LETTERS

Molecular basis of photoprotection and control of photosynthetic light-harvesting

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In order to maximize their use of light energy in photosynthesis, plants have molecules that act as light-harvesting antennae, which collect light quanta and deliver them to the reaction centres, where energy conversion into a chemical form takes place. The functioning of the antenna responds to the extreme changes in the intensity of sunlight encountered in nature[1–3]. In shade, light is efficiently harvested in photosynthesis. However, in full sunlight, much of the energy absorbed is not needed and there are vitally important switches to specific antenna states, which safely dissipate the excess energy as heat[2,3]. This is essential for plant survival[4], because it provides protection against the potential photo-damage of the photosynthetic membrane[5]. But whereas the features that establish high photosynthetic efficiency have been highlighted[6], almost nothing is known about the molecular nature of the dissipative states. Recently, the atomic structure of the major plant light-harvesting antenna protein, LHCII, has been determined by X-ray crystallography[7]. Here we demonstrate that this is the structure of a dissipative state of LHCII. We present a spectroscopic analysis of this crystal form, and identify the specific changes in configuration of its pigment population that give LHCII the intrinsic capability to regulate energy flow. This provides a molecular basis for understanding the control of photosynthetic light-harvesting.

The reversible switch between these two antenna states of energy harvesting and energy dissipation has been well-characterized physiologically[8]. It is known as non-photochemical quenching (NPQ), because, by decreasing the excitation level, it reduces the yield of (or quenches) chlorophyll fluorescence. NPQ covers a range of responses operating on different timescales and with different strengths of quenching, which tune the antenna to the prevailing light conditions[9]. The antenna is composed of specialized membrane-bound light-harvesting pigment–protein complexes, in which chlorophylls and carotenoids are organized in a very ordered manner at significant density. The precise molecular mechanisms through which the antenna could reversibly switch between fundamentally different states remain controversial[10–12]. In particular, there is little understanding of how pigment function could be altered within these complexes so as to form efficient energy quenchers.

When removed from the photosynthetic membrane, the main trimeric light-harvesting antenna complex, LHCII, is highly fluorescent, indicating a low rate of energy dissipation. The lifetime of the excited state is typically around 4 ns (ref. 13; see also below). However, when the complexes self-associate into oligomers or aggregates, the fluorescence is highly quenched[14], with a range of lifetimes between 0.2 and 1.5 ns (refs 13, 15). The increase in energy dissipation within LHCII oligomers compared to trimers provides a model for understanding *in vivo* NPQ[16], because of the strong similarities between the two quenching processes[2]. In the atomic structure of LHCII, derived from X-ray crystallography[7], the contacts between adjacent trimers within the proteoliposome vesicle are minimal, with only two pairs of chlorophylls in weak van der Waals interaction. No transmembrane domains are involved in the contact (Fig. 1). Therefore, each trimer in the crystal is almost functionally separate. We were therefore surprised to find that the fluorescence of LHCII in these crystals is quenched (Fig. 2). To measure quenching, we have used FLIM (fluorescence lifetime imaging microscopy), a form of laser-scanning microscopy that excites chromophores only within single pixels. For each pixel, a

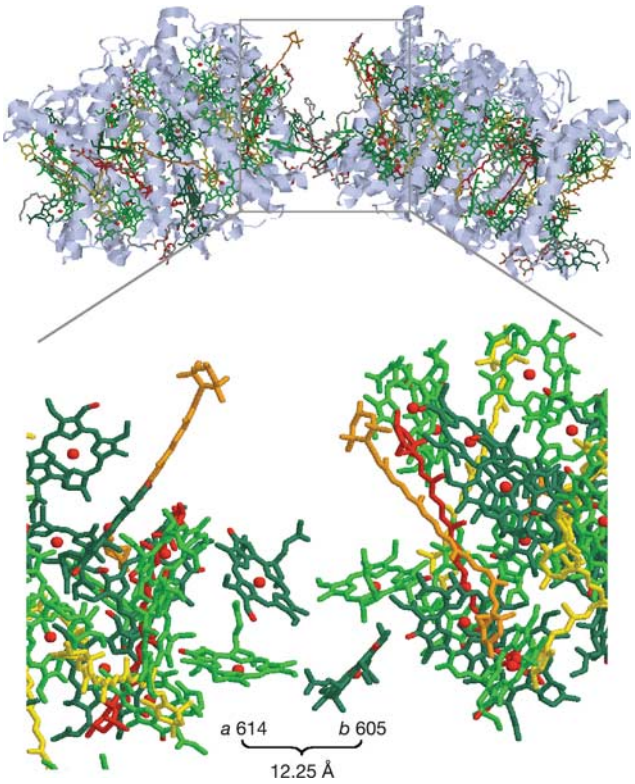

Figure 1 | Crystal structure of LHCII. Two interacting trimers are shown from the icosahedral vesicle in the crystal[7]. The enlargement shows the two pairs of their closest peripheral pigments, Chl*a* 614 and Chl*b* 605.

