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From red to blue to far-red in Lhca4: How does the protein modulate the spectral properties of the pigments?

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ABSTRACT

The first event of photosynthesis is the harvesting of solar energy by a large array of pigments. These pigments are coordinated to proteins that organize them to assure efficient excitation energy transfer. The protein plays an essential role in tuning the spectroscopic properties of the pigments, by determining their site energy and/or by favoring pigment–pigments interactions. Here we investigate how the protein modulates the pigment properties by using a single-point-mutation approach. We monitor changes in the low-energy absorption/emission band of Lhca4, which is well separated from the bulk absorption and thus represents an attractive model system. Moreover, it was recently shown that Lhca4 exists in at least two conformations, a dominating one emitting at 720 nm and a second one emitting at 685 nm (Kruger et al. PNAS 2011). Here we show that a single amino-acid substitution (from Asn to Gln, which are both chlorophyll-binding residues and only differ for one C—C bond), moves the equilibrium almost completely towards the 685-nm conformation. This indicates that small changes in the protein can have a large effect on the properties of the pigments. We show that His99, which was suggested to coordinate a red-absorbing chlorophyll (Melkozernov and Blankenship, JBC 2003), is not a chlorophyll ligand. We also show that single amino-acid substitutions nearby the chlorophylls allow to tune the emission spectrum of the pigments over a wide range of wavelengths and to modulate the excited-state lifetimes of the complex. These findings are discussed in the light of previously proposed non-photochemical quenching models.

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1. Introduction

Photosynthesis is the process in which light energy is used to convert CO_2 into organic compounds. It occurs in plants, algae and photosynthetic bacteria. In the first steps light is absorbed by pigments, which transfer excitation energy to the reaction center (RC), where charge separation occurs. The pigments are phycobilins, carotenoids and chlorophylls (Chls) [e.g. [1]]. The pigments are generally coordinated by proteins, which keep them at appropriate orientation and distance with respect to each other and with respect to the protein itself, and hence determine the pigment environment. The environment influences the excited-state energy level of the pigments leading to blue/red shifts and broadening of their absorption and emission spectra [e.g. [2–5]]. In this way a small variety of pigments can be used to absorb light over a broad spectral range.

Pigment selection and tuning can improve the match between the absorption spectrum of the photosynthetic complexes and the irradiation spectrum, as is nicely demonstrated for photosynthetic microorganisms living in different spectral niches [6]. Furthermore, tuning of the Chl excited-state-energy level is important for the efficiency of excitation-energy transfer to the RC. For instance, in purple photosynthetic bacteria chemically identical bacteriochlorophylls *a* can absorb light at very different wavelengths, which ensures that light energy absorbed by external antennas is efficiently funneled "downhill" to the inner antenna, and onto the RC [e.g. [7]]. Interestingly, it has been shown that the absorption properties of these antennas can be largely modified by the introduction/removal of an H-bond between the protein and the pigment [3,4].

In higher plants and green algae Chl *a*, Chl *b* and carotenoids (Cars) are coordinated by light-harvesting complexes (Lhcs). Upon absorption of a photon by an Lhc, excitation energy is efficiently transferred to the RC located in the core complex of Photosystem I (PSI) or Photosystem II (PSII). There are four PSI antennas (Lhca1–4), which are organized as two hetero-dimers: Lhca1/4 and Lhca2/3 [8–11]. The six PSII antennas

Abbreviations: CT, Charge-transfer; Chl, Chlorophyll; DAS, decay associated spectra; Lhc, light-harvesting complex; Lhca, Light-harvesting complex of Photosystem I; Lhcb, Light-harvesting complex of Photosystem II; Photosystem, PS; RC, reaction centre; TCSPC, time-correlated single photon counting

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are named Lhcb1–6. Lhcb1–3 form the major trimeric antenna, called LHCII. Lhcb4–6 are the minor monomeric Lhcs, also named CP29, CP26 and CP24, respectively [12].

All Lhcs are encoded by the Lhc gene family [13]. Based on the available Lhca and Lhcb crystal structures, it can be inferred that they share their protein fold and have a similar Chl organization [10,14–17]. In Lhcbs the Chls *a* Q_y transitions show a maximum in the 660–680 nm range [18,19]. For Lhcas this range is extended to the red. The most red-shifted absorption band is found in Lhca4, at 708 nm [20]. Absorption at long wavelengths can be important under a canopy where light is enriched in $\lambda > 700$ nm [21]. Furthermore, these low-energy Chls have a high probability to be populated upon excited-state equilibration and can thus play an important role in photoprotection [22–24].

In the past decade it was discovered that the low-energy Chls, also called red forms, of all four Lhcas originate from the low-energy band of the excitonically coupled Chl *a* 603–609 dimer [nomenclature as in [15]] [25–27], mixed with a charge-transfer (CT) state [28,29]. Mixing with the CT state explains the large change of dipole moment in the excited state, which leads to: (i) a large contribution of optical transitions into higher vibronic sub-states, (ii) a large Stokes shift, and (iii) a large homogeneous broadening by strong coupling of the electronic transition to the phonons of the protein [2,20]. It has also been proposed that an additional Chls, coordinated to H99 is involved in the low energy absorption [30].

The red forms give a strong contribution to the fluorescence emission spectra. For all Lhcbs the emission maxima are found at ~680 nm, while for Lhca4 the emission maximum is observed at 720 nm at 283 K [31] and shifts to 732 nm at 77 K [32]. Based on time-resolved fluorescence measurements it was proposed that Lhca4 can occur in different conformations: a "blue" (maximum at 680–690 nm) conformation with a relatively short excited state lifetime and a "red" (720 nm) one with a long lifetime [31]. The existence of different emission states was recently confirmed by single molecule fluorescence spectroscopy, that showed that the "red" emission from an Lhca complex can occasionally switch to a typical "blue" Lhcb spectrum, while a "blue" Lhcb complex can switch to a typical "red" Lhca emitting state [33]. The reversible disappearance of the red band was explained by a conformational change of the protein, involving an increase of the inter-pigment distance and/or a change of the orientation of the Chls responsible for the red forms such that the CT-exciton state is lost. The red emission from Lhcb complexes can be explained in an identical manner, with a conformational change bringing Chls 603 and 609 closer together. For Lhca complexes the equilibrium lies at the red-emitting side, while for Lhcb complexes it lies at the blue-emitting one [33,34].

In this work we investigate the origin and the extent of these changes, by modifying the protein in or close to the binding site of the red forms. Site-directed mutagenesis and *in vitro* reconstitution were used to obtain Lhca4 complexes with single amino acid substitutions. Different effects that can influence the Chl excited-state-energy level have been investigated:

(i). Influence of changes in the pigment organization. How large the change in pigment organization and thus in the protein structure must be to lose the red forms of Lhca4 and thus to convert a typical Lhca complex into a typical Lhcb complex? This is particularly relevant because the flexibility of Lhc complexes is a matter of discussion. On the one hand quenching of Lhc complexes is assumed to be caused by a conformational change which switches the complex from a light-harvesting into a quenched state [35,36]. On the other hand, based on the low crystallographic temperature factor and the high similarity of LHCII structural models obtained from crystals grown under different conditions and originating from different organisms, it was argued that LHCII is very rigid and therefore unlikely to undergo conformational changes [37].

(ii). Direct influence of the protein environment. We study the effect of the presumably negatively charged glutamic acid (E) 94 residue, located near Chl609, by changing it into the neutral glutamine (Q). Due to their CT character red forms are sensitive to the presence of charges in their environment and might thus be influenced by such a mutation.

The results show that we can tune the absorption and emission properties of the red forms and their excited state lifetime over a wide range by small changes in the protein.

2. Materials and methods

2.1. Mutagenesis and reconstitution of Lhca4 complexes

A modified pET-28a (+) vector containing the sequence coding for the mature protein of *A. thaliana* Lhca4 has been mutated by site directed mutagenesis using the Stratagene QuikChange Site Directed Mutagenisis Kit. The following mutations were introduced: in N47Q the Chl603 ligand (N47) was substituted for another Chl ligand Q, in E94Q the acidic water mediated Chl-606 ligand E is mutated into the neutral water mediated Chl-606 ligand Q, in H99A the putative Chl binding residue H is mutated into Alanine (A) a residue that cannot coordinate a Chl. WT and mutant apoproteins were overexpressed in Rosetta2(DH3) strain of *E. coli*. The proteins were purified as inclusion bodies, as described in ref. [38]. Reconstitution was done as described in ref. [39]. The reconstituted complexes were purified by His-tag Ni-affinity chromatography and sucrose density ultracentrifugation, on a 0.1–1 M sucrose gradient (with 0.06% n-dodecyl-α-D-maltoside, 10 mM Hepes pH 7.6) at 41.000 rpm (Beckman Coulter, SW41 rotor) at 4 °C for at least 18 h.

