Single-Molecule Identification of Quenched and Unquenched States of LHCII

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ABSTRACT. In photosynthetic light harvesting, absorbed sunlight is converted to electron flow with near-unity quantum efficiency under low high conditions. Under high light conditions, plants avoid damage to their molecular machinery by activating a set of photoprotective mechanisms to harmlessly dissipate excess energy as heat. To investigate these mechanisms, we study the primary antenna complex in green plants, light-harvesting complex II (LHCII), at the single-complex level. We use a single-molecule technique, the Anti-Brownian Electrokinetic (ABEL) trap, which enables simultaneous measurements of fluorescence intensity, lifetime, and spectra in solution. With this approach, including the first measurements of fluorescence lifetime on single LHCII complexes, we access the intrinsic conformational dynamics. In addition to an unquenched state, we identify two partially quenched states of LHCII. Our results suggest that there are at least two distinct quenching sites with different molecular compositions, meaning multiple dissipative pathways in LHCII. Furthermore, one of the quenched conformations significantly increases in relative population under environmental conditions mimicking high light.

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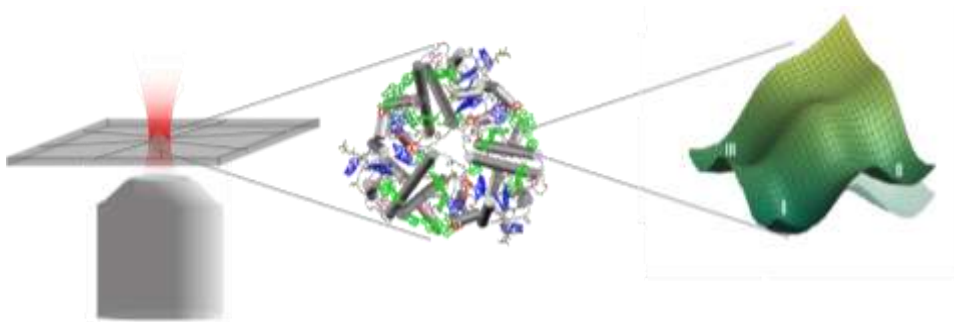
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Photosynthetic organisms convert absorbed sunlight to electron flow with remarkable near-unity quantum efficiency[1, 2]. This is achieved by transporting absorbed energy through a network of light-harvesting pigment-protein complexes, the antenna, to reach the reaction center [3, 4]. In the reaction center, the excitation energy initiates an electron transfer chain to drive downstream biochemistry [2]. Notably, higher plants regulate the energy transport process so that the amount of excitation energy does not exceed the capacity of the reaction center [5-8]. This regulation, known as non-photochemical quenching (NPQ), protects the reaction center by preventing a build-up of absorbed energy, which would in turn generate deleterious photoproducts [9, 10]. One component of NPQ is the dissipation of excess energy during periods of intense sunlight. Although the antenna complexes are the site of dissipation, which antenna complex and what is the molecular mechanism of dissipation remain under debate [5, 6, 11].

In plants and green algae, the primary antenna complex is light-harvesting complex II (LHCII), which binds over 50% of terrestrial chlorophyll [12]. LHCII plays a key role in light harvesting, as it is responsible for most of initial absorption and energy transfer events. Other antenna complexes, known as the minor complexes, may play a role in dissipation (quenching) [13]. However, LHCII has been observed to quench energy [14, 15], and, because of its ubiquity, serves as relatively well-characterized system to study quenching within light harvesting complexes.

LHCII is a trimeric complex, where each monomer contains fourteen chlorophyll, eight Chl-*a* and six Chl-*b*, and four carotenoids, two luteins (Lut), one neoxanthin (Neo) and substoichiometric amounts of zeaxanthin (Zea) or violaxanthin (Vio), surrounded by a protein matrix[12, 16]. Multiple carotenoid quenching sites in LHCII have been proposed