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fluorescence decay curve is measured by time-correlated single photon counting. Fluorescence lifetimes are independent of chromophore concentration and scatter, thus readily allowing comparison of the fluorescence lifetimes and monitoring of the sample homogeneity. The fluorescence lifetimes of each pixel are displayed to give the false colour image in Fig. 2b. The homogeneity of the image indicates that the lifetime was remarkably uniform and that there is only very minor variation within the crystal (Fig. 2b). The average fluorescence decays from the crystal fit a single exponential and indicate a lifetime of 0.89 ns (Fig. 2c), resembling the oligomeric rather than the trimeric state, indicating the presence of energy traps not present in the trimer. (Using the same apparatus, the lifetime of LHCII oligomers was found to be 0.65 ns.)

In order to identify the molecular basis of the change in excitation lifetime, a spectroscopic analysis of the LHCII crystal was carried out. The fluorescence emission spectrum of crystals showed a strong broadening and shift to the red compared to the spectrum of the solubilized trimer (Fig. 3a, b); it also showed several distinct fluorescence peaks, indicating the presence of different emitting species arising from changes in chlorophyll configuration. This spectrum is similar to that found for LHCII oligomers[17], the higher level of structure found in the spectrum of the crystal reflecting greater homogeneity of the crystal form.

Resonance Raman spectroscopy provided further evidence of major differences in the pigment configuration and pigment–protein interactions in the crystal compared to the trimer. In the spectra produced with 488.0 nm excitation (Fig. 3c), there was a significant enhancement of the modes at 951 and 955 cm^{-1} in the crystals, indicative of a twisting of the neoxanthin molecule as compared to the free trimer[18]. In LHCII crystals these modes are even more

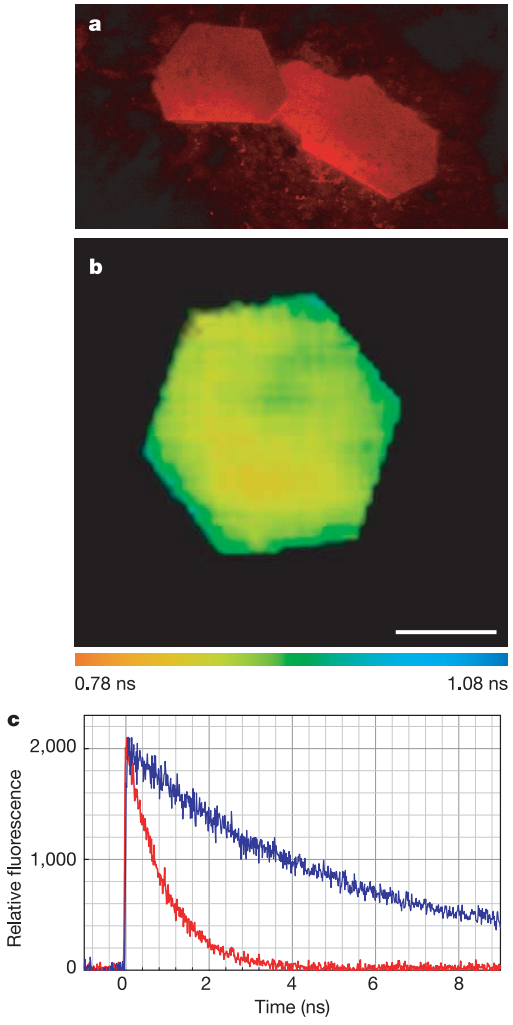

Figure 2 | Quenching of chlorophyll fluorescence in LHCII. **a**, Confocal fluorescence image of LHCII crystals. **b**, Fluorescence lifetime image of an LHCII crystal. The false colours indicate the fluorescence lifetimes in the corresponding pixels, and the colour code is presented below the panel. One picture consists of 64 × 64 pixels. Scale bar, 50 μm. **c**, Fluorescence decay curves of trimers (blue trace) and crystals (red trace), the latter obtained by averaging decay curves from an area of 11 × 11 pixels within the crystal.

