

The specificity of controlled protein disorder in the photoprotection of plants

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Light-harvesting pigment-protein complexes of photosystem II of plants have a dual function: they efficiently utilize absorbed energy for photosynthesis at limiting sunlight intensity and dissipate the excess energy at saturating intensity for photoprotection. Single-molecule spectroscopy studies on the trimeric LHCII complex demonstrated that environmental control of the intrinsic protein disorder could in principle explain the switch between their light-harvesting and photoprotective conformations in vivo (Krüger et al. 2012. *Biophys J.* 102:2669-2676). However, the validity of this proposal depends strongly on the specificity of the protein dynamics. Here, a similar study has been performed on the minor monomeric antenna complexes of photosystem II (CP29, CP26, and CP24). Despite their high structural homology, similar pigment content and organization compared to LHCII trimers, the environmental response of these proteins was found to be rather distinct. A much larger proportion of the minor antenna complexes were present in permanently weakly fluorescent states under most conditions used; however, unlike LHCII trimers the distribution of the single-molecule population between the strongly and weakly fluorescent states showed no significant sensitivity to low pH, zeaxanthin or low detergent conditions. The results support a unique role for LHCII trimers in the regulation of light harvesting by controlled fluorescence blinking and suggest that any contribution of the minor antenna complexes to photoprotection would probably involve a distinct mechanism.

Keywords: nonphotochemical quenching (NPQ), light-harvesting complex, single-molecule spectroscopy, fluorescence blinking, protein dynamics

The structural flexibility of proteins is an important property that enables their regulatory switching between active and inactive functional states. Some proteins possess the ability to switch between different functional states, thereby exhibiting multifunctionality. The peripheral light-harvesting complexes of plant photosystem (PS) II are such examples: under normal conditions they ensure highly efficient transfer of absorbed solar energy to the reaction center, while under stress conditions, most of the absorbed energy is dissipated as heat (1, 2). This rapidly reversible, Δ pH-dependent process is commonly referred to as qE. The properties of this regulatory function are strongly contested, in particular the site and molecular mechanism, as recently reviewed in Ref. (3).

It is commonly accepted that one or more of the peripheral antenna complexes are involved in qE. These macromolecules are composed of six homologous pigment-protein complexes from the Lhcb multigenic family (4). The first three form heterotrimers of different compositions (5, 6) and are known as the major antennae, often referred to as LHCII, while the other three complexes exist naturally as monomers (7) and are known as the minor antennae CP29, CP26, and CP24. The proteins of these complexes bind the same pigments, but in different ratios. The crystal structures of only LHCII and CP29 have been resolved (8-10). The first indicated that each monomeric subunit of LHCII binds 14 chlorophylls (Chls) and 4 carotenoids (Cars) at fixed binding sites (8). While one less Chl has been resolved for CP29 (9), only 8 Chls are normally identified by *in vitro* studies (11-14), suggesting that conventional isolation techniques of CP29 lead to a loss of Chl pigments, or that a specific subset of CP29 complexes was crystallized. The number of Chls in CP26 (9 Chls) and CP24 (10 Chls) may similarly be underestimated by *in vitro* studies. The minor antenna complexes bind the same xanthophyll Cars as LHCII – lutein (Lut), neoxanthin (Neo), and violaxanthin (Vio) or zeaxanthin (Zea) – but with a different occupancy (15-17).

Under high incident photon fluxes the energy delivered to the PSII reaction center can no longer be utilized efficiently, and the luminal pH drops from typically ~8.0 to ~5.5. The resulting transmembrane pH gradient triggers qE, induces the enzymatic de-epoxidation of Vio into Zea within the PSII antennae (18), and protonates the antenna proteins and the PsbS protein (19, 20). In addition, a rearrangement and aggregation of the antennae occurs within the membrane (21). All these processes enhance qE (1, 22).

Several molecular mechanisms have been proposed for qE, the following three being the most recurrent: (i) energy transfer from the terminal emitter Chls of the LHCII trimer to the S₁ state of the neighboring Lut 1 molecule, followed by rapid internal conversion (23-26); (ii) formation of a Chl-Chl charge-transfer state in LHCII and subsequent charge recombination (27); (iii) formation of a Zea-Chl or Lut-Chl charge-transfer state in one or more of the minor antenna complexes, followed by rapid charge recombination (28-30). In each case, protein dynamics were suggested to underlie the switch between light-harvesting and photoprotective states.

Single, isolated LHCII trimers have been observed to possess the intrinsic capability to quench their fluorescence rapidly, reversibly and for times that scale from milliseconds to minutes (31, 32). Although such intermittent behavior – commonly referred to as fluorescence blinking – is shared by diverse fluorescing systems, ranging from other photosynthetic multichromophoric light-harvesting complexes (33-36) to semiconductor nanocrystals (quantum dots) (37) and single dye molecules (38), for LHCII it manifests some unique properties (39).

