Antenna proton sensitivity determines photosynthetic light harvesting strategy

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Abstract

Photoprotective non-photochemical quenching (NPQ) represents an effective way to dissipate the light energy absorbed in excess by most phototrophs. It is often claimed that NPQ formation/relaxation kinetics are determined by xanthophyll composition. We, however, found that, for the alveolate alga Chromera velia, this is not the case. In the present paper, we investigated the reasons for the constitutive high rate of quenching displayed by the alga by comparing its light harvesting strategies with those of a model phototroph, the land plant Spinacia oleracea. Experimental results and in silico studies support the idea that fast quenching is due not to xanthophylls, but to intrinsic properties of the Chromera light harvesting complex (CLH) protein, related to amino acid composition and protein folding. The $pK_a$ for CLH quenching was shifted by 0.5 units to a higher pH compared with higher plant antennas (light harvesting complex II; LHCII). We conclude that, whilst higher plant LHCII is better suited for light harvesting, CLHs are 'natural quenchers' ready to switch into a dissipative state. We propose that organisms with antenna proteins intrinsically more sensitive to protons, such as C. velia, carry a relatively high concentration of violaxanthin to improve their light harvesting. In contrast, higher plants need less violaxanthin per chlorophyll because LHCII proteins are more efficient light harvesters and instead require co-factors such as zeaxanthin and PsbS to accelerate and enhance quenching.

Keywords: Chromera velia, in vitro quenching, light harvesting strategy, non-photochemical quenching, NPQ kinetics, photoprotection, quenching $pK_a$, violaxanthin.

Introduction

Although under low light more than 83% of absorbed photons can be converted into chemical energy (e.g. Jennings et al., 2005; Wientjes et al., 2013), prolonged high light exposure rapidly switches photosystems to energy-dissipating states that release excess energy as heat (Demmig-Adams, 1990; Kaňa and Vass, 2008; Ruban et al., 2012). The switch from light-harvesting to energy-dissipation mode has long been investigated, resulting in various models for various autotrophs (Demmig-Adams, 1990; Horton et al., 1996; Kaňa et al., 2012; Pinnola et al., 2013; Erickson et al., 2015; Büchel, 2015).

In higher plants, several processes contribute to excess light energy dissipation, but only the pH-dependent one, the...
so-called energy-dependent quenching mechanism (non-photochemical quenching; NPQ) is considered photoprotective (Demmig-Adams and Adams, 1992; Ruban, 2013). Proof of a strict connection between NPQ and pH is that reverse ATPase activity can stimulate NPQ even in the dark (Gilmore and Yamamoto, 1992). Besides controlling xanthophyll cycle activity, in several phototrophs pH exerts a direct control on NPQ. This is thought to act via a regulation of antennas (e.g. Dekker and Boekema, 2005; Horton et al., 2008; Peers et al., 2009; Grossman et al., 2010). Indeed, a very similar thermal dissipation process to that in vivo can be induced in vitro in purified antennas by lowering pH and detergent concentration (Ruban et al., 1994a). Starting from this evidence, it was proposed that antenna aggregation is at the basis of the NPQ process (Horton et al., 1996), and subsequent findings employing liposomes started to clarify how pH and ions together with lipids and lipid to antenna ratios control the ‘aggregation state’ of antennas (Moya et al., 2001; Kirchhoff et al., 2008; Akhtar et al., 2015; Kafà and Govindjee, 2016; Natali et al., 2016; Crisafi and Pandit, 2017). In higher plants, antennas are the site of energy dissipation, whilst xanthophylls and the PsbS protein seem to be simply controllers of the process (Noctor et al., 1991; Walters et al., 1994; Li et al., 2000; Betterle et al., 2009). Evidence that npq1, a mutant lacking zeaxanthin, and npq4, a mutant without PsbS, could both perform NPQ indicated their dispensability, thus placing antennas and pH as the only key elements of the process (Niyogi, 1999; Johnson et al., 2009; Johnson et al., 2011). Nevertheless, xanthophylls play an important role modulating the kinetics of NPQ activation and dissipation (Johnson et al., 2010). Pre-conditioning of leaves with light exposure, for instance, makes NPQ fast and persistent because of the conversion of violaxanthin into zeaxanthin (Ruban and Horton, 1999). Zeaxanthin, a highly hydrophobic pigment, in turn, makes antennas more dehydrated and therefore sensitive to pH and prone to quench compared with violaxanthin–enriched antennas. Interestingly, this idea was put forward not only for higher plant antennas (Ruban et al., 1994a), but also for antennas from distant organisms such as diatoms (Guidermann and Büchel, 2008), brown algae (Ocampo-Alvarez et al., 2013) and alveolates (Kafà et al., 2016).

The state-of-the-art model of NPQ for plants claims that, under high light, lumen acidification induces antenna protonation, which in turn triggers protein conformational changes, aggregation and energy dissipation. However, it seems that pre-aggregation in vivo can affect efficiency of antenna protonation and vice versa (e.g. Petrou et al., 2014). Optical changes induced by aggregation can be visualized spectrscopically (Lokstein et al., 2002), specifically as an increase in the fluorescence yield of red-shifted emission from antennas at low temperatures (Ruban et al., 1991; Bassi and Dainese, 1992; Miolosilvina et al., 2008; Belgio et al., 2012). Based on dicyclohexylcarbodiimide (DCCD) binding and mutagenesis work (Ruban et al., 1998; Belgio et al., 2013; Ballottari et al., 2016), it was concluded that sensors for low pH are negatively charged residues located in a lumen–exposed antenna protein loop and in the C-terminus. Once protonated, those residues become neutral, thus making the whole protein more hydrophobic and easier to aggregate and quench. Although in vitro fluorescence quenching as a function of pH has been observed for various types of antennas (Guidermann and Büchel, 2012; Kafà et al., 2012; Schaller-Lauldel et al., 2015), identification of putative protonable residues so far concerned mainly antennas from the green lineage (Ruban et al., 1998; Li et al., 2004; Liguori et al., 2013; Belgio et al., 2013; Ballottari et al., 2016).

Despite the progress in our understanding of NPQ in higher plants, this subject has been less explored in algae. The alveolate Chromera velia represents an interesting system in this context, as it shows efficient non-photochemical quenching (Kotabová et al., 2011; Quigg et al., 2012; Mann et al., 2014) with similarities on the one side to higher plants, and on the other to brown algae and diatoms (see below). Isolated from stony corals from Sidney harbor, this facultative symbiont is globally distributed in the marine environment at depths not exceeding 5 m (Obornik and Lukes, 2013). The phylogenetic origin of the alga is complex. C. velia is an alveolate, and therefore closely related to dinoflagellates and other algae in the SAR clade (such as diatoms and brown algae), but all phylogenetic analyses have invariably demonstrated its genuine relationship to apicomplexan parasites (Obornik et al., 2016). In any case, C. velia is considered a ‘red-clade’ alga, i.e. an alga whose chloroplast was obtained by secondary endosymbiosis from a red algal ancestor (Kotabová et al., 2011; Sobotka et al., 2017). In C. velia, NPQ is connected to the xanthophyll cycle (Kotabová et al., 2011) as in brown algae (Ocampo-Alvarez et al., 2013); however, differing from them (Garcia-Mendoza et al., 2011) but similar to diatoms (Ruban et al., 2004; Lavaud and Kroth, 2006; Grouneva et al., 2008), its activation is extremely fast (almost monophasic) and pH-dependent (see Belgio et al., 2018).

In the present paper, we investigated the reasons for the characteristic high rate of quenching displayed by the alga. We compared the NPQ of C. velia with that of a higher plant (a well-known system) and showed that the mechanism of heat dissipation and in particular NPQ activation is different in the two evolutionarily distant phototrophs. Our data indicated that the Chromera light harvesting complex (CLH) is more sensitive to protons than the higher plant antenna (light harvesting complex II; LHCII). We propose that protonation of the antenna is the basis of the ‘constitutively’ fast NPQ found in C. velia and, as previously suggested for diatoms (Lavaud and Kroth, 2006; Lavaud and Lepetit, 2013), ΔpH by itself is important for NPQ activation. This conclusion might also explain the unusual high light acclimation strategy recently reported for C. velia, consisting of a decrease in reaction centers whilst still maintaining a full antenna content (Belgio et al., 2018).

Materials and methods

Plant material

Chromera velia (strain RM12) was grown in artificial sea water with additional f/2 nutrients (Guillard and Ryther, 1962). Cells were cultivated in glass tubes at 28 °C, in a continuous light regime of 200 μmol m−2 s−1 while aerated with air.

Spinacia oleracia (spinach) was purchased from a local supermarket. Intact chloroplasts were prepared as previously described (Crouchman et al., 2006).
Isolation of C. velia and plant light harvesting complexes

C. velia cells were broken and solubilized as described in Kříža et al. (2016) and then loaded on a fresh, continuous 5–15% sucrose density gradient prepared using a home-made gradient maker in buffer containing 25 mM HEPES pH 7.8 and 0.04% n-dodecyl β-D-maltoside (β-DM). The ultracentrifugation was performed at 140 000 g at 4 °C for 20 h (with rotor SW28, for 40 ml tubes, of an L-8 M ultracentrifuge; Beckmann, USA). The resulting band no. 2 contained a strong double band at 18 and 19 kDa, previously identified as ‘fucoxanthin chlorophyll a/c binding protein (FCP)-like antenna’ (Tichy et al., 2013). The band analysis by Pan et al. (2012) and Tichy et al. (2013) placed this antenna protein within the main FCP-like group of light-harvesting complexes and so it was named Chnenon light harvesting complex (CLH).

After separation by sucrose gradient, the antenna protein was desalted using a PD10 column (GE Healthcare) in a buffer containing 20 mM HEPES (pH 7.6) and 0.01% (w/v) β-DM. Spinach LHCCIb was isolated as previously described (Ruban et al., 1994b) and then purified, desalted and eluted in the same buffer as CLH. In both cases, antennas were isolated from samples dark-adapted for 30–45 min.

Non-photochemical fluorescence quenching in native cells and isolated chloroplasts

Chlorophyll fluorescence was measured using a double modulation fluorometer FL-3000 (Photon System Instruments, Czech Republic). A multiple turnover saturating flash was applied to measure the maximum quantum yield of the photochemistry of photosystem II (Fm/Fm′) according to (Fm−F0)/Fm′, where the difference between the maximum (Fm) and minimum (F0) fluorescence is used to calculate the variable fluorescence (Fv) (van Kooten and Snel, 1990). Cells were then illuminated with an orange actinic light (625 nm, 500 µmol photons m−2 s−1), during which periodic saturating flashes were applied. NPQ was calculated as [(Fm−Fv)/Fm′] or Fv′, where Fv′ is the maximum fluorescence measured in the presence of actinic light. Non-photochemical quenching of fluorescence was measured in whole cells of C. velia (chlorophyll concentration 0.7 µg ml−1) and isolated spinach chloroplasts (chlorophyll concentration 1.4 µg ml−1). NPQ formation rates (NPQ as a function of time) in different xanthophyll cycle de-epoxidation states (DEPSs) were determined according to (Ruban et al., 1994b; Belgio et al., 2014) by pre-conditioning leaves with 350 µmol photons m−2 s−1 under 98% N2 for 20–40 min for 20% and 40% DEPS, respectively. For C. velia, 10 min illumination with 500 µmol photons m−2 s−1 was sufficient to obtain 40% DEPS, in agreement with what has been previously published (Kotabová et al., 2011). DEPS was assessed by immediate incubation in methanol followed by HPLC analysis (see ‘Pigment extraction and HPLC analysis’ section).

In silico studies

For in silico studies, the LHCCIb structure resolved at 2.5 Å resolution (PDB code: 2BH4; Standfuss et al., 2005) was employed. The structure of the CLH polypeptide (Cvelia_19753, taken from Tichy et al. (2013)) was predicted using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) and YASARA software (http://www.yasara.org). The protonation states of protein ionizable groups were computed in both cases using the H++ program (http://biophysics.cs.vt.edu), an automated system that calculates pK values of ionizable groups in macromolecules and adds missing hydrogen atoms according to the specified pH of the environment. Results shown for LHCCIb are relative to chain A, but results for chains B and C were very similar, in agreement with Xiao et al. (2012). As recommended for typical physiological conditions and deeply buried residues, the external dielectric value was set to 80, the internal dielectric value to 4, salinity to 0.15 and pH to 7.5.

Data analysis and model fitting

NPQ formation rates (NPQ in function of time; see Fig. 1) were determined using a well-established methodology valid for both algae and vascular plants (see Šeródiová and Lavda (2011) concerning the applicability of the Hill equation to NPQ in algae). Briefly, average data from three to six independent measurements of C. velia cells and spinach chloroplasts in different DEPSs were fitted using the sigmoidal Hill equation three-parameter implementation in SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA, USA). The standard error of the estimate was between 0.02 and 0.08, meaning that ~95% of the data fell within 2% of the fitted line; moreover R2 values were above 0.97, thus confirming the appropriateness of the approach.

In order to determine the quenching pKs of antennas, we used a method previously established for various antennas including mutants (Ruban et al., 1994a; Ruban and Horton, 1999; Belgio et al., 2013; Zaks et al., 2013). Briefly, the relationship between quenching kinetics and pH (see Fig. 4) and relative parameters (Table 1) were obtained from experiments like the one shown in Fig. 3 as follows. Quenching kinetics were generated at each pH point by fitting the measured traces (Fig. 3) with the three-parameter hyperbolic decay function: y = (y0+a)/(1+b+x) where 1/b is the rate of the process. Then the data points from Fig. 4 were fitted by the sigmoidal Hill equation y = [a(b+x)]/[b+1+x] in order to obtain Hill coefficients (b), pK values (c) and quenching kinetics at pH 4.97 (see also Johnson et al., 2012; Petrov et al., 2014). The standard error of the estimate was again low (below 0.1) and R2 above 0.9, confirming the validity of the approach.
Results

The kinetics of non-photochemical quenching (NPQ) activation were studied for *C. velia* in different xanthophyll de-epoxidation states (DEPSs) and compared with that of *Spinacia oleracea* (spinach) (Fig. 1). NPQ formation rate positively correlated with the DEPS in spinach as 10, 20, and 40% de-epoxidation yielded significantly different NPQ formation rates of 0.006, 0.011, and 0.05 s⁻¹, respectively (Fig. 1B), in agreement
Proton sensing controls light harvesting strategy

with current NPQ models and previous results from various phototrophs (see e.g. (Demmig-Adams, 1990; Ruban et al., 1994b; Jahns and Holzwarth, 2012; Goss and Lepetit 2015). In contrast, NPQ in C. velia formed quickly regardless of the de-epoxidation state (Fig. 1A). Values between 0.04 and 0.05 s\(^{-1}\) were thus found for both 10 and 40% DEPS, showing fast NPQ formation, independent of xanthophyll content (Fig. 1C).

The lack of an evident kinetic effect of xanthophylls in C. velia can also be seen from the shape of the NPQ formation curve. Whilst in spinach the increase in zeaxanthin (zea) concentration from 10 to 40% reduced curve sigmoidicity from 3.2 to 1.8 (Fig. 1B; Table S1), in C. velia no evident change could be seen (Fig. 1A) and the Hill coefficients were not significantly different in the two conditions (3.0 ± 0.4 versus 2.5 ± 0.3; see Supplementary Table S1). The increased de-epoxidation (from 10% to 40%; Fig. 1A), therefore, did not seem to affect NPQ kinetics as strongly as in spinach, but it stimulated the total NPQ (NPQ\(_{max}\); see Table S1). This is in agreement with a previous report (Kotabová et al., 2011) showing NPQ enhancement by zeaxanthin in C. velia.

In C. velia, NPQ was induced almost instantaneously with the turning on of the actinic light, and we therefore used NH\(_4\)Cl to investigate the possibility that lumen acidification was the basis of fast NPQ. As with spinach, in C. velia NH\(_4\)Cl reversed fluorescence quenching independent of its addition time during irradiation (Fig. 2A), proving a strict link between protons and NPQ in C. velia. However, the kinetics of NPQ relaxation at different time points were very different from each other and from those of spinach (Supplementary Fig. S1A). Whilst in spinach NPQ relaxed almost immediately after NH\(_4\)Cl injection, with 70% fluorescence recovery within 10 s, it took at least 500 s to achieve a similar recovery in C. velia (Fig. 2B). Interestingly, in C. velia, the later NH\(_4\)Cl was added, the faster NPQ relaxed (Fig. 2B). This is in strict contrast to higher plants (Supplementary Fig. S1A), where faster relaxation kinetics were observed at the beginning of NPQ formation (see e.g. Fig. 3A in (Ruban et al., 2004), suggesting a different sensitivity of NPQ to lumen acidification. The connection between NPQ and protons was further investigated in vitro using isolated antennas.

