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REGULAR PAPER

Carotenoid–chlorophyll coupling and fluorescence quenching in aggregated minor PSII proteins CP24 and CP29

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Abstract It is known that aggregation of isolated light-harvesting complex II (LHCII) in solution results in high fluorescence quenching, reduced chlorophyll fluorescence lifetime, and increased electronic coupling of carotenoid (Car) S_1 and chlorophyll (Chl) Q_y states, as determined by two-photon studies. It has been suggested that this behavior of aggregated LHCII mimics aspects of non-photochemical quenching processes of higher plants and algae. However, several studies proposed that the minor photosystem II proteins CP24 and CP29 also play a significant role in regulation of photosynthesis. Therefore, we use a simple protocol that allows gradual aggregation also of CP24 and CP29. Similarly, as observed for LHCII, aggregation of CP24 and CP29 also leads to increasing fluorescence quenching and increasing electronic Car S_1–Chl Q_y coupling. Furthermore, a direct comparison of the three proteins revealed a significant higher electronic coupling in the two minor proteins already in the absence of any aggregation. These differences become even more prominent upon aggregation. A red-shift of the Q_y absorption band known from LHCII aggregation was also observed for CP29 but not for CP24. We discuss possible implications of these results for the role of CP24 and CP29 as potential valves for excess excitation energy in the regulation of photosynthetic light harvesting.

Keywords CP24 · CP29 · LHCII · Aggregation · Electronic coupling · Fluorescence quenching · Chlorophylls · Carotenoids

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Introduction

Plants evolved regulation mechanisms to achieve optimal usage of available light under changing light conditions during a day. Typically, solar irradiation of plants can vary up to several orders of magnitude on different time scales (Demmig-Adams and Adams 2002). Plants need to switch quickly between highly efficient light-harvesting performance under low irradiation conditions and effective protection of their photosynthetic apparatus from excessively absorbed light energy under high irradiation conditions (see for overviews Croce and van Amerongen 2014; Rochaix 2014). The mechanisms that lead to dissipation of excess energy as harmless heat are typically described by the expression non-photochemical quenching (NPQ) (Horton et al. 1994; Müller et al. 2001; Niyogi and Truong 2013; Papageorgiou and Govindjee 2014). Essential not only for light harvesting, in general, but also for all of the suggested NPQ mechanisms are the photosynthetic pigments and the electronic interactions among them. Different theories whether and how chlorophylls and carotenoids contribute to NPQ have been extensively discussed (Demmig-Adams 1990; Horton et al. 1996; Young and Frank 1996; van Amerongen and van Grondelle 2001; Holt et al. 2004; Standfuss et al. 2005; Ruban et al. 2007; Ahn

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et al. 2008; Miloslavina et al. 2008; Holleboom and Walla 2014). However, the exact mechanisms of NPQ, which pigments are involved, and an accurate location of NPQ sites in thylakoid membranes are still a matter of debate. An early idea suggested xanthophyll cycle carotenoids being key players in NPQ. Based on direct energy transfers between chlorophyll Q_y states and the lowest excited singlet states of carotenoids (Car S_1), a switch between violaxanthin and zeaxanthin could provide a lower lying zeaxanthin S_1 state which was suggested to function as a trap for excess energy (Frank et al. 1994; Demmig-Adams and Adams 1996). More recently, energy transfer from chlorophylls to lutein was also proposed to play a role in the quenching (Ruban et al. 2007; Ilioaia et al. 2011; Duffy et al. 2013). Other suggestions include a charge transfer leading to carotenoid radical cations and an excited Chl–Car radical pair as a transition state in NPQ mechanisms (Holt et al. 2005; Cheng et al. 2008; Avenson et al. 2008; Ahn et al. 2008). Further findings led to the proposal of a kind of fast alternating equilibrium or even excitonic mixing of these two states (van Amerongen and van Grondelle 2001; Bode et al. 2009; Liao et al. 2012; Holleboom and Walla 2014) since increased energy transfer in both directions under quenched conditions was observed (Liao et al. 2010b; Holleboom and Walla 2014), and a strongly reduced fluorescence lifetime in quenched samples could be explained by this theory (van Amerongen and van Grondelle 2001). Besides these carotenoid–chlorophyll interaction theories, also a chlorophyll–chlorophyll charge transfer state contribution to energy quenching was proposed (Miloslavina et al. 2008; Müller et al. 2010; Wahadoszamen et al. 2012). Obviously, the precise nature of the regulation mechanisms in plants and algae is still not fully resolved, and regarding the different theories, it also can be considered possible that there might be even more than one single mechanism contributing to NPQ.

