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ORIENTATION OF PIGMENTS IN THE THYLAKOID MEMBRANE AND IN THE ISOLATED CHLOROPHYLL-PROTEIN COMPLEXES OF HIGHER PLANTS

II. LINEAR DICHROISM SPECTRA OF ISOLATED PIGMENT-PROTEIN COMPLEXES ORIENTED IN POLYACRYLAMIDE GELS AT 300 AND 100 K

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The absorption and linear dichroism (LD) spectra (380–780 nm) of isolated light-harvesting complex (LHC), Photosystem I (PS I), Photosystem II (PS II), as well as intact thylakoids have been determined at 300 and 100 K. The samples were oriented in squeezed polyacrylamide gel. The low-temperature spectra of LHC and PS I present LD signals which are characteristic enough to be recognized in the LD spectrum of thylakoids. Tentative assignments of the various features of the LD spectra to the major photosynthetic pigments are discussed. A shoulder in the low-temperature absorption spectra is observed at about 673 nm in all the systems under investigation. The absence of an associated LD signal suggests that this ubiquitous chlorophyll (Chl) *a* form is non-dichroic. Furthermore, in the three isolated chlorophyll-protein complexes described in this study the sign of the LD signal indicates that both the Q_y transition of the Chl *a* and the carotenoid molecules are preferentially oriented parallel to the largest dimension(s) of the particles.

Introduction

Analysis of the orientation of the pigments in isolated pigment-protein complexes is an important step towards our understanding of the mechanism of excitation transfer and trapping in green plant photosynthesis. In the first paper of this series [1], we have assessed a variety of orientation techniques, applicable to thylakoids and isolated pigment-protein complexes. We concluded that the polyacrylamide gel squeezing technique of Abdourakhmanov et al. [2] gives oriented samples with minimal optical artefacts, negligible pigment disruption and is suitable for low-temperature measurements [1,3].

The information we can derive from LD measurements on such systems includes: (i) comparison of LD and absorption spectra for band assignment, (ii) determination of the angle between the chromophores and the axis of orientation by analysing LD/A or dichroic ratios ($A_{\parallel}/A_{\perp}$), (iii) calculation of the orientation of different chlorophyll forms, in the isolatable particles, when Gaussian deconvolution is performed simultaneously on $A_{\parallel}$ and $A_{\perp}$ spectra, and (iv) analysis of the relative contribution of the LD of the various particles to that of intact thylakoids. In this paper we present only the first level of analysis.

Gagliano et al. [4] were the first to report the

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Abbreviations: Chl, chlorophyll; PS, photosystem; LHC, light-harvesting complex; LD, linear dichroism; Tricine, *N*-tris(hydroxymethyl)methylglycine.

LD spectrum of an isolated pigment-protein complex. In this work [4], a PS I particle was oriented by a pulsed electric field; however, this technique does not allow low-temperature spectroscopy [1]. More recently, Biggins and Svejkovsky [5] have reported the low-temperature LD spectrum of submembrane fractions oriented in stretched poly(vinyl alcohol) films. However, because of our recent observations on the presence of optical artefacts and possible pigment disruption in such systems [1], we feel justified in presenting a second and more detailed analysis.

In this paper we present and discuss room-temperature (300 K) and low-temperature (100 K) LD spectra and absorption spectra of thylakoids, LHC, PS I and PS II, oriented in polyacrylamide gel. These studies demonstrate that a precise organization of the pigments prevails in all the isolated complexes. A comparison of characteristic LD signals occurring both in the intact thylakoid and in the isolated chlorophyll-protein complexes suggests that the native orientation of the photosynthetic pigments is preserved during the isolation process.

Materials and Methods

Pea thylakoids were used to isolate LHC according to the procedure of Burke et al. [6]. A PS I particle was isolated by the same procedure and is characterized by Mullet et al. [7] as PS I_{110}. PS II was prepared using Triton X-100 (Sigma Chem. Co.) prewashed thylakoids which were subsequently treated with digitonin and octylglucopyranoside (Sigma Chem. Co.). The details of this procedure are given in Ref. [8].