‘Fluorescence quenching titration’ is an efficient way to systematically study the pH dependency of quenching in vitro, by injecting purified antennas into buffers of increasingly acidic pH (Ruban et al., 1994b; Wentworth et al., 2000; Kafka et al., 2012; Belgio et al., 2013). This method was employed

Fig. 3. Quenching of Chromera velia antennas is highly sensitive to pH. Representative fluorescence time course of CLH (A) and LHCII (B) as a function of pH. Samples were injected into a buffer containing 0.0005% β-DM, 10 mM HEPES and 10 mM sodium citrate (final concentrations). Each buffer had been HCl-buffered to the pH indicated in the figure, prior to sample injection. Data were normalized to the fluorescence maximum. Inset: absorption spectrum of CLH at high (solid line, pH 7.8) and low (dashed line, pH 4.5) pH. (This figure is available in colour at JXB online.)

Fig. 4. Comparison of pH titration curves for fluorescence quenching of LHCII and CLH. The relationship between percentage of quenching and pH was obtained from traces like those shown in Fig. 3 fitted as described in the ‘Data analysis and model fitting’ section of ‘Materials and methods’, using a previously established model (see e.g. Ruban and Horton 1999). Circles, mean averages from at least three independent replicates; solid lines, fittings; dashed lines, 95% confidence intervals. The error bar shows typical standard deviation.
to assess the hypothesis that faster NPQ activation (Fig. 1) related to antenna protonation, rather than to zeaxanthin content. Therefore LHCII and CLH complexes were isolated from dark-adapted material and the absence of zeaxanthin was confirmed by HPLC analysis (see Supplementary Table S1 at JXB online).

Upon injection, CLH displayed a progressive quenching proportional to the acidity of the buffer (Fig. 3A). Sample integrity was constantly monitored by absorption spectroscopy (Fig. 3, inset) and by reversibility of the quenching after detergent addition (data not shown). Besides the general similarity of the process, pointing to a fundamentally conserved quenching mechanism, the differences between the two types of sample are notable. At each pH value, fluorescence quenching was consistently higher in CLH compared with LHCII, with the biggest difference found around pH 6.0. From the traces in Fig. 3, a titration curve of quenching kinetics as a function of pH was constructed (Fig. 4). It shows that, to attain the same rate of fluorescence quenching, a lower pH is required in LHCII compared with CLH. In particular, almost 50% of maximum quenching rate was observed at pH 5.5 in CLH, whilst a pH of 5.0 was necessary to get the same quenching rate in spinach. Similarly, CLH showed almost the maximum quenching rate (90 ± 5%) at pH 4.97, whereas for LHCII it was only 50%. This was reflected in a shift by 0.5 pH unit to higher pH values in the calculated quenching pK_a of CLH compared with LHCII, i.e. from 5.5 ± 0.1 to 5.0 ± 0.1 (Table 1). The pK_a value for LHCII was in good agreement with that previously reported (see e.g. pK_a = 4.9 in Petrou et al. (2014)).

The Hill coefficient for CLH was not significantly different from that of LHCII (7.2 ± 1.9 and 7.5 ± 1.1 for LHCII and CLH, respectively; Table 1) and in both cases they were higher than those for in vivo quenching (see Fig. 1, consistent with the absence of zeaxanthin (see Supplementary Table S1 and Discussion). In summary, the shift in quenching pK_a confirmed a higher proton sensitivity of CLH compared with LHCII, independent of xanthophylls.

In order to address possible reasons behind the higher pH sensitivity found in CLH, a comparative in silico analysis was conducted using the amino acid sequences of CLH and LHCII (Supplementary Fig. S2). A schematic overview of the two proteins is presented in Supplementary Fig. S3. We have explored in particular the protein lumenal loop to identify residues that are protonable within the physiological range. The protein structure predicted for CLH is presented in Fig. 5. We found 24 negatively charged amino acidic residues in total (i.e. aspartic and glutamic acids) in CLH, four of which are located in the luminal loop (Glu-93, Asp-107, Asp-113, Asp-119) and one in the C-terminus (Glu-205).

