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Time-resolved fluorescence analysis of the recombinant photosystem II antenna complex CP29

Effects of zeaxanthin, pH and phosphorylation

Massimo Crimi[1], Dieter Dorra[2], Carola S. Bösinger[2], Elisabetta Giuffra[1], Alfred R. Holzwarth[2] and Roberto Bassi[1]

[1]*Dipartimento Scientifico e Tecnologico, Università di Verona, Italy;* [2]*Max-Planck-Institut für Strahlenchemie, Mülheim a.d. Ruhr, Germany*

Nonradiative dissipation of excitation energy is the major photoprotective mechanism in plants. The formation of zeaxanthin in the antenna of photosystem II has been shown to correlate with the onset of nonphotochemical quenching *in vivo*. We have used recombinant CP29 protein, over-expressed in *Escherichia coli* and refolded *in vitro* with purified pigments, to obtain a protein indistinguishable from the native complex extracted from thylakoids, binding either violaxanthin or zeaxanthin together with lutein. These recombinant proteins and the native CP29 were used to measure steady-state chlorophyll fluorescence emission and fluorescence decay kinetics. We found that the presence of zeaxanthin bound to CP29 induces a ≈35% decrease in fluorescence yield with respect to the control proteins (the native and zeaxanthin-free reconstituted proteins). Fluorescence decay kinetics showed that four components are always present but lifetimes (τ) as well as relative fluorescence quantum yields (rfqy) of the two long-lived components (τ_3 and τ_4) are modified by the presence of zeaxanthin. The most relevant changes are observed in the rfqy of τ3 and in the average lifetime (≈ 2.4 ns with zeaxanthin and 3.2–3.4 ns in the control proteins). When studied *in vitro*, no significant effect of acidic pH (5.2–5.3) is observed on chlorophyll *a* fluorescence yield or kinetics. The data presented show that recombinant CP29 is able to bind zeaxanthin and this protein-bound zeaxanthin induces a significant quenching effect.

Keywords: fluorescence quenching, light-harvesting complex, photoprotection, xanthophylls.

The photosynthetic apparatus of higher plants is composed of two photosystems, I and II (PSI and PSII, respectively), each of them consisting of a core complex moiety binding chlorophyll (Chl) *a* and β-carotene, and a peripheral antenna system binding Chl *a*, Chl *b* and xanthophylls. The large absorption cross-section of the antenna systems allows plants to grow in dim light. However, light intensity seen by a plant varies largely over relatively short time periods. When irradiance is over-saturating with respect to the electron-transfer capacity, a decrease in the quantum efficiency of photosynthesis is observed, due to an enhancement of nonradiative deactivation at the level of PSII. The down-regulation can be observed by its quenching effect on Chl fluorescence. This process is called 'nonphotochemical quenching' (NPQ). It occurs mainly in the light-harvesting antennae but to some part also in the reaction centre of PSII [1]. It protects PSII from irreversible photoinhibition (reviewed by Horton *et al. and* Bassi *et al.* [2,3]).

Correspondence to M. Crimi, Dipartimento Scientifico e Tecnologico, Università di Verona, Strada le Grazie, Cà Vignal 1, 37134 Verona, Italy. Fax: + 39 45 8027929, Tel.: + 39 45 8027924;
E-mail: crimi@sci.univr.it, http://www.sci.univr.it/~crimi

Abbreviations: Chl, chlorophyll; LHC, light-harvesting complex; nCP29, native CP29; NPQ, nonphotochemical quenching; PSI, photosystem I; PSII, photosystem II; rCP29, recombinant CP29; rfqy, relative fluorescence quantum yield.

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Photoprotection in higher plants is achieved by several different mechanisms. State 1–State 2 transition causes variations of antenna size leading to a redistribution of excitation energy between the two photosystems [4]. This process is rather slow (half-time of dark-relaxation around 5 min) and correlates with the phosphorylation of light-harvesting complex (LHC) II antenna proteins. Nonradiative dissipation is induced upon higher excess of excitation energy; it is catalysed by faster mechanisms involving the formation of a trans-thylakoid ΔpH, and the de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin. Although these processes have been shown to correlate with the phenomenon of the so-called qE-quenching or NPQ-quenching *in vivo and* in thylakoids [5,6], the detailed mechanisms at a molecular level are still poorly understood.

