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Orientation of pigments and pigment-protein complexes in the diatom *Cylindrotheca fusiformis*. A linear-dichroism study

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The orientation of pigments and pigment-protein complexes of the marine diatom *Cylindrotheca fusiformis* was studied by linear dichroism at 77 K. The technique of polyacrylamide gel squeezing was used to orient the diatom intact cells, their isolated thylakoid membranes and the three pigment-protein complexes: chlorophyll *ac*-fucoxanthin, chlorophyll *ac* and PS I complexes. The data indicate that specific orientation of various pigments exists at all structure levels. Tentative assignments of various features of the linear-dichroism spectra to the major photosynthetic pigments are presented. The orientation of the three pigment-protein complexes with respect to the thylakoid membrane plane and the major axis of the cell is also discussed.

Introduction

The pigments of photosynthetic membranes are bound in discrete pigment-protein complexes [1]. Some of these complexes contain photochemical reaction centers, while others serve only as light-harvesting antenna. Since the membranes are functionally organized to give high efficiency of excitation energy transfer, and the mutual orientation of the pigments is a major factor making the optimization of the transfer [2], an analysis of the arrangement of the pigments is thus an important step toward our understanding of the energy-transfer mechanisms.

Linear dichroism is a valuable tool to study the orientation of pigments. Information has been obtained in this way in the photosynthetic membranes as well as the isolated pigment-protein complexes of various organisms including higher plants [3–5], alga [6,7] and bacteria [8,9].

Among the algal classes, the nonchlorophyte algae, particularly the diatom, play a major and important role in global carbon fixation [10]. However, data on the structural organization of its pigments are still not available. In the past few years, procedures to isolate the light-harvesting antenna complexes as well as the Photosystem I complex from diatom had been developed [11–13]. It provides the first opportunity to investigate the pigment organization in these complexes of the algal group.

In the present report, we study low-temperature linear dichroism on intact cells of the marine diatom *Cylindrotheca fusiformis*, their isolated thylakoid membranes and the three pigment-protein complexes. We believe that this is the first

Abbreviations: Chl, chlorophyll; LD, linear dichroism; PS, Photosystem; P-700, reaction center of Photosystem I.

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report concerning the pigment orientation of the algal group containing mainly Chl *a*, Chl *c* and carotenoid fucoxanthin. The algae also provide a system which demonstrates the relationship of the pigment organization among various levels of structure from intact cells to pigment-protein complexes.

Materials and Methods

Plant material. The marine diatom *C. fusiformis* was grown at 20 °C in an artificial seawater medium [14] under a 12/12 h day/night cycle at 30 $\mu E \cdot m^{-2} \cdot s^{-1}$. Cultures were bubbled with air and harvested in late log growth phase by centrifugation at $17000 \times g$ for 2 min.

Preparation of thylakoid membranes. Pelleted cells were resuspended in a homogenation medium containing 100 mM Tris-borate (pH 8) and 0.4 M sucrose. They were disrupted by sonication (Brnason sonifier 200, setting 7) for 15 s followed by centrifugation at $1900 \times g$ for 3 min to remove unbroken cells and debris, which were resuspended and subjected to sonication again. More than 70% of the cells were disrupted after 4 times of repeated sonication and centrifugation. The thylakoid membranes were then pelleted at $180000 \times g$ for 30 min, washed twice and resuspended in 100 mM Tris-borate (pH 8). This crude thylakoid preparation was purified by a discontinuous sucrose gradient essentially as described by Owens and Wold [11]. Centrifugation was done for 90 min at $180000 \times g$ on a Hitachi RPV50T rotor. The purified thylakoid membranes collected from the sucrose gradient were pelleted and resuspended in 100 mM Tris-borate (pH 8).

Isolation of pigment-protein complexes. The isolation was accomplished using the procedures described in Ref. 11. The thylakoid membranes wre solubilized in 1% Triton X-100 (Triton/Chl *a* = 25 : 1). The pigment-protein complexes collected from sucrose gradient were concentrated by ultrafiltration (Amicon) against 100 mM Tris-borate (pH 8) containing 0.1% Triton X-100. Concentrations of Chl *a* and *c* were determined in 90% (v/v) acetone extracts using the equation of Jeffrey and Humphrey [15]. The polypeptide composition of the isolated pigment-protein complexes was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Lee et al. [16].