A direct relationship between qE and fluorescence blinking was recently proposed, based on single-molecule studies of LHCII complexes (40). It was demonstrated that each of the investigated qE-associated environmental conditions affected the fluorescence blinking such that the time- and population-averaged intensities decreased and the average dwell time in quenched states increased. The connection between qE and fluorescence blinking suggests that the intrinsic dynamic disorder of LHCII trimers, which is reflected by the observed intensity fluctuations, can be controlled by the local environment of the complexes. On a molecular scale this control features as a subtle conformational change, which shifts the population equilibrium between quenched and unquenched states. In this view, new dissipative states do not need to be invoked; instead, as more qE-related conditions are incorporated, the probability for accessing one or

more dissipative states increases, in agreement with the population-shift model of protein functionality (41).

The experimental blinking behavior of LHCII trimers was reproduced quantitatively by a conformational diffusion model (42). Here, the protein structural motion in the terminal emitter domain was described to contain a specific, relatively slow, diffusive component that is modulated by the local environment. In the context of the Chl-to-Lut energy-transfer model (23), the probability of energy transfer from the terminal emitter Chls to the electronically coupled Lut 1 molecule fluctuates extensively and on a similar timescale as fluorescence blinking, thus supporting the notion that fluorescence blinking is an intrinsic property of LHCII and associated with one of the qE mechanisms.

The protein dynamics described by this model could be extended on the same principles to other proteins of the Lhcb family, implying that the relationship between blinking and the qE-associated conditions proposed for LHCII might be a general property of all these proteins. Indeed, *in vitro* ensemble measurements have indicated that isolated minor antenna complexes respond qualitatively in a similar manner to qE-related conditions as isolated LHCII trimers and exhibit an even more pronounced quenching (43, 44). On the other hand, only small or negligible quenching effects were observed for isolated minor antennae in some other studies (29, 45-47).

In this study, the intrinsic quenching capacity of the minor antenna complexes is investigated by comparing the environmental sensitivity of their fluorescence blinking behavior to that of LHCII trimers. An important part of the study involves the contribution of the “dim fraction”, i.e. complexes in moderately quenched states during the entire duration of the measurement. The results support a unique role for LHCII in qE and provide an explanation for the varying quenching behavior reported in different *in vitro* bulk studies on isolated minor antennae. In addition, the findings suggest that artifactual or single-molecule enhanced quenching effects have a negligible contribution to the observed environmental changes, thus supporting the significance of single-molecule studies to the investigation of physiological energy-dissipating processes.

MATERIALS AND METHODS

Sample preparation

The complexes enriched in Vio were isolated by iso-electric focusing from PSII-particles obtained from dark-adapted spinach leaves (13). To prepare the Zea-containing complexes, thylakoids were treated with 40 mM ascorbate at pH 5.5 before isolation and purification of the PSII-particles (13). The pigment composition of the complexes is shown in Table 1, indicating that the Vio and Zea contents were elevated as compared to standard purification protocols. Complexes were solubilized in 20 mM HEPES at pH 8.0, 1 mM MgCl₂ and 0.03% (w/v) n-dodecyl- β ,D-maltoside (β -DM), which collectively mimicked the light-harvesting environment. After dilution to a few pM, a small volume was deposited onto a layer of poly-L-lysine (Sigma, Schnellendorf, Germany) and flushed with the experimental environment. The environmental variables consisted in the amount of de-epoxidation (either Vio- or Zea-enriched complexes), the pH (either 8 or 5.5), and the detergent concentration (either 0.03% (w/v) or significantly lowered). Sodium citrate (15 mM) was added when a pH of 5.5 was used, and the detergent

Table 1. Pigment composition of isolated photosystem II antenna proteins.

	Complex	Neo	Vio	Ant	Lut	Zea	DES	Chl <i>a/b</i>
Vio-enriched	CP24	28.3(18)	25.0(12)	0.0	46.7(28)	0.0	0%	1.6(1)
	CP26	31.3(15)	23.7(15)	0.0	46.0(23)	0.0	0%	2.5(2)
	CP29	23.0(14)	36.0(24)	0.0	41.0(16)	0.0	0%	3.4(1)
	LHCII	27.0(11)	18.0(13)	0.0	55.0(16)	0.0	0%	1.3(1)
Zea-enriched	CP24	27.3(17)	10.3(22)	2.0(4)	46.3(19)	14.0(19)	57%	1.6(1)
	CP26	30.0(20)	8.0(13)	2.7(3)	47.3(20)	12.0(13)	56%	2.5(1)
	CP29	24.3(12)	17.0(18)	3.7(7)	42.3(12)	12.7(9)	40%	3.4(1)
	LHCII	27.0(12)	2.0(2)	2.0(2)	54.0(22)	15.0(21)	84%	1.3(1)

PSII light-harvesting complexes purified by iso-electric focusing from spinach plants, obtained from the thylakoids of plants that were dark adapted for 12 hours (Vio-enriched) and by prior de-epoxidation of the thylakoids at pH 5.5 in the presence of 40 mM ascorbate (Zea-enriched). Neo, Vio, Ant, Lut, Zea, DES and Chl *a/b*: neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin, de-epoxidation state $(Z + 0.5A) / (V + A + Z)$ and chlorophyll *a/b* ratio. Data are presented as (xanthophyll / total xanthophyll) (in %) and are means from four replicates; DES is presented as $(Zea + 0.5Ant) / (Vio+Zea+Ant)$; Chl *a/b* is presented as a molar ratio. Numbers in parenthesis denote standard deviations, as applied on the last significant digit(s), taking into account variations between different purifications.

