

## Molecular Mechanisms of Nonphotochemical Quenching in the LHCSR3 Protein of *Chlamydomonas reinhardtii*

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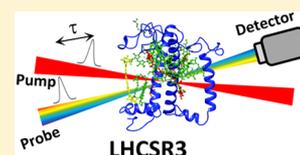
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### Supporting Information

**ABSTRACT:** Photosynthetic organisms possess photoprotection mechanisms from excess light conditions. The fastest response consists in the pH-triggered activation of a dissipation channel of the energy absorbed by the chlorophylls into heat, called nonphotochemical quenching. In green algae, the pigment binding complex LHCSR3 acts both as a chlorophyll quencher and as a pH detector. In this work, we study the quenching of the LHCSR3 protein *in vitro* considering two different protein aggregation states and two pH conditions using a combination of picosecond time-resolved photoluminescence and femtosecond transient absorption in the visible and NIR spectral regions. We find that the mechanisms at the basis of LHCSR3 quenching activity are always active, even at pH 7.5 and low aggregation. However, quenching efficiency is strongly enhanced by pH and by aggregation conditions. In particular, we find that electron transfer from carotenoids to chlorophylls is enhanced at low pH, while quenching mediated by protein–protein interactions is increased by going to a high aggregation state. We also observe a weak pH-dependent energy transfer from the chlorophylls to the S<sub>1</sub> state of carotenoids.



Photosynthetic organisms depend on the harvested sunlight to fuel their metabolic energy consumption. However, high-intensity illumination can be harmful to the organism. Under excess light conditions, the activity of the photosynthetic electron transfer chain saturates, inhibiting the photochemical relaxation of chlorophyll (Chl) singlet excited states (<sup>1</sup>Chl\*). These states can then undergo intersystem crossing to the Chl excited triplet state (<sup>3</sup>Chl\*), and the consequent formation of singlet oxygen (<sup>1</sup>O<sub>2</sub>\*) causes photodamage or even cell death.<sup>1,2</sup> Photosynthetic organisms evolved a variety of photoprotection mechanisms to prevent such harmful effects: differential gene expression and metabolic changes act on the long-term,<sup>2</sup> while at the molecular level short-term mechanisms are rapidly activated upon increased irradiances.<sup>3–5</sup>

One of these short-term responses is called nonphotochemical quenching (NPQ) and consists in the opening of channels for safe dissipation of the excitation energy as heat. NPQ consists of several processes, the fastest of which is called energy-dependent quenching (qE).<sup>3–8</sup> Several nonmutually exclusive molecular mechanisms for qE were proposed, all involving activation of a fast (~ps) quenching process of <sup>1</sup>Chl\*. Strong electronic coupling of two adjacent Chls was suggested to lead to the formation of a charge transfer state that dissipates energy.<sup>9,10</sup> All other qE mechanisms involve the interaction of Chls with carotenoids (Cars), which are suitable quencher molecules due to their low-energy short-lived dark states.<sup>11,12</sup> One is energy transfer from <sup>1</sup>Chl\* to a lower energy excited dark state of the Car, such as S<sub>1</sub><sup>13–15</sup> or the more recently proposed S<sub>q</sub><sup>16</sup> with short (~ps) lifetime; another is

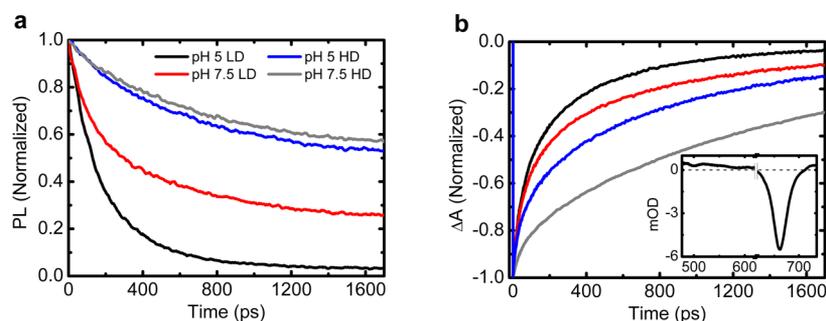
electron transfer from the Car to the photoexcited Chl, generating a Car radical cation and a Chl anion;<sup>17–20</sup> a third mechanism is Car-Chl excitonic coupling, with the coupled state acting as excitation quencher.<sup>21</sup> Often Cars quenching is related to the xanthophyll cycle (conversion of Car violaxanthin to zeaxanthin), which upregulates the NPQ process.<sup>22–24</sup>