The estimated in situ pK_a values were calculated and compared with LHCII (2.5 Å resolution structure from Standfuss et al. (2005)) and are presented in Table 2. Results for LHCII are in good agreement with a previous report (Xiao et al., 2012), where two residues in particular (Glu-107 and Asp-215) were indicated as putative pH sensors for NPQ as their quenching pK_a values are within the thylakoidal physiological range (3.9–7.5). The same analysis applied to CLH revealed the presence of three plausible protonable residues: Asp-107, Asp-119 and Glu-205 (see Table 2, Fig. 5 right). Furthermore, their pK_a values were shifted to higher pH values compared with LHCII, confirming that the lumenal loop is more sensitive to protonation in CLH (see Asp-107, Asp-119 and Glu-205 and their pK_a in Table 2).

An overall comparison between LHCII and CLH protein structures (Table 3) indicated that, despite a similar number of total protonable residues (~11.4–11.5% in both cases), CLH displayed a lower protein charge than LHCII at pH 7.6, that is ~6 versus ~24, respectively. This means that LHCII tends to be more charged than CLH and a stronger protein–protein repulsion is expected for LHCII at pH 7.6 (see Discussion). In agreement with this, the CLH isoelectric point was ~0.4 higher than LHCII, implying that ~30 times fewer protons are required to neutralize negatively charged residues compared with LHCII. In summary, the in silico results supported the experimental data well and provided theoretical explanations for the faster, more efficient quenching found for CLH.

### Discussion

In the present paper, we investigated reasons for fast NPQ activation in *C. vela*. In higher plants, the kinetics of NPQ induction are influenced by xanthophyll composition (Fig. 1B). Demmig-Adams (1990) was the first to provide evidence for a connection between the xanthophyll cycle and NPQ. She showed that the conversion of violaxanthin into zeaxanthin, stimulated under light by lumen acidification, strongly enhanced NPQ. Later it was noticed (Ruban and Horton, 1999) that the NPQ of zeaxanthin–enriched samples was much faster, as zeaxanthin changed the NPQ dependency (cooperativity) as a function of ΔpH, from sigmoidal (violaxanthin) to hyperbolic (zeaxanthin) (see also Horton et al., 2000; Johnson et al., 2009). Here, we confirmed with a control sample (spinach) that the transition into the quenched state is slower for leaves enriched in violaxanthin compared with zeaxanthin (Fig. 1B) as the Hill coefficient

| Sample | Hill coefficient | Estimated pK_a | Quenching kinetics at pH 4.97 |
|--------|------------------|----------------|-------------------------------|
| LHCII  | 7.2 ± 1.9        | 5.0 ± 0.1      | 0.50 ± 0.01                   |
| CLH    | 7.5 ± 1.1        | 5.5 ± 0.1      | 0.90 ± 0.05                   |

Titrations were performed by fitting measured traces like those represented in Fig. 3, described in the ‘Materials and methods’ section of ‘Materials and methods’. Standard fitting errors were provided by SigmaPlot software. (For more details, see Ruban et al. 1994a; Ruban and Horton 1999; Petrou et al. 2014.)
decreased in the presence of zeaxanthin. In *C. velia* however, we found a different behavior. Although NPQ was greater in zeaxanthin-enriched samples, confirming the first observations (Kotabová et al., 2011), its rate was insensitive to xanthophyll composition (Fig. 1A, C), indicating that the reason for the fast NPQ in *C. velia* resided elsewhere. The NH₄Cl-infiltration experiment (Fig. 2) following the procedure of Ruban et al. (2004) and Lavaud et al. (2002), suggested that fast NPQ related to lumen acidification and protons. Incomplete diffusion of the uncoupler was in fact ruled out by previous evidence of efficient NH₄Cl penetration in *C. velia* cells (see Fig. 4b in Belgio et al., 2018). The titration of NPQ as a function of pH confirmed that CLH was significantly more sensitive to acidification and protons. Incomplete diffusion of the uncoupler was in fact ruled out by previous evidence of efficient NH₄Cl penetration in *C. velia* cells (see Fig. 4b in Belgio et al., 2018). The titration of NPQ as a function of pH confirmed that CLH was significantly more sensitive to acidification and protons. Incomplete diffusion of the uncoupler was in fact ruled out by previous evidence of efficient NH₄Cl penetration in *C. velia* cells (see Fig. 4b in Belgio et al., 2018). The titration of NPQ as a function of pH confirmed that CLH was significantly more sensitive to acidification and protons. Incomplete diffusion of the uncoupler was in fact ruled out by previous evidence of efficient NH₄Cl penetration in *C. velia* cells (see Fig. 4b in Belgio et al., 2018). The titration of NPQ as a function of pH confirmed that CLH was significantly more sensitive to acidification and protons. Incomplete diffusion of the uncoupler was in fact ruled out by previous evidence of efficient NH₄Cl penetration in *C. velia* cells (see Fig. 4b in Belgio et al., 2018). The titration of NPQ as a function of pH confirmed that CLH was significantly more sensitive to acidification and protons. Incomplete diffusion of the uncoupler was in fact ruled out by previous evidence of efficient NH₄Cl penetration in *C. velia* cells (see Fig. 4b in Belgio et al., 2018). The titration of NPQ as a function of pH confirmed that CLH was significantly more sensitive to acidification and protons. In incomplete diffusion of the magnitude of the NPQ was found (Table 3), corresponding to 30–40 less protons required for charge shielding. Considering the in vitro and in silico results together, we suggest that the increased NPQ formation kinetics relate to built antenna properties, in terms of