concentration was lowered by replacing the environment in which the immobilized, micelle-embedded complexes were present with a detergent-free buffer. Prior to flushing, oxygen was scavenged in the flushing solution by copious flushing of nitrogen gas while an enzymatic system of 200 $\mu\text{g}/\text{mL}$ glucose oxidase, 7.5 mg/mL glucose, and 35 $\mu\text{g}/\text{mL}$ catalase was allowed the time to react with oxygen. Measurements were performed in a hermetically sealed sample cell at 5°C and repeated on at least one other day to reduce the heterogeneity caused by slight day-to-day variations. A new sample preparation was performed for every experimental environment. Only freshly prepared samples, subject to at most one freeze-thaw cycle, were used. The freeze-thaw cycle did not notably affect the spectroscopic behavior.

Single-molecule spectroscopy

The experimental setup used to perform single-molecule fluorescence spectroscopy was described earlier (32, 48). All complexes were excited with an intensity of ca. 250 W/cm^2 at 632.8-nm, originating from a continuous-wave He-Ne laser (JDS Uniphase, Eindhoven, The Netherlands). The linear polarization of the light was changed into a near-circular state by a Berek polarization compensator (5540M; New Focus, Santa Clara, CA) to further reduce Chl-specific excitation. Fluorescence counts were registered in 10-ms consecutive bins with an avalanche photodiode (SPCM-AQR-16; Perkin-Elmer Optoelectronics, Waltham, MA).

Data analysis

For each type of complex in each of the experimental environments, data sets consisting of intensity–time traces of a few hundred individually measured complexes were collected and subjected to similar data screening as previously employed on LHCII trimers (31, 40). In short, the single-molecule identity of the complexes was established by single-step transitions into fully quenched or photobleached states. Three additional filtering criteria involved excluding complexes exhibiting spectral blueing and intensity traces characterized by photonbursts or excessive fluctuations. Data sets containing at least 10% of such filtered-out traces were disregarded altogether. In addition, only complexes surviving longer than typically one-third of the average survival time of ~ 30 s were considered. Intensity traces were trimmed to the last intensity level exceeding a pre-defined intensity threshold, the latter of which was set relatively high for Figs. 1, 2, and 3, *A–C*, thereby excluding the so-called dim fraction of complexes for these figures. Screened data sets contained information from at least 100 complexes. Intensity bins of 20 ms were used, giving signal-to-noise ratios of typically 6–10 for the fully emitting states. Intensity levels were resolved for data in Fig. 1, employing the algorithm described in Ref. (31). Intensities were calibrated based on the best alignment of the optical setup. Presented data constitute averages from at least three different data sets and at least two batches from different protein purifications. Comparisons with in vitro ensemble results were done by calculating the time- and population-averaged intensity $\bar{I} = \sum_i I_i \tau_i / \sum_i \tau_i$ for data sets consisting of N intensities I_i and associated dwell times τ_i . All calculations were implemented in MATLAB (The MathWorks, Natick, MA).

RESULTS

Environmental sensitivity of intensity distributions

Under continuous illumination, single complexes of CP29, CP26, and CP24 were found to display the familiar phenomenon of fluorescence blinking. Fig. 1 displays a typical intensity–dwell-time distribution, shown here only for CP29 complexes under conditions that mimic the harvesting of light in vivo, i.e., Vio-enriched complexes at pH 8.0 at a detergent concentration of 0.03% β -DM. The accessed intensity levels can be broadly separated into three groups: unquenched, intermediate, and quenched, or alternatively, bright, dim, and dark. This distinction is based on the observation that the intermediate intensity states were accessed for significantly shorter average times than the quenched and unquenched states. Note that this figure includes only the results from complexes that during the measurement exhibited emission in the unquenched “band” (above ≈ 48 c/20 ms), i.e., excluding those complexes that were “dim” during the whole period of measurement. The latter property will be considered later.

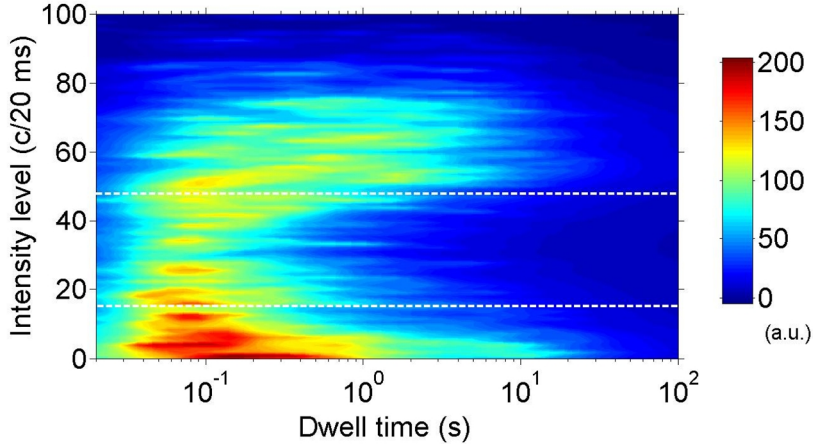


Fig. 1. Fluorescence intensity distribution of individually measured CP29 complexes in the light-harvesting-mimicking environment, excluding the dim fraction. Density map shows the resolved intensity levels and corresponding time of residence for a set of ~ 100 complexes. Dashed lines separate three categories, referred to as bright, dim, and dark states.