Different hypotheses have been put forward in order to explain zeaxanthin-dependent quenching. Direct quenching of Chl *a* singlet excited states by zeaxanthin is potentially an attractive model from a photophysical point of view [7], but the mechanism has never been experimentally proven. As the minor light-harvesting complexes CP29, CP26 and CP24 bind the highest amount of xanthophylls, e.g. up to 85% in maize [8], they should be the ideal location of zeaxanthin-dependent quenching. Unfortunately, up to now, no experimental demonstration of *in vitro* quenching in an isolated antenna containing protein-bound zeaxanthin has been reported. Recently, violaxanthin and zeaxanthin have been shown to have essentially the same energy of the S0 → S1 transition thus making unlikely a differential role on Chl a excited states quenching [9].

Table 1. Pigment composition and pigment/protein stoichiometry of the reconstituted proteins and the native CP29.

Sample	Chl	Chl *a*/*b*	Carotenoid	Chl/carotenoid	Lutein	Neoxanthin	Violaxanthin	Zeaxanthin
nCP29	8 ± 0.4	3 ± 0.08	2 ± 0.15	4 ± 0.1	1 ± 0.05	0.35 ± 0.05	0.65 ± 0.05	–
rCP29 viola	8 ± 0.4	3 ± 0.08	2 ± 0.15	4 ± 0.1	1 ± 0.05	0.4 ± 0.05	0.5 ± 0.05	–
rCP29 zea	8 ± 0.4	3 ± 0.08	2 ± 0.15	4 ± 0.1	0.5 ± 0.05	0.4 ± 0.05	0.2 ± 0.02	0.9 ± 0.05

An indirect action of zeaxanthin in the quenching mechanism was proposed [2] acting through the carotenoid-induced control of the aggregation state of LHCII and of the minor LHCs [10,11]. *In vitro* experiments have suggested that zeaxanthin, added to solubilized complexes, favours LHC aggregation while violaxanthin exerts the opposite effect. However, LHC aggregation has not been demonstrated unequivocally so far *in vivo* and, in particular, during NPQ.

In both hypotheses a trans-thylakoid ΔpH is required to trigger the quenching mechanism. In fact, low luminal pH is required for activation of the violaxanthin de-epoxidase, the enzyme catalysing de-epoxidation of violaxanthin to zeaxanthin [12] and therefore LHCII oligomerization [13]. In a somehow alternative view, a low luminal pH has been proposed to be the main factor in NPQ, thus acting through a conformational change of antenna proteins [14] or through the induction of the formation of LHCII aggregates in the membrane even in the absence of zeaxanthin [2].

CP29, together with CP26, is the most likely candidate for the role of a regulatory antenna protein as suggested by many reports. It undergoes reversible phosphorylation at a threonine residue in the N-terminal region when cold-resistant plants are exposed to low temperatures in the presence of light thus inducing conformational changes [15] and protection from photoinhibition [16]. It can be reversibly protonated at a specific glutamate residue (E166), involved in the binding of a Chl *b*, as revealed by *N*,*N*′-dicyclohexylcarbodiimide (DCCD) binding [17,18]. As DCCD is a powerful inhibitor of the energy-dependent quenching [19], this binding is indicative of a direct involvement of CP29 in the energy dissipation process. The minor antenna proteins CP29 and CP26 are located between the major LHCIIs and the PSII core in the PSII unit [20,21]. Thus, all the excitation energy transferred has to pass through the minor antennae on its way to the PSII reaction centre. This situation provides the opportunity for the regulation of energy flux toward the reaction centre.

In order to elucidate the effects of protein-bound zeaxanthin, low pH and phosphorylation on the fluorescence properties of isolated CP29, we have used a recombinant CP29 (rCP29) protein obtained by over-expression of the *Lhcb4* gene from maize in *E. coli* and subsequent *in vitro* refolding of the apoprotein with purified pigments [22]. This system, besides yielding a pigment protein indistinguishable from the native complex extracted from thylakoids, allows point mutation of the protein [17,18] and chromophore modification [23]. We have used the latter approach and obtained a recombinant reconstituted CP29 protein binding either violaxanthin (rCP29 viola) or zeaxanthin (rCP29 zea) together with lutein and neoxanthin in the two internal binding sites (namely L1 and L2).

A detailed spectroscopic study of these rCP29 preparations as well as the native protein (nCP29), under various experimental conditions, is presented here. We report a clear evidence for zeaxanthin-induced quenching in the isolated protein. It should be noted that in our experiments zeaxanthin is incorporated into the protein. Acidic pH (measurements at pH 5.3) or the presence of the phosphorylated threonine in the native protein did not induce significant changes in the fluorescence yield and decay kinetics.