2.2. Steady state spectroscopy and pigment analysis

Absorption spectra were recorded on a Varian Cary 4000 UV–visible spectrophotometer (Varian, Palo Alto, CA). For 77 K measurements, a homebuilt liquid-nitrogen cooled low-temperature device was used. Fluorescence spectra were recorded at 77 K and 283 K on a Fluorolog 3.22 spectrofluorimeter (HORIBA Jobin Yvon-Spex, Longumeau, France). Samples were diluted to an absorbance of 0.04 cm^{-1} at 680 nm. The fluorescence quantum yield (Φ_{Fl}) was determined as described in ref. [11], based on spectral integration from 640 nm to 850 nm. CD spectra were recorded at 283 K on an AVIV 62ADS spectropolarimeter (Aviv Associates, Lakewood, NJ). All measurements were performed in 10 mM Tricine, pH 7.8, 0.03% α-DM and 0–0.5 M sucrose (283 K) or 67% (w/v) glycerol (77 K).

Pigment analysis was based on fitting of the absorption spectra of the acetone-extracted pigments with the spectra of the individual pigments, as described in ref. [40].

2.3. Time-correlated single-photon counting

Time-correlated single-photon counting (TCSPC) of fluorescence was performed using a FluoTime200 picosecond fluorescencemeter (PicoQuant). The samples were diluted to an OD of 0.05 cm^{-1} at the Q_y maximum, stirred in a 3.5 ml cuvette with a path length of 1 cm, and kept at 283 K. Excitation was provided by a 468 nm laser diode, operated at a repetition rate of 10 MHz. The instrument response function (~80 ps FWHM) was obtained with pinacyanol iodide in methanol, with a 6 ps fluorescence lifetime [41]. The fluorescence was detected at 670 nm to 760 nm with 10 nm steps and an 8 nm slit width. The channel time spacing was 8 ps, and a time window of 20 ns was used for data analysis. The steady-state fluorescence spectra were used to calculate the decay associated spectra (DAS).

3. Results

3.1. Introduced mutations

Fig. 1 shows the mutations introduced in Lhca4. All mutations are targeting residues located nearby/or suggested to coordinate the Chls responsible for the red-shifted absorption and emission of Lhca4. In the H99A mutant the putative Chl binding site H99, which has been proposed to coordinate one of the Chls responsible for the red forms [proposed by 30] was substituted for A, which cannot coordinate the central Mg of a Chl. The second mutation is directed to asparagine N47, the ligand of Chl 603. It has been shown previously that substitution of N47 for H induces a strong blue-shift of the red forms (emission maximum from 732 nm to 700–704 nm at 77 K) [25]. This was explained by the larger dimensions of H as compared to N, which increases the Chl 603–609 distance and thus lowers the interaction energy between these pigments. Here, we substitute N47 for glutamine (Q), thus increasing the side-chain lengths of the Chl ligand by one C—C bond, but without further changes in the amino acid characteristics. The last mutation is E94Q, this mutation is expected to change the Chl *a*/*b* occupancy of a neighboring Chl-binding site (607 or 609) [42] and remove a negative charge.

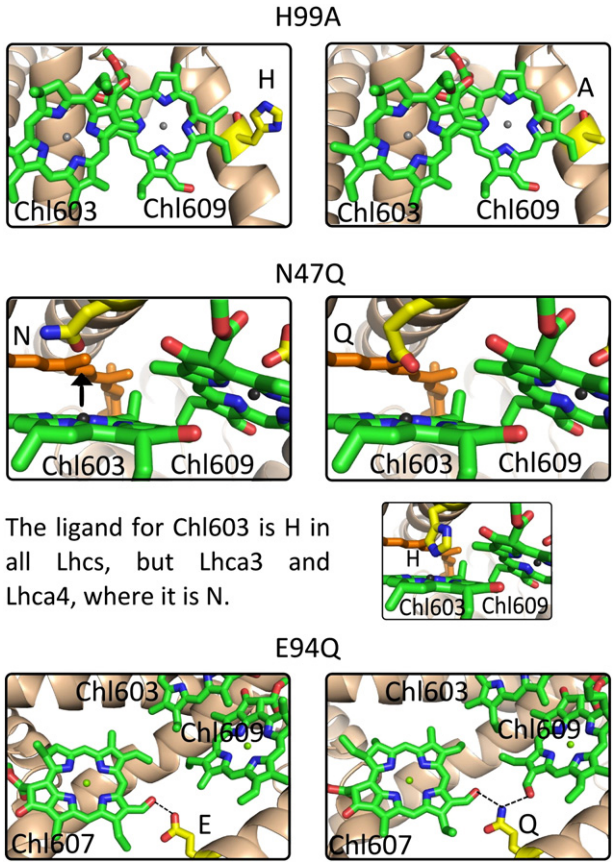

Fig. 1. Molecular models of the introduced mutations – Models are based on the structure of LHCII [15] or for the left lower panel of CP29 [17]. The LHCII and CP29 structures are used because they were solved to higher resolution than the one of Lhca4 [in the higher plant PSI structure, [16]]. Note that the Chl *a*/*b* occupancy in Lhca4 can be different from that in LHCII and CP29. Only pigments nearby the mutated site are shown, the phytol chains of the Chls are omitted. The Chls and the mutated residue are colored by element: nitrogen (blue), oxygen (red) and carbon (green/yellow). In the middle panel a Car is shown in orange, and the black arrow indicates that in Lhca4 Chl603 is located closer to its ligand (N) compared to the figure, which is based on the LHCII model where the Chl603 ligand is an H (small cartoon). The dashed lines in the lower panel show hydrogen bonds. Figures were prepared with PyMOL (DeLano, WL. The PyMOL Molecular Graphics System, 2002).

Table 1
Pigment composition. Chl *a*/*b* and Chl/Car ratio of Lhca4 samples, based on two repetitions, the standard deviation (SD) in the Chl/Car ratio is <0.2 and in the Chl *a*/*b* ratio <0.1.

Sample	Chl/Car	Chl *a*/*b*
WT	4.7	2.1
H99A	4.7	2.0
N47Q	5.2	2.0
E94Q	4.8	1.8

3.2. Pigments composition

The Chl *a*/*b* and Chl/Car ratios of the samples are reported in Table 1. In Lhca4-WT the Chl/Car ratio indicates that ~2 Cars are present per Lhca4 complex assuming a total of 10 Chls, in agreement with previous results [43]. The Chl/Car ratio of H99A is similar to that of WT, while a decrease to 4.2 would be expected if one Chl was lost. This suggests that H99 is not a Chl-binding residue. The Chl/Car ratio of E94Q is also similar to that of WT, while for N47Q this ratio is somewhat higher, suggesting that this complex has a reduced affinity for Cars. Most likely the Car binding in the 621 (L2) site, nearby Chl 603 and 47Q (Fig. 1), is slightly destabilized by the mutation. It can be concluded that none of the mutations lead to loss of Chls.

The difference in Chl *a*/*b* ratio between WT and E94Q indicates that in the mutant 0.3 Chls *a* are replaced by Chls *b*. In the other two mutant complexes the number of Chls *a* per complex is the same as in the WT (the difference is ±0.1, which is smaller than the error of the measurement) and it can thus be concluded that these mutations do not affect the affinity of the binding sites for Chl *a* or *b*.

3.3. Absorption properties

Fig. 2 shows the 77 K absorption spectra of the Lhca4 complexes and their second derivatives. In the Soret region the main spectral

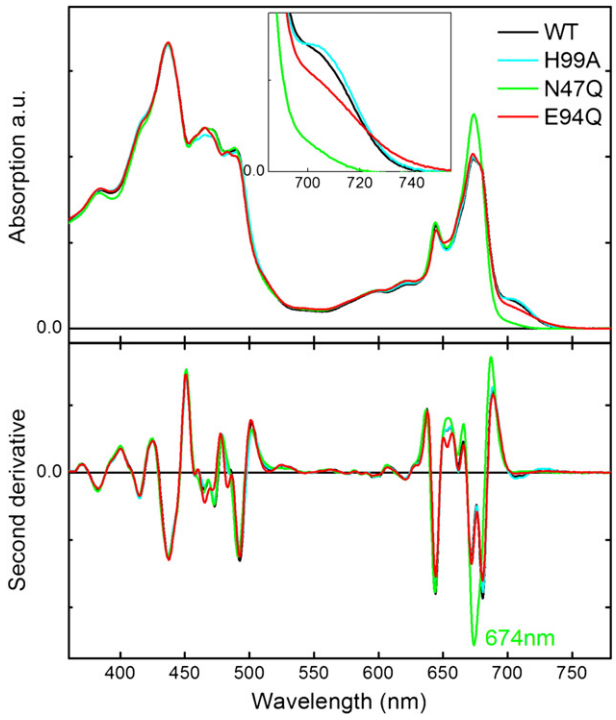

Fig. 2. 77K absorption spectra of Lhca4 complexes. Upper panel: absorption spectra normalized to same area in the Q_y region. Lower panel: second derivative of the absorption spectra.