Considering from Fig. 1 that the complexes spent most time in either unquenched (bright) or quenched (dark) states, a two-state intensity model provides a reasonable approximation to describe the primary intensity dynamics of these complexes. In addition, if the intensity threshold used to separate the two states, I_{thr} , is chosen well above zero (the background), the influence of shot noise on the intensity distributions is reduced significantly (31, 40, 49). This suggests that for such a two-state model, the accessed intensity levels do not have to be resolved when considering the environmental sensitivity of the intensity distributions.

For each of the three types of minor antennae, the changes on the intensity distributions incurred by the three environmental conditions that were associated with qE in a former study on LHCII trimers (40) were investigated, viz., Zea-containing complexes at pH 5.5 and a considerably lower detergent concentration. Distributions of 20-ms binned fluorescence intensities are shown in Fig. 2, *A-D* for minor antenna complexes that were exposed to different combinations of these qE-associated conditions, and compared to the major antennae in Fig. 2, *E* and *F*. While the distributions of the minor antennae follow mostly similar trends, two distinct differences are evident when compared to LHCII: under “light-harvesting” conditions (Vio, pH 8.0, 0.03% DM), the minor antennae spent on average considerably more time in quenched states than LHCII, and the acidic environment caused a large equilibrium shift into the opposite direction than for LHCII, enhancing the unquenched states considerably. The effect of the other two environmental factors was less dramatic. First, Zea caused a small population shift to the quenched states for LHCII, while for the minor antennae the effect was marginal (this being the reason for investigating its effect in less detail for CP26 and CP24). Second, by reducing the detergent concentration, quenched states were favored for all complexes, though the extent of the equilibrium shift was smaller for CP29 than for the other complexes.

In Fig. 3, *A-C*, the population shifts that resulted from the different environmental changes are quantified by the changes in $\Sigma\tau_Q$, denoting the total time spent in quenched states. The strong divergence in the sensitivity to pH between the minor and major antennae is evident, and also the varying sensitivity of the complexes to detergent concentration. In addition, while LHCII trimers exhibited a small, positive (i.e., decreasing) Zea effect, the minor antennae showed the opposite

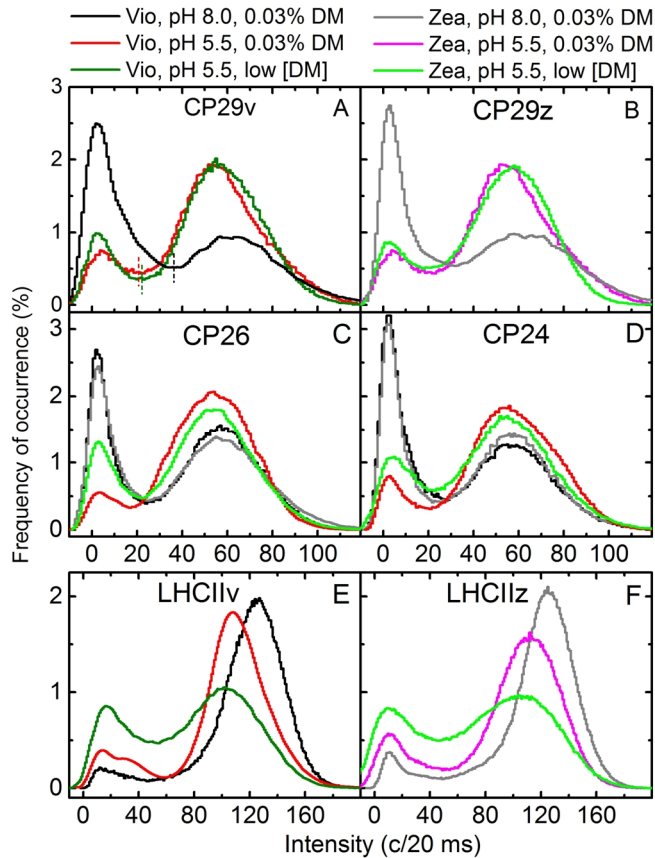


Fig. 2. Environmental effect on fluorescence intensity distributions of individually probed PSII peripheral antennae. Histograms were constructed from consecutive 20-ms bins without resolving the intensity change points and excluding the dim fraction of complexes. Negative intensities stem from shot noise after background subtraction. Quenched and unquenched states are separated by I_{thr} , defined as the local minima between the histogram peaks (vertical lines in Fig. 2A). Zea, z: Zea-enriched; Vio, v: Vio-enriched; Low [DM]: low detergent concentration

indication, i.e., slightly favoring unquenched states for Zea-containing complexes (Fig. 3C). The two-state intensity equilibrium is quantified further by the values of $\Sigma\tau_Q$ displayed in Table S1 in the Supporting Material.

