Crystal structure of plant light-harvesting complex shows the active, energy-transmitting state

Tiago Barros¹, Antoine Royant^{2,3},
Jörg Standfuss¹, Andreas Dreuw⁴
and Werner Kühlbrandt^{1,*}

¹Department of Structural Biology, Max Planck Institute of Biophysics, Frankfurt am Main, Germany, ²Laboratoire de Cristallogénèse et Cristallographie des Protéines, Institut de Biologie Structurale J-P Ebel, UMR 5075 CNRS-CEA-UJF, Grenoble, France, ³European Synchrotron Radiation Facility, Grenoble, France and ⁴Institute for Physical and Theoretical Chemistry, Johann Wolfgang Goethe-University Frankfurt, Frankfurt am Main, Germany

Plants dissipate excess excitation energy as heat by non-photochemical quenching (NPQ). NPQ has been thought to resemble *in vitro* aggregation quenching of the major antenna complex, light harvesting complex of photosystem II (LHC-II). Both processes are widely believed to involve a conformational change that creates a quenching centre of two neighbouring pigments within the complex. Using recombinant LHC-II lacking the pigments implicated in quenching, we show that they have no particular role. Single crystals of LHC-II emit strong, orientation-dependent fluorescence with an emission maximum at 680 nm. The average lifetime of the main 680 nm crystal emission at 100 K is 1.31 ns, but only 0.39 ns for LHC-II aggregates under identical conditions. The strong emission and comparatively long fluorescence lifetimes of single LHC-II crystals indicate that the complex is unquenched, and that therefore the crystal structure shows the active, energy-transmitting state of LHC-II. We conclude that quenching of excitation energy in the light-harvesting antenna is due to the molecular interaction with external pigments *in vitro* or other pigment–protein complexes such as PsbS *in vivo*, and does not require a conformational change within the complex.

The EMBO Journal (2009) 28, 298–306. doi:10.1038/emboj.2008.276; Published online 8 January 2009

Subject Categories: plant biology; structural biology

Keywords: chlorophyll; fluorescence; light harvesting; non-photochemical quenching; photosynthesis

Introduction

Under normal light conditions, plants convert the absorbed solar energy into biologically useful chemical energy by photosynthesis. However, under high-light conditions, the excess excitation energy is dissipated in the form of heat by non-photochemical quenching (NPQ), without the risk of causing damage to the photosynthetic apparatus. The major

light harvesting complex of photosystem II (LHC-II) participates in energy-dependent quenching (qE), the most significant component of NPQ (Ruban and Horton, 1994). The main characteristics of qE are a drop in chlorophyll (Chl) fluorescence yield and average fluorescence lifetime at ambient temperature, and an increase in 700 nm fluorescence at liquid nitrogen temperature (77 K), as observed in isolated thylakoids (Ruban *et al*, 1991; Gilmore *et al*, 1995) or plant leaves (Ruban and Horton, 1994).

The spectroscopic signature of unquenched LHC-II in detergent solution is strong fluorescence at 680 nm with long corresponding lifetimes (Ide *et al*, 1987; Vasil'ev *et al*, 1997; Moya *et al*, 2001; Palacios *et al*, 2002). When LHC-II forms aggregates, either by cation-induced precipitation (Burke *et al*, 1978) or detergent removal, both the fluorescence yield and lifetime drop by an order of magnitude, whereas the 700 nm fluorescence at 77 K increases (Mullineaux *et al*, 1993; Ruban *et al*, 1995; Vasil'ev *et al*, 1997), similar to qE. For these reasons, *in vitro* aggregation quenching has long been used as a model for qE *in vivo*, although their molecular mechanisms are most likely to be different. Quenching in LHC-II or the minor LHCs is widely assumed to be caused by a conformational change that switches the complex from the active, energy-transmitting state to a quenched, dissipative state (Moya *et al*, 2001; Pascal *et al*, 2005; Ruban *et al*, 2007; Yan *et al*, 2007; Ahn *et al*, 2008). As yet there is no conclusive evidence for such a conformational change.

Using mutants of LHC-II that lack the Chl pairs or carotenoids proposed to be involved in quenching (Pascal *et al*, 2005), we show that these pigments have no special role in energy dissipation. The reported change in neoxanthin (Neo) conformation associated with qE (Pascal *et al*, 2005) does not require an internal rearrangement of the rigid LHC-II structure. Calculations indicate that all LHC-II trimers in the crystals are excitonically coupled. The crystals used for determining the structure of the pea complex (Standfuss *et al*, 2005) exhibit strong low-temperature fluorescence with an emission maximum at 680 nm and lifetimes that are very much longer than those for quenched aggregates. Therefore, the structure of the pea complex in our crystals is that of the unquenched, energy-transmitting LHC-II. As all available structures of LHC-II are the same, they all show the unquenched state. qE is probably the result of a close interaction of LHC-II with another pigment protein complex, most likely PsbS, that does not involve a conformational change within the antenna complex. In LHC-II aggregates, this process is mimicked by close interactions of pigments on the outer perimeter of the LHCs.