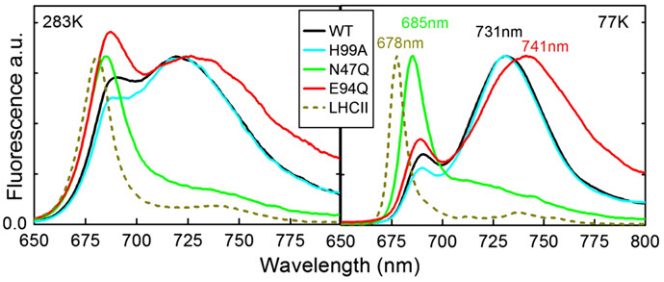

Fig. 3. Fluorescence emission spectra of Lhca4 complexes. Emission spectra recorded at 283 K and 77 K after excitation at 500 nm. Spectra were normalized to their (red) maximum.

features are not affected by the mutations, however in the Q-region large differences can be appreciated. In the N47Q mutant the absorption above 700 nm is strongly decreased, while it increases at 674 nm. The 674 nm band can be attributed to the absorption of "monomeric" Chl 603 and Chl 609, i.e. in absence of the strong excitonic interaction, in agreement with previous results [25]. The absorption spectrum of E94Q shows a decrease in the amplitude at $\lambda < 722$ nm, compared to that of the WT, but an increase at longer wavelengths (Fig. 2, inset), thus suggesting that the red forms band is broadened in the mutant. The H99A mutant has an absorption spectrum that is almost indistinguishable from that of WT (only in the red a slight increase in absorption is observed).

3.4. Fluorescence emission

Because the red forms have a high probability to be populated, they strongly contribute to the fluorescence emission, with the result that the effect of the mutations on the red forms can be better appreciated in fluorescence. Fig. 3 shows the emission spectra of the complexes at 283 K and 77 K. For H99A the properties of the red forms (emission maximum and band-width) are not different from those of the WT. The only difference is a small change in the ratio between the "blue" (685 nm) and the "red" (720 nm) emission, which suggests that the H99A mutant favors the red conformation somewhat more than the WT complex. Similarly, the emission spectra of the N47Q mutant show that this complex favors the "blue" emitting Lhcb-like conformation. However, the emission in the red is stronger than for Lhcb complexes, suggesting that part of the complexes can still form an excitonic-CT state. The red band of the E94Q emission spectrum is red-shifted and broadened compared to the WT, in agreement with the broader red absorption band.

3.5. Time-resolved fluorescence

To get further inside in the effect of the mutations on the emission of the Lhca4 complexes we performed time-resolved fluorescence measurements. The decay associated spectra (DAS) are presented in Fig. 4. Four lifetimes were needed to describe the fluorescence decay kinetics of all complexes. In the WT complex the shorter lifetimes of 0.34 and 1.3 ns are associated with "blue" emission spectra ($\lambda_{MAX} = 690$ nm), the 2.7 ns DAS has a "red" emission maximum ($\lambda_{MAX} = 720$ nm), the low amplitude 4.3 ns component has maxima in the "blue" and in the "red" part of the spectrum. In the H99A mutant the "red" 2.7 ns component has an increased amplitude, compared to the WT complex, at the cost of the "blue" DASs, in agreement with the idea that this mutant favors the "red" conformation. The average lifetime is slightly increased to 1.99 ns, compared to 1.84 ns for WT. All DASs of the N47Q mutant have a "blue" emission maximum, although the normalized spectra (data not shown) show that the amplitude in the "red" is slightly higher for the 2.4 ns and 4.6 ns component, indicating that a small fraction of the complexes could have a "red" emission maximum. The shorter lifetimes dominate the fluorescence decay, resulting in an average lifetimes of 1.16 ns for this mutant. In the E94Q mutant the 4.6 ns DAS is almost absent and all other DASs have an increased amplitude in the "red" compared to the WT. The average lifetime of this complex (1.56 ns) is reduced compared to the WT complex. It can thus be concluded that all three mutations affect the fluorescence lifetime. To investigate this further we determined the fluorescence quantum yields (Φ_{Fl}) and calculated the emitting dipole strengths. The Φ_{Fl} of the H99A mutant is increased to 10.6% compared to 8.8% in the WT complex. The changes in the emission spectrum and in fluorescence lifetimes were only small, meaning that the emitting dipole strengths is larger for this mutant (Table 2). This can be explained by the increased red form content, which have been shown to have a large emitting dipole strengths [44]. The N47Q and E94Q mutants both show a reduced Φ_{Fl} and are thus quenched, however, the emitting dipole strengths of N47Q is the same as in the WT complex, while this

