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The negatively charged amino acids in the luminal loop influence the pigment binding and conformation of the major light-harvesting chlorophyll *a/b* complex of photosystem II

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ABSTRACT

The major chlorophyll (Chl) *a/b* complexes of photosystem II (LHCIIb), in addition to their primary light-harvesting function, play key roles in the organization of the granal ultrastructure of the thylakoid membranes and in various regulatory processes. These functions depend on the structural stability and flexibility of the complexes. The luminal side of LHCIIb is exposed to broadly variable pH environments, due to the build-up and decay of the pH gradient during photosynthesis. Therefore, the negatively charged amino acids in the luminal loop might be of paramount importance for adjusting the structure and functions of LHCIIb. In order to clarify the structural roles of these residues, we investigated the pigment stoichiometries, absorption, linear and circular dichroism spectra of the reconstituted LHCIIb complexes, in which the negatively charged amino acids in the luminal loop were exchanged to neutral ones (E94G, E107V and D111V). The mutations influenced the pigment binding and the molecular architecture of the complexes. Exchanging E94 to G destabilized the 3₁₀ helix in the luminal loop structure and led to an acquired pH sensitivity of the LHCIIb structure. We conclude that these amino acids are important not only for pigment binding in the complexes, but also in stabilizing the conformation of LHCIIb at different pHs.

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1. Introduction

The major light-harvesting chlorophyll (Chl) *a/b* complex of photosystem (PS) II (LHCIIb) contains more than half of the Chls in granal chloroplasts. LHCIIb collects excitation energy and transfers it to the reaction centers, primarily to PSII. It maintains the lateral sorting of the protein complexes between the stromal and granal regions of the thylakoids and mediates grana stacking [1–4]. It regulates the energy distribution between PSII and PSI via LHCIIb phosphorylation that triggers state transition [5,6]. It also plays a key role in protecting the photosynthetic apparatus from photodamage by

dissipating excessive excitation energy into heat mainly via the energy-dependent non-photochemical fluorescence quenching (qE) [4,7,8]. It has been proposed that LHCIIb can switch between light harvesting and energy dissipation under the continuously changing environmental conditions. Although the exact roles of the various protein components of qE are still unresolved, it is clear that carotenoids as quenchers and conformational changes in light-harvesting antenna proteins of PSII play key roles in this ΔpH-dependent regulatory process [4,8–11].

LHCIIb is found to be in a trimeric form both in vitro and in vivo [12,13]. These complexes are found in two large domains: extended ordered arrays of LHCII-PSII supercomplexes and LHCII-only macrodomains [3,14]. The LHCII-containing chirally ordered macrodomains appear to be capable of undergoing reversible structural changes under illumination [15]. Under certain conditions (e.g. high temperature or excessive light) LHCIIb can undergo trimer–monomer transition [16,17]. Monomerization of LHCIIb may be of physiological significance for its proteolytic degradation under physiological conditions [18].

LHCIIb binds 14 Chls (8 Chl *a*, 6 Chl *b*) and 4 carotenoid molecules. The complex also contains at least 2 lipids. It is organized in three transmembrane (TM) α-helices (helices A, B, C), an amphiphilic α-helix

Abbreviations: CD, Circular dichroism; Chl, chlorophyll; DM, *n*-dodecyl-β-D-malto-side; LD, linear dichroism; LHCIIb, the major Chl *a/b* complexes of photosystem II; Lut, lutein; MD, molecular dynamics; Neo, neoxanthin; OG, *n*-octyl-β-D-glucopyranoside; PAGE, Partially denaturing polyacrylamide gel electrophoresis; PG, phosphatidylglycerol; PS, photosystem; qE, non-photochemical fluorescence quenching; RMS, root-mean-square; RMSD, RMS deviations; RMSE, RMS fluctuations; SDS, sodium dodecyl-sulphate; TM, transmembrane α-helices; WT, wild type

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(helix D) and a short 3_{10} -helix (helix E) at the luminal side. Between helix E and the TM helix C, there is a peptide folding into two anti-parallel strands which are stabilized by an inter-strand ionic pair (D111–H120) and H-bonds [19]. Mutagenesis analysis showed that the anti-parallel strands play important roles in the structural and functional relationship of LHClIb [20]. The structure between the TM helices B/C is very important for regulating the tertiary structure of LHClIb, because, as proposed by the two-stage folding mechanism of Popot and Engelman [21], the links between the TM α -helices contribute to the formation of the tertiary structure. Mutagenesis analysis has shown that the folding state of the luminal loop influences the thermal stability of the reconstituted monomer [22].

Due to the build-up and decay of the pH gradient during photosynthesis, the luminal loop of LHClIb is exposed to broadly variable pH environments. Hence, the negatively charged amino acids in the luminal loop might play important roles in adjusting the structure and functions of LHClIb. Further, one of the key regulatory mechanisms of the light-harvesting antenna functions and of LHClIb in particular, the energy-dependent non-photochemical quenching is dependent on the acidification of the thylakoid lumen. In LHClIb, there are three negatively charged amino acids (E94, E107 and D111) in the luminal loop between TM helices B/C, two of which (E94, D111) form ion pairs (E94–Q103, D111–H120) which, according to the crystal structure, are very important for keeping the secondary structure of the lumen-exposed region between the TM helices B/C [19]. However, less is known about how these negatively charged amino acids affect the organization of the pigment molecules and the structural stability of the complexes in neutral and low pH environments.

