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## pH sensitivity of chlorophyll fluorescence quenching is determined by the detergent/protein ratio and the state of LHCII aggregation <sup>☆</sup>

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### ABSTRACT

Here we show how the protein environment in terms of detergent concentration/protein aggregation state, affects the sensitivity to pH of isolated, native LHCII, in terms of chlorophyll fluorescence quenching. Three detergent concentrations (200, 20 and 6 μM *n*-dodecyl β-D-maltoside) have been tested. It was found that at the detergent concentration of 6 μM, low pH quenching of LHCII is close to the physiological response to lumen acidification possessing pK of 5.5. The analysis has been conducted both using arbitrary PAM fluorimetry measurements and chlorophyll fluorescence lifetime component analysis. The second led to the conclusion that the 3.5 ns component lifetime corresponds to an unnatural state of LHCII, induced by the detergent used for solubilising the protein, whilst the 2 ns component is rather the most representative lifetime component of the conformational state of LHCII in the natural thylakoid membrane environment when the non-photochemical quenching (NPQ) was absent. The 2 ns component is related to a pre-aggregated LHCII that makes it more sensitive to pH than the trimeric LHCII with the dominating 3.5 ns lifetime component. The pre-aggregated LHCII displayed both a faster response to protons and a shift in the pK for quenching to higher values, from 4.2 to 4.9. We concluded that environmental factors like lipids, zeaxanthin and PsbS protein that modulate NPQ *in vivo* could control the state of LHCII aggregation in the dark that makes it more or less sensitive to the lumen acidification. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: Keys to Produce Clean Energy.

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### 1. Introduction

Photosynthetic organisms first appeared and evolved in the water environment, which primarily prevents high light input into the photosynthetic machinery. Therefore, photosynthetic bacteria and algae have developed light harvesting systems (LHC), or antennae that bind numerous pigments capable of efficiently absorbing and delivering photon energy to the photosynthetic reaction centres where primary charge separation occurs. Hence, the photosynthetic antennae function to increase input of light energy into the photosynthetic reaction centres [1]. Occupation of land by photosynthetic organisms resulted in their more frequent exposure to rapid and large fluctuations in light intensity [2,3]. High light exposure leads to the increase in the fraction of “unused” excitation energy in the photosynthetic membrane [2,3].

This excess energy can cause damage to the photosynthetic reaction centres, particularly those of photosystem II (PSII), leading to the sustained photoinhibition of its efficiency and eventual damage to the photosynthetic membrane [4–7]. In order to avoid photoinhibition, photosynthetic organisms evolved a molecular strategy within the PSII light harvesting antenna that enables them to control absorbed energy fluctuations and dissipate any excess energy into heat [8–10]. The energy dissipation is often indirectly monitored as a decline in the antenna chlorophyll fluorescence yield of photosystem II (PSII) – a phenomenon known as non-photochemical quenching (NPQ) [10]. The kinetics of NPQ formation and fluorescence recovery reveals a complex picture. The major component is energy-dependent quenching (qE) that forms within several minutes and relaxes equally quickly in the dark. It is this component of NPQ that is believed to be of a photoprotective nature. In addition, some slower components of NPQ also reflect photoprotective energy dissipation and could be due to the formation of zeaxanthin (qZ) and entrapment of protons within the quenched antenna [9,10]. The mentioned fast (qE) and slow photoprotective components of NPQ are frequently referred to as photoprotective NPQ [9,10]. In addition, a part of slowly-reversible NPQ is attributed to the onset of photodamage – photoinhibitory quenching (qI). What distinguishes NPQ from other ordinary *in vitro* quenching mechanisms (like, for example, the light-induced quenching

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of antenna complexes initially observed in [11] and [12]) is: i) a dependence from the  $\Delta\text{pH}$  across the photosynthetic membrane; ii) the reversibility of the process. Consequently, NPQ can be induced and sustained in the darkness by artificially inducing  $\Delta\text{pH}$  across the photosynthetic membrane [13].

Apart from  $\Delta\text{pH}$ , two more key elements that control photo-protective NPQ in the light harvesting antenna are the *de-epoxidation state* of the xanthophyll cycle carotenoids, and the *PsbS* protein [14–18]. Despite a long history of NPQ research, its exact molecular features are not well understood, although the majority of the modern NPQ theories agree that structural changes within the peripheral PSII antenna system, LHCII, are behind the mechanics of the process [10,19]. *PsbS* protein was suggested to play a role of pH sensor that transduces a signal into LHCII system, triggering its transition into the quenched state [18–21]. All theories tend to agree that this transition involves lateral remodelling of the antenna [21–23]. This remodelling has been recently and carefully documented in intact, unisolated PSII membranes using freeze-fracture electron microscopy [24]. It was shown that the transition into the NPQ state required a clustering of LHCII complexes, leading to the establishment of quenching aggregates, as was previously proposed [25]. The degree of this aggregation was found to be promoted by the presence of the xanthophyll cycle carotenoid zeaxanthin that emerged as a result of violaxanthin *de-epoxidation* [26]. These recent data directly confirmed a long standing idea proposed by Horton's group [25] that aggregation of the light harvesting antenna complexes lies at the heart of the mechanism of protective NPQ. This idea has been developing for a number of years with more evidence emerging that, in principle, both, trimeric and monomeric LHCII's, could be involved in the process [27–31] and that all the antenna complexes, including those isolated from diatoms, can in principle undergo an intrinsic transition into the inefficient, quenched state that is triggered by environmental elements such as lipid/protein ratio, protein overcrowding in the membrane, magnesium and low pH resulting in protein aggregation [10,19,21,32–37]. Indeed, it was shown that the aggregation is rather a consequence of the conformational transition of the LHCII complex into the quenched state and happens only at high protein concentration, such as that in the thylakoid membrane [38–42]. The mechanism of *PsbS* action in NPQ has not been revealed. However, recent work with the use of  $\Delta\text{pH}$  enhancer across the thylakoid membrane, diaminodurene (DAD), has demonstrated that the protein is not strictly required for qE, provided the lumen pH reaches a somewhat lower level than that of the control one, since the pK for quenching in the absence of *PsbS* was found to be shifted from 5 to 4.2–4.7, depending on the xanthophyll cycle carotenoid composition [41]. Therefore it was proposed that *PsbS* somehow increases sensitivity of the LHCII system to lumen pH, bringing the pK up to 6.0 in mutants overexpressing *PsbS* [10]. It was suggested that since *PsbS* was found to promote LHCII aggregation *in vivo* [22,24] it is possible that partially aggregated LHCII becomes more responsive to protons [10]. The mechanism of this enhanced sensitivity was proposed to be the increase in the hydrophobic environment of proton-receiving lumen-exposed amino acids [10]. Indeed, there is clear experimental evidence that hydrogen bonding, steric hindrance and the dielectric constant of the environment can all affect the pK of amino acids [42–44]. For example the pK of the carboxyl group on aspartate can be as low as 2.4 in a water environment, due to hydrogen bonding, while in a hydrophobic environment, the pK can be as high as 6.4 [44] It has recently been demonstrated that the pK of LHCII can be shifted towards higher values by single-point mutagenesis of the acidic, lumen-exposed residue glutamate E94, when this is replaced by neutral Glycine, providing a hint of where the potential regulatory epitope can be localised within LHCII [40,45]. In order to address the idea of the link between the environment and indeed aggregation of LHCII and pH sensitivity of the fluorescence quenching we have used an *in vitro* quenching approach [27,34,40,53] to determine the pH response of LHCII trimers incubated at different LHCII concentrations of detergent and in different aggregation

states. Although a number of papers dealt with the effect of the detergent on the spectroscopic properties of LHCII such as changes in time resolved triplet-minus-singlet signal [46] or variable linear dichroism and circular dichroism signals [47], no studies so far have been focused on measuring the pK for quenching of LHCII in different detergent environments and upon variations of the aggregation state. Previously, pH titrations of quenching in isolated LHCII trimers showed an increase in pK in the complexes that contained zeaxanthin in comparison to those that were enriched in violaxanthin [48], both xanthophylls bound to the extrinsic V1 site [49]. This was a first indication that other molecules could potentially interact with LHCII like membrane lipids or indeed detergents in isolated complexes would affect the quenching pK. However, this has never been specifically tested on LHCII in the case of lipids or detergents. In addition, the effect of LHCII pre-aggregation on pK has never been shown and quantified. In this work we demonstrated increased sensitivity of pH-induced quenching upon the decrease in detergent/protein ratio, as well as the increase in the extent of aggregation. Hence, our findings provide an explanation of how the state of LHCII antenna aggregation could make its efficiency more or less sensitive to the lumen pH, making the idea that the mechanism of this control could lie within the alteration in the hydrophobicity of the environment of proton-receiving amino acids of LHCII more plausible.

## 2. Materials and methods

### 2.1. LHCII isolation from spinach leaves

Unstacked thylakoids were prepared from 100 g dark-adapted spinach leaves with the mid-rib removed. Leaves were homogenised in 300 ml of icy grinding medium (0.33 M sorbitol, 10 mM  $\text{Na}_4\text{P}_2\text{O}_7 \cdot \text{H}_2\text{O}$  and 130 mg *D*-iso-ascorbate; pH 6.5) and the homogenate filtered through a bi-layer of muslin cloth, followed by a secondary filtration through 4 layers of muslin interlaid with cotton wool. Thylakoids were then centrifuged (4000  $\times$ g) for 10 min and the pellet gently re-suspended in washing medium (0.33 M sorbitol and 10 mM MED) before additional centrifugation. The pellet was then re-suspended in re-suspension medium (0.33 M sorbitol, 1 mM EDTA, 50 mM HEPES; pH 7.6) and osmotically shocked by mixing in 50 ml of break medium (10 mM HEPES; pH 7.6). After 30 s osmotic potential was returned to normal with the addition of 50 ml of osmoticum medium (0.66 M sorbitol, 40 mM MES; pH 6.5) and thylakoids centrifuged (4000  $\times$ g) for 10 min. The final pellet was re-suspended in re-suspension medium and aliquots frozen immediately in liquid nitrogen.

LHCII trimers were isolated from unstacked spinach thylakoids using isoelectric focussing [50–52]. The protein band corresponding to LHCII was collected and eluted in elution buffer containing 0.01% *n*-dodecyl  $\beta$ -*D*-maltoside ( $\sim 200$   $\mu\text{M}$ ), then followed by size exclusion purification to remove ampholites (PD-10 columns, GE Healthcare). Aliquots of LHCII prepared at the same concentration (OD = 6) were immediately frozen in liquid nitrogen for later use in fluorescence analyses.

### 2.2. *In vitro* chlorophyll *a* fluorescence quenching of isolated LHCII trimers

Chlorophyll *a* fluorescence was measured using Dual PAM 100 (Walz GmbH, Effeltrich, Germany) chlorophyll fluorescence photosynthesis analyzer. Illumination was provided by low blue light ( $<20$   $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) and the sample was stirred continuously. The experimental set up was similar to the one described in [53]. Briefly, quenching was induced by diluting an aliquot of isolated LHCII into buffer containing 10 mM Hepes pH 7.4, 10 mM tri-sodium citrate with 6  $\mu\text{M}$ , 20  $\mu\text{M}$  or 200  $\mu\text{M}$  *n*-dodecyl  $\beta$ -*D*-maltoside ( $\beta$ -DM, final concentrations) [34,48]. The pH adjustments (values between 7.33 and 3.5) were made by the addition of small amounts of HCl after 1 min of recording fluorescence and fluorescence quenching measured for an additional 4 min (see Supplemental Fig. S1). Absorption spectra were recorded following lifetime fluorescence measurements to ensure

no formation of pheophytin/sample denaturation as a result of acidification and pH measured to confirm final pH of sample [52].

### 2.3. Fitting of quenching kinetics

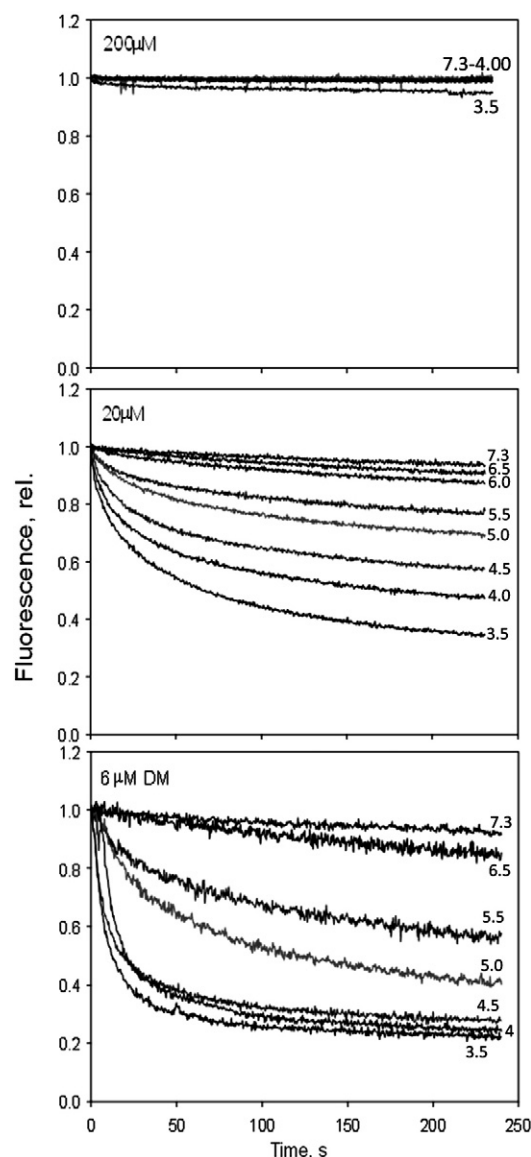
Fluorescence quenching data were fitted with a 3-parameter hyperbolic decay [ $y = y_0 + (ab) / b + x$ ] using SigmaPlot software (SPSS, Chicago, IL, USA), where  $a$  is the maximum  $F$ ,  $b$  is the  $x$  value at which  $a = 50\%$  (pK) and  $y_0$  is the amplitude of fluorescence quenching does not relax within 300 s [48]. Quenching parameters (fluorescence quenching percentage and average fluorescence lifetime) derived from the pH titrations were then plotted as a function of pH and  $\Delta$ pH and the values fitted with a Hill function [ $y = (ax^b) / (c^b + x^b)$ ], where  $a$  is the theoretical maximum,  $c$  is the  $x$  value at which  $a = 50\%$  (pK) and  $b$  is the sigmoidicity parameter (Hill coefficient) [54].