MATERIALS AND METHODS

Purification and biochemical characterization

Native CP29 was isolated from maize thylakoid membranes as previously described [24,25]. Reconstitution of CP29 from the recombinant apoprotein with Chl *a and b* and carotenoids has been performed as reported by Giuffra *et al.* [22] and different carotenoid mixtures have been used for reconstitution. The amount of zeaxanthin, purified by HPLC from a *Synechocystis* 6083 pigment extract, can be varied by changing the xanthophyll mixture during reconstitution without any modification of the Chl *a*/*b* ratio of the final CP29 product.

For the rCP29 zea used for the studies in this paper, the relative carotenoid composition of the mixture used for reconstitution was 3.5 mol neoxanthin + violaxanthin, 12 mol lutein, 7.8 mol β-carotene and 23.7 mol zeaxanthin. These values are referred to 100 mol Chl *a* and 11 mol Chl *b*. The products of reconstitution do not contain any free pigments after the additional purification steps described previously [22]. The phosphorylated CP29 complex (also called CP34) was isolated and purified as described in [15] after cold treatment during illumination of maize plants.

The Chl *a*/*b* ratio and carotenoid content were determined as in Bassi *et al.* [8]. The pigment analysis was performed by HPLC on a reverse-phase C_{18} bondclone column detected with a Beckman system (Gold 126) equipped with a 168 element diode array [22]. The pigment composition is shown in Table 1.

Spectroscopy

Absorption spectra were measured on an Aminco DW 2000 spectrophotometer; steady-state fluorescence emission spectra were measured on a Jasco spectrofluorimeter model FP 777 and/or on a Spex Fluorolog [26]. CD spectra were obtained at 8 °C with a Jasco J-600 spectropolarimeter.

Time-resolved fluorescence spectroscopy was carried out at 10 ± 2 °C using the single-photon-timing method as described previously [27]. The time-resolution was about 5 ps. To avoid anisotropy effects magic angle detection was used. Data taken at about 5 nm intervals across the fluorescence spectrum were summed up typically to 20 000 counts in the peak channel and analyzed by global lifetime analysis [28]. All spectroscopic measurements have been performed in the same buffer containing 10 mM Hepes, pH 7.6 (or acidic pH in 50 mM acetate buffer, where mentioned specifically), 0.06% dodecyl maltoside and 20% glycerol.

To prevent photodamage of the samples by singlet oxygen during the time-resolved fluorescence measurements, oxygen molecules were enzymically trapped by the addition of glucose oxidase and catalase (200 and 40 $\mu g \cdot mL^{-1}$, respectively) to the buffer in the presence of glucose as a substrate [29]. The sample ($D_{650} \approx 0.2$) was kept oxygen-free in a cuvette with a 1.5-mm window. In this oxygen-free system the stability of the samples under illumination is increased from tens of minutes to hours at room temperature.

RESULTS

Biochemical and steady-state spectroscopic characterization of native and recombinant CP29 proteins

The absorption spectra of nCP29, the recombinant control (rCP29 viola) and the zeaxanthin-containing (rCP29 zea) reconstituted complexes are shown in Fig. 1. These spectra demonstrate that the reconstituted and the native protein are essentially identical with absorption maxima at 677 nm, 640 nm, 465–467 nm and 437 nm. The most significant difference is observed in the protein reconstituted in the presence of zeaxanthin, rCP29 zea, where the modified contribution of the xanthophylls to the absorption spectrum can be seen in the region around 450–500 nm.

The pigment composition and the pigment/protein stoichiometry of nCP29 and rCP29 used in the measurements are reported in Table 1. It should be noted that the Chl content, the Chl *a*/*b* ratio and the total carotenoid content were the same within the error limits for the three samples with a total number of eight Chl and two xanthophyll molecules per CP29 polypeptide and a Chl *a*/*b* ratio of three. The only difference was therefore the carotenoid composition; the rCP29 zea showed an increased zeaxanthin and a decreased violaxanthin and lutein content with respect to nCP29 and rCP29 viola (Table 1). These results indicate that rCP29 is able to specifically bind zeaxanthin during refolding in the presence of other xanthophylls including violaxanthin (molar ratio zeaxanthin/xanthophylls = 1). To confirm that pigment binding occurs in a specific and correct way, CD spectroscopy was carried out (Fig. 2). Again, the spectra of the control CP29 and of the same complex reconstituted with zeaxanthin are very similar if not identical with only, possibly, a small difference in the relative amplitude of the two negative signals in the region around 467 nm.