Polyacrylamide gel electrophoresis, used to determine the polypeptide composition of isolated complexes was carried out on sodium dodecyl sulphate (SDS)-treated preparations [9].

Thylakoids and isolated complexes were oriented by the polyacrylamide gel squeezing technique [1–3]. As before [1], gels were squeezed to 60–70% of their original length and no attempt has been made to quantify the extent of orientation as a function of the degree of squeezing. LD spectra were determined using the instrument described in Ref. 10. This instrument and the Cary 17 (Varian) spectrophotometer, used to record the absorption, were fitted with a variable-temperature cryostat (SMC, France). $A_{\parallel}$ and $A_{\perp}$ refer to the absorption of light polarized parallel and perpendicular, respectively, to the longer axis of the oriented object, i.e., that axis orientating perpendicularly to the direction of squeezing as assumed in Ref. 1.

By separately measuring $A_{\parallel}$ and $A_{\perp}$ [10,11] it is possible to calculate the absorption spectrum of an oriented sample. However, technical constraints prevented us from taking these measurements at 100 K, but we have been able to determine dichroic ratios ($A_{\parallel}/A_{\perp}$) at 300 K. We present these dichroic ratios in order that some assessment of the magnitude of our LD spectra (which are presented in arbitrary units) can be made. Because of our technical inability to measure 100 K absorption spectra in gels, we have determined 300 and 100 K absorption spectra of particles and thylakoids in 60% glycerol/buffer (10 mM Tricine-NaOH, pH 7.8). This approach is valid, since we have confirmed that the 300 K absorption spectra of the different samples are identical in this medium and in unsqueezed polyacrylamide gels.

Results and Discussion

The 300 K absorption and LD spectra of: (a) intact thylakoids, (b) LHC, (c) PS I and (d) PS II are presented in Fig. 1. To allow for a better comparison between some of the features (especially those which appear in the 100 K spectra) in both LD and absorption, we have normalized all these spectra to the same height at the red maximum. The polypeptide profiles of all of these samples, as determined by SDS-polyacrylamide gel electrophoresis, are given in Fig. 2.

In thylakoids (Fig. 1a) the major absorption peak is observed at 679 ± 1 nm with a corresponding LD signal at 680 ± 1 nm. This LD spectrum is identical to that presented in Fig. 1 of Ref. 1. The spectra of LHC (Fig. 1b) at 300 K are also in agreement with previously published data [1,6]. The long-wavelength absorption peaks at 675 ± 1 nm, while the LD maximum is at 680 ± 1 mm.

PS I (Fig. 1c) has an absorption spectrum identical to that given in Ref. 7 with a long-wavelength peak at 681.5 ± 1 nm. The LD spectrum is in broad agreement with data produced by Gagliano