The most abundant light-harvesting complex (LHC) in thylakoid membranes is LHCII. Unlike other LHCs, this integral thylakoid membrane protein is organized in trimers (Schmid 2008). Besides its central function in light harvesting and energy transfer being connected to PSII and also to PSI (Wientjes et al. 2013), LHCII is moreover suggested to play a key role in light-harvesting regulation (Pascal et al. 2005; Ruban et al. 2007). It was shown that this protein complex and, in particular, its aggregation in solution provides an interesting model system which reflects properties of different NPQ levels of living plants (Horton et al. 1991, 2005; van Oort et al. 2007; Ruban et al. 2007; Johnson and Ruban 2009; Liao et al. 2010a; Holleboom and Walla 2014). Besides LHCII, minor light-harvesting antennas can be found associated to PSII. These proteins, named CP24, CP26, and CP29, belong to the same protein family (Lhcb) (Schmid 2008). Like LHCII, the minor antennas also carry chlorophylls and carotenoids but the protein primary structure and the pigment content vary. According to the crystal structure, a monomer of LHCII binds 14 chlorophylls and 4 carotenoids (Liu et al. 2004; Standfuss et al. 2005) compared to 13 chlorophylls and 3 carotenoid binding sites in CP29 (Pan et al. 2011). Whereas the number of chlorophylls associated with CP29 and the chlorophyll a/b ratio differ in the isolated complexes when compared to the crystal structure (Dainese and Bassi 1991; Irrgang et al. 1991; Pascal et al. 1999; Pan et al. 2011), three carotenoid binding sites were also observed in the recombinant complexes with lutein in the L1 site, violaxanthin in L2, and neoxanthin in N1 (Caffarri et al. 2007). It was also shown that all carotenoids are active in transferring excitation energy to the chlorophylls (Croce et al. 2003), while only L1 and L2 participate in triplet quenching (Mozzo et al. 2008a). The crystal structure of CP24 is not yet available, but both recombinant and native CP24 were shown to coordinate 10 chlorophylls (5 chlorophylls a and 5 chlorophylls b) and 2 carotenoids, lutein in the L1 site and violaxanthin in the L2 site (Passarini et al. 2009, 2014), while the N1 site is empty in agreement with the absence of the tyrosine that in the other complexes stabilizes the binding of neoxanthin (Caffarri et al. 2007).

The recently discussed roles of these minor proteins in photosynthesis range from structural functions influencing the stability and shape of the PSII core complex up to acting as possible quenchers in NPQ. From the two minor proteins investigated in this work, CP29 was suggested to be essential for the stability of the PSII core dimer (Andersson et al. 2001), whereas CP24 is suggested to influence the binding affinity of LHCII trimer M to the PSII supercomplex and even to dissociate from the PSII core upon light stress which correlates with NPQ and thus seems to be important for photo protection in higher plants (Kovács et al. 2006; de Bianchi et al. 2008; Betterle et al. 2009; Caffarri et al. 2009; van Oort et al. 2010). In addition, for all minor proteins, carotenoid radical cations were detected (Cheng et al. 2008; Ahn et al. 2008; Avenson et al. 2008, 2009).

Most studies propose that carotenoid–chlorophyll interactions are key players in the regulation. A convenient approach to gain detailed insights in electronic interactions between carotenoids and chlorophylls is provided by two-photon excitation (TPE) techniques. TPE allows selective excitation of the dipole forbidden S_1 state of carotenoids (Shreve et al. 1990; Walla et al. 2000a, b, c; 2002; Shima et al. 2003; Wehling and Walla 2005). A comparison of the chlorophyll fluorescence observed after selective excitation of either the carotenoid or the chlorophyll provides a direct measure closely related to differing and changing

electronic interactions between carotenoids and chlorophylls even in intact plants (Bode et al. 2009). Measuring the fluorescence after selective one-photon excitation (OPE) of chlorophylls, F^{OPE}, and selective TPE of carotenoids, F^{TPE}, allows calculation of an electronic coupling parameter which is a measure for the extent of Car $S_1 \rightarrow$ Chl Q_y energy transfer (see Bode et al. (2009) for further details):

$$\Phi_{\text{Coupling}}^{\text{Car}S_1-\text{Chl}} = \frac{F^{\text{TPE}}}{F^{\text{OPE}}} \quad (1)$$

Such TPE studies provided evidence for increasing electronic interactions between carotenoids and chlorophylls in LHCII aggregates, grana membranes as well as entire plants that were closely correlated to increasing fluorescence quenching in these samples (Bode et al. 2009; Liao et al. 2010a; Holleboom et al. 2013). But so far the parameter $\Phi_{\text{Coupling}}^{\text{Car}S_1-\text{Chl}}$ was not determined for the minor complexes CP24 and CP29 as a function of quenching.