### 2.4. Chlorophyll fluorescence lifetime

Time-correlated single photon counting measurements were performed using a FluoTime200 picosecond fluorometer (PicoQuant, Germany) [55]. Excitation was provided by a 470 nm laser diode at a repetition rate of 10 MHz. Fluorescence was detected at 682 nm with a 2 nm slit width. Fluofit software (PicoQuant, Germany) was used for the analysis of the fluorescence lifetime data and the quality of the fit determined by the  $\chi^2$  parameter [55].

## 3. Results and discussion

Fig. 1 displays pH-dependent chlorophyll fluorescence quenching experiments performed on LHCII complexes at three different  $\beta$ -DM concentration (200, 20 and 6  $\mu$ M). It must be mentioned that pH-induced quenching of Fm level has been first observed on isolated thylakoids from spinach and possessed similar properties to that of the isolated LHCII [48,54,56]. Here we undertook a similar approach for isolated LHCII, using the  $cmc$  as a threshold reference value. An example of the methodology used is reported in Supplemental Fig. S1 showing the timing for sample injection, acidification and lifetime measurements. 200  $\mu$ M was also the concentration of detergent in the samples before injection. Upon acidification, at detergent concentration above  $cmc$  (200  $\mu$ M) only a little fluorescence quenching was observed, whilst at the concentrations below  $cmc$  (20 and 6  $\mu$ M) the progressive quenching followed the gradual decrease in the buffer pH. In particular, the sample diluted into 6  $\mu$ M  $\beta$ -DM buffer possessed higher sensitivity to low pH than the sample incubated at 20  $\mu$ M detergent displaying faster and larger quenching than that induced in the sample incubated at 20  $\mu$ M  $\beta$ -DM (Fig. 1, Table 1). From these traces a pH titration curve of quenching was calculated and is presented in Fig. 2. Comparison among the three detergent conditions revealed a significant shift towards higher levels of pH for samples incubated at lower concentration of detergent. Interestingly, despite the fact that both the 20 and 6  $\mu$ M  $\beta$ -DM concentrations were below  $cmc$ , LHCII incubated at the lower concentration of detergent responded to low pH more readily than the sample incubated at 20  $\mu$ M of  $\beta$ -DM. This fact suggests that not only the detergent micelle state but also the absolute amount of free detergent in the buffer determines the protein sensitivity to protons. Accordingly, whilst the pK of the 20  $\mu$ M  $\beta$ -DM sample was found to be around 4.2 – a fairly low value compared to the physiological lumen range – the one for the 6  $\mu$ M  $\beta$ -DM sample increased to 5.5 – a more dramatic effect than the one induced by zeaxanthin replacement by zeaxanthin in isolated LHCII that was previously reported [48]. At  $\beta$ -DM of 200  $\mu$ M only a very little quenching (see Fig. 1, Table 1) occurred only at pH of 3.3. This is about 4 pH units if calculated for  $\Delta$ pH *in vivo* which is a rather improbable value that has never been reported before. On the other hand, the pK of 5.5 estimated for LHCII quenching in 6  $\mu$ M  $\beta$ -DM was perfectly within the physiological range



**Fig. 1.** Time course of chlorophyll a fluorescence of LHCII trimers suspended in 200, 20 and 6  $\mu$ M n-dodecyl  $\beta$ -D-maltoside ( $\beta$ -DM) buffer. Traces shown are following the adjustment of the pH through the addition of HCl. Traces are the average of at least four independent measurements.

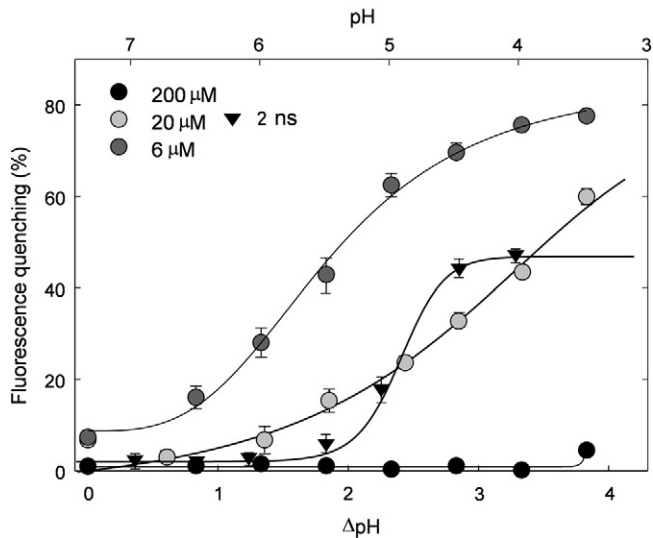
and close to the pK of qE for chloroplasts quenching (5.0) and is identical to the one reported for LHCII isolated from *Arabidopsis* [54].