To study the effects of zeaxanthin bound to CP29 on the energy equilibration and on its possible involvement in the quenching mechanism, we have investigated these proteins by fluorescence spectroscopy.

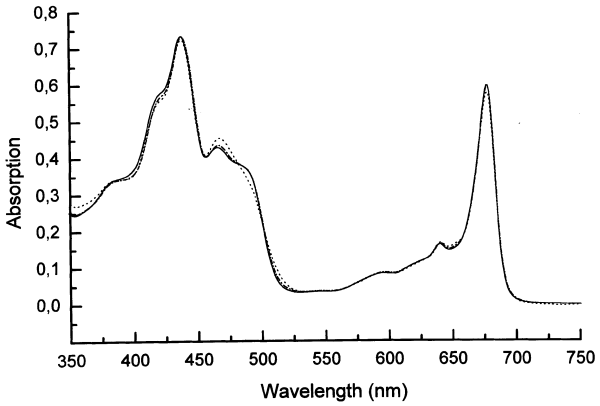

Fig. 1. Absorption spectra of nCP29 (solid line), the recombinant control protein (dashed-dotted line) and zeaxanthin-containing (dotted line) reconstituted complexes.

Steady-state fluorescence

Fluorescence excitation spectra (Fig. 3) show that both Chl *b* and xanthophylls efficiently contribute to Chl *a* emission which, in turn, appears as a single peak at 681 nm irrespectively from excitation at either 440 nm (Chl *a*) or 475 nm (Chl *b*). Small differences in both absorption and fluorescence excitation spectra at around 480–490 nm are to be ascribed to the violaxanthin versus zeaxanthin absorption.

Steady-state fluorescence emission spectra are shown in Fig. 4. Upon Chl *a* excitation (440 nm), nCP29 and rCP29 viola show identical emission spectra characterized by a 680-nm peak. Also, the fluorescence yield was the same within the error limits. The rCP29 zea sample, although showing a very similar emission spectrum, differed pronouncedly in the fluorescence yield. Under these conditions the relative fluorescence yield of rCP29 zea was reduced by 35% [quenching (Fmax − Fx/)Fx = 1.85 ± 0.3] as

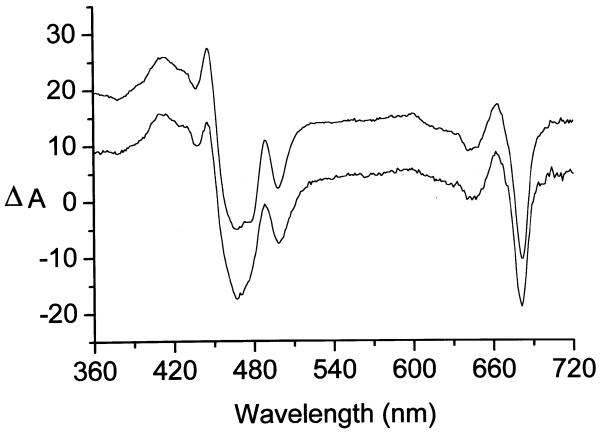

Fig. 2. CD spectra in the visible range of control rCP29 viola (upper) and rCP29 zea (lower). The spectra were shifted on the *y*-axis for better clarity.

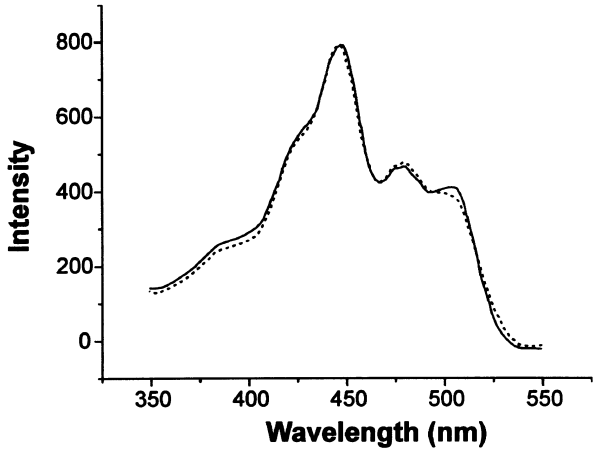

Fig. 3. Fluorescence excitation spectra (680 nm emission) of rCP29 viola (solid line) and rCP29 zea (dotted line).

compared to those of rCP29 viola or nCP29. Essentially identical results were obtained with Chl *b* excitation at 475 nm (data not shown). This decrease in the fluorescence yield indicates that the binding of zeaxanthin induces a quenching of fluorescence emission, possibly through activation of a heat dissipation mechanism as occurring *in vivo* under qE-quenching conditions.