Therefore, we here conducted a systematic investigation of the electronic couplings of CP24 and CP29 during quenching. We first tested if the increasing quenching in these complexes can be gradually achieved by detergent removal in a similar manner as previously shown for LHCII. Subsequently, we determined $\Phi_{\text{Coupling}}^{\text{Car}S_1-\text{Chl}}$ for LHCII, CP24, and CP29 as a function of the gradually increased quenching to test if these data provide further indications for the potential roles of CP24 and CP29 for the proposed regulation mechanisms described above. The direct comparison with LHCII unveiled that in the absence of aggregation quenching, the coupling in CP24 as well as CP29 is significantly higher than in LHCII. Similar to LHCII, both complexes also showed increased couplings with increasing aggregation quenching. The coupling increased even stronger for the minor proteins than for LHCII of comparable quenching levels. These results provide a systematic insight into the effects of aggregation quenching in three of the most important light-harvesting complexes and support a role also of the minor complexes CP24 and CP29 in photosynthesis regulation.

Materials and methods

Protein samples

Samples of native LHCII were prepared from spinach as previously described (Kühlbrandt et al. 1983). LHCII was diluted in 50 mM tris buffer at pH 7.5 and containing 0.3 % NG (*n*-nonyl-β-D-glucopyranoside). CP24 and CP29 were overexpressed in *E. Coli* using the constructs described, respectively, by Passarini et al. (2009) and Caffarri et al. (2007) and reconstituted in vitro with spinach pigments (see Natali et al. (2014) for a detailed protocol of the reconstitution). The chlorophyll a/b ratio was 2.28 for CP29 and 1.01 for CP24. CP24 was measured in 10 mM HEPES buffer at pH 7.5 containing 0.5 M sucrose and 0.04 % β-DDM (*n*-dodecyl-β-D-maltoside). For CP29, the same buffer was used containing 0.4 M sucrose and 0.02 % β-DDM.

Aggregation

Aggregations of LHCII, CP24, and CP29 were performed using a detergent removal method described before by Liao et al. (2010a). Briefly, detergent was removed from the solution with SM-2 absorbent (BioRad), and aggregation was monitored by chlorophyll fluorescence observation with a conventional PAM fluorometer (FMS1, HansaTech). After aggregation, the chlorophyll concentration of the samples represented by optical densities (OD) at Chl Q_y maximum was controlled and if necessary corrected to consistent values. Final OD (at $Q_{y,max}$) of different series of measurements varied between 0.095 and 0.140 mm^{-1}.

Electronic Car S_1–Chl Q_y coupling measurement

Experimental determination of the electronic coupling parameter was performed as described before by Bode et al. (2009). For measuring fluorescence after OPE, the modulation light of a conventional PAM fluorometer (FMS1, HansaTech) with a wavelength of 594 nm was used. The feasibility of simultaneous detection of fluorescence after TPE in the same sample was achieved by a confocal setup. The two-photon light source was an ultrafast laser system composed of an optical parametric amplifier OPA 9450 which was pumped by a combination of a Vitesse Duo and a RegA 9000 (all components from Coherent Inc.). The OPA was adjusted to provide IR light with a wavelength of 1188 nm for TPE of carotenoids. The visible part of the OPA output was blocked by a hot mirror (L46-386, Edmund Optics) and a combination of a 900 nm and a 1100 nm long-pass filter (FEL900 and FEL1100, Thorlabs) in order to avoid any one-photon excitation of chlorophylls. To ensure a detection of TPE-induced fluorescence using our setup, control experiments were performed monitoring the quadratic dependence of fluorescence signals on the excitation power as it is expected for TPE (data not shown). Fluorescence detection was achieved using an ultrafast photodiode and selective amplification by a lock-in amplifier (EG&G 5205). The lock-in was triggered by a mechanical chopper positioned in the excitation beam.

Normalization to chlorophyll content

As mentioned above, the chlorophyll concentration of the investigated samples of one series of measurement was adjusted to similar values. Additionally, intensities observed after OPE and TPE were corrected to the calculated chlorophyll content of each sample. Considering the different chlorophyll a/b ratio of the investigated proteins, this correction was done based on the area beneath the absorption spectra in the Q_y region (630–750 nm). With respect to the different extinction coefficients, a chlorophyll b to chlorophyll a extinction ratio of 0.7 was assumed based on values measured in solvents (Sauer et al. 1966). For LHCII, a chlorophyll a/b ratio of 1.33 and for CP24 and CP29, the above-mentioned values (1.01 and 2.28) were used. However, it is important to note that variations in sample concentration are not influencing the electronic Car S_1–Chl Q_y coupling, since these concentrations are canceled in the calculation of $\Phi^{\mathrm{Car}S_1-\mathrm{Chl}}_{\mathrm{Coupling}}$ (Eq. 1).

