Structural and Functional Analysis of the Antiparallel Strands in the Luminal Loop of the Major Light-harvesting Chlorophyll $a/b$ Complex of Photosystem II (LHCIIb) by Site-directed Mutagenesis*

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The light-harvesting chlorophyll $a/b$-binding protein of photosystem II (LHCIIb) fulfills multiple functions, such as light harvesting and energy dissipation under different illuminations. The crystal structure of LHCIIb at the near atomic resolution reveals an antiparallel strands structure in the luminal loop between the transmembrane helices B/C. To study the structural and functional significances of this structure, three amino acids (Val-119, His-120, and Ser-123) in this region have been exchanged to Phe, Leu, and Gly, respectively, and the influence of the mutagenesis on the structure and function of LHCIIb has been investigated. The results are as follows. 1) Circular dichroism spectra of the mutations reveals that the antiparallel strands in the luminal region are very important for adjusting pigment conformation in the neoxanthin domain of LHCIIb. Although the mutagenesis causes only a slight loss of the Neo binding in the complexes (V119F, 0.09; S123G, 0.19; and H120L, 0.27), it imparts remarkable changes to the pigment conformation. 2) Substituting Ser-123 with Gly results in a higher susceptibility to photodamage, an increased tendency to aggregate, and enhanced fluorescence quenching induced by the medium acidification. These results demonstrate that this antiparallel strands domain plays an important role in regulating the pigment conformation and in adjusting the aggregation and the fluorescence yield of LHCIIb.

In addition to absorbing sunlight and delivering excitation energy to the photosystem (PS)$^3$ II core complex, the major light-harvesting chlorophyll (Chl) $a/b$-binding protein (LHCIIb) of PSII plays an important role in the adaptation processes under excessive light conditions (1, 2). Under strong light conditions, the function of LHCIIb is to regulate a balanced distribution of absorbed energy between different PSs through its ability to migrate between PSI and PSII (3), or to dissipate overexcited energy (non-photochemical quenching (NPQ)) via different mechanisms, e.g. its conformational change (4), the xanthophyll cycle (5, 6), or a charge transfer heterodimer complex between Chl and zeaxanthin (7). The proton gradient over the thylakoid membrane (ΔpH) caused by reduced carbon dioxide fixation under strong light might be one of the sensors that initialize the protonation of integral photosynthetic proteins, which in turn, increases NPQ as a consequence (8). It has been observed that the two negatively charged residues (Glu) in the luminal region of PsbS are protonation sites, which induce a series of physiological processes and then increase the NPQ under excessive light conditions (9, 10). LHCIIb is claimed to be one of the candidates for protonation because there are several potential protonation sites in the luminal region (11). It has been suggested that LHCIIb exists in two different states (the U state: unquenched state, and the Q state: quenched state) (12). Protonation controls the balance of the two states of LHCIIb (13). Unfortunately, solid evidence for this proposal is still lacking. More research is needed to elucidate the molecular mechanism regulating the different functions of LHCIIb.

As is revealed by the LHCIIb crystal structure at near atomic resolution (5, 14), LHCIIb is composed of three homo- or hetero monomers, each binding 14 Chls (8 Chl$^a$, 6 Chl$^b$), 4 carotenoid molecules (2 luteins (Lut), one neoxanthin (Neo), one violaxanthin (Vio) (or zeaxanthin (Zea)), and 2 lipids. The two Luts, which locate in the center of LHCIIb, form an internal cross-brace interacting with helices A and B. The Vio has low affinity to LHCIIb and readily migrates out of LHCIIb during the xanthophyll cycle (15). Binding Neo is a peculiar character-

dodecylsulfate; HPLC, high performance liquid chromatography; LDS, lithium dodecylsulfate; LHCIIb, light-harvesting chlorophyll $a/b$ complexes of PSII; Lut, luteins; OG, octyl-$b$-D-glucopyranoside; PG, 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-1-glycerol; Neo, neoxanthin; NPQ, non-photochemical quenching; TM, transmembrane; Vio, violