Photosystem I light-harvesting complex Lhca4 adopts multiple conformations: Red forms and excited-state quenching are mutually exclusive

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ABSTRACT

In this work we have investigated the origin of the multi-exponential fluorescence decay and of the short excited-state lifetime of Lhca4. Lhca4 is the antenna complex of Photosystem I which accommodates the red-most chlorophyll forms and it has been proposed that these chlorophylls can play a role in fluorescence quenching. Here we have compared the fluorescence decay of Lhca4 with that of several Lhca4 mutants that are affected in their red form content. The results show that neither the multi-exponentiality of the decay nor the fluorescence quenching is due to the red forms. The data indicate that Lhca4 exists in multiple conformations. The presence of the red forms, which are very sensitive to changes in the environment, allows to spectrally resolve the different conformations: a "blue" conformation with a short lifetime and a "red" one with a long lifetime. This finding strongly supports the idea that the members of the Lhc family are able to adopt different conformations associated with their light-harvesting and photoprotective roles. The ratio between the conformations is modified by the substitution of lutein by violaxanthin. Finally, it is demonstrated that the red forms cannot be present in the quenched conformation.

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1. Introduction

Photosystems I (PSI) and II (PSII) are multi-protein complexes located in the thylakoid membranes of higher plants and algae. Their role is to harvest sunlight energy and to transform it into chemical energy which is used for the reduction of CO_2 into carbohydrates. Both Photosystems are composed of a core complex and an outer antenna system. The core complex contains the reaction centre with the primary donors P700 in PSI and P680 in PSII, all the electron transport cofactors and Chls a and β-carotene molecules. The outer antenna is composed of pigment–protein complexes which harvest light-energy and transfer the resulting excited-state energy to the reaction centre where it is used for charge separation. It has been shown that in high-light conditions, when the harvested energy exceeds the needs of the chloroplast, the outer antenna complexes are involved in energy dissipation processes, which protect plants from photodamage [1,2]. These complexes thus have the dual role of harvesting light in low-light conditions and of dissipating excess absorbed energy in high-light. All antenna complexes of PSI and PSII are members of the Lhc (light-harvesting complex) multigenic family [3]. They show high structural homology [4,5] and coordinate Chls a, Chls b and various carotenoid molecules [6]. Despite the similarities in structure and composition, their spectroscopic properties differ substantially, with all antenna complexes of PSI (Lhca) showing red-shifted fluorescence as compared to those of PSII (Lhcb). The difference in wavelength can be as big as 55 nm, with Lhcbs emitting at 680 nm and Lhca4, the most red-shifted Lhca complex, at 735 nm at 77 K [7]. The presence of Chls absorbing and emitting at low-energy is typical for PSI complexes [8–10]. In PSI–LHCI supercomplexes a large part of the excitation energy arrives at these Chls (called red forms), which are at lower energy than the primary electron donor. To be used for charge separation the excitation energy is transferred from the red forms to the primary donor in a thermally activated process [11]. Considering that the low-energy Chls represent an energy sink, it was proposed that they might be active in photoprotection via energy dissipation thus diverting the excess excitation energy away from the RC [9,12,13].

The red forms of the Lhca complexes originate from the mixing of the lowest excitonic state of two interacting Chls, 603 and 609 (Fig. 1) (nomenclature of LHCII from [4]) [14–17], with a charge transfer state (CT) [18,19]. However, the emission maximum of these forms is different in the individual Lhca's (690 nm for Lhca1, 701 for Lhca2, 726 for Lhca3 and 732 for Lhca4 at 77 K[20,21]), and depends primarily on the nature of the ligand of Chl 603: when the ligand is an Asn, as in
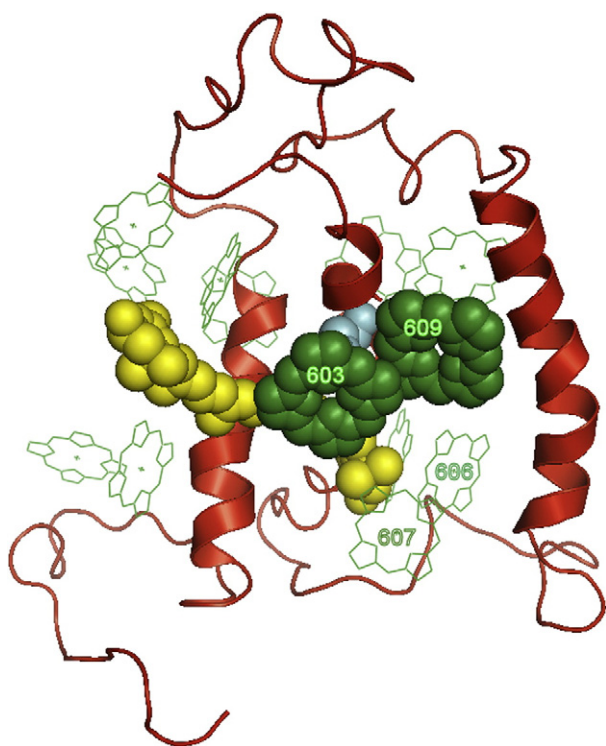
Fig. 1. A molecular model of Lhca4 from the PSI–LHCI structure [5]. The low-energy Chls, Chl603 and Chl609 are in space-filling style. The position of the carotenoid in the L2 site (yellow, space filling), which is not resolved in the structure of Lhca4, is taken from the structure of LHCII [4]. The ligand of Chl 603 (Asn) is in cyan.

the case of Lhca3 and Lhca4, the observed red shift is larger than when the ligand is a His, as is the case for Lhca1 and Lhca2 [22].

It was shown that the excited-state lifetime of Lhca complexes is shorter than that of Lhcbs and that the fluorescence decay of Lhca is multiphasic [23–27], which is puzzling because for an equilibrated system only one decay lifetime is expected. A strong correlation was observed between the excited-state lifetime of the complexes and the presence of the red emission forms, e.g. the complexes with the red-most shifted emission showed the shortest lifetime [26]. The CT state might in principle be responsible for the lower fluorescence yield of the Lhca complexes as compared to Lhcb. Recently, it was also suggested that a CT state is involved in the quenching of Lhcb complexes [28] and several experiments show an enhancement of the red absorption of LHCII in its quenching state [29,30] as compared to its light-harvesting state.

In this work we have investigated the origin of the fast fluorescence decay of Lhca4 and of its multi-exponential character with particular attention for the role of the red forms. To this aim we have compared Lhca4-WT with mutants affected in the low-energy forms [16]. This analysis leads to the conclusion that neither the multi-exponential decay nor the short excited-state lifetime of Lhca4 is due to the presence of the red forms. The complex appears to adopt different conformations which are associated with different degrees of quenching.

2. Material and methods

2.1. Mutagenesis and reconstitution of recombinant Lhca4 complexes

A modified pET-28a (+) vector containing the sequence coding for the mature protein of *Arabidopsis* Lhca4 has been mutated by site-directed mutagenesis as follows: in Lhca4-N47L, Chl603-binding residue (N47) has been substituted by a L, which is a non-binding

residue with similar steric hindrance, whereas in Lhca4-N47H, the same residue has been changed to a H (as in [22]), which is a Chl-binding residue. WT and mutant apoproteins were overexpressed in Rosetta2(DH3) strain of *E. coli* and purified as inclusion bodies. Reconstitution and purification of pigment–protein complexes were performed as described in [31] using a mix of purified pigments with Chl a/b ratio of 2.9 and Chl/Car ratio of 2.7; in the case of the WT and Chl-binding mutants a total carotenoid extract from spinach thylakoids was used, only lutein was used in the case of Lhca4-Lut. The purification of the refolded complex from the unfolded protein and the excess of pigments was performed in three steps: (1) sucrose gradient ultracentrifugation, which also allowed to check the monomeric state of the complex; (2) anionic-exchange chromatography, and (3) a second sucrose gradient. In the case of the Chl-binding mutants a faster procedure consisting of His-tag Nickel affinity chromatography followed by a sucrose gradient has been applied.

2.2. Absorption and steady state fluorescence

77 K absorption spectra were recorded using a Cary4000 (Varian Inc.) spectrophotometer at a Chl concentration of about 6 μg/ml in 10 mM HEPES pH 7.6, 0.5 M sucrose, 0.03–0.06% β-DDM and 70% v/v glycerol. Fluorescence emission spectra were measured at 283K and 77 K using a Fluorolog (Jobin Yvon) spectrofluorimeter and corrected for the instrument response. Samples were diluted to a Chl concentration of 0.3 μg/ml in 10 mM HEPES pH 7.6, 0.02% β-DM (70% v/v glycerol at 77 K). Bandwidths were 5 nm (10 nm at 77 K) in excitation and 2.5 nm in emission.

2.3. Pigment analysis

The pigment complement of the complexes was analysed by fitting the acetone extract spectrum with the spectra of the individual pigments [32] and by HPLC analysis [33]. The Chl a/b ratio of Lhca4-WT is 1.88 and the Chl/Car ratio is 4.8. Normalization of the pigment content to 10 Chls [22] indicates that it binds 1.6 molecules of lutein and 0.4 of violaxanthin. Lhca4-Lut shows a Chl a/b ratio of 2.46 and a Chl/Car ratio of 4.6, thus binding 2.1 molecules of lutein per 10 Chls. Lhca4-N47H and Lhca4-N47L have respectively a Chl a/b ratio of 1.82 and 2.56 and a Chl/Car ratio of 4.7 and 4.9. The former binds on average 1.6 molecules of lutein and 0.5 of violaxanthin per 10 Chls, whereas the latter binds 1.2 molecules of lutein and 0.2 of violaxanthin per 7 Chls (to be considered as the maximum number of Chls, see [16]). Data are expressed as average of at least 3 repetitions, maximum standard deviation is 0.1.

2.4. Time-resolved fluorescence

Time correlated single photon counting (TCSPC) was performed with a home-built setup, as described previously [34]. The samples were diluted in 10 mM Hepes pH 7.6, 0.03% β-DM to an OD of 0.1 cm^{-1} at 475 nm, stirred in a 3.5 ml cuvette with a path length of 1 cm, and kept at 283 K. The excitation was at 475 nm with a repetition rate of 3.8 MHz. Pulse energies of sub-pJ were used with pulse duration of 0.2 ps and a spot diameter of ~1 mm. The instrument response function (~60 ps FWHM) was obtained with pinacyanol iodide in methanol, with 6 ps fluorescence lifetime [35]. Fluorescence was collected at 90° through a long-pass (530 nm) and interference filters with FWHM of 10–15 nm and transmission maxima as indicated in the decay-associated spectra (DAS) (Schott, Mainz, Germany or Balzer B-40, Rolyn Optics, Covina, CA, USA). Individual photons were detected at magic angle polarisation by a microchannel plate photomultiplier, and arrival times were stored in 4096 channels of a multichannel analyzer, the channel spacing was 5 ps, resulting in a 20 ns time-
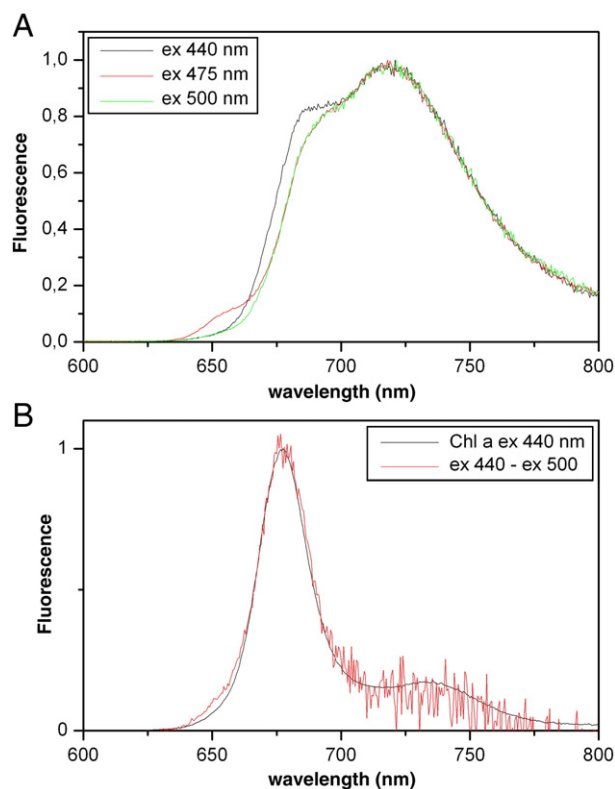
window. Excitation intensity was reduced to obtain count-rates of 30 000 s^{-1}, and care was taken to minimize data distortion [36].

The data was globally analysed using home-built software [37]. The fit quality was evaluated from the χ^2, and from plots of the weighted residuals and the autocorrelation thereof. The steady state fluorescence emission spectra were used to calculate the DAS.

3. Results

Fluorescence emission spectra of Lhca4-WT were measured at 283K upon excitation at 440 nm, 475 nm and 500 nm (Fig. 2A), which leads to increased excitation of Chls *a*, Chls *b* and carotenoids, respectively [38]. The difference between the spectra upon excitation at 440 nm and 500 nm is identical to the spectrum of "free" Chl *a* measured in the same solution (Fig. 2B), thus indicating the presence of a small amount of unconnected Chl *a* in the preparation. On the other hand, the difference between the spectra upon excitation at 475 nm and 500 nm is identical to the spectrum of "free" Chl *b* (data not shown). The areas of the "free" Chl *a* and Chl *b* correspond respectively to 4% and 1.5% of the total emission. Considering that the average lifetime in solution is 6 ns for Chl *a* and 3.6 ns for Chl *b*, while that of Lhca4 is 2 ns (see below), the presence of "free" Chls is estimated to be around 2%. The fact that the emission difference spectra appear to be entirely due to "free" Chls, also demonstrates that the shape of the emission spectrum of intact Lhca4 is independent of excitation wavelength.

Time-resolved fluorescence of Lhca4-WT was measured at room temperature (RT) with a TCSPC setup. The sample was excited at 475 nm, to minimize the effect of the disconnected Chls, and the fluorescence was detected in the 670–770 nm range. The fluorescence decay curves were globally fitted with four exponential components with lifetimes of 0.33 ns, 1.4 ns, 2.6 ns and 4.5 ns (Fig. 3). No
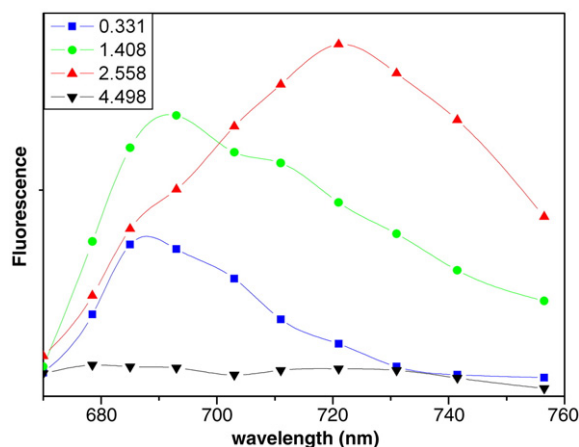
Fig. 3. Decay associated spectra of Lhca4-WT. The measurements were performed at 283K and the sample excited at 475 nm. The spectra were obtained by global analysis of the kinetic traces (lifetimes are in ns).

significant improvement of the fit quality was achieved by including an additional lifetime component, but fitting with 3 components led to a fit quality that was clearly less good. All components show positive amplitudes in the whole spectral region, indicating that they are associated with pure decay processes. The excitation energy transfer processes occur in less than 5 ps in Lhca4 [39] and are not resolved in our measurements. The four decay-associated spectra (DAS) differ: the 0.33 ns component peaks at 685 nm and shows little contribution above 700 nm (it is called "blue" component in the following), while the 2.6 ns component has a maximum at 720 nm and little amplitude below 700 nm ("red" component) (Fig. 3). The 1.4 ns and the 4.5 ns components show both "blue" and "red" contributions. Comparison of the spectra suggests that "free" Chl a contributes for 8% to the 4.5 ns spectrum. These results confirm the multi-exponential character of the decay of Lhca4 [26]. The data are also presented as time-resolved emission spectra (TRES), which show that the fluorescence of Lhca4-WT evolves in time toward the red (Fig. 4A), in agreement with previous results. This was attributed to a slow equilibration between the bulk Chls and the red forms [40] (but see below). However, the data also clearly show (Fig. 4B) that a strong reduction of the fluorescence intensity occurs in the first hundreds ps, thus indicating the presence of at least one fast decay channel.

Previous analyses of Lhca complexes showed multi-exponential decay curves and it has been suggested that the multi-exponentiality is due to variation in the pigment composition and the red-pigment content of the complexes [26]. It was also proposed that interactions between red forms and carotenoids may play a primary role in shortening the fluorescence lifetimes of Lhca complexes as compared to those of Lhcbs [26].

To test these hypotheses a mutant of Lhca4, Lhca4-N47H, which does not contain the red-most form, while maintaining the same pigment composition as the WT [22], was analysed. In this mutant the natural ligand of Chl 603 (Fig. 1), an Asn, was mutated into a His. The absorption and fluorescence spectra at 77 K of the mutant are presented in Fig. 5 where they are compared with those of the WT. The Lhca4-N47H mutant loses the lowest energy absorption form at 705 nm and its emission is shifted from 735 nm in the WT to 700 nm (Fig. 5B), due to a decrease in excitonic interaction between Chls 603 and 609 [22]. If the short lifetime of Lhca4-WT is related to the presence of the low-energy Chls, the lifetime of the mutant should be longer. Moreover, if the multi-exponentiality of the decay is mainly reflecting the slow equilibration between red forms and bulk, it should not be present in the mutant. The fluorescence decay of the mutant was measured in the same conditions as the WT. As for the WT, four components were needed for the best fitting of the decay of Lhca4-N47H (Fig. 6A). The lifetimes of the components (Table 1) are

Fig. 2. Fluorescence emission spectra of Lhca4-WT at 283K. (A) Emission spectra upon excitation at 440 nm (black), 475 nm (red) and 500 nm (green). (B) Difference emission spectra, ex 440-ex 500 nm (red), and emission spectra of free Chls in the same buffer (black). The spectra are normalized to the maximum.
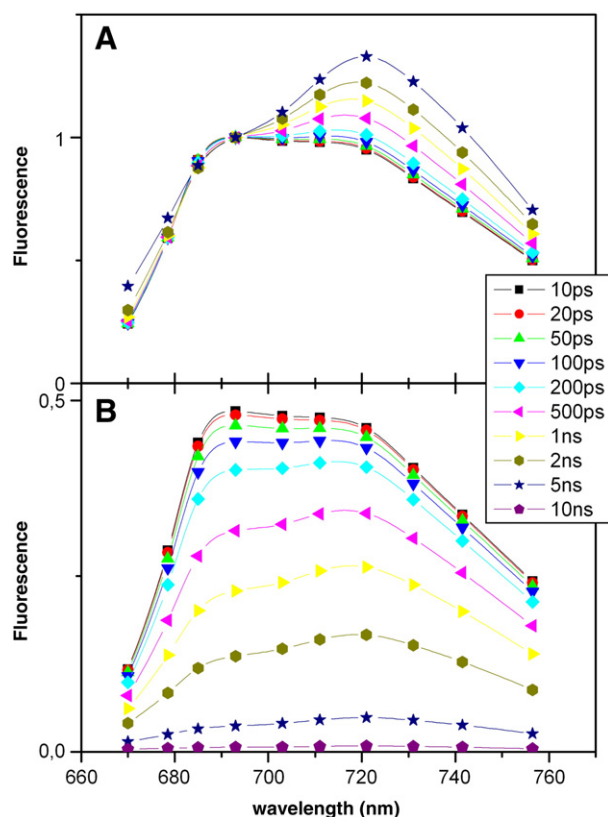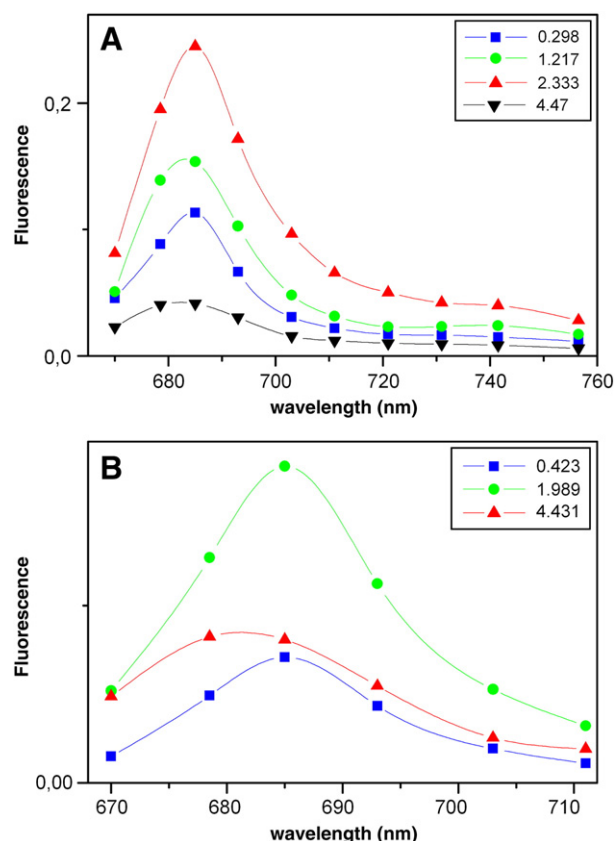
Fig. 4. Time-resolved emission spectra (TRES) of Lhca4-WT. (A) TRES spectra at different times, normalized at 690 nm. (B) The same spectra as in A but not normalized.

Fig. 6. Decay associated spectra of Lhca4-N47H (A) and Lhca4-N47L (B). The measurements were performed at 283K and the samples excited at 475 nm. The spectra were obtained by global analysis of the kinetic traces (lifetimes are in ns).

slightly shorter (10%) as compared to those of Lhca4-WT, but their relative amplitudes are conserved. Due to the absence of the red-most form all components have similar spectra. The average lifetime of the mutant is 1.84 ns vs. 1.96 ns of the WT. The data clearly show that neither the multi-exponentiality of the decay nor the short average lifetime of Lhca4 changes in the absence of the red-most form, indicating that the latter does not play a role in these processes. However, the fluorescence properties of Lhca4-N47H are very similar

to those of Lhca2 (fluorescence max at 700 nm at 77 K). It was shown that the low-energy emission of Lhca2 originates from the same two Chls that in Lhca4 are responsible for the 735 nm emission and probably also involve a CT state [15]. Moreover, it was shown that Lhca2 is quenched as compared to LHCII [26]. Therefore, the possibility exists that in Lhca4-N47H a CT state is still present and it can act as a quencher.
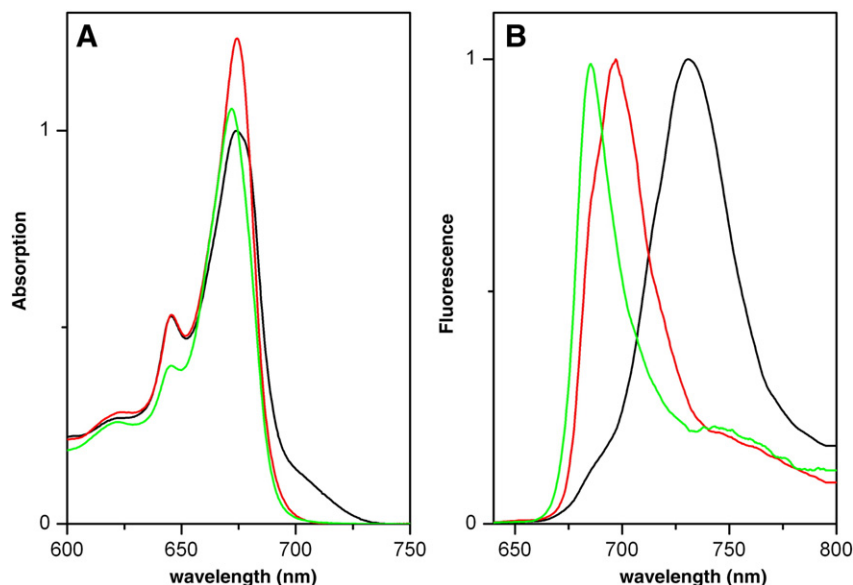
Fig. 5. Absorption (A) and fluorescence emission (B) spectra at 77 K upon excitation at 500 nm of Lhca4-WT (black/solid), Lhca4-N47H (red/dash) and Lhca4-N47L (green/dot). The absorption spectra are normalized to the Chls, and the fluorescence spectra to the maximum.

Table 1

Sample	Chl a/b	τ_{av}[a] (ns)	τ_1(ns)/A1 (%)	τ_2/A2	τ_3/A3	τ_4/A4
Lhca4-WT	1.88 ± 0.03	1.96	0.331/14.8	1.408/34.2	2.558/44.4	4.498/6.6
Lhca4-N47H	1.82 ± 0.02	1.83	0.298/18.6	1.217/27.2	2.333/45.6	4.47/8.6
Lhca4-N47L	2.56 ± 0.01	2.37	0.423/19.8	1.989/52.0	–	4.431/28.0
Lhca4-Lut	2.46 ± 0.01	1.41	0.322/29.9	1.294/43.3	2.6/24.6	4.896/2.3

[a] The average lifetime τ_{av} is calculated according to $\Sigma A_n \tau_n$ where A is the relative area of the DAS and τ is the lifetime.

To investigate this possibility, a mutant lacking Chl 603 [16] was measured. In this complex the natural ligand for Chl 603 has been substituted with a Leu, a residue that cannot coordinate Chls and as a result the mutant (Lhca4-N47L) is lacking Chl 603 and shows a fluorescence maximum at 690 nm (Fig. 4). The fluorescence decay of this mutant can be satisfactorily described with three components, leading to an average lifetime of 2.37 ns (Fig. 6B). A sub-nanosecond decay component is still present in this mutant and it has similar amplitude as for the WT. The data thus clearly indicate that Chl 603 is not at the origin of the multiphasic decay and that it is not "the" quenching site in Lhca4, although the increase of lifetime suggests that it can be "a" quenching site.

It has been proposed that the lower fluorescence yield observed for Chls bound to proteins as compared to Chl in solution is due to Chl/carotenoid interactions [41,42] and it was also recently proposed that these interactions lie at the basis of the process of non-photochemical quenching which protects plants from high-light damage, via fast dissipation of excess energy [30,43].
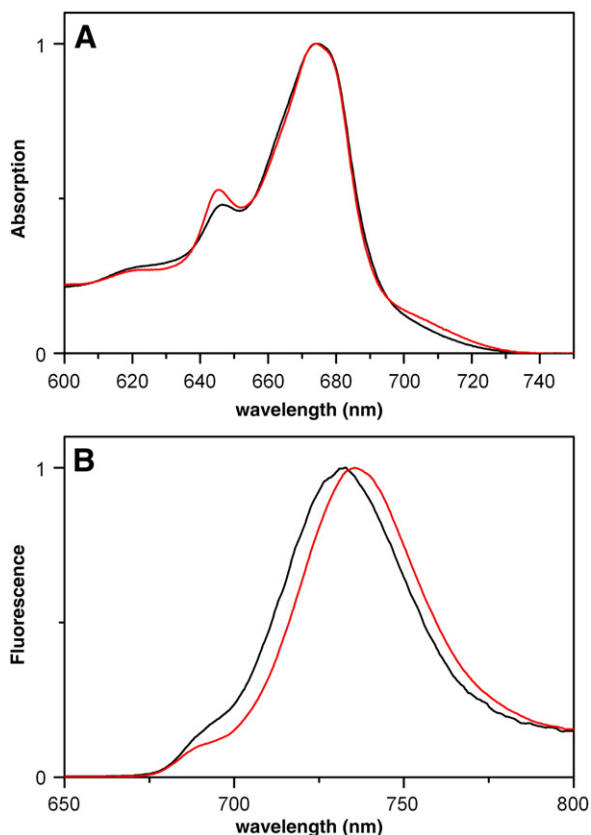

Fig. 7. Absorption (A) and fluorescence emission (B) spectra at 77 K upon excitation at 500 nm of Lhca4-Lut (black/solid) and Lhca4-WT (red/dash). The spectra are normalized to the maxima.
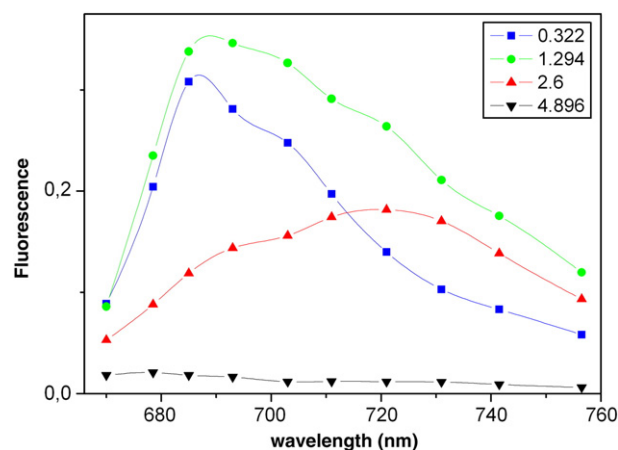

Fig. 8. Decay associated spectra of Lhca4-Lut. The measurements were performed at 283K and the sample excited at 475 nm. The spectra were obtained by global analysis of the kinetic traces (lifetimes are in ns).

In Lhca4-WT site L2 is occupied by lutein or violaxanthin [16,44]. The mixed occupancy of site L2 can thus contribute to the multi-exponentiality of the decay of Lhca4, with violaxanthin and lutein interacting differently with the neighbouring Chls thus leading to different degrees of quenching as recently observed for CP24 [31]. To test this possibility, a complex reconstituted in the absence of violaxanthin and thus containing only lutein, (Lhca4-Lut) was analysed. The Chl/car ratio is identical to that of the WT indicating that lutein has fully substituted violaxanthin in this complex. Lhca4-Lut shows two peculiar features: its Chl a/b ratio is higher (Table 1) than that of the WT and the absorption above 700 nm is lower (Fig. 7A). The difference absorption spectrum WT-Lut shows two positive contributions in the Q_y region at 707 and 644 nm, which represent respectively the absorption of the red forms and that of Chl (s) b affected by the presence of lutein/absence of violaxanthin. The fluorescence emission at 77 K (Fig. 7B) has a maximum at 732 nm vs. 735 nm of Lhca4-WT, also indicating a change in the red forms.

The fluorescence decay of Lhca4-Lut is best described as a sum of four decay components (Fig. 8), the lifetimes being very similar to those of the WT, indicating that the multi-exponential fluorescence decay of the complex is not due to heterogeneity in the carotenoid binding. However, the amplitude of the fast-blue component has increased for the mutant as compared to the WT, leading to a shortening of the average lifetime from 1.96 ns to 1.4 ns.

4. Discussion

4.1. Multi-exponential decay

All fluorescence decay measurements on Lhca complexes show multi-exponential behaviour [23,25,26,45–47], which is puzzling because the excitation energy transfer between the pigments in Lhca4 occurs very rapidly, in less than 5 ps [39] and in an equilibrated system only one decay component is expected. For different systems it has been proposed that the multi-exponentiality is due to protein relaxation and the red shift of the emission spectra in time was used as an argument to support this hypothesis [48]. A red shift of the emission in time is also observed for Lhca4, but it is not anymore present in the mutants lacking the red forms, although their decay is still multi-exponential with very similar lifetimes. This indicates that protein relaxation is not responsible for the observed multi-exponential decay of Lhca4.

The presence of a multi-exponential decay for LHCI preparations has been attributed to heterogeneity of the sample (for preparations containing several different Lhca complexes—[25,46]), to the presence of aggregation [23] or to the presence of complexes having different

pigment compositions or having different conformations [26]. Moreover, it was expected that this phenomenon was coupled to differences in pigment–pigment interactions, and that the red forms play a primary role [26].

In the present study we have analysed the product of a single Lhca gene, Lhca4, in its monomeric state, in the total absence of aggregates. Still, four lifetimes are necessary to describe the fluorescence decay. Moreover, it is found that even in mutants lacking the red forms the decay remains multi-exponential. These data suggest that the different components are associated to pigment–protein complexes having different pigment compositions or being present in different conformations, as was previously proposed for Lhcbs [49]. The peculiarity of Lhca4-WT is that the different lifetime components also correspond to different spectra, thus indicating that the complexes differ in their red form content. One of the possible explanations is that site 609 coordinates both Chl a and Chl b, while site 603 only coordinates Chl a as has been proposed previously [16]. The presence of Chl a in the 609 site would lead to large excitonic splitting between the absorption bands of the 603–609 dimer, accounting for the 720 nm emission and the slow decay. The occupancy of the 603 site by a Chl b would instead lead to a smaller apparent excitonic splitting, whereas the chemical difference between Chl a and Chl b might lead to an increased CT character, with the CT state acting as a quencher, thus explaining the short lifetime of the "blue" conformation. However, this would also imply a difference in the Chl a/b ratio for the two populations and thus selective excitation in the Chl a or Chl b bands would produce different emission spectra. This is not the case, as the emission spectra are independent of excitation wavelength, apart from some contribution from free Chls (Fig. 2). It should be concluded that the difference in red forms in the individual decay components is not due to a different occupancy of site 609 by Chl a or Chl b.

The data also indicate that the presence of a multi-exponential decay is not due to variation in the carotenoid occupancy, because the same decay components are observed both in the presence of lutein and violaxanthin and in the presence of only lutein. In the complex containing only lutein the quenched component has higher amplitude, indicating that the carotenoid identity can influence the relative occurrence of different conformations.

It can thus be concluded that the multi-exponentiality of the fluorescence decay does not depend on heterogeneity in the pigment binding of the complexes. Apparently, the pigment–protein complex can exist in different conformations, in which the relative organization of the pigments can differ. In addition, the protein environment controls the interaction strength within the excitonic dimer responsible for the red forms, thus associating a "blue" or a "red" spectrum to the different conformations. The "red forms" can thus be seen as a very sensitive tool to detect conformation changes. Indeed, the mixing of the lowest state of the Chl–Chl dimer with a CT state makes the red forms energetically very flexible, and small changes in orientation and distance of the interacting Chls, would have a strong effect on the spectra.

We have to underline that the multi-exponential decay is a typical property of Lhca complexes *per se*, which is not related to the reconstitution procedure: also the dimers purified from plants exhibit a similar behaviour although in the native complexes the spectrum is dominated by the long decay (Wientjes, in preparation). This difference might be due to the higher structural flexibility of monomers in comparison to dimers. This is supported by the fact that the unfolding temperature of monomeric Lhca complexes is 12 °C below that of dimers [44]. A similar effect can also be observed for monomeric and trimeric LHCII, with the monomers showing a larger contribution of a 200 ps decay component [50].

Differences in the emission spectrum of Lhca4-WT were previously reported upon non-selective (400–690 nm) and selective excitation of the red forms (>700 nm), with the latter spectrum showing enhanced red emission [40]. This difference was ascribed to slow-energy transfer from the red to the bulk Chls. However, this interpretation is based on the assumption that the sample is homogeneous and that it can reach an almost equilibrated state. It is demonstrated here that this is not the case, and that the complex exists in different conformations characterised by different amounts of red emission. The data of Jennings et al. [40] can thus be interpreted considering that excitation above 700 nm is selective for the "red conformation", which explains the fact that the spectrum is more red than the spectrum obtained upon non-selective excitation when indeed the excitation is distributed over all conformations.

4.2. Are the red forms a quencher?

Previous data showed a strong correlation between the presence of red forms in the various Lhca complexes and the shortening of the lifetime, thereby suggesting a quenching effect by a red CT state [26]. However, the analysis of Lhca4 mutants depleted in red forms demonstrates that the red forms are not acting as a quencher in Lhca4 in solution: the lifetimes of the mutants are very similar to those of the WT.

The results also show that the red forms, although not influencing the lifetime of the complex, can only be accommodated in the "slow" conformation. This means that the geometry that allows for a strong 603/609 interaction is incompatible with the "quenched" conformation. Considering that Chl 603 is very close to the xanthophyll in the L2 site (Fig. 1), as shown by triplet-minus–singlet experiments [51], it is likely that a change in the occupation of L2 site has an effect on the geometry of Chl 603 and 609, thus changing their absorption properties. This is exactly what is observed when the occupancy of the site is changed from violaxanthin to lutein. Indeed, Lhca4-Lut shows a difference in the relative amplitudes of the blue-fast and red-slow components, which is inverted as compared to the WT. Moreover, Lhca4-Lut also shows less red absorption and less red-shifted fluorescence in comparison to the WT. These results strengthen the idea that the different DAS components originate from different conformations, suggesting that the presence of lutein disfavours the red conformation, shifting the equilibrium toward the blue conformation.

It is interesting to observe that the Chl a/b ratio of Lhca4-Lut is higher than that of the WT, indicating that the presence of lutein is influencing the binding of a Chl b, or the occupancy of a mixed binding site. Taking into account previous results which showed that Chl b is involved [52] or is important for the stability of the red-emitting forms [21], it can be suggested that a Chl b is needed to stabilize the conformation which leads to the red forms and that its binding is destabilized by the presence of lutein/absence of violaxanthin in the L2 site. This Chl could be Chl 606 and/or 607 (Fig. 1), which is located close to site L2, making plausible that a different occupancy of the L2 site has an effect on their binding selectivity.

5. Conclusions

In summary:

(1) Lhca4 exists in multiple conformations that are characterised by different fluorescence lifetimes and spectra.
(2) The "blue conformation" has a short lifetime, while the long lifetime is associated with the "red conformation".
(3) The red-emitting forms can only be present in the "slow conformation" but the "slow conformation" does not depend on the red forms as it is also present in their absence. (Mutants Lhca4-N47H and Lhca4-N47L).
(4) The quencher in the "fast-blue conformation" is also independent of the red forms, because it is still present in their absence. In general, the quenched conformation and the red forms are mutually exclusive.

(5) The substitution of violaxanthin with lutein changes the equilibrium between the conformations, favouring the quenched one. The decrease of red forms in the presence of lutein is due to a change in the equilibrium between the conformations.

Based on our results we can thus exclude that the short lifetime of Lhca4 is related to the presence of red forms. Thus another pigment or pool of pigments is responsible for the quenching. Interestingly, a 200–300 ps component was observed also in LHCII [50,53] and in the minor antenna complex CP24 [31]. Considering the high homology of the spectroscopic properties of most of the pigments in Lhca and Lhcb complexes, it thus seems plausible that the quenching might have the same origin in all members of the family.

Acknowledgements

References

[1] B. Demmig-Adams, W.W. Adams, Photoprotection and other responses of plants to high light stress, Ann. Rev. Plant Physiol. Plant Mol. Biol. 43 (1992) 599–626.

[2] P. Horton, A.V. Ruban, Molecular design of the photosystem II light-harvesting antenna: photosynthesis and photoprotection, J. Exp. Bot. 56 (2005) 365–373.