In order to study the roles of the negatively charged amino acids in the luminal loop, we have investigated the structural characteristics in detergent environment of three different mutant versions of LHClIb, E94G, E107V, and D111V, each with one negatively charged amino acid being exchanged to a non-charged one. We found that the negatively charged amino acids in the luminal loop play important roles not only in pigment binding but also in the structural response to low pH conditions.

2. Materials and methods

2.1. *Lhcb1* mutants

Mutants of *lhcb1*2* (E94G, E107V, D111V) were obtained by random mutagenesis [23] of a fragment (coding for the luminal loop domain) of an expression plasmid expressing the mature pea Chl *a/b* protein gene *Lhcb1*2* ("AB80") [24,25]. In order to add a C-terminal hexahistidyl tag (His₆-tag) to these proteins, the 447 bp EcoRI–BstEII fragments of their coding sequences, containing the mutations, were substituted for the corresponding segment in the plasmid C3.2 h [26] coding for the His₆-tagged wild type (WT) LHClIb. The plasmids were transformed into *E. coli* JM 101 and the proteins were overexpressed as described in [25].

2.2. *In vitro* reconstitution

Monomeric LHClIb was reconstituted by the detergent-exchange method [27]. 1 mg *Lhcb1* protein dissolved in 2 mL solubilising buffer containing 100 mM TRIS–HCl (pH 9.0), 5 mM ϵ -amino-capronate, 1 mM benzamidine, 12.5% (w/v) sucrose, 2% sodium dodecylsulphate (SDS) and 20 mM β -mercaptoethanol, was mixed by vortex with thylakoid pigments, containing 1.5 mg Chl, dissolved in 200 μ L ethanol [25]. Then 10% (w/v) *n*-octyl- β -D-glucopyranoside (OG) (Gerbu Biotechnik, Gailberg, Germany) was added to a final concentration of 1%. After 5 min incubation at room temperature, 1 M KCl was added to a final concentration of 0.2 M; the mixture was incubated on ice for 5 min and then SDS was pelleted by 2 min centrifugation at 23,000 g at 4 °C. The supernatant was collected and incubated on ice for 15 min and the mixture was centrifuged again (23,000 g, 5 min, 4 °C). The

supernatant was applied to a Ni²⁺-chelating Sepharose fast-flow column (chelating Sepharose fast flow: Amersham Biosciences, Uppsala Sweden; column: 0.8 cm \times 4 cm, Bio-Rad, Hercules, CA, U.S.A.) equilibrated with OG buffer, containing 1% (w/v) OG, 100 mM TRIS–HCl (pH 9.0), and 12.5% (w/v) sucrose, then incubated in dark for 30 min at 4 °C. The column was washed with 1 mL OG buffer and 2 mL TX buffer (0.05% (w/v) Triton X-100 (Roche, Basel, Switzerland), 0.1 mg/mL phosphatidylglycerol (PG) and 100 mM TRIS–HCl (pH 7.5)). Complexes were eluted with elution buffer (0.05% Triton X-100, 0.1 mg/mL PG, 10 mM TRIS–HCl (pH 7.5) and 300 mM imidazol). The material eluted from the Ni²⁺-chelating column was further purified by sucrose-density-gradient ultracentrifugation (\sim 0.1–1 M sucrose, 0.1% (w/v) *n*-dodecyl- β ,D-maltoside (DM), 5 mM TRIS–HCl, pH 7.5) at 450,000 g (SW-41 rotor, Beckman, Palo Alto, CA) for 17 h at 4 °C. The bands corresponding to LHClIb trimers and monomers were collected. In order to investigate the possible role of Mg²⁺ on the structural stability of different LHClIb mutants, reconstitution was carried out in the presence of Mg²⁺. In that case, 2 mM MgCl₂ was added to all buffers throughout the whole reconstitution and isolation procedures.

2.3. Pigment analysis

Sucrose-density-gradient bands containing trimeric LHClIb were analyzed for their pigment contents by HPLC. The pigments were extracted with 2-butanol according to the method described in [28]. 10 μ L 2-butanol extract was mixed with 20 μ L 80% (v/v) acetone, applied to an RP-18 HPLC column (Chromolith SpeedROD, Merck, Darmstadt, Germany) in a HPLC and eluted by a gradient from 70%–100% (v/v) acetone, flow rate 1.5 mL/min. The pigments were quantified as described in [25]. For comparison of mutant with WT, the pigment composition was calculated based on pigment per 2 lutein (Lut) level, assuming that each protein binds approximately 2 Lut molecules.

2.4. Partially denaturing polyacrylamide gel electrophoresis (PAGE)

10% polyacrylamide gels were run in a running buffer containing 12 mM TRIS, 0.15% (w/v) Deriphat (Henkel, Ambler, PA, USA) and 48 mM glycine.

2.5. Circular (CD) and linear dichroism (LD) measurements

CD and LD spectra were recorded with a modified CD6 dichrograph (Jobin-Yvon, France) that contained an additional modulator board optimized for LD measurements. The spectra were plotted normalized to the maximal amplitude in the red region. For LD measurements the samples were embedded in polyacrylamide gel, containing 5% acrylamide:bis-acrylamide (30:1), polymerized with 0.2% ammonium persulfate and 0.2% *N,N,N',N'*-tetramethylethylenediamine, and aligned by two-dimensional squeezing [29]. For CD measurements, the optical path length was 1 cm and the Chl content was adjusted to 10 μ g/mL.

