Journal of Fluorescence, Vol. 14, No. 2, March 2004 (©2004)

The Effect of Pea Chloroplast Alignment and Variation of Excitation Wavelength on the Circularly Polarized Chlorophyll Luminescence

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Received October 2, 2003; revised November 24, 2003; accepted November 24, 2003

Circularly polarized luminescence (CPL) is a powerful technique to study the macroorganization of photosynthetic light-harvesting apparatus *in vivo* and *in vitro*. It is particularly useful for monitoring environmental stress induced molecular re-organization of thylakoid membranes in green leaves. The current study focuses on two questions which are important to perform and interpret such experiments: how does CPL depend on the excitation wavelength and how on the orientation of the granal thylakoids. CPL and circular dichroism (CD) of pea chloroplasts were complementarily applied when chloroplasts were either in suspension or trapped in a polyacrylamide gel (PAAG) after alignment in a magnetic field. In contrast to the CD spectrum, the CPL signal was found to be independent of the excitation wavelength in both the Soret and the Q_y absorption region for chloroplasts in both suspension and PAAG. The improved resolution of luminescence measurements revealed a relatively small negative CPL band in addition to the previously described large positive band. No effect of photoselection upon excitation on the CPL spectra was detected. The CPL intensity at 690 nm at the edge of the granal thylakoids was found to be higher than at the face of the grana suggesting the CPL anisotropy.

KEY WORDS: chiral macroaggregates of LHCII; anisotropy of CPL; circular dichroism of chloroplasts.

INTRODUCTION

Chiral macroaggregates of the photosynthetic light-harvesting chlorophyll *a*/*b* pigment–protein complexes (LHCII) have been shown to be involved in the lateral separation of the two photosystems [1]. The aggregates of LHCII constitute the basis for long-range migration of excitation energy in light harvesting antenna, and for the dissipation of the excessive absorbed light energy; all that might protect the photosynthetic apparatus against photoinhibitory damage [1–3]. This indicates the important role of LHCII aggregates in the photosynthetic machinery. The formation of chiral LHCII macroaggregate is affected by the presence of magnesium ions and sorbitol in the suspension medium of chloroplasts, as well as by short or prolonged photoinhibitory illumination of the suspension [1–5].

Chiral macroaggregates of LHCII can be detected in isolated thylakoids and chloroplasts by means of large

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ABBREVIATIONS: CD, circular dichroism; CPL, circularly polarized luminescence; Chl, chlorophyll; LHCII, light-harvesting chlorophyll *a*/*b* pigment–protein complex; g_{ab} and g_{em}, absorption and emission anisotropy factors, respectively; PAAG, polyacrylamide gel.

1053-0509/04/0300-0207/0

anomalous non-conservative circular dichroism (CD) signals in the Q_y and Soret absorption region [1,5]. The anomalous CD signals have been shown to be sensitive to orientation, macroorganization and size of the grana [6,7].

The total fluorescence of a chiral molecule is expected to contain a circularly polarized part even upon non-polarized excitation (circularly polarized luminescence, CPL) [8,9]. Quantitatively CPL is expressed by the emission anisotropy factor, g_{em}, which is a weighed difference between the left- and right-handed circularly polarized components of the total emitted light:

$$g_{em} = 2\Delta f/f \quad (1)$$

where f and Δf are, respectively, the total luminescence intensity and circularly polarized part, which is the difference between left- and right-handed circularly polarized emission. More detailed information about the CPL method is available in several reviews [8–10].

The comparison of the emission anisotropy factor (1) and absorption anisotropy factor:

$$g_{ab} = 2\Delta\varepsilon/\varepsilon \quad (2)$$

where $\Delta\varepsilon$ and ε are CD and extinction coefficient, respectively, and g_{ab} characterizes the weighed circular dichroism, indicates that the CD and CPL methods are in fact complementary. CPL was interpreted to provide information on the excited state molecular stereochemistry and transition properties from the excited to the electronic ground state [11]. On the other hand, CD yields information on the ground state molecular stereochemistry and transition properties from the ground to an excited state. It is important to note that in contrast to CD, CPL may be applied for both transparent and non-transparent samples, which allows in particular, *in vivo* studies of green leaves, both attached and detached, as demonstrated previously [12].

In isolated chloroplasts, the LHCII chiral macroaggregates can be detected by means of CD as well as by monitoring the CPL signal at 690 nm when excited in the Soret band (436 nm) [13,14]. It is well known that a chlorophyll molecule in solution fluoresces from the lowest Q_y singlet electronic level, even after excitation in the Soret band because of internal conversion. An acetone solution of chlorophyll does not show any CPL signal because of the high symmetry of the molecule [14,15]. The big CPL signal appears for chlorophylls in LHCII, which in turn are organized in chiral macroaggregates. This is a typical example of induced asymmetry, which leads to chiral properties of the chromophores in the excited electronic state. While the behavior of g_{ab} over the absorption spectrum and that of g_{em} over the emission spectrum have been studied before [8,10,11], the dependence of g_{em} on the excitation wavelength has not been considered so far.

There are at least two reasons why g_{em} can depend on the excitation wavelength. Firstly, the CD of LHCII chiral macroaggregates depends to a large extent on wavelength. Secondly, the linear polarization spectrum of chlorophyll *a* strongly depends on the excitation ranging from 0 to 0.2 in the Soret band to about 0.4 in the Q_y-band [16]. Therefore, for properly interpreting the changes in CPL, we have studied the excitation-wavelength dependence of the CPL. The present study indicates that the CPL signal of LHCII chiral macroaggregates does not depend on the excitation wavelength. This appears to be true for granal thylakoids in pea chloroplasts both in suspension and when first aligned magnetically and then trapped in a polyacrylamide gel (PAAG). It is also found that the CPL spectrum, besides the strong positive band around 690 nm, has a negative band in the 665-nm range.

A previous study on chloroplasts has revealed that the alignment of granal thylakoids in a magnetic field leads to significant changes of the CD spectrum [5]. Moreover, microscopic CD measurements revealed that the grana spots and spots close to the edge of magnetically aligned chloroplasts give rise to different CD signals [6]. Hence, if the anisotropy of granal thylakoids is reflected in CD measurements, then it might also be revealed by CPL. Our results indicated that there is anisotropy of the CPL signal, being higher when measured from the edge side (as opposed to the face side) of granal thylakoids.

EXPERIMENTAL

Chloroplasts

Chloroplasts were isolated from pea leaves of 2-week old plants as described elsewhere [5]. Isolated chloroplasts (0.5–3 mg/mL) were suspended in a standard buffer of 20 mM tricine-NaOH of pH 7.6, 0.4 M sorbitol, 5 mM $MgCl_2$ and 10 mM KCl, kept in ice and used within 4–5 hr after isolation. The Chl a/b ratio was about 2.3–2.5. The Chl$(a + b)$ concentration was determined according to the method of Arnon [17].

Chloroplasts were aligned in a magnetic field **H** of 1.2 T (Institute of Superconductivity, Bar Ilan University, Ramat Gan, Israel) and trapped in PAAG [18,19] as indicated below. Control experiments of randomly oriented chloroplasts were performed with chloroplasts trapped in PAAG without alignment with magnetic field. An amount of 10–60 μL of chloroplast suspension was added to 3 mL of standard buffer with 5% acrylamide and 0.17%. After alignment of chloroplasts in the magnetic field, the polymerization reaction was initiated by adding

freshly prepared ammonium persulfate (APS) solution and tetramethylethylenediamine (TEMED), to obtain a final concentration of 0.2% for both. Both trapping the chloroplasts and CD and CPL measurements were performed in an all-side polished 10.0-mm cuvette. For the chloroplasts alignment, the cuvette was kept between the poles of a magnet till the polymerization reaction was complete (approximately 2 min after adding APS and TEMED). For the CD and CPL measurements, the cuvette was put in the respective instruments such that chloroplasts were (in average) oriented either parallel or perpendicular with respect to the luminescence excitation and emission collection directions (CPL) or the measuring light beam (CD). It is known that the short axis of chloroplast orients preferentially along the magnetic field vector **H** [18]. During the measurements no magnetic field was applied to the sample.

CD and CPL Measurements

CD spectra were measured with an AVIV model 62A DS Circular Dichroism spectrometer (Lakewood, NJ). A concentration of 10–20 μg/mL Chl and a 10.0-mm pathlength cuvette were used for CD measurements.

CPL with either 7.4 or 20 nm spectral resolution was measured with the setup described earlier [14,20,21] where the excitation was performed at 180 or 90° with respect to the direction of the emission collection. Blue light excitation (436 nm) was provided by a 150 W mercury lamp, and red light excitation was provided by a Thorlabs laser diode module of 635 nm, 2.5 mW. In these experiments, a 10.0-mm pathlength cuvette was used.

High resolution (5 nm) CPL spectra and CPL excitation spectra were measured with a home-built setup as shown in Fig. 1. Excitation in the red region (615–690 nm) was provided by a DCM-dye laser pumped with an argon ion laser. The excitation wavelength was adjusted with an increment of 5 nm in the red region. The luminescence spectrum was measured, starting 5 nm higher than the excitation wavelength.