Results and discussion

With one exception, LHC-II pigments are rigid

The X-ray structures of spinach and pea LHC-II were determined independently from crystals grown either at pH 5.4

*Corresponding author. Department of Structural Biology, Max Planck Institute of Biophysics, Max-von-Laue-Strasse 3, Frankfurt am Main 60438, Germany. Tel.: +49 69 6303 3000; Fax: +49 69 6303 3002; E-mail: werner.kuehlbrandt@mpiibp-frankfurt.mpg.de

Received: 6 August 2008; accepted: 3 December 2008; published online: 8 January 2009

Figure 1 Stereo diagrams of four Chl pairs and closest carotenoids in spinach (lighter colours; PDB code 1RWT) and pea (darker colours; PDB code 2BHW) LHC-II. Phytyl chains have been omitted for clarity. Cyan, Chl *a*; green, Chl *b*; orange, lutein and neoxanthin.

(Standfuss *et al*, 2005) or 7.5 (Liu *et al*, 2004). The question arises whether these structures show the same or different states, and whether they correspond to a quenched or unquenched conformation of LHC-II. If the two structures were to represent different states, significant differences in the arrangement of the LHC-II pigments would be expected. The r.m.s.d. between the C α coordinates of spinach and pea LHC-II polypeptide is 0.35 Å. If the coordinates of the chlorin rings are compared, the r.m.s.d. is only 0.25 Å (Figure 1). The structure of pea LHC-II in two-dimensional membrane crystals (Kühlbrandt *et al*, 1994), although at lower resolution, does not show any significant differences to the X-ray structure of the pea complex (Standfuss *et al*, 2005). The available structures of LHC-II from two different plant species, and especially the position and orientation of the Chl molecules attached to the LHC-II monomer, are thus identical within experimental error and therefore show the same state of the complex.

Resonance Raman spectroscopy suggested that the LHC-II-bound carotenoid Neo is twisted in 3D crystals, aggregates

and leaves under qE conditions, compared with its conformation in the detergent-solubilized complex (Pascal *et al*, 2005; Ruban *et al*, 2007). The Neo twist has been suggested to have a pivotal function in switching the complex from the unquenched to the quenched state (Pascal *et al*, 2005). We prepared LHC-II mutants (Rogl and Kühlbrandt, 1999) (Figure 1; Supplementary Table SI) lacking pigments that have been implemented in the quenching mechanism (Pascal *et al*, 2005) and tested their fluorescence properties upon aggregation. In each case, the 680 nm fluorescence yield at room temperature dropped by at least an order of magnitude (Figure 2), as in native LHC-II, demonstrating that these pigments are not required for aggregation quenching. The Δ Chl 13 mutant was largely deficient in Neo and violaxanthin (Vio) (Supplementary Table SII), demonstrating that neither carotenoid has a specific role in this process. Examining the crystallographic temperature (B) factor, which reflects the relative flexibility of the structure, we found that the hydrophobic core of LHC-II containing the Chls, luteins (Lut) and Vio is the most rigid part of the complex (Figure 3). The only

Figure 2 Room temperature fluorescence emission spectra of refolded LHC-II mutants each lacking a specific Chl-binding site (see Supplementary Table S1) and refolded wild-type complex. **(A)** Refolded complexes in detergent solution (solid lines) and in the aggregated state on the same scale (dashed lines, at the bottom). The spectra of individual Chl variants have not been normalized. **(B)** Same spectra of refolded complexes in the aggregated state as in **(A)** on an enlarged scale.

Figure 3 Stereo diagram of the temperature factor distribution in the structure of spinach LHC-II (PDB code 1RWT). The colour gradient ranges from blue (lower temperature factors, rigid) to red (higher temperature factors, flexible). The only part of the structure with a high temperature factor, indicating significant flexibility, is the protruding part of Neo (arrows). The asterisk indicates Chl 2. Detergent, lipids and phytol chains have been omitted for clarity. The approximate position of the lipid bilayer is indicated in grey.

part with a significant degree of flexibility is the portion of Neo that protrudes into the surrounding lipid or detergent. The Neo conformation is therefore most likely to be sensitive to contacts between LHC-II trimers in aggregates, crystals or in the thylakoid membrane. This would account for any twist observed by resonance Raman spectroscopy, which thus seems to be a diagnostic of close intermolecular contacts, rather than a cause of quenching in LHC-II.

Fluorescence emission of single crystals

We recorded low-temperature (100 K) fluorescence emission spectra of single 3D crystals of pea LHC-II (Figure 4). As each crystal was rotated around an axis perpendicular to the incident laser beam, the emission peak at 680 nm varied from being predominant, as in detergent-solubilized trimers (Figure 4), to virtually absent (Supplementary Figure S1). The intensity of the red-shifted emission peak at ~700 nm showed a similar, roughly orthogonal variation. With the beam focused on the crystal surface, the fluorescence recorded on the same side (90° position in Figure 5A) showed a strong peak at 680 nm. When the detector was placed on the opposite side (270° position in Figure 5A), or the crystal was rotated by 180°, emission at 680 nm was weak or absent (Figure 5B and E), and the broad 700-nm band was observed instead. The converse effect was found when the laser was focused in the crystal interior near its opposite surface (Figure 5C and F). An absorption spectrum of the same crystal (Figure 5D) indicated virtually total absorption

Figure 4 Fluorescence emission spectra of a type-I crystal from native pea LHC-II grown at pH 5.5 at 100 K (red) and of detergent-solubilized recombinant LHC-II recorded at 77 K (green).

between ~640 and 678 nm, due to the extremely high Chl concentration of 170 mM in crystalline LHC-II. The transmittance increased steeply between 678 and 692 nm, being negligible at 680 nm but close to 100% at 700 nm. There was no indication of light-induced radiation damage during our measurements. In fact, several crystals diffracted X-rays to better than 3 Å after being exposed to the intense laser beam for about 1 h (not shown).