Low luminal pH was reported to be necessary for fluorescence quenching *in vivo* and phosphorylation of CP29 at threonine 83 was reported to affect the protein conformation and induce quenching *in vivo* [16]. We thus checked the effect of these conditions on CP29 fluorescence in solution.

Compared to the emission spectra measured at neutral pH (7.5–7.6) the emission spectra measured under the same conditions but at acidic pH (5.3) show a very small quenching effect in all cases, close to the measuring error of $\approx$ 5%. Other mechanisms are then required for the *in vivo* low pH dependence of NPQ (e.g. a more acidic lumenal pH and the presence of the phospholipid bilayer). The phosphorylated CP29 protein (also called CP34) also did not show a significant variation of the fluorescence yield.

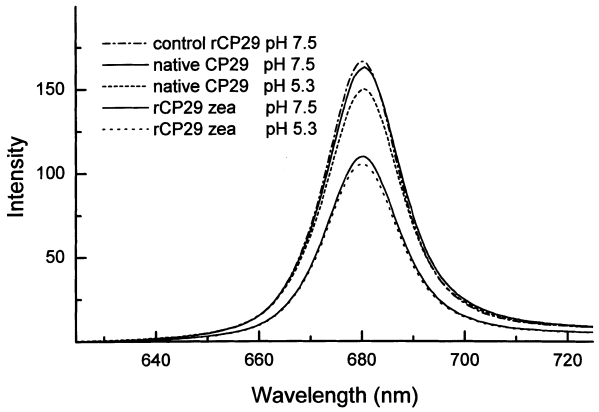

Fig. 4. Steady-state fluorescence emission spectra (λ_{exc} = 440 nm) of the three different CP29 proteins. Measurements were made at neutral pH (7.5) and acidic pH (5.3) in 50 mM citrate buffer.

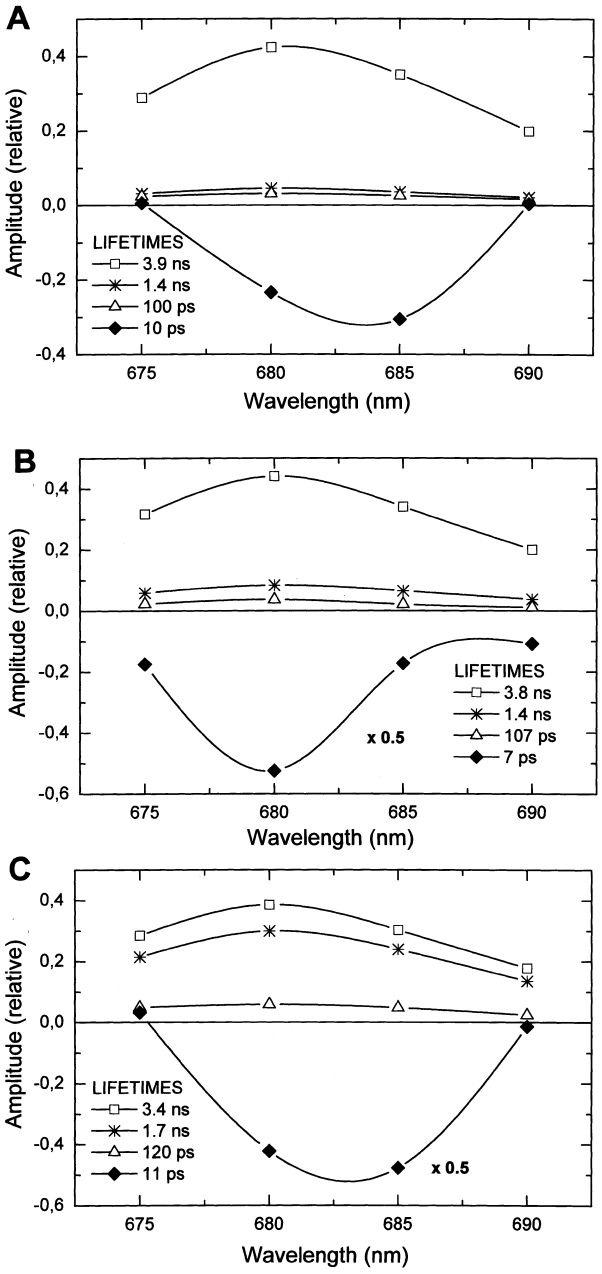

Fig. 5. Decay-associated spectra of the time-resolved fluorescence spectra of the three complexes (λ_{exc} = 650 nm). (A) Native CP29. (B) Control recombinant protein (rCP29 viola). (C) Zeaxanthin-containing recombinant protein (rCP29 zea).