Absorption and fluorescence spectra

Absorption spectra and OD measurements were carried out using a Perkin-Elmer Lambda 25 UV–Vis spectrometer. Fluorescence spectra were recorded with a Varian Cary Eclipse fluorescence spectrometer at room temperature using an excitation wavelength of 670 nm.

Results

Fluorescence quenching

As described for LHCII by Liao et al. (2010a, b), protein aggregation in a detergent solution can be induced by adsorbing the detergent on surfaces of bio beads and thus removing it from the solution. The aggregation of such light-harvesting proteins is accompanied by a decrease of chlorophyll fluorescence yield of the investigated protein. Figure 1 shows exemplarily a quenching of chlorophyll fluorescence of LHCII, CP24, and CP29, achieved by the detergent removal method. Although the aggregations of the minor proteins were done from a different detergent solution, the fluorescence quantum yield for all three proteins could be significantly reduced.

Absorption spectra and Q_y absorption band shift

The absorption spectra of the investigated proteins LHCII, CP24, and CP29 normalized to their chlorophyll content are shown in Fig. 2. A major difference is a weaker pronounced chlorophyll b Q_y absorption band observed for

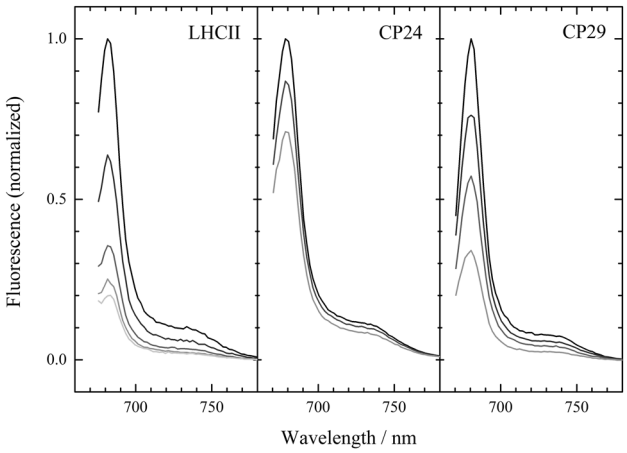

Fig. 1 Aggregation-induced chlorophyll fluorescence quenching in LHCII (*left*), CP24 (*middle*), and CP29 (*right*). *Black lines* represent unquenched protein. Aggregation-quenched samples are shown as gray lines (*dark* to *light gray* with increasing quenching level). All spectra are normalized to the corresponding unquenched protein

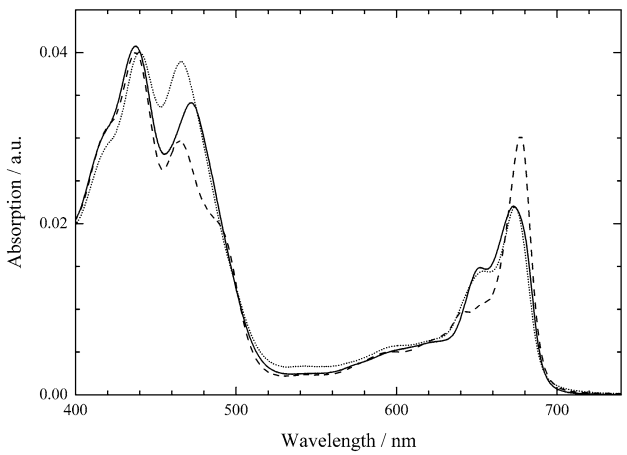

Fig. 2 Absorption spectra of LHCII (*solid*), CP29 (*dashed*), and CP24 (*dotted line*). The spectra are normalized to chlorophyll content

CP29 compared to the other two proteins, which is explained by CP29 having a higher chlorophyll a/b ratio (see "Materials and methods" section). Figure 3 shows absorption spectra of LHCII and CP29 in different quenched states around the Q_y absorption band ($\sim$680 nm) as well as absorption difference spectra obtained by subtracting the spectra of the unquenched from the quenched states. The spectra of LHCII and CP29 exhibit a red-shift of the Q_y band, which was already known for LHCII (Liao et al. 2010a). This shift increases for both proteins with the level of aggregation, or compared to the fluorescence spectra above (Fig. 1), with the extent of chlorophyll fluorescence quenching. In a range of moderate NPQ values, this red-shift even correlates linearly with the extent of chlorophyll fluorescence quenching as has been published before for