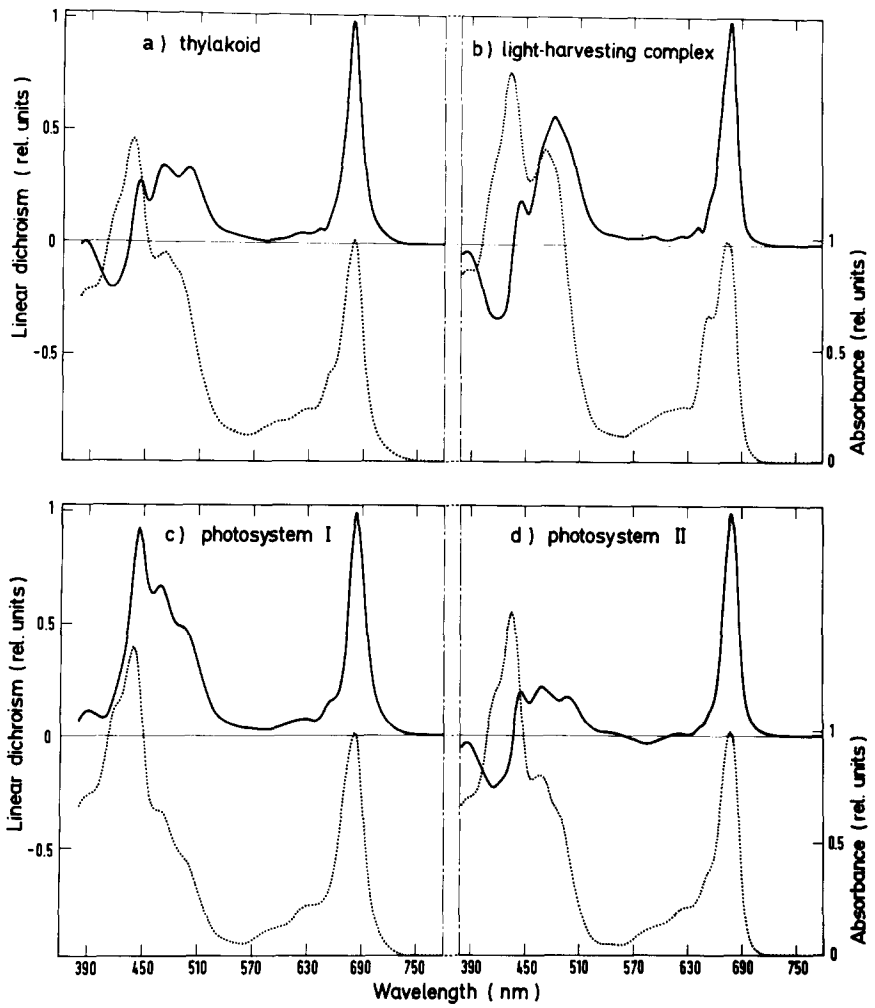

Fig. 1. The 300 K absorption (·····) and LD (———) spectra of, (a) thylakoids, (b) LHC, (c) PS I and (d) PS II. Absorption spectra were determined on samples in 60% glycerol/buffer, LD was determined upon samples oriented in polyacrylamide gels with dichroic ratios ($A_{\parallel}/A_{\perp}$) at the red maximum of 1.2–1.4 for thylakoids, 1.3–1.6 for LHC, 1.15–1.25 for PS I and 1.15–1.3 for PS II.

et al. [4] using an SDS-extracted CP I complex, oriented by a pulsed electric field. In Fig. 1c, there is a large positive long wavelength LD signal at 684.5 ± 1 nm, with a shoulder at 640–650 nm. Unlike LHC, there is a recognizable component between 710 and 730 nm, a signal also observed in intact thylakoids (Fig. 1a).

In contrast to LHC and PS I which appear as pure preparations in the SDS-polyacrylamide gel electrophoresis profile of Fig. 2, PS II, as characterized elsewhere [8], is contaminated by the presence of LHC. As a consequence, therefore, it is difficult to be rigorous in the interpretation of the spectral data obtained for this sample. The absorption spectrum in Fig. 1d is in close agreement with previously published data [12]. Neither this spectrum nor the LD spectrum at 300 K resembles that of LHC. This suggests that the pigments of PS II are themselves specifically oriented, and give rise to a long-wavelength Chl *a* signal at 679.5 ± 1 nm in the LD spectrum.

When samples are cooled to 100 K, spectral resolution is increased and some blue shifts in absorption occur. The 100 K absorption and LD