Upon injection in a buffer with lower detergent concentration than the one required for solubilisation, any antenna complex displays

**Table 1**  
pH versus quenching titration curve fitting parameters in LHCII trimers.<sup>a</sup>

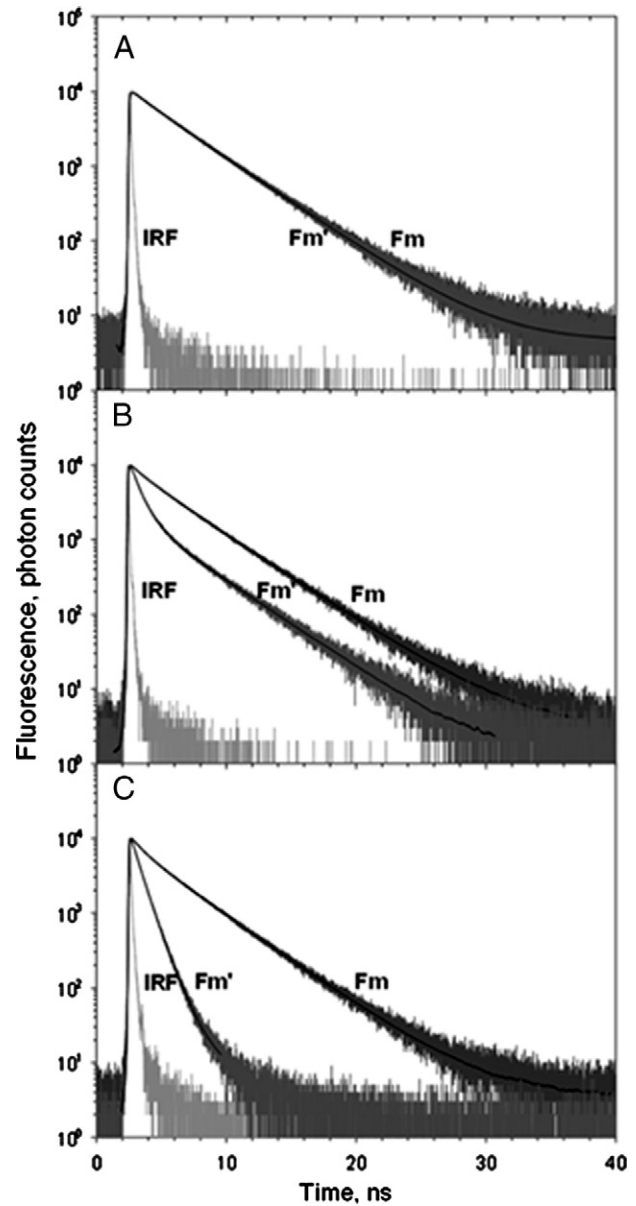
DM concentration	Hill coefficient	Estimated pK	% quenching max (experimental)	% quenching max (theoretical)
200 $\mu$ M	–	–	4.6	–
20 $\mu$ M	2.8	4.2	65.7	80.6
6 $\mu$ M	3.2	5.5	77.6	77.3
20 $\mu$ M, 2 ns	4.0	4.9	41.2	43.0

<sup>a</sup> Titration parameters for data presented in Fig. 2 were determined by fitting the data with the equation [% quenching = quenching<sub>max</sub> $\Delta$ pH<sup>n</sup> / ( $\Delta$ pH<sup>n</sup> +  $\Delta$ pH<sup>n</sup><sub>0</sub>)], where quenching<sub>max</sub> is the theoretical maximum fluorescence quenching,  $\Delta$ pH is the change in pH from the control (7.33),  $\Delta$ pH<sub>0</sub> is the level of  $\Delta$ pH at which quenching = 0.5 quenching<sub>max</sub>, and  $n$  is the sigmoidicity parameter (Hill coefficient). pK was estimated by converting  $\Delta$ pH<sub>0</sub> to lumen pH by subtracting from the control pH (7.33).



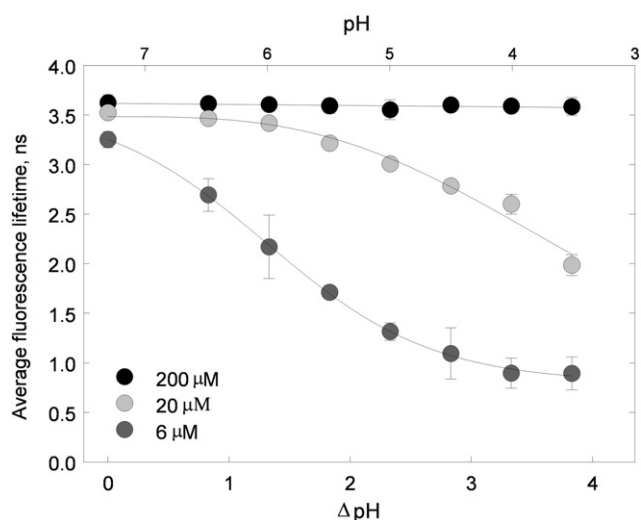
**Fig. 2.** Effect of pH on fluorescence quenching in native LHCII trimers suspended in 200, 20 and 6  $\mu\text{M}$  of  $\beta\text{-DM}$ . Quenching was induced by the addition of HCl acid at a range of pH values between 7.33 and 3.5. Lines represent the curve fit using the Hill function. Data represent average of four independent experiments  $\pm$  SE.