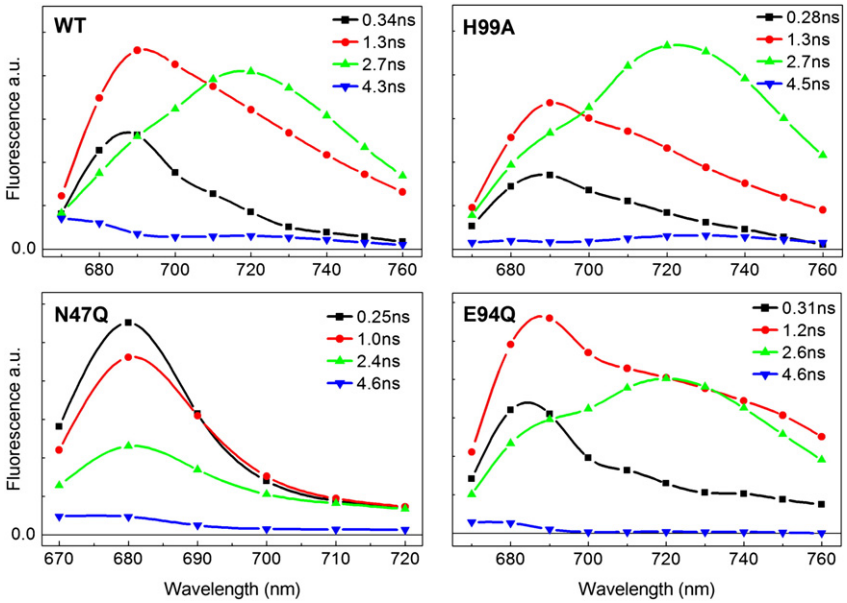

Fig. 4. Decay associated spectra of Lhca4 complexes. The measurements were performed at 283 K, excitation was at 468 nm. The fast excitation-energy transfer component (~6 ps lifetime [53]) was not resolved.

Table 2
Fluorescence quantum yield of Lhca4 complexes. Φ_{Fl} determined for two repetitions, SD is given between brackets. The Φ_{Fl} of LHCII was also determined. *The Φ_{Fl} of Chl a in 100% acetone is taken from [55]. The average fluorescence lifetime ($<\tau_{Fl}>$) is obtained according to $<\tau_{Fl}> = \Sigma A_i\tau_i$, with A_i the relative area of the DAS with lifetime τ_i. The radiative rate (k_{rad}) is obtained according to: $k_{rad} = \Phi_{Fl}/<\tau_{Fl}>$ and the relative emitting dipole strengths by: $|\mu^2| = 3\varepsilon_0 hc^3 k_{rad}/n16\pi^3\upsilon^3$, with ε_0 the vacuum dielectric constant, h the Planck's constant, c the speed of light in vacuum, n the refractive index, and υ the mean emission frequency, see [44,56] for further details.

	WT	H99A	N47Q	E94Q	LHCII	Chl a
$<\tau_{Fl}>$ (ns)	1.84	1.99	1.16	1.57	3.74	5.73
Φ_{Fl} (%)	8.8 (0.1)	10.6 (0.2)	6.1 (0.3)	5.6 (0.2)	20.5 (0.2)	30*
k_{rad} (ns^{-1})	0.048	0.053	0.053	0.036	0.055	0.052
$<\upsilon>$ (s^{-1})	4.20×10^{14}	4.17×10^{14}	4.33×10^{14}	4.20×10^{14}	4.35×10^{14}	$4.42v10^{14}$
Relative $\|\mu^2\|$	1.09	1.24	1.10	0.81	1.13	1.00

value is decreased for the E94Q mutant. This means that the quenching must have a different origin in the two complexes.

3.6. Pigment–pigment interaction—circular dichroism

The CD spectrum (Fig. 5) of the E94Q mutant shows, compared to the WT, an increase in the amplitudes in the Soret region. The difference spectrum (E94Q-WT, not shown) has maxima at 443 nm (+) and 473 nm (−), suggesting that an additional Chl *a*/*b* interaction has been created in the mutant. The spectrum of the N47Q mutant is similar to that of the WT in the Soret region, indicating that the mutation does not change the overall pigment organization of the complex. The slight decrease of amplitude around 500 nm is probably related to the small loss of Cars. Large differences are observed in the red part ($\lambda > 700$ nm) of the spectrum, where the mutant clearly lacks the (−) signal, which has been shown to arise from the excitonic interaction responsible for the red forms [25]. The CD spectra of the H99A mutant and the WT complex are nearly indistinguishable.