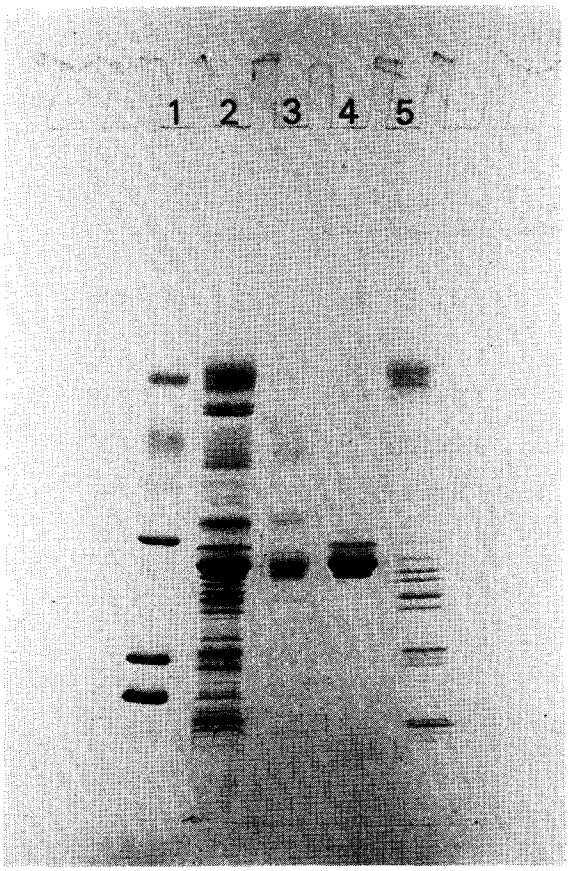

Fig. 2. SDS-polyacrylamide gel electrophoresis of samples used for optical studies; lane 1, standards: bovine serum albumin (65 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), myoglobin (17 kDa), cytochrome *c* (12.4 kDa). Lane 2, thylakoids; lane 3, PS II; lane 4, LHC; and lane 5, PS I.

spectra of intact thylakoids are presented in Fig. 3a. The main Chl *a* Q_y absorption peak is centered at 678 ± 0.5 nm, coincident with an LD signal at 677.5 ± 0.5 nm. As with the room-temperature spectra (Fig. 1a), there is a significant absorption and LD signal between 710 and 730 nm. The absorption spectrum of intact thylakoid has a shoulder at 673 ± 1 nm, which appears to have no corresponding LD signal but a shoulder does appears in the LD spectrum at 664 ± 1 nm. The Chl *b* absorption peak at 650 nm is associated with a complex LD signal between 640 and 652 nm.

The 100 K absorption and LD of LHC are presented in Fig. 3b. Comparison with Fig. 1b shows the increased resolution occurring upon lowering of temperature. The major Q_y Chl *a* transition peaks at 676.5 ± 0.5 nm in the LD spectrum and at 677 ± 0.5 nm in the absorption spectrum. As in the thylakoid absorption spectrum (Fig. 3a), there is a shoulder at 673 ± 1 nm which has no corresponding LD signal. An absorption shoulder at 664 ± 0.5 nm does have a corresponding LD signal at 664 ± 1 nm, while a complex group of LD signals between 640 and 652 nm is present under the main Chl *b* absorption band at 650 ± 0.5 nm. This array of LD peaks as well as the shoulder at 664 ± 1 nm is identical to signals observed in thylakoids and we interpret this array as a 'fingerprint' for the presence of LHC. Assignment of a source of each of these components is difficult at this stage. Undoubtedly Chl *b* is involved, but it is possible that Chl *a* may play some role. The extreme 'sharpness' of the negative component at 648 nm in the LD spectrum, for which we estimate a full-width at half-maximum height of 5 ± 1 nm, suggests that it might arise as the sum of two close absorption bands exhibiting dichroism of opposite sign. Although several origins can be found for such bands (e.g., two populations of Chl *b* with different orientations), we tentatively suggest that the complex set of LD bands at 650 nm, together with the shoulder at 664 nm, can be related to the excitonic components of the Chl *b* trimers described by Knox and Van Metter [13] and Shepanski and Knox [14]. In this last model, two of the three excitonic components are largely degenerate and lie at 652 nm, while the third is located at 665 nm (in room-temperature spectra). Although such a model can explain most of the features in our LD spectra in the 640–670 nm region, further spectroscopic investigations on the same material are required to test its validity.

In Ref. 1 we demonstrated the presence of a scattering artefact in the room-temperature LD spectrum of thylakoids oriented in poly(vinyl alcohol) stretched films as compared to thylakoids oriented by the gel squeezing technique. We are now able to compare the low-temperature LD spectra of both thylakoid and LHC oriented in polyacrylamide gel with the equivalent data obtained by Biggins and Svejkovsky [5] using poly(vinyl alcohol) films. Again we can observe several differences which are consistent with an increased scattering artefact in the poly(vinyl al-

