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LINEAR DICHROISM OF MICROALGAE, DEVELOPING THYLAKOIDS AND ISOLATED PIGMENT-PROTEIN COMPLEXES IN STRETCHED POLY(VINYL ALCOHOL) FILMS AT 77 K

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Summary

A variety of unicellular algae, thylakoids from higher plants in different stages of maturity and isolated pigment-protein complexes were oriented in stretched polyvinyl alcohol films. Low temperature linear dichroism (LD) spectra of *Chlorella pyrenoidosa* and higher plant thylakoids in the films were very similar to those obtained after orientation of similar samples using magnetic or electric fields.

Positive LD bands corresponding to Chl *a* (670) and (682) and negative bands due to Chl *a* (658) and Chl *b* (648) were resolved in spectra of the light harvesting Chl *a*/*b* protein. Chl *b* (648) and Chl *a* (658) and (670) were not seen in the LD spectrum of thylakoids from plants grown in intermittent light, the Chl *b*-less mutant of barley, *Euglena gracilis* or the cyanobacteria, *Phormidium luridum* and *Anacystis nidulans*, but did appear upon chloroplast maturation in Romaine lettuce and during the greening of etiolated and intermittent light plants. The highly oriented long wavelength Chl *a* (682) in the light-harvesting complex may represent residual PS II whose peak dichroism is centered at 681 nm. The PS I preparation had a Chl *a*/*b* ratio of approx. 6 and the LD spectrum was positive with a maximum at 690—694 nm and a band of lower amplitude at 652 nm. The minor LD band was not observed in PS I preparations from organisms that lack Chl *b* such as the cyanobacteria, intermittent light plants and the Chl *b*-less mutant of barley. We suggest that the 652 nm band is due to Chl *b* molecules associated with the antenna of PS I and are distinct from those on the light harvesting complex whose orientation is different. We also conclude that all the Chl *a* forms are oriented and that the long

Abbreviations: PS I, Photosystem I; PS II, Photosystem II; Chl, chlorophyll; LD, linear dichroism.

geometric axes of the pigment-protein complexes, as deduced from the configuration they assume in the stretched films, are axes that normally lie parallel to the plane of the native thylakoid.

Introduction

A detailed analysis of the molecular architecture of the thylakoids of photosynthetic organisms is essential for a complete understanding of the mechanism of energy transfer and quantum conversion in the photosynthetic units. It has been established that all the Chl on the thylakoid is present in association with protein in the form of discrete pigment-protein complexes [1] and the structure, composition and dynamic interactions of these membrane sub-units have been described in some detail [2,3].

At least 50% of the Chl on the thylakoids of higher plants and algae is present in combination with a Chl *a*/*b* protein (light harvesting complex) which is functional in both light harvesting and cation-induced thylakoid stacking phenomena [2]. Improved methods for membrane disruption and electrophoretic separation of the complexes [4—11] have now led to the resolution of additional complexes in light harvesting and also a complex that is possibly the reaction center of PS II [6,11]. Since the original isolation of CP1 by Thornber et al. [12] there have been many reports of preparations that are derived from PS I. These range in complexity from reaction center preparations that are considerably enriched in *P*-700 and contain few pigment molecules [13] to those that retain the antenna of PS I [14].

The object of the present work was to investigate the orientation of the pigments on the thylakoid. Previous studies on oriented algae, thylakoids and subchloroplast fractions utilizing techniques of linear dichroism and polarization of fluorescence have established that the longer wavelength ($\geqslant$680 nm) Chl *a* forms are oriented with the Q_y transition of the porphyrin mainly in the plane of the membrane [15—22] whereas the corresponding transition for Chl *b* is perpendicular to the plane [17,19,21,23]. As the spectral envelope of the bulk Chl absorption in the red comprises multiple forms of Chl *a* [24—31] it is now necessary to investigate the orientation of the individual forms that contribute to the overall LD signal and relate them to the specific pigment-protein complexes referred to above. Such data might also be helpful in determining the topology of the complexes in the thylakoid itself.

To conduct LD experiments it is necessary to align the specimens of interest with relation to the polarized measuring beam and for cells and thylakoids orientation using a magnetic field is the most convenient technique [16]. Unfortunately small pigment-protein complexes cannot be magneto-oriented [20] but have been aligned in electric fields [20,32] and in flow gradients [33]. More recently, a number of investigators have studied the optical properties of pigment-protein complexes and reaction center preparations from photosynthetic bacteria in gelatin [34] or poly(vinyl alcohol) [35] films that have been stretched to induce orientation. It has been shown that such preparations in stretched films are not physiologically impaired, at least to the extent that they retain photochemical activity in primary quantum conversion.

This report describes the LD of preparations from various algae, developing systems and pigment-protein complexes in mechanically stretched poly(vinyl alcohol) films. The LD spectra were acquired at the temperature of liquid nitrogen to increase spectral resolution. The data show that most likely all the Chl *a* forms are oriented, including those with absorption maxima shorter than 680 nm, and that two populations of Chl *b* are apparent; one is associated with the light harvesting complex as expected and one, which assumes a different orientation, is associated with the antenna of PS I.

Methods

Chlorella pyrenoidosa (UTEX 395), *Anacystis nidulans* (UTEX 625), and *Phormidium luridum* (UTEX 426) were obtained from the University of Texas Culture Collection of Algae, Austin, Texas and grown autotrophically as described previously [36]. Photoheterotrophically grown *Euglena gracilis* was provided by Dr. A.W. Holowinsky of Brown University. Seeds of the wild type and Chl *b*-less mutant of barley were obtained from Dr. K. Miller of Harvard University, cucumber strian Beit Alpha MR from FMC Co. Modesto, CA and peas, Progress number 9 from W. Atlee Burpee Co., Warminster, PA. The higher plants were grown in a controlled environment chamber and intermittent light plants were grown by exposing 7-day-old etiolated plants to a 24-h regime of 2 h light, 2 min darkness. Romaine lettuce was obtained from a local market.

Thylakoids were isolated from the higher plant tissues as described previously [37] and from *Phormidium luridum* by protoplast lysis [38]. Pigment protein complexes were isolated from EDTA-washed thylakoids according to the procedure of Burke et al. [39]. The membranes were solubilized in 0.5% Triton X-100 and then subjected to isopycnic density gradient ultracentrifugation. The banding pattern of the fractions in the gradient was identical to that reported by Burke et al. [39]. The light harvesting complex was recovered after aggregation using 10 mM $MgCl_2$. The fraction immediately below the light harvesting complex in the gradient, which is primarily PS II, and the pellet, which is the PS I reaction center and its antenna, were used without further treatment.

Linear dichroism measurements were made on algae, thylakoids and pigment-protein complexes after orientation of the specimens in poly(vinyl alcohol) films by mechanical stretching. The samples in their respective isolation media were mixed with poly(vinyl alcohol) (20% w/v aqueous, final concentration) and films were prepared following the procedure developed by Bolt and Sauer [35]. The films were stretched mechanically to a stretch ratio of 3 or greater. Stretching of the films induces orientation such that the long axis of the biological specimens lies parallel to the stretch axis of the polymer in the film.

Spectroscopic measurements were made on the stretched poly(vinyl alcohol) films using instrumentation described previously [40] and modified as follows. A single monochromatic measuring beam of 3 nm width at half height was modulated at 1 kHz using a tuning fork chopper (Model L-40, Bulova Watch Co., Flushing, NY) that was positioned at the entrance slit of the monochromator. Immediately prior to traversing the specimen the modulated beam was vertically polarized and then passed through a photoelastic modulator (PEM-3,

Morvue Electronic Systems, Newberg, OR). The drive voltage of the PEM was fixed to produce half-wave retardation at 680 nm. This further modulated the measuring beam to give alternatively vertically and horizontally polarized light at 100 kHz. The transmitted light was detected using a 2 inch end-on photomultiplier (EMI 9558B). The linear dichroism signal (ΔA) was obtained by demodulation of the 100 kHz component of the waveform using a lock-in amplifier (Princeton Applied Research, Model 126) to give $A_{\parallel} - A_{\perp}$ directly. Recovery of the 1 kHz component of the signal using a second lock-in amplifier gave the absolute transmission of the sample. Both LD and transmission spectra were obtained simultaneously by scanning the monochromator and were displayed on separate *X-Y* recorders. Spectra were obtained at room temperature and close to that of liquid nitrogen using a low temperature assembly similar to that described by Estabrook [41].