from previous experimental results. One proposal involves one of the luteins, Lut 1, and its neighboring Chl-*a*. Structural information from x-ray crystallography suggests a conformation exists in which the Lut moves closer to one of the Chl-*a* [17]. Ultrafast measurements have shown that the terminal emitter Chl-*a* populates the S_1 state of Lut under conditions that mimic high light [14]. The identification of this quenching site has further been supported by single-molecule studies with LHCII electrostatically attached to a poly-lysine coated surface [18-20], by Stark-fluorescence experiments on quenched LHCII [21], and resonance Raman data [22]. In previous single-molecule experiments, the emission from individual LHCII complexes exhibited blinking, which represents reversibly entering into and recovery from a dark state. This behavior was shown to increase with conditions that mimic high light, and was proposed to arise from quenching at this site[19, 20, 23]. A second proposal involves Zea. When Vio is converted to Zea, quenching and carotenoid-Chl coupling increase under conditions that mimic high light [15], which could lead to dissipation via the carotenoid. A third proposal involves Neo and its neighboring Chl-*b*. In this proposal, the Neo gets pushed towards the Chl-*b* to give rise to a quenching site [24, 25]. Finally, quenching charge transfer states have also been proposed, where oligomerization or conformational changes lead to the formation of quenching Chl-Chl charge transfer states [21, 26, 27]. Which and how many of these different sites and mechanisms is responsible for biologically relevant quenching remains controversial.

One challenge to characterizing the photophysics of these complexes arises from their sensitivity to molecular configuration. The excited-state energies and dynamics are tuned by pigment-pigment and pigment-protein electrodynamic interactions, and so are

exquisitely sensitive to changes in the molecular configuration of the complex [28]. As a result, conformational changes produce significantly altered photophysics [28-33]. In an ensemble measurement, the weighted average of all possible conformations is recorded, obscuring the identification and characterization of different conformations.

To understand the photophysics of individual antenna complexes, and how the dissipation pathways emerge with conformational changes, we employ a single-molecule approach, in which information about the individual conformations and their dynamics becomes accessible. To explore the conformational heterogeneity, we use the Anti-Brownian ELectrokinetic (ABEL) trap. The ABEL trap merges microscopy and microfluidics to implement a closed loop feedback system that achieves extended observation times of single fluorescent particles in solution [34, 35]. We present the first measurements of fluorescence lifetime of individual LHCII complexes, and perform correlated measurements of fluorescence intensity (brightness or emission rate), fluorescence (excited state) lifetime, and fluorescence (emission) spectrum. Using the correlation between fluorescence intensity and lifetime, we show that photophysical changes occur in individual LHCII complexes that lead to dissipation. We discover that there are two distinct quenched conformations and that the relative population of one of these quenched conformations significantly increases under conditions that mimic high light. The two different quenched conformations most likely correspond to two different dissipative sites within LHCII, partially reconciling the multiple effects observed in ensemble measurements. These results suggest multiple pathways for dissipation of excess energy, which may have a photoprotective role in plants.
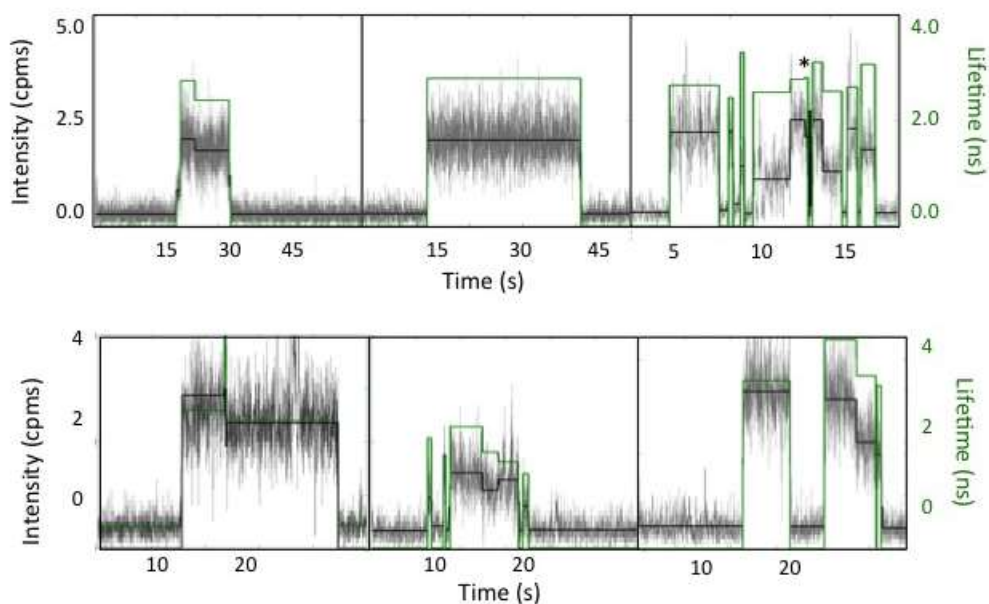
Figure 1. Representative fluorescence intensity-lifetime traces of six single LHCII complexes. The dynamics of the emission intensity levels (black, left axis) are identified by a change-point–finding algorithm on the data binned at 10 ms (gray, left axis), and the lifetime (green, right axis) is calculated by binning all photons for a given intensity level. Three traces of individual LHCII complexes at pH 7.5 are shown in the upper panel. Concomitant changes in fluorescence intensity and lifetime are observed. However, occasionally large changes in intensity are accompanied by small changes in lifetime as indicated with a star in the upper right. In the lower panel, representative traces are shown of LHCII at pH 5.5 (left), pH 7.5 and Zea-enriched (center), and pH 5.5 and Zea-enriched (right).