[3] S. Jansson, A guide to the Lhc genes and their relatives in *Arabidopsis*, Trends Plant Sci. 4 (1999) 236–240.

[4] Z. Liu, H. Yan, K. Wang, T. Kuang, J. Zhang, L. Gui, X. An, W. Chang, Crystal structure of spinach major light-harvesting complex at 2.72 A resolution, Nature 428 (2004) 287–292.

[5] A. Amunts, O. Drory, N. Nelson, The structure of a plant photosystem I supercomplex at 3.4 Angstrom resolution, Nature 447 (2007) 58–63.

[6] H. van Amerongen, R. Croce, Structure and function of photosystem II light-harvesting proteins (Lhcb) of higher plants, in: G. Renger (Ed.), Primary Processes of Photosynthesis, vol. 1, Royal Society of Chemistry, Cambridge, 2008, pp. 329–368.

[7] V.H.R. Schmid, K.V. Cammarata, B.U. Bruns, G.W. Schmidt, In vitro reconstitution of the photosystem I light-harvesting complex LHCI-730: heterodimerization is required for antenna pigment organization, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 7667–7672.

[8] B. Gobets, R. van Grondelle, Energy transfer and trapping in photosystem I, Biochim. Biophys. Acta 1057 (2001) 80–99.

[9] N.V. Karapetyan, Protective dissipation of excess absorbed energy by photosynthetic apparatus of cyanobacteria: role of antenna terminal emitters, Photosynth. Res. 97 (2008) 195–204.

[10] M. Mozzo, M. Mantelli, F. Passarini, S. Caffarri, R. Croce, R. Bassi, Functional analysis of photosystem I light-harvesting complexes (Lhca) gene products of *Chlamydomonas reinhardtii*, Biochim. Biophys. Acta 1797 (2010) 212–221.

[11] R.C. Jennings, G. Zucchelli, R. Croce, F.M. Garlaschi, The photochemical trapping rate from red spectral states in PSI–LHCI is determined by thermal activation of energy transfer to bulk chlorophylls, Biochim. Biophys. Acta-Bioenerg. 1557 (2003) 91–98.

[12] A.N. Melkozernov, J. Barber, R.E. Blankenship, Light harvesting in photosystem I supercomplexes, Biochemistry 45 (2006) 331–345.

[13] A.N. Melkozernov, R.E. Blankenship, Structural and functional organization of the peripheral light-harvesting system in photosystem I, Photosynth. Res. 85 (2005) 33–50.

[14] T. Morosinotto, S. Castelletti, J. Breton, R. Bassi, R. Croce, Mutation analysis of Lhca1 antenna complex—low energy absorption forms originate from pigment–pigment interactions, J. Biol. Chem. 277 (2002) 36253–36261.

[15] R. Croce, T. Morosinotto, J.A. Ihalainen, A. Chojnicka, J. Breton, J.P. Dekker, R. van Grondelle, R. Bassi, Origin of the 701-nm fluorescence emission of the Lhca2 subunit of higher plant photosystem I, J. Biol. Chem. 279 (2004) 48543–48549.

[16] T. Morosinotto, M. Mozzo, R. Bassi, R. Croce, Pigment–pigment interactions in Lhca4 antenna complex of higher plants photosystem I, J. Biol. Chem. 280 (2005) 20612–20619.

[17] M. Mozzo, T. Morosinotto, R. Bassi, R. Croce, Probing the structure of Lhca3 by mutation analysis, Biochim. Biophys. Acta-Bioenerg. 1757 (2006) 1607–1613.

[18] J.A. Ihalainen, M. Ratsep, P.E. Jensen, H.V. Scheller, R. Croce, R. Bassi, J.E.I. Korppi-Tommola, A. Freiberg, Red spectral forms of chlorophylls in green plant PSI—a site-selective and high-pressure spectroscopy study, J. Phys. Chem. B 107 (2003) 9086–9093.

[19] E. Romero, M. Mozzo, I.H.M. van Stokkum, J.P. Dekker, R. van Grondelle, R. Croce, The origin of the low-energy form of photosystem I light-harvesting complex Lhca4: mixing of the lowest exciton with a charge-transfer state, Biophys. J. 96 (2009) L35–L37.

[20] V.H.R. Schmid, S. Potthast, M. Wiener, V. Bergauer, H. Paulsen, S. Storf, Pigment binding of photosystem I light-harvesting proteins, J. Biol. Chem. 277 (2002) 37307–37314.

[21] S. Castelletti, T. Morosinotto, B. Robert, S. Caffarri, R. Bassi, R. Croce, Recombinant Lhca2 and Lhca3 subunits of the photosystem I antenna system, Biochemistry 42 (2003) 4226–4234.

[22] T. Morosinotto, J. Breton, R. Bassi, R. Croce, The nature of a chlorophyll ligand in Lhca proteins determines the far red fluorescence emission typical of photosystem I, J. Biol. Chem. 278 (2003) 49223–49229.

[23] A.N. Melkozernov, V.H.R. Schmid, G.W. Schmidt, R.E. Blankenship, Energy redistribution in heterodimeric light-harvesting complex LHCI-730 of photosystem I, J. Phys. Chem. B 102 (1998) 8183–8189.

[24] A.N. Melkozernov, S. Lin, V.H.R. Schmid, H. Paulsen, G.W. Schmidt, R.E. Blankenship, Ultrafast excitation dynamics of low energy pigments in reconstituted peripheral light-harvesting complexes of photosystem I, FEBS Lett. 471 (2000) 89–92.

[25] B. Gobets, J.T.M. Kennis, J.A. Ihalainen, M. Brazzoli, R. Croce, L.H.M. van Stokkum, R. Bassi, J.P. Dekker, H. Van Amerongen, G.R. Fleming, R. van Grondelle, Excitation energy transfer in dimeric light harvesting complex I: a combined streak-camera/fluorescence upconversion study, J. Phys. Chem. B 105 (2001) 10132–10139.

[26] J.A. Ihalainen, R. Croce, T. Morosinotto, I.H.M. van Stokkum, R. Bassi, J.P.X. Dekker, R. van Grondelle, Excitation decay pathways of Lhca proteins: a time-resolved fluorescence study, J. Phys. Chem. B 109 (2005) 21150–21158.

[27] E.C.M. Engelmann, G. Zucchelli, F.M. Garlaschi, A.P. Casazza, R.C. Jennings, The effect of outer antenna complexes on the photochemical trapping rate in barley thylakoid photosystem II, Biochim. Biophys. Acta-Bioenerg. 1706 (2005) 276–286.

