CHLOROPHYLLS AND CAROTENOIDS STATES IN VIVO

I - A LINEAR DICHROISM STUDY OF PIGMENTS ORIENTATION IN SPINACH CHLOROPLASTS

J. Breton and E. Roux
From the Department of Biology - C.E.N. SACLAY
B.P. 2 - 91 GIF-SUR-YVETTE

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SUMMARY

Using a spectropolarimeter for measuring the linear dichroism of pigments in oriented spinach chloroplasts we found a composite signal that could be interpreted neither as textural dichroism nor as a sample birefringence. We detected a fairly good orientation of pigments with respect to the normal at the plane of chloroplast lamellae. We failed to show any orientation axe in this plane. We found that all the Ca 683 is oriented, its Y direction being parallel to, or lying in the lamellae plane. Ca 673 is either unoriented or is oriented with its Y direction making an angle of 55° with the normal. If Ca 673 is unoriented, then the X direction of Ca 683 could be space positionned at about 45° of the lamellae plane. Carotenoids are oriented in the lamellar plane or close to it. Cb is equally oriented.

INTRODUCTION

A great deal of studies have been published upon chlorophylls orientation in vivo (1-16). Many conflicting hypotheses emerged from the results obtained by various techniques. Some authors (2-5) think that the stacking of lamellae in the grana might induce the observed signal (textural dichroism). Others suppose that the phenomenon they observed in the red part of the spectrum could be interpreted as Ca orientation. Some of them (9-13,15) have shown that only the Ca that absorbs in the spectral range 695-705 nm is strongly oriented. In contrast a few others (14,16) have described the Ca 683 as partly oriented. Furthermore only Goedheer (7,8) reported an orientation of carotenoids in vivo.

Those studies that led to different models (11,16) of molecular organisation of the pigments in the lamellae are of interest in terms of structure and function of the photosynthetic apparatus.

METHODS

Chloroplasts are extracted according to the classical techniques in aqueous medium (sucrose 4.10^{-1}M ; tris pH 7.8, 2.10^{-2}M).

Isolated lamellae are prepared as in ref 17 or by ultrasonic desintegration

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Abbreviations : Ca, chlorophyll a ; Ca 673, chlorophyll a form that absorbs near 673 nm ; Cb, chlorophyll b ; L.D. linear dichroism

(Bronwill Biosonik III. 20 KHz . 80% full power output. 3 x 15 s) of osmotically shocked chloroplasts.

Orientation of chloroplasts (or lamellae) is obtained by picking up with a hair brush a small quantity of chloroplasts (or lamellae) contained in a centrifugation pellet, and spreading them over a glass plate (type 1 orientation). Good orientations are easily obtained by a slow motion of the brush, forward and backward until the hardening of the preparation. A slight admixture of polyvinyl alcohol to the chloroplasts makes easier the spreading and improves the results. We also employed another procedure (type 2 orientation) that is easier to deal with : one drop of a lamellae suspension in sodium ascorbate (5mM) is deposited on an optically polished glass plate and is allowed to dry in the cold room and in darkness.

We measure LD by means of an unmodified commercially available spectropolarimeter (Fica). Let us briefly recall the principle of these measurements that has been described, as well as some of the theoretical aspects, in a previous paper (18).

If plane polarized monochromatic light crosses an isotropic sample that is optically active, the polarization plane rotates and, by varying the wavelength, we record an ORD spectrum.

If we put a sample, containing oriented pigments, perpendicularly to the beam of a spectropolarimeter with the direction of the oriented transitions lying in the polarization plane (or perpendicular to it) we still record an ORD spectrum. But if we incline at an angle θ the direction of the oriented transitions with the polarization plane (the plane of the preparation standing perpendicular to the light beam), then the polarized light is not equally absorbed along this direction ($A_{//}$) and the perpendicular direction ($A_{\perp}$). Therefore a rotation α of the polarization plane occurs, its value is :

$$\text{eq (1)} \quad \underset{\text{(degrees)}}{\alpha} = \frac{180}{4\pi \log e} \sin 2\theta \, (O.D._{//} - O.D._{\perp}) \quad + \text{ORD signal}$$

Looking at eq (1) we can see that α could not be influenced by unoriented pigments (ORD being not taken into account).

We record α as a function of wavelength. A special sample holder, permitting an accurate measurement of θ is used.

Absorption spectra are recorded with a Cary 14 spectrophotometer.

RESULTS

The LD spectrum ($O.D._{//} - O.D._{\perp}$) for a type 1 preparation is shown together with absorption spectrum in the fig. 1.