The blue excitation was provided directly by the different lines of the Argon ion laser, (457, 479, 488, 496, 501, and 515 nm) which were separated by a prism. A small part of the excitation light was directed to a monochromator via a beam splitter to monitor the excitation wavelength. The rest of the beam passed through an optical chopper, which modulated the excitation light at a frequency of 200 Hz. A depolarizer was used to scramble the polarization of the excitation light. The depolarized beam was directed to the sample at 180 or 125° with respect to the direction of luminescence detection. The angle 125° was taken as the magic angle at which the photoselection must be minimized (see section "Photoselection is Not the Case"). The 180° setup was used previously for isolated chloroplast studies [13,14]. That's why we also used this setup in order to compare the data obtained at the magic angle and at 180°. A 2-mm pathlength rectangular glass cuvette and a cylindrical glass cuvette of 4 mm internal diameter were used, for the 180 and 125° excitation, respectively (see Fig. 1). A set of red filters with a low-pass wavelength in the 664 to 715-nm range was used to cut off the excitation light behind the sample.

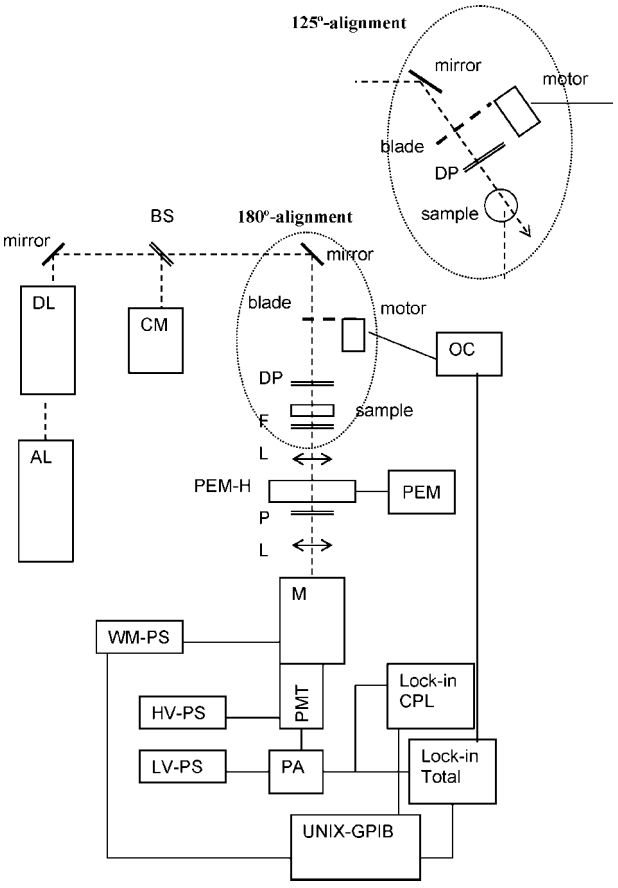

Fig. 1. A scheme of the setup for measurement of circularly polarized luminescence (CPL). Solid bold lines show flat mirrors. The dashed bold line represents a chopper blade rotated by a motor. AL, Coherent Innova 300 Argon laser; DL, a Coherent CR-599 dye laser; BS, beam splitter; CM, Jobin Yvon monochromator for the excitation wavelength control; OC, Bentham model 218 variable frequency optical chopper; DP, depolarizer; F, cutoff glass filter; L, lenses; PEM, Hinds International PEM-80 Photoelastic Modulator System controller; PEM-H, photoelastic modulator optical head; P, polarizer; M, analytical Oriel model 77250 monochromator; PMT, EMI 9658R photomultiplier; HV-PS, high voltage power supply; LV-PS, 12 Vdc power supply; PA, Ortec 9301 pre-amplifier; Lock-in CPL, EG&G model 5209 lock-in amplifier; Lock-in Total; Itacho model 391A lock-in amplifier; WM-PS, wavelength-drive motor power supply; UNIX-GPIB, Unix computer for the GPIB-mediated control and reading collection.

Chl fluorescence was collected by a lens and passed through a photoelastic modulator (PEM) by which the intensity of the circularly polarized emission component was modulated at 50 kHz. The doubly-modulated (200 and 50 kHz) emission was focused onto the entrance slit of a monochromator. The monochromatic light was detected by a photomultiplier. The photocurrent was pre-amplified and directed to two lock-in amplifiers locked to the optical chopper frequency of 200 Hz (for measurement of total fluorescence) and to the PEM frequency of 50 kHz (for detection of the circularly polarized emission), respectively. The output signals were read and further processed by the computer. The final CPL signal was presented as a ratio between the circularly polarized component and the total fluorescence.

Both CPL instruments were calibrated using (1R)-(−)-camphorquinone (Aldrich) in chloroform which is known to have a g_{em}-value of -8.4×10^{-3} in the 490 to 530-nm range [10,22,23].

Because the excitation light provided more than 100 μmol m^{-2} s^{-1} illumination, the chloroplasts were in a photosynthetically active form, and the total luminescence intensity corresponded to F_v [24].