In some experiments algae and thylakoid preparations were oriented in a magnetic field of 18 kG and then trapped by freezing in liquid nitrogen as described by Vermeglio et al. [19].

Results

Prior to investigating the LD of pigment-protein complexes in poly(vinyl alcohol) films it was necessary to establish that the poly(vinyl alcohol) and the technique of film preparation did not significantly alter the LD of the pigments.

Fig. 1 (top left panel) shows 77 K absorption and LD spectra of pea thylakoids in stretched poly(vinyl alcohol) films. The LD spectrum is virtually identical to those that have been obtained upon orientation of thylakoids using either magnetic [17] or electric fields [20] showing that the membranes align with the stretch axis of the polymer as expected, and that poly(vinyl alcohol) and the preparation of the film does not modify the pigment orientation on the native membrane. The resolution at the lower temperature is greater due to contraction of the absorption bands and the LD spectrum was identical to that following magneto-orientation and trapping in liquid nitrogen (not shown). The LD spectrum is characterized by a large positive signal at 682 nm with long and short wavelength asymmetries, and negative peaks at 648 and 657 nm. Previous investigators have concluded from such spectra that the longer wavelength Chl *a* forms are oriented with the Q_y transition of the porphyrin in the plane of the membrane [17,18]. The negative linear dichroism at 648 nm has been assigned to Chl *b* whose red transition moments are oriented outside the membrane plane [17,19,21,23].

Fig. 1 also shows that whole cells may be oriented in the poly(vinyl alcohol) stretched films. The top right panel illustrates the 77 K LD spectrum for the green alga *Chlorella pyrenoidosa*. This is very similar to that of the higher plant thylakoids (top left panel). The lower panels show spectra for the cyanobacterium *Anacystis nidulans* and the Euglenoid *Euglena gracilis*. The positive LD maximum for *Anacystis* and *Euglena* are considerably red shifted in comparison to the higher plants and *Chlorella*. *Anacystis* also shows an additional very broad band at 645 nm due to the phycobilins. As expected no negative signal at 648 nm was resolved for *Anacystis*, which totally lacks Chl *b*, or *Euglena* that