The 10 different type-I crystals that were measured showed essentially identical emission spectra, with the same strong

Figure 5 Self-absorption in fluorescence spectra of LHC-II crystals. (A) Schematic diagram of the experiment showing the two directions in which fluorescence spectra were collected, with the detector in two alternative positions at 90° or 270° in the plane defined by the incident laser beam and the rotation axis of the crystal. (B) Fluorescence emission spectra of a crystal in two opposite directions, as defined in (A), with the exciting laser focused on the crystal surface facing the detector at the 90° position. (C) Spectra recorded of the same crystal in two opposite directions as in (B), but with the laser focus near the surface that faces the detector at the 270° position. (D) Absorption spectrum of a LHC-II crystal at 100 K. Individual absorption bands cannot be identified due to the high Chl concentration of 150 mg/ml. The sharp drop in absorption between 680 and 700 nm is evident. (E, F) Detailed drawings of the experiments in (B, C). Depending on the position of the detector (90° or 270°), or on the position of the crystal with respect to the laser focal spot, the 680 nm emission is either reabsorbed or not.

orientation and position dependence, as did all of a similar number of cubic crystals of pea LHC-II measured in the same way (not shown). The large unit cell and symmetry of the cubic crystals (Kühlbrandt, 1987) indicate that they were of the same type as those used by Pascal *et al* (2005) and Yan *et al* (2007). Every crystal could be oriented such that a dominant 680-nm emission band was observed. In other orientations or focal positions, the same crystals showed emission at 700 nm. Fluorescence of crystalline LHC-II therefore does not depend on crystal type, plant species or age. This is consistent with our finding that the structures of pea and spinach LHC-II are identical within experimental error (Figure 1). In this context, it is interesting to note that the fluorescence properties of the crystalline complex are unchanged at a pH where quenching occurs *in vivo*. Polarization effects as an explanation of the orientation dependence of the crystal spectra can be excluded, as an earlier study revealed only minor linear dichroism in LHC-II crystals as a function of crystal tilt (Kühlbrandt *et al*, 1988). The orientation dependence of the LHC-II emission spectrum is thus clearly due to self-absorption. In agreement with our observations, self-absorption is also apparent in the precipitated complex where the 680 nm emission is progressively removed with increasing optical path lengths (Vasil'ev *et al*, 1997). We conclude that the striking disappearance of the 680 nm fluorescence peak cannot be taken to signify that the crystal structure shows a quenched state of the complex, as has been

claimed on the basis of similar observations (Pascal *et al*, 2005; Yan *et al*, 2007; Supplementary Figures S2 and S3).

Fluorescence lifetimes

Apart from the red shift of the fluorescence maximum, earlier assertions that the crystal structure shows the quenched state (Pascal *et al*, 2005; Yan *et al*, 2007) were based on the ~1 ns lifetime of the fluorescence emitted by LHC-II crystals. We carried out a detailed investigation of the fluorescence lifetimes of LHC-II in single crystals, aggregates and detergent solution by time-correlated single-photon counting (TCSPC), using the same experimental device for all measurements. Single crystals at 100 K indicated two main emitting species at 680 and 700 nm with average fluorescence lifetimes of 1.31 and 2.63 ns, respectively (Supplementary Figure S4; Supplementary Table SIII). The average fluorescence lifetime of the total emission was 1.98 ns. Under the same conditions, the average total fluorescence lifetime of LHC-II aggregates was 1.43 ns, but only 0.39 ns for the main 680-nm emission band (Figure 6; Supplementary Table SIII), close to the time resolution limit of our apparatus. The total emission from a frozen drop of dilute LHC-II solution at 100 K indicated two decay components (Supplementary Table SIII), with an average lifetime of 5.22 ns, in agreement with the literature (Palacios *et al*, 2002).

The total emission of LHC-II crystals and aggregates appears overall similar (Supplementary Table SIII). This is

largely due to the 700-nm region where lifetimes are long, and which is hardly affected by quenching or self-absorption. There is, however, a very clear difference in the average lifetime of the critical 680 nm emission, which is much longer than expected for a quenched state in crystalline LHC-II, but consistent with literature values for LHC-II aggregates. At room temperature, the average fluorescence lifetime of crystals dropped to ~ 1 ns, in good agreement with fluorescence lifetime imaging of a spinach LHC-II crystal (Pascal *et al*, 2005). These lifetimes are on average 5–10 times longer than those reported recently for quenched aggregates (van Oort *et al*, 2007). We conclude that the strong 680 nm fluorescence and the long associated lifetimes observed in single 3D

crystals are inconsistent with a quenched state, and indicate that the crystal structure of LHC-II must be that of the energy-transmitting, unquenched complex.

