Characterisation of LHC II in the aggregated state by linear and circular dichroism spectroscopy

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Abstract

Absorption, linear dichroism and circular dichroism spectroscopy at 293 and 77 K have been used in order to further explore the process of energy quenching in LHC IIb, the main light-harvesting complex of photosystem II. Upon aggregation there was an enhancement of linear dichroism bands in the $Q_y$ absorption region of chlorophyll $b$. The absorption spectrum at 77 K of aggregates revealed new bands around 656 nm and 680 nm, characterised by positive linear dichroism and negative circular dichroism signals. In the circular dichroism spectrum of aggregates a characteristic change was seen in the carotenoid and chlorophyll $b$ regions, an increase of the chlorophyll $a$ transition at 438 nm ($-$) and decrease of the red most negative band at around 677 nm. The amplitude of this band was in a tight correlation with a fluorescence quenching occurring upon LHC II aggregation. A new transition appeared at 505 nm with positive linear dichroism signal. It is suggested that protein aggregation causes a change in conformation and association of some chlorophyll $a$, chlorophyll $b$ and xanthophyll molecules. These features of the linear dichroism spectrum of the aggregates were also detected for thylakoids in which they were particularly enhanced at low pH, suggesting that at least part of the light harvesting complex in the thylakoid membrane is in an aggregated form and the extent of aggregation in vivo can be controlled by the thylakoid pH gradient. © 1997 Published by Elsevier Science B.V.

Keywords: Photosynthesis; Thylakoid membrane; Light-harvesting complex; Linear dichroism; Circular dichroism; Chlorophyll fluorescence quenching

1. Introduction

An important feature of plant photosynthesis is the regulation of the transfer of excitation energy from the light harvesting system of photosystem II to the reaction centre [1]. For PS II in higher plants there is evidence that this control results from the dynamic properties of the peripheral light harvesting pigment protein complexes [2–4]; these are called LHC IIa, LHC IIb, LHC IIc and LHC IId [5]. LHC IIb is the major complex, binding about 40% of leaf chlorophyll and is frequently referred to as just LHC II. This LHC II is isolated as a trimer of polypeptides with apparent molecular weights of 25–28 kDa. Each monomer binds 7–8 chlorophyll $a$ and 6 chlorophyll

Abbreviations: LHC II, light harvesting complex II; Chl, chlorophyll; DM, n-dodecyl β-D-maltoside; OD optical density; LD, linear dichroism; CD, circular dichroism; qE, rapidly reversible non-photochemical fluorescence quenching

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b molecules [6,7]. Analysis of the chlorophyll organisation in LHC II trimer has been carried out by low-temperature absorption, linear dichroism, circular dichroism, fluorescence and polarised excitation spectra [8–11]. A number of \( Q_y \) electronic transitions have been characterised and assigned in terms of the orientation of the dipole moments and the excitonic interactions between the pigments.

The dynamic properties of LHC II are observed when trimers form higher order aggregates upon exposure to a variety of conditions and this gives rise to large changes in spectroscopic properties of the bound pigments. Whilst the yield of chlorophyll fluorescence of the LHC II trimer is high, the fluorescence of LHC II in the aggregated state is quenched [12,13]; almost all excitation energy is rapidly dissipated as heat. The physiological significance of this rests upon the evidence that LHC II exists in a partially aggregated state in vivo [14–16] and that regulation of LHC II in vivo is detected as the decline in yield of chlorophyll fluorescence [1]. Therefore it has been suggested that this feature of LHC II is the basis for the control of the light-harvesting function of the photosynthetic membrane [1–4]. Time-resolved fluorescence measurements on aggregated LHC II revealed a rate constant of 110 ps\(^{-1} \) for deexcitation [17]. Measurements of 293 K absorption spectra and 77 K fluorescence excitation spectra have shown that, upon aggregation, the LHC II absorption bands at 435 nm and 470 nm were decreased and that new bands appeared absorbing at 505–540 nm, 640–660 nm and 685–690 nm [13]. In the 77 K fluorescence spectrum of LHC II aggregates a set of new bands around 700 nm appeared, which displays a strong temperature dependence [13,18]. These features indicate that at least some of the electronic transitions of the pigments bound to the protein have been changed by aggregation. Further support for this view comes from Raman spectroscopy which showed new H bond to Chl a and Chl b and a twisting of carotenoid, upon LHC II aggregation [19].