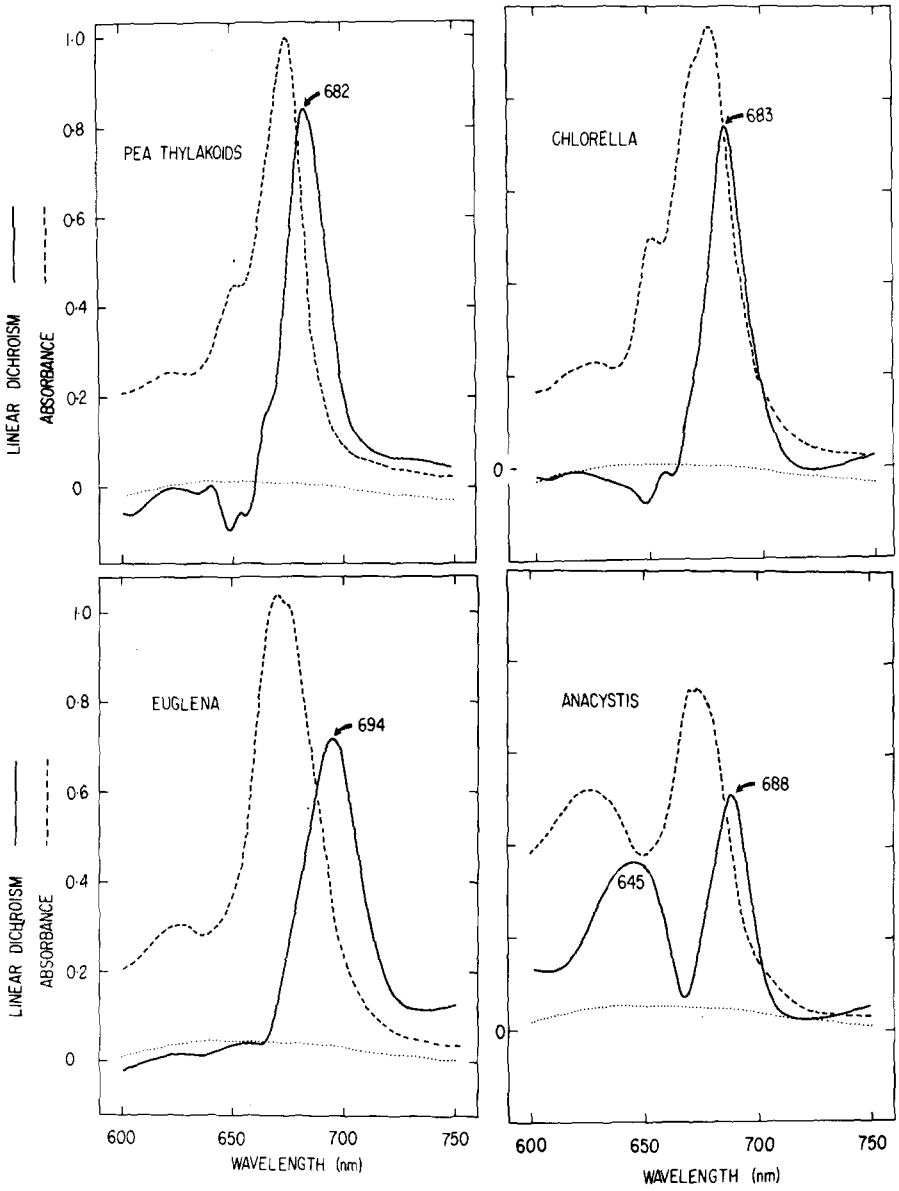

Fig. 1. Linear dichroism spectra (———) and absorption spectra (- - - - - -) of pea thylakoids and microalgae in stretched poly(vinyl alcohol) films at 77 K. The linear dichroism spectra are uncorrected for the base line which is shown.

has a very high Chl *a*/*b* ratio.

Thus microalgae may also be studied by this technique and the orientation of cells results in LD spectra that are similar to those of isolated thylakoids. Small cells such as *Anacystis* cannot be magneto-oriented but they do have the necessary geometric anisotropy to align in the stretched film. No LD signals were observed from unstretched control films.

We have previously reported that the addition of low concentrations of divalent cations such as Mg^{2+} and Ca^{2+} to magneto-oriented thylakoids modi-

fied the LD spectrum [40]. Specifically, we reported an increase in amplitude of the LD signal and introduction of a long wavelength asymmetry. We suggested that the change in LD spectrum was due to the reorientation of a long wavelength component that was concerned with the regulation of the photoreactions. Using the poly(vinyl alcohol) stretched film technique for thylakoid orientation during the course of the present work we were unable to demonstrate a similar cation-induced modification in the LD spectrum. It is possible that the poly(vinyl alcohol) itself or the preparation of the film interferes with the cation-induced physiological changes in the thylakoid. Alternatively, as will be argued below, the modification of the LD spectrum by cations might be due to changes in the polarized selective light scattering of the membranes because such effects would have been minimized in the poly(vinyl alcohol) films.

Changes in LD signal during chloroplast development

The first indication that short wavelength Chl *a* forms were contributing to the overall LD signal were noted upon studying pigment orientation during chloroplast development. Chloroplasts of varying degrees of maturity can be isolated from the inner, middle and outer leaves of a Romaine lettuce shoot. Henriques and Park [42] showed that in such a system the immature chloroplasts from the inner leaves are fully competent in photosynthesis but lack the light harvesting complex. During chloroplast development light harvesting complex is incorporated into the thylakoid and its full complement is present in plastids isolated from the outer leaves.