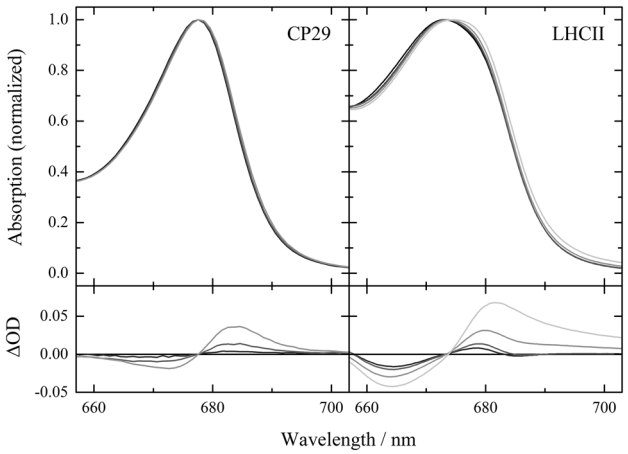

Fig. 3 Absorption band shift in CP29 (*left*) and LHCII (*right*). In the upper section, normalized Q_y absorption bands of the unquenched proteins (*black line*) and increasing aggregation levels (*dark* to *light gray lines*) are displayed. In the lower section, the corresponding absorption difference spectra ($OD_{aggregated} - OD_{unaggregated}$) are shown

LHCII (Liao et al. 2010a). Here, we show that this effect is also observable for CP29 (Fig. 4). For comparability, ΔOD, as a measure for absorption band shift, and NPQ were determined just like before by Liao et al.: ΔOD represents the maximum of the red peak in the lower section of Fig. 3, and NPQ was calculated as $F_m/F_m^{'}-1$ (Lichtenthaler et al. 2005) with F_m, fluorescence of unquenched protein, and $F_m^{'}$, fluorescence of the respective aggregation-quenched sample. Interestingly, the correlation between absorption band shift and fluorescence quenching, which can be observed for LHCII and CP29, cannot be found for CP24. For CP24, no significant red-shift of the absorption band occurs after aggregation. The absorption spectra of CP24 show rather a slight blue shift (data not shown). This is an important difference of CP24 aggregation quenching in comparison with that of CP29 and LHCII. Usually, redshifts are observed upon quenching in many samples. This observation is a supportive indication for increased couplings of the chlorophylls or the lowest energy chlorophyll with neighboring molecules such as carotenoids and is thus supporting the general idea of molecular interactions as quenching valve. However, in the case of aggregation, also other effects have to be considered and can lead to different shifts in the absorption spectra.

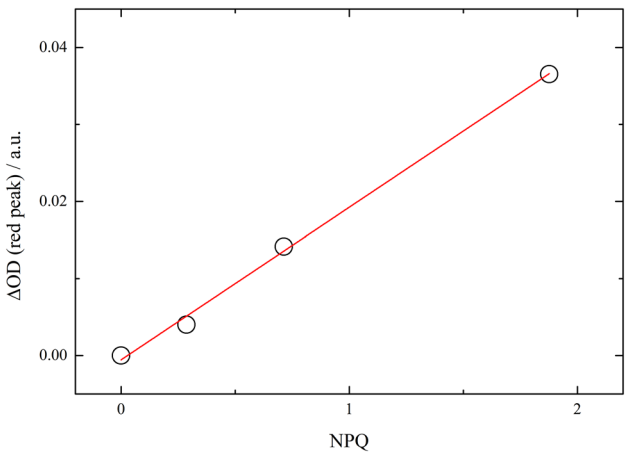

Fig. 4 Correlation of Q_y absorption band shift and NPQ value of CP29 in differently quenched states. For comparability, ΔOD and NPQ were determined analogous to Liao et al. (2010a). See text for details

Electronic Car S_1–Chl Q_y coupling

The extent of electronic coupling between carotenoids and chlorophylls is evaluated by the same procedures as described in our previous studies. Following Eq. 1, the chlorophyll fluorescence observed after selective two-photon excitation of the carotenoids divided by the amount of chlorophyll fluorescence after common one-photon excitation provides a parameter which is proportional to the quantum yield of energy transfer between Car S_1 and Chl Q_y. Thus, this parameter is a direct measure for the electronic coupling of these two states. The comparison of the three unquenched proteins reveals a significant lower fluorescence yield of the investigated samples of minor complexes. In OPE measurements using PAM fluorometer, unquenched CP24 showed only about 50 % and CP29 about 66 % of the fluorescence obtained by unquenched LHCII (Fig. 5a, F^{OPE}). Additionally, the electronic pigment coupling was found to be 10–20 % stronger in the minors (Fig. 5b). It has to be mentioned that the buffer