Figure 1 (upper panel) shows representative fluorescence intensity-lifetime traces

of three single trapped LHCII complexes at pH 7.5 with excitation at 650 nm, which

primarily excites the Chl-*b*. Extensive ultrafast studies have shown that energy rapidly

transfers from the Chl-*b* and higher energy Chl-*a* to the lower energy Chl-*a* [36-42], and so

emission (centered at 680 nm) occurs from the longer wavelength Chl-*a* band. Periods of

constant intensity are found using a change-point finding algorithm (shown by black lines

in Fig. 1) [43], and we define these as "levels". All the photons from each level are used to

extract an excited state lifetime (green lines in Fig. 1). Several levels are typically

observed for a time of many seconds before a single molecule photobleaches or leaves

the trap. Intensity and lifetime exhibit concomitant, and primarily positively correlated, changes (left, right). However, sometimes small changes in lifetime are accompanied by a large change in intensity (right, starred). Additionally, periods of stability are observed (center). Despite a lower count rate than many other light harvesting complexes [29, 31, 44], the dynamics of single LHCII complexes can be explored through the relatively long emission times with single complexes continuing to emit for times on the order of 10 s, due to impressive photostability, or a low photobleaching quantum yield [45].

By observing large numbers of these dynamical changes (typically thousands of events), we build up statistics to characterize the behavior. By pooling each period of constant intensity and its accompanying lifetime for approximately a thousand single complexes, we construct a data set of correlated measurements each of which can be thought of as a point in intensity-lifetime space. Because a scatter plot of these points can be hard to visualize, a kernel density estimation is applied to this data set to determine the probability distribution in intensity-lifetime space. The resulting probability distribution of intensity levels in intensity-lifetime space is shown in Fig. 2A, with the number of intensity levels contributing written in the upper left corner.

From the correlations between intensity and lifetime, three different clusters are visible. These clusters are labeled as I, II, and III (Fig. 2A) and for convenience we will now refer to them as "states" of the system, which can be characterized to a resolution defined by the limitations of our measurement. State I appears to be the bright unquenched state. State II is a dimmer form, but with similar emission lifetime, and state III is a state with a range of correlated lifetimes and brightnesses, which are all quenched relative to state I. In an ensemble fluorescence measurement, these scatter plots would be

essentially integrated, preventing identification of three distinct states. While, for example, multiple components of the fluorescence lifetimes can be measured, in an ensemble measurement there is no way to identify if each complex exhibits multi-exponential fluorescence decay or if it is complex-to-complex heterogeneity.