In this paper, LHC II aggregation is investigated by absorption, linear dichroism and circular dichroism spectroscopy at 293 K and 77 K. The results show the appearance of absorption bands at 505 nm, 636 nm and 680 nm, a considerable enhancement of the fine structure of the LD spectrum in the Chl b region and a relatively strong change in the orienta-

tion of some xanthophyll transitions. New signals appear in Soret band region from chlorophyll \( a \) and carotenoid in CD spectra of aggregates suggesting the change in the conformation of these pigments. The extent of decrease in negative CD band at around 680 nm induced by aggregation was in linear correlation with the relative fluorescence yield of LHC II. Comparison of LD spectra for thylakoids and aggregates of LHC II revealed a remarkable similarity between them, particularly in the Chl b \( Q_y \) region, providing further evidence that LHC II in vivo is at least partially aggregated. The presence of zeaxanthin and low pH induced a further increase in the Chl b LD feature.

2. Materials and methods

LHC IIb, hereafter referred to as just LHC II, was prepared by isoelectric focussing following solubilisation of PS II BBY membranes with \( n \)-dodecyl-\( \beta \)-D-maltoside (Sigma) and characterised as described in [13,20]. For preparation of LHC II aggregates with highly reduced light scattering (small aggregates), almost comparable to that for LHC II trimers, the solubilised LHC II was approx. 30 times diluted in 70% glycerol buffer, containing 20 mM HEPES with no DM.

Intact spinach chloroplasts were prepared from either dark-adapted leaves or leaves preilluminated to induce zeaxanthin synthesis [21], and treated to induce non-photochemical fluorescence quenching for 5 min as described in [22]. To prevent reversibility of the quenching during the preparation of samples for LD measurements, the pH of incubation medium was lowered at the end of the treatment from 7.6 to 5.5.

Linear dichroism and circular dichroism spectra were recorded in a home-built spectrophotometer [8]. For LD, orientation of samples was achieved by the two-dimensional squeezing of a 1.25 \( \times \) 1.25 cm polymerised polyacrylamide gel simultaneously in two perpendicular directions into a 1.0 \( \times \) 1.0 cm cuvette. The LD spectrum was obtained from the difference in the absorption spectra with light polarised parallel and perpendicular to the stretching direction of the gel. The composition of the gel was 66% (w/v) glycerol, 14.5% (w/v) acrylamide, 0.5% (w/v) bisacrylamide, 0.05% DM (only for solubilised LHC
II), polymerised with 0.03% (w/v) ammonium persulphate and 0.03% TEMED. For CD measurements at 293K samples were placed in 1 x 1 x 3 cm quartz cuvettes; the samples contained 70% glycerol. 77 K absorption spectra were recorded in a Beckman DU650 spectrophotometer (USA) using an Oxford optical cryostat (UK). Fluorescence and fluorescence excitation spectra were measured in Spex Fluorolog spectrofluorimeter (Jobin Ivon, USA).

3. Results

3.1. LHC II characterisation

The preparation of LHC IIb used in these experiments was prepared by isoelectric focussing and it has no detectable contamination from other Lhc proteins [20] and, because the samples were prepared from dark adapted spinach leaves, the level of phosphorylation is likely to be very low. Upon formation of small aggregates, the fluorescence of the sample was approx. 80% quenched compared to the trimer. The low-temperature fluorescence spectrum of these aggregates was characterised by a maximum at 680 nm and a shoulder at 700 nm (Fig. 1). Electron microscopy of these aggregates showed them to be small two dimensional plates of approx. 30 nm diameter; with a trimer diameter of approx. 10 nm each aggregate would contain approx. 10 LHC II trimers.