Fig. 2 shows low temperature LD spectra of thylakoids isolated from leaves in such a developing sequence. For immature chloroplasts from the inner leaves the LD spectrum is characterized by a single broad positive band of maximum wavelength 682 nm and 20 nm width at half height. Thylakoids isolated from the middle leaves have a lower Chl *a*/*b* ratio and the LD spectrum shows a main

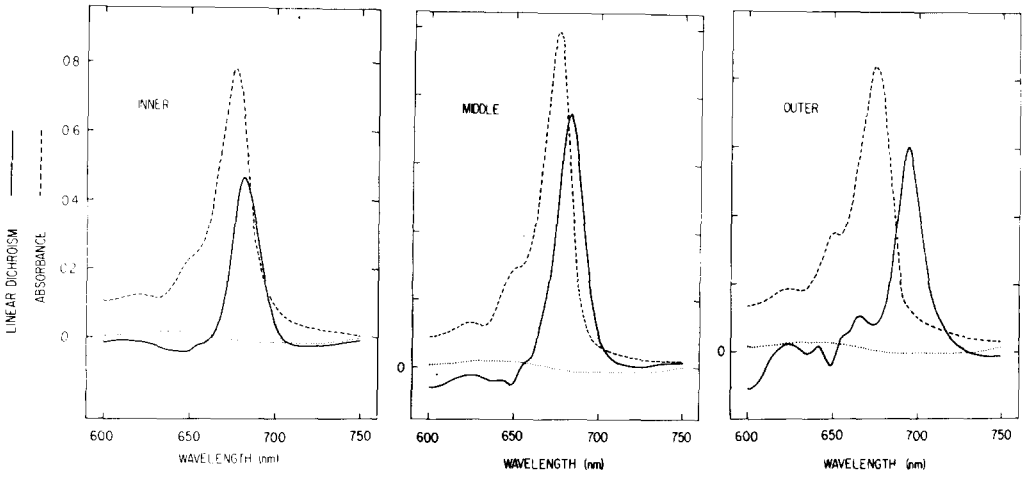

Fig. 2. Low temperaure linear dichroism spectra (———) and absorption spectra (- - - - -) of thylakoids isolated from the inner, middle and outer leaves of a romaine lettuce shoot.

positive peak at about the same wavelength and, as expected, a small negative band at 648 nm due to Chl *b*. In addition the positive signal shows a pronounced short wavelength shoulder. The LD spectrum of the mature thylakoid (right trace) is more complex. The amplitude of the 648 nm band due to Chl *b* is greater and the short wavelength shoulder on the positive band is now resolved as a maximum at 666 nm. Unlike pea thylakoids and *Chlorella* the main positive LD band in romaine lettuce was at 690 nm which permitted resolution of the shorter wavelength pigment. Similar results were found during the greening of etiolated bean seedlings (not shown).

We suggest that as the light harvesting complex is laid down on the thylakoids during chloroplast maturation the Chl constituent on this complex contributes to the dichroism at 648 nm due to Chl *b* and at 666 nm due to a short wavelength Chl *a*. As Chl on the light harvesting complex represents greater than 50% of the total Chl on the mature thylakoid it may be noted that the value $\Delta A/A$ at 666 nm is small in comparison to that at 690 nm and therefore, the light harvesting complex pigments assume a different angle of orientation from the longer wavelength forms that are associated with the two reaction centers and the antenna of PS I.

Linear dichroism of pigment-protein complexes

Further evidence that the pigments of light harvesting complex and shorter wavelength Chl *a* forms are oriented is provided from a study of the isolated thylakoid complexes in stretched films. Room temperature absorption and 77 K LD spectra are shown in Fig. 3 for fractions separated by density gradient centrifugation following solubilization of the EDTA-washed membranes using Triton X-100. The banding pattern of the pigment-protein complexes in the gradient was identical to that described by Burke et al. [39] and the room temperature absorption spectra of the light harvesting complex and PS I fractions are very similar. The fraction immediately below the light harvesting complex in the gradient was construed to be PS II based on the preponderance of polypeptides in the 42 000—48 000 dalton range that have previously been related to PS II activity (Arntzen, C.J., personal communication). As anticipated the light harvesting complex shows dichroism due to Chl *b* at 648 nm and an additional negative band at 658 nm, presumably due to a short wavelength form of Chl *a* of similar orientation. This short wavelength form is also seen in the LD spectrum of the intact thylakoid at approx. 657 nm (Fig. 1). The positive signal is centered at 682 nm with a well defined shoulder at around 668 nm. The 682 nm component could represent a population of long wavelength pigment oriented at a different angle relative to the Chl *a* (668) or might possibly be due to some residual PS II, whose main peak dichroism is also at approx. 681 nm (center panel), that has not been removed from the light harvesting complex by the detergent.