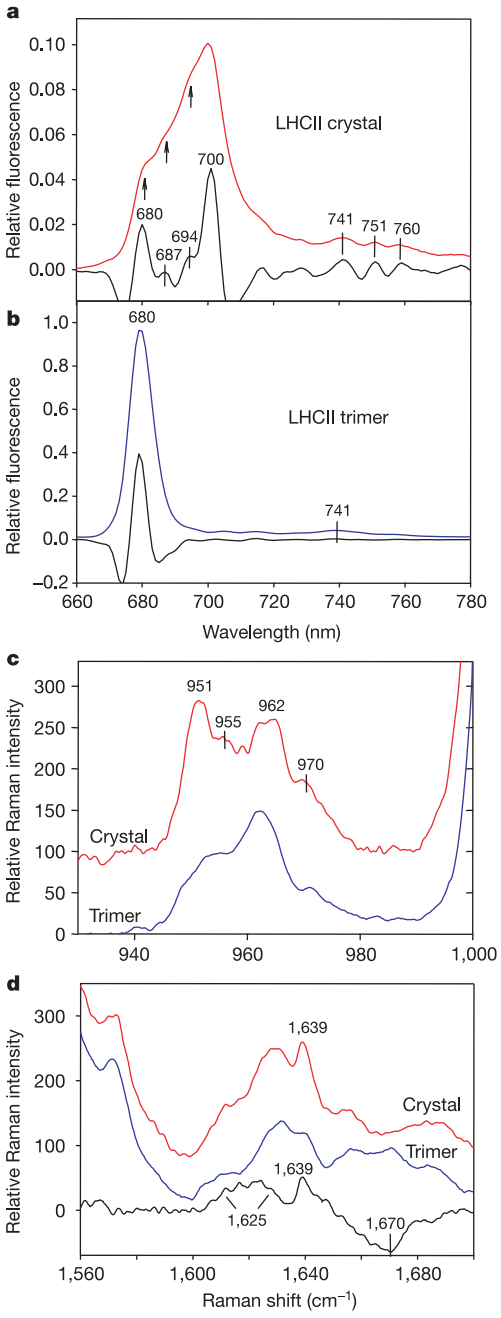

Figure 3 | Spectroscopic analysis of LHCII crystals. **a**, **b**, Low-temperature (77 K) fluorescence spectra of LHCII in crystalline (**a**, red) and trimeric (**b**, blue) forms, and second derivatives (black, multiplied by −1). Arrows show distinct shoulders on the crystal spectrum. **c**, **d**, Molecular configuration of LHCII-bound neoxanthin and chlorophyll *b* measured by resonance Raman spectroscopy of crystals (red) and trimers (blue). **c**, Excitation at 488.0 nm: neoxanthin-selective spectra. **d**, Excitation at 441.6 nm: chlorophyll *b* spectra. Also shown is the calculated difference spectrum (black, crystals minus trimers).

prominent than those previously found in oligomers[18,19]. Similarly, the interactions between the chlorophyll *b* molecules and their environment are very different in the crystal compared to the trimer (Fig. 3d; note in particular the appearance of the prominent mode at 1,639 cm^{-1}). The spectral changes show that the formyl carbonyl group of at least one chlorophyll *b*, free from interactions and in a non-polar environment in the trimer, becomes involved in a hydrogen bond in the crystal[18]. It is of interest that the mode at 1,639 cm^{-1} has an unusually small bandwidth (6–7 cm^{-1}), indicating a high degree of homogeneity in H-bonding strength for this new interaction. These changes in pigment configuration are observed in the near absence of protein–protein contacts and therefore can not be the result of inter-trimer interactions involving pigment molecules bound on the outside of the protein. For instance, the neoxanthin molecule only comes within 17 Å of the neighbouring trimer—the molecular twist cannot arise from any direct interaction (Fig. 1).We conclude that there is a protein conformational change leading to a reorganization of the structure within each of the interacting trimers.