3.2. OD spectra

Absorption spectra at 77 K for the aggregated and trimeric states of LHC II and the difference between them are shown in Fig. 2, for Soret band and Fig. 3, for red region. The changes upon aggregation in the low-temperature fluorescence spectrum of these aggregates was characterised by a maximum at 680 nm and a shoulder at 700 nm (Fig. 1). Electron microscopy of these aggregates showed them to be small two dimensional plates of approx. 30 nm diameter; with a trimer diameter of approx. 10 nm each aggregate would contain approx. 10 LHC II trimers.

Fig. 1. 77 K fluorescence spectra of LHC II in aggregated (solid line) and trimeric (dashed line) form. Excitation wavelength was 435 nm, excitation optical bandwidth was 3.6 nm, and the emission optical bandwidth was 1.8 nm. The Chl concentration was 6 µM and the DM concentration for trimers was 0.01% and for aggregates was 0.0003%.

7% for the change at 505 nm, 6% at 488 nm and 8% at 437 nm. The red absorption region of the 77 K aggregate-minus-trimer difference spectrum (Fig. 3B) has a strong positive band around 680 nm (approx 25% change in absorption), increased absorption at 642 nm and 655–660 nm (6% change at 656 nm) and negative peaks at 648 nm and 672 nm; the latter being approx. 5% of the absorption. The second derivative spectrum of aggregated LHC II clearly shows an increase in a shoulder around 680 nm (see Fig. 4, arrow). In the difference derivative spectrum aggregated-minus-trimer this feature emerges as a distinct band (Fig. 4B).

The changes in absorption properties of LHC II observed upon aggregation were confirmed by measurements of the fluorescence excitation spectra and calculation of aggregated-minus-trimer difference spectra. In both the Soret band and red region (dotted lines on Fig. 2B and Fig. 3B, respectively) the difference excitation spectrum and the difference absorption spectrum were very similar. The near identity of these spectra conclusively exclude any effect of light scattering artefacts in our experiments. There are slight differences in the 450–470 nm region between Soret band absorption difference spectrum and fluorescence excitation spectrum.
3.3. LD spectra

Fig. 5 shows the LD spectra for LHC II measured at 77 K. Aggregation causes a strong increase in the Soret/carotenoid absorption region. Fig. 5B shows a difference LD spectrum aggregated-minus-trimeric. Upon aggregation a new band appears at 440 nm, apparently originating from Chl a. The structural components of xanthophyll region at 485 and 495 nm become more pronounced and a shoulder at around 505 nm is clearly seen. The positive value of the LD means that the angle of the transition dipole of these bands to the stretching direction of the gel (presumably the plane of the membrane) is smaller than 35° (or larger than the magic angle, 55°, with the normal of the membrane). Upon aggregation the main positive LD band in the red region shifts from 676 nm to 680 nm, corresponding to the absorption band at 680 nm in aggregates (Figs. 3 and 4).

The most conspicuous change in the LD spectrum upon aggregation was observed in the absorption region of the Chl b Q_y transitions (Fig. 5). This Chl b region consists of 642 and 65 nm (+) bands and 648 and 658 nm (−) bands. The inset of the Fig. 5A shows this specific region for small aggregates, trimers and large aggregates. The spectra for small and large aggregates show the same structure, although for small aggregates the amplitudes of 642 nm (+) and 648 nm (−) bands are smaller than for large ones. This suggests that the features which increase upon aggregation are not due to polarised
scattering artefacts [23,24]. There is also a distinct vibronic structure in the spectra for both types of aggregates: 592 nm (+), 606 nm (−), 614 nm (+), 618 nm (−), 622 nm (+), 626 nm (−), 630 nm (+) and 634 nm (−) bands (not shown). The LD features of chlorophylls increased upon aggregation are clearly seen in aggregated-minus-trimer difference spectrum (Fig. 5B). The enhancement of the Chl b region points to a change in the conformation of some Chl b pigments upon aggregation, which could be due to changes in their environment or to excitonic interactions between certain Chl b molecules.