2.6. Absorption spectra

Absorption spectra were measured with a UV-1601 spectrophotometer (Shimadzu, Japan) at room temperature.

2.7. pH treatment

In order to check how the conformations of the WT LHClIb and its different mutants change in media with different pHs, the CD spectra of WT and mutant LHClIb species measured at low pH (pH 5.4) were compared with those at neutral pH (pH 7.5). For this purpose, the eluates of the Ni²⁺-chelating sepharose column were loaded onto a sucrose density gradient (\sim 0.1–1 M sucrose, 0.1% (w/v) DM, and 20 mM phosphate buffer (pH 5.4 or 7.5, respectively)). The complexes were centrifuged at 485,000 g (SW-60 rotor, Beckman, Palo Alto, CA) for 17 h

at 4 °C. The bands corresponding to LHCIIb trimers were collected and pigment stoichiometries and the CD spectra were measured.

2.8. Heat treatment

The sucrose-density-gradient solution containing trimeric LHCIIb, either in presence or in the absence of MgCl₂, was incubated for 30 min at different temperatures (40, 50, 55 and 60 °C) and then used for PAGE and CD measurements.

2.9. Molecular dynamics simulations

The molecular dynamics (MD) simulations were performed using the GROMACS package version 3.2.1 [30,31] with the GROMOS96 force field [32,33]. The original structure of luminal loop (A86–I124) in LHCIIb was extracted from the crystal structure (PDB code, 1RWT). MD simulations were performed on two models, the luminal loop of the WT LHCIIb and its E94G mutant. Both models were protonized before MD simulation to reflect the acidic environment. Afterwards, the models were solvated with the simple point charge water model [34]. To maintain the systems at a constant temperature of 300 K, the Berendsen thermostat [35] was applied using a coupling time of 0.1 ps. The pressure was maintained by coupling to a reference pressure of 1 bar. A coupling time of 1.0 ps was used for the simulations in bulk water. The values of the isothermal compressibility were set to 4.5×10^{-5} bar⁻¹ for water simulations. All bond lengths including hydrogen atoms were constrained by the LINCS algorithm [36]. Electrostatic interactions between charge groups at a distance less than 9 Å were calculated explicitly; long-range electrostatic interactions were calculated using the particle-mesh Ewald method [37] with a grid width of 1.2 Å and a fourth-order spline interpolation. A cutoff distance of 10 Å was applied for the Van der Waals interactions. The simulation cell in both systems was a rectangular periodic box; the minimum distance between the protein and the box walls was set to more than 20 Å so that the protein does not directly interact with its own periodic image given the cutoff in every system. Numerical integration of the equations of motion used a time step of 2 fs with atomic coordinates saved every 10 ps for analysis. Four water molecules were replaced by four Cl⁻ ions to neutralize the simulation systems. The position restraints on two terminals of the simulated systems (A86 and I124) were used to approximate the movement of the terminal residues. Finally, two 9.7-ns MD simulations were performed on the two models.

3. Results

For LHCIIb, the substitution of any one of the three negatively charged amino acids, E94, E107 and D111, with non-charged amino acids, G, V and V, respectively, did not significantly influence the ability of the proteins to fold into functional monomeric or trimeric complexes, and like the WT proteins, all the mutants were capable of binding pigments *in vitro*.

3.1. Pigment stoichiometries of LHCIIb mutant

As shown in Table 1, the WT LHCIIb bound, on average, 6.8 Chl *a*, 5.6 Chl *b* and nearly one Neo per 2 Luts. Since it is difficult to separate

Table 1
Pigment stoichiometry in WT and mutant LHCIIb trimers determined by HPLC

Type of LHCIIb	Neo ^a	Chl <i>a</i>	Chl <i>b</i>	Chl <i>a</i> /Chl <i>b</i>
WT	0.85 ± 0.035 ^b	6.82 ± 0.32	5.56 ± 0.19	1.22 ± 0.062
E94G	0.67 ± 0.017	6.61 ± 0.03	5.29 ± 0.11	1.25 ± 0.024
E107V	0.41 ± 0.043	7.60 ± 0.37	5.73 ± 0.37	1.33 ± 0.127
D111V	0.86 ± 0.022	7.58 ± 0.07	6.36 ± 0.13	1.19 ± 0.025

^a mol pigments per 2 mol Lut.

^b Mean ± SD from 6–10 independent experiments.

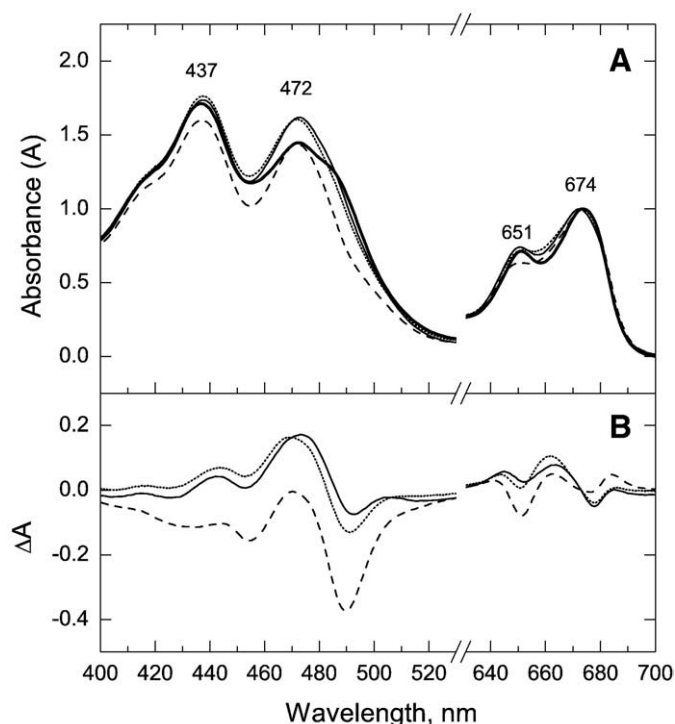


Fig. 1. Absorption spectra (A) and difference spectra (B) of reconstituted LHCIIb trimers measured at room temperature. All absorption spectra are normalized to the red-most maximum. WT, thick line; E94G, solid line; E107V, dashed line; D111V, dotted line.

the reconstituted LHCII complexes from the non-pigmented apoprotein, we estimated the pigment stoichiometries based on the assumption that each LHCIIb monomer binds 2 Luts [19], which are obligatory for maintaining a stable complex. In this way, the pigment stoichiometries can be compared between WT and the mutated LHCIIb complexes. It has been demonstrated via detailed spectroscopic and biochemical analyses that the properties of recombinant LHCIIb, including the orientation of pigments, the excitonic interaction between chromophores, organization and folding of the reconstituted complexes are all similar to “native” LHCIIb although the recombinant complexes may contain fewer Chls [38].

Interestingly, pigment binding and stoichiometry were noticeably affected by the mutations of negatively charged amino acids in the luminal loop, although the pigment binding sites are distant from the mutated positions. In particular, the mutations not only influenced the Neo binding, but also altered the Chl stoichiometries of LHCIIb. E107V contained only half of the Neo found in the WT LHCIIb and E94G about three fourths. D111V bound almost as much Neo as the WT. Notably, in comparison to the reconstituted WT, the mutations in D111 and E107 caused the complexes to bind more Chls: the former bound nearly two more Chls (1 Chl *b* and 1 Chl *a*), and the latter nearly one more Chl *a*. The apparent increase in Chl binding in the mutants, discussed further below, is not likely to be caused by some loss of Lut because no corresponding increase was observed (not shown) of other carotenoids that can replace Lut [39]. We can only measure about 0.1 violaxanthin in each monomer, because it is a loosely bound pigment in LHCIIb, which can be removed by very mild detergents [40]. No significant difference in violaxanthin binding was detected, either between the WT and the mutants, or under different pH conditions.

3.2. Absorption spectra

The absorption spectra of the different LHCIIb mutant complexes, normalized to the Chl *a* absorption maxima in the Qy region, are presented in Fig. 1. The main absorption maximum of the

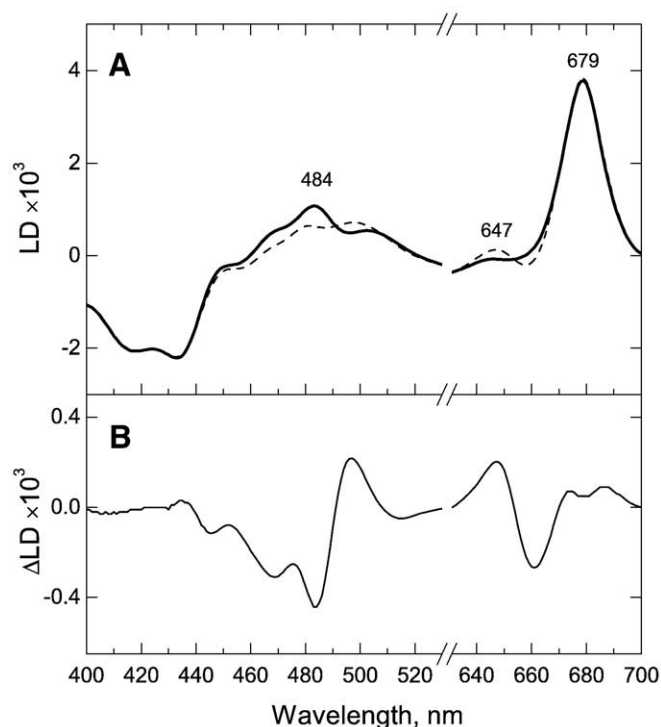


Fig. 2. Linear dichroism spectra of reconstituted LHCIIb trimers measured at room temperature. The spectra are normalized to the maximum in the red. (A) LD spectra. WT, solid line; E107V, dashed line. (B) Difference spectrum of the spectra in A.

reconstituted WT LHCIIb in the red region was found at 674 nm (Chl *a* absorption) with a shoulder at 651 nm (Chl *b* absorption). The Chl *b* absorption at 651 nm in E107V was decreased, relative to the principal absorbance band at 674 nm (Fig. 1, dashed line), which is in accordance

with the alterations in the Chl *a/b* ratio (Table 1). Of note, the absorption spectrum of E107V in the red region resembled that of Neo-deficient LHCII [41,42].

The mutations more significantly influenced the absorption of the complexes in the Soret region (440–520 nm). The absorption bands of the mutant LHCIIbs were narrower than those in the WT complexes. The difference spectrum (Fig. 1B) shows a decrease in the absorption at 488 nm of E107V, which presumably is due to the loss of Neo [41,42].