This LD spectrum has been corrected for the ORD signal that always accounts for less than 5% in such a spectrum. The shape of this LD signal is remarquably constant in all the tested chloroplasts preparations (over 75 with 2 or 3 samples a preparation). Its intensity varies from sample to sample (usually no more than

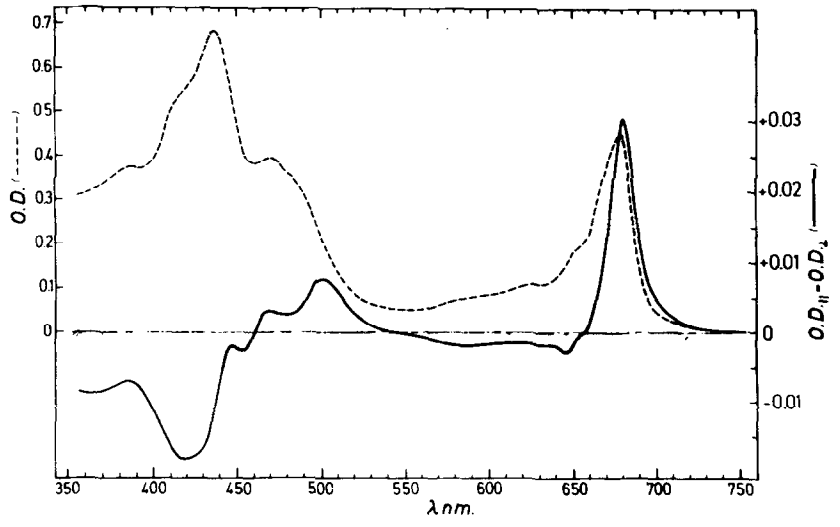

Fig. 1

Absorption (- - -) and linear dichroism (—) spectra for spinach chloroplasts oriented by spreading.

twice for a standard absorption), but it seems to only depend on the spreading, not on the chloroplasts themselves.

For type 1 preparations the shape of the LD spectrum is the same whether chloroplast lamellae or intact chloroplasts are used. For type 2 preparations we needed to tilt the plate with respect to the beam in order to record the LD signal. (If the plate were perpendicular to the beam we should only record ORD spectrum whatever θ would be). In those conditions the recorded LD spectrum has the same characteristics as for type 1 preparations.

Moreover, due to the sensitivity of the apparatus, the signal is very easy to detect and could be amplified (10 times with no detectable noise) when needed.

DISCUSSION

We first notice that the shape of the LD spectrum and particularly the opposite signs at 682 and 420 nm rule out an effect of textural dichroism alone (the expected spectrum would then be the absorption spectrum). Moreover, the identity of the signals for intact chloroplasts and for lamellae preparations shows that textural dichroism is not very large.

On the other hand, a distortion of the signal by the sample birefringences might occur. We checked some type 1 preparations with another good LD measurement technique (19) that is much less affected by birefringences than our. The recorded spectra are similar in shape and magnitude (the observed differences, less than 5% of the signal, could be explained by base-line discrepancies).

Although we cannot completely eliminate the textural dichroism and the birefringence effects, we assume that the spectrum indeed represents essentially the contribution $O.D._{//} - O.D._{\perp}$ of oriented forms of chloroplasts pigments.

When spreading chloroplasts in type 1 preparations their great axe is oriented parallel to the spreading axe. As many electron microscopic pictures show that the lamellae planes are lying parallel to the great axe of the chloroplast, we conclude that when spreading isolated lamellae we orient their planes parallel to the spreading axe. In type 2 preparations we postulate that when water is evaporating, more lamellae settle with their plane parallel to the glass plate than perpendicular to it.

As type 1 and type 2 preparations yield the same results we conclude that in type 1 preparations we are unable to reveal any orientation axe in the lamellae plane. May be this axe does not exist, may be we are unable to orient it.

In a previous publication (18), working with chlorophylls oriented on nitrocellulose fibers, we have discussed a model where transitions moments are isotropically distributed in all the planes tangent to a cylinder. In the present study we use for lamellae oriented in type 1 preparations a model that is analogous. In such a model (fig. 2), the plane of chloroplasts lamellae are tangent to cylinders the axe of which are the spreading axe. As a matter of fact the only pigments we can reveal are those that are oriented in relation with the normal to the lamellae plane. For those preparations we have checked that the attenuation tensor (18) is uniaxe.

In such a case, as it has been shown (18), a transition whose direction makes an angle φ with the normal at the plane, and which is isotropically distributed round this normal will give a dichroic signal that is φ dependent :

$$\text{eq (2)} \quad O.D._{//} - O.D._{\perp} = \frac{4\pi \log e}{180 \times \sin 2\theta} \times \alpha \times \frac{1 - 3\cos^2 \varphi}{2}$$

From eq (2) we can see that a perfectly oriented transition making an angle $\varphi_1 = 55°$ $(3\cos^2 \varphi_1 - 1 = 0)$ is completely undetectable by linear dichroism. The signal has an opposite sign for $0 < \varphi < 55°$ and for $55° < \varphi < 90°$.