| Residue | pKᵣ | Residue | pKᵣ |
|---------|------|---------|------|
| Glu-94  | 1.5  | Glu-90  | 1.0  |
| Glu-107 | 4.4  | Asp-107 | 5.9* |
| Asp-111 | 3.4  | Asp-113 | 2.9  |
| Glu-207 | 2.9  | Asp-119 | 3.9* |
| Asp-211 | 3.5  | Lys-211 | 7.4  |
| Asp-215 | 5.3  | Glu-205 | >7*  |

The putative residues that can be protonated within the physiological pH range (3.9–7.5) are in shown bold. Residues in CLH with a higher pKᵣ than LHCII have been marked with an asterisk. Set values in the simulation were: for internal dielectric, 4; external dielectric, 80; and salinity, 0.15; in agreement with Xiao et al. (2012). Predicted sequences and protein structures are shown in Supplementary Figs S2 and S3, respectively.

Fig. 5. Predicted protein structure for CLH. Predicted structure of the CLH antenna based on sequence homology with LHCII. A zoom of the stranded lumenal loop is shown on the right. The putative residues involved in triggering NPQ are labelled in black. Blue, transmembrane helices; green, lumenal loop region; yellow, stromal loop. For the prediction of protein structure and the pKᵣ of residues, YASARA and H++ programs were used, respectively.
both a higher number of lumen–exposed protonable residues and an overall increased protein hydrophobicity. We hypothesize that this applies also to other similar antennas, such as diatom FCPs. In fact, although CLH binds only chlorophyll $a$ and xanthophylls (see Kotabová et al., 2011), due to its structural properties, this protein was classified as ‘FCP-like’, i.e. closely related to antennas from dinoflagellates, brown algae, and diatoms (Lepetit et al., 2010; Pan et al., 2012; Tichy et al., 2013). Moreover diatoms can also be characterized by fast NPQ activation (Ruban et al., 2004; Lavaud and Kroth, 2006; Grouneva et al., 2008).

It was experimentally shown for brown algae (Nitschke et al., 2012), alveolates (Belgio et al., 2018), and other microalgae (Goss and Jakob, 2010) that the habitat and particularly the light conditions affect NPQ capabilities of algae from the SAR clade. As a coral symbiont, C. velia is expected to be mainly exposed to rather ‘moderate’ light intensities. However, as this organism can be also found ‘free-living’ outside the coral, at depths of 3–5 m, light intensities of up to 1000 $\mu$mol m$^{-2}$ s$^{-1}$ are normally experienced on a sunny day (Behrenfeld et al., 1998; Oborník et al., 2011). We can speculate that, due to fast quenching of antennas, in C. velia there was no selective pressure towards proteins capable of enhancing NPQ rate such as PsbS or Lhcsr (Pan et al., 2012). These proteins in fact have a role as NPQ enhancers in vascular plants and green microalgae, respectively (Goss and Lepetit 2015). Spinach and C. velia seem therefore to have evolved very different ‘antenna behaviors’ in relation to different acclimation strategies. They can be summarized as follows (Fig. 6):

- Chromera velia carries antenna proteins that are ‘natural quenchers’; PsbS, a strong NPQ enhancer (Li et al., 2000), is absent (Pan et al., 2011) and the thylakoid membrane is highly enriched in violaxanthin, an ‘anti-quenching’ pigment (Kaňa et al., 2016). As a consequence its NPQ kinetics are characterized by fast formation/slow relaxation.
- Higher plant antenna proteins, here represented by spinach LHCII, are ‘natural light harvesters’, so PsbS is required for effective but in particular fast quenching (Johnson and Ruban, 2011) and little or no free violaxanthin is present the membranes (Dall’Osto et al., 2010; Xu et al., 2015). The NPQ kinetics are characterized by slow formation/fast relaxation.