Ensemble intensities

From Fig. 2 it is evident that the environmental changes not only affected the population equilibrium between quenched and unquenched states but also the intrinsic brightness of the complexes, indicated by the peak intensity of the broad histogram that designates the unquenched states. Evidently, both these factors influence \bar{I} , referred to below as the ensemble intensity. Changes in this parameter can be compared directly to the extent of qE, considering that upon the simultaneous excitation of billions of complexes in an in vivo environment, their combined fluorescence signal is averaged into a single intensity level.

In Fig. 3, D-F, the effect of each of the three environmental factors on \bar{I} is quantified. For all three minor antennae, an acidic environment increased the ensemble intensity considerably when neglecting the dim fraction (*open circles*), in stark contrast to LHCII. This indicates that the changes in the intrinsic brightness were not large enough to counterbalance the strong population

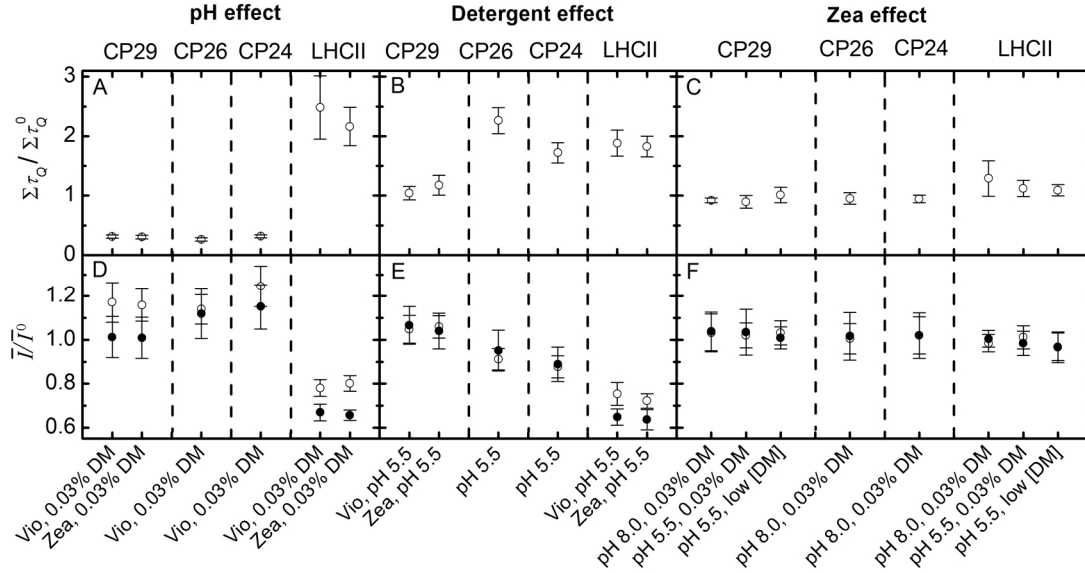


Fig. 3. Effect of the three qE-related factors on the fluorescence intensity dynamics of individually investigated PSII peripheral antennae. (A-C) Fraction of increase in $\Sigma\tau_Q$ induced by the environment, where $\Sigma\tau_Q^0$ and $\Sigma\tau_Q$ denote the total dwell time in quenched states for complexes in the “light-harvesting” and quenching environment, respectively. X-axes denote environmental conditions that remained constant, assuming for the detergent effect of CP26 and CP24 that Zea and Vio exhibited a similar behavior. Calculations are based on data for bright complexes in Table S1. Error bars reflect standard errors. Zea: Zea-enriched; Vio: Vio-enriched. (D-F) Similar to Fig. 3, A-C but for the time- and population-averaged intensities (\bar{I}) when the dim fraction was neglected (*open circles*) and included (*filled circles*). \bar{I}^0 denotes the value of \bar{I} before the quenching environment was introduced. Error bars denote standard deviations, including calibration uncertainties of absolute intensities. See text for details.

shift to unquenched states. However, the intrinsic brightness was such that the lack of detergent had a marginal effect on \bar{I} for CP29 and a positive (i.e. decreasing) effect on \bar{I} for CP26 and CP24, although the extent was significantly smaller than for LHCII. Furthermore, Zea had a minor or negligible effect on all the complexes.

Effect of the dim fraction

The increase in \bar{I} when the pH was lowered does not concur with the findings of previous in vitro bulk studies (43-45, 47). An important property that has thus far been neglected in the current study is the dim fraction of complexes. To include their contribution, the intensity thresholds designating the “bright” population (see Fig. 1, *top line*) were halved. The consequent fluorescence intensity distributions are displayed in Fig. 4 for Zea-containing CP29 and LHCII complexes, the former of which represent the general trend of all the minor antennae.

Strikingly, in the presence of a high pH and 0.03% detergent (i.e., “light-harvesting” conditions), the minor antennae consisted of a very large dim fraction (Fig. 4A), in stark contrast to LHCII (Fig. 4D). Moreover, the dim fraction decreased strongly as more environmental changes were made, while for LHCII it increased considerably. For the minor antennae the ensemble intensity of the dim fraction, \bar{I}_{dim} , was constant within the experimental error margins, and so was the average intensity of the whole ensemble, \bar{I}_{all} , due to the dominating dim fraction. However, for

LHCII, \bar{I}_{dim} displays a more complicated behavior, while \bar{I}_{all} decreased strongly with a changing environment. The latter property was enhanced by the increasing dim fraction, shifting the intensity population more strongly to quenched states (see Fig. 3, *D* and *E*, *filled circles*). In contrast, for the minor antennae the dim fraction reduced the shift in the intensity population (Fig. 3, *D* and *E*, *filled circles*).