Linear dichroism measurements. To orient particles of sizes ranging from intact cell to pigment-protein complex, the technique of polyacrylamide gel squeezing was chosen [17]. It has been shown that the technique, besides being able to orient particles of very different sizes, has several other advantages [18]: minimal scattering distortion, feasibility of low-temperature spectroscopy and no degradation of biological materials. The gel mixture contained 50% (v/v) glycerol, 10% (w/v) acrylamide, 0.26% (w/v) *N,N'*-methylenebisacrylamide, 0.03% (v/v) *N,N,N',N'*-tetramethylethyldiamine and 0.05% (w/v) freshly prepared ammonium persulfate in distilled water. Alignment of the samples was achieved by uniaxially squeezing the gel to 65% of its original length in a 1-cm cuvette. Linear dichroism is defined as the difference in absorption between light that is linearly polarized parallel ($A_{\parallel}$) and perpendicular ($A_{\perp}$) to the long axis of the oriented object, i.e., that axis orientating perpendicualrly to the direction of squeezing as assumed in Ref. 9. Absorption spectra $A_{\parallel}$ and $A_{\perp}$ were measured separately using a Hitachi double-wavelength/double-beam spectrophotometer (Model 557). Linear dichroism ($A_{\parallel} - A_{\perp}$) was then calculated by a microcomputer connected to the spectrophotometer.

Results and Discussion

Sucrose gradient fractionation of the Triton X-100 solubilized thylakoid membranes resulted in five pigmented bands essentially the same as described in Ref. 11. The absorption spectra (see below) and the pigment composition of the five bands were also similar to those in Ref. 11. We therefore suggest the following identifications from top to bottom of the density gradient: band 1 (yellow-green), free pigments; band 2 (green), Chl *a/c* complex; band 3 (brown-orange), Chl *a/c*-fucoxanthin complex; band 4 (brown), partially dissociated thylakoid membranes; band 5 (dark green), PS I complex.

The thylakoid membrane had a Chl *a* : *c* ratio of about 4.2 and the ratio of Chl *a* to P-700 was 780. The Chl *a/c* complex, which was suggested to be a light-harvesting complex [11], had a slightly

higher Chl a : c ratio (4.6), but contained no detectable P-700. The Chl a/c-fucoxanthin complex, another light-harvesting complex, enriched in Chl c (Chl $a/c = 2.8$) and had no P-700. The PS I complex, on the other hand, had little Chl c (Chl $a/c = 9.1$), but enriched in P-700 (Chl a/P-700 = 180). The protein composition of the thylakoid membranes and the three pigment-protein complexes was analyzed by SDS-polyacrylamide gel electrophoresis as shown in Fig. 1.

The 77 K absorption and LD spectra of: (a) isolated thylakoid membranes, (b) Chl a/c-fucoxanthin ocmplex, (c) Chl a/c complex and (d) PS I complex are presented in Fig. 2. It is worth noting that, in a squeezed gel, the object is oriented with its long axis perpendicular to the direction of squeezing. Thus, in the case of the thylakoid membranes, the LD spectrum analyzes the pigment orientation with respect to the membrane plane, while in the isolated complexes, the spectrum describes the orientation with respect to the largest dimension of the complex itself [19].

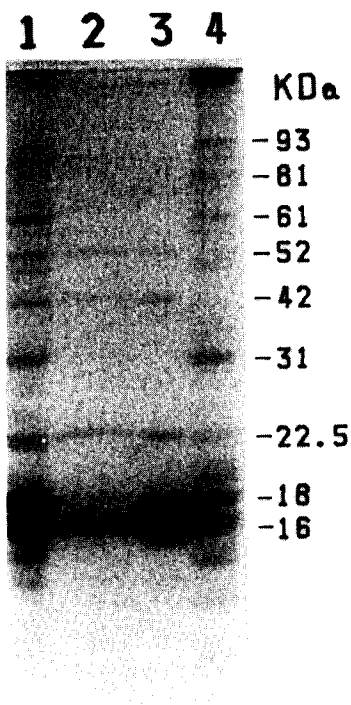

Fig. 1. SDS-polyacrylamide gel electrophoresis of samples used for optical studies; lane 1, thylakoid membranes; lane 2, Chl *ac* complex; lane 3, Chl *ac*-fucoxanthin complex; lane 4, PS I complex.