3.3. Linear dichroism spectra

In order to investigate how the mutations influence the orientation of the absorption transition dipole moments of the pigment molecules, we measured LD spectra of different LHCIIb mutants and compared it with the WT. The LD spectra of the WT and the E107V mutant, normalized to the LD maximum in the red, are presented in Fig. 2. The LD spectrum of WT in the red region is characterized by a positive peak at 679 nm and a small shoulder at 647 nm. The main characteristics of the LD spectrum of the WT LHCIIb are in good agreement with earlier reports [43] and, similarly to those data, can be correlated with the crystal structure [19]. It can be seen that the mutations did not affect the overall shape of the LD spectrum. Nevertheless, they induced minor but well discernible alterations in the spectra. In comparison to the WT complexes, the LD spectra of the mutants exhibited an increased amplitude of the 647 nm band. This effect was observed in all three mutants but to a lesser extent in E94G and D111V (not shown). The LD spectra of the mutant proteins compared with WT LHCIIb were different in the Soret region as well. E107V showed clear increase at 498 nm and a decrease at 484 nm compared to the WT LHCIIb, along with the decrease of some minor bands between 440 and 470 nm (Fig. 2B). In agreement with the data of Croce et al. [43], the decrease in the LD amplitude around 484 nm can be accounted for by the partial loss of Neo in E107V. Neo-deficiency, via changes in the orientation and/or absorbance of the respective dipoles, has also been shown to induce changes in the Chl *b*

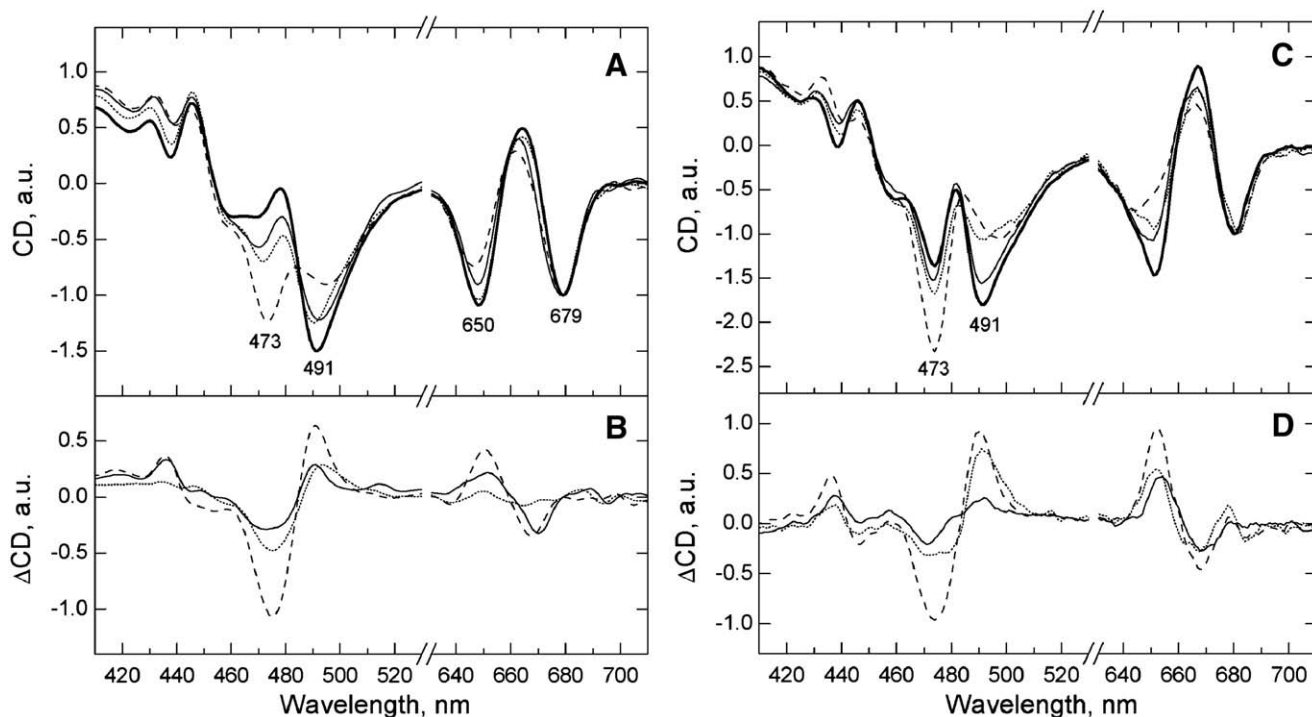


Fig. 3. Circular dichroism spectra and difference spectra of reconstituted LHCIIb monomers (A and B) and trimers (C and D) measured at room temperature. The spectra are normalized to the red-most absorption. (A) CD spectra of monomeric complexes; (B) difference spectra of monomeric complexes; (C) CD spectra of trimeric complexes; (D) difference spectra of trimeric complexes. WT, thick line; E94G, solid line; E107V, dashed line; and D111V, dotted line.

