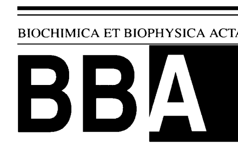
Biochimica et Biophysica Acta 1412 (1999) 173–183



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Xanthophyll pigments in light-harvesting complex II in monomolecular layers: localisation, energy transfer and orientation

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Received 16 November 1998; received in revised form 31 March 1999; accepted 3 May 1999

Abstract

Monomolecular layers of the largest light-harvesting pigment-protein complex of Photosystem II (LHCII) were formed at the argon-water interface. The molecular area of the LHCII monomer in monomolecular layers determined from the isotherms of compression is found to be close to 14 nm^2, which corresponds well to the molecular dimensions of the protein evaluated on the basis of crystallographic studies. Monolayers of LHCII were deposited on a glass support by means of the Langmuir-Blodgett technique and subjected to spectroscopic studies: electronic absorption spectrophotometry and spectrofluorometry. The fluorescence excitation spectra of chlorophyll *a* in monolayers of LHCII were analysed using gaussian deconvolution. Comparison of the absorption and fluorescence excitation spectra enabled calculation of the rate of excitation energy transfer in the system. Excitation energy was found to be transferred to chlorophyll *a* from chlorophyll *b* with 97% efficiency, from neoxanthin with 85%, from lutein with 62% and from violaxanthin with at least 54% efficiency. The analysis of the position of the 0-0 absorption band of the xanthophylls revealed that neoxanthin is located in the same protein environment as lutein but in a different environment than violaxanthin. The analysis of fluorescence excitation spectra of chlorophyll *a* in LHCII, recorded with the excitation light beam polarised in two orthogonal directions, enabled the determination of the mean orientation angle of the accessory xanthophyll pigments with respect to the plane of the sample. The mean orientation of lutein found in this study (approx. 51°) corresponds well to the crystallographic data. Neoxanthin was found to adopt a similar orientation to lutein. The transition dipole moment of violaxanthin was found to form a mean angle of 71° with the axis spanning two polar regions of the protein, perpendicular to the plane of the monolayer, suggesting planar orientation of this pigment with respect to the plane of the thylakoid membrane. These experimentally determined xanthophyll orientations are discussed in terms of importance of peripheral xanthophyll pigments in supramolecular organisation of LHCII and the operation of the xanthophyll cycle within the thylakoid membrane. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Xanthophyll; Light-harvesting complex II; Linear dichroism; Monolayer

1. Introduction

The main light-harvesting pigment-protein antenna complex of Photosystem II (LHCII) is one of the

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PII: S0005-2728(99)00055-9

largest photosynthetic proteins comprising accessory pigments. Its molecular structure was resolved in detail by electron crystallography [1,2]. According to the model based on the crystallographic studies the monomer of LHCII contains about 15 molecules of chlorophyll *a* and *b* and at least two xanthophyll molecules (most probably lutein) that occupy a central position in the complex and adopt an orientation with an angle between 45° and 50° with respect to the axis normal to the plane of the thylakoid membrane [2]. Detailed chromatographic analyses of the xanthophyll composition of trimeric LHCII preparations have shown that two additional xanthophyll pigments are associated with the pigment-protein complex: neoxanthin and violaxanthin, in a mean molar ratio close to 0.33 and 0.17 [3], or 0.5 and 0.12 [4,5] or 0.5 and 0.125–0.165 [6] relative to lutein, respectively. Interestingly, violaxanthin was reported to be almost absent in a monomeric LHCII, suggesting binding of this xanthophyll in a peripheral region of LHCII upon trimer formation [4]. Spectroscopic properties of LHCII have been the subject of numerous precise studies, including the polarised light technique [7–14]. In most cases the pigment-protein was oriented in a squeezed polyacrylamide gel. In the present work, the spectroscopic properties of LHCII are examined in monomolecular layers of the protein deposited onto a solid support by means of the Langmuir-Blodgett technique. The defined orientation of LHCII with respect to the solid support makes it possible to determine a mean orientation of the accessory photosynthetic pigments in the protein on the basis of linear dichroism measurements. In particular, the orientation of the peripheral accessory xanthophyll pigments may be determined by taking into consideration the known carotenoid composition of LHCII and the position of the absorption maxima of these pigments in LHCII determined by Peterman and co-workers [4]. The transition dipole moment of the $1A_g^1$-$1B_u^1$ electronic transition of carotenoids responsible for light absorption in the blue spectral region is determined by the conjugated double bond system and its orientation is very close to the molecular axis of a rod-shaped carotenoid molecule [15]. A recent study shows that the transition dipole moment forms an angle of 15° with the molecular axis of the polyenes defined by the conjugated double bond system [16]. The peripheral xanthophyll pigments of LHCII, particularly those involved in the xanthophyll cycle, have been proposed to play an important role in regulation of an organisation pattern of the protein complexes. This has been shown to be linked with the process of quenching of excess excitations under light stress conditions [17–19]. The stoichiometry of the peripheral xanthophyll pigments in trimeric LHCII, particularly violaxanthin (less than one molecule per monomer of the protein) and the absence of violaxanthin in monomeric LHCII, seems to suggest a role of this pigment in the formation of molecular assemblies of the protein. Thus an investigation of the localisation and orientation of the accessory xanthophyll pigments in LHCII seems to be relevant to the subject of organisation of molecular assemblies of these antenna proteins and overall protection against overexcitation and light-induced damage.