Similarly, neither quenching nor the changes in the fluorescence spectrum can arise from the formation of inter-trimer associations of chlorophyll. The only pair of chlorophylls at close distance between trimers is chlorophyll *a* 614 and chlorophyll *b* 605 (Chl*a* 614 and Chl*b* 605; Fig. 1). The centre-to-centre distance between them is 12.25 Å, and the closest distance is 3.93 Å. It can be calculated that they are only weakly coupled[7]. Therefore, the only likely scenario is that a conformational change in LHCII upon crystallization causes subtle changes in the distances/orientations between its pigments, leading to the formation of quenching sites. The red-shift in fluorescence emission for LHCII crystals (Fig. 3a) suggests that these quenching centres could be chlorophyll dimers or excimers, which are known to have the potential to be powerful quenchers[20]. There are a number of such pairs of chlorophylls observed in the LHCII structure. Figure 4 depicts two chlorophyll–chlorophyll pairs of particular significance. The first, on the stromal side of the complex, has been shown to be the site of lowest energy[21] and can be referred to as the terminal emitter locus, which is an acceptor of the energy delivered to all LHCII pigments. It contains the Chl*a* 611/Chl*a* 612 pair, lutein 620 (Lut 620) and Chl*a* 610 (Fig. 4a). The presence of lutein in this domain is also consistent with the proposed role of carotenoids in energy dissipation[6]. The terminal emitter locus has previously been considered as a possible quenching site[22].

A second chlorophyll pair, Chl*b* 606 and Chl*b* 607, is located on the lumenal side of the complex and constitutes the most closely associated dimer (Fig. 4b). The closest distance between these molecules is 3.5 Å, between atoms of their macrocycles. The ligand of the central Mg of Chl*b* 607 is a water molecule (water 308), which hydrogen-bonds the formyl group of Chl*b* 606. In addition, the formyl group of Chl*b* 607 interacts with Gln 131, a residue that also forms an H-bond to the coordinating water 310 of Chl*b* 606 (ref. 7). These two chlorophylls thus constitute a special pair, with sandwiched interactions. Interestingly, these molecules are close to neoxanthin, the carotenoid whose configuration changes when LHCII adopts the dissipative state and which has been shown to have strong electronic interactions with the chlorophyll *b* molecules[23]. The change observed in chlorophyll *b* interactions (Fig. 3d) could well be interpreted as the formation of the H-bond between water 308 and the formyl group of Chl*b* 606. Indeed, the unusual homogeneity of this H-bond is fully consistent with its playing an integral part in formation of the quenching centre. That this pair of chlorophyll molecules is the site of excitation quenching in LHCII is thus an attractive hypothesis.

The formation of the quenched antenna state, both in LHCII oligomers[2] and in NPQ *in vivo*[24], is controlled by the carotenoids of the xanthophyll cycle, violaxanthin and zeaxanthin. It should be noted that the crystal does not contain any zeaxanthin, which has been frequently proposed to be directly involved in NPQ. However, it is important to point out that the violaxanthin binding site is in close proximity to the above two hypothetical quenching centres (Fig. 4c). Here violaxanthin (and also a phospholipid) is sandwiched between two chlorophylls, Chl*a* 611 and Chl*b* 601. Therefore, we now have a framework for understanding the roles of the various molecular partners that control NPQ. Exactly as predicted previously[2,16], de-epoxidation of violaxanthin into zeaxanthin, which stimulates energy dissipation, could modulate the structural changes in LHCII: the replacement of violaxanthin by zeaxanthin at this binding site could make the transition easier or pigment interactions could be further enhanced. The protein PsbS is necessary for the appearance of a part of NPQ *in vivo*[25], and it appears to be responsible for sensing the increase in acidification of the thylakoid lumen (ref. 26), the principal factor that signals the occurrence of excess light energy and therefore the need for transition to the dissipative state. We propose that at low lumen pH, PsbS interacts with LHCII in the photosynthetic membrane, promoting the conformational change and quenching in the same way as occurs *in vitro* upon oligomerization and crystallization.

In conclusion, the LHCII molecule behaves as a natural nanoswitch that controls the emission or transfer of incoming light quanta. It can exist in very different functional states, the interconversion of which involve changes in pigment configuration brought about by a protein conformational change. We have provided the first