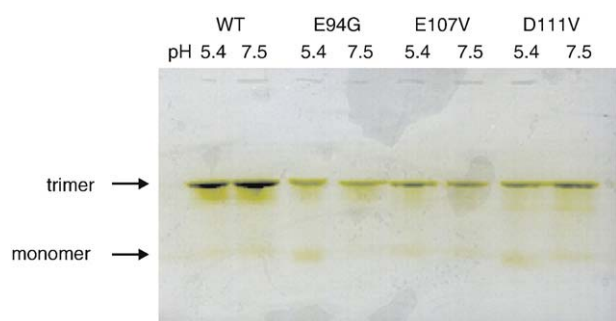


Fig. 4. Partially denaturing polyacrylamide gel electrophoresis of reconstituted WT and mutant LHCIIb in neutral pH (pH=7.5) and acidic pH (pH=5.4) environments. For the same mutants at the two pHs, similar protein amounts were loaded on the gel.

region due to losses in Neo-Chl *b* interactions [41]. Hence, these changes in the red and additional variations between about 440 and 470 nm and around 495 nm indicate conformational changes in the Neo-domain in E107V, which at least in part can be accounted for by a partial loss of Neo.

3.4. Circular dichroism spectra

In order to investigate the influence of the negatively charged amino acids in the luminal region on the structural organization of LHCIIb and the pigment–pigment interactions in LHCIIb, CD spectra of the mutants were compared with the WT LHCIIb, and also to LHCIIb reconstituted without Neo [44]. Fig. 3 shows the CD spectra of WT and mutant LHCIIb monomers (Fig. 3A, B) and trimers (Fig. 3C, D), normalized to the (–)679 nm band. The CD spectrum of the WT LHCIIb monomers exhibits negative bands at 491, 650 and 679 nm and a weak band at 473 nm. The CD spectrum of the trimers was similar to the monomer spectrum except for the larger amplitude of the (–)473 nm band, which is specific for trimeric complexes [45]. A shoulder at 640 nm also appeared, though it was not always clearly visible. The CD spectra of all three mutants were markedly different from that of the WT, especially in the Soret region. The ratio of the amplitudes of the negative CD bands at (–)473 nm and (–)493 nm (CD_{473}/CD_{493} signal) increased to different extents in different mutants, both in the monomers and the trimers. Hobe et al. [44] have shown that the CD_{473}/CD_{493} signal is negatively correlated with the amount of bound Neo in the complexes. In our experiment, this ratio in different mutants presented an order of $E107V > E94G > D111V$. In agreement with the finding of Hobe et al., the mutant with the lowest Neo content (E107V) had the highest CD_{473}/CD_{493} signal ($CD_{473}/CD_{493}=2.2$). However, the ratio was substantially higher in D111V than in the WT ($CD_{473}/CD_{493}=1.56$ in D111V vs 0.75 in WT), although the content of Neo in D111V was not reduced compared with the WT (Table 1). This result is similar to observation of Liu et al. [20].

Besides the changes in the amplitude of the (–)473 nm band, the trimer specific negative band at 640 nm [45] became more dominant in the Chl *b* region in the mutants, eminently in E107V and D111V. In E107V, the (–)493 nm band was shifted to 498 nm both in the trimers and the monomers while in D111V a new band appeared at 498 nm.

Table 2

Pigment stoichiometry in WT and mutant LHCIIb trimers determined by HPLC after low pH (pH=5.4) treatment

Type of LHCIIb	Neo ^a	Chl <i>a</i>	Chl <i>b</i>	Chl <i>a</i> /Chl <i>b</i>
WT	0.92±0.02 ^b	7.39±0.19	6.13±0.23	1.21±0.08
E94G	0.79±0.01	7.21±0.12	6.20±0.18	1.16±0.01
E107V	0.37±0.02	7.41±0.41	5.80±0.34	1.28±0.14
D111V	0.82±0.01	7.38±0.01	6.19±0.02	1.19±0.01

^a mol pigments per 2 mol Lut.

^b Mean±SD from 4–6 independent experiments.

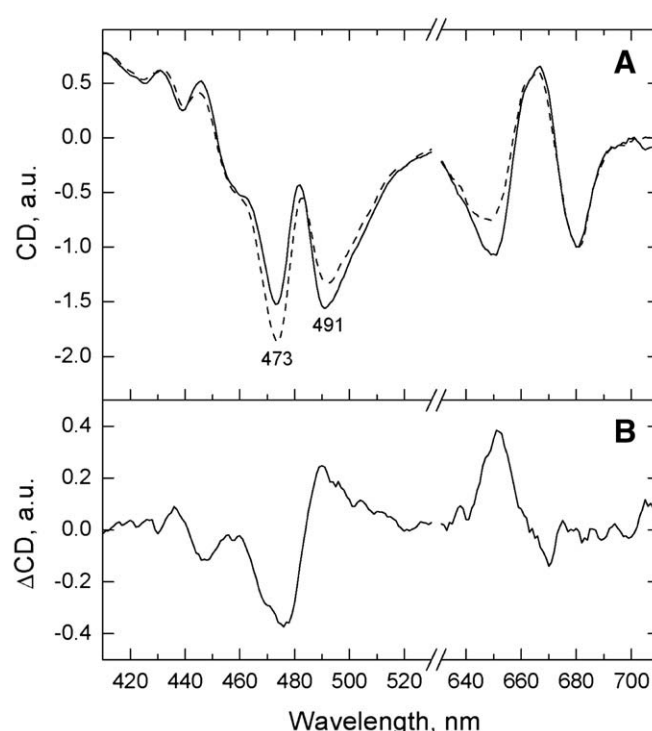


Fig. 5. Circular dichroism spectra of reconstituted trimeric LHCIIb measured at different pH environments. The CD spectra are normalized to the red-most maximum. (A) CD spectra measured in neutral (pH 7.5) and acidic (pH 5.4) environment, Neutral pH, solid lines; acidic pH, dashed lines. (B) Difference CD spectra of the spectra in A.