**Table 3.** Comparison of total charges between LHCII and CLH

|          | LHCII | CLH |
|----------|-------|-----|
| Protonable residues | 25/218 (11.5%) | 24/211 (11.4%) |
| $p_I$    | 4.61  | 4.97 |
| Charge at pH 7.6 | $-24$ | $-6$ |

Total number of protonable residues, the isoelectric point ($p_I$) and the total protein charge of LHCII and CLH antenna proteins, as predicted by the H++ program. Relative sequences and protein structures are shown in Supplementary Figs S2 and S3, respectively.

*Fig. 6.* Scheme showing the different light harvesting strategies of C. velia and higher plants. The C. velia thylakoid membrane carries CLH proteins that are ‘natural quenchers’ with three protonable lumen-facing residues, D107, D119, and E205 (indicated by small protrusions). The membrane is highly enriched in unbound, ‘anti-quenching’, violaxanthin pigments, and PsbS protein is absent. The higher plant thylakoid membrane supports the LHCII protein, a ‘natural light harvester’ with two protonable lumen-facing residues. PsbS protein is required for effective quenching, and the amount of unbound violaxanthin in the membrane is negligible. The scheme does not represent real stoichiometries/proportions. For more details, see main text.
In this scenario, ‘free’ violaxanthin plays a role of quenching inhibitor, particularly important for \( C.\) \( \text{velia} \) and less crucial for LHCII. A similar role of violaxanthin was previously suggested for some brown algae (Ocampo-Alvarez et al., 2013). It explains the abundance of violaxanthin in algae like \( C.\) \( \text{velia} \), where the violaxanthin to Chl \( a \) ratio is \( \approx 0.36 \) (mol mol\(^{-1}\)), \( \approx 8 \) times higher than in plants (see e.g. Kotabová et al., 2011), which is supported by work showing quenching modulation by ‘free’, i.e. not firmly bound to protein, xanthophylls (Ruban et al., 1994a; Lepetit et al., 2010; Mann et al., 2014; Xu et al., 2015; Kaňa et al., 2016).

Finally, the model presented (Fig. 6) provides an explanation also for the unusual acclimation strategy observed in \( C.\) \( \text{velia} \): whilst plants (carrying ‘natural harvester’ antennas) protect themselves from high light by reducing their antenna size (see Kouřil et al., 2013), in \( C.\) \( \text{velia} \) (characterized by ‘natural quencher’ antenna proteins) the antenna size is unaffected even after days of exposure to high light (Belgio et al., 2018). This evidence, at first puzzling, seems now more logical in view of the results presented here.

**Conclusions**

In conclusion, we have shown a similar quenching mechanism in antennas from a higher plant compared with those from an alveolate. In both cases the trigger is low pH and the likely sensors are protonatable luminal residues. However, the actual sensitivity to lowering pH is different for the two proteins as CLH is more sensitive to protons than LHCII. We propose that this is due to subtle differences in the amino acid composition of the protein luminal loop. As a result, CLH switches into a dissipative quenched state more easily than LHCII and therefore the higher plant antenna protein can be considered a ‘natural light harvester’ whilst the CLH protein is a ‘natural quencher’.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. \( \text{NH}_4\text{Cl} \) induces fast NPQ relaxation in spinach chloroplasts.

Fig. S2. Sequence of LHCIIb and CLH used in the present study.

Fig. S3. Schematic overview of LHCII (left) and CLH (right) antenna protein structures used in the present study.

Table S1. Pigment composition of antennas isolated from \( C.\) \( \text{velia} \) and spinach.

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**Author contributions**

RK, EKT and EB conceived the project; EKT and EB performed the experiments and analyzed the data; AMY provided HPLC technical assistance; RS provided experimental advice concerning protein isolation; EB, RK and EKT wrote the article with contributions from all the co-authors; EB, EKT and AMY prepared the Figures. AVR supervised and complemented the work.

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