Under high light conditions, dissipation processes are triggered by an accumulation of protons in the lumen. The resulting pH drop induces the xanthophyll cycle (the enzymatic conversion of Vio to Zea) and protonation of the lumen residues of the proteins [5, 6]. The short-time, energy-dependent component of NPQ is known as qE. To explore the molecular mechanism behind qE, we characterize how the behaviors of individual LHCII complexes change in response to pH and Zea enrichment. Fig. 1 (lower panel) shows representative fluorescence intensity-lifetime for LHCII at pH 5.5 (left), Zea-enriched LHCII at pH 7.5 (center), and Zea-enriched LHCII at pH 5.5 (right). Under all conditions, LHCII shows similar concomitant changes in intensity and lifetime.
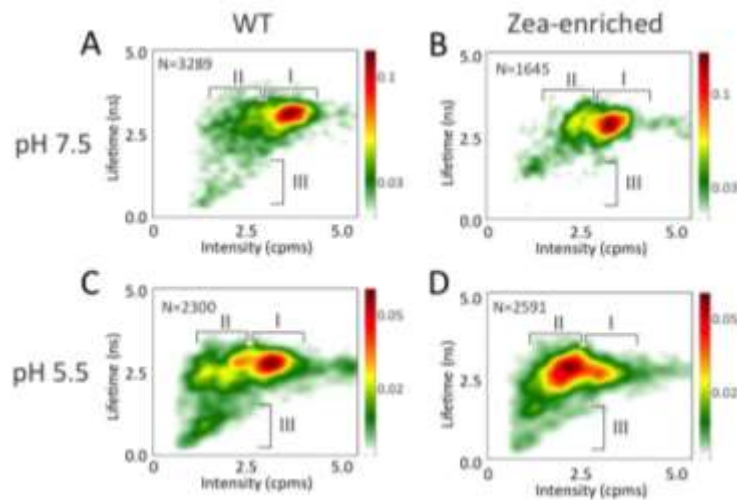

Figure 2. Intensity-lifetime probability distributions of individual LHCII complexes reveal three states. The probability distribution is calculated using all periods of constant intensity and their accompanying lifetime. The calculation is performed for four different combinations of pH and carotenoid composition. The number of events used to determine each probability distribution is shown in the upper left of each plot. Three states are observed, and labeled as I, II, and III. The brackets serve as a guide to the eye.

These states are observed under all conditions, but state II increases relative amplitude under high light conditions (lower pH and Zea-enriched).

In Fig. 2, the probability distributions for all four conditions are displayed, where LHCII and Zea-enriched LHCII are in the left and right columns, respectively, and pH 7.5 and pH 5.5 are in the top and bottom rows, respectively. The same three clusters are visible under all conditions, although the relative populations change. Both Zea-enriched LHCII (Fig. 2B) and LHCII at pH 5.5 (Fig. 2C) show an increased population of state II. Although the average fluorescence intensity appears the same with a pH drop in ensemble measurements, a single-molecule experiment characterizes individual complexes free from the background of the average behavior, which can reveal properties obscured in ensemble experiments. The drop in pH produces an intensity decrease of ~30% in 5-10% of the LHCII complexes observed. This would produce an intensity decrease of only 1-2% in an ensemble fluorescence measurement.

Overall, the relative population of state II increases with conditions mimicking high light, where the largest relative population in state II is observed in Zea-enriched LHCII at pH 5.5 (Fig. 2D). This shows that, with respect to state II population, the Zeaxathin and pH effects are additive. In these measurements, the relative population of state III appears to increase slightly with a pH drop as well.

As the intensity-lifetime traces directly follow the transitions from state to state, we quantify the connectivity between the three states by determining the order of magnitude of the rate of transitions between states, defined as the number of transitions from state i to state j divided by the total time in state i. The rate of switching in both directions between states I and II and states I and III is ~0.1 s^{-1}. In contrast, the rate of switching in both directions between states II and III is ~0.01 s^{-1}, showing that II and III

have a much larger free energy barrier between them. While the equilibrium populations change, the rates remain within the same order of magnitude. Our results show that single LHCII complexes transition between all three states. That is, there is no evidence of subpopulations. Notably, these transitions are all reversible, showing that the dynamics do not arise from a permanent degradation process, as observed in Fig. 1, upper right.

The most likely molecular mechanism behind the three states of LHCII is three different structural conformations, as small conformational changes can have a significant effect on the excited state energies and dynamics [46]. These states correspond to one unquenched (I) and two partially quenched states (II and III), where quenching is an increased amount of non-radiative decay, meaning dissipation as heat. The relationship between fluorescence intensity and lifetime can identify the possible molecular origin of the quenching change. If the fluorescence intensity and lifetime changes are directly proportional (with a positive slope and a y-intercept at the origin), the emissive state is quenched due to an increase in the rate of non-radiative decay. These photophysics are summarized in Fig. S3.

By considering the crystal structure of LHCII combined with previous spectroscopic measurements on the complex, our observations make it possible to speculate on the possible quenching mechanisms. State II shows an increased relative population under conditions that mimic high light, most likely by stabilization of the state II conformation by both protonation of LHCII and insertion of Zea.