The same phenomenon appeared also in the Neo-deficient LHCIIb observed by Hobe et al. [44].

3.5. pH-dependent conformational changes

Since negatively charged amino acids in the luminal loop are potential targets for protonation during lumen acidification, it is of special interest to study the conformation of different LHC IIb mutants at neutral and acidic pH. Different recombinant LHCIIb species were ultracentrifuged overnight in near-neutral (pH 7.5) and acidic (pH 5.4) media. From the partially denaturing PAGE results, we can see that none of the LHCIIb mutants showed significant dissociation after the ultracentrifugation at low pH (Fig. 4). Pigment stoichiometries and CD spectra were also measured using the sucrose gradient band corresponding to trimers. Table 2 presents the pigment stoichiometries after the low pH treatment. From a comparison of these data with Table 1, we can see that there are no significant changes in pigment compositions upon lowering the pH.

Fig. 5 presents the CD spectra of WT and different LHC IIb mutations at neutral and acidic pH. The CD spectra of WT, D111V and E107V showed only minor changes after the low pH treatment. In contrast, E94G exhibited a strong increase in the amplitude of the (–)473 nm CD band, despite the fact that there was not major loss in Neo (Table 2). Hence, it appears that the E94 residue is important in maintaining the conformation for LHCIIb at different pHs.

4. Discussion

4.1. Stability of LHCIIb trimers is not affected by the mutations of negatively charged amino acids in the luminal loop

It has been shown earlier that exchanging one of the negatively charged amino acids in the luminal loop did not hamper the ability of the apoproteins to bind pigments and be reconstituted to functionally active complexes [22]. We also found that the thermal stability of the

trimers, monitored by CD in the visible range, was not at all affected by the mutations of the negatively charged amino acids of the luminal loop, neither in the presence nor in the absence of MgCl_2 (not shown), showing that these residues and Mg^{2+} ions which might be bound at these sites do not play stabilizing role in trimers. Earlier observations showed that the monomeric pigment–protein complexes of the same mutant proteins exhibited a lower thermal stability than the WT [22].

4.2. The negatively charged amino acids in the luminal loop are important for pigment binding and conformation

We found that exchanging any of the negatively charged amino acids in the luminal loop altered the pigment stoichiometry of the reconstituted complexes (Table 1), despite the fact that these residues are distant from the pigment ligands [19]. One evident effect is that two mutants (D111V and E107V) bound more Chls (Table 1) than the WT. This is not likely to be due to a partial loss of Lut, because we have not observed the corresponding increase of other carotenoids that can replace Lut [39]. It should also be noted that the recombinant LHCIIB studied here was isolated under sufficiently stringent conditions that less than the full complement of Chl molecules was bound per 2 Lut, presumably some of the less tightly bound Chls were dissociated during the isolation procedure. Consequently, the increased Chl/Lut ratio in the mutants may be due to slightly higher affinities of one or several more labile Chl binding sites.

Spectroscopic data in this study revealed that the orientation and dipole–dipole interactions of the transition moments of some pigments in the mutants were changed as well. Since the luminal loop connects helices B and C, it seems plausible that pigments localized within the scaffold created by these two helices are primarily affected. According to the crystal structure of LHCIIB, this domain contains five of the six Chl *b* molecules, one Chl *a* and the carotenoid Neo (Fig. 6). Changes in the conformation of the luminal loop may

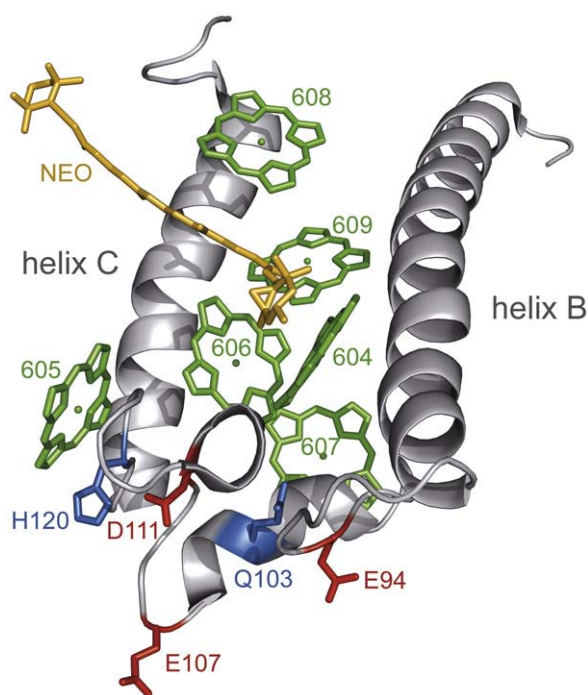


Fig. 6. Representation of the helices B/C domain of LHCIIB and the connecting luminal loop based on the crystal structure of Liu et al. [19]. The Chls are represented only with their porphyrin (in green) and labelled according to the nomenclature of Liu et al. [19]. The negatively charged residues in the luminal loop (E94, E107, D111) are shown in red, and their ion-pair counterparts (Q103, H120) are shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