The PS I preparation shows a much longer wavelength maximum in the room temperature absorption spectrum and the low temperature LD spectrum features a main positive peak at 690—694 nm and a peak of lower amplitude at 652 nm. It will be shown below that this small peak is most likely due to a population of Chl *b* molecules associated with the antenna of PS I and that

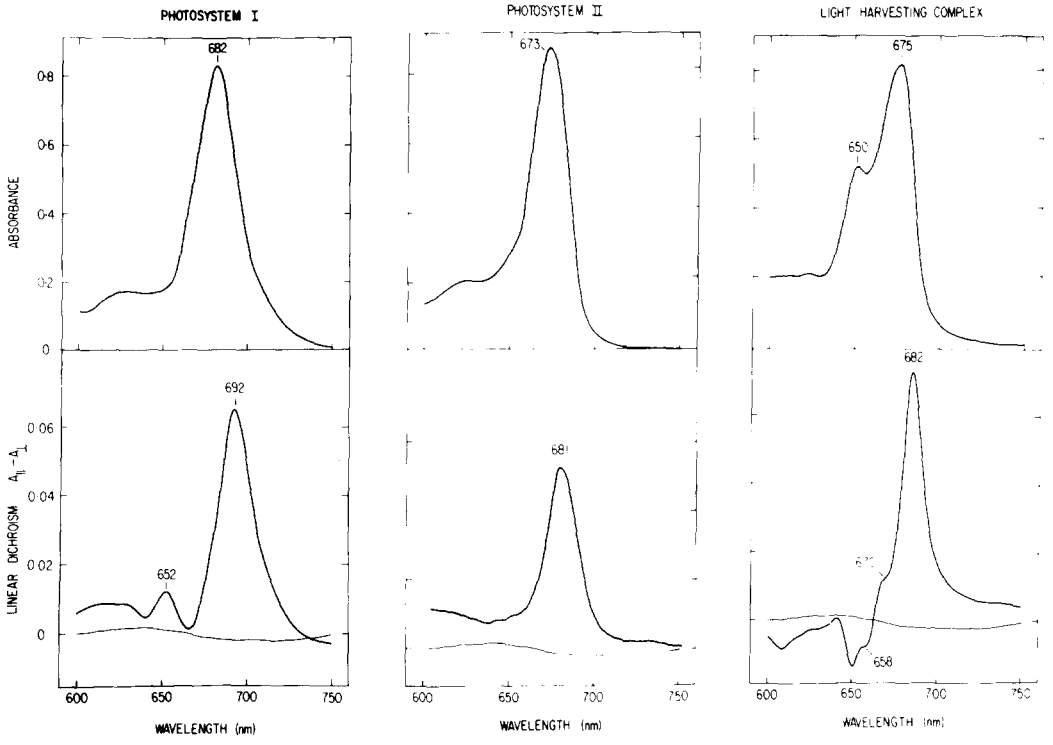

Fig. 3. Room temperaure absorption spectra (upper panels) and low temperature linear dichroism spectra (lower panels) of pigment-protein complexes isolated from pea thylakoids.

they assume a different orientation from the Chl *b* on the light harvesting complex.

Analysis of PS I preparations from units that lack chlorophyll-b

Previous investigators have assigned the 648 nm negative dichroism in the LD signal of thylakoids to Chl *b* based upon the absence of such a signal in the Chl *b*-less mutant of barley [23] and the LD of the electochromic shift in magneto-oriented thylakoids [21]. Our studies confirm this assignment as the signal was also not detected in intermittent light plants or in cyanobacteria. Pursuant to our suggestion that the positive 652 nm band in the signal from PS I preparations is also Chl *b*, a variety of Chl *b*-less systems were studied. Fig. 4 shows low temperature LD spectra of PS I preparation from the Chl *b*-less barley, intermittent light cucumbers and a cyanobacterium. It can be seen that the 652 nm peak is not evident in such preparations, but is clearly present in the PS I fraction from the wild-type barley and the mature cucumber control derived by greening the intermittent light plants in continuous light for 36 h. The longer LD wavelength maximum of 652 nm may reflect a different interaction of Chl *b* with PS I relative to that associated with the light harvesting complex. The fact that the peak wavelength is different from the Chl *b* associated with the light harvesting complex also indicates that the band is not due to contamination of the PS I preparations with light harvesting complex.