2. Materials and methods

The largest light-harvesting pigment-protein complex of photosystem II (LHCII) was isolated from 10-day-old rye leaves *Secale cereale* L., c.v. Pastar, according to the procedure described previously [20,21]. The chlorophyll *a* to chlorophyll *b* molar ratio in our present preparation is close to the value 1.20 and suggests that seven to eight molecules of chlorophyll *a* and six molecules of chlorophyll *b* were present per single monomer of LHCII. For the purpose of monolayer formation the calculation of the LHCII concentration was based on the assumption that a single monomer of the protein contained six molecules of chlorophyll *b*. HPLC analysis of xanthophyll pigment composition in our preparation revealed a violaxanthin to lutein molar ratio of 0.063 ± 0.004 and a neoxanthin to lutein molar ratio of 0.45 ± 0.03. The composition of the protein in our preparation is very close to that reported elsewhere [3–6]. This implies that on average not more than one molecule of a peripheral xanthophyll is present per LHCII monomer. Monomolecular layers of LHCII were formed at the surface of doubly distilled water and then distilled a third time with $KMnO_4$ to eliminate any organic impurities. LHCII was deposited at the argon-water interface from 25% (v/v) isopropanol solution in water. Such a system was found

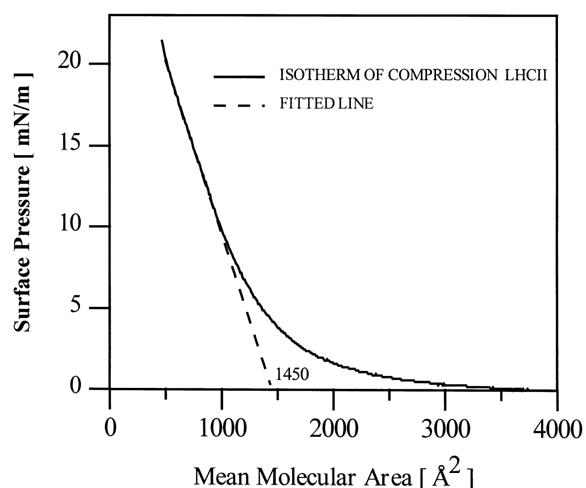

Fig. 1. Surface pressure-mean molecular area isotherm of compression of the monomolecular layer of LHCII at the argon-water interface. The line fitted to the linear part of the curve and extrapolated to the zero surface pressure indicates the mean molecular area of LHCII monomer in the monolayer.

to dissolve large molecular aggregates of the protein but not to induce pigment extraction or disruption of the excitation energy transfer from chlorophyll *b* to chlorophyll *a* within the complex as tested by fluorescence. The concentration of LHCII to be deposited as a monolayer was evaluated on a chlorophyll basis as mentioned above. An initial mean molecular area as large as 5000 $Å^2$ per LHCII monomer was applied in order to assure entire relaxation of the protein at the air-water interface. Monomolecular layers were formed and compressed in a 35 cm long by 10 cm wide Teflon trough at 21°C. A film was compressed along the longer side of the trough with a speed of 0.5 mm/s. This was controlled by a computer. Surface pressure was monitored with a Wilhelmy plate-based tensiometer, produced by Nima Technology (model PS3, Coventry, UK). Monolayers of LHCII were deposited onto a glass support using the Langmuir-Blodgett technique with the FL-1E film-lift produced by Lauda Film (Koenigshofen, Germany). LHCII films were deposited at a constant computer-controlled surface pressure at a speed of 1 cm/min. Monolayers were transferred to a glass support at a surface pressure of 20 mN/m. The deposition ratio was close to 1 under such conditions. In order to avoid pigment-protein destruction, monolayer compression and deposition to a glass support were carried out in darkness and under argon atmosphere. Other details of the preparation of LHCII monolayers were described previously [22]. All spectroscopic measurements of deposited LHCII monolayers were carried out directly after deposition of the monolayer. Absorption spectra were recorded with a Shimadzu 160A-PC spectrophotometer, and fluorescence spectra were recorded with a Shimadzu RF 5001-PC spectrofluorometer equipped with Shimadzu polarisation filter setup. Absorption and fluorescence measurements were carried out at 21 ± 1°C. Fluorescence spectra were corrected for the lamp intensity and for the internal dichroism of the apparatus.

3. Results and discussion

Fig. 1 presents a typical isotherm of the compression of the monomolecular layer of LHCII deposited at an air-water interface. Extrapolation of the linear portion of the isotherm to zero surface pressure yields a mean molecular area close to 1450 $Å^2$, which corresponds well to the molecular dimensions of this protein revealed by crystallographic studies: 30 Å × 50 Å elliptical cross-section [1,2]. Fig. 2 presents

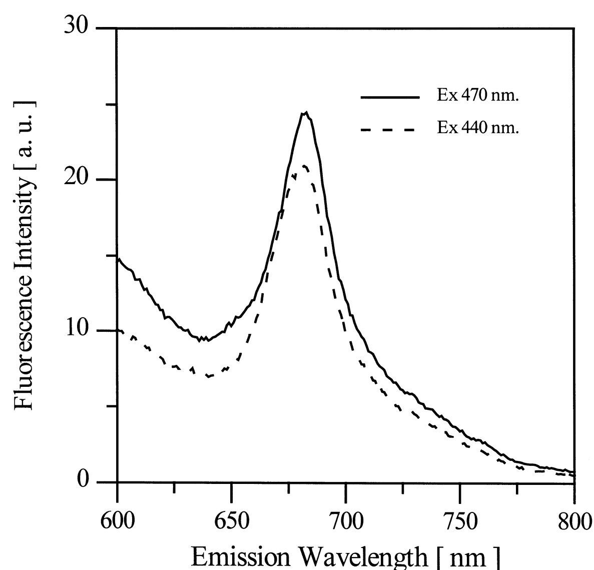

Fig. 2. Emission spectra of chlorophyll *a* fluorescence in LHCII in monolayer excited at 470 nm and 440 nm, the wavelength maxima characteristic of absorption of chlorophyll *b* and chlorophyll *a*, respectively. A single emission band with a maximum at 680 nm is observed in both cases. Excitation and emission slits were set to 3 nm.