4. Discussion

Proteins have the intriguing ability to alter the optical properties of pigments. This modulation is caused by changes in pigment–pigment and pigment–protein interactions and lies at the basis of the efficient functioning of the light-harvesting process and allows its regulation.

It has been shown that both Lhca and Lhcb complexes can occasionally switch between states that are characterized by differences in emission maxima as large as 50 nm, suggesting that the Chl excited-state energy levels can be strongly modified by spontaneous changes of the protein conformation [33]. If the protein conformational changes that can alter the Chl energy levels are small, it should also be possible to alter the energy levels by making small changes in the protein. It has indeed been observed that the single amino acid N47H substitution in Lhca4 can blue shift the red emission maximum by 30 nm (from 732 nm to 700–704 nm), while the number of coordinated Chls remains the same [25]. In this work we tested different hypotheses with the aim to improve the understanding of how Lhcs regulate the excited-state energy levels of Chls.

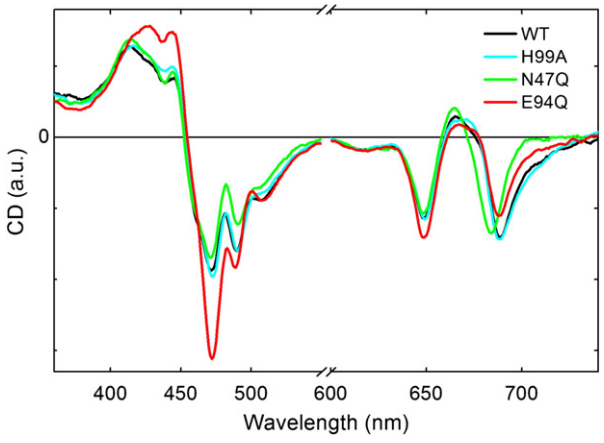

Fig. 5. CD spectra of Lhca4 complexes. Spectra are normalized to the same absorption in the Q_y region.

4.1. H99 is not a Chl binding residue

It was proposed that H99, which is unique for Lhca4, coordinates a Chl to form a pigment cluster with the nearby located Chls 603 and 609 being thus involved in the red absorption [30]. To fully clarify the origin of the red absorption in order to be able to mutate their environment, we have tested this hypothesis. Our analysis shows that substitution of H into a residue that cannot coordinate a Chl does not induce a Chl loss, leading to the conclusion that H99 is not a Chl ligand in monomeric Lhca4. In addition, the data show that the substitution of the polar H with a unipolar residue does not substantially change the properties of the red form, thereby demonstrating that this residue is also not involved in the modulation of the energy level of Chl 603 and 609.

4.2. Changing Lhca into Lhcb—how flexible are Lhcs?

It has been observed, with single-molecule fluorescence spectroscopy, that an Lhca4 complex can occasionally switch off its entire red emission band [33]. This is explained by a conformational change. The red forms can be lost by a combination of factors: (i) an increase in inter-pigment distance, (ii) a decrease of the pigment orientation factor, and (iii) the lost capability to form a CT state. But, how large should the change be to lose the red forms? To the best of our knowledge there are no structure-based calculations available that can correctly predict the excited-state energy levels of the red forms and therefore their tuning. To answer this question we have thus tried to move the equilibrium between the conformations towards the blue by mutating amino-acid residues located close to the Chls responsible for the red forms and of course making sure that the mutation did not induce Chl loss. It is shown that increasing the side chain length of the Chl603 ligand by one carbon–carbon bond (1.54 Å) is enough to lose the red forms in most of the complexes. In fact, the mutant shows characteristics that resemble more that of a typical Lhcb complex than that of an Lhca complex. First, its emission maximum is located at 685 nm, only 5 nm red-shifted as compared to the Lhcbs (and 45 nm blue shifted compared to Lhca4 WT). Second, the width of the main fluorescence emission band is more similar to that of Lhcbs than to that of Lhcas (Fig. 3). Third, the mutant shows only little absorption and CD signal for $\lambda > 700$ nm. The increase in distance, between the backbone C_α and the oxygen that coordinate the central Mg of the Chl, depends on the side-chain conformation; for the models shown in Fig. 1 it is 0.74 Å. This suggests that a rather small change in the protein scaffold can have a very large effect on the spectroscopic properties of the pigments. This is in agreement with the findings of Brecht et al. [45] which show that even at 1.4 K, where large scale motions are frozen out, considerable changes of site-energies occur in PSI.