Energy transfer

To understand energy transfer and dissipation in crystalline LHC-II, it is necessary to consider not only intramolecular Chl distances but also those between Chls in neighbouring trimers on the crystal lattice. In our type-I crystals (Standfuss *et al*, 2005), the closest π - π distance of an inter-trimer Chl pair (Chl 2:Chl 2') is 14.8 Å (17.6 Å centre-to-centre; Figure 7), yielding a dipole coupling constant of 11 cm^{-1} (see Materials and methods). In the rhombohedral crystal form (Liu *et al*, 2004), the shortest π - π distance between Chl 8 and Chl 14' is 5.4 Å, well within the range of Chl distances in the LHC-II monomer. The centre-to-centre distance is 12.3 Å, so that the dipole coupling constant is 60 cm^{-1} (Liu *et al*, 2004). Excitonic coupling of the trimers in these crystals is thus about 30% stronger than between monomers in a trimer. From these distances, we estimate Förster transfer rates between LHC-II trimers of 1065 ns^{-1} for rhombohedral crystals and 80 ns^{-1} for type-I crystals (see Materials and methods). In the thylakoid membrane, the shortest centre-to-centre distance between Chls in adjacent trimers would be ~ 13.5 Å (Figure 7), yielding a transfer rate of $\sim 100 \text{ ns}^{-1}$, which evidently allows rapid energy transfer *in vivo*. LHC-II trimers in both crystal forms are thus similarly or more strongly coupled than in the photosynthetic membrane, so that the energy absorbed by any one spreads rapidly to its neighbours.

Compared with single crystals, the fluorescence lifetimes of LHC-II in detergent solution at room temperature are

Figure 6 TCSPC histograms of the fluorescence decays of LHC-II in dilute solution (blue), single crystals (red) and aggregates (black), with corresponding fits for the 680-nm emission band. All experiments were performed at 100 K.

Figure 7 Interactions between neighbouring LHC-II trimers. (A) In the membrane similar to type-I crystals from pea LHC-II, neighbouring trimers are symmetrically related within each crystalline layer on a up and down manner. (B) Model for the *in vivo* interaction within the thylakoid membranes. The same arrangement as in the type-I crystals was used, and one of the trimers was flipped by 180° so that both trimers are pointing in the same direction as in thylakoid membranes. Additionally, we restrained the model so that the distances between atoms that are in close contact is identical in (A, B). This is necessary to avoid clashes between trimers. The centre-to-centre distance between the closest Chls in neighbouring trimers are shown. Grey, polypeptide; cyan, Chl *a*; green, Chl *b*; dark orange, lutein; light orange, neoxanthin; yellow, violaxanthin; pink, lipids.

Figure 8 Energy transfer and dissipation by LHC-II *in vitro*. (A) Solubilized LHC-II trimers in detergent solution are functionally independent and re-emit absorbed light as fluorescence. (B) LHC-II crystals show similar fluorescence, although its intensity and spectral range are modified by re-absorption. Conceivably, occasional tight interaction of LHC-II trimers with one another, for example, at grain boundaries, or with contaminating free pigments can generate quenching centres, to which the energy flows through the excitonically coupled complexes. (C) Randomly compacted trimers in LHC-II aggregates generate a high density of quenching centres, resulting in reduced lifetimes and conversion of the absorbed energy into heat.

longer by a factor of ~ 4 . This is explained by the long distances between emitting trimers and the absence of nearby quenching centres in dilute solution. By contrast, LHC-II trimers on a crystal lattice are in close contact, so that the absorbed excitation energy flows rapidly from one to the other, until it is finally dissipated by randomly present quenching sites. Such quenching centres might arise, for example, through irregular molecular contacts at domain boundaries, occasional free pigments or spurious impurities (Figure 8). The shorter fluorescence lifetimes of LHC-II crystals compared with dilute solution are thus fully accounted for by excitonic coupling. The same would be true of LHC-II reconstituted into proteoliposomes, where indeed similar lifetimes have been measured (Moya *et al*, 2001). The state of LHC-II in single crystals thus seems to resemble its native state in the membrane.

Mechanism of aggregation quenching

The irregular, tight aggregates of LHC-II (Figure 8) produced by detergent removal or salt precipitation are an artificial, non-physiological state of the complex, as are dilute detergent solutions at the other end of the scale. LHC-II aggregates are known to be quenched, with average lifetimes ranging from 0.1 to 0.6 ns, depending on the method of preparation (Vasil'ev *et al*, 1997; van Oort *et al*, 2007). We attribute these short lifetimes primarily to the formation of quenching centres through random, tight interaction of pigments exposed on the outer surface of the complex (Figure 8). This would create a large number of quenching centres that will effectively dissipate the excitation energy in aggregates (Beddard *et al*, 1976; Dreuw *et al*, 2003; van Oort *et al*, 2007).

Our proposed mechanism of aggregation quenching explains the well-documented fluorescence lifetime heterogeneity found in LHC-II aggregates (Barzda *et al*, 2001). This is especially pronounced in aggregates of LHC-II monomers (van Oort *et al*, 2007), in which several more Chls are

exposed on the surface of the complex than in trimers. Moreover, monomer solutions inevitably contain significant amounts of free pigment, giving rise to yet more quenching centres upon aggregation. The increase in the number of quenching centres per unit volume from LHC-II in detergent solution to crystals and finally, aggregates would result in an increase in energy transfer, and easily account for the observed associated reduction in fluorescence yield and lifetimes.