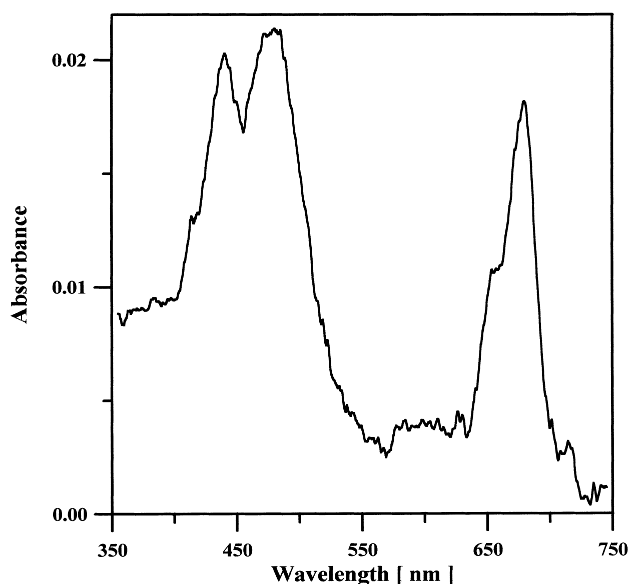

Fig. 3. Absorption spectrum of two monolayers of LHCII deposited at two sides of a glass slide at a surface pressure of 20 mN/m.

fluorescence emission spectra of chlorophyll *a* in the LHCII monomolecular film deposited on a glass support and excited at 440 nm and 470 nm, the characteristic wavelengths of absorption by chlorophyll *a* and chlorophyll *b*, respectively. The single chlorophyll *a* emission band centred at 680 nm, as may be seen in Fig. 2, is an indication of efficient excitation energy transfer from chlorophyll *b* to chlorophyll *a* in LHCII Langmuir-Blodgett film (LHCII-LB film). Unimpaired excitation energy transfer between both chlorophyll forms is an important indication of the native conformation of this antenna protein in the model system. A concentration of isopropanol higher than 30% resulted in a decrease of the excitation energy transfer efficiency from chlorophyll *b* to chlorophyll *a* as demonstrated by the appearance of a second emission band in the region of 650 nm characteristic for chlorophyll *b* (not shown). The absorption spectrum of the LHCII-LB film (see Fig. 3) displays all the characteristic features typical of LHCII. On the other hand, the very low absorbance of a single monolayer makes it difficult to carry out precise linear dichroism measurements. This problem may be overcome by the application of the fluorescence excitation technique. The gaussian deconvolution analysis of the spectra was applied in this study to understand the function of excitation energy transfer from accessory pigments to chlorophyll *a* and to determine the orientation of these pigments. The gaussian components centred at 440 ± 0.3 nm (half-width 26 nm), 382 ± 0.5 nm (half-width 20 nm), 467 ± 0.7 nm (half-width 30 nm), and 414 ± 0.6 nm (half-width 24 nm) correspond to the B_x and B_y absorption bands of chlorophyll *a* and chlorophyll *b*, respectively (see also Table 1). The three bands centred at 494 nm, 510 nm and 486 nm, and half-width 20 nm each represent the 0-0 absorption bands of lutein, violaxanthin, and neoxanthin, respectively, in accordance with the findings of Peterman et al. [4]. Fig. 4 presents the 100% minus transmission spectrum ($100-T$) of the LHCII-LB film in the Soret region and the chlorophyll *a* fluorescence excitation spectrum recorded from the same sample. Excitation spectrum of the fluorescence emission of chlorophyll *a* detected at 680 nm represents the absorption of all the accessory pigments of LHCII involved in the efficient excitation energy transfer, including the xanthophylls. As may be seen from Fig. 4B, the fluorescence excitation spectra

Table 1
Mean orientation of the transition dipoles of photosynthetic pigments in LHCII

Pigment		Position of the band (nm) (cm^{-1})	Mean dichroic ratio $Ex_{\parallel}/Ex_{\perp}$	Mean orientation angle ± S.D. (°)
Lutein		494 (20 242)	0.70	66 ± 1
Violaxanthin		510 (19 607)	0.62	71 ± 3
Neoxanthin		486 (20 576)	0.74	64 ± 4
Chlorophyll *a*	B_x	440 (22 750)	0.76	63 ± 1
	B_y	382 (26 200)	0.74	64 ± 1
Chlorophyll *b*	B_x	467 (21 400)	0.81	61 ± 1
	B_y	414 (24 160)	0.76	63 ± 1

Mean of 12 independent samples.