Time-resolved fluorescence measurements

In order to further characterize the quenching effect observed by steady-state fluorescence, time-resolved fluorescence measurements have been performed on the above mentioned CP29 samples.

Fig. 5 shows the decay-associated spectra of the three complexes upon excitation at 650 nm. Free running global fits require four lifetimes in all cases. Lifetimes are summarized in Table 2. The first relevant result is the finding that the native protein extracted from leaves, and

Table 2. Lifetimes and relative fluorescence quantum yields of native and recombinant CP29 proteins and of the phosphorylated form of CP29 at neutral pH and of the native protein at pH 5.3. τ, lifetime, relative amplitudes at 680 nm in brackets.

Sample	τ_1[a] (ps)	τ_2 (ps)	$rfqy_2$	τ_3 (ns)	$rfqy_3$	τ_4 (ns)	$rfqy_4$	Average τ (ns)
nCP29	10	100 (6)	< 1	1.4 (9)	4	3.9 (85)	96	3.4
rCP29 viola	7	107 (7)	< 1	1.4 (15)	7	3.8 (78)	93	3.2
rCP29 zea	11	120 (8)	< 1	1.7 (40)	28	3.4 (52)	72	2.4
nCP29 pH 5.2	2	61 (11.3)	< 1	0.899 (7.4)	2	3.9 (81.3)	98	3.3
CP29 phosphate	6	57 (6)	< 1	0.733 (8)	2	3.6 (86)	98	3.2

[a] Rise component, due to the negligible contribution it was omitted in the calculation of the rfqy.

the recombinant protein, refolded *in vitro*, exhibit very similar, if not identical, decay components, thus supporting the suitability of rCP29 for spectroscopical work. From the time-resolved fluorescence measurements the following observations can be made: the shortest component of $\tau_1 = 7–11$ ps is always positive at emission wavelengths shorter than 675 nm (data not shown) and negative at lower energies, suggesting this component represents an energy transfer term between different Chl pools in agreement with our previous studies on the homologous protein LHCII [10–31]. All other components have positive amplitudes and show a similar spectrum with a maximum at 680 nm. The component with the highest amplitude has a lifetime of $\tau_4 = 3.7–3.9$ ns in the native and the reconstituted wild type as well as in the zeaxanthin-binding form of rCP29. The major difference between zeaxanthin- and violaxanthin-binding rCP29 is the largely different amplitude of the τ_3 component ($\tau_3 \approx 1.4–1.7$ ns) which is very small (4–7% of the rfqy) in the violaxanthin-containing samples while it is responsible for ≈ 30% of the rfqy in rCP29 zea. CP29 measured in acidic buffer as well as the phosphorylated protein show minor variations only in the lifetimes of two components (τ_2 and τ_3), but the relative contribution of these decay terms to the total emission yield remains very small (Table 2).

DISCUSSION

Nonradiative dissipation of excitation energy is a photoprotective mechanism in plants by which the quantum yield of PSII is decreased when photosynthesis is saturated. The mechanism of nonradiative dissipation has been the subject of intense investigation yet controversial hypotheses are proposed, as pointed out in the introduction. A crucial point deals with the capacity of zeaxanthin to quench excitation energy independently of other factors such as low luminal pH or aggregation of pigment-binding proteins. Furthermore the molecular mechanism of this quenching is poorly understood. Previous *in vitro* work by Horton and coworkers pointed to the effect of exogenously added zeaxanthin on the aggregation behavior of the major LHCII [32] and minor Chl proteins [33]. More recently, fluorescence quenching induced by external zeaxanthin in nonaggregating conditions has been observed [34]. However, we have previously shown that xanthophyll cycle pigments are bound to proteins *in vivo* and, in particular, are located in the minor complexes CP26, CP24 and CP29 [8–35]. Recent work has shown that only 15–20% of total zeaxanthin is not firmly bound to Chl proteins in both control and photoinhibited plants [36], suggesting that the protein-bound zeaxanthin might be responsible for the *in vivo* effects.