Whereas a conformational change is the most likely mechanism for stabilizing state II, the photophysical mechanism behind the reduced fluorescence intensity remains unknown. There are several possibilities, which may also be acting in combination to

give rise to the observed photophysics. One potential mechanism is quenching in the Chl-*b*. The most likely molecular site for the conformational change behind the switching between states I and II is the region around the Zea, because state II has an increased relative population with Zea-enriched LHCII. State II has a similar fluorescence lifetime, but lower fluorescence intensity than state I. Assuming a constant absorbance, the lower fluorescence intensity indicates that state II has increased quenching (fewer photons emitted per absorbed photon). Because the fluorescence lifetime for state II is the same as for state I, the photophysical pathways out of the emissive state are most likely unchanged. Instead, the probability of quenching the excitation energy could increase at a site before the emissive state, assuming the site is disconnected from the emissive state. Zea most likely binds to one of the Lut sites, Lut 1 [12, 47]. Therefore, we suggest the quenching in state II could arise from a conformation where the Chl-*b* that neighbors the Lut 1 site, Chl-*b* 608, couples more strongly to the Zea. The S_1 states of carotenoids exhibit rapid non-radiative decay, thus increasing the probability of quenching the excitation through mixed Chl-car states or energy transfer to the carotenoid [48]. For the intensity decrease observed, energy would transfer to the carotenoid with a rate competitive with the transfer to the Chl-*a* band (femto to picoseconds), which is reasonable given the short distances and resulting strong electrodynamic coupling between the pigments [12]. Another process that could quench the Chl-*b* is through the formation of a charge transfer state, either in Chl-Chl or Chl-car dimer. Charge transfer states have been observed previously in LHCII, and correlated with quenching [21, 49]. However, quenching of the Chl-*b* also requires energy would not be able to transfer back to the Chl-*b* from the emissive site. This would require that the Chl-*b* involved in the

quenching site be more disconnected from the Chl-*a* than in the crystal structure conformation. Other potential mechanisms are an increased contribution from a triplet state and a conformational change that alters both the intrinsic radiative rate and the amount of quenching at the emissive state. We discuss these possibilities in detail in the Supporting Information.

The results presented here suggest that part of the role of high light conditions is to change the equilibrium populations, giving rise to increased populations of quenched LHCII as shown in Fig. 2. The equilibrium populations of these three states are reasonably independent of excitation intensity. The intensity-lifetime plots at ~300 nJ/cm^2 and ~900 nJ/cm^2 are displayed in the supporting information (Fig. S2). Notably, this is in contrast to the dramatic dependence of distinct emissive states on excitation intensity observed for other photosynthetic light harvesting complexes [29, 44, 50]. Similarly to previously observed spectral dynamics of single LHCII complexes,[19, 20] this indicates that the system appears to control the equilibrium between conformations via changes in the near environment, as opposed to conformational changes triggered directly through light intensity.

Finally, the most likely molecular mechanism of the switch between states I and III is a conformational switch at the emissive site. Upon switching between states I and III, fluorescence intensity and lifetime changed in a directly proportional manner. This could be a partial conformational motion towards the fully off state observed previously [31, 51]. Based on the direct proportionality, State III arises from increased quenching on the emissive state. The lowest energy states are localized on a trimer of Chl-*a,* Chl-*a* 610, 611, and 612 [36, 52-54]. This trimer neighbors the Lut 1 binding site [12], which is one of the

previously proposed quenching sites [14]. These pigments are shown in Fig. 3C. A small

structural change could produce an increased probability of quenching by increasing the

coupling between the carotenoid in the Lut 1 site and the Chl-*a*. This could occur by

increasing the oscillator strength of the S_1 state of the Lut, decreasing the distance

between the Lut and the Chl-*a*, or changing the energy the Lut S_1 state relative to the

lowest exciton state. If both states II and III involve the carotenoid in the Lut 1 binding

pocket, switching between these two states would require the carotenoid move from one

side of the pocket to the other. This could be the molecular basis of the slow transition

rate between states II and III. Overall, the position of the carotenoid in the Lut 1 binding

pocket (Lut in WT-LHCII and Zea in Zea-enriched LHCII) would control the state of the

complex. If the carotenoid moves to one side of the pocket, the complex switches to state