Previous studies demonstrated that qE is triggered by lumen acidification caused by saturation of the photosynthetic apparatus: Its onset thus requires both a sensor of the luminal pH and a quencher for energy dissipation. In higher plants, the pH sensor is the nonpigment-binding protein PSBS,<sup>25,26</sup> while other proteins from the light harvesting complex gene family are responsible for the energy dissipation process.<sup>9,13,19</sup> In algae, pigment binding complexes named Light Harvesting Complex Stress Related (LHCSR)<sup>27</sup> proteins are responsible for both pH detection and Chl quenching. In the model organism for green algae *Chlamydomonas* (*C.*) *reinhardtii* two LHCSR isoforms, LHCSR1 and LHCSR3, were observed to accumulate in the thylakoid membrane of the chloroplasts under excess illumination, with the latter being the main trigger for qE activation.<sup>27</sup> LHCSR are trihelical proteins with Chl a, Chl b, and Car molecules bound to the helices and acid functional groups attached facing the luminal part of the chloroplasts.<sup>28–30</sup> High light stress conditions generate a pH

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**Figure 1.** LHCSR3 kinetics traces measured by (a) TRPL (pump at 430 nm, integrated from 650 to 760 nm) and (b) TA at 680 nm (pump at 630 nm). Inset: TA spectrum at 500 fs (pH 5 LD).

gradient across the thylakoid membrane, which is sensed by the acid groups of LHCSR3 and triggers its quenching activity.<sup>29–32</sup>

The molecular quenching mechanisms in LHCSR proteins are still intensively studied and debated.<sup>22,28,31</sup> A zeaxanthin-dependent and a zeaxanthin-independent quenching process were identified in the LHCSR1 from the moss *Physcomitrella (P.) patens*, involving, respectively, energy transfer to the Car  $S_1$  state and formation of a Car radical cation.<sup>22</sup> Both quenching mechanisms, energy transfer to Car  $S_1$  and Car radical cation formation, have been also recently observed *in vivo* in the microalga *Nannochloropsis oceanica* strictly related to the presence of both zeaxanthin and the complex LHCX1, homologous to LHCSR subunits.<sup>33</sup> Differently, in the case of *C. reinhardtii*, zeaxanthin has been demonstrated to be unnecessary for qE induction,<sup>30</sup> opening the question about the quenching mechanisms underlying LHCSR3 activity *in vivo*. In this complex, a zeaxanthin-independent Car radical cation quenching mechanism was previously reported, being triggered at low pH.<sup>30</sup> However, this mechanism was measured *in vitro*, resulting in protein complexes with a fluorescence lifetime in the ns scale, incompatible with the strong quenching on tens to hundreds of ps time scales observed upon activation of LHCSR3-dependent quenching *in vivo*<sup>33,34</sup> or *in vitro* in isolated PSII-LHCII-LHCSR supercomplexes.<sup>32</sup> Recently, site directed mutagenesis<sup>31</sup> allowed to obtain *C. reinhardtii* LHCSR3 variants with reduced quenching activity and pH inducibility *in vivo*. However, similar reduced quenching efficiency *in vitro* was obtained only when partial aggregation of the protein was induced by reducing the detergent concentration.<sup>31</sup> It is known that qE induction *in vivo* is accompanied by a change in protein–protein interactions,<sup>35</sup> so these results suggest that protein–protein interactions induced *in vitro* by partial aggregation could simulate the quenching conditions occurring *in vivo*, even if the neighboring interacting proteins are not exactly the same in both cases. In this work, we employ ultrafast optical spectroscopy to understand the quenching mechanisms in LHCSR3 under different pH and aggregation conditions. We detect the onset of quenching pathways in LHCSR3 *in vitro* by changing the pH from 7.5 (neutral) to 5 (acid medium) and by reducing the concentration of detergent from high to low. We identify distinct pH- and aggregation-dependent mechanisms that are responsible for the qE activity of the protein.