As isolation of native zeaxanthin-containing pigment proteins from high-light treated plants in sufficient amount is complicated and difficult to reproduce, we have used recombinant proteins. The reconstitution of a recombinant antenna system with pigments allows the study of these proteins *in vitro* by performing a controlled modification of the pigment composition. When present during reconstitution, zeaxanthin is bound to rCP29. This is in agreement with the experimental evidence for binding of this xanthophyll in the native protein in plants exposed to photoinhibitory conditions. Thus, it is feasible that the reconstituted zeaxanthin complex has the same pigment composition as the native complex formed after light stress in plants.

In order for the reconstituted complexes to be 'spectroscopically useful' for elucidating the *in vivo* quenching mechanism, it is necessary to demonstrate that pigment binding occurs in a specific and correct way and that the complexes are functionally competent. As far as the control rCP29 is concerned, it has been previously shown that it is essentially identical to the native complex extracted from leaves as assessed by a number of biochemical and spectroscopical techniques, namely pigment/protein stoichiometry and pigment composition [18–22], absorption spectroscopy at room and low temperature, fluorescence emission and excitation, CD, Chl photo-oxidation kinetics and energy equilibration [23].

When the complex was reconstituted in the presence of zeaxanthin, the absorption spectrum in the Q_y transition was not modified with respect to the control protein supporting the view that the Chls are correctly bound to the protein. This is confirmed by CD spectroscopy, which provides a very sensitive measure for correct pigment placement within a pigment-protein complex. Again, the CD spectra of the control CP29 and of the same complex reconstituted with zeaxanthin are very similar except for a small difference in the relative amplitude of the two negative signals in the region around 467 nm (Fig. 2). Fluorescence excitation spectra show that both Chl *b* and xanthophylls efficiently contribute to Chl *a* emission (Figs 3 and 4). The differences in both absorption and fluorescence excitation spectra at around 480–490 nm are to be ascribed to the violaxanthin versus zeaxanthin absorption. From the above data, we conclude that pigment

arrangement in both violaxanthin- and zeaxanthin-containing reconstituted CP29 closely reflects that in the nCP29 complex. Nevertheless, fluorescence measurements demonstrate a decrease in emission yield in rCP29 zea, with respect to both nCP29 and rCP29 viola, of ≈ 35%.

Fluorescence decay kinetics give an insight into the energy equilibration and quenching mechanism in pigment proteins. The fluorescence lifetimes are identical for the native and the reconstituted wild-type CP29 within the error limits. One can thus conclude that these complexes are also functionally identical.

The CP29 zea complex shows a prominent component with a lifetime of ≈ 1.7 ns that seems to be characteristic for the presence of this xanthophyll. The average lifetime τ_{av} is shortened as compared to the control proteins. This shortening of τ_{av} in the zeaxanthin complex (≈ 30–35%) is, within the limits of error, consistent with the decrease in the relative fluorescence yield (35%) observed in stationary emission. It is important to note that no additional short-lived fluorescence component is observed with zeaxanthin present, which might also be expected. We can exclude that such a potential fast component escaped our time-resolution (5 ps). This may be concluded as the sum of the amplitudes of the fluorescence decays of all components is the same within the error limits for all complexes, i.e. we are not missing any ultrafast component in the zeaxanthin-containing complex which could also explain the yield decrease of 35%. Thus, the zeaxanthin-induced quenching is 'slow' (i.e. ns).