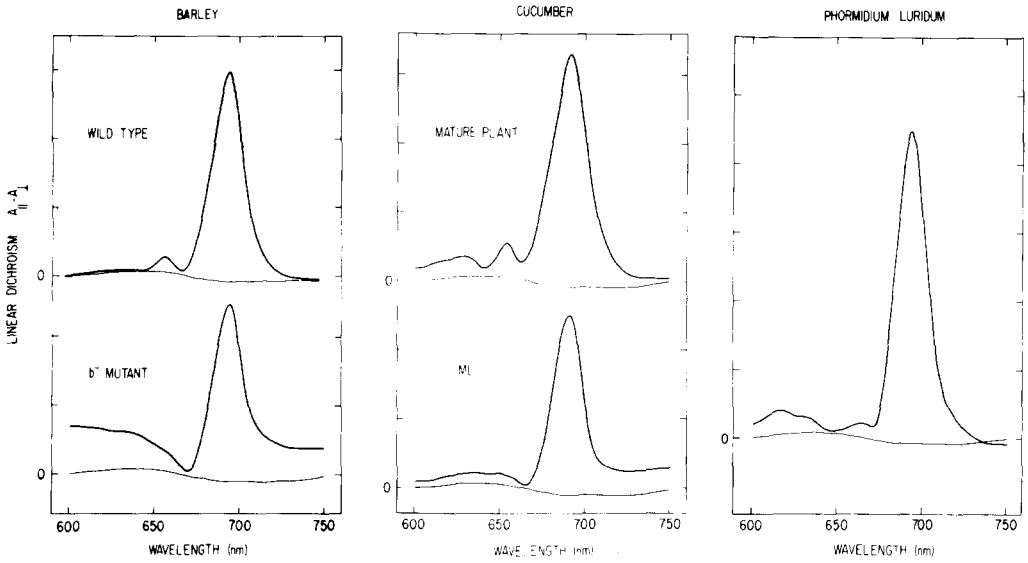

Fig. 4. Low temperature linear dichroism spectra of PS I preparations isolated from photosynthetic organisms that lack Chl *b* and corresponding controls that have a normal complement of Chl *b* (upper panels). IML, intermittent light plant.

Discussion

These data show that the LD spectra of photosynthetic membranes from higher plants and algae aligned in stretched poly(vinyl alcohol) films are very similar to those spectra of membranes and cells oriented in either magnetic [17—19] or electric fields [20]. The stretched films have an advantage over the other techniques for orientation in that they are optically very clear glasses and the contribution due to the light scattering of whole cells and large membrane fragments is considerably reduced.

We previously reported that the addition of low concentrations of cations to magneto-oriented thylakoids results in a change in the room temperature LD spectrum [40]. A long wavelength asymmetry was noted upon addition of 3 mM Mg^{2+} or Ca^{2+}. We suggested that the divalent cation-induced asymmetry represented a change in membrane conformation and that this was related to the regulation of excitation transfer within the photosynthetic unit [43,44]. We were unable to show a similar divalent cation effect in the present work using the stretched film technique. It is possible that the physiological effect does not occur in poly(vinyl alcohol) or is abolished during film preparation, or that the previously reported effect is a consequence of light scattering changes as a result of the cation-induced change in thylakoid stacking. Such a change in scattering would have been minimized in the work presented here. On the other hand, previous investigators have argued that selective polarized scattering does not substantially contribute to the LD spectra of thylakoids. Such arguments were based upon the similarity of LD spectra of large thylakoids and small subchloroplast fragments [20], lack of distortion of the LD signal following changes in the refractive index of the support medium [17], similarity between

the LD spectra of qranal and agranal chloroplasts [45] and a detailed analysis of the polarized scattering of magneto-oriented *Chlorella* cells [46]. Despite these assurances our data indicate that further studies are warranted to clarify this point.

A number of investigators have shown that the absorption envelope of the red Chl band is a composite of several forms of Chl *a* [24—31]. There is substantial agreement on the peak wavelengths based on work relying upon either higher derivative analysis or deconvolution of the envelope into gaussian components and band assignments have been made for subchloroplast pigment-protein complexes. In a theoretical analysis of the optical properties of photosynthetic membranes Paillotin and Breton [47] reported that although the longer wavelength forms of Chl *a* are oriented, several pigments contribute to the LD in the wavelength range 677—690 nm. The work presented here shows that most likely all the Chl *a* forms are oriented, including those with wavelength maxima shorter than 677 nm.

