorption changes due to repetitive laser flash excitation of a suspension of nonoriented chloroplasts, at −50° and −170°C. Chlorophyll concentration: 150 $\mu g/ml^{-1}$. The spectra have been normalized for an identical ΔA at 703 nm.

**TABLE I. Dichroic Ratio (D) for Absorption Changes Measured at Different Wavelengths, at −50°C and −170°C**

| | λ nm | 520 | 685 | 690 | 703 | 820 |
|---|---|---|---|---|---|---|
| D | − 50° | 1.2 ± 0.1 | 1.3 ± 0.1 | 1.2 ± 0.1 | 1.6 ± 0.05 | 1.6 ± 0.1 |
| | −170° | − | 1.3 ± 0.05 | 1.5 ± 0.05 | 1.5 ± 0.05 | 1.5 ± 0.05 |

measurement of linear dichroism spectra have been discussed elsewhere (8). In the measurement of flash-induced absorption changes, additional artifacts may occur. At first, a photoselection effect might have occurred with our polarized laser flash. As a control the cuvette holder was positioned in the magnet (at room temperature, before cooling) in such a way that, in the flash spectroscopy equipment, the membranes were perpendicular to the direction of the measuring beam (face-viewing orientation). In this case the absorption change is exactly the same for the two polarizations of the measuring beam (Fig. 4, bottom left), a result which rules out the preceding artifact and also indicates an identical optical path for the two polarizations. As another potential source of error, we considered partial bleaching by the measuring beam which might be different for the two polarizations, especially at low temperature, because randomization by chloroplast rotation cannot occur. This effect was found to be present at high intensities of the measuring beam. In the results presented here, the intensities were low enough so that this effect was negligible.

The measured dichroic ratios (Table I) seem to belong to two classes. A relative large value was obtained at 703 nm. In agreement with previous measurements at room temperature (10, 11, 20), this indicates that the long-wavelength oscillator of P700 lies mostly parallel to the membrane plane. An equally large value was obtained at 820 nm,

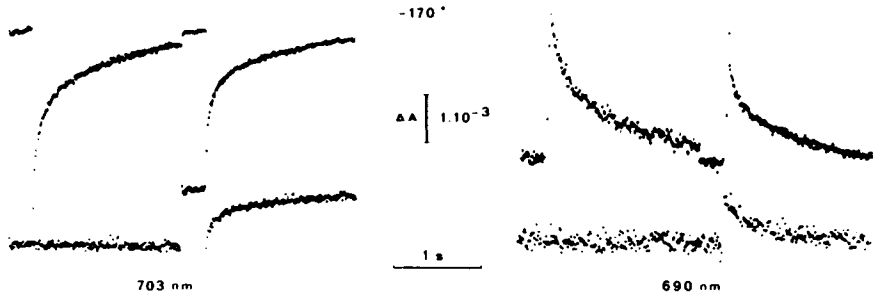


Fig. 6. Absorption changes due to repetitive laser flash excitation of a suspension of oriented chloroplasts, at −170°C. Average of 80 flashes for each polarization, at 703 and 690 nm. The traces are obtained as in Fig. 3. Chlorophyll concentration: 500 $\mu g/ml^{-1}$.

at the absorption peak of the radical cation of chlorophyll *a* in P700. This indicates a similar orientation of the long-wavelength oscillator in the cation and in the parent chlorophyll molecule(s), as predicted by molecular-orbital calculations on chlorophyll *a* (R. H. Felton, personal communication) and bacteriochlorophyll (29). By contrast, a low dichroic ratio was obtained at 690, 685, and 520 nm. At this last wavelength the absorption change probably reflects the same carotenoid shift as at room temperature, for which a rather small polarization has also been reported (19). At 690 and 685 nm, the absorption changes may be due to secondary bands of P700 and to a shift of the absorption bands of chlorophyll *a*, resulting from a light-induced transmembrane electric field (compare Ref. 30).

**Measurements at −170°C.** At −170°, the absorption changes present the same basic features as at −50°. Typical kinetics are presented in Fig. 5 (for 703 and 690 nm), the difference spectrum is plotted in Fig. 4, and the dichroic ratios are reported in Table I. It must be pointed out that at the flash frequency of 0.2 Hz a large fraction of the Photosystem-1 reaction centers remain undetected, as only a partial back-reaction (about 50%) occurs in 5 sec. No measurement was possible at 520 nm because absorption changes linked to Photosystem-1 activity are very small at this temperature (31). In the red, the peaks at 690 and 685 nm are large and narrow, in agreement with previous reports (32, 33). The increasing amplitude and the narrowing of these bands follow a progressive evolution with decreasing temperature. At 703 and 820 nm, the dichroic ratio is slightly smaller than at −50°, probably due to the formation of some cracks in the cuvette. As a general trend, the dichroic ratio at these wavelengths follows the same evolution as for the antenna pigments. By contrast, at 690 nm the dichroic ratio is larger than at −50° and the same as at 703 nm. Several interpretations have been proposed to explain the red part of the P700 difference spectrum at room temperature (34, 35) as well as at low temperatures (32). The identical dichroic ratios observed at 703 and 690 nm at low temperatures are not sufficient to discriminate between these hypotheses, although they indicate a planar orientation of the corresponding transition moments.

## CONCLUSION

This study was intended to investigate the possibility of trapping and studying at low temperature a suspension of chloroplasts oriented by a magnetic field at room temperature. Our results show that this is indeed possible, although the orientation is smaller than at room temperature. The main factor responsible for the loss of orientation is the

addition of glycerol. The comparison between the orientation of antenna pigments and of transition moments relative to P700 indicate a parallel loss of orientation, which is probably due to a low orientation of the chloroplasts rather than to a disorientation of the transition moments relatively to the membrane plane. It must be pointed out that we never observed any decrease of the orientation within a few hours at low temperature. This stability allows for various spectroscopic measurements on the same oriented sample.

This technique of studying the orientation of membrane components at low temperature is of great potential interest. We have already used it for measuring the orientation of the primary donor of Photosystem-2, a chlorophyll molecule whose Qy transition moment is nearly parallel to the membrane plane (22). A similar study at room temperature would have been impossible because of time resolution. Other membrane components, such as cytochrome f, cytochrome $b_{559}$, and C-550, can be conveniently studied at low temperature, and their orientation might be measured by linear dichroism. The technique might also be used for the intermediate steps in the photoconversion of rod outer segments, which can be oriented by a magnetic field (36).

Apart from studies by absorption spectroscopy, the technique of trapping oriented membranes may present some interest when coupled with ESR measurements. It has been shown recently that some specific information can be gained from spin-labeled probes included in artificial membranes oriented with respect to the magnetic field of an ESR spectrometer (see Refs. 37, 38). In primary photosynthetic reactions, many paramagnetic species have been characterized by their ESR spectrum at low temperature (see Ref. 39 for a review). It is expected that additional structural information will be gained from similar measurements performed on oriented membranes.

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