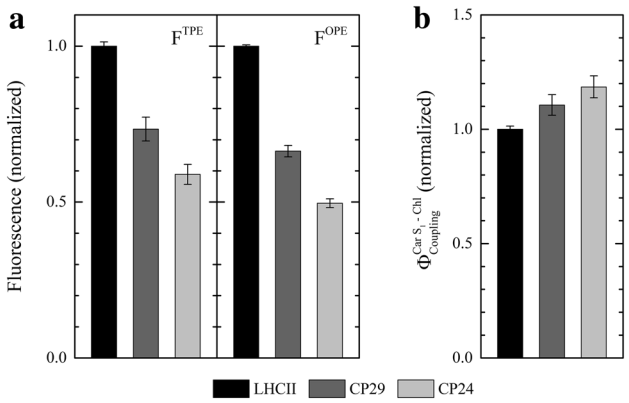

Fig. 5 **a** Fluorescence intensities of unaggregated LHCII, CP24, and CP29 detected after selective two-photon (*left*) and one-photon excitation (*right*). **b** Calculated electronic Car S_1–Chl Q_y coupling following Eq. 1. All data are normalized to LHCII as a reference

system used for CP24 and CP29 was different than the one used for LHCII, which was the same as in our previous studies on LHCII (Bode et al. 2009; Liao et al. 2010a, b, 2011, 2012; Holleboom et al. 2013). Thus, it cannot be completely excluded that the different conditions might influence the quantum yield of non-aggregated proteins. Nevertheless, the aggregation of the light-harvesting proteins causes a reduction in the overall chlorophyll fluorescence capability. This can be seen for all three proteins in the upper section of Fig. 6. The bars beneath the fluorescence values indicate the electronic coupling, and all three proteins obviously show an increase in electronic Car S_1–Chl Q_y coupling upon aggregation-induced fluorescence quenching. This correlation that has already been reported for LHCII (Liao et al. 2010a) is very similar for the two minor PSII proteins CP24 and CP29.

Coupling–quenching correlation

Besides LHCII, also the two minor proteins CP24 and CP29 display a strong correlation of fluorescence quenching and electronic Car S_1–Chl Q_y coupling upon aggregation in the same range of fluorescence yield (Fig. 7). Here, the fluorescence after OPE and the coupling parameter of all samples were normalized to an unquenched LHCII reference. Thus, the *x*-axis in Fig. 7 directly reflects the fluorescence quantum yield of the sample compared to non-aggregated LHCII. This includes the remarkably lower fluorescence which was obtained from the minor complexes at similar concentrations. The aggregations of CP29 and CP24 result in a very similar coupling–quenching correlation as the aggregation of LHCII. Taking into account that the data of the minors indicate curves lying slightly above the data of LHCII, CP29 and especially CP24 seem to show already a slightly stronger coupling for less quenched samples. Nevertheless, an essential result of our study based on the presented data is a general similarity between the investigated two minor proteins CP24 and CP29 and LHCII in aggregation-induced quenching and its correlation to electronic Car S_1–Chl Q_y coupling.

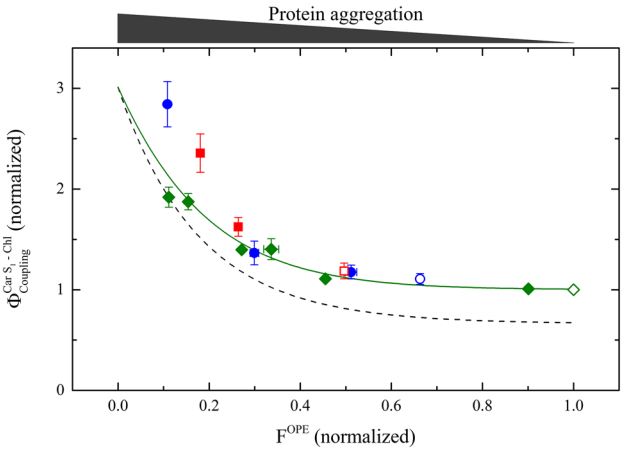

Fig. 7 Correlation of Car S_1–Chl Q_y coupling and chlorophyll fluorescence quenching upon aggregation of LHCII (*green diamonds*), CP24 (*red squares*), and CP29 (*blue circles*). Open symbols represent the unaggregated proteins. Corresponding data of LHCII (*green line*) and grana membranes (*black dashed line*) were taken from Holleboom et al. (2013) for comparison. All data are normalized to fluorescence yield and electronic coupling of unquenched LHCII as a reference