In the thylakoid membranes (Fig. 2a), the long-wavelength absorption peaks at 670 nm, while the LD maximum is at 674 nm. The LD spectrum has a shoulder at 683 nm, which corresponds to an absorption shoulder around 684 nm. The positive LD in this region indicates that most of the Q_y transitions of Chl a lie close to the membrane plane. In the spectra of the Chl a/c-fucoxanthin complex (Fig. 2b), the main Q_y absorption peak of Chl a and the corresponding LD peak are both located at 668 nm. The major Q_y transitions are nearly parallel with the long axis of the complex as indicated by the positive sign of the LD signal. However, a small dip observed at 684 nm in the LD spectrum suggests the presence of a transition dipole tilted at less than 55° from the direction of squeezing. The Chl *ac* complex (Fig. 2c) has the long-wavelength absorption peak at 667 nm, with a corresponding LD signal at 666 nm. There is an LD shoulder at 677 nm which appears to have no corresponding absorption signal. The negative LD in the region suggests that the major Q_y transitions of Chl a, contrary to those of the Chl *ac*-fucoxanthin complex, are tilted away from the long axis of the complex. In the spectra of the PS I complex (Fig. 2d), the major absorption peak appears at 671 nm, which corresponds to an LD shoulder at the same wavelength. The major LD peak is located at 677 nm, which correlates to a shoulder at 678 nm in the absorption spectrum. In addition, there is a small LD shoulder at 697 nm which has no absorption counterpart.

In the spectral range 600–640 nm of the thylakoid membranes, two absorption bands are observed. One around 632 nm can be assigned to Chl c. A corresponding LD signal appears as a shoulder at 636 nm, indicating that the Q_y transition of Chl c is also oriented close to the membrane plane. The other absorption peak is around 617 nm. We tentatively asisgn it to the vibrational $Q_{y\,0\text{-}1}$ transition of Chl a, which has a corresponding LD peak at 615 nm. The absorption spectrum of the Chl *ac*-fucoxanthin complex in this range is similar to that of thylakoid membranes with the two bands located at 631 nm and 615 nm, respectively. The corresponding LD signals appear as a peak at 635 nm and a shoulder at