[28] Y. Miloslavina, A. Wehner, P.H. Lambrev, E. Wientjes, M. Reus, G. Garab, R. Croce, A.R. Holzwarth, Far-red fluorescence: a direct spectroscopic marker for LHCII oligomer formation in non-photochemical quenching, FEBS Lett. 582 (2008) 3625–3631.

[29] M. Wentworth, A.V. Ruban, P. Horton, Thermodynamic investigation into the mechanism of the chlorophyll fluorescence quenching in isolated photosystem II light-harvesting complexes, J. Biol. Chem. 278 (2003) 21845–21850.

[30] S. Bode, C.C. Quentmeier, P.N. Liao, N. Hafi, T. Barros, L. Wilk, F. Bittner, P.J. Walla, On the regulation of photosynthesis by excitonic interactions between carotenoids and chlorophylls, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 12311–12316.

[31] F. Passarini, E. Wientjes, R. Hienerwadel, R. Croce, Molecular basis of light harvesting and photoprotection in CP24: unique features of the most recent antenna complex, J. Biol. Chem. 284 (2009) 29536–29546.

[32] R. Croce, G. Canino, F. Ros, R. Bassi, Chromophore organization in the higher-plant photosystem II antenna protein CP26, Biochemistry 41 (2002) 7334–7343.

[33] A.M. Gilmore, H.Y. Yamamoto, Zeaxanthin formation and energy-dependent fluorescence quenching in pea chloroplasts under artificially mediated linear and cyclic electron transport, Plant Physiol. 96 (1991) 635–643.

[34] O.J.G. Somsen, G. Trinkunas, M.N. de Keijzer, A. van Hoek, H. van Amerongen, Local diffusive dynamics in DNA—a time-resolved fluorescence and molecular-dynamics study of dinucleotides with 2-aminopurine, 15th International Conference on Dynamical Processes in Excited States of Solids, Shanghai, Peoples R China, 2005, pp. 100–104.

[35] B. van Oort, A. Amunts, J.W. Borst, A. van Hoek, N. Nelson, H. van Amerongen, R. Croce, Picosecond fluorescence of intact and dissolved PSI–LHCI crystals, Biophys. J. 95 (2008) 5851–5861.

[36] A. Van Hoek, A.J.W.G. Visser, Artefact and distortion sources in time correlated single photon counting, Anal. Instrum. 14 (1985) 359–378.

[37] A.V. Digris, V.V. Skakoun, E.G. Novikov, A. van Hoek, A. Claiborne, A.J. Visser, Thermal stability of a flavoprotein assessed from associative analysis of polarized time-resolved fluorescence spectroscopy, Eur. Biophys. J. 28 (1999) 526–531.

[38] R. Croce, G. Cinque, A.R. Holzwarth, R. Bassi, The soret absorption properties of carotenoids and chlorophylls in antenna complexes of higher plants, Photosynth. Res. (2000) 221–231.

[39] K. Gibasiewicz, R. Croce, T. Morosinotto, J.A. Ihalainen, I.H.M. van Stokkum, J.P. Dekker, R. Bassi, R. van Grondelle, Excitation energy transfer pathways in Lhca4, Biophys. J. 88 (2005) 1959–1969.

[40] R.C. Jennings, G. Zucchelli, E. Engelmann, F.M. Garlaschi, The long-wavelength chlorophyll states of plant LHCI at room temperature: a comparison with PSI–LHCI, Biophys. J. 87 (2004) 488–497.

[41] H. Van Amerongen, R. van Grondelle, Understanding the energy transfer function of LHCII, the major light-harvesting complex of green plants, J. Phys. Chem. B 105 (2001) 604–617.

[42] K.R. Naqvi, Carotenoid-induced electronic relaxation of the first excited state of antenna chlorophylls, in: G. Garab (Ed.), XIth International Congress on Photosynthesis—Mechanisms and Effects, Budapest, Hungary, 1998, pp. 265–270.

[43] S.S. Lampoura, V. Barzda, G.M. Owen, A.J. Hoff, H. Van Amerongen, Aggregation of LHCII leads to a redistribution of the triplets over the central xanthophylls in LHCII, Biochemistry 41 (2002) 9139–9144.

[44] R. Croce, T. Morosinotto, S. Castelletti, J. Breton, R. Bassi, The Lhca antenna complexes of higher plants photosystem I, Biochim. Biophys. Acta-Bioenerg. 1556 (2002) 29–40.

[45] L.O. Palsson, S.E. Tjus, B. Andersson, T. Gillbro, Ultrafast energy-transfer dynamics resolved in isolated spinach light-harvesting complex-I and the Lhc-I-730 subpopulation, Biochim. Biophys. Acta-Bioenerg. 1230 (1995) 1–9.

[46] E. Engelmann, G. Zucchelli, A.P. Casazza, D. Brogioli, F.M. Garlaschi, R.C. Jennings, Influence of the photosystem I-light harvesting complex I antenna domains on fluorescence decay, Biochemistry 45 (2006) 6947–6955.

[47] I. Mukerji, K. Sauer, Energy transfer dynamics of an isolated light harvesting complex of photosystem I from spinach: time-resolved fluorescence measurement at 295 K and 77 K, Biochim. Biophys. Acta 1142 (1993) 311–320.

[48] J.R. Lakowicz, On spectral relaxation in proteins, Photochem. Photobiol. 72 (2000) 421–437.

[49] I. Moya, M. Silvestri, O. Vallon, G. Cinque, R. Bassi, Time-resolved fluorescence analysis of the photosystem II antenna proteins in detergent micelles and liposomes, Biochemistry 40 (2001) 12552–12561.

[50] B. van Oort, A. van Hoek, A.V. Ruban, H. van Amerongen, Equilibrium between quenched and nonquenched conformations of the major plant light-harvesting complex studied with high-pressure time-resolved fluorescence, J. Phys. Chem. 111 (2007) 7631–7637.

[51] R. Croce, M. Mozzo, T. Morosinotto, A. Romeo, R. Hienerwadel, R. Bassi, Singlet and triplet state transitions of carotenoids in the antenna complexes of higher-plant photosystem I, Biochemistry 46 (2007) 3846–3855.

[52] V.H. Schmid, P. Thome, W. Ruhle, H. Paulsen, W. Kuhlbrandt, H. Rogl, Chlorophyll b is involved in long-wavelength spectral properties of light-harvesting complexes LHC I and LHC II, FEBS Lett. 499 (2001) 27–31.

[53] M.A. Palacios, J. Standfuss, M. Vengris, B.F. van Oort, I.H. van Stokkum, W. Kuhlbrandt, H. van Amerongen, R. van Grondelle, A comparison of the three isoforms of the light-harvesting complex II using transient absorption and time-resolved fluorescence measurements, Photosynth. Res. 88 (2006) 269–285.