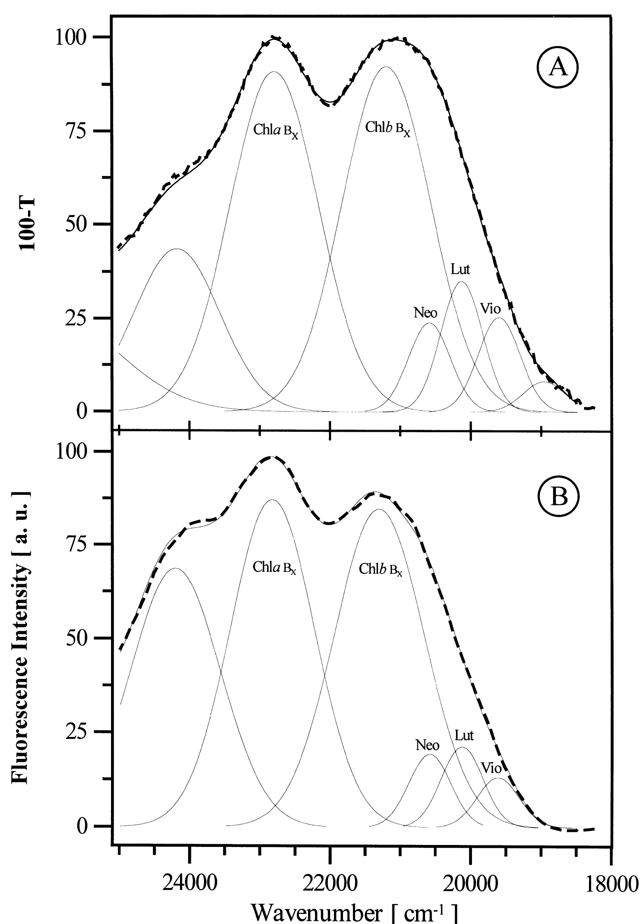

Fig. 4. 100% minus transmission (100−*T*) spectrum of LHCII monomolecular film (A) and chlorophyll *a* fluorescence excitation spectrum in the same LHCII film deposited to the glass slide. Emission was recorded at 680 nm. Excitation and emission slits were set to 3 nm and 10 nm respectively. Original spectra are represented by dotted lines and the gaussian components and the results of spectra reconstruction are represented by solid lines. The 100−*T* spectrum is a mean of five different spectra of the same sample.

reproduced on the basis of the gaussian components fit very well into the experimental results in the long wavelength spectral region (see Fig. 4B). Deconvolution of the spectra with a larger number of components representing the 0-1 and 0-2 transitions of the xanthophylls does not change the results of the calculation, of the xanthophyll orientation based only on the 0-0 transition. Therefore we decided to minimise the number of gaussian components necessary to reproduce the experimental excitation spectra. As may be seen in Fig. 4A, the 100−*T* spectrum could also be successfully reproduced with the same main gaussian components as the fluorescence excitation spectrum. An exception is the long wavelength region, where an additional component centred at 18 953 cm^{-1} (528 nm) had to be considered. This component is not present in the fluorescence excitation spectrum (Fig. 4B), showing that this spectral form is not active in the excitation energy transfer to chlorophyll *a*. The relatively low intensity of the gaussian band corresponding to lutein suggests that the 528 nm band may represent this pigment, present in an environment in the monolayer, which is different to that characteristic of lutein in a native pigment-protein environment (see also below).

The absorption maxima position of a pigment is closely correlated to the polarisability of the environment of the chromophore [23]. Fig. 5 presents such a correlation for 0-0 transition of lutein, neoxanthin and of violaxanthin. First, neoxanthin and violaxanthin show very similar linear dependence of the 0-0 transition versus the polarisability term. Second, both positions of the absorption maxima of lutein and neoxanthin in LHCII point exactly to the same environment, as characterised by the polarisability term close to 0.315 (a refractive index $n = 1.54$). In

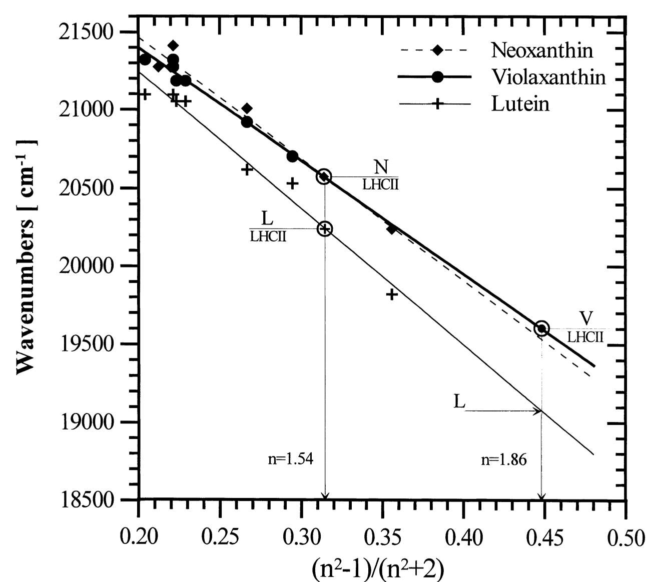

Fig. 5. Position of the 0-0 absorption band of violaxanthin, lutein and neoxanthin in different organic solvents of the refractive index *n* and in LHCII versus the polarisability term. Data for the plot are based on [4] for LHCII, and on ref. measurements [24] and our own measurements for the xanthophylls. See the text for further explanations.