RESULTS

Invariance of the CPL Spectrum

To reveal whether CPL depends on the excitation wavelength, the CPL spectra of pea chloroplasts were measured upon excitation in the Q_y and Soret absorption regions (Fig. 2) when the excitation beam was at 125°

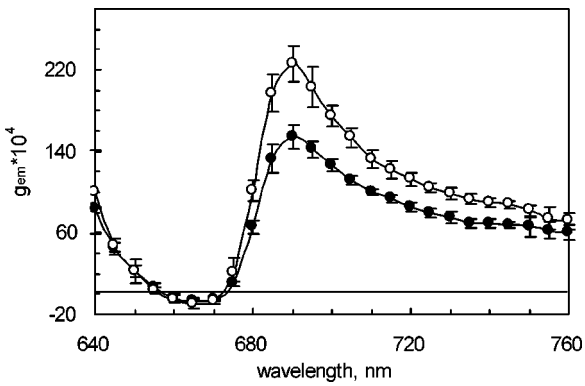

Fig. 2. The averaged CPL spectra of isolated pea chloroplasts in the standard buffer (0.4 M sorbitol, 20 mM tricine, 5 mM $MgCl_2$, 10 mM KCl, pH 7.6) at the excitation in the Q_y band (615 through 690 nm every 5 nm; open circles) and Soret band (457, 479, 488, 496, 501, and 515 nm; filled circles). The excitation light beam was at 125° to the direction of the fluorescence collection. Error bars show standard deviation. Pea chloroplasts for the red and blue excitation were of different batches.

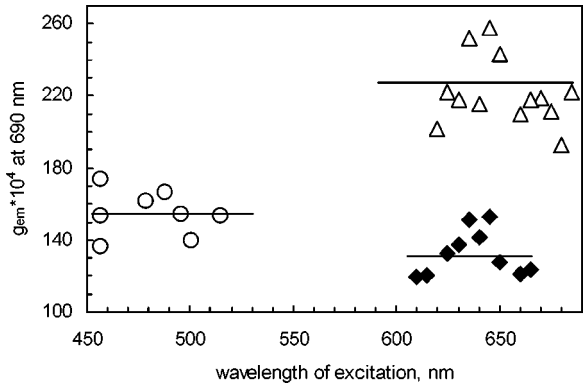

Fig. 3. Dependence on the excitation wavelength of chlorophyll fluorescence of CPL at 690 nm for suspension of the isolated pea chloroplast. Open and filled symbols represent setups for 125 and 180°, respectively, between directions of excitation and emission collection. Different symbols correspond to different batches of chloroplasts.

with respect to the direction of the fluorescence collection as shown in Fig. 1. Each spectrum in Fig. 2 is a mean (with standard deviation) after averaging the CPL spectra (spectral resolution of 5 nm), that were obtained after excitation at six wavelengths in the Soret band and from 615 to 690 nm with increment of 5 nm for the Q_y band.

The plot of the CPL intensity at 690 nm versus the wavelength of excitation for both the Soret and Q_y bands (excluding the 690 nm excitation; Fig. 3) indicates that the distribution of the CPL values inside each band was random. This was the case for both setups with the angle between excitation and emission collection being either 125 or 180°. Hence, the CPL was independent of the excitation wavelength. Because of this invariance, the CPL spectra measured at different excitation wavelengths were averaged separately for the Soret and Q_y bands in Fig. 2.

The spectra closely resemble the CPL spectra obtained before [13,14] (spectral resolution of 20 nm, excitation wavelength 436, 180° angle between directions of excitation and detection). The present CPL intensity $g_{em} = (154 \pm 4) \times 10^{-4}$ at 690 nm (excitation in the Soret band) is within the range of values obtained earlier at 20-nm resolution (g_{em} from 90×10^{-4} to 170×10^{-4} depending on the sample [13,14]).

Figs. 2 and 3 show that upon excitation in the Q_y band, the CPL intensity was higher, $g_{em} = (225 \pm 7) \times 10^{-4}$, than upon excitation in the Soret band for 125° setup. When the 180° setup was used, the g_{em}-factor of $(134 \pm 12) \times 10^4$ was significantly lower. In these three cases, however, different chloroplast preparations were used. When CPL was measured for chloroplasts from the same batch, no difference in the CPL intensity at 690 nm was found after excitation at either 436 or 635 nm (Table I). This was the case for chloroplasts both in suspension and

Table I. The g_{em}-Factor at 690 nm of Pea Chloroplasts Magnetically Oriented in a Polyacrylamide Gel or in Suspension