3.4. CD spectra

The circular dichroism spectra for LHC II trimers and aggregates are very different (Fig. 6). A new negative band is formed upon aggregation in the chlorophyll a region around 438 nm. A small negative shoulder at 455–463 nm in the spectrum of trimers became more pronounced in aggregates. Fig. 5B shows the difference CD spectrum aggregated-minus-trimer. A positive band at 472 nm and negative band at around 475 nm appear upon aggregation in the chlorophyll b absorption region. There are also a few bands in the carotenoid region: 485 nm (+), 498 nm (+) in this spectrum.

In the red region of the CD spectrum for aggregates, the negative doublet of bands at 651 is slightly red-shifted and broadened. Further aggregation reveals clearer splitting and a decrease in intensity of this group of bands (not shown). The negative band around 677 nm is significantly decreased, broadened and red shifted by about 3 nm. The change in amplitude of this band was taken as a parameter to com-
pare with the extent of chlorophyll fluorescence quenching occurring during LHC II aggregation. A number of samples were prepared at different detergent concentrations of 0.1–0.0003% of DM. Fig. 7 shows a plot of the amplitude of CD signal at approx. 680 nm and the fluorescence yield of LHC II for four different concentrations of DM. It can be seen that these parameters are in good linear correlation. Similar dependency was observed for the fluorescence yield and the negative CD band at 438 nm (not shown).

3.5. 77K LD spectra for thylakoids

Fig. 8 shows the 77 K LD spectra for thylakoids. These spectra reveal a good resemblance with the LD spectrum of small aggregates of LHC II, particularly in the region from 630 to 680 nm (see Fig. 5), suggesting that the pigment orientation in aggregates of LHC II is similar to that in thylakoid membrane. It was interesting to further explore the possible dynamics of LD structure in this region modulated by conditions known to promote non-photochemical quenching of chlorophyll fluorescence in vivo — zeaxanthin formation and the generation of a ΔpH across the thylakoid membrane [1–4]. Fig. 8 shows LD spectra for thylakoids prepared from leaves which have been either dark-adapted (no zeaxanthin) or light-treated (60% conversion of the xanthophyll cycle pool to zeaxanthin). Whilst the amplitudes of the main maximum at 679 nm are the same, those for Chl b bands are slightly different. For thylakoids containing zeaxanthin the characteristic 642 and 654 nm positive and 648 and 658 nm negative bands are more resolved and the amplitudes of 648 nm (–) and 654 nm (+) bands are increased. There is also slight increase in the width of Chl a band at 679 nm in thylakoids containing zeaxanthin. Clear increases in the amplitudes of carotenoid bands were observed in
Soret region at 485 and 505 nm. The difference LD spectrum shown in Fig. 8B reveals more clearly that changes occurred in zeaxanthin containing membranes. A chlorophyll $a$ band appeared at 442 nm, similar to the 440 nm band observed upon LHC II aggregation (Fig. 5B). In the red region of the difference spectrum bands at 642 and 654 nm also resemble the aggregation LD pattern and also there is a major band at 682 nm consistent with the aggregation-induced change around 680 nm. Illumination of thylakoids to induce $\Delta p$H-dependent non-photochemical quenching brought about a much more pronounced enhancement of the amplitudes of 642 and 652 nm (+) bands and 648 and 658 nm (-) bands around the Chl $b$ region (Fig. 9). These features resemble those observed in the spectrum for large aggregates (see Fig. 5A, inset) and the amplitudes of bands at 642 and 652 nm match that of the shoulder at 662 nm, again in a similar way as in the spectrum of large aggregates.

4. Discussion

The results described in this paper have revealed a strong and specific dependence of the spectral properties of the pigment population in the main peripheral light-harvesting complex of plants upon the aggregation of the apoprotein. The combined measurements of absorption, linear dichroism and circular dichroism provide a sensitive tool for monitoring of the dynamic behaviour of LHC II pigments in relation to the regulation of the dissipation of excitation energy into heat via a change in the state of aggregation. Although the aggregation of LHC II by altering detergent concentration cannot be the same as the changes that may occur upon the induction of energy dissipation in vivo, a number of observations indicate that the modulation of the properties of the bound pigments may be similar in both cases [4].