LHCSR3 holoprotein was obtained upon pigment addition in an *in vitro* refolding process of recombinant apoprotein overexpressed in *E. coli*, as previously described.<sup>30</sup> The refolded protein was characterized by an absorption spectrum with a peak in the Chl Qy region at 679.2 nm (Figure S1 in the

Supporting Information, SI) and fluorescence peaking at 683 nm (Figure S2), consistent with previous findings.<sup>30</sup> Pigment binding properties of the refolded complex are reported in Table S1. To induce protein aggregation, the concentration of the detergent alpha-dodecyl maltoside ( $\alpha$ -DM) was decreased from 0.03% to 0.003% (see Materials and Methods in the SI). For simplicity, these conditions are referred to as high detergent (HD) and low detergent (LD) in the following. In principle, different pH conditions can induce different aggregation levels, so we tested the amount of aggregation using the fluorescence spectra at 77 K. Figure S3 reveals the formation of oligomers characterized by increased far-red fluorescence only in the case of LD samples, with the strongest aggregate emission found in pH 5 LD. Hence, the HD samples act as controls for the effect of pH alone. We study the photophysics of the samples following photoexcitation of the Chls, combining time-resolved photoluminescence (TRPL), to determine the lifetime of  $^1\text{Chl}^*$ , and transient absorption (TA), to identify the formation and decay of dark excited states such as triplets, the Car  $S_1$ , and radical cation states.

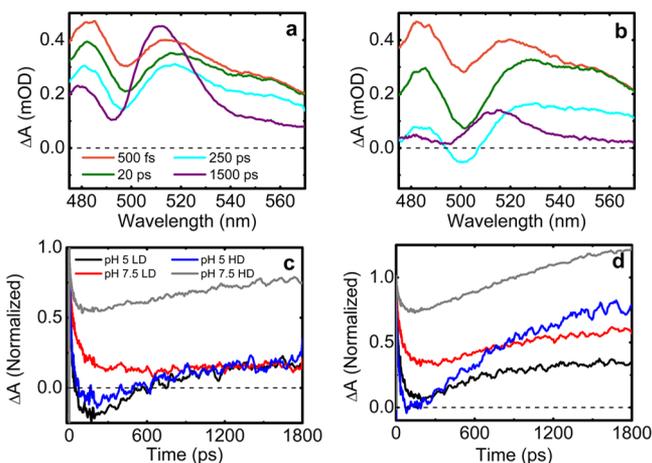
Figure 1a shows the normalized TRPL decay kinetics integrated over the 650–760 nm spectral range under different pH and aggregation conditions. The HD sample at pH 5 shows a faster decay compared to pH 7.5, a trend that is also present under LD conditions with even shorter lifetimes (see Table S2 for time constants obtained by biexponential fits). This confirms that both aggregation and pH play a role in the overall quenching mechanism, with the detergent concentration being the most important factor, as the pH 7.5 LD sample is more quenched than the pH 5 HD one. Similar behavior was observed in the related protein LHCSR1 from *P. patens*.<sup>36</sup> TRPL maps and spectra integrated over the entire temporal window of the experiment are reported in Figures S4 and S5, respectively.

Figure 1b displays the TA kinetics at 680 nm of all samples (for TA maps and spectra in the visible, see Figures S6–S8) following excitation at 630 nm. For all samples we used the same optical density and pump fluence, set at  $6 \mu\text{J}/\text{cm}^2$  to minimize bimolecular annihilation effects<sup>37</sup> (see Figure S9 for fluence-dependent dynamics of all quenched samples). At early times, the TA spectra of all samples consist of a negative peak around 680 nm, due to ground state bleach (GSB) and stimulated emission (SE) of  $^1\text{Chl}^*$ , and a broad positive band from 470 to 600 nm corresponding to photoinduced absorption (PA) from  $^1\text{Chl}^*$ . The GSB/SE dynamics follow the same trend observed in TRPL: shorter lifetimes at low pH and LD, with the aggregation state prevailing. In the TA data a fast component is resolved in the pH 5 HD and LD samples, whereas in TRPL measurements this difference is not observed

due to the lower temporal resolution (20 ps as compared to 100 fs).

Most of the reported qE mechanisms involve interaction of the Chls with Cars. In our TA experiments, selective excitation of  $^1\text{Chl}^*$  ensures that any signal from the Car molecules must be due to an interaction with the photoexcited Chl. We thus focus on the 470–570 nm wavelength range (for TA maps and spectra, see Figures S7 and S8b) that contains the Car GSB (peaking at 500 nm) and the Car triplet–triplet PA (peaking at 510 nm),<sup>11,38</sup> besides the previously mentioned broad PA of the  $^1\text{Chl}^*$ .