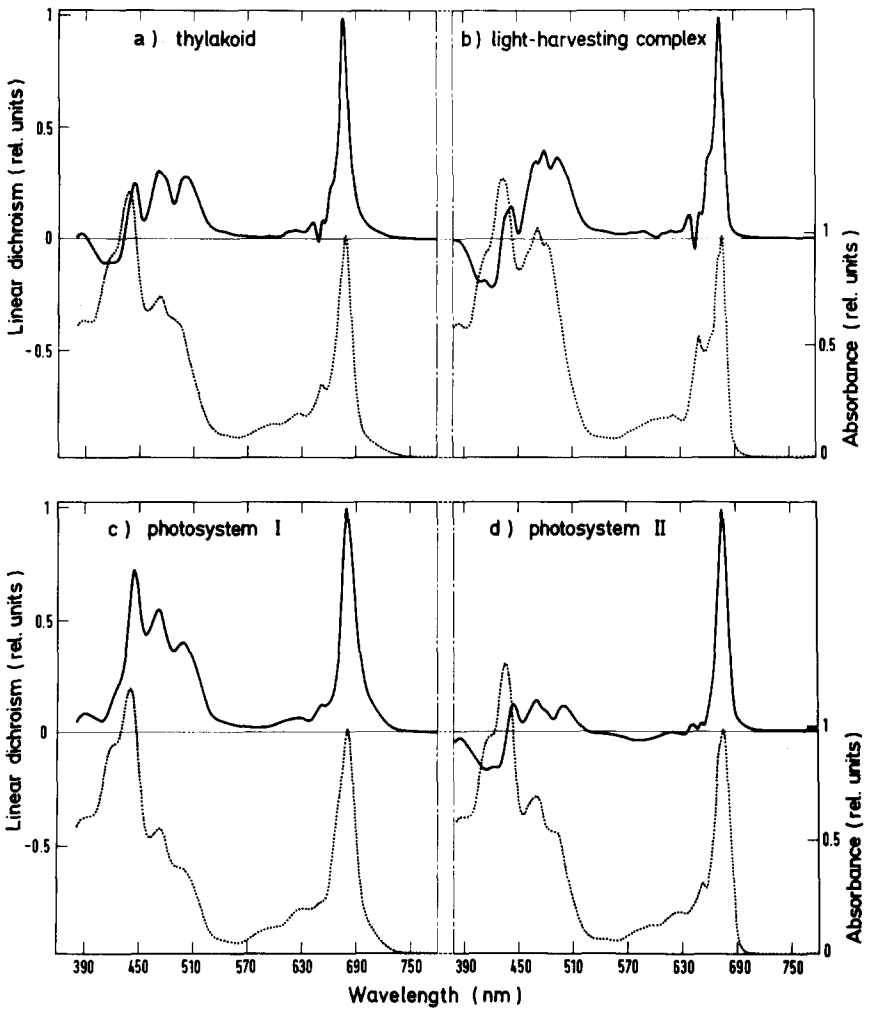

Fig. 3. the 100 K absorption (·····) and LD (———) spectra of (a) thylakoids, (b) LHC, (c) PS I and (d) PS II. Spectra were determined as described in Materials and Methods.

cohol) films. These differences, which are most evident in the case of LHC, include a negative LD signal between 600 and 660 nm, a strong positive contribution around 750 nm and a 5–6 nm red shift in the LD peak. In a recent paper, however, Biggins [15] has obtained a low-temperature spectrum of thylakoids, suspended in glycerol/buffer and oriented by a magnetic field, which more closely resembles that of Fig. 3a.

The 100 K spectra of our isolated PS I fraction are shown in Fig. 3c. The absorption and LD spectra both show a signal between 710 and 730 nm which is also observed in thylakoids (Fig. 3a). It seems likely that this signal reflects the presence of a long-wavelength antenna component, which is unaltered by the isolation procedure [8]. The major Q_y transition has both LD and absorption peaking at 681 ± 1 nm. As with previous low-temperature absorption spectra, there is a shoulder at 673 ± 1 nm which has no corresponding LD signal. The absorption spectrum in the 620–660 nm region is composed of a broad shoulder between 625 and 640 nm and a smaller shoulder centered at 648 ± 0.5 nm. This small feature, which may result from Chl *b* absorption, was recently described by Brown and Schoch [16]. The LD spectrum in the same