Significant changes were observed in the Chl Soret and xanthophyll absorption region of aggregated LHC II. In agreement with earlier spectroscopic work [13], a new absorption band was found at about 505 nm, which is most likely caused by red-shifted xanthophyll absorption. It is characterised by a positive LD (i.e., by an orientation of the transition dipole prefer-
entially parallel to the plane of the membrane). As was shown previously [25], absorption bands above 500 nm can be induced in a number of LHC II xanthophylls in water-ethanol mixtures and indicate an aggregation state of carotenoid. Steady-state absorption spectroscopy on thylakoid membranes shows that bands above 500 nm are formed upon illumination and induction of non-photochemical quenching [22,25,26] and therefore have been used as indicators of quenching-related conformational changes in LHC II. Resonance Raman spectroscopy revealed a number of low-frequency vibrations around 950 cm$^{-1}$ upon excitation in the 500–535 nm region (Ruban and Robert, unpublished data). Together with the CD data shown here this suggests that aggregation causes distortion of xanthophyll molecules. The more short wavelength carotenoid transitions also undergo some changes upon aggregation. Absorption bands at 455 and 488 nm decrease in intensity upon aggregation (Fig. 2). As follows from LD measurements the orientation of corresponding transitions is also changed (Fig. 5). CD measurements show that the region from 456 to 498 nm is altered upon aggregation, which could be an indication of conformational change of a carotenoid molecule (Fig. 6). This conclusion is also consistent with Resonance Raman measurements in the 950 cm$^{-1}$ region with 488 nm excitation. These measurements show the appearance of a 951 cm$^{-1}$ band in aggregated LHC II, which suggests a twisted conformation of at least one xanthophyll molecule with an absorption transition around 488 nm [19].

The positive band signs of LD and negative of CD are observed for the Chl $\alpha$ absorption bands around 438 and 680 nm in the LHC II aggregates. The excitation fluorescence spectrum of LHC II aggregates shows that the Chl $\alpha$ Soret region around 435 nm undergoes a decrease in absorption upon aggregation, indicating that the absorption change does not arise from artefacts due to scattering. A similar 438 nm band in the CD spectrum was observed in the experiments of Bassi et al. in the spectrum of aggregates prepared by ultracentrifugation of LHC II solubilised by octylglucoside [27]. It is clear from our experiments that the 438 nm band forms in parallel with changes in the xanthophyll CD region and is independent of detergent type and aggregation method. Low pH and magnesium ions have also been used to induce fluorescence quenching and aggregation and the band at 438 nm was present in all cases (not shown).

Aggregation is also clearly correlated with a change in Qy region for Chl $\alpha$ at around 680 nm. The second derivative of the absorption spectrum of large aggregates revealed a band at 680 nm at 77 K, which is consistent with the observation of a positive band at the same position in the absorption difference spectrum, aggregated-minus-trimer. This band is the same as the 685 nm absorption band observed at room temperature and is seen in the difference excitation spectra for aggregation (Fig. 2). As deduced from LD, this transition is parallel to the membrane plane (Fig. 5). From absorption, LD and CD spectra, it appears that the 680 nm band is formed, at least partially, at the expense of the 676 nm band of trimers. In the CD spectrum of small aggregates, the 677 nm band is reduced and slightly red-shifted compared to trimers. The extent of the decrease in this region was in very close correlation with the decrease in the relative fluorescence yield (Fig. 7). This feature, along with formation of 438 nm negative band, are clear ‘fingerprints’ in the chlorophyll $\alpha$ region of the CD spectrum that are associated with a quencher.

Unlike the preparations studied by Barzda et al. our preparations of aggregated LHC II did not exhibit anomalous CD signals in this region [28,29]. This is referred to as psi-type CD which gives rise to a large (−) CD signals around 680 nm. The psi-type CD requires macrohelical domains which are perhaps only formed in the multilayer lamellar sheets of LHC II used by these authors. Rather special conditions are required to form LHC II aggregates that show psi-type CD and the conditions used here are unlikely to create such higher order aggregates (G. Garab, personal communication). In fact, the aggregates used here are not multilayer lamelli [13], most likely as a result of being formed in the absence of Mg$^{2+}$ [23]. It is important to note however that significant extents of fluorescence quenching are found in these aggregates (Figs. 1 and 7).