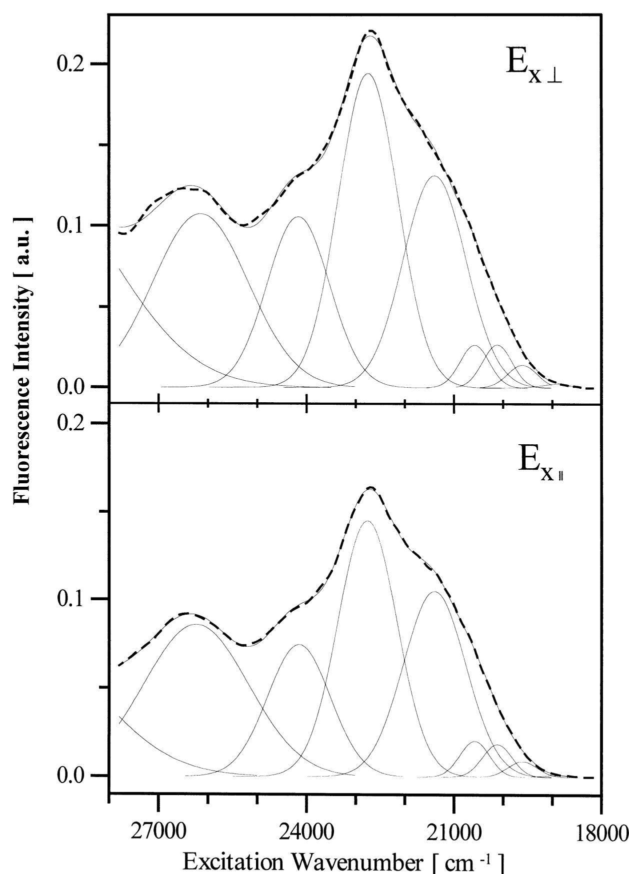

Fig. 6. Fluorescence excitation spectra of chlorophyll *a* emission (at 680 nm) in a monomolecular layer of LHCII deposited on a glass slide at a surface pressure of 20 mN/m and excited with light polarised perpendicular (upper panel) and parallel (lower panel) to the plane of incidence (dashed line). Results of gaussian deconvolution of the excitation spectra are also presented along with the results of the reconstitution of excitation spectra with gaussian components (solid line). Geometric conditions of measurements with polarised light are presented in Fig. 7. Excitation and emission slits were set to 3 nm and 10 nm respectively. The spectra with different polarisation of the excitation beam were recorded separately from the same fixed sample.

contrast, violaxanthin in LHCII is surrounded by a different environment ($n = 1.86$). Interestingly, this interpretation is consistent with the correlation presented in Fig. 5, where the additional long-wavelength absorption band observed in monolayers at 528 nm represents lutein displaced from its original place during sample preparation to the environment originally occupied exclusively by violaxanthin.

Relative mobility of violaxanthin within the thylakoid membrane and weak binding of this particular xanthophyll to the protein suggest that it is most probably located in the peripheral region of the complex. Eventual displacement of neoxanthin to the same region would result in the enhancement of the absorption band attributed to violaxanthin. This is most probably also the case, as there is a relatively high intensity of the violaxanthin band in the $100-T$ spectrum. The comparison of the intensities of the same bands in the $100-T$ spectrum and the fluorescence excitation spectrum in Fig. 4 leads to the conclusion that the excitation energy is transferred from chlorophyll *b* to chlorophyll *a* with 97% efficiency, consistent with the conclusion based on Fig. 2. The efficiency of the excitation energy transfer from the xanthophyll pigments to chlorophyll *a* in LHCII-LB films is lower, calculated as 85% for neoxanthin, 62% for lutein and 54% for violaxanthin.

Fig. 6 presents gaussian deconvolution of the fluo-

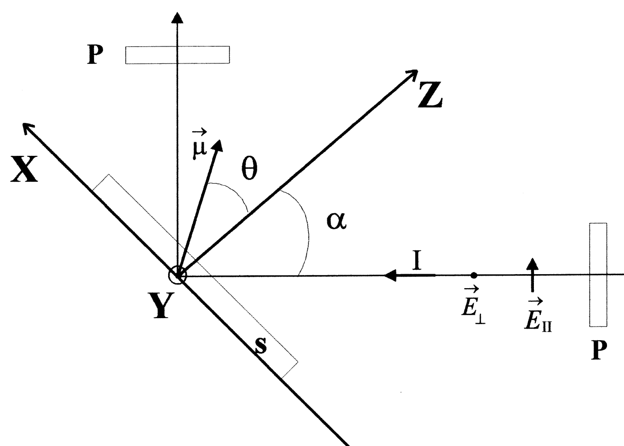

Fig. 7. Geometry of dichroic measurements. S, single monolayer of LHCII deposited to a glass support; I, light beam; $Ex_{\parallel}$ and $Ex_{\perp}$, electric vector of excitation light polarised parallel and perpendicular to the plane of incidence defined by the direction of an incident light beam and the axis normal to the sample. The plane of incidence is represented in Fig. 4 by the plane of the drawing. The Z-axis is normal to the plane of the sample, the X-axis is the intersection of the plane of the sample with the plane of incidence, the Y-axis is in the plane of the sample and is perpendicular to the plane of incidence, α is the angle of incidence, 45°. Magic angle-oriented polarisation filter was placed at the front of the fluorescence detector in order to avoid fluorescence excitation spectra distortion due to photoselection. P is the projection of the polarisation filters to the plane of incidence. The plane of this polariser is perpendicular to the emission beam. The direction of transmission of the electric vector of light in this polarisation filter forms the magic angle with axis Y. μ is the projection of the dipole moment of a pigment to the plane of incidence.