In the case of the light harvesting complex, which is laid down on the thylakoid during chloroplast maturation, the negative dichroism is due to Chl *b* at 648—650 nm and a short wavelength Chl *a* form at 658—660 nm. Both of these LD bands are seen in whole cells and intact thylakoids indicating that the long geometric axis of the complex, that is assumed to be parallel to the stretch axis of the film, is also parallel to the plane of the native membrane. Other absorption maxima that have been shown to be constituents of the absorption envelope of the light harvesting complex lie at approx. 670, 677 and 684 nm [30, 31]. Chl *a* (670) is clearly seen as a distinct shoulder on the main positive LD signal and fully resolved in the LD spectrum of thylakoids isolated from mature lettuce leaves (Fig. 2). It is also apparent that the longer wavelength Chl *a* forms are oriented at a different angle in the light harvesting complex because the value $\Delta A/A$ is much greater at 682 nm than the corresponding ratio at shorter wavelengths.

Absorption maxima at 670 and 683 nm have been detected in PS II preparations [30]. Both forms could be accomodated in the 77 K LD signal for this fraction whose maximum is at 681—682 nm. It is possible that these same forms also contribute to the light harvesting complex spectrum and represent contaminating PS II that has not been stripped off by the Triton X-100. Again, the positive LD signal indicates that the long geometric axis of the PS II complex in the stretched films is an axis that is normally parallel to the plane of the intact thylakoid. The peak value $\Delta A/A$ for this complex was much greater than that for the light harvesting complex.

The LD spectrum for the PS I preparation is a positive broad signal (25—30 nm) centered at 690—694 nm. This is in contrast to the spectra of PS I preparation reported by Gagliano et al. [22] whose room temperature LD maximum was at 686 nm. Curve analysis of the absorption spectrum of PS I has revealed the presence of absorption maxima at 686, 690 and 695 nm (26,27,29,30]. We suggest that most likely all these forms are oriented and contribute to the PS I LD signal and the Chl *a* (695) is more highly oriented [32].

In addition a short wavelength absorption band at 652 nm has been reported in PS I [26,27,30,31] and the data above show that it has a positive dichroism. Our observation that this LD band is not evident in PS I preparations from

organisms that lack Chl *b* such as cyanobacteria, the Chl *b*-less mutant of barley and intermittent light plants, suggest that it might be due to a population of Chl *b* associated with the antenna of PS I. Alternatively the 652 nm LD band could have originated by contamination of the PS I preparation by some light harvesting complex. This is unlikely because we did not observe accompanying negative LD bands at 670 and 682 nm. The LD maximum at 652 nm, slightly longer relative to that of the light harvesting complex, could indicate a greater state of aggregation of these Chl *b* molecules, or reflect a different interaction with the protein fraction. The orientation of the postulated PS I Chl *b* is similar to that of the bulk Chl *a* forms which is opposite to the Chl *b* on light harvesting complex. This is of considerable interest with regard to the synthesis of Chl *b*. If it is synthesized from a pre-existing Chl *a* on the thylakoid as it has been suggested [48] then the orientation of the precursor porphyrin should be the same as that of the Chl *b* in each type of complex, i.e. perpendicular to the plane of the membrane for the light harvesting complex Chl *b* and parallel to the plane of the membrane for the PS I Chl *b*. For the light harvesting complex Chl *b* a possible candidate for the precursor is Chl *a* (658) whose dichroism is also negative. We did note an inverse correlation between the amplitudes of these two LD bands in the light harvesting complex preparations. It is conceivable that a different population of precursor Chl *a* exists for the PS I Chl *b*.

From the data presented it is not possible to quantitatively evaluate the angles of orientation of the various Chl with respect to the membrane plane and, in view of the additional complexities of the pigment orientations reported here, it may be necessary to revise some of the previous assignments [47]. To achieve this it will be necessary to precisely determine the contribution of each pigment form to the absorption and LD spectral envelopes.

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