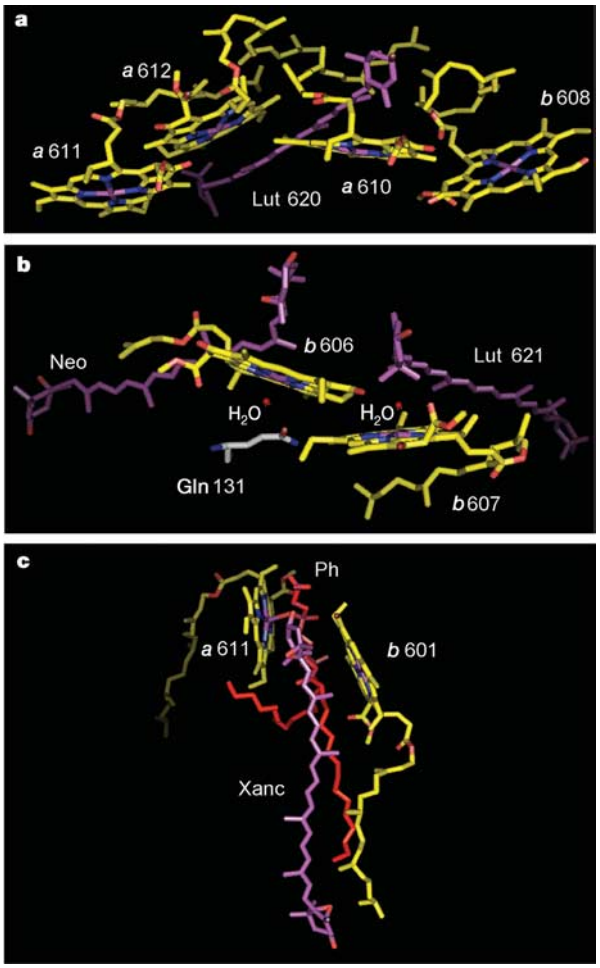

Figure 4 | Pigment–pigment interaction domains in LHCII. a, The terminal emitter domain; **b**, neoxanthin/chlorophyll *b* domain; **c**, the xanthophyll cycle carotenoid binding site. Carotenoids are shown in purple, phospholipid (Ph) in red, chlorophylls in yellow and a glutamine residue (Gln 131) in grey. Also shown are two water molecules (red spheres). Xanc, xanthophyll cycle carotenoid; Neo, neoxanthin; Lut, lutein. Chlorophylls (*a* 611, *b* 608, and so on) are named as in ref. 7

insights into the molecular design of a dissipative state of LHCII, probably made up of one or two chlorophyll pairs, which provides a channel for the safe dissipation of energy. Many questions are raised by these findings: how much of *in vivo* NPQ is due to this mechanism; what changes in protein conformation induce these pigment configurational changes; how is the extent of dissipation quantitatively controlled; are there other conformational states with different quenching strengths; does replacement of violaxanthin by zeaxanthin induce further structural change or just catalyse the conformational change; and what is the pigment configuration in the unquenched state?

METHODS

Crystals were obtained and soaked in a cryoprotectant solution as described[7]. Trimeric and oligomeric LHCII were obtained as described[27]. Raman and fluorescence measurements were obtained from samples frozen on glass plates, as described[17,28]. For crystalline LHCII, about 10 crystals were closely positioned in the centre of the excitation beam. Fluorescence emission spectra were measured using a SPEX Fluorolog FL3-22 spectrophotometer (Jobin-Yvon) equipped with xenon lamp excitation at 435 nm, defined by the double grating monochromator and photomultiplier detection. Resonance Raman spectra were measured using a Jobin-Yvon U1000 Raman spectrophotometer equipped with a liquid nitrogen-cooled CCD detector (Spectrum One, Jobin-Yvon). Excitation at 488.0 nm and 441.6 nm was provided by Coherent argon (Innova 100) and Liconix helium-cadmium lasers, respectively. Confocal images were taken with a Nikon TE300 inverted microscope. Excitation was with the 476 nm argon laser line, focused by a Plan Apochromat ×20 objective lens (numerical aperture 0.75) and fluorescence was measured with an internal detector through a HQ590LP long-pass filter, yielding images of 512 × 512 pixels (0.554 μm × 0.554 μm pixel size). For FLIM, a Bio-Rad Radiance 2100 MP system with a Nikon TE300 inverted microscope was used. A Ti:sapphire laser (Coherent Mira) pumped by a 5 W Coherent Verdi laser generated two-photon excitation pulses (860 nm, 150 fs) at a repetition rate of 76 MHz. The excitation light (0.3 mW) was directly coupled into the microscope and focused into the sample using a Plan Apochromat ×20 objective lens (numerical aperture 0.75). Fluorescence light was detected using non-descanned single photon counting detection with a Hamamatsu R3809U MCP PMT, with a time resolution of ~50 ps (in a time window of 12.5 ns, with 1,024 channels), through a HQ700/75m bandpass filter, yielding images of 62 × 62 pixels (3.5 μm × 3.5 μm). The fluorescence decay curves of each pixel were fitted with a triple exponential decay model. The lifetimes did not change with excitation densities up to 16 times higher, excluding singlet–singlet and singlet–triplet annihilation, which may lead to decreased lifetimes[29].

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