fluorescence quenching, a process called “spontaneous quenching” [48]. It has been previously demonstrated that spontaneous quenching and low-pH induced quenching are not separate effects in LHCII, but part of a continuum of quenched states of the complex arising from the same mechanism, where acidification accelerates the rate at which the state of maximum quenching is reached (see 40 and ref. within). Therefore, during the time-course of chlorophyll fluorescence of Fig. 1, a slowly ongoing spontaneous quenching process always sums up to the pure effect of proton addition on LHCII (see Supplementary Fig. S2). Because the technique allows for resolving the lifetime components originating from the different fluorescence states in the studied LHCII complexes, we used the fluorescence lifetime component analysis to better clarify how much the spontaneous quenching could affect the accuracy of pH-induced quenching [48]. In addition, there has never been a detailed analysis of the effect of quenching induced by acidification on the sample fluorescence lifetime components, or, in other words, a pH titration experiment for fluorescence lifetimes of samples incubated at different detergent concentrations. Fluorescence lifetimes were measured for comparison before and after the induction of low-pH fluorescence quenching. Fig. 3 shows typical fluorescence decay curves of LHCII measured before ( $F_m$ , fluorescence level of the sample diluted in low fluorescence buffer) and after addition of HCl to achieve pH 4 ( $F_m'$ ). It is clear that the amount of spontaneous quenching in LHCII incubated at 20 and 6  $\mu\text{M}$   $\beta\text{-DM}$  ( $F_m$  levels) was relatively small with average lifetimes being respectively 3.5 and 3.2 ns (see Fig. 4, points corresponding to pH 7.33) and therefore not more than 10% of the level of LHCII incubated at 200  $\mu\text{M}$   $\beta\text{-DM}$ . Hence, the arbitrary fluorescence measurements performed using PAM fluorimetry can be considered as reasonably accurate. In addition, it is interesting to notice that the pH titration profiles of the average chlorophyll fluorescence lifetime matched fairly well those performed using the steady-state fluorescence (compare Fig. 2 with Fig. 4). The whole quenching process shown in Fig. 1 was therefore fully characterised by measuring at each pH point the relative fluorescence lifetime, yielding a titration curve of lifetime against pH in the three detergent conditions in a similar way as done for the total arbitrary quenching (see Fig. 4 for average lifetimes and Fig. 5 for the single component analysis). According to the analysis, initial average lifetime value for all detergent conditions before addition of HCl was around 3.5 ns — much higher than the average LHCII antenna lifetime recently measured *in vivo* [45] (see also below). The component analysis presented in Fig. 5 revealed that up to 4 different lifetimes were



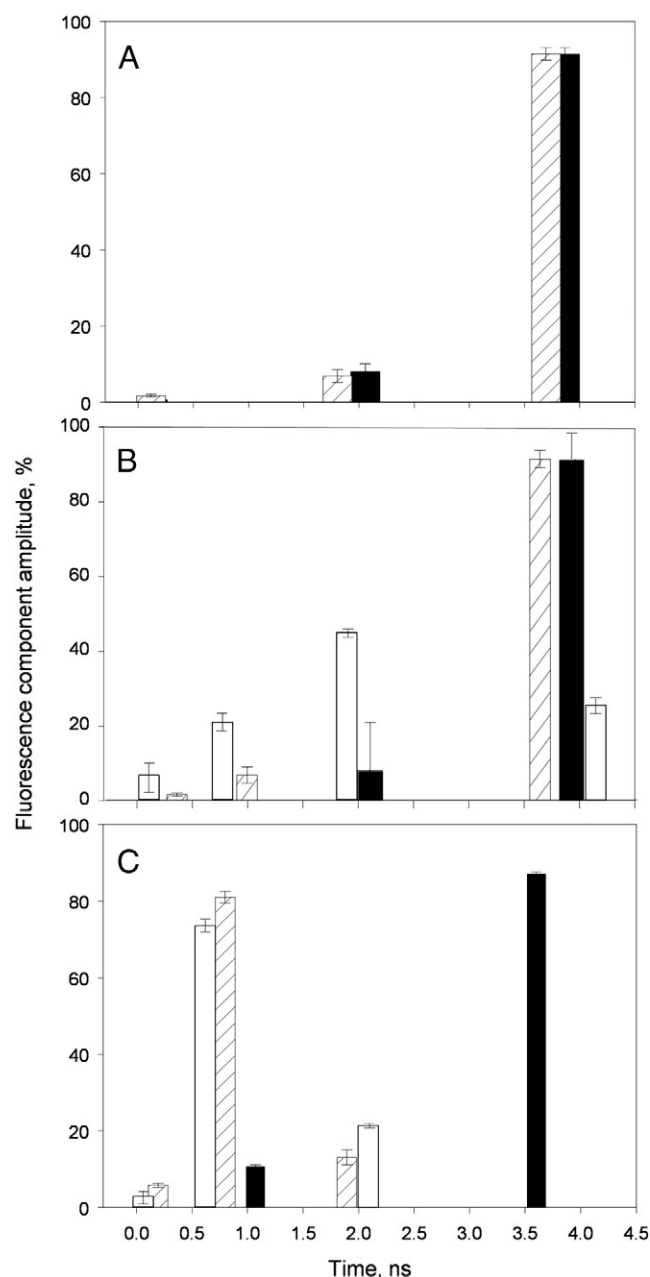
**Fig. 3.** Time correlated single photon counting analysis of chlorophyll fluorescence lifetime of LHCII complexes suspended in (A) high, (B) medium and (C) low detergent buffer (200, 20 and 6  $\mu\text{M}$ , respectively) at pH 7.33 ( $F_m$ ), pH 4.0 ( $F_m'$ ).