Buffer	Excitation, nm	Angle[a]	∥	⊥	Random
Standard, suspension	635	90			108.1 ± 1.2
Standard, PAAG	635	90	35.1 ± 4.1	18.4 ± 1.3	21.6 ± 2.8
Tricine 20mM, pH 7.6	635	90	0.9 ± 0.6	-3.9 ± 1.5	1.0 ± 0.9
Standard, suspension	436	90			111.9 ± 1.6
Standard, suspension	436	180			122.5 ± 2.5
Standard, PAAG	436	90	36.6 ± 1.8	19.9 ± 2.8	24.2 ± 1.3
Standard, PAAG	436	180	26.1 ± 2.9	95.4 ± 1.8	52.1 ± 2.3
Tricine 20mM, pH 7.6	436	90	2.4 ± 1.3	-2.2 ± 2.7	-2.5 ± 1.3
Standard, suspension	CD ratio[b]	180			-0.69 ± 0.01
Standard, PAAG	CD ratio[b]	180	0.22 ± 0.04	-0.58 ± 0.07	-0.17 ± 0.04

Note. ∥ and ⊥, excitation light beam was parallel or perpendicular, respectively, to the magnetic field applied during the polyacrylamide gel formation as shown in Fig. 4.

[a] Angle in degrees between excitation beam and the direction of emission collection (see Fig. 5). Random orientation is supposed to occur in the absence of a magnetic field. Standard buffer consisted of 20 mM tricine-NaOH, 0.4 M sorbitol, 5 mM $MgCl_2$ and 10 mM KCl, pH 7.6. Each figure is an average value of 3–7 samples with standard deviation.

[b] Ratio of minimum (negative CD band) to maximum (positive CD band) of the CD spectrum in Q_y absorption band (see Fig. 5A).

in PAAG (after alignment) and was independent of the angle between excitation and emission collection directions.

Therefore, the position, shape, and intensity of the CPL spectrum of pea chloroplasts do not vary in the range from 430 to 690 nm.

Negative CPL Band

The high spectral resolution and the narrow excitation bandwidth in the present study have allowed detecting a small negative CPL intensity at 665 nm of $(-8.5 \pm 1.2) \times 10^{-4}$ (Fig. 2), which was impossible to detect before when a resolution of 20 nm was used [14]. The CPL intensity of this negative band was also found to be independent of the excitation wavelength both in the Soret and the Q_y regions (Fig. 2) similar behavior of the positive band.

Circular Dichroism of Chloroplasts with Aligned Granal Thylakoids

Fig. 5A shows the CD spectra of pea chloroplasts in suspension and in PAAG, in which granal thylakoids were face- or edge-aligned or randomly oriented with respect to the circularly polarized light beam of the CD spectrometer (see Fig. 4 for chloroplast alignment with respect to excitation beam, magnetic field, and 180° detection). The CD spectra in the Q_y region showed two major bands related to the chiral macroaggregates in the thylakoid membranes [1]. The ratios of the negative and positive band intensities are given in Table I.

When granal thylakoids were face-aligned (the light beam is parallel to the magnetic field), the negative CD band at about 675 nm was transformed into a positive CD band, looking like a strong shoulder. This closely corresponds to the data reported for magnetically aligned chloroplasts [5]. For randomly oriented grana, either in suspension or trapped in the PAAG, the CD spectrum and the intensity ratio were intermediate between the edge- and face-aligned ones indicating that the CD of randomized granal thylakoids is a superposition of that of edge- and face-aligned grana. It should be noted that the CD variations are mainly related to the changes in the negative band intensity while the positive band changes to a lesser extent.

The embedding of chloroplasts with randomly oriented granal thylakoids in the PAAG resulted in a reduced CD intensity and smaller negative-to-positive band ratio when compared with the thylakoids in suspension. Similar changes were reported and attributed to a partial perturbation of the thylakoid membrane by the PAAG [19].

CPL of Aligned Granal Thylakoids

Fig. 5B and Table I show CPL spectra and averaged g_{em}-factor values for chloroplasts either in suspension or trapped in a PAAG with granal thylakoids oriented randomly or face- and edge-aligned with respect to the direction of the excitation light beam (see Fig. 4 for the alignment definitions). The CPL spectra were measured with a resolution of 20 nm in this case masking the 665-nm negative band detected with a resolution of 5 nm as described above (Fig. 2). However, it is evident that the spectra