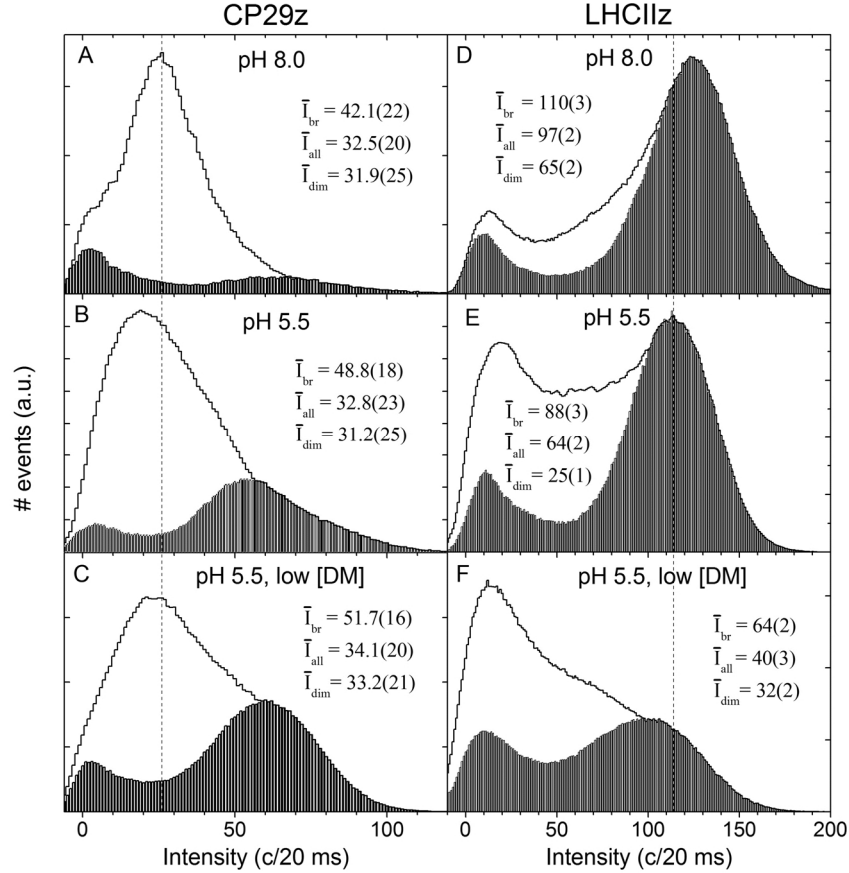


Fig. 4. Effect of the dim fraction on the intensity distributions of PSII peripheral antennae. Histograms of consecutive 20-ms bins of fluorescence counts are shown for Zea-containing CP29 complexes (*A-C*) and Zea-containing LHCII trimers (*D-F*) under three different environmental conditions when the dim fraction was excluded (*bars*) and included (*histogram contours*). Vertical dashed lines serve as guides to monitor peak shifts. Ensemble intensities are displayed for analyses excluding the dim fraction (\bar{I}_{br}), including the dim fraction (\bar{I}_{all}), and for the dim fraction only (\bar{I}_{dim}). Numbers in parentheses denote standard deviations, as applied on the last significant digit(s).

Fig. 2 shows that when neglecting the dim fraction, under “light-harvesting” conditions the minor antennae spent on average considerably more time in quenched states than LHCII (see also Table S1). When the dim fraction is included, this difference increases substantially (see also Fig. 4, *A* and *D*). Also of interest is that the average intensity of LHCII under light-harvesting conditions is about three times that of the minor antennae, in agreement with their relative effective absorption cross-sections.

Evidently, energy states associated with intermediate intensity levels play an important role for the minor antenna complexes. Hence, while the two-state intensity model provides a reasonable approximation for LHCII trimers in a light-harvesting environment, it is not valid for the minor antennae.

DISCUSSION

Sources of quenching

The dynamic fluorescence intensity fluctuations of the PSII peripheral antenna complexes point to an intrinsic capability to dissipate absorbed energy to different extents and also indicates considerable underlying disorder. The differences in the blinking behavior between LHCII trimers and the minor antennae, in particular their differing responses to local environmental changes, strongly indicate different distributions of energy traps. In this section the relationship between the possible sources of quenching and the primary sources associated with qE will be examined by comparing this environmental sensitivity with the results from previous *in vitro* bulk studies, considering that the *in vivo* behavior of the complexes should be reflected to a reasonable extent in a qE-mimicking environment. Moreover, taking into account that all these complexes are structurally and compositionally related, valuable information on some fundamental properties of protein functionality can be obtained.

It should first be noted that triplet states as an explanation for blinking can be excluded, based on their much shorter lifetimes with respect to the experimental time resolution (39). However, accessing a dark state, such as a radical cation, via the triplet state could play a role, as demonstrated for the molecular dye R6G (50). It is unlikely that this is a dominant mechanism for the complexes investigated in this study, as this would require excitation of a Car triplet state, which has a negligible probability under the utilized conditions.