region consists of a single peak at 652 ± 1 nm, which appears very different from the fingerprint signal of LHC in this region. A similar signal observed in PS I oriented in poly(vinyl alcohol) films was attributed to Chl *b* by Biggins and Svejkovky [5]. However, the PS I spectrum presented in this case is very different from that of Fig. 3c. Biggins and Svejkovsky [5] have reported a long-wavelength Chl *a* peak which is 10 nm higher than that of Fig. 3c, together with a large 'dip' around 660 nm. We attribute these differences to the presence of scattering artefacts in poly(vinyl alcohol) films, as discussed in Ref. 1, and/or the contribution of free pigments to their LD signal as observed by Gagliano et al. [4].

The 100 K absorption and LD spectra of PS II are presented in Fig. 3d. The long-wavelength peak is centered at 676.5 ± 0.5 nm in both absorption and LD spectra. As with the other samples, there is an absorption shoulder at 673.5 ± 1 nm which has no corresponding LD signal. A Chl *b* absorption band is observed at 650 ± 0.5 nm, similar to that seen in thylakoids (Fig. 3a). This Chl *b* absorption is associated with a weak LHC fingerprint in the LD spectrum, consistent with the observation of a limited LHC contamination in this preparation (Fig. 2). One curiously unique feature of the PS II LD, seen in both Fig. 1d and 3d, is a broad negative signal centered at approx. 580 nm. The source of this signal is as yet unknown.

The absorption and LD spectra in the 400–500 nm spectral range are poorly resolved, even at 100 K, and are therefore difficult to interpret. The pigment absorption in this region arises from both Chl *a* and *b* as well as various species of carotenoids. Since most of the Chl *b* in the thylakoid is associated with LHC [6], we might expect to see a Chl *b* signal mainly in LHC and those samples which contain LHC, i.e., thylakoids and PS II. In Fig. 3a, b and d we observe a positive LD signal at 483 ± 2 nm, the size of which is clearly related to the size of the LHC fingerprint signal at 648 nm, which in turn can be related to the Chl *a*/*b* ratio of the sample. We therefore feel confident in assigning this LD signal to Chl *b*, which is in agreement with an earlier LD study involving a Chl *b*-less mutant of barley [17]. The corresponding Chl *b* absorption peak in LHC appears to be at 484.5 ± 1 nm.

Carotenoid absorption in vivo is not well characterized, but we might expect three absorption peaks in this region. Fortunately, each of these absorption peaks arises from different vibrational levels of the same transition and so we would expect both absorption and LD spectra to be of similar shape. Since we know carotenoid to be present in all our samples [6,7], we might expect

TABLE I

SPECTRAL FEATURES OF LD SPECTRA AT 100 K FOR THYLAKOIDS, LHC, PS I AND PS II

Values are expressed in nm. Features: (p+), positive peak; (p−), negative peak; (s), shoulder.

Thylakoids	LHC	PS I	PS II	Possible assignments
710–730 (s)		710–730 (s)		PS I long-wavelength antenna
677.5 ± 0.5 (p+)	676.5 ± 0.5 (p+)	681 ± 1 (p+)	676.5 ± 0.5 (p+)	Long-wavelength Chl *a*
664 ± 1.5 (s)	664 ± 1 (s)			Chl *b* (?)
653.0 ± 0.5 (p+)	653.0 ± 0.5 (p+)	652 (p+)	653.0 ± 0.5 (p+)	Chl *b*
648.0 ± 0.5 (p−)	648.0 ± 0.5 (p−)		648.0 ± 0.5 (p−)	Fingerprint for LHC
642.0 ± 1.0 (p+)	642.0 ± 1.0 (p+)		642.0 ± 1.0 (p+)	
			583 ± 2 (p−)	
501 ± 2 (p+)	497 ± 1 (p+)	500 ± 2 (p+)	500 ± 2 (p+)	Carotenoid
481 ± 2 (s)	483 ± 1 (p+)		483 ± 2 (s)	Chl *b*
472.5 ± 1 (p+)	473 ± 2 (p+)	472 ± 2 (p+)	469.5 ± 1 (p+)	Carotenoid
445 ± 1 (p+)	445 ± 1 (p+)	446 ± 1 (p+)	445.5 ± 1.0 (p+)	Carotenoid/Chl *a*
421 ± 1 (s)	423 ± 1 (p−)	422 ± 2 (s)	428 ± 2 (p−)	Chl *a*
411 ± 1 (s)	411 ± 2 (p−)	409 ± 2 (s)	416 ± 2 (p−)	