The fluorescence decay behavior of Chl proteins is not fully understood. In fact, a single long decay should be expected for a pigment protein in which rapid energy equilibration occurs before fluorescence emission. Nevertheless, we have observed several lifetime components in both native and recombinant proteins. This is in agreement with many previous results with the homologous protein LHCII [31–37]. The presence of multiple lifetime components may be accounted for by hypothesizing some structural heterogeneity of protein complexes. In the case of CP29, the chromophore pattern includes six Chl *a,* two Chl *b and* two xanthophylls per CP29 [22,23], although three different xanthophyll species (lutein, neoxanthin and violaxanthin) are found in CP29 from dark-adapted leaves in nonstoichiometric amounts and ratios. Upon de-epoxidation, heterogeneity is increased by partial conversion of violaxanthin to zeaxanthin and antheraxanthin. Native CP29 may thus consist of a mixed population binding a varying combination of xanthophylls in the two central sites of the complex (namely, L1 and L2). In the case of the rCP29 zea an average of 0.9 mol zeaxanthin per mol of apoprotein was found. This implies that at most one zeaxanthin is bound per CP29. Assuming a random occupancy of the two sites by the different xanthophylls and excluding cooperativity, a 0.2 probability for double occupancy and a 0.5 probability for single occupancy by zeaxanthin on the two central xanthophyll sites would be obtained, while 30% of the complexes would bind no zeaxanthin. In this model, the population without zeaxanthin is responsible for the ≥ 3.4 ns lifetime component (i.e. no quenching) in the rCP29 zea sample. The lifetimes of complexes with one or two zeaxanthin molecules cannot be distinguished as both contribute to the ≈ 1.7 ns lifetime component and this explains the heterogeneity in the long-lived fluorescence decay. Thus, the two long lifetimes may represent an average of the three expected components. Alternatively, the heterogeneity is restricted to CP29 with or without one zeaxanthin in the L2 carotenoid-binding site and the observed two lifetimes are then representing these two populations (quenched and unquenched states, respectively). Mutation analysis of CP29 has shown that xanthophyll site L1 binds preferentially lutein while site L2 binds violaxanthin, neoxanthin or zeaxanthin [18]. Competition studies on the homologous protein LHCII showed that the site L1 has the highest affinity for lutein while site L2 has a wider specificity and may bind violaxanthin or zeaxanthin *in vitro* [38]. These data support the hypothesis that reconstitution in the presence of both lutein and zeaxanthin leads to preferential binding of zeaxanthin or violaxanthin to site L2. If this is the case then the zeaxanthin-mediated quenching effect should be more likely due to the presence of zeaxanthin in site L2 rather than in site L1.

These observations correlate well with the 35% decrease in fluorescence yield and with the fraction of molecules fluorescing with a lifetime of ≈ 1.7 ns. Our data strongly support the view that tightly bound zeaxanthin is responsible for the observed Chl *a* fluorescence quenching in CP29. Accordingly, previous work in isolated chloroplasts has shown that short-lived fluorescence is induced by increased zeaxanthin concentration [39]. This suggests that the zeaxanthin effect observed in rCP29 *in vitro* may reflect one of the *in vivo* mechanisms of nonphotochemical quenching. Such a quenching effect of bound zeaxanthin on the isolated solubilized complex is shown here for the first time. We propose that the binding of zeaxanthin induces and stabilizes a quenched state of CP29. This means that the xanthophyll composition is responsible for the structural and functional heterogeneity observed in antenna proteins. In agreement with this hypothesis, we observed the presence of multiple lifetimes both in nCP29 and rCP29.

Upon lowering the pH to 5.3, fluorescence yield and fluorescence decay kinetics of either control or rCP29 zea are minimally affected, suggesting that this pH value (*per se*) does not induce fluorescence quenching. The recent finding by Niyogi and coworkers [40] that *psbS* gene deletion leads to strong decrease in NPQ may reflect a possible need for *psbS* in the induction of CP29 fluorescence quenching. In fact, pigment binding to *psbS* was not proven experimentally and most of the residues shown to bind Chl in LHCs are not conserved. In the isolated protein, changes in pH or phosphorylation were found to have only minor effects either on the steady-state fluorescence or on the time-resolved fluorescence for the three forms of CP29 studied here. This indicates that these factors are not directly responsible for the quenching in the isolated system but might be important *in vivo* by mediating protein–protein interaction leading to the amplification of quenching.

CONCLUSION

We describe that protein-bound zeaxanthin is effective in inducing Chl *a* fluorescence quenching in a nonaggregated isolated Chl protein, namely the minor antenna protein CP29, reconstituted *in vitro*. Concerning the mechanism of action, recent results by Walla and coworkers [41] indicate

that the S1 energy level of xanthophylls can well be above the Qy transition of Chl *a* and therefore direct quenching of Chl *a* excited states by zeaxanthin might be an inefficient way of de-excitation. Moreover, Polivka and coworkers [9] showed that violaxanthin and zeaxanthin have very similar S1 state levels thus making de-epoxidation unlikely as a differential source of direct quenching. Our results show that violaxanthin and zeaxanthin, bound to internal sites of the protein, have differential effect on fluorescence yield of CP29 and thus suggest that mechanisms other than direct quenching may be induced by zeaxanthin binding. Recently, external addition of zeaxanthin has been shown to induce quenching in the absence of aggregation of the antenna proteins [34]. Both the present results and those of Wentworth and coworkers point to conformational changes of CP29 protein structure rather than direct Chl → carotenoid energy transfer as the source of quenching. The availability of recombinant proteins binding either violaxanthin or zeaxanthin now opens the possibility to study the detailed molecular mechanism of zeaxanthin-induced quenching, including the effects of modified protein domains on the quenched conformation by using mutation analysis [18–44].

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