Radical-state formation in photosynthetic complexes is more frequently associated with electron transfer. This photochemical pathway was assumed to explain fluorescence blinking of allophycocyanin complexes (51, 52). Moreover, each of CP24, CP26 and CP29 was observed to exhibit the formation of Car radicals after exciting their Chl *b*'s with a high excitation repetition rate (29). It was claimed that these radicals were formed by electron transfer to nearby Chls (28), after rapid relaxation of a charge-transfer (CT) state that was delocalized over the Car and Chls (29, 30). The cations were attributed primarily to Zea (29) but also to Lut (53). A couple of findings challenge the notion to relate such radical formation to the primary source of fluorescence blinking: (i) Only a very small fraction (<0.5 %) of the complexes showed evidence for Car radicals. Their probability should increase by at least two orders of magnitude under the utilized single-molecule conditions if they were to be associated with the primary source of blinking. (ii) The short lifetimes (~150 ps) of these radical cations (30) make them unfit to account for the observed long dwell times in quenched states. Although the preceding CT states relax even faster, evidence for long-lived CT states was found for LHCII trimers under similar single-molecule conditions (32). However, these states were mostly unquenched and attributed to only ~5% of the complexes. (iii) The negligible or negative Zea effect in the present study opposes its association with quenching. In addition, due to the relatively strong coordination of Zea and Vio inside the minor antenna complexes (54, 55), their detachment from the complexes is deemed an unlikely explanation for this lack of Zea dependence.

A more likely way in which the xanthophylls in these complexes are involved in quenching is via direct energy transfer from the Q_y energy levels of nearby Chl *a*'s. It is known that the S_1 energies of most of these xanthophylls are lower than the Chl *a* Q_y energies in these protein environments (56, 57). As a consequence, close proximity and proper orientation between some Chl *a*'s and xanthophylls will lead to fast energy transfer from the Chl to the Car and a

subsequent rapid relaxation of the S_1 states, thereby dissipating the energy as heat. Such a situation is likely to be achieved by the abundant structural disorder of the local protein microenvironments. Indeed, the quantitative description of LHCII's fluorescence blinking by a model that is based on such a mechanism (42) supports the notion that this is an important source of quenching in LHCII trimers and likely the primary mechanism that underlies fluorescence blinking of all PSII peripheral antenna complexes. This mechanism would be highly sensitive to the specific protein microenvironment, type of xanthophyll, and the positions and relative orientations of the xanthophyll and neighboring Chls. The minor differences between the structurally homologous Lhcb complexes are expected to be sufficient to explain the differences in their blinking behaviors and hence their quenching capabilities.

Comparison with previous in vitro bulk studies

It should first be noted that the major and minor antenna complexes are structurally and compositionally very similar but showed a very different environmental sensitivity of their blinking dynamics, in particular with regards to the pH and the dim fraction. This signifies that the population equilibrium shifts are unlikely to be coincidental, artifactual effects caused by the single-molecule environment.

At first sight our results on the lack of sensitivity of the emission from single minor antenna complexes to a low detergent concentration, low pH, and Zea would seem to contradict previous ensemble studies (43, 44). However these studies all examined the effect of Zea and/or low pH in driving acceleration of quenching against a background of low-detergent induced aggregation of antenna complexes in solution. The conditions used in our study are quite distinct in that they evaluate the effect of each environmental condition in the absence of aggregation of the antenna complexes. One explanation is that the very large population of (permanently) dim complexes efficiently quenches the smaller population of bright complexes upon aggregation of the sample in the ensemble studies and that Zea and low pH may simply facilitate this effect by promoting aggregation. Indeed, the small effect of Zea on the population distribution of PSII antenna complexes between quenched and unquenched states in single-molecule studies supports the view that these molecules affect qE in vivo by controlling protein-protein interactions (58, 59). In those ensemble studies that have examined the effect of Zea and low pH in the absence of protein aggregation it is noteworthy that these conditions had only very minor quenching effects (K_D of <0.5) (29, 45-47). The large dim fractions in the minor antenna samples also provide an explanation for the considerable yield of short fluorescence lifetime components compared to LHCII trimers in detergent solution (47, 60).

qE based on specifically controlled disorder

Proteins are inherently dynamic and sample a vast ensemble of conformations, a concept well illustrated by the protein free-energy landscape (61), which consists of different conformational substates in dynamic equilibrium. Proteins respond to environmental changes by redistributing the relative populations of these preexisting substates, a model commonly known as "conformational selection" (41). An elegant example that this is also a fundamental property of photosynthetic light-harvesting proteins is our finding that isolated Lhcb complexes, which normally emit at around 680 nm, were found to occasionally fluoresce in the region around 700–

730 nm, typical for Lhca complexes, while the latter complexes performed the exact opposite behavior (62).

The conformational selection model implies that all physiological states of the PSII peripheral antenna complexes can be accessed under any environmental conditions. Only the equilibrium between these states, and thus the access probability, may differ from the *in vivo* situation. This context strongly supports the proposition that qE is based on some environmental control of the protein's structural disorder (40) and suggests that the observed intensity dynamics should at least partially account for the primary mechanism underlying qE.