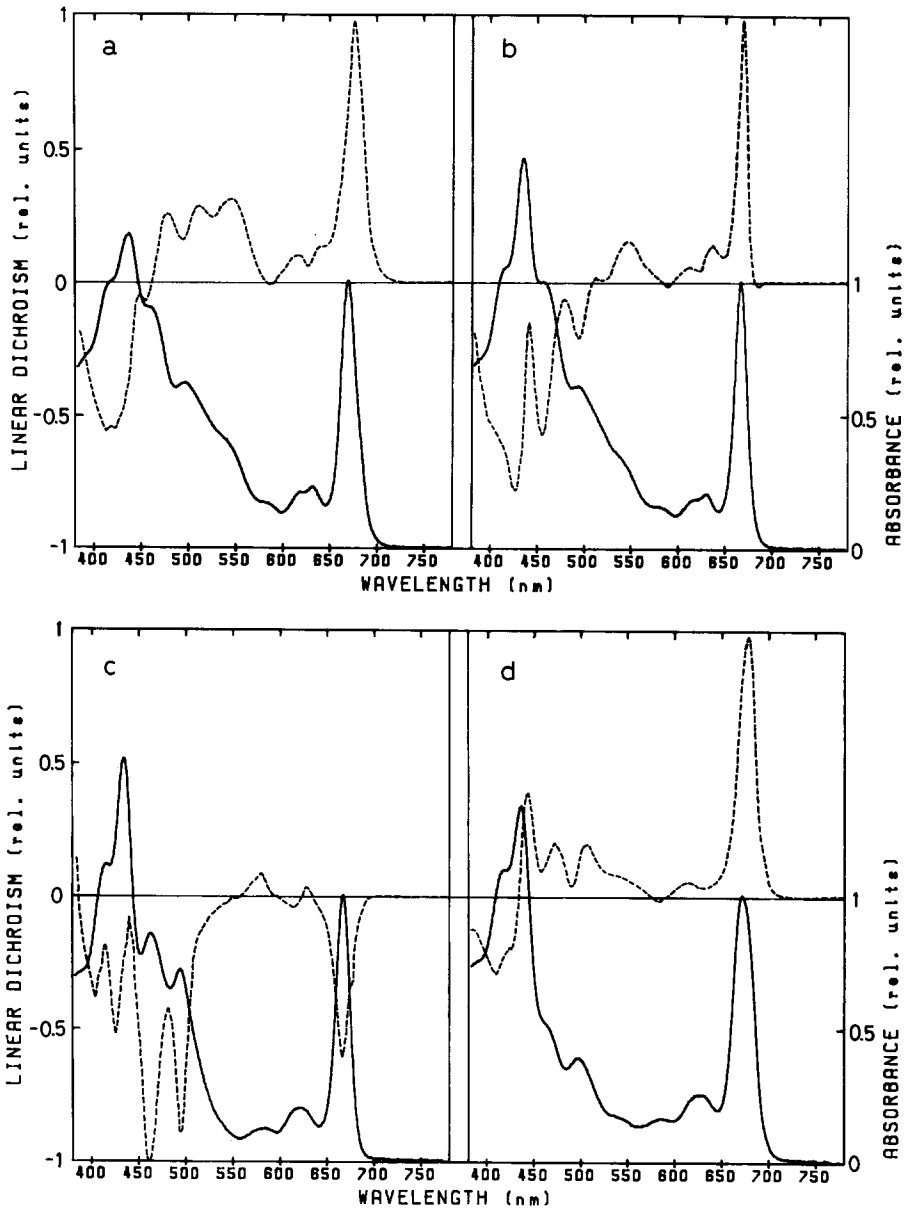

Fig. 2. The 77 K absorption (———) and linear dichroism (- - - - -) spectra of (a) thylakoid membranes, (b) Chl *ac*-fucoxanthin complex, (c) Chl *ac* complex and (d) PS I complex. Spectra were determined as described in Materials and Methods.

611 nm. The two absorption bands are not well resolved in the spectrum of the Chl *ac* complex. However, the corresponding LD spectrum shows a positive peak at 628 nm and a negative peak at 615 nm. The positive peak can be assigned to the Q_y transition of Chl *c* which differs from that of Chl *a*, having a parallel orientation with respect to the major axis of the complex. The negative peak can be attributed to the $Q_{y\,0-1}$ transition of Chl *a*, whose orientation is consistent with that of the major Q_y transition and thus reinforces the previous assignment of this band. The absorption band of the PS I complex in this spectral range is also not well resolved. Nevertheless, the LD spectrum appears to have only one peak at 614 nm due to the $Q_{y\,0-1}$ transition of Chl *a*. The absence of LD

signal corresponding to Chl *c* is in accordance with the low Chl *c* content of this complex.

In all the spectra shown in Fig. 2, there is an absorption band around 583 nm with a corresponding LD signal at the same wavelength. The LD signals are negative in the thylakoid membranes, the Chl *ac*-fucoxanthin complex and the PS I complex, but is positive in the Chl *ac* complex. We assign this band to the Q_x transition of Chl *a*, which appears to orient perpendicularly to the Q_y transition. Similar spectral features corresponding to the Q_x transition have been observed on various pigment-protein complexes isolated from higher plants and alga [4,5,7].

An absorption band at 544 nm is seen in the spectra of the thylakoid membranes and the Chl *ac*-fucoxanthin complex, which has a very prominent LD counterpart. The same signals are much smaller in the spectra of the PS I complex and are completely absent in those of the Chl *ac* complex. Judging from the signal wavelength and the pigment composition of each sample, we assign this band to fucoxanthin. The major axis of this molecule is oriented close to the thylakoid membrane plane or the long axis of the Chl *ac*-fucoxanthin complex.

In the spectral range 380–520 nm, highly dichroic pigments are present in all samples. Apart from their vertical positions, all the LD spectra have troughs around 493 nm, 457 nm and 424 nm. In addition, there are peaks around 508 nm, 476 nm and 442 nm (except for the Chl *ac* complex where the first peak is absent). Since two functionally different pools of carotenoids have been recognized in the photosystems [20,21], we suggest that the LD signals in this region originate mainly from the two populations of carotenoids with dichroism of opposite signs. The vertical positions of the LD signals are determined by the relative proportion of the two pigment groups present in the samples. The pool which exhibits an orientation perpendicular to the main axis of the complex is dominant in the two light-harvesting complexes, whereas the other pool with parallel orientation is prevailing in PS I complex. Similar spectral features corresponding to carotenoids have been widely observed on many pigment-protein complexes from various organisms [4,5,7,8], suggesting a common structure of these carotenoids.

