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Regulation of Light Harvesting in the Green Alga *Chlamydomonas reinhardtii*: The C-Terminus of LHCSR Is the Knob of a Dimmer Switch

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Supporting Information

ABSTRACT: Feedback mechanisms that dissipate excess photoexcitations in light-harvesting complexes (LHCs) are necessary to avoid detrimental oxidative stress in most photosynthetic eukaryotes. Here we demonstrate the unique ability of LHCSR, a stress-related LHC from the model organism *Chlamydomonas reinhardtii*, to sense pH variations, reversibly tuning its conformation from a light-harvesting state to a dissipative one. This conformational change is induced exclusively by the acidification of the environment, and the magnitude of quenching is correlated to the degree of acidification of the environment. We show that this ability to respond to different pH values is missing in the related major LHCII, despite high structural homology. Via mutagenesis and spectroscopic characterization, we show that LHCSR's uniqueness relies on its peculiar C-terminus subdomain, which acts as a sensor of the lumenal pH, able to tune the quenching level of the complex.

The capture and storage of light energy by photosynthetic organisms is the process that sustains virtually all life on earth, but it is also a hazardous business. If the absorbed energy exceeds the capacity of the metabolic reactions, it can result in photo-oxidation events that can ultimately result in the organism's death.[1] Plants and algae have evolved elaborate mechanisms to protect themselves against oxidative damage.[1,2] Collectively known as non-photochemical quenching (NPQ), these multicomponent mechanisms serve to dissipate excess absorbed energy as heat. It is known that this process is triggered by low luminal pH,[1] an indication that the electron transport chain in the photosynthetic apparatus is under stress, but the exact action mechanism is a matter of debate.

Members of a subfamily of light-harvesting complex (LHC) genes are known to be major players in this process.[2] While the PsbS protein required for qE, the fast component of NPQ, is constitutively expressed in higher plants and does not bind pigments,[1] algae and mosses[3] require the expression of a stress-related pigment-binding complex previously indicated as LI818.[4,5] LHCSR, as it is now known, has recently been identified as the key component to activate qE in the model organism *Chlamydomonas reinhardtii*.[3]

LHCBs, the light-harvesting antennae of photosystem II (PSII), were also suggested to have a role in the quenching process as sites of chlorophyll energy dissipation.[6,7] Currently, the hypothesis that PsbS and LHCSR represent active triggers of a conformational switch after sensing lumen acidification is the most accepted one.[8,9] This switch is in turn hypothesized to initiate a functional rearrangement of the whole PSII, including conformational changes in LHCb antennae, leading to energy dissipation.[10−12] However, the nature of the quencher still remains a matter of debate,[6,13−15] and the fact that LHCSR binds pigments, while PsbS does not, suggests different quenching mechanisms in plants and algae. Nevertheless, in all organisms, the necessary condition to induce structural interconversion is the availability of pH sensors. PsbS has been shown to possess two lumen-exposed acidic residues which are necessary for its function in qE in plants.[16] Although the availability of one or two pH-sensitive residues was also reported for nearly every LHCb,[17−19] a self-assisted conformational switch to a dissipative state upon lumen acidification for the single PSII antenna has not been clearly demonstrated. Indeed, most of the studies showing pH-dependent quenching of LHCs[19−22] have been performed upon detergent removal, thus inducing aggregation. Oligomerization is well known to cause high degrees of quenching,[23,24] with the dissipation magnitude depending on the size of the aggregates.[24,25] This makes it impossible to discriminate between the direct effect of the pH on observed quenching and that of aggregation, and it is then easy to understand the primary importance of elucidating the direct effect of pH on the induction of energy dissipation. In this work, we employed a new methodology to investigate *in vitro* the response and sensitivity to pH variations of two different systems. First, we studied the main LHC complex, LHCII, in both trimeric and monomeric forms, aiming to characterize its sensitivity to its environment. Next we focused on the pH response of the stress-related LHCSR from *C. reinhardtii*, with the aim of understanding its action mechanism in triggering qE activation.[3] Finally, we investigated possible bridges between its optical properties and structural features by mutating all protonable residues in its C-terminus.

To obtain reliable data, it is essential to be able to perform the experiments at different pH values without incurring aggregation or misfolding of the complexes. Indeed, aggregation is not the only undesired side effect deriving from an acid

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environment. There is risk of protein misfolding with consequent loss and degradation of pigments (at pH ≈ 5.5, chlorophylls are already over the threshold of pheophytinization).[26] Therefore, for experiments in stress conditions (pH ≪ 6), solubilizing protein in common detergents (β-DM or α-DM) was not reliable; this method systematically resulted in a significant loss of pigments, especially in the case of monomers. To reduce these undesirable effects, we made use of neutral amphipols (called NAPols),[27] which are synthetic polymers that keep membrane proteins stable in aqueous solutions. Replacement of detergent (α-DM or β-DM) with NAPol resulted in stable pigment-binding complexes at all experimental conditions tested here, while at the same time not affecting the optical properties of the samples (Supporting Information (SI) Figures S1 and S2).

Briefly, after exchanging pigment−protein complexes in detergent for NAPol (methods described in SI), we varied the acidity of the solution first by dilution from a neutral solution buffer (HEPES at pH 7.6) to the desired ones (MES at pH 5.5 and citrate buffer at pH 4.4). Next, the samples were loaded on sucrose density gradients with different pH buffers. This step sets the final pH and reveals the oligomeric state of the complexes. In general, one band corresponding to monomeric or trimeric complexes was observed in all gradients. We noticed that some aggregates were formed, in the case of LHCII monomers and LHCSR, as expected only at lower pH. To check that no aggregation was taking place in the monomeric/trimeric samples, we acquired the 77 K fluorescence spectra of all samples. The eventual appearance of a fingerprint-emitting band around 700 nm would have indicated that aggregation processes had taken place.[23] Measurements were repeated after 24 h to be sure that any eventual process of oligomerization was complete. The formation of aggregates was not detected in the spectra from the collected monomeric or trimeric bands in any case (Figures 1A,B and 2A,B).

We measured time-resolved fluorescence, via time-correlated single-photon counting (TCSPC) technique, to test the effect of pH on the chlorophyll singlet excited state lifetime of the complexes. Emission was recorded after selective excitation at 470 nm and monochromatic acquisition at 685 nm for LHCSR (emission maxima centered at ∼685 nm) and 680 nm for

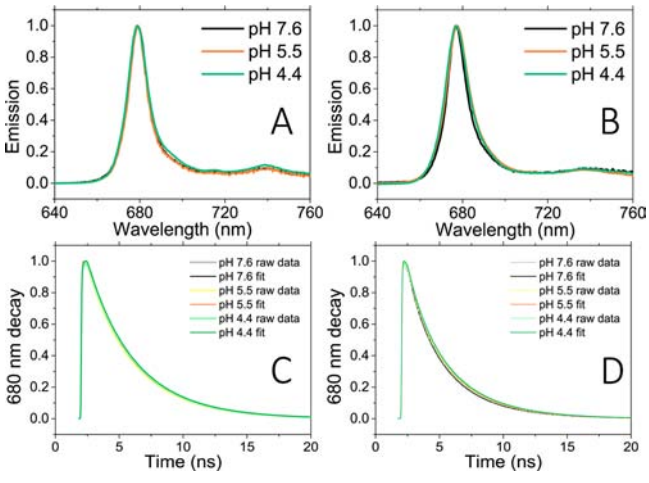

Figure 1. 77 K emission spectra of LHCII (A) trimers and (B) monomers at indicated pH conditions. Raw and fitted traces acquired via TCSPC of LHCII (C) trimers and (D) monomers at indicated pH conditions.

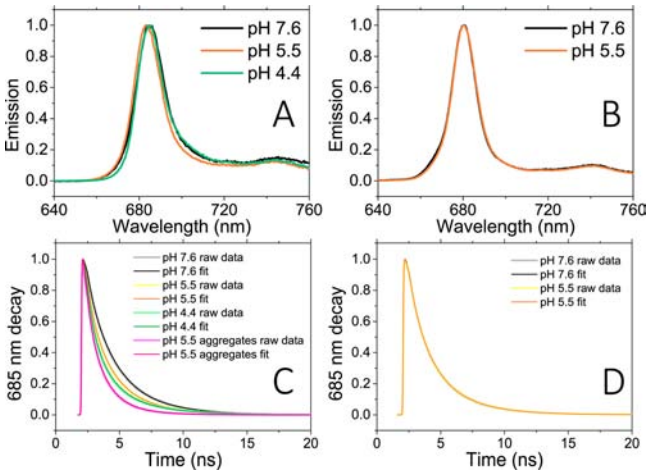

Figure 2. (A) 77 K emission spectra of LHCSR-wildtype and (B) LHCSR-NEUTAIL, under different pH conditions. Raw and fitted traces acquired via TCSPC of (C) LHCSR-wildtype and (D) LHCSR-NEUTAIL, again under different pH conditions.

LHCII (emission maxima at ∼680). In all conditions, we found that three components were sufficient for an optimal global fit of the fluorescence kinetics. As shown in Tables S1, the lifetimes obtained for LHCII trimeric and monomeric complexes at pH 7.6 were ∼3.3 and ∼2.6 ns, respectively (Figure 1C,D, Table S1), highly comparable to the values reported in the literature,[28,29] and in no case were they visibly shortened at lower pH. For each sample, we calculated the percentage of quenching as the ratio between the lifetime in stress conditions (pH 5.5 or 4.4) over the lifetime at pH 7.6. Virtually no quenching was observed at any of the pH's for LHCII (both trimers and monomers) (Table S1).

To support our findings, we additionally measured the relative fluorescence quantum yield for all systems under investigation (Table S2). This value is expressed as the ratio between the quantum yields at lower pH (5.5 or 4.4) and at pH 7.6 (see SI). Again, no significant changes (<10%) in quantum yield were detected (Table S2). To confirm that NAPol did not affect the response of the antennae to environment conditions, we also tested LHCII trimers in α-DM at the different pH conditions. Again, we found a lifetime of ∼3.3 ns at all pH values (Figure S6, Table S4). No visible degradation was detected at low pH conditions in detergent, testament to the reputation of LHCII trimers as extremely stable complexes.

These results altogether strongly indicate that pH drop alone cannot induce conformational changes to a dissipative state in LHCII monomers or trimers. This leads to the conclusion that quenching previously reported in LHCb complexes upon acidification and detergent removal[19−22] was caused by aggregation.

Strikingly different was the scenario depicted for LHCSR. The decay kinetics collected in low-light-mimicking conditions (pH 7.6) were satisfactorily described with three components, the shortest being ∼300 ps (Figure 2C, Table S1), while no lifetimes faster than 100 ps were actually found, at variance with previous results.[9] The calculated average lifetime was ∼1.9 ns, a value similar to that of the PSI antennae in unquenched conditions,[30] and significantly different from the 0.9 ns previously reported.[9] This latter value is more similar to that of an aggregate-like antenna, and it was indeed used to suggest that LHCSR is in a permanent quenching state in the

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membrane.[9] On the contrary, the LHCSR chlorophyll lifetime found in our study presents relaxation time scales for the nonstress conditions (pH 7.6) largely corresponding to a light-harvesting conformation and not to that of a quencher,[9] which suggests that LHCSR is not constantly quenched in the membrane.

In an acidic environment (pH 5.5 and 4.4), LHCSR displayed a clear shortening of the fluorescence lifetime, with the amplitude correlating with the drop in pH (Figure 2C, Table S1). A 23% quenching activity is observed at pH 5.5, whose magnitude increases to 32% at pH 4.4. Interestingly, upon quenching induction in LHCSR, we obtained a significant increase of the amplitude of the shortest component (~300–400 ps), indicating that the protonation stabilizes the quenched conformation. Again, confirming the trends registered via TCSPC acquisition, a quantum yield reduction of 28% was calculated at pH 5.5, reaching values of 49% at pH 4.4 (Table S2). As was the case for LHCb monomers, a small amount of LHCSR aggregates was present in the sucrose gradient at low pH. Fluorescence lifetime measurements on LHCSR aggregates (Figure 2C, Table S1) clearly showed that the quenching extent was simply increased upon aggregation.

To check if the pH-dependent quenching of LHCSR is reversible, we again acquired time-resolved fluorescence on a sample previously placed at pH 4.4 and consequently diluted back to pH 7.6 (methods in SI). Lifetime increased from ~1.5 ns (pH 4.4) back to ~2.1 ns at pH 7.6 (Figure S5, Table S3), confirming that the pH-dependent "switch" of LHCSR is completely reversible. The small difference in lifetimes compared to the previous measurements is attributed to a small amount of free pigments in the preparation at both pH's.

From the dependence of the extent of quenching on the pH, we derive that the nature of the conformational switch for this protein is different than an "on–off mechanism" activated once the pH is below a certain threshold. The protein scaffold of LHCSR seems to functionally tune pigment interactions due to a folding conformation that is sensitive to the acidity of the environment and determines the extent of the quenching.

To understand the origin of the pH sensitivity of LHCSR, we compared the primary sequences of LHCSR with those of two model antennae, LHCII and CP29. Although most of the pigment-binding sites and secondary structure elements are conserved,[9] there is an obvious difference in the C-terminus region (Figure 3A). LHCSR presents nine acidic residues compared to only two in LHCII. Figure 3B shows a model rendered after alignment of LHCSR and LHCII, suggesting that these acidic residues are exposed to the luminal space.

We hypothesized an involvement of these residues in the pH-dependent sensitivity and conformational switch peculiar of LHCSR. To test this hypothesis, we designed a mutant LHCSR-NEUTAIL in which the nine acidic residues have been exchanged for neutral amino acids with no capability to sense pH (glutamic acid to glutamine, aspartic acid to asparagine).[16] After overexpression of the apoprotein in *Escherichia coli*, the pigment–protein complex was reconstituted in vitro with pigments (see SI). Spectroscopic characterization of the mutant shows that the LHCSR-NEUTAIL is virtually identical to the wildtype (WT) (Figure S3A,B). However, the pH response of the mutant differs from that of the WT: LHCSR-NEUTAIL was incapable to respond to pH changes (Figure 2D, Table S1). The average lifetime calculated from the fit of the fluorescence decay kinetics (~1.8 ns), as well as the lifetime and the amplitude of the three decay components, was the same at pH

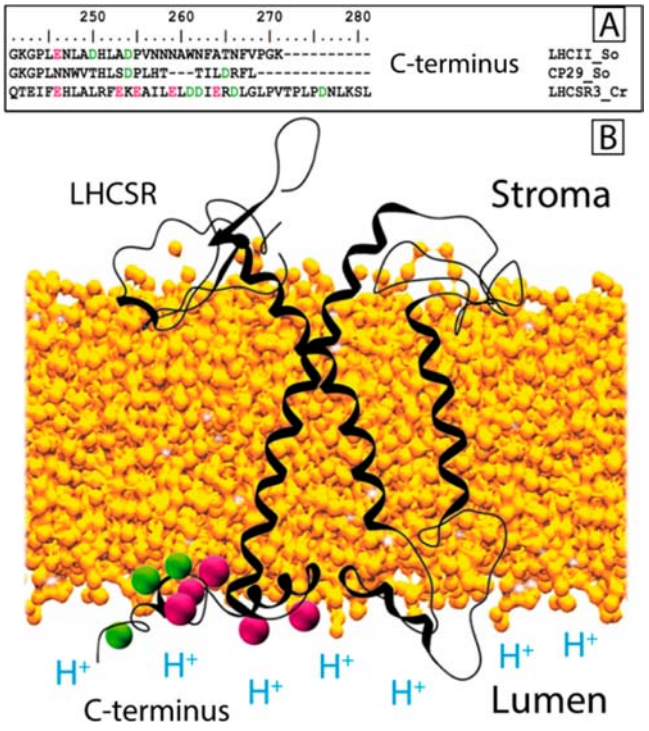

Figure 3. (A) Protein sequence alignment of the C-terminus of LHCSR from *Chlamydomonas reinhardtii* (Cr) and the related LHCII and CP29 from *Spinacia oleracea* (So). Evidenced in green and mauve are aspartic (D) and glutamic (E) acids. Rendered in (B), a 3D structure of LHCSR obtained from structure homology modeling based on the crystal structure of LHCII from spinach. From the overall black structure of the protein, we can distinguish at the C-terminal (same color scheme as in (A)) eight different acid residues (the ninth one belongs to a portion of the C-terminus longer than the LHCII corresponding domain and therefore excluded from this homology model). In yellow is rendered a simplified section of a model thylakoid membrane.

7.6 and 5.5. Relative quantum yield measurements confirmed that pH sensitivity in LHCSR-NEUTAIL was abolished by our mutations (Table S2). In the case of LHCSR-NEUTAIL, a release of pigments was observed at pH 4.4 (Figure S4), which hindered a clear interpretation of the data collected (Tables S1 and S2). Pigment disconnection could be due to the pH conditions being too harsh for the mutated protein, perhaps because one or more of the mutated residues may be important for stabilization under acidic conditions.

Summarizing, the data suggest that the LHCII antennas are optimized for light capture and excitation energy transfer[31] and do not have the in-built capability to change conformation in response to pH changes. Instead, they require external triggers to switch to a dissipative conformation in stress conditions.[1,3,8,32] On the contrary, LHCSR is able to switch from a light-harvesting to a dissipative conformation simply in response to a pH change, while it is in a monomeric state and without the need of zeaxanthin binding. Interestingly our data show that, in contrast to what was previously suggested, at neutral pH LHCSR exists in a light-harvesting conformation and can thus participate in the energy transfer dynamics of PSII when normal physiological conditions are restored in the organism. Then, as a chameleon protein, LHCSR possesses the ability to convert to a nonaggregated quenched conformation under stress conditions, with the amplitude of the energy-

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dissipating activity depending on the degree of pH-drop in the thylakoid lumen. Presumably, this switch in conformation can in turn trigger a conformational switch in other LHC partners,[11] with LHCSR in the double role of trigger and quencher. What is clear is that the unique characteristic of the LHCSR C-terminus, being rich in acidic residues, confers the ability to sense pH variations and drive NPQ in the model organism *Chlamydomonas reinhardtii*.

■ ASSOCIATED CONTENT

Supporting Information

Complete experimental details and further analyses as indicated in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): G.D. holds a patent on nonionic amphiphilic homopolymers [Pucci, B.; Popot, J.-L.; Sharma, K. S.; Bazzacco, P.; Durand, G.; Giusti, F. Polymers comprising a majority of amphiphilic monomers intended for trapping and manipulation of membrane proteins. FR Patent 2,952,642, Nov 16, 2009].

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■ NOTE ADDED AFTER ASAP PUBLICATION

In text related to Figure 2C and Table S1, the description of amplitude of the shortest component was corrected from ∼300−400 fs to ∼300−400 ps. The revised version was re-posted on November 27, 2013.