It should be pointed out that the 680 nm transition was also observed in trimeric LHC II (Fig. 4 and [8,11,30]). Several possibilities were discussed, among them partial aggregation of samples and, as an alternative, that the 680 nm transition represents the
delocalised state of the trimer [30]. Considering the latter possibility, one may suggest that 680 nm transition should be sensitive to aggregation, probably due to distortion of the coupled state, which probably favours excitation to dissipate efficiently through the vibrational or phonon modes. Alternatively, if 680 nm band requires an involvement of Chl \( a \) absorbing at 676 nm to form aggregates (see Figs. 2 and 5A), then fluorescence quenching can be explained by formation of low fluorescence chlorophyll dimers from 676 nm chlorophylls responsible for the highly fluorescent 680 nm band in trimers [8]. The quenching-associated chlorophyll bands in CD spectrum of aggregates (Fig. 6) could indicate that at least some chlorophyll \( a \) molecules adopt different conformation from others. This may reflect a change in pigment environment, including formation of pigment–pigment interaction. Indeed, some in vitro chlorophyll associates have similar spectral features to those described in this study. Chl \( a \) aggregates with absorption at 685 nm and negative CD at 440 nm were recently observed in aqueous tetrahydrofuran [31]. Water-acetonitrile Chl \( a \) aggregates also possess a distinct negative band in the Soret region and a low fluorescence yield [32]. It is interesting to note that in some previous work similar decreases in the CD signal at 680 nm have been observed with treatment of LHC II with sodium dodecyl sulfate [33]; in this case the loss of this band is not due to aggregate formation, but rather to disruption of pigment interactions by detergent. In both contrasting conditions excitonic interactions and/or Chl conformation giving rise to the CD signal are altered.

In the absorption region of the \( Q_y \) transitions of Chl \( b \) a strong enhancement of the fine structure of the LD spectra was observed upon aggregation of LHC II. The most drastic changes involve the amplitudes of the positive bands near 642 nm and 654 nm and the negative bands at 648 and 657 nm (Fig. 5). Bands around 642 and 656 nm, which correspond to the positive LD transitions, are also seen in the OD difference spectrum aggregated-minus-trimer and the corresponding second derivative spectra (Figs. 3 and 4). The CD in this region seems to be also altered towards better resolution of these components, particularly in large aggregates (not shown), which is consistent with the measurements of Ide et al. [34]. The interpretation of the observed spectral features in Chl \( b \) region of aggregated LHC II is not straightforward: the bands at 640 and 654 nm could have an excitonic origin or they may arise from change in Chl \( b \) environment [35], such as co-ordination of Chl via hydrogen bonding to an aminoacid or to other Chl. This suggestion finds support in Resonance Raman experiments on aggregated LHC II, which revealed formation of a H-bond to a formyl group of small population of Chl \( b \) molecules [19]. It was remarkable to find that in the LD spectra for chloroplast membranes this region of Chl \( b \) is also very pronounced and almost identical to that of small LHC II aggregates (Figs. 5 and 8). Moreover, this LD feature was enhanced in thylakoids showing non-photochemical quenching (Fig. 9), revealing a correlation between fluorescence quenching and LD. Thus, LD is an important indicator of the state of LHC II in photosynthetic membranes along with many other techniques such a Resonance Raman [19], singlet-triplet annihilation [15], CD [36], low-temperature fluorescence [26,37] and fluorescence excitation spectroscopy [37]. Each of these provides evidence that LHC II in vivo exists in a higher order of organisation than the trimeric state. Furthermore, the data provides further evidence for the suggestion that the modulation of the extent of oligomerisation of Lhcb proteins is a key feature of the dynamics of the photosynthetic membrane because it can provide an explanation of the mechanism by which excess excitation energy in the PS II antenna can be dissipated as heat [1–4]. The detection of changes in the LD spectrum of thylakoids associated with \( \Delta pH \) and zeaxanthin, the physiological factors controlling energy dissipation in vivo [4], provide direct evidence in support of this suggestion.

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References