Moreover we want to emphasize that all the transition moments, with a given φ, do not equally contribute to the rotation of the polarization plane depending on the geometrical arrangement of the chloroplast lamellae. When transition moments are contained in the planes tangent to a cylinder that are perpendicular to the light beam they will not rotate the polarization plane in contrast to those that lie in the planes tangent parallel to the light beam who will give a strong contribution to the LD signal.

Transition moments isotropically distributed in a plane yield a four time larger dichroic signal than when they are distributed exactly in the same way upon a cylindrical surface (reduction factor = 1/4).

That explains why for type 1 preparations the signal is not very high in comparison with absorption, much more transition moments being involved in absorption than in LD.

By tilting type 1 preparations with respect to the light beam we have been able to check the model (fig. 2). We found it is valid though there is a slight anisotropy round the spreading axe, some more lamellae being oriented with their plane parallel to the glass plate.

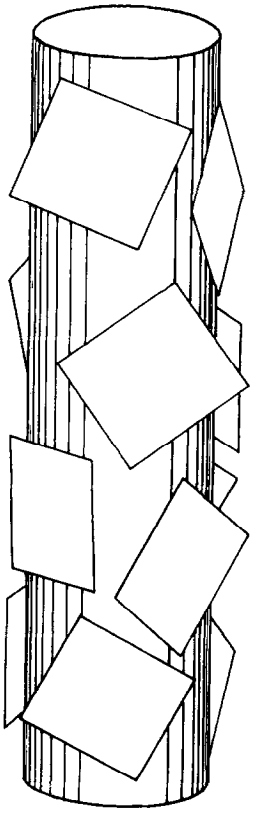

Fig. 2

Suggested arrangement of chloroplast lamellae oriented by spreading.

By comparison between absorption and LD spectra in the best oriented samples and by taking into account the factor of reduction above mentionned we find that the transitions Qy of the chlorophyll a molecules that absorb in the spectral range 680-730 nm must lie quite parallel to the lamellae plane (or lying in it).

The chlorophyll a molecules that absorb near 673 nm do not give a noticeable LD signal. Their Qy transition seem to be distributed either at random or precisely oriented near 55° of the normal to the lamellae plane. By comparing the results obtained with isolated chlorophyll a oriented in films (18) we attribute the complex

band observed in the range 450-400 to transitions of chlorophyll a molecules polarized mainly in the X direction. If one wants to calculate the angle φ for that direction it is necessary to take into account the Ca 673.

If Ca 673 is randomly distributed then we can calculate the X direction orientation of Ca 680-730. We found it must be quite near 45°.

If Ca 673 is oriented as described before, then we only know that the overall orientation of the X direction for all the chlorophylls a is near 45°.(In fact the hypothetical optical density we choose for oriented Ca at 420 nm is not very important as $1 - 3\cos^2\varphi$ vary very sharply when varying φ round 45°).

We must notice that the shoulder located at 385 nm on the absorption spectrum of chloroplasts that corresponds to an Y polarized transition of Ca (18) appears in our LD spectrum as expected with the same sign as the band situated at 682 nm. Equally we attributed the negative sign of the dichroism in the region 650-600 nm to the Qx transitions of chlorophyll a.

The little positive signal we observed near 650-655 nm seems to belong to Cb showing a Qy transition lying with $55° < \varphi < 90°$.

In the region 550-450 nm there is a lot of overlapping bands as expected because Ca, Cb and carotenoids absorb in this region. If the 530-500 nm part of this spectrum is attributed mainly to carotenoids, then they must lie in the lamellae plane (or close to it).

Studies upon the non gaussian shape of the LD signal in the range 670-740 nm are currently under progress and will appear, together with some more details upon Ca 673 and the nature of the orientation of some other chloroplasts chromophores, in a further publication.

Using techniques of absorption with polarized light, various authors found difficulties in obtaining strong orientation signal. It is obvious that in oriented chloroplasts preparations the largest part of pigments will be inactive in polarized absorption. Our method is advantageous because it detects only the oriented species (unoriented one will not rotate the polarization plane). In the expression of our results we use the difference $A_{//} - A_{\perp}$ instead of the more classical dichroic ratio $A_{//} / A_{\perp}$. For an unique oriented pigment it does not matter, but for a mixture of differently oriented (and unoriented) species with overlapping absorption bands the dichroic ratio will be dependent on the absorption of the pigments that are unoriented (or oriented with a low $A_{//} - A_{\perp}$). This could somewhat explained why various authors (9-13,15) found that the oriented Ca species absorb maximally round 700 nm.

However in the red bands of Ca we agree quite well with the published curves of Sauer (15) and of Thomas et al. (16) who found a difference signal $A_{//} - A_{\perp}$ that is maximum between 680 and 685 nm. Sauer and Calvin (11) published a curve $A_{//} / A_{\perp}$

versus λ in the spectral range 350-750 where some features of our spectrum could be detected (if we expressed our results as $(A_{//} - A_{\perp})/A$), though they failed to detect an opposite sign in the red and in the spectral range 460-350 nm.

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