necessary to fit the various sample conditions. The longest lifetime component at 3.6–4.0 ns was the main and almost the only component (>96%) besides a  $\sim$ 2.0 ns component (<5% in amplitude) of LHCII in 200  $\mu\text{M}$  detergent, similar to that reported earlier [57]. Similar components have been observed for LHCII incubated in 20  $\mu\text{M}$   $\beta\text{-DM}$  at pH 7.33. For the sample in 6  $\mu\text{M}$  detergent buffer the 2 ns component was replaced by the 1 ns one. Acidification to pH 4.0 caused the appearance of an additional, 100–200 ps component (2–5% in amplitude) in all the samples in agreement with previously reported measurements [57]. Apart from this very fast component, a  $\sim$ 0.8–1.0 ns component emerged upon acidification of the LHCII incubated at 20  $\mu\text{M}$  and especially at 6  $\mu\text{M}$   $\beta\text{-DM}$ . This component appears to be the major cause of the pH-induced fluorescence quenching of the 6  $\mu\text{M}$   $\beta\text{-DM}$  sample, which is also the one attaining the most complete quenching, and it has also been found in the isolated LHCII crystals as the only fluorescence lifetime component [58,59]. The fact that up to 80% of the photons emitted by a quenched LHCII sample comes from only one main fluorescence decay component points towards the existence of a single protein



**Fig. 4.** Effect of pH on average fluorescence lifetime in native LHCII trimers suspended in high, moderate and detergent free buffer (final detergent concentrations of 200, 20 and 6  $\mu\text{M}$ , respectively). Quenching was induced by the addition of HCl acid at a range of pH values between 7.33 and 3.5. Data represent average of four independent experiments  $\pm$  SE.

conformation in the quenched LHCII. This prevailing single component was observed in all the quenched samples analysed and was interpreted as a strong indication of the absence of any source of heterogeneity. The absence of heterogeneity in the quenched sample is also in agreement with the previous conclusion of size uniformity of LHCII aggregates based on the migration profile of native “green” gels (see Supplemental material of ref. [38]). Interestingly, the time-resolved fluorescence analysis of plants grown on lincomycin and containing very few photosystem complexes (<10% of control) revealed total absence of the 3.5–4.0 ns component with only 2.0–2.5 ns and 1.0–1.3 ns components being present in the dark-adapted leaves [60]. This finding suggests that the LHCII 3.5 ns state is never attained *in vivo* and is rather an artefactual consequence of the detergent/protein interaction, and that the actual state of the antenna complex in the natural proteolipidic environment corresponds to the 2.0–2.5 ns component. Relevant to this conclusion is the fact that the circular dichroism spectra of trimers solubilized in  $\beta$ -DM, *n*-octyl- $\beta$ -D-glucopyranoside, or Triton X-100 were drastically different in the Soret region to the one of LHCII in the thylakoid membranes [47]. Solubilization of LHCII is also always accompanied by profound changes in the linear dichroism signal compared to the one from thylakoids. These data support the notion that the detergent, 4 ns solubilised state of LHCII does not retain the physiological, native organisation of LHCII in the *in vivo* thylakoid membrane. On the other hand the CD spectrum of lamellar aggregates of LHCII was found to closely resemble those of unstacked thylakoid membranes [47]. The above observation is consistent with an earlier reported fluorescence lifetime data obtained on the LHCII incorporated into liposomes that possessed a specific 2 ns component [32]. Thus it appears that an *in vivo* conformation of LHCII exists that does not correspond to a typical state of the complex in the detergent micelle. In liposomes, in thylakoid membranes of lincomycin-treated plants as well as in solution, LHCII was found to be partially aggregated [32,60]. In order to further explore the conformational state of LHCII that gives 2 ns fluorescence lifetime component LHCII was incubated in 20  $\mu\text{M}$  detergent buffer at pH 7.33 for 150 min to achieve gradual decrease (by spontaneous quenching) of the average fluorescence lifetime from 3.5 to 2.0 ns. As shown in Supplemental Fig. S3, the 77 K fluorescence spectrum of this sample revealed a significant shoulder at 700 nm – a typical sign of a partially quenched state often linked with LHCII aggregation which was further enhanced upon acidification (Fig. S3, trace 3) [25]. This pre-aggregated LHCII possessed the main lifetime component of 2.0 ns