Discussion

Besides demonstrating that the minor light-harvesting complexes CP24 and CP29 can also be gradually

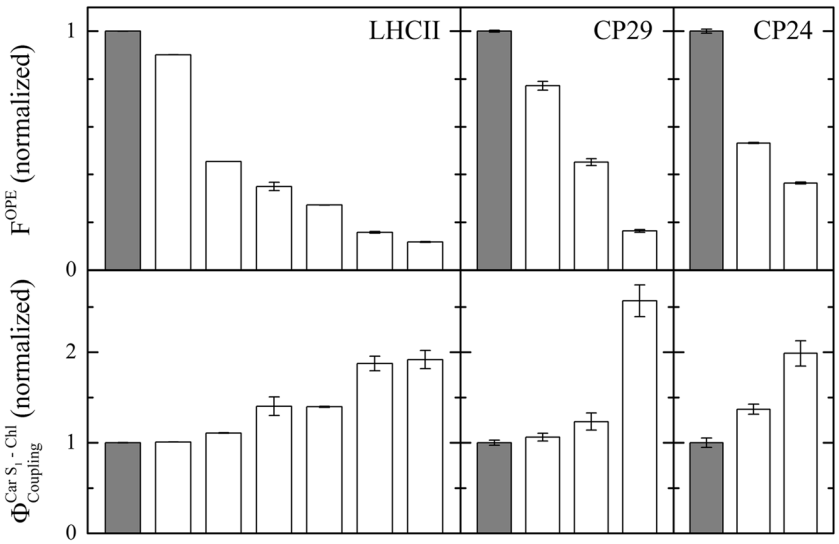

Fig. 6 Fluorescence intensities after selective one-photon excitation of chlorophylls F^{OPE} (*top*), and Car S_1–Chl Q_y coupling (*bottom*) in various aggregation states of LHCII (*left*), CP29 (*middle*), and CP24 (*right*). All data are normalized to the corresponding unquenched sample

aggregated by detergent removal in the present study, three key observations were made in a direct, quantitative comparison with the major light-harvesting complex LHCII: (1) Even without any aggregation, the electronic Car S_1–Chl Q_y couplings in CP24 and CP29 are apparently higher than in LHCII with CP24 having the highest observed electronic Car S_1–Chl Q_y coupling (Fig. 5b). The difference in the fluorescence quantum yield between the minor complexes CP24 and CP29 and LHCII is even larger. Both complexes have a fluorescence quantum yield that is significantly lower than that of LHCII (Fig. 5a, F^{OPE}). Both parameters were quantitatively determined by directly comparing samples of similar chlorophyll content, and additionally, fluorescence intensities were corrected by the calculated chlorophyll content of the samples (see "Materials and methods" section). It should be noted, however, that different contributions of excitonic states seriously mixed with charge transfer states to the observed differences cannot be entirely excluded. 2. While the differences in the electronic coupling were initially small, they become much more apparent upon aggregation quenching. Regardless if the couplings are compared for different quenching degrees relative to the original fluorescence quantum yield of each individual unaggregated complex (Fig. 6), or for relative fluorescence quantum yields between the complexes (Fig. 7), they are much higher for CP24 and CP29 when quenched by aggregation (see right most couplings, $\Phi^{\mathrm{Car}S_1-\mathrm{Chl}}_{\mathrm{Coupling}}$, for each of the complexes in Fig. 6 and left most couplings for the complexes in Fig. 7). 3. Similarly, as for LHCII, there is an aggregation-dependent red-shift of the Q_y band observable for CP29 but not for CP24.