rescence excitation spectra of LHCII-LB as recorded with a polarised excitation beam (the geometry of the fluorescence excitation measurements with polarised light is depicted in Fig. 7). The main absorption bands characteristic of the LHCII accessory pigments could be distinguished in the excitation spectra of both polarised and non-polarised light (see Fig. 4). On the other hand, the proportion of the gaussian components differs, which represents photoselection due to the orientation of the pigments in the sample and the polarisation of the excitation beam. As was shown in our previous report [25], the difference in the absorption of light polarised in two orthogonal directions, parallel (‖) and perpendicular (⊥) to the plane of incidence, can be expressed by the dichroic ratio (r) in the following form:

$$r = A_{\parallel}/A_{\perp} = 1/2 + \cot^2 \nu \quad (1)$$

where ν denotes the mean angle formed by the axis normal to the plane of the sample and the transition dipole moment corresponding to light absorption by a chromophore. Eq. 1 follows directly from Eq. 1 of [25] after substitution of 45° as the angle of incidence α (see Fig. 7). A typical dichroic ratio spectrum of our preparations is shown in Fig. 8. In general, except from the fine structure, the shape of the dichroic ratio spectrum observed in the present study is close to the linear dichroism spectra of LHCII oriented in a gel [7,8,14]. The different method to align LHCII in a sample and the different orientation of the plane of the sample with respect to the measuring light beam (45° in the present study versus normal orientation in the previous one) are most probably responsible for certain differences observed. Table 1 presents the orientation angle values of the LHCII pigments calculated on the basis of the dichroic ratio values determined for all the gaussian bands presented in Fig. 6.

The deposition of the polar part of the protein onto the polar surface of the glass support using the Langmuir-Blodgett technique assures that the orientation angles determined in this model study represent an orientation of the accessory xanthophyll pigments in LHCII similar to that in the natural thylakoid membrane. The mean orientation of both axes x and y of chlorophyll a and chlorophyll b (61–64°) corresponds well to the general picture based on crystallographic studies [1,2]. The same holds true in the case of lutein, which was assumed to occupy a

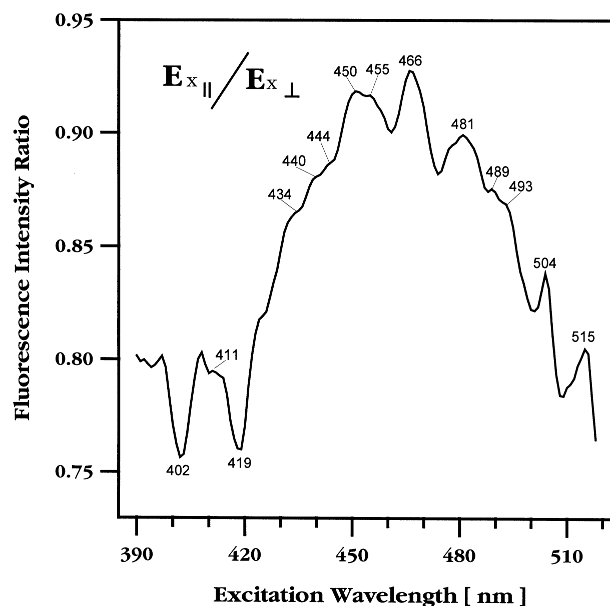

Fig. 8. Dichroic ratio spectrum of chlorophyll a fluorescence excitation in LHCII monomolecular layer deposited on the glass slide recorded under geometric conditions presented in Fig. 6. Some characteristic wavelengths are indicated.

central position in the LHCII monomer and is oriented at an angle between 45° and 50° with respect to the axis of the complex. In this case, we have taken into account that the transition dipole moment forms an angle of 15° with the symmetrical molecule [16], as indicated in Fig. 9A. On the other hand, the asymmetric localisation of the double bonds in the terminal rings of lutein (4′-5′ bond not conjugated to the entire system) most probably results in an angle, between the molecular axes and the transition dipole moment, of less than the 15° reported for linear polyenes [16]. This implies that on the basis of the crystallographic data (molecular orientation 45°–50° [2]), one may expect the orientation of the transition dipole moment of lutein to be between 60° and 65° or slightly lower. This assumption for lutein corresponds well to the results presented in Table 1. The determined orientation angle of the transition dipole moment of neoxanthin is very close to that of lutein. This fact along with a very similar localisation of both lutein and neoxanthin, concluded above, indicates that these pigments may play similar roles in LHCII. They supply energy to chlorophyll and play important roles in photoprotection and in the structural stabilisation of the complex. In fact, it was