**Fig. 5.** Relative time resolved fluorescence lifetime component amplitudes of LHCII complexes suspended in 200 (A), 20 (B) and 6 (C)  $\mu\text{M}$  detergent buffer at pH 7.33 (black) and pH 4.0 (dashed). Open bars in (B) and (C) correspond to the components of pre-aggregated LHCII in 20  $\mu\text{M}$  detergent at pH 7.33 and following acidification at pH 4.0, respectively. Data represent mean of four independent measurements  $\pm$  SEM.

with minor contributions from a longer (3.8 ns) and two shorter (0.8 ns and 0.1 ns) components typical of the quenching states normally achieved upon acidification (see Fig. 5, B and C). Acidification to pH 4 of the pre-aggregated LHCII caused a very similar effect on the lifetime component profile as in the case of LHCII quenched by low pH in the 6  $\mu\text{M}$  detergent buffer (Fig. 5C). However, the sample in the pre-aggregated state was found to be more readily responsive to acidification than the 3.5 ns one, although the detergent concentration of the two was identical. Accordingly, the rate of quenching formation in the “2 ns” sample was much faster (see Supplemental Fig. S4). The sample with the 2 ns average lifetime revealed a peculiar pH titration curve of the fluorescence quenching (Fig. 2, triangles, Table 1). The titration curve was much steeper than the one of the sample with 3.5 ns average lifetime and pK was shifted to higher pH values, from 4.2 (unquenched

sample) to 4.9 of the pre-quenched state. However, the maximum amount of quenching in the sample with 2 ns average lifetime was only about 43% in comparison to ~77% of the sample possessing 3.5 ns lifetime. This is not surprising taking into account that the capacity for the fluorescence quenching in LHCII complexes in the experimental system used was found to be always limited to a certain level [48] and that at 2 ns the state of the complex is already partially quenched. In return, the 2 ns pre-quenched and pre-aggregated LHCII gained a better sensitivity to the pH of the medium. It is worth mentioning in this context that the higher pH sensitivity found for LHCII in aggregated state has also been observed in other proteins, like the fucoxanthin chlorophyll protein a (FPCa) isolated from diatoms [35]. In that case the observed behaviour was even more pronounced since the protein showed a pH dependence of the fluorescence yield only when proteins were aggregated (see also Fig. 5B of that report), once more pointing to the importance of the aggregation state of a protein in the NPQ mechanism.

Our fluorescence lifetime analysis shows that a conformation/state that is characterised by ~2 ns fluorescence lifetime is likely to correspond to a physiological condition of the LHCII antenna *in vivo* in the dark. It is possible that zeaxanthin causes a small reduction of this lifetime to about 1.6–1.8 ns [61]. Acidification of LHCII caused the appearance of a new lifetime component at around 0.6–1 ns, that corresponds to a new, quenching conformation of the complex [59,60] the structure of which has been solved by X-ray crystallography [49]. The transition between the two states/conformations can be triggered by low pH but also the decrease in detergent/protein ratio in solution or lipid/protein ratio in the liposomal membrane [32]. Can the same transition be induced *in vivo*? Recent freeze fracture electron microscopy provided evidence of clustering of LHCII trimers induced by  $\Delta$ pH that is modulated by the xanthophyll cycle [24]. So far there has not been any evidence obtained to suggest that the decrease in lipid/protein ratio *in vivo* can result in the further LHCII aggregation and fluorescence quenching leading to the decrease in the quantum efficiency of PSII. However, an extensive study of the effect of thylakoid lipids on LHCII aggregation *in vitro* revealed a complex picture of effects of various types of lipids on the process in many ways different from that of detergents, suggesting that different types of lipids can play an important role in LHCII, stabilising both efficient light harvesting and photoprotective states of LHCII [62]. Further studies on the quenching and structural properties of LHCII incorporated into various types of liposomes along with PsbS protein as was recently performed by Wilk et al. [63] but with an absolute requirement of induction of  $\Delta$ pH are needed for the further understanding of the role of environmental factors, protons and cations in the process of conformational switching of LHCII complexes that underlies the mechanism of NPQ.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbabo.2013.11.018>.

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