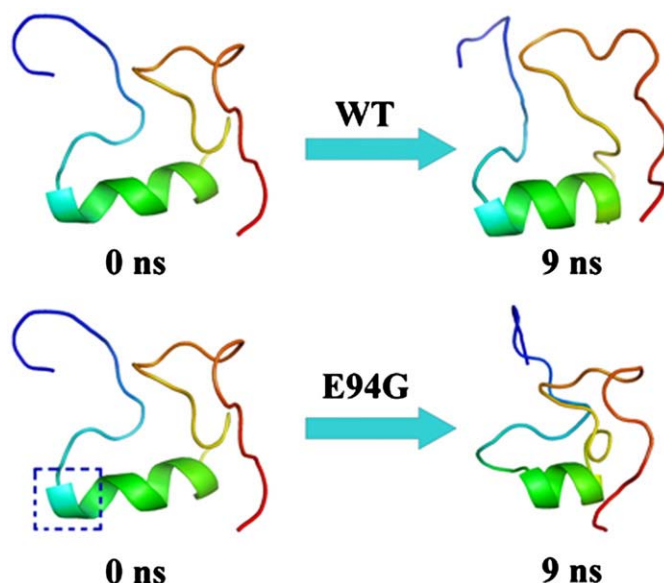


Fig. 7. The comparison of the conformation at the 0 ns and 9 ns in the MD simulation of the luminal loop in WT LHCIIB and its E94G mutant.

affect those pigments that form direct interactions with the amino acids inside the loop, e.g. the Neo, Chl $\alpha 604$, and Chl $\alpha 605$, which form hydrogen bonds with Y112, L113 and V119, respectively.

The most striking effect on pigment binding is the loss of Neo, especially by the mutant E107V, in which the amount of Neo was reduced by half. It is surprising that the mutation in E107V influenced the Neo binding and Chl conformation so strongly despite the fact that it is the outermost of the three negatively charged amino acids (Fig. 6) and does not have any direct interaction with any pigment, according to the high resolution crystal structures [4,19]. E107 is located in the loop between helix E and the anti-parallel strands upstream of helix C. Because of its negative charge and the hydrophilic character, E107, together with the neighbouring amino acids (SEGG), forms a hydrophilic loop which extrudes with its carboxylic acid group into the lumen. Exchanging E107 to V not only eliminates one negative charge but also changes the hydrophilic character in the region. This may influence the orientation of Y112 which interacts with Neo via a hydrogen bond and then, in turn, alter the Neo conformation in the complexes and weaken the Neo binding.

The loss of Neo led to drastic changes in the Soret region of the CD spectra, particularly, the increase of the amplitude of the negative CD band at 473 nm, as reported by Hobe et al. for a reconstituted WT LHCIIB lacking Neo [44]. However, the changes of the (–)473 nm CD band are not strictly correlated with the Neo content in our mutants (Fig. 3 and Table 1). Consequently, changes other than loss of Neo contribute to the altered CD signal in the Soret region. Indeed, based on theoretical analyses by Georgakopoulou et al. [46], it was concluded that the absence of Neo could be satisfactorily mimicked by omitting the Neo-related bands. In other terms, the removal of this xanthophyll per se caused no significant conformational changes in the protein. Further, according to the model of Georgakopoulou et al., not only the dipole–dipole interactions of the transition moment of Lut or Neo with Chls contribute to the CD signals in the Soret region but Chls *b* are also involved and interactions among the pigments in different monomeric subunits play a key role in the $\text{CD}_{473}/\text{CD}_{493}$ signal. Based on these data, it is plausible to propose that the mutation in the luminal loop caused changes in the conformation of the Neo-domain. It could also be seen that the conformational changes already occurred in the monomeric complexes, as revealed by changes in the CD spectra, especially at (–)473 nm, of the different monomeric LHCIIB mutants (Fig. 3).

4.3. The negatively charged amino acid E94 plays an important role in maintaining the secondary structure of the luminal loop at low pH

The importance of residue E94 in maintaining the structure of the luminal loop could be addressed by MD simulations on the WT luminal loop of LHCIIb and its E94G mutant. The comparison of root-mean-square deviations (RMSD) of the C α atoms versus simulation time between the WT and E94G demonstrated that the conformations experienced dramatical change when residue E94 is mutated to G, as shown in Supplementary Fig. 1. The RMS fluctuations (RMSF) of residues (Supplementary Fig. 1B) disclosed that segments G93–A95 and W97–Q103 become significantly more flexible after E94 was mutated into G. The cause of this phenomenon could be inferred from the MD trajectory. The whole MD simulation trajectory demonstrated that the residue E94 could form important hydrogen bonds with the residues S102, Q103, and S106 in the 3₁₀ helix, as shown in Supplementary Figs. 2A–D. These hydrogen bonds may contribute to the stability of E94 and its nearby residues, as well as residues near the area S102–Q103 which is part of 3₁₀ helix. By contrast, when residue E94 is mutated to G, these hydrogen bonds disappear, so these residues will be more flexible. In our simulation, part of the 3₁₀ helix was unstable and eventually became destroyed at the end of the simulation if E94 was mutated into G. Consequently, the two loops on either side of the α -helix approach and interact with each other (Fig. 7 and Supplementary Fig. 3B), while in the WT, the 3₁₀ helix remained stable (Supplementary Fig. 3A), demonstrating the importance of this residue in keeping the secondary structure of the luminal loop.

Based on these data, we propose that the negatively charged amino acids in the luminal loop of LHCIIb play important structural roles in pigment binding and also in keeping a stable LHCIIb conformation under low pH conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.bbabo.2008.08.009.

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