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Direct observation of large chiral domains in chloroplast thylakoid membranes by differential polarization microscopy

(photosynthesis/circular dichroism/linear dichroism/scattering)

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ABSTRACT **Long-range chiral organization of the pigment–protein complexes in mature granal chloroplasts has been established by differential polarization imaging and local circular dichroism spectra. Linear and circular dichroism images of oriented chloroplasts were obtained in a confocal differential polarization microscope. The circular dichroism images display signals of opposite signs emerging from discrete regions with local dichroic values much larger than anticipated, indicating *domains* in the thylakoid membranes having long-range chiral organization. These domains are associated with positive and negative circular dichroism bands obtained at specific locations on the chloroplasts. Surprisingly, the local circular dichroism spectra do not display the excitonic shape of spectra obtained for macroscopic suspensions, but the latter can be produced by superposition of two local spectra of opposite sign. These data are evidence for the existence of long-range chiral order of the pigment–protein complexes in thylakoid membranes. The possible role of the long-range chiral domains in the efficiency of energy delocalization through the thylakoid membranes is discussed.**

The efficiency of the conversion of light energy into chemical energy is largely determined by the macromolecular organization of the photosynthetic pigment molecules. Several hundred of these antenna molecules, associated with a photochemical reaction center, absorb light and funnel its energy into the reaction centers, where primary charge separation takes place. In chloroplasts, these molecules (chlorophyll a, chlorophyll b, and carotenoids) are found in pigment–protein complexes embedded in thylakoid membranes in a highly ordered fashion (1, 2). The transition dipoles of chlorophyll are oriented parallel to the membrane planes, favoring a long-range diffusion of excitation energy along these planes (2).

Recent circular dichroism (CD) (3, 4) and circular intensity differential scattering (5) experiments suggest that pigment–protein complexes in the thylakoid membranes are organized in chiral macrodomains the dimensions of which are a sizeable fraction of the wavelength of visible light. In this paper, differential polarization imaging (6–8), which permits the spatial resolution of the optical anisotropy of chiral objects, is used to map chirally organized domains in thylakoid membranes. CD spectra of these domains (local CD) have also been recorded to study the wavelength dependence of this anisotropy. These results provide new evidence of the long-range structural organization of the pigment–protein complexes in the thylakoid membranes of chloroplasts.

MATERIALS AND METHODS

Chloroplasts were isolated from spinach (*Spinacia oleracea*) leaves (9), aligned in a 1.5-T magnetic field and trapped in a polyacrylamide gel between two coverslips. Because chloroplasts align with their thylakoid membranes perpendicular to a magnetic field (10), placing the coverslips either parallel or perpendicular to the magnetic field results in an edge-on or face-up view of their membranes, respectively (Fig. 1 *a* and *b*).

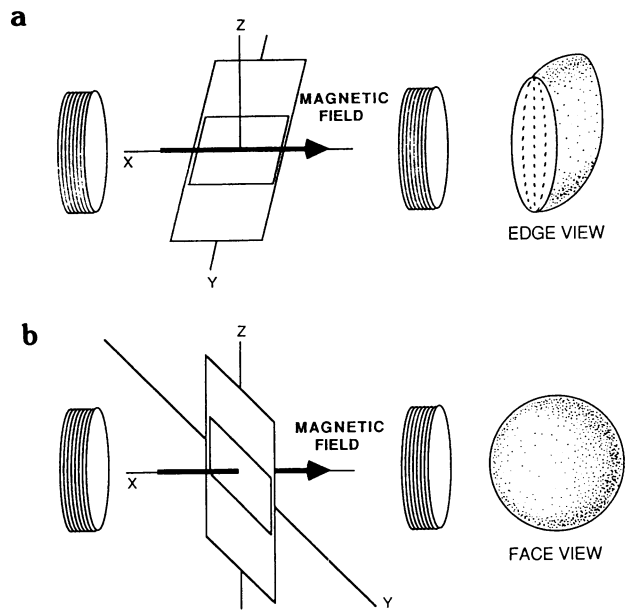

FIG. 1. Schematic representation of the chloroplast alignment setup. (*a*) The chloroplast appears edge-on under the microscope, and the membranes run parallel to the long axis of the chloroplast edge. (*b*) The chloroplasts appear as flat disks lying on one of their faces.

With a confocal scanning differential polarization microscope (11), the sample was illuminated point by point using alternating orthogonal polarizations of the light, I_{p1} and I_{p2}. The photomultiplier tube signal for each pixel position in the image was electronically integrated to measure the total transmitted intensity, $I_{p1} + I_{p2}$, while a lock-in amplifier measured the difference in transmitted intensity between the two orthogonal polarizations. To obtain a differential polarization image, ratios of these two signals were then taken for every point in the object according to the relationship:

$$\frac{I_{p1} - I_{p2}}{I_{p1} + I_{p2}}.$$

All chloroplasts were tested for correct alignment, and all CD images were checked for linear dichroism (LD) contributions. High resolution images were obtained using a dye

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Abbreviations: LD, linear dichroism; CD, circular dichroism.
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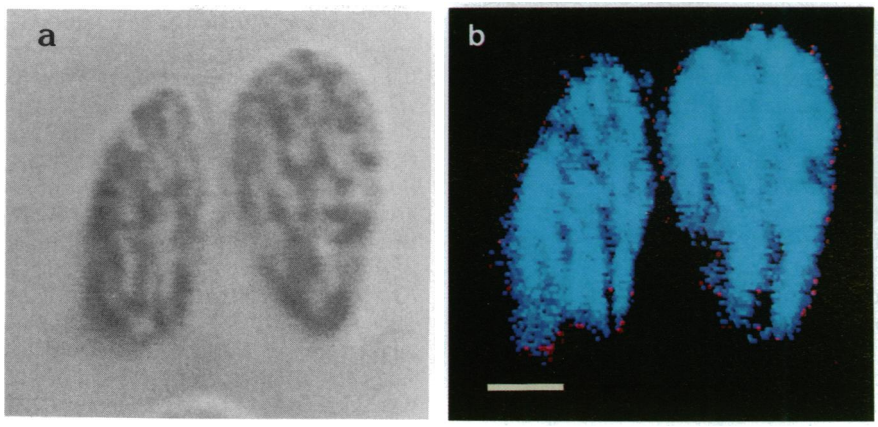

FIG. 2. (*a*) Nonpolarized image of two edge-aligned chloroplasts; (*b*) LD image $I_H - I_V/I_H + I_V$; horizontal polarization (H) is defined parallel to the bottom edge of this figure. The largest positive value of the dichroic ratio measured in this LD image was 0.11. Color scale for the dichroic values is shown. For this image the lightest shade of blue in the color bar corresponds to a dichroic ratio of 0.11. (Bar = 2 μm.)

laser (Spectra-Physics model 375B) pumped by an argon-ion laser (Lexel model 98).

Local CD spectra of chloroplasts were recorded with a Jasco 40-C spectropolarimeter, specially adapted and modified to be used with the microscope. The top pinhole of the microscope determines the area from which light is collected by the photomultiplier tube. The CD spectra magnitude, in degrees ($\theta°_\lambda$), is related to the values of the dichroic ratios obtained in the CD images by the relation: $\theta°_\lambda = 6600\,[(I_R - I_L)/(I_R + I_L)]$, where I_R and I_L indicate the intensity of right and left circularly polarized light, respectively.

RESULTS

Test of Alignment. Correct alignment of the chloroplasts was verified by using LD images. These can be interpreted similarly to the macroscopic LD spectra of suspensions of chloroplasts (1, 2) on the basis of strength and orientation of the absorbing dipoles relative to the plane of the membranes. Fig. 2 *a* and *b* shows the regular nonpolarized and LD image of two "edge"-aligned chloroplasts obtained at 435 nm (2, 12). In these figures the thylakoid membranes, containing in-plane oriented dipoles, lie parallel to the vertical polarization in the laboratory frame. Thus, the polarization parallel to the membranes (vertical) is absorbed more than the perpendicular (horizontal) polarization, and the difference $I_H - I_V$, which determines the sign of the dichroic ratio, is positive. In the pseudocolor scale used here and in Fig. 3, positive values of this dichroic ratio are encoded in blue, whereas negative values are depicted in red; zero LD values are encoded in black. Notice that the thylakoid membranes running parallel to one another are resolved as closely appressed, lighter blue strips and reveal a correct alignment. This orientation gives the largest average LD value, i.e., the whole chloroplast shows, for the most part, one sign of LD over the whole imaged shape. Fig. 3 *a* and *b* represent the regular image and the LD image at 435 nm of a "face"-aligned chloroplast, respectively. In this orientation, the thylakoid membranes are perpendicular to the incident light. Because within the coherence area of the light (the coherence area of the light is proportional to the square of the wavelength and, therefore, is about the minimum resolvable area in the microscope), the chromophores are randomly oriented in the plane of the membranes, and the preferential absorption averages to zero. At the edges of the chloroplast, however, the curvature of the membrane introduces preferential absorption by dipoles aligned tangentially to the outer boundary of the chloroplast. This gives rise to four circular sectors of alternating LD sign, surrounding a region of zero LD values at the center of the chloroplast. The color pattern shows correct face-alignment of the chloroplast.

Low-Resolution CD Images. Low-resolution CD images of both edge- and face-aligned chloroplasts were taken to de-

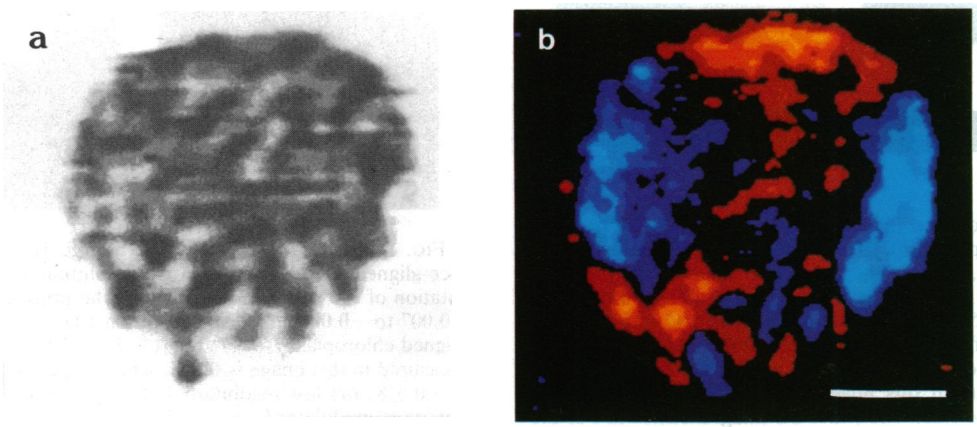

FIG. 3. (*a*) Nonpolarized image of a face-aligned chloroplast; (*b*) LD image $I_H - I_V/I_H + I_V$, of the same chloroplast at 435 nm. The largest positive value of the dichroic ratio was 0.014, and the largest negative value −0.0094. For this image the lightest shade of blue in the color bar corresponds to a dichroic ratio of 0.014, whereas the lightest shade of yellow corresponds to −0.014. (Bar = 2 μm.)

termine if any correlation existed between the *average* CD signal emerging from the chloroplast as a whole and that recorded in macroscopic measurements of chloroplast suspensions. The CD images, obtained at 670 nm (1, 4), were also tested for LD contributions by comparing the CD images taken with orthogonal positions of the polarizer–modulator unit with a corresponding pair of LD images. CD signals are invariant to rotation of the polarizer–modulator unit, whereas LD signals invert sign upon 90° rotation of the polarizer–modulator (7).

Fig. 4*a* shows the low-resolution CD image of an edge-aligned chloroplast. Positive values of the dichroic ratio are encoded in white and light gray, zero values in gray, and negative values in dark gray and black. The spatial distribution of CD values is unchanged upon 90° rotation of the polarizer–modulator (4*b*), whereas the same operation inverts the sign of the LD image (Fig. 4 *c* and *d*). Thus, the CD signals have no LD contributions. On the other hand, these images reveal unexpected, anomalous features of the microscopic CD. Both positive and negative CD signals can be observed at 670 nm, originating from different regions of the chloroplast. Local dichroic values are much higher than anticipated based on macroscopic measurements (see Fig. 4 caption). The positive and negative CD almost cancel, although inspection of a large number of images shows that the negative CD signals prevail after integrating the signals over the entire chloroplast. This result is consistent with macroscopic data obtained in suspensions of edge-aligned chloroplasts that exhibit a negative band peaking at 670 nm (L.F., C.B., and G.G., unpublished data).

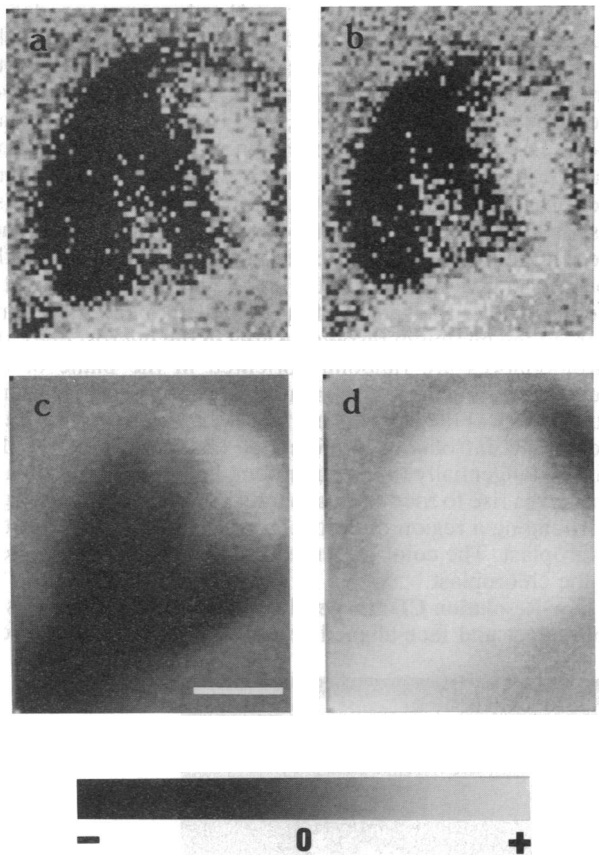

FIG. 4. Low-resolution (*a*) CD image, $I_R - I_L/I_R + I_L$ of an edge-aligned chloroplast; (*b*) CD image upon 90° rotation of the polarizer–modulator, $I_R - I_L/I_R + I_L$. The range of CD values is +0.003 to −0.0048; (*c*) LD image, $I_{45} - I_{-45}/I_{45} + I_{-45}$, of the same chloroplast. The +45 linear polarization is defined as a 45° counterclockwise rotation of the horizontal polarization. (*d*) LD image upon 90° rotation of the polarizer–modulator, $I_{-45} - I_{45}/I_{45} + I_{-45}$. The largest positive value in this image is 0.028. Gray scale for the dichroic values of the black and white CD images is shown. For the CD and LD images in this figure white color corresponds to a dichroic ratio of 0.0048 and 0.028, respectively, whereas black corresponds to a dichroic ratio of −0.0048 and −0.028. All four ratios were recorded at 670 nm. (Bar = 2 μm.)

CD images of face-aligned chloroplasts (Fig. 5 *a* and *b*) also are unchanged on 90° rotation of polarizer–modulator, whereas the LD image (Fig. 5*c*) inverts sign (5*d*). The CD image displays similar characteristics to those of Fig. 4 *a* and *b*; very large local positive and negative CD values are present. However, positive CD values dominate the face-aligned images in accordance with macroscopic data from suspensions of face-aligned chloroplasts (4). These results were corroborated by CD spectra of gel-trapped suspensions of edge- or face-aligned chloroplasts that revealed no major LD contributions (unpublished data, G.G.).

High-Resolution CD Images and Local CD Spectra. High-resolution CD images were generated to better resolve the anomalous features of the large signals detected at low resolution. Fig. 6 shows a high-resolution CD image of an edge-aligned chloroplast and its corresponding LD image, while Fig. 7 shows high-resolution CD and LD images of a face-aligned chloroplast. The LD images show correct alignment and absence of correlation with the CD images. The CD signals seem to originate from "islands" or domains of very strong ellipticity. These islands appear even more clearly in Fig. 8, depicting the CD image of another edge-aligned chloroplast taken with a longer integration time. The diameters of these islands are between 0.3 and 0.6 μm, in

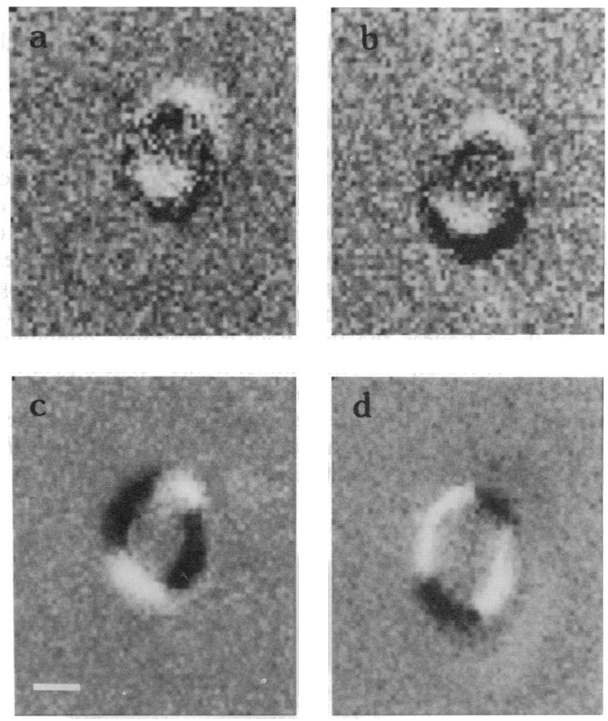

FIG. 5. (*a*) Low-resolution CD image $I_R - I_L/I_R + I_L$ of a face-aligned chloroplast; (*b*) low-resolution CD image upon 90° rotation of the polarizer–modulator. The range of the CD values is +0.007 to −0.0049; (*c*) low-resolution LD image of the same face-aligned chloroplast $I_{45} - I_{-45}/I_{45} + I_{-45}$. The largest positive value measured in this image is 0.018, whereas the largest negative value is −0.018; (*d*) low-resolution LD image upon 90° rotation of the polarizer–modulator $I_{-45} - I_{45}/I_{45} + I_{-45}$. For the CD and LD images in this figure, white corresponds to a dichroic ratio of 0.007 and 0.018, respectively, and black corresponds to a dichroic ratio of −0.007 and −0.018, respectively. All four images were recorded at 670 nm. (Bar = 2 μm.)

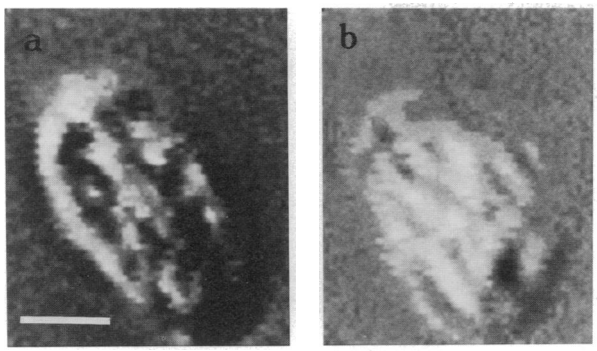

FIG. 6. (*a*) High-resolution CD image $I_R - I_L/I_R + I_L$ of an edge-aligned chloroplast. The range of CD values is +0.001 to −0.009; (*b*) high-resolution LD image $I_{45} - I_{-45}/I_{45} + I_{-45}$ of the same chloroplast. Maximum dichroic value = +0.024. For the CD and LD images in this figure, white corresponds to a dichroic ratio of 0.009 and 0.024, respectively, and black corresponds to a dichroic ratio of −0.009 and −0.024. (Bar = 2 μm.)

agreement with the size of 0.4 μm of the chiral domains estimated from circular intensity differential scattering measurements on suspensions of granal chloroplasts (5).

The identification of the chiral domains seen in CD images of chloroplasts as the chirally organized macrodomains in the thylakoid grana is also strongly supported by CD spectra recorded on individual islands by using the microscope. Local CD spectra showed large single sign bands (Fig. 9 *a* and *b*) unlike the spectra of macroscopic CD of pigment–protein complexes or of chloroplast suspensions (2); surprisingly, adding the spectra from these domains yielded a much smaller spectrum (Fig. 9*c*) that resembled macroscopic measurements and had the same excitonic-like characteristics described in the literature. The maximum amplitude of the local CD bands recorded on all the chloroplasts examined ranged from 150 to 300 m°. This value corresponds to a CD signal from a suspension of thylakoids with an OD of 1–2 at 678 nm (3).

DISCUSSION AND CONCLUSIONS

The mosaic-like distribution of the microscopic CD of chloroplasts reveals the presence of large chiral domains in the thylakoid membranes (0.4–0.6 μm), which show ellipticities much larger than those measured in solutions of nonaggregated chromophores. Moreover, the circular intensity differential scattering patterns obtained from suspensions of chloroplasts (5) and the theory of scattering of chiral particles (13)

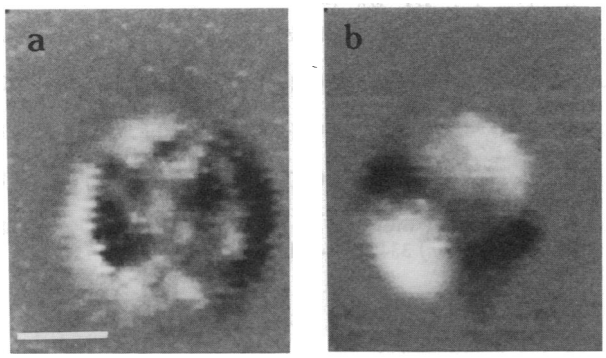

FIG. 7. (*a*) High-resolution CD image $I_R - I_L/I_R + I_L$ of a face-aligned chloroplast. The range of CD values is +0.016 to −0.008. (*b*) High-resolution LD image $I_{45} - I_{-45}/I_{45} + I_{-45}$ of the same chloroplast. The range of the LD values is +0.078 to −0.04. For the CD and LD images in this figure, white corresponds to a dichroic ratio of 0.016 and 0.078, respectively, and black corresponds to a dichroic ratio of −0.016 and −0.078. (Bar = 2 μm.)

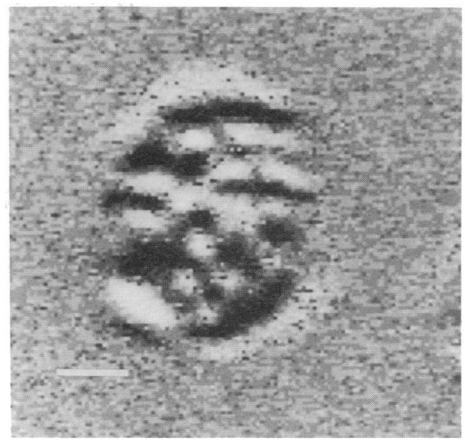

FIG. 8. High-resolution CD image of an edge-aligned chloroplast taken with a longer integration time than that used in Figs. 6 and 7. (Bar = 2 μm.)

are consistent with the presence of long-range chiral domains in the thylakoids.

In addition, we found that the local CD spectra of these domains do not resemble the spectra of macroscopic measurements performed on oriented suspensions of chloroplasts. Their wavelength dependence, displaying a large single broad band, closely resembles the *psi-type* spectra described for large chiral aggregates (14). The theory of *psi-type* circular dichroism (14–16) has shown that these properties are characteristic of long-range chiral structures with dimensions comparable to the wavelength of light. These large chiral domains permit the efficient delocalization of the excitation throughout the entire aggregate, and their presence can be advantageous in the efficient photosynthetic utilization of the light energy (2, 17).

There is substantial evidence linking these domains to the chlorophyll a/b light harvesting pigment–protein complex of photosystem II in granal chloroplasts (4). Recent experiments have shown that the light harvesting complex II is necessary to observe anomalous CD signals in chloroplasts (3). UV CD, visible CD, and circular intensity differential scattering studies indicate that the macro-array assembly is governed by electrostatic interactions among the pigment–protein complexes (J. Kieleczawa, G.G., L.F., C.B., J. C. Sutherland, and G. Hind, unpublished data). In view of these observations and the results presented here, we propose that these domains correspond to clusters of light harvesting complex II in the grana whose chirality results from some type of asymmetric adhesion.

Finally, we propose that the spectra obtained in the macroscopic measurements result from the superposition of signals originating in different regions of each chloroplast. The existence of nonuniform optical activity within the chloroplast, displaying characteristic wavelength dependence and sign, can have several interpretations. Possibly these signals relate to two different molecular entities or, more likely, the signals might represent different orientations of a single type of chiral domain. Regardless of their origin, the two bands of opposite sign described in macroscopic CD spectra of chloroplasts at 680 nm and traditionally attributed to the excitonic nature of the chromophore interactions may have an alternative explanation: they might result from the overlapping and substantial cancellation of different signals of large magnitude, coming from spatially distinct regions inside chloroplasts.

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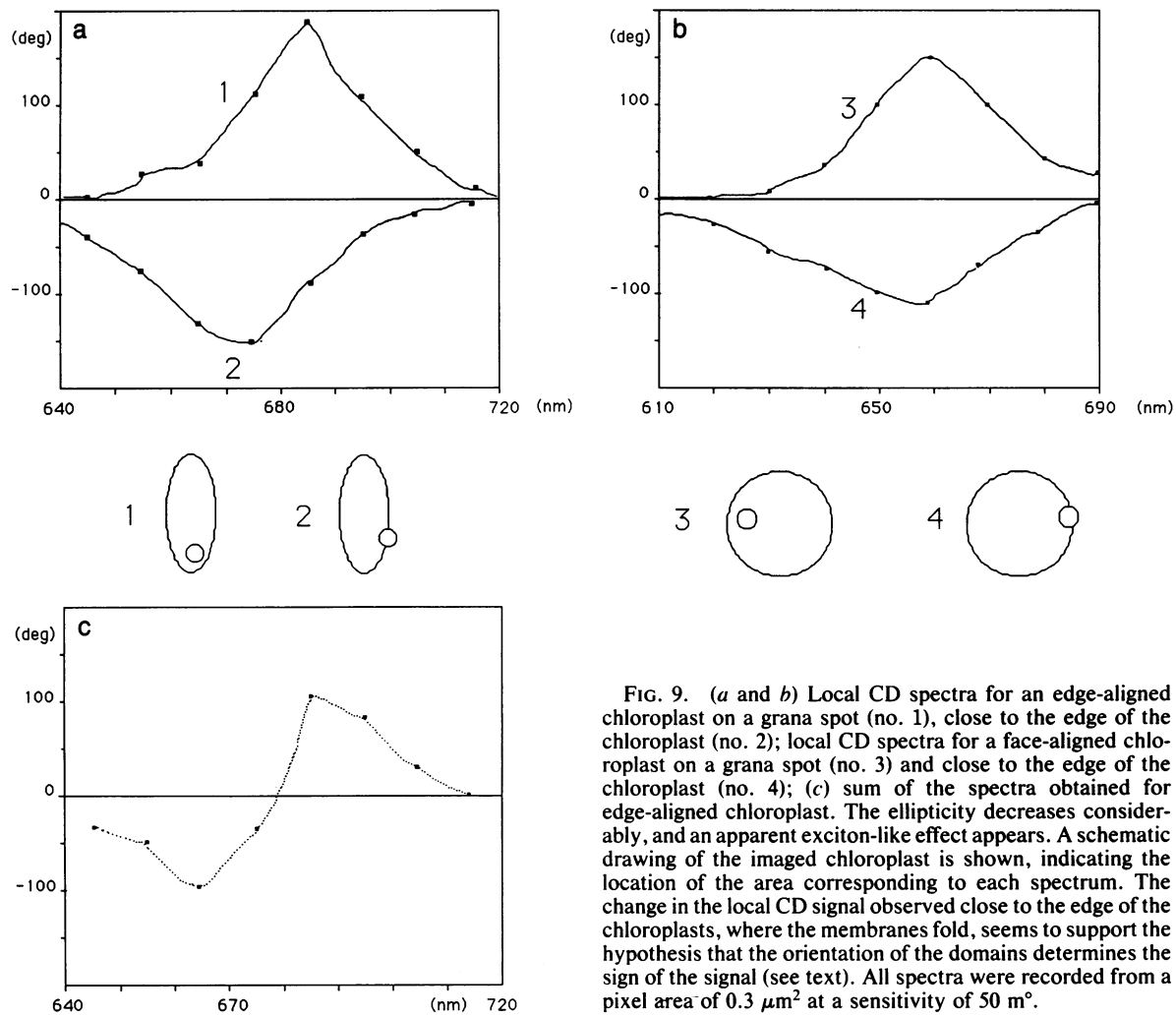

FIG. 9. (*a* and *b*) Local CD spectra for an edge-aligned chloroplast on a grana spot (no. 1), close to the edge of the chloroplast (no. 2); local CD spectra for a face-aligned chloroplast on a grana spot (no. 3) and close to the edge of the chloroplast (no. 4); (*c*) sum of the spectra obtained for edge-aligned chloroplast. The ellipticity decreases considerably, and an apparent exciton-like effect appears. A schematic drawing of the imaged chloroplast is shown, indicating the location of the area corresponding to each spectrum. The change in the local CD signal observed close to the edge of the chloroplasts, where the membranes fold, seems to support the hypothesis that the orientation of the domains determines the sign of the signal (see text). All spectra were recorded from a pixel area of 0.3 μm^2 at a sensitivity of 50 m°.

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