Finally, our model also explains the results of an earlier study (Ruban *et al*, 1995), which found that the drop in fluorescence yield upon LHC-II aggregation is surprisingly reversed at 4 K. This indicates that, at very low temperature, the absorbed energy is lost as fluorescence before it can be transmitted to any quenching centres, which suggests that these must be physically distant from the emitting pigments. If emitter and quencher were located in the same monomer, as postulated by the conformational switch model, quenching should be equally effective at 4 K and at room temperature, which is clearly not the case. Although we cannot rule out the possibility that there might be other quenching mechanisms that are in themselves temperature dependent, our conclusion that controlled energy dissipation plant photosynthesis is not brought about by a conformational switch within the antenna is nevertheless sound. If the atomic structures of the quenched and unquenched states of the complex were indeed different, then LHC-II aggregates or crystals might contain a mixture of both. For crystals this can be excluded, as we show here that the chlorophyll configurations in the two independently determined X-ray structures are identical to within 0.25 Å. This would evidently not be the case if they were to represent mixed occupancies, let alone two different mixed occupancies of two different states. If the LHC-II aggregates were to harbour two different conformational states, then the recovery of the fluorescence yield at very low temperature would not be complete, as the quenched state would remain

quenched at 4 K. This can also be ruled out, as detergent-solubilized LHC-II trimers and aggregates have the same fluorescence yield at 4 K (Ruban *et al*, 1995).

It is worth noting that a conformational switch within the antenna as a quenching mechanism could turn into a major disadvantage *in vivo*, as a large part of the photosynthetic apparatus might become quenched as a result of random changes in the pigment configuration of a single complex. For this reason, light-harvesting pigment-protein complexes need to be rigid, and this is indeed borne out by all known structures of such assemblies.

Implications for the mechanism of qE

A well-documented effect of NPQ is the conversion of Vio to Zea in the xanthophyll cycle. Indeed, there is some evidence that Zea itself may function as a quencher (Dreuw *et al*, 2003; Holt *et al*, 2005; Ahn *et al*, 2008; Avenson *et al*, 2008). However, recent data show that simple substitution of Zea against Vio in isolated LHC-II does not significantly influence the Chl excited state lifetime in the complex (Amarie *et al*, 2007; Bode *et al*, 2008). More recently, it has been proposed that a pH-induced conformational change in PsbS is transduced to the minor LHCs, promoting excitation energy quenching, through a Zea radical cation (Ahn *et al*, 2008). Although a carotenoid radical cation has been observed in isolated minor LHCs enriched in Zea (Ahn *et al*, 2008; Avenson *et al*, 2008), its role in the quenching mechanism *in vivo* is unclear, nor does it account for any contribution of LHC-II in qE.

We show here that a conformational switch in LHC-II can be excluded as part of the quenching mechanism. As the simple substitution of Vio by Zea in the complex does not bring about energy dissipation, the controlled interaction of pigment-protein complexes in the membrane appears to be the most probable mechanism for qE. The likely interaction partner with the LHC-II or the minor LHCs is the PsbS protein, which is required for qE in *Arabidopsis* (Li *et al*, 2000), and has been shown to interact with LHC-II and CP29 *in vivo* (Teardo *et al*, 2007). PsbS is a dimer at neutral pH that dissociates into monomers at low pH, where qE occurs (Bergantino *et al*, 2003), probably in response to the protonation of lumenally exposed glutamates (Li *et al*, 2004). Low pH also triggers the conversion of Vio into Zea in the xanthophyll cycle. The PsbS protein seems to be stable in the absence of pigments (Funk *et al*, 1995; Dominici *et al*, 2002; Bonente *et al*, 2008), although it has been reported to bind Zea *in vitro* (Aspinall-O'Dea *et al*, 2002), which has however been challenged (Bonente *et al*, 2008). Pigment binding by PsbS thus appears to be transient, and may occur only under qE conditions.

On the basis of these considerations, we propose a simple, although speculative, feedback mechanism for qE in thylakoid membranes, based on the mechanism previously suggested by Szabo *et al* (2005). When the lumenal pH drops as a result of high photosynthetic activity, Vio is converted into Zea, which partitions into the lipid phase of the membrane. At the same time, PsbS monomerizes. Presumably, the dimer-to-monomer transition uncovers the Zea-binding site on the hydrophobic surface of PsbS. We propose that Zea is bound in a position where, upon interaction with LHC-II or one of the minor LHCs, it is brought into van der Waals contact with a pigment exposed on the hydrophobic,

membrane-embedded surface of the complex, most likely Chl 2 (or its equivalent in the minor LHCs), which is thought to be the terminal emitter in the antenna. This specific interaction would form the quenching Zea-Chl heterodimer that has been observed by femtosecond time-resolved spectroscopy of thylakoid membranes and has the very short 0.1–1 ps relaxation time expected of an efficient quencher (Holt *et al*, 2005). At lower light levels, the lumenal pH rises, PsbS dimerizes, the released Zea is converted back into Vio and the system reverts to normal photosynthetic activity (Supplementary Figure S5). In LHC-II aggregates, the tight interaction of LHC-II trimers with one another or with pigment impurities mimics this NPQ, although it is then significantly slower. Neither quenching process requires a conformational change within LHC-II.