some common features in our spectra. The features of the 100 K LD spectra, presented in Fig. 3a–d, are summarized in Table I. Several LD peaks are common to all samples, these are at 498 ± 3, 471 ± 2 and 445.5 ± 0.5 nm. Having already assigned the Chl *b* signal at 483 nm, we tentatively suggest that the two longest wavelength features, i.e., 498 ± 3 and 471 ± 2 nm, are associated with carotenoid absorption . The observed variation in these peak maxima might reflect small changes in the carotenoid composition in the samples or differences in carotenoid environment.

The third of these common features is a strong positive peak at 445.5 ± 0.5 nm. This could be the third band of carotenoid absorption. However, in the PS I LD spectrum (Fig. 3c) the amplitude of this third peak in relation to the two carotenoid bands at 498 and 471 nm is much larger than could be expected for carotenoid absorption. At this stage we tentatively attribute most of the 445.5 ± 0.5 nm LD signal to a *Y*-polarized transition from the Soret region of Chl *a*. In all the above LD spectra we observed components around 420 and 410 nm exhibiting negative LD. They are probably related to the *X*-polarized transitions from the Soret region of Chl *a*. We have no explanation, however, for the broad positive contribution to the LD signal of PS I in this region.

The LD data presented in Fig. 3a–d are summarized in Table I. We have already noted several common dichroism features in our samples which we have associated with carotenoid and chlorophyll absorption. These features are rather specific, since they occur at almost the same wavelengths in all samples. However, there are other important properties of the LD spectra which need further consideration. Firstly, the common positive LD signals in the 680 nm region indicate that the Q_y transition moments of Chl *a* in each of the particles are mostly oriented parallel to a direction lying along the largest dimension(s) of the particle. Similarly, LD bands of carotenoids are positive in all the samples, this indicates that the carotenoids are also preferentially oriented parallel to the longest dimension(s) of the particle. This observation is in agreement with a previous discussion of Junge et al. [18]. In contrast, the negative components around 410 and 420 nm, which we have tentatively assigned to the *X*-polarized transitions of Chl *a*, suggest that the plane of the oriented Chl *a* molecules lies perpendicular to the longest dimension(s) of the particles. Finally, close examination of the absorption spectra presented in Fig. 3a–d shows the presence of an absorption shoulder at 673 ± 1 nm in all samples. The presence of this shoulder has been observed previously in thylakoids [4] and PS I [16]. We are now able to report that this Chl *a* species is common to all the major pigment-protein units. Furthermore, the absence of an associated LD signal demonstrates that this 673 nm band is a non-dichroic Chl *a* form common to all our samples. By analyzing the polarization of absorbance changes linked to the Stark effect occurring in chloroplasts, Breton and Paillotin [19,20] have shown that a Chl *a* species absorbing at 670–675 nm was oriented near the 'magic angle' (35° away from the membrane plane). It is most probable that this species corresponds to the ubiquitous 673 nm Chl *a* form described in the present study.

The LD studies presented here reveal that a precise organization of the pigments, demonstrating an anisotropic distribution of orientation of transition moments, prevails in all the isolated complexes. Furthermore, several of the features of the low-temperature LD spectra of thylakoids correspond precisely with LD signals in the isolated complexes (notably the LHC signal around 650 nm and the PS I signal at 710–730 nm). This indicates that the in vivo orientation of the various photosynthetic pigments is probably not greatly disturbed during the isolation. In a subsequent paper [21], we have further explored this property by analyzing the relative contributions of each of the three particles to the LD signal of the intact thylakoid.

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