Figure 2a,b shows sequences of TA spectra for the most contrasting samples: pH 7.5 HD and pH 5 LD, respectively. At



**Figure 2.** TA of LHCSR3 exciting  $^1\text{Chl}^*$  at 630 nm for (a) pH 7.5 HD and (b) pH 5 LD conditions. Kinetics measured at (c) 500 nm, showing the buildup of Car GSB, and (d) 510 nm, showing the buildup of Car triplet–triplet PA.

early times, a negative peak overlapped in the broad PA spectrum of  $^1\text{Chl}^*$  is evident at 500 nm in both samples; as it matches the 0–0 peak of the  $S_0 \rightarrow S_2$  Cars transition, we assign it to Cars GSB. These results highlight coupling between Chls and Cars, previously observed<sup>21,22</sup> and assigned to an ultrafast electrochromic shift of the carotenoid in response to the presence of the excited state of the Chl a. In Figures 2a,b we observe a buildup of the Cars GSB over tens of picoseconds, which is a trend we find on the other samples too (Figure 2c). This is more strongly present in the low pH samples, with the Car GSB becoming strong enough to prevail over the Chl PA and cause a change of sign of the overall TA signal. On the nanosecond time scale, we observe in Figure 2d the formation of a PA band peaking at 510 nm, attributed to the absorption of  $^3\text{Car}^*$ .<sup>22</sup> This state is populated via triplet–triplet energy transfer from the Chl to the Car, following intersystem crossing (ISC) in the Chl, according to the scheme  $^1\text{Chl}^* \rightarrow ^3\text{Chl}^* \rightarrow ^3\text{Car}^*$ .<sup>36</sup> Therefore, a longer lifetime of the  $^1\text{Chl}^*$  state corresponds to a higher efficiency for the ISC process and to a higher  $^3\text{Car}^*$  signal. Consistently, the formation of the  $^3\text{Car}^*$  PA band in Figure 2d is more pronounced in the less quenched samples, with the highest triplet PA signal seen in pH 7.5 HD. The TA data in the Car region was adequately fitted with three time constants for all samples using a global analysis routine (see Figure S10 for the quality of the fits).<sup>39</sup> The fit results are summarized in Table 1, and decay-associated spectra (DAS) of all samples are shown in Figure S11. We assign the calculated time constants to a fast multiexponential buildup of the

**Table 1.** Time Constants from the Global Fit Analysis of the TA Data from 480 to 570 nm

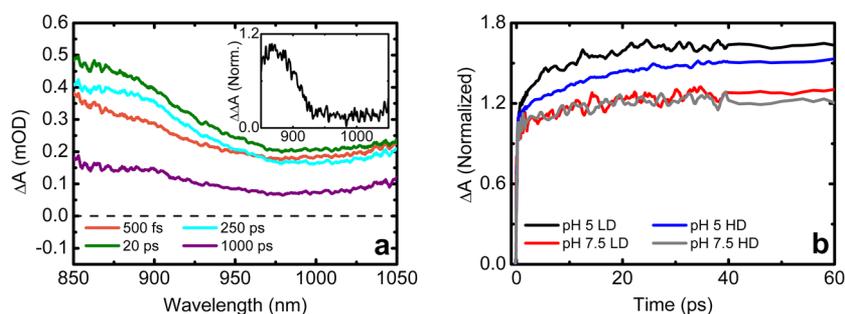
	$\tau_1$ (ps)	$\tau_2$ (ps)	$\tau_3$ (ps)
pH 7.5 HD	$13.4 \pm 0.1$	$106 \pm 1$	$1559 \pm 13$
pH 5 HD	$10.3 \pm 0.1$	$81 \pm 1$	$718 \pm 6$
pH 7.5 LD	$11.3 \pm 0.2$	$101 \pm 1$	$684 \pm 7$
pH 5 LD	$9.0 \pm 0.1$	$69 \pm 0.5$	$477 \pm 2$

carotenoid GSB ( $\sim 10$  ps,  $\tau_1$ ; 70–100 ps,  $\tau_2$ ), followed by the rise of the triplet state in a longer time scale (480–1600 ps,  $\tau_3$ ), which becomes faster in the more quenched samples due to the shorter lifetime of the  $^1\text{Chl}^*$  state.