In a former study, we investigated the influence of the protein packing density in thylakoid membranes on the electronic Car S_1–Chl Q_y coupling. In this study, it was observed that—depending on the protein packing density—the fluorescence was quenched and the electronic Car S_1–Chl Q_y coupling also increased with increasing quenching. A curve fitted to these corresponding data is visualized as black dashed line in Fig. 7. In addition, the corresponding curve of the study for aggregation-quenched LHCII is shown as green curve. The aggregation-dependent data of LHCII observed in the present study (green circles) match to a high degree the data of our former study. The in vivo situation of native membranes corresponds to chlorophyll to lipid ratios for which in our previous grana membrane study already significant fluorescence quenching was observed (see the range of 0 up to 0.3 on the x-axis in Fig. 7). Apparently, even without adaptation to excess light conditions and regulation processes, the fluorescence of the light-harvesting proteins in native membranes is already quite quenched by a high packing density (Holleboom et al. 2013). Anyway, while the LHCII data (green curve) seemed to converge for large quenching (low F^{OPE}) with the density-dependent membrane data (black dashed line), the actual slope of the grana membranes is much steeper in the critical range corresponding to 0–0.3. In contrast to LHCII, the increase of $\Phi^{\mathrm{Car}S_1-\mathrm{Chl}}_{\mathrm{Coupling}}$ with increasing quenching (decreasing F^{OPE}) seems to be also much steeper for CP24 (red squares) and CP29 (blue dots). These observations might indicate that at high quenching, minor complexes potentially play major roles in the quenching by being effectively coupled to the light-harvesting network at higher membrane protein packing densities. The fact that the minor proteins exhibit intrinsically lower fluorescence quantum yields already in an unaggregated state (Fig. 6 and open symbols in Fig. 7) further corroborates that they might play a role as quenching centers that need to be coupled closer to the LHCII light-harvesting network. It has also been stated that isolated proteins reveal lower quenching or less influence on quenching of typical NPQ triggering factors as, for example, addition of zeaxanthin or ΔpH compared to samples including protein–protein interactions (Crimi et al. 2001; Avenson et al. 2008; Amarie et al. 2009; Krüger et al. 2013).

Though in all presented data the similarities of the investigated LHCs are predominant, we also observed some differences especially in the behavior of CP24. One prominent difference is the lack of an aggregation-dependent red-shift of the Q_y absorption band in CP24 in comparison with the data observed with LHCII or CP29. A different spectral behavior of CP24 was also found by Mozzo et al. in a mutational study on LHCII and the CP's (Mozzo et al. 2008b). It was reported that in both CP24 and CP29, a cluster consisting of two chlorophylls (Chl 611/612) and a lutein (in the L1 binding site) exists, which is supposed to be the site of probable chlorophyll–carotenoid interaction quenching in LHCII (Ruban et al. 2007; Mozzo et al. 2008b; Passarini et al. 2009). Furthermore, it was also shown that the pair of the two mentioned chlorophylls in this site generates the lowest energy state in all these complexes, but with a larger contribution at higher energy in the case of CP24, suggesting a slightly different pigment organization (Mozzo et al. 2008b). If the observed red-shift in LHCII and CP29 is associated with this cluster as indeed suggested (Johnson and Ruban 2009), then the different geometry in CP24 could explain the absence of a red-shift. Additionally, it was proposed that in CP24, the interactions of a chlorophyll (Chl 603) and the carotenoid bound to L2 potentially account for an energy quenching capability instead of the above-mentioned cluster (Passarini et al. 2009). Considering that CP24 binds less chlorophylls a than LHCII and CP24, an alternative explanation for the absent absorption shift in CP24 could also

be that the shift is caused by an increased chlorophyll–chlorophyll interaction upon aggregation involving a chlorophyll a that is missing in CP24. However, the difference in the aggregation-dependent spectral shift of CP24 might also arise from a different aggregation size upon the same treatment.

A closer look to pigment binding sites of the examined proteins provides another basis for discussion of the higher coupling of the minor proteins compared to LHCII. While there is no discrepancy in the L1 site of the investigated proteins (all bind lutein), the L2 site is reported to bind violaxanthin in CP24 and CP29 instead of lutein in LHCII (Caffarri et al. 2007; Passarini et al. 2009; 2014). Thus, a stronger coupling of the minor proteins could also originate from stronger interactions of the chlorophyll–violaxanthin pair in L2.

In summary, a protocol is presented, which allows gradual aggregation quenching also of minor complexes in a similar manner as it has previously shown for LHCII. A quantitative comparison of the electronic carotenoid–chlorophyll interactions in CP24 and CP29 determined by selective excitation of the Car S_1 states, $\Phi^{\mathrm{Car}S_1-\mathrm{Chl}}_{\mathrm{Coupling}}$, provides evidence that similar to LHCII, the interactions increase with increasing quenching. However, both minor light-harvesting complexes, CP24 as well as CP29, are more quenched than LHCII already in the absences of aggregation and these differences become even more prominent when aggregated. A quantitative comparison of these data with corresponding data of grana membranes of varying protein density supports a model in which the fluorescence of LHCs in native membranes is already significantly quenched by protein interactions even under dark-adapted conditions and that minor light-harvesting complexes can contribute to down-regulation of photosynthetic light harvesting by protein–protein interactions in the membrane and increased carotenoid–chlorophyll interactions.

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