The LD signal around 410 nm probably is also related to the X-polarized transitions from the Soret region of Chl *a*. In the LD spectra shown in Fig. 2, only that of Chl *ac* complex, whose X-polarized transition of Chl *a*, contrary to other two complexes, have a parallel orientation with respect to the major axis of the complex, is raised toward positive.

It is worth noting that the LD spectrum of the Chl *a*/*c*-fucoxanthin complex resembles that of PS II complex isolated from the alga *Chlamydomonas reinhardtii* [7]. Along with the presence of some high molecular-mass polypeptides in the complex (52 kDa, 42 kDa and 22.5 kDa, besides 18 kDa and 16 kDa of LHC, see Fig. 1), it seems to suggest that the complex may actually contain PS II reaction centers, although no PS II photochemical activity has been detected. Another interesting feature of this complex is that, in the LD spectrum, the peak at 668 nm disappears upon incubating the complex casted in polyacrylamide gel at room temperature for more than 15 min. A new negative peak appears at 660 nm. A very similar observation has been made on PS I complex and attributed to denaturation [5]. This phenomenon rules out the possibility that Chl *ac* complex is a denatured product of Chl *ac*-fucoxanthin complex, because of the difference in their peak positions (666 nm vs. 660 nm). This is in agreement with Ref. 11. In addition, the LD spectrum of the PS I complex resembles the ones reported for the same particles isolated from higher plant and algae, except that the signals corresponding to the X-polarized transitions, in our spectrum, appear to be more negative [5]. This indicates that the PS I complexes from various organisms have a similar structure. Nevertheless, the LD spectrum of the Chl *a*/*c* complex is completely different from that of the light-harvesting Chl *ab* complex from higher plants [4]. The differences are mainly due to their opposite orientations of the Y- and X-polarized transitions with respect to the major axes of the complexes.

By comparing the LD sepctra shown in Fig. 2, it seems clear that the Chl *ac*-fucoxanthin complex and the PS I complex are oriented with their long axes close to the thylakoid membrane plane. But the orientation of the Chl *ac* complex is not so obvious, because its transition dipoles of Chl *a*

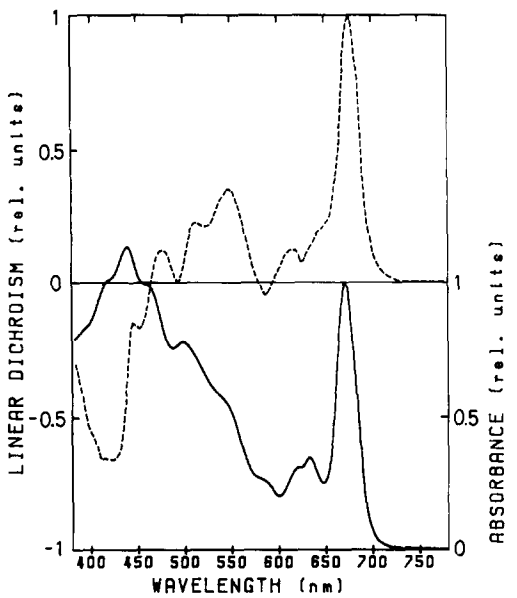

Fig. 3. The 77 K absorption (———) and linear dichroism (- - - - -) spectra of the intact cells of *C. fusiformis*.

and carotenoids cannot match those of thylakoid membranes at the same time. Nevertheless, we favor a parallel relationship between the long axis of the complex and the plane of the thylakoid membranes, since the LD signals of carotenoids appear to be the major features in the spectrum. If this is the case, then the main Q_y transitions of Chl *a* are tilted out of the membrane plane, which, though not very common, has been observed in the photosystem complexes derived from *Prosthecochloris aestuarii* [8].

The absorption and LD spectra of intact cells are presented in Fig. 3. Under electron microscope, the shape of the cell *C. fusiformis* was found to be rod-like. The nucleus is located at the center of the cell, whereas the two chloroplasts reside on either side of the nucleus [22]. The similarity between the LD spectra of intact cells and the thylakoid membranes indicates that the plane of the membrane runs parallel with the long axis of the cell, i.e., chloroplasts have a fixed orientation with respect to the cell. It also suggests that the organization of pigments in the thylakoid membranes is not altered by the isolation procedures.

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