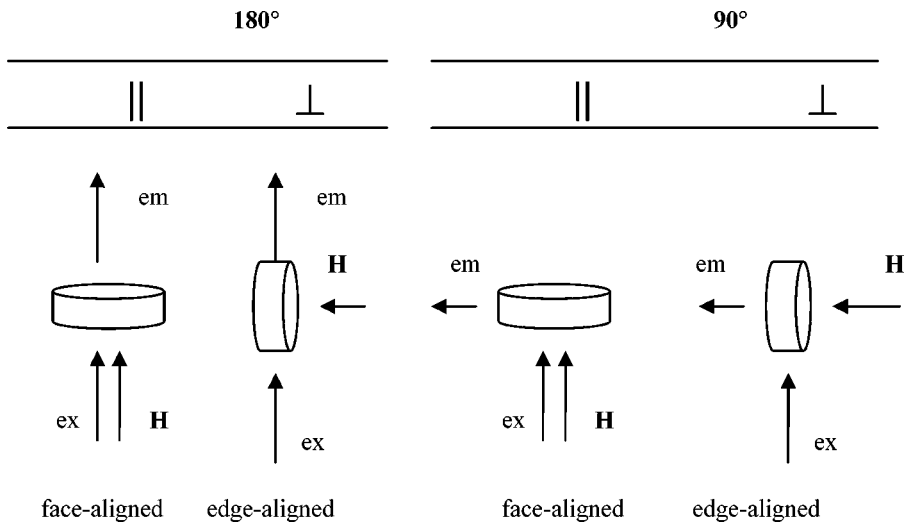

Fig. 4. Mutual orientation of excitation (ex), emission collection (em), and magnetic field (H) directions in experiments with chloroplasts trapped in a polyacrylamide gel. The "puck" indicates a thylakoid membrane plastid where a face side of the plastid is the top or bottom, and the edge side of the plastid is the side of the puck.

measured with lower and higher resolution are not significantly different and both have a maximum at 690 nm, which is used to characterize the CPL behavior.

The g_{em}-factor for granal thylakoids depended on the thylakoid alignment with respect to the excitation beam and direction of emission collection (Table I). Firstly, chloroplasts trapped in PAAG in tricine without magnesium and sorbitol, showed a g_{em}-factor value close to zero (within the error of measurement) indicating the absence of chiral macroaggregates in the sample. At the same time, in sorbitol and magnesium when the LHCII macroaggregates are known to exist, these values were positive. Such effects were reported earlier [14]. No negative CPL of isolated chloroplasts at 690 nm was detected. Hence in PAAG, CPL reflects chiral macroaggregates similar to those in suspension [13,14].

Secondly, the CPL of randomly oriented chloroplasts in the standard buffer suspension did not depend on the direction of emission collection. Chloroplasts trapped in PAAG in standard buffer (sorbitol, magnesium, and potassium) with randomly oriented granal thylakoids had a g_{em}-factor that was lower than that for a suspension of the same buffer. Probably, the level of the chiral macroorganization of LHCII in PAAG was lower than in suspension. This correlates with the smaller CD signal in the red region and lower negative to positive CD band intensity ratio (Table I).Thus, PAAG influences the macroorganization of light-harvesting antenna in thylakoid membranes.

Thirdly, in all cases, the g_{em}-factor for the randomly oriented granal thylakoids was intermediate between the values for the face-aligned and edge-aligned grana. This was independent of the fact whether the emission was collected at 180 or 90°. Hence, CPL of randomly oriented granal thylakoids should be a superposition of CPL of face- and edge-aligned grana similar to CD (see above).

Fourthly, at the 180° collection of luminescence, which relates to the CD measurements, the CPL intensity was higher for edge-aligned and lower for the face-aligned granal thylakoids with respect to the excitation light beam (see alignment in Fig. 4). This fully agrees with the CD data (Table I) according to which the negative-to-positive band ratio was higher for edge-aligned and lower for face-aligned chloroplasts. When the emission was collected at 90°, the maximal CPL intensity was found for face-aligned granal thylakoids. Thus, CPL was maximal when excited to the edge of the thylakoid and collected also from the edge side (180° configuration) and minimal when excited to the edge and emission is collected from the face side of the chloroplast (90° configuration). The intermediate intensity was obtained with face-aligned excitation and either edge-side (90° configuration) or face-side detection (180° configuration).

DISCUSSION

CPL Does Not Depend on The Excitation Wavelength

Chlorophyll molecules fluoresce from the lowest Q_y singlet electronic level even when being excited in the Soret band because of the internal conversion of the

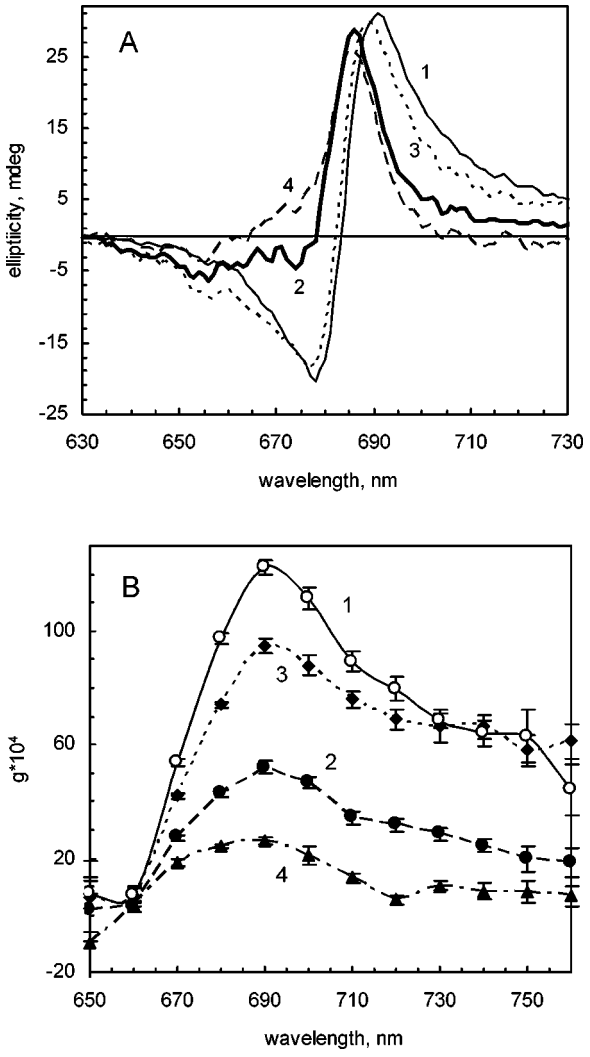

Fig. 5. CD (panel A) and CPL (panel B) spectra of randomly oriented (2) or edge (3) and face (4) aligned chloroplasts in 5% polyacrylamide gel or in suspension (1) in the standard buffer (0.4 M sorbitol, 20 mM tricine, 5 mM $MgCl_2$, 10 mM KCl, pH 7.6). Emission as collected at 180° in respect to the excitation beam.

excitation energy. For isolated LHCII particles, the quantum yield of total luminescence is not a function of excitation wavelength in the 600 to 700-nm range [25]. Chlorophylls of light harvesting antenna are connected via excitation energy transfer and spectral equilibration of excitations is complete within hundreds of femtoseconds [26,27]. Most fluorescence photons are emitted from the equilibrated states [28]. CPL is a weighed part of the total luminescence and hence may also possess these properties. Indeed, our data have confirmed that the CPL of pea chloroplasts in the presence of sorbitol, magnesium and potassium does not depend on excitation wavelength, both in suspension and in PAAG, and both in the blue (430–520 nm) and red (615–690 nm) wavelength regions.

It is important to emphasize that in these wavelength regions, the CD of chloroplasts in suspension and in PAAG vary significantly, even reversing sign. In general, for a single chromophore both the emission g_{em} and absorption anisotropy factor g_{ab} (Eqs. (1) and (2)), are expected to be the same and to be wavelength-independent across a single absorption band [8]. The sign of g_{ab} is necessarily the same as that of CD. In contrast, the CPL values for isolated pea chloroplasts did not change sign and even remained constant across the excitation wavelength range while the CD did change sign. This different behavior of the CPL excitation and the CD spectrum might in principle originate from both artificial effects (photoselection, insufficient alignment of chloroplasts in magnetic field, effect of PAAG) and real chiral properties of the electronic states of chlorophyll chromophore in thylakoids.

Photoselection Is Not the Case

Photoselection, which is the preferential absorption of left- or right-handed circularly polarized light, might affect the emission because of non-zero CD. Even if the excitation is performed with non-polarized light, photoselection can lead to circularly polarized emission, which is not "real" CPL. The fact that CPL does not depend on the excitation wavelength indicates that photoselection via absorption is negligible because otherwise the CPL sign would have followed the sign of CD which is not the case.

It is known [8] that when the angle between excitation and emission collection directions is the magic angle (54.75 or 125.25°), photoselection becomes negligible (theoretically, equal to zero). Our results indicate no effect of the angle between excitation and emission collection on both the CPL spectral shape and the value of the g_{em}. This provides additional evidence for the absence of photoselection.

The absence of photoselection also allows us to conclude that the circularly polarized emission of magnetically aligned chloroplast is not due to an artifact, related to alignment-induced photoselection but should be ascribed to "real" CPL stemming from LHCII chiral macroaggregates.

Two-Band Shape of the CPL Spectrum

In general, for a single chromophore both the emission g_{em} and absorption anisotropy factor g_{ab} (Eqs. (1) and (2)), are expected to be the same and g_{em} is expected to be wavelength-independent across a single absorption

band [8]. The CPL of chloroplasts has more complicated properties; especially it exhibits a negative and a positive band. The CD spectrum of isolated chloroplasts in the Q_y absorption band is split into a positive and a negative part. The CPL spectrum was expected to have a similar structure, but this had not been observed before.

Our new CPL results, which were obtained with a spectral resolution of 5 nm, clearly showed a negative and a positive band. The presence of both a positive and a negative CPL band was also recently reported for pea green leaves [12]. Therefore, the CPL spectrum was now found to be similar to the CD spectrum, the negative and positive bands of which were attributed to different islands inside the chloroplast [6]. In analogy, one would expect that positive and negative CPL signals originate from distinctive parts of chloroplasts.

Effect of PAAG

In our experiments the total chlorophyll luminescence of pea chloroplast samples in 20 mM tricine, pH 7.6 was about 40–45% lower in 5% PAAG than in suspension: the ratio of luminescence intensities in PAAG versus in suspension was 0.60 ± 0.05 and 0.66 ± 0.07 at the 635 and 436 nm excitation, respectively.

In contrast, the chlorophyll luminescence of pea chloroplasts prepared in standard buffer containing sorbitol, magnesium and potassium, was not quenched in a 5% PAAG: the ratio of the total luminescence intensity of chloroplasts in PAAG versus that in suspension at the same concentration of chlorophyll was 1.17 ± 0.06, 1.05 ± 0.08, and 1.14 ± 0.12 for chloroplasts with randomly oriented, face- and edge-aligned grana thylakoids, respectively (635-nm excitation). These ratios were 0.99 ± 0.04, 0.90 ± 0.07, and 0.95 ± 0.08, respectively, upon 436-nm excitation. Therefore, in this case the LHCII particles are organized into stable chiral macroaggregates, the structure of which is not disturbed upon polymerization.

It was proposed before [19] that PAAG affects thylakoid membrane organization, thereby lowering the CD intensity and changing the spectrum. Obviously, APS and TEMED free radicals also affect thylakoids during the polymerization process. Our present CD measurements show a similar effect of PAAG. The overall CD intensity and ratio of the negative and positive band intensities bec**o**me smaller. The CPL signal of randomly-oriented chloroplasts in PAAG was also smaller than of those in suspension. Taken together with the quenching result mentioned above, the lowering of CPL signal in PAAG supports the PAAG-induced perturbation effect on the LHCII chiral macroaggregates, probably, because of some membrane deformation and free radical effects. However, although the effect of PAAG is significant, it should be the same for magnetically aligned and randomly oriented chloroplasts. So the difference between the CPL signals for aligned and non-aligned chloroplasts is not caused by the PAAG effect.

Spatial Anisotropy of Thylakoid CPL

Substantial fluorescence anisotropy of granal thylakoids was observed using linearly polarized luminescence microscopy on thylakoids that were oriented in a magnetic field [18,29]: the luminescence was maximal when the polarization was parallel to the plane of the granum while minimal (almost zero) when the polarization was perpendicular. A preferential orientation of chlorophyll molecules in thylakoids was also shown using different polarized-light techniques [30,31]. So, on average, dipole moments of chlorophyll chromophores also have a preferential orientation with respect to the edge side of the granum. In agreement with the results on grana, it was also reported that LHCII, which is the main constituent of the granum, has its transition dipole moments of the main Q_y band almost parallel to the plane of the membrane [32].

It should be noted that the anisotropy cannot be connected with a well-known sieve effect which may depend on whether the light is incident on the edge or front of thylakoid (variation of the absorption cross section because of the chloroplast space anisotropy): g_{em}-factor is a ratio of fluorescence intensities which is independent on the absorption and also on the excitation wavelength as shown above.

Micro- and macroscopic CD measurements on spinach chloroplasts aligned in a magnetic field and then trapped in PAAG have also demonstrated different CD spectra for edge- and face-aligned grana [6,33]. Our results show a similar anisotropy for CPL: it is higher from the edge side and lower from the face side of the granum. When both excitation and emission were performed from the edge side (180° setup), the g_{em}-factor had the highest value in PAAG (Table I and Fig. 4). At the same time, the direction of excitation seemed to affect the CPL intensity to a lesser extent. We conclude that the anisotropy of both CPL and CD probably result from a preferential orientation of chlorophyll dipole moments in the thylakoids.

CONCLUSIONS

In contrast to the CD spectrum, the CPL spectrum is independent on the excitation wavelength in both the Soret and the Q_y absorption region for chloroplasts in

suspension and PAAG. Photoselection due to chloroplast alignment does not affect CPL. The improved spectral resolution of the measurements revealed a negative CPL band around 665 nm. The CPL signals appeared to be most intense when detected from the edge, reflecting spatial anisotropy of the transition dipole moments of the chlorophyll chromophores.

ACKNOWLEDGMENTS

Prof. E. Haas, Bar Ilan University is acknowledged for enabling the use of the CD and CPL instruments, which were supported in part by the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities. Prof. R. van Grondelle is acknowledged for providing the equipment and lab facilities for a part of CPL experiments at Faculty of Sciences, Vrije Universiteit Amsterdam. We thank Prof. Y. Yeshurun and Dr. X.N.Xu, Institute of Superconductivity, Bar Ilan University, for using the magnetic field equipment. VB was supported by an EMBO long-term fellowship and a travel grant of the European Science Foundation Programme "Biophysics of Photosynthesis." EEG was supported by an EMBO short-term fellowship. M.I. was supported by a training fellowship from the Center of International Cooperation (MASHAV), the Ministry of Foreign Affairs, Israel.

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