A likewise small change in the other direction can explain the sporadic occurrence of red emitting LHCII complexes [33]. In our opinion the possibility of Lhcs to undergo conformational changes is not necessarily in contrast with the low-temperature factor of the LHCII structure [37]. The changes are small compared to the resolution of the structure and only a small fraction of the Lhcs is in another conformation. Furthermore, the spectral changes occur rarely, suggesting a high-energy barrier between the different states, and once a complex is switched it is stable in the other state, suggesting that both states can be considered rather rigid. This means that Lhcs are flexible enough to undergo conformational changes that have a strong effect on their spectroscopic properties and could as such play a role in non-photochemical quenching.

4.3. Red-shifting the Lhca4 emission—the E94Q mutant

The emission of the E94Q mutant is strongly red-shifted and broadened as compared to the WT. This can be explained by an increased CT character and thus a stronger electron–phonon coupling. There are a few possibilities how this mutation could increase the CT character. (i) The effect can be due to the removal of a negative charge in the surrounding of Chl 609 that can make the environment of Chl603–609 asymmetric and as such increase the CT character. (ii) The substitution of E, which can at most stabilize the binding of one Chl *b*, into Q which can stabilize two Chls *b*, slightly increased the amount of Chl *b* in the complex (Table 1). This additional Chl *b* can in principle be located in sites 609 or 607. If it is located in the 609 site, the excitonic Chl 603–Chl 609 pair becomes a Chl *a*-Chl *b* dimer (indeed the CD indicates an additional Chl *a*/*b* interaction (Fig. 5)), which could in theory favor the CT state by enhancing the asymmetric character of the pair. However, the large difference between the excited-state energy of these pigments will result in a smaller exciton splitting than in the WT complex. Thus, mixing of the exciton state with the CT state should strongly lower the energy to be able to explain the 10 nm red shift. (iii) If the extra Chl *b* is located in the 607 site it might also influence the red forms. It has been suggested that Chl *b* is involved in [46] or required for the stabilization of [47] the red forms of Lhca4, and coordination of Chl *b* in this site has been proposed to stabilize the red Lhca4 conformation [31].

4.4. Optimized light harvesting, the precision of nature

An efficient Lhc is able to rapidly transfer excitation energy to nearby complexes, while the loss of excitation energy should be minimal. This means that the complex should have a high radiative rate with respect to the rates of the loss processes and hence have a high Φ_{Fl}. Interestingly, when the concentration of Chls in organic solvents is as high as the Chl concentration in Lhcs (~0.25 M in LHCII) the emission is almost completely quenched [48]. This means that the protein plays an important role to keep the Chls at such orientation with respect to each other that concentration dependent quenching is circumvented [49]. In addition, the Cars present in the Lhcs should be close enough to the Chls to be able to quench the dangerous triplet state, but at the same time the Chl–Car inter-pigment distance should be large enough to avoid Chl Q_y–Car S_1 mixing. That it is not trivial to keep the Φ_{Fl} high is clear from the fact that two of the single amino acid substitutions resulted in a considerable decrease of Φ_{Fl} as compared to the WT complex (Table 2).

For N47Q the quenching might be explained by the higher flexibility (compared to N and H) of the long Q side-chain which can bring Chl 603 close enough to the Car in the L2 site for Chl Q_y–Car S_1 mixing to take place. As the lifetime of Cars is short, even a small degree of mixing will seriously shorten the Chl excited-state lifetime [50] but it will not affect the emitting dipole strengths as this will have mainly the characteristics of the emitting Chls. Such interactions were proposed to lie at the basis of non-photochemical quenching, which protects plants from high-light damage [51,52].

The E94Q seems to have an increased CT character. It has been proposed that mixing with a dark CT can quench the excited state lifetime of Lhc complexes by lowering the emitting dipole strengths [53] and as such protects the plants from high-light damage [54]. For the native Lhca1/4 dimer such quenching was not observed [44], however the emitting dipole strengths of the E94Q mutant is clearly decreased (Table 2). It can therefore be suggested that Nature has selected for a red absorbing antenna, which still has a rather high Φ_{Fl} and thus proper light-harvesting characteristics. Most likely this is generally true for the red forms in PSI. The large decrease of Φ_{Fl} caused by single amino acid substitutions show that it is not trivial to keep antennas in the active light-harvesting state at such high pigment concentrations.

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