Materials and methods

LHC-II isolation and crystallization

Pea LHC-II was purified and crystallized as described (Standfuss *et al*, 2005). Briefly, hexagonal plates were obtained by vapour diffusion at 15–22°C at 10–20% polyethyleneglycol 350 monomethyl ether, 10–20% glycerol, 50 mM MES buffer pH 5.3–5.6 and 20 mM sodium chloride. Crystals appeared after 2 weeks and grew to a final size of around $150 \times 150 \times 15 \mu\text{m}^3$ within 4 weeks. Thin hexagonal plates were harvested within 2–4 weeks, washed at least $2 \times$ with a Chl-free cryoprotectant solution (polyethyleneglycol 350 monomethyl ether and glycerol added to the mother liquor at a final concentration of 20% each) and flash frozen in liquid nitrogen or immediately used for room-temperature measurements. Cubic crystals of pea LHC-II were grown as described (Kühlbrandt, 1987). Cryo-loops used for handling the crystals were thoroughly washed with ethanol and water to exclude contamination with free LHC-II or pigments.

Fluorescence measurements of crystals, solutions and aggregates of native LHC-II

Fluorescence data on LHC-II crystals were recorded at the Cryobench of the ESRF, France (Royant *et al*, 2007). Fluorescence lifetime measurements were performed using TCSPC. Histograms were recorded until at least 10^4 photons had accumulated on the channel of highest intensity. A 440-nm laser with a $\sim 15\text{-}\mu\text{m}$ focal spot was used for excitation. A total of ~ 20 crystals were measured. Precise positioning of the excitation spot on the crystal, and of crystal orientation with respect to the incident laser beam was achieved with a computer-operated goniometer head. The wavelength dependence of the fluorescence lifetimes was analysed by placing band-pass filters with 10-nm bandwidth in front of the detector, sampling the 670–750 nm range in 10-nm intervals. For room temperature measurements, a 0.5 μl droplet of cryoprotectant containing one single crystal was sandwiched between two glass cover slips, and sealed by silicone grease to prevent evaporation, taking care not to damage the crystal. TCSPC data were analysed using either FluoTimeFit (Royant *et al*, 2007) or PeakFit (Seasoft Software Inc., San Jose, CA). To ensure that the spectra originated from the crystals and not the surrounding buffer, droplets of the cryoprotectant solution in which the crystals had been washed were checked for fluorescence, but none was detected.

Samples of detergent-solubilized LHC-II with Chl *a*/Chl *b* ratio (*n/n*) of 1.35 were centrifuged for 10 min at 14 000 g to remove any aggregates, immediately before measurement as described above. Chl concentration was measured according to Porra *et al* (1989). For low-temperature measurements, a 0.2 mm nylon loop was used to hold a small volume of the LHC-II solution, which was then frozen in the cryo stream. Ice formation was prevented by adding glycerol to a final concentration of 30%. LHC-II aggregates were prepared by diluting 5 μl of concentrated stock 100-fold with distilled water, and adding MgCl_2 to a final concentration of 50 mM. The precipitate was collected by centrifugation at 14 000 g and washed twice with 50 mM MgCl_2 , before being resuspended in 500 μl buffer (Tris pH 7.5; 10 mM MgCl_2). A cryo-loop was used to hold a small volume of the suspension for low-temperature measurements, as above.

Preparation of refolded LHC-II

Pea LHC-II variants WT, Δ Chl 2 (N183A), Δ Chl 8 (H212A), Δ Chl 12 (E139A) and Δ Chl 13 (Q131A) derived from the *lhcb1*2* gene were expressed, purified and refolded with pigments extracted from spinach leaves as described (Rogl *et al*, 1998) with modifications (Standfuss and Kühlbrandt, 2004). As some mutants yielded only low amounts of trimers, the monomeric fraction was collected from the sucrose gradients and used for the fluorescence measurements of all variants. Pigment analysis was performed by HPLC (Gilmore and Yamamoto, 1991).

Fluorescence measurements on refolded LHC-II

Room temperature emission spectra of LHC-II variants were recorded on a Hitachi F-4500 fluorimeter with the excitation wavelength at 441 nm. For spectra of detergent-solubilized variants, sucrose density gradient fractions were diluted with 50 mM Tris, pH 7.5 containing 0.05% DDM. For spectra of the aggregated variants, the same fractions were diluted with equal amounts of detergent-free buffer with 50 mM MgCl_2 .

Coupling strength and Förster energy transfer rates calculations

A good estimate of the coupling strength V between individual Chls in LHC-II can be obtained from the equation (van Amerongen and van Grondelle, 2001)

$$V = \frac{\alpha \kappa}{R^3} \quad (1)$$

where α corresponds to 90 or 75 nm³ cm⁻¹ for Chla:Chla or Chla:Chlb pairs, respectively. κ is the orientation factor

$$\kappa = \vec{\mu}_1 \cdot \vec{\mu}_2 - 3(\vec{\mu}_1 \cdot \vec{r})(\vec{\mu}_2 \cdot \vec{r}) \quad (2)$$

with $\vec{\mu}_1$, $\vec{\mu}_2$ and $\vec{r}$ being the normalized vectors of the transition dipole moments of the individual Chls and of the centre-to-centre distance. The corresponding Förster energy transfer rates are obtained from

$$K_{ET} = \frac{\beta \kappa^2}{\eta^4 R^6} \quad (3)$$

where η is the refractive index (~ 1.55) and β is 32.26 or 9.61 nm⁶ ps⁻¹ for Chla:Chla or Chla:Chlb pairs, respectively.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Anke Terwisscha van Scheltinga for valuable discussions, Heidi Betz for technical assistance and Philippe Carpentier for help with data fitting. TB is supported by the fellowship SFRH/BD/21440/2005 from Fundação para a Ciência e Tecnologia. AD and JS acknowledge funding from Sonderforschungsbereich 472.

References

- Ahn TK, Avenson TJ, Ballottari M, Cheng Y-C, Niyogi KK, Bassi R, Fleming GR (2008) Architecture of a charge-transfer state regulating light harvesting in a plant antenna protein. *Science* **320**: 794–797
- Amarie S, Standfuss J, Barros T, Kühlbrandt W, Dreuw A, Wachtveitl J (2007) Carotenoid radical cations as a probe for the molecular mechanism of nonphotochemical quenching in oxygenic photosynthesis. *J Phys Chem B* **111**: 3481–3487
- Aspinall-O'Dea M, Wentworth M, Pascal A, Robert B, Ruban A, Horton P (2002) *In vitro* reconstitution of the activated zeaxanthin state associated with energy dissipation in plants. *Proc Natl Acad Sci USA* **99**: 16331–16335
- Avenson TJ, Ahn TK, Zigmantas D, Niyogi KK, Li Z, Ballottari M, Bassi R, Fleming GR (2008) Zeaxanthin radical cation formation in minor light-harvesting complexes of higher plant antenna. *J Biol Chem* **283**: 3550–3558
- Barzda V, de Grauw CJ, Vroom J, Kleima FJ, van Grondelle R, van Amerongen H, Gerritsen HC (2001) Fluorescence lifetime heterogeneity in aggregates of LHCII revealed by time-resolved microscopy. *Biophys J* **81**: 538–546
- Beddard GS, Carlin SE, Porter G (1976) Concentration quenching of chlorophyll fluorescence in bilayer lipid vesicles and liposomes. *Chem Phys Lett* **43**: 27–32
- Bergantino E, Segalla A, Brunetta A, Teardo E, Rigoni F, Giacometti GM, Szabo I (2003) Light- and pH-dependent structural changes in the PsbS subunit of photosystem II. *Proc Natl Acad Sci USA* **100**: 15265–15270
- Bode S, Quentmeier CC, Liao P-N, Barros T, Walla PJ (2008) Xanthophyll-cycle dependence of the energy transfer between carotenoid dark states and chlorophylls in NPQ mutants of living plants and in LHC II. *Chem Phys Lett* **450**: 379–385
- Bonente G, Howes BD, Caffarri S, Smulevich G, Bassi R (2008) Interactions between the photosystem II subunit PsbS and xanthophylls studied *in vivo* and *in vitro*. *J Biol Chem* **283**: 8434–8445
- Burke JJ, Ditto CL, Arntzen CJ (1978) Involvement of the light-harvesting complex in cation regulation of excitation energy distribution in chloroplasts. *Arch Biochem Biophys* **187**: 252–263
- Dominici P, Caffarri S, Armenante F, Ceoldo S, Crimi M, Bassi R (2002) Biochemical properties of the PsbS subunit of photosystem II either purified from chloroplast or recombinant. *J Biol Chem* **277**: 22750–22758
- Dreuw A, Fleming GR, Head-Gordon M (2003) Chlorophyll fluorescence quenching by xanthophylls. *Phys Chem Chem Phys* **5**: 3247–3256
- Funk C, Adamska I, Green BR, Andersson B, Renger G (1995) The nuclear-encoded chlorophyll-binding photosystem II-S protein is stable in the absence of pigments. *J Biol Chem* **270**: 30141–30147
- Gilmore AM, Hazlett TL, Govindjee (1995) Xanthophyll cycle-dependent quenching of photosystem II chlorophyll *a* fluorescence: formation of a quenching complex with a short fluorescence lifetime. *Proc Natl Acad Sci USA* **92**: 2273–2277
- Gilmore AM, Yamamoto HY (1991) Resolution of lutein and zeaxanthin using a non-endcapped, lightly carbon-loaded C18 high-performance liquid chromatographic column. *J Chromatogr A* **543**: 137–145
- Holt NE, Zigmantas D, Valkunas L, Li XP, Niyogi KK, Fleming GR (2005) Carotenoid cation formation and the regulation of photosynthetic light harvesting. *Science* **307**: 433–436
- Ide JP, Klug DR, Kühlbrandt W, Giorgi LB, Porter G (1987) The state of detergent solubilized light-harvesting chlorophyll-*a/b* protein complex as monitored by picosecond time-resolved fluorescence and circular-dichroism. *Biochim Biophys Acta* **893**: 349–364
- Kühlbrandt W (1987) Three-dimensional crystals of the light-harvesting chlorophyll *a/b* protein complex from pea chloroplasts. *J Mol Biol* **194**: 757–762
- Kühlbrandt W, Becker A, Mantele W (1988) Chlorophyll dichroism of three-dimensional crystals of the light-harvesting chlorophyll *a/b*-protein complex. *FEBS Lett* **226**: 275–279
- Kühlbrandt W, Wang DN, Fujiyoshi Y (1994) Atomic model of plant light-harvesting complex by electron crystallography. *Nature* **367**: 614–621
- Li X-P, Björkman O, Shih C, Grossman AR, Rosenquist M, Jansson S, Niyogi KK (2000) A pigment-binding protein essential for regulation of photosynthetic light harvesting. *Nature* **403**: 391–395
- Li XP, Gilmore AM, Caffarri S, Bassi R, Golan T, Kramer D, Niyogi KK (2004) Regulation of photosynthetic light harvesting involves intrathylakoid lumen pH sensing by the PsbS protein. *J Biol Chem* **279**: 22866–22874
- Liu Z, Yan H, Wang K, Kuang T, Zhang J, Gui L, An X, Chang W (2004) Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution. *Nature* **428**: 287–292
- Moya I, Silvestri M, Vallon O, Cinque G, Bassi R (2001) Time-resolved fluorescence analysis of the photosystem II antenna proteins in detergent micelles and liposomes. *Biochemistry* **40**: 12552–12561

- Mullineaux CW, Pascal AA, Horton P, Holzwarth AR (1993) Excitation-energy quenching in aggregates of the LHC II chlorophyll-protein complex: a time-resolved fluorescence study. *Biochim Biophys Acta* **1141**: 23–28
- Palacios MA, de Weerd FL, Ihalainen JA, van Grondelle R, van Amerongen H (2002) Superradiance and exciton (de)localization in light-harvesting complex II from green plants? *J Phys Chem B* **106**: 5782–5787
- Pascal AA, Liu Z, Broess K, van Oort B, van Amerongen H, Wang C, Horton P, Robert B, Chang W, Ruban A (2005) Molecular basis of photoprotection and control of photosynthetic light-harvesting. *Nature* **436**: 134–137
- Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim Biophys Acta* **975**: 384–394
- Rogl H, Kosemund K, Kühlbrandt W, Collinson I (1998) Refolding of *Escherichia coli* produced membrane protein inclusion bodies immobilised by nickel chelating chromatography. *FEBS Lett* **432**: 21–26
- Rogl H, Kühlbrandt W (1999) Mutant trimers of light-harvesting complex II exhibit altered pigment content and spectroscopic features. *Biochemistry* **38**: 16214–16222
- Royant A, Carpentier P, Ohana J, McGeehan J, Paetzold B, Noirclerc-Savoye M, Vernede X, Adam V, Bourgeois D (2007) Advances in spectroscopic methods for biological crystals. 1. Fluorescence lifetime measurements. *J Appl Cryst* **40**: 1105–1112
- Ruban A, Horton P (1994) Spectroscopy of non-photochemical and photochemical quenching of chlorophyll fluorescence in leaves; evidence for a role of the light harvesting complex of photosystem II in the regulation of energy dissipation. *Photosynth Res* **40**: 181–190
- Ruban AV, Berera R, Iliaia C, van Stokkum IH, Kennis JT, Pascal AA, van Amerongen H, Robert B, Horton P, van Grondelle R (2007) Identification of a mechanism of photoprotective energy dissipation in higher plants. *Nature* **450**: 575–578
- Ruban AV, Dekker JP, Horton P, van Grondelle R (1995) Temperature dependence of chlorophyll fluorescence from light-harvesting complex II of higher plants. *Photochem Photobiol* **61**: 216–221
- Ruban AV, Rees D, Noctor GD, Young A, Horton P (1991) Long-wavelength chlorophyll species are associated with amplification of high-energy-state excitation quenching in higher plants. *Biochim Biophys Acta* **1059**: 355–360
- Standfuss J, Kühlbrandt W (2004) The three isoforms of the light-harvesting complex II: spectroscopic features, trimer formation, and functional roles. *J Biol Chem* **279**: 36884–36891
- Standfuss J, Terwisscha van Scheltinga AC, Lamborghini M, Kühlbrandt W (2005) Mechanisms of photoprotection and non-photochemical quenching in pea light-harvesting complex at 2.5 Å resolution. *EMBO J* **24**: 919–928
- Szabo I, Bergantino E, Giacometti GM (2005) Light and oxygenic photosynthesis: energy dissipation as a protection mechanism against photo-oxidation. *EMBO Rep* **6**: 629–634
- Teardo E, de Laureto PP, Bergantino E, Dalla Vecchia F, Rigoni F, Szabo I, Giacometti GM (2007) Evidences for interaction of PsbS with photosynthetic complexes in maize thylakoids. *Biochim Biophys Acta* **1767**: 703–711
- van Amerongen H, van Grondelle R (2001) Understanding the energy transfer function of LHCII, the major light-harvesting complex of green plants. *J Phys Chem B* **105**: 604–617
- van Oort B, van Hoek A, Ruban AV, van Amerongen H (2007) Aggregation of light-harvesting complex II leads to formation of efficient excitation energy traps in monomeric and trimeric complexes. *FEBS Lett* **581**: 3528–3532
- Vasil'ev S, Irrgang KD, Schrotter T, Bergmann A, Eichler HJ, Renger G (1997) Quenching of chlorophyll *a* fluorescence in the aggregates of LHCII: steady state fluorescence and picosecond relaxation kinetics. *Biochemistry* **36**: 7503–7512
- Yan H, Zhang P, Wang C, Liu Z, Chang W (2007) Two lutein molecules in LHCII have different conformations and functions: insights into the molecular mechanism of thermal dissipation in plants. *Biochem Biophys Res Commun* **355**: 457–463