II, if it moves to the other side the complex switches to state III, and in the center (crystal

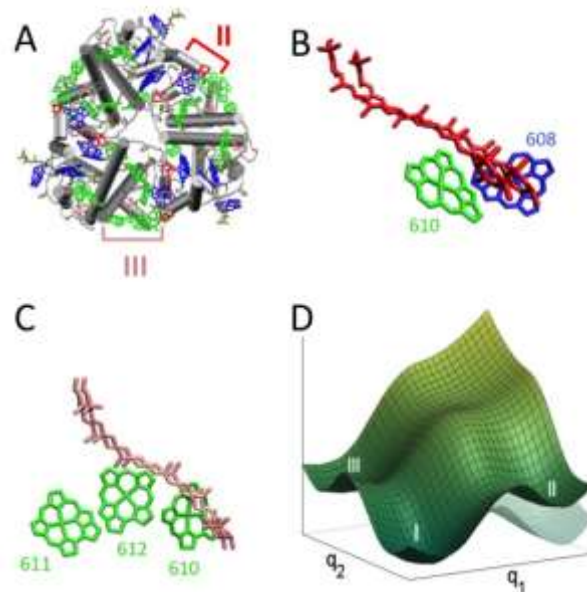structure) position the complex is in state I.

Figure 3. **Molecular and simplified excited state structure of LHCII**. (A) Structural
model from x-ray crystallography of LHCII [12]. The Chl-*a* rings are shown in green, Chl-*b*
rings in blue, one Lut and Neo in gray, the Lut near the emissive site in pink, and the Vio

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in red. The numbers indicate the proposed quenching sites. (B) Side view of proposed quenching site on the Chl-*b*, consisting of Zea (with two configurations shown), the neighboring Chl-*b* ring, and one of the Chl-a rings that contributes to the emissive state. The numbers of the Chl binding pockets are written on the figure. (C) Side view of proposed quenching on the emissive state, consisting of Lut and neighboring Chl-*a* rings. (D) Potential energy surface as a function of two generalized nuclear coordinates (q_1 and q_2). Individual complexes can transition between I and II and between I and III. However, the high barrier between II and III means individual complexes rarely transfer between these states. Conditions that mimic high light lower the potential energy well for state II, as illustrated by the second potential energy surface, increasing the relative population in state II.

We present a simplified diagram of these three states, and the barriers between them, as a function of two generalized nuclear coordinates in Fig. 3D. Individual LHCII complexes are able to switch back and forth between states I and II and between states I and III. However, the barrier between states II and III is large. The local environment controls the relative depth of the wells, changing the relative populations. This type of environmentally-induced change in the equilibrium between conformations has been observed previously in LHCII for states with different emissive energies and intensities [19, 20]. To explore the quenching dynamics, here we identify conformations using the first measurements of fluorescence lifetime on single LHCII complexes. Most notably, we identify that the relative population of one of the quenched states (state II) increases under conditions mimicking high light intensities. The model of these results supports the proposal that the carotenoid in the Lut binding pocket that neighbors the emissive state is responsible for quenching within LHCII. While the physiological membrane-bound environment, and thus the relative populations, may be slightly different, this changing equilibrium reflects an ability of LHCII to tune the conformation in response to pH and carotenoid composition. In this way, the system is able to use the local environment to

implement an active feedback loop to regulate the system, with downstream effects of regulating flux to the reaction center.

In this work, in addition to the dominant state, we have simultaneously observed two quenched states in LHCII, the primary light-harvesting complex in green plants. By performing correlated measurements of fluorescence intensity, lifetime and spectra on single LHCII complexes, we are able to characterize these intrinsic states, and their interconversions, which would be inseparable in an ensemble measurement. The two quenched states may correspond to two molecular sites that quench excess energy, a mechanism that resolves some seemingly contradictory proposals of quenching sites. Furthermore, the relative population of one of the quenched states increases under high light conditions, indicating the surrounding environment acts directly on LHCII to control the conformation. Thus, we were able to observe both quenching and conformational dynamics. Overall, these results serve to reveal the complexity of the multi-protein and multi-timescale process by which plants flourish under multiple light intensities.