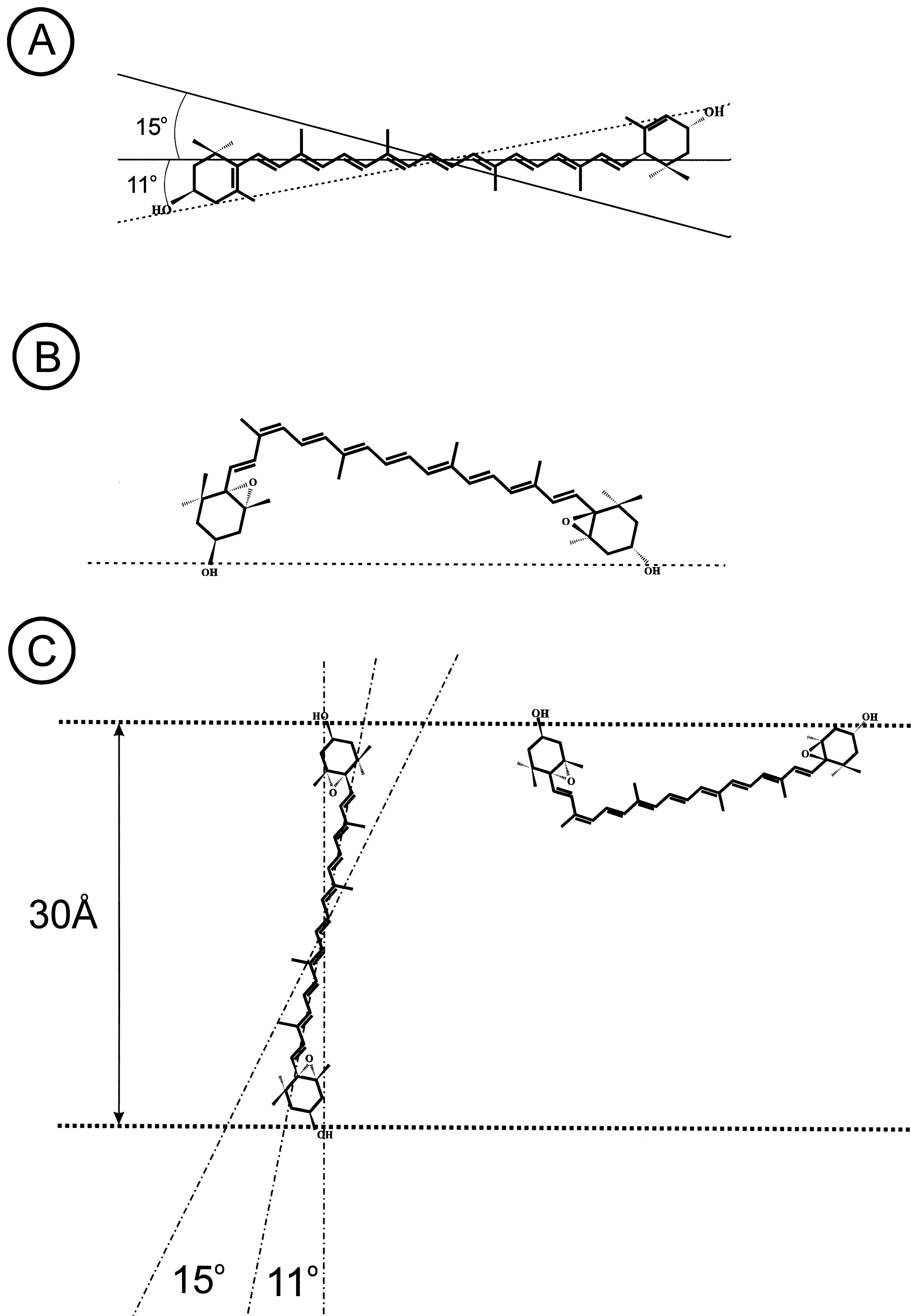
A
15°
11°
HO
OH
B
O
O
OH
OH
C
30Å
HO
O
O
OH
OH
O
O
OH
15°
11°

Fig. 9. (A) Chemical structure of lutein with schematic representation of the characteristic molecular axes: orientation of the dipole moment transition tilted by 15° with respect to the molecular shape defined by the direction of the conjugated double bond system and axis connecting polar groups tilted by 11°. (B) Stereochemical structure of 9-*cis*-violaxanthin. (C) Model of the hydrophobic core of the lipid membrane (30 Å thick) with incorporated *all-trans*-violaxanthin and 9-*cis*-violaxanthin. The characteristic axes are indicated. See the text for further explanations and discussion.
←

reported that lutein might be successfully replaced by neoxanthin in order to reconstitute LHCII [26]. According to this report neoxanthin appeared to be the only xanthophyll pigment necessary for the functional reconstitution of the complex. It was also reported that the lutein-deficient *Arabidopsis* mutants were able to synthesise functionally active pigment-protein complexes containing increased amounts of the other xanthophyll pigments [27]. As seen in Table 1, the orientation of the transition dipole moment in violaxanthin (71°) is clearly larger than that of lutein (66°). Assuming the peripheral localisation of violaxanthin with respect to the protein, a vertical orientation with respect to the membrane might be expected, similar to the orientation of the same pigment is a lipid membrane [25,29]. The almost perpendicular orientation of the xanthophyll pigments with respect to the plane of the thylakoid membrane may be expected from the comparison of the thickness of the hydrophobic core of the membrane (approx. 30 Å [1]) and the distance of the most separated polar groups in the xanthophyll molecule (close to 30 Å [28]), provided that in the thylakoid membrane this group of pigments remains in close contact with the lipid environment. The principles of such a localisation and orientation of xanthophylls in thylakoid membrane is presented in Fig. 9C. Xanthophyll pigments incorporated into lipid membranes, in general, adopt an orientation determined by the condition of localisation of opposite polar groups within the two opposite polar zones of a lipid bilayer [25,28–30]. This should result in the orientation of violaxanthin close to 25°, which is the angle of the molecular axis connecting the opposite hydroxyl groups and the direction of the transition dipole moment (see Fig. 9C). Interestingly, violaxanthin [31,32] as well as neoxanthin [33] have been reported to adopt a *cis* conformation in LHCII. The molecular distance of the most separated polar groups in the *all-trans* xanthophyll molecule (30 Å [28]) is enough to span the hydrophobic core of the thylakoid membrane (30 Å [1]). In order to locate the polar groups in the opposite head-group regions of the bilayer the pigments must be in the *all-trans* conformation. This criterion does not hold for a pigment in its *cis* conformation. In the case of violaxanthin, it is not possible to place the two opposite side-located polar groups of the pigment in contact with the same polar membrane zone. A *cis* conformation shortens the distance between the opposite side-located polar groups of a xanthophyll molecule, but on the other hand makes it able to be anchored in the same polar region of a membrane, as proposed in early work by Yamamoto and Bangham [34]. The planar orientation of the molecular axis of violaxanthin connecting the opposite hydroxyl groups will yield a relatively large angle in orientation of the transition dipole moment with respect to the normal axis of the sample (90° minus 15°, see Fig. 9). Such a model is consistent with the violaxanthin orientation found in the