Comparison between TA spectra in Figures 1b and 2c reveals a correlation between the quenching activity of LHCSR3 and the build-up Car GSB, pointing to the involvement of Cars in the qE process. However, this observation does not provide conclusive evidence of the quenching mechanism as we cannot distinguish if the process is associated with energy or charge transfer from Chls to Cars. Careful inspection of the TA dynamics at 560 nm for the pH 5 samples (both HD and LD), shown in Figure S12, reveals a weak increase of the PA signal. As this probe wavelength matches the peak of the characteristic intense  $S_1 \rightarrow S_n$  PA band of Cars,<sup>36</sup> this demonstrates the presence of a weak quenching channel for  $^1\text{Chl}^*$  via energy transfer to the Car  $S_1$ . This channel is activated solely at low pH since it is not observed in the pH 7.5 HD or LD samples.

Because Cars radical cations absorb in the NIR spectral range, following previous studies,<sup>22,30</sup> we can directly assess the presence of electron transfer to the photoexcited Chls by extending our TA probe to the 850–1050 nm spectral range.

TA spectra at different pump–probe delays are reported in Figure 3a (see Figure S13 for the complete maps). At early times one observes a broad positive band, which is assigned to PA of the photoexcited Chl;<sup>17</sup> on a 20 ps time scale the signal shows a clear build-up, which is more pronounced at 880 nm with respect to 1050 nm. Since the Chl population decays on this time scale (see Figure 1), the signal build-up must be associated with the formation of a new species. To estimate the spectrum of this species, we take the difference of the TA spectra recorded at 20 ps (when most of the build-up has occurred) and 500 fs. The resulting spectrum (inset of Figure 3a) peaks at 870 nm, in excellent agreement with the PA spectrum of the lutein radical cation.<sup>40</sup> Due to our limited spectral coverage, we cannot exclude partial involvement of the other Car present in LHCSR3, violaxanthin, for which the radical cation PA peaks at 830 nm.<sup>40</sup> The TA dynamics at 880 nm, reported in Figure 3b, show that the Car radical cation is formed even at pH 7.5, and in similar amounts regardless of the detergent concentration. At pH 5 the formation of radical cation is enhanced, especially so for LD conditions. Global analysis of the NIR TA revealed three time constants for all samples (Table 2 and Figure S14), which were assigned to a fast multiexponential buildup of the Car radical cation ( $\sim 10$  ps,  $\tau_1$ ;  $\sim 70$  ps,  $\tau_2$ ) followed by a longer decay ( $>400$  ps,  $\tau_3$ ), which reflects both Car radical cation decay and Chl excited state decay. These observations correlate very well with the buildup of the Car GSB at 500 nm seen in Figure 2c. Therefore, we conclude that the buildup of the PA signal in the NIR and of the Cars GSB at 500 nm are predominantly associated with the formation of the Car radical cation, and that electron transfer from Car to Chl is an important



**Figure 3.** (a) NIR TA spectra of LHCSR3 for the pH5 LD sample following Chl excitation at 630 nm. Inset: difference TA spectrum obtained by subtraction of the spectra at 20 ps and 500 fs. (b) TA kinetics at 880 nm normalized by their average value over the first 300 fs following excitation.

**Table 2. LHCSR3 Time Constants Obtained by a Global Fit of the Data in the 880–1050 nm Spectral Range**

	$\tau_1$ (ps)	$\tau_2$ (ps)	$\tau_3$ (ps)
pH 7.5 HD	$9.7 \pm 0.2$	$74.7 \pm 3.1$	$906.9 \pm 12.5$
pH 5 HD	$10.1 \pm 0.1$	$56.4 \pm 0.5$	$772.9 \pm 2.9$
pH 7.5 LD	$20.0 \pm 0.5$	$91.3 \pm 4.4$	$559.2 \pm 8.7$
pH 5 LD	$10.2 \pm 0.1$	$56.0 \pm 1.0$	$409.7 \pm 1.1$

quenching mechanism in LHCSR3 enhanced by pH and aggregation.

Our results demonstrate the pH-dependent activation of electron transfer from Car to Chl in LHCSR3 and a concomitant weak excitation energy transfer to Car  $S_1$ . These findings can be compared to what has been recently reported *in vivo* in the case of LHCX1 from *N. oceanica*,<sup>33</sup> where both quenching mechanisms were observed but only in the presence of zeaxanthin. Chl to Car energy transfer has been previously reported also *in vitro* for the LHCSR1 from *P. patens*, again only in the presence of zeaxanthin,<sup>22</sup> or in the case of trimeric LHCII in aggregation conditions only.<sup>13</sup> The case of LHCSR3 from *C. reinhardtii* herein reported is peculiar since Car radical cation formation and Chl to Car  $S_1$  energy transfer are both related to low pH and occur even in absence of zeaxanthin or aggregation. Electron transfer from Car to Chl is, however, further enhanced at pH 5 and LD conditions, where LHCSR3 activity was previously correlated to its *in vivo* activity.<sup>31</sup>

The radical cation formation and the energy transfer to the Car  $S_1$  are, however, insufficient to fully explain the quenching activity of LHCSR3. Indeed, these processes are, respectively, enhanced and activated at low pH, while our data consistently show that the pH 7.5 LD sample is more quenched than the pH 5 HD one. Hence, there must be an additional quenching mechanism related to protein–protein interactions occurring in the LD samples, the condition where a closer correlation was found with the *in vivo* situation by analyzing the LHCSR3 activity in mutant variants.<sup>31</sup> Aggregation-dependent quenching mechanisms have been previously reported both *in vitro* and *in vivo* in the case of different LHC complexes in higher plants,<sup>9,13,41,42</sup> being revealed by the appearance of far-red fluorescence in quenched leaves.<sup>43</sup> In the case of *C. reinhardtii* such far-red fluorescence was not detected in quenched cells.<sup>44</sup> However, we note that upon NPQ induction a strong LHCSR3-dependent reduction of PSI fluorescence was also recently reported,<sup>45</sup> likely masking the possible appearance of aggregation-dependent far-red fluorescence. Considering the substoichiometric content of LHCSR3 per PSII,<sup>46</sup> LHCSR proteins upon protonation likely change their conformation

inducing a different interaction with other LHC subunits rather than forming oligomers themselves.

In our LHCSR3 measurements, TRPL and TA data consistently show decay components for the  $^1\text{Chl}^*$  signal with hundreds of ps time constant in LD conditions, with increased amplitude at low pH (see Figure 1), indicating the presence of a quenching mechanism related to aggregation and consequent protein–protein interactions. This is consistent with previous TRPL work on LHCSR1 from *P. patens*<sup>36</sup> showing in LD conditions the presence of a 80 ps decay component with a red-shifted emission spectrum, which was attributed to the formation of aggregates. In the case of LHCII aggregates *in vitro*, besides energy transfer to Car  $S_1$ , the main quenching mechanism proposed is the formation of a Chl–Chl charge transfer state,<sup>9</sup> which could also be at the basis of the strong quenching observed in the case of LD samples here.

In conclusion, we presented a comprehensive characterization of the ultrafast photophysical mechanisms underlying qE in LHCSR3 using TRPL and TA in the visible and NIR regions with selective excitation of the Chls. The existence of signals attributable to the quenching species in all samples implies that the mechanisms of LHCSR3 quenching are always active, even at pH 7.5 and low aggregation. However, quenching efficiency is strongly enhanced by the pH and by aggregation conditions. In particular, we find that both energy transfer to the  $S_1$  of Car and electron transfer from Car to Chl are enhanced at low pH, with the latter being further increased by aggregation, the condition where a strong correlation was found between *in vitro* LHCSR3 activity and *in vivo* NPQ induction.<sup>31</sup> Since *in vivo* the lumen acidification is the trigger for LHCSR-dependent quenching in *C. reinhardtii*, it is important to note that protein–protein interactions are also tuned upon protonation of different thylakoidal proteins occurring at low pH. In a final model we can thus propose that lumen acidification induces qE by switching LHCSR3 to a strongly quenched state correlated to increased charge transfer from carotenoids to chlorophylls and increased protein–protein interactions. Quenching mechanisms in LHC proteins appear to be differently distributed among LHC subunits, with different quenching species being formed in different subunits and/or in different conditions. Possible quenching species were indeed observed in all the different LHC proteins investigated up to now, opening the unsolved question of how it is possible for these complexes with a high pigment density to maintain a stable and efficient light harvesting function rather than always causing excitation energy quenching. Considering the strong effect of the distance between Chl–Chl and/or Chl–Car for the formation of quenching species, fine-tuning of protein

conformation and pigment organization is at the base of the light harvesting vs energy quenching activity of LHC subunits.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcllett.9b01184.

Additional experimental data and global fits, materials and methods (PDF)

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### Notes

The authors declare no competing financial interest.

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