Experimental Methods

Sample Preparation. Purified LHCII complexes from wild type and the NPQ2 strain [55] of *A. Thaliana* were prepared as described previously [47, 56]. The Zea-enriched LHCII trimers were isolated from the NPQ2 strain, which lacks zeaxanthin epoxidase (the enzyme that converts Zea back to Vio). Table S1 contains a pigment composition chart for both samples. The stock solution of 4 μM WT-LHCII and 2.5 μM NPQ2-LHCII were kept at −80 °C and thawed before experiments. The sample was diluted to a concentration of ~10 pM in 20 mM HEPES buffer with 0.075 wt% *n*-Dodecyl α-D-maltoside. 25% glycerol was added in the solution. An enzymatic oxygen-scavenging system was added to the

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buffer for a final concentration of 2.5 mM of protocatechuic acid and 50 nM

protocatechuate-3,4-dioxygenase [57]. The pH was adjusted using KOH/HCl, and sample

integrity was confirmed with ensemble fluorescence lifetime, fluorescence spectra, and

linear absorption measurements.

Fused silica ABEL trap microfluidic cells with 700-nm cell depth were used,

which achieves confinement in the z-direction. They were prepared as described

previously [29, 44]. The internal surface of the cell was coated with two to four pairs of

layers of the polyelectrolytes polyethyleneimine and polyacrylic acid, terminating in

polyacrylic acid, to prevent adsorption [44].

Excitation and Detection Optics. A pulsed excitation source centered at 650 nm with a

peak excitation fluence of ~900 nJ/cm^2 per pulse was used. A mode-locked Ti:sapphire

laser (Mira 900-D; Coherent; tuned to 750 nm, 200 fs pulse length, 76-MHz repetition

rate) pumped a nonlinear photonic crystal fibre (FemtoWhite 800, Newport) to generate a

supercontinuum, from which the desired window was selected by spectrally filtering

(FF01-650/13-25; Semrock and 3RD630-650; Chroma). The pulse length was stretched

to ~10 ps by propagation through a 20 m optical single-mode fiber (PMJ-3S3S-633-

4/125-3-20-1; Oz Optics). The ABEL trap apparatus has been described in detail

previously [34, 58, 59]. In brief, the excitation pulse traveled through an orthogonal pair of

acousto-optic modulators (46080-3-LTD; NEOS) driven by a field-programmable gate

array (FPGA), which deflected the beam with a frequency of 9.8 kHz to produce a 32-

point grid of focal spots with 0.4 μm spacing in the sample plane. The excitation pulse

enters an Olympus IX71 inverted microscope with a silicon oil immersion objective

(UPASAPO60XS, N.A. 1.30, 60x; Olympus). Further details of the trap operation are

described below. Sample fluorescence is collected back through the same objective, passes through a dichroic (z532/658rpc; Chroma), a 400 μm pinhole to reject out-of-focus background fluorescence, two long-pass filters (HQ665LP and HHQ665LP; Chroma), one short-pass filter (SP01-785RS-25; Semrock), and is focused onto a photon-counting avalanche photodiode (SPCM-AQR-15; Perkin-Elmer). Time-correlated single-photon counting is implemented with a timing module (PicoHarp 300; PicoQuant). An instrument response function of width 0.25 ns was measured from scatter off a quartz coverslip. Details of the fitting procedure to extract lifetime have been reported elsewhere [29, 44, 50].

Implementation of ABEL trap algorithm. A real-time estimate of the object's position is found by taking the beam position in the beam scan pattern at the time each photon is detected. The position estimate is improved by applying a Kalman filter algorithm using the previous estimates, applied voltage and the estimated transport parameters (diffusion coefficient and mobility). This approach enables estimates of these transport parameters to be found as well [58-60]. The algorithm is implemented with a Labview interface on a field-programmable gate array (FPGA; PCI-783R; National instruments). Feedback voltages in x and y are calculated and then applied to the sample cell to induce electrokinetic flow that cancels diffusion (Brownian motion). These voltages are produced by amplifying the FPGA analog output with two home-built high-voltage amplifiers (OPA453; Texas Instruments) and are delivered by four platinum electrodes inserted into the sample cell. The resulting electric field is approximately uniform across the shallow trapping region, and this field (~5 V across ~20 μm distance) has been shown to not alter the fluorescence spectrum or intensity for solubilized complexes [21, 44].

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Notes

The authors declare no competing financial interests.

Supporting Information: This material is available free of charge via the Internet at

http://pubs.acs.org

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