Inclusion of the dim fraction of LHCII trimers resulted in a stronger correlation between quenching and qE-related conditions than observed before (40), supporting the notion that qE and fluorescence blinking share the same primary mechanism(s) for this complex. The lack of such a correlation for the minor antennae indicates that these complexes do not utilize their intrinsic capability of quenching in the same way as LHCII trimers. In fact, these complexes appear remarkably insensitive to the conditions we have used to simulate qE. We therefore conclude that LHCII is more sensitive to environmental changes than the minor antennae and that the structural disorder underlying blinking in all these complexes is controlled in a specific way for LHCII to exhibit a biologically relevant functionality related to qE. Of course, *in vivo*, when all the antenna complexes are organized in the grana membrane associated in the PSII supercomplexes, it is likely that the intrinsic dynamicity is constrained (22). Indeed, the structural changes that have been observed upon qE formation (21) suggest that these changes are necessary to "release" the LHCII trimers and shift the equilibrium towards increased quenching. CP29 and CP26 are tightly bound to the supercomplex and therefore probably do not have such freedom, further constraining their dynamic range. In this case, any qE-related quenching probably occurs by a different mechanism, in agreement with previous *in vitro* bulk studies (63-65).

CONCLUSION

It was demonstrated that the minor antenna complexes of plant PSII exhibit a distinct fluorescence blinking behavior and dim fraction with respect to the structurally and compositionally related LHCII trimeric complexes. Hence, the observed environmental responses of fluorescence blinking cannot be attributed to a general property of photosynthetic light-harvesting complexes. The same conditions that, on average, induced a strong quenching of single LHCII complexes had no or only a small quenching effect on the minor antenna complexes when the dim fraction was taken into account. This indicates that the minor antennae possess an intrinsically lower degree of dynamicity than LHCII trimers and that additional environmental conditions must change to appropriately shift their population equilibrium to quenched states. In addition, the results strongly support that for LHCII trimers the principal molecular mechanism underlying qE and fluorescence blinking is shared. Considering in addition that only subtle conformational changes are possible in these dense complexes, these findings strongly indicate that the environmentally controlled disorder underlying qE is a sensitive, finely tuned and highly specific mechanism. Hence, the findings provide insights on some fundamental properties of protein dynamics in relation to protein multifunctionality.

We have also challenged the involvement of radical cations as a primary source of fluorescence blinking in these complexes, and related the phenomenon of blinking instead to excitonic

interactions between Chls and Cars that are controlled by their disordered protein microenvironments.

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Table S1 Fluorescence intermittency properties of PSII antenna complexes in different environments, neglecting and including the dim fraction.

Complex	Environment	Bright		Bright + Dim
		$\Sigma\tau_Q$ (%)	\bar{I}_{br} (c/20 ms)	\bar{I}_{all} (c/20 ms)
CP29	Vio, pH 8.0, 0.03% DM	52.8(15)	40.8(27)	31.3(18)
	Zea, pH 8.0, 0.03% DM	48.6(16)	42.1(22)	32.5(20)
	Vio, pH 5.5, 0.03% DM	16.5(13)	47.8(20)	31.7(23)
	Zea, pH 5.5, 0.03% DM	14.8(13)	48.8(18)	32.8(23)
	Vio, pH 5.5, low [DM]	17.2(12)	50.1(22)	33.8(12)
	Zea, pH 5.5, low [DM]	17.4(19)	51.7(16)	34.1(12)
CP26	Vio, pH 8.0, 0.03% DM	38.8(28)	41.4(17)	30.9(18)
	Zea, pH 8.0, 0.03% DM	37.0(26)	41.6(23)	31.4(28)
	Vio, pH 5.5, 0.03% DM	10.3(7)	47.2(20)	34.6(29)
	Zea, pH 5.5, low [DM]	23.3(16)	43.0(14)	32.9(17)
CP24	Vio, pH 8.0, 0.03% DM	40.8(18)	39.8(26)	30.4(20)
	Zea, pH 8.0, 0.03% DM	38.5(20)	40.6(21)	31.0(24)
	Zea, pH 5.5, 0.03% DM	12.9(8)	49.6(18)	35.0(21)
	Zea, pH 5.5, low [DM]	22.2(17)	43.5(19)	31.1(20)
LHCII	Vio, pH 8.0, 0.03% DM	6.2(15)	111.9(32)	96.4(33)
	Zea, pH 8.0, 0.03% DM	8.0(10)	110.2(30)	96.8(15)
	Vio, pH 5.5, 0.03% DM	15.4(14)	87.3(34)	64.5(29)
	Zea, pH 5.5, 0.03% DM	17.3(14)	88.3(30)	63.5(21)
	Vio, pH 5.5, low [DM]	29.0(21)	65.8(37)	41.8(14)
	Zea, pH 5.5, low [DM]	31.6(15)	63.7(20)	40.4(26)

Time- and population-averaged intensity (\bar{I}), and total dwell time in quenched (dark) states ($\Sigma\tau_Q$), excluding the dim fraction (Bright) and including this fraction (Bright + Dim). Numbers in parenthesis denote standard deviations, as applied on the last significant digit(s). See main text for details.