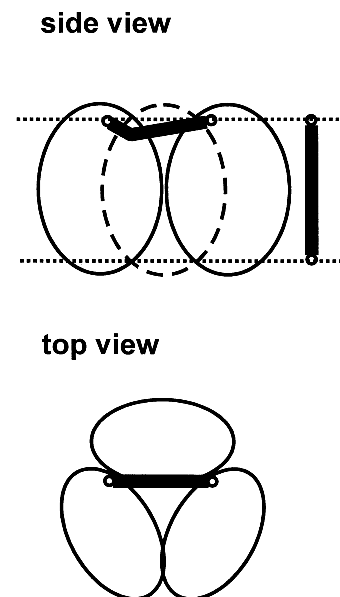

Fig. 10. Model illustrating the localisation of violaxanthin within a trimeric LHCII and the thylakoid membrane. The side view and top view are indicated.

present study (71 ± 3°). This result indicates that violaxanthin in a *cis* conformation is in a protein environment when present in the LHCII complex, whereas, the *all-trans* conformation would highly favour a localisation at the lipid-protein interface. Another indication for such a localisation is the refractive index of the violaxanthin environment in LHCII ($n = 1.86$, see Fig. 5), which is very different from the typical lipid membrane environment ($n = 1.44$ [35]).

As mentioned above, Peterman et al. [4] reported recently that violaxanthin was present only in the LHCII trimers but not in the protein monomers. This important finding agrees with the hypothetical arrangements of violaxanthin proposed in Fig. 10.

The model, assuming a planar orientation of the protein-bound violaxanthin, is also interesting from the point of view of functionality of the xanthophyll cycle within the thylakoid membrane. Deepoxidation of two opposite ends of a violaxanthin molecule in the thylakoid membrane requires operation of a flip-flop mechanism of carotenoids due to the localisation of the deepoxidase enzyme exclusively at the inner side of the membrane [36–38]. On the other hand such a mechanism is highly unfavourable for energetic reasons, as it requires all polar groups of a carotenoid molecule to penetrate the hydrophobic membrane core. A transient interaction of the carotenoid with LHCII in which the pigment molecule adopts *cis* conformation [32] and the planar orientation with respect to the surface of the thylakoid membrane (this report) followed by the detachment of the pigment from the protein environment towards the lipid phase where it adopts roughly vertical orientation [25,29], would potentially facilitate reorientation of violaxanthin within the thylakoid membrane (see Fig. 10). Previously, we reported the epoxidase activity in the LHCII preparation isolated by means of a selective cation precipitation [39]. However, we have not been able to reproduce this reaction with the preparation of the same protein isolated by means of gel separation (G. Jackowski, Z. Krupa, W.I. Gruszecki, not published). This means that the epoxidase enzyme was co-purified along with the LHCII rather than being an integrated part of the LHCII. However, the efficient excitation energy transfer from the newly formed violaxanthin to chlorophyll *a* in LHCII (isolated by means of a selective cation precipitation), as demonstrated by chlorophyll *a* fluorescence excitation analysis, demonstrated the pigment coupling to the antenna protein [32], most probably due to the close interaction of LHCII with the epoxidase enzyme [40]. This pigment coupling reaction, together with the light-induced [31,32] and heat-induced [41] uncoupling of violaxanthin from LHCII, provides a framework for a reversible epoxy-carotenoid binding to the protein that appears to be important not only for the organisation of LHCII aggregates [17–19] but also for the operation of the xanthophyll cycle itself by allowing a flip-flop pigment reorientation in order to complete the deepoxidation reaction. A weak binding of violaxanthin, which could occur, for example, in cavities between the LHCII monomers, is one of the main structural determinants of such a model.

Violaxanthin is a xanthophyll pigment relatively weakly bound to the protein environment. Under strong illumination of the photosynthetic apparatus, violaxanthin becomes detached from the antenna complexes and is subjected to the enzymatic deepoxidation in the thylakoid membrane [36–38]. This implies a relative mobility of this pigment within the thylakoid membrane. As mentioned above, both violaxanthin [31,32] and neoxanthin [33] were reported to adopt a *cis* conformation within LHCII. In contrast to neoxanthin, violaxanthin was, however, reported to be present almost entirely in the *all-trans* configuration after extraction from the complex [42]. It is possible that the *cis* conformation of violaxanthin is the only one which 'fits' the protein and that the process of isomerisation to the conformation *all-trans* is combined with the pigment detachment from the complex. Violaxanthin detachment from the LHCII complex is often discussed in terms of making the pigment available for the enzymatic deepoxidation within the lipid phase of the thylakoid membrane [31,32]. In this context an important open question is whether pigment detachment is followed by alteration of the organisation of LHCII (trimer monomerisation followed possibly by monomer aggregation)? We plan to address this question by studying how the oligomerisation state of LHCII in monomolecular layers may be modified by the presence of exogenous xanthophyll pigments in different stereochemical conformations.

Acknowledgements

This work was financially supported by the State Committee of Scientific Research of Poland under project P04A0212 and by the Swiss National Science Foundation within a co-operation program (CEEC/NIS). The authors are thankful to the Referee of *Biochim. Biophys. Acta* for several valuable suggestions concerning the interpretation of our spectroscopic data and to Trevor Swartz from the Department of Chemistry and Biochemistry, University of California Santa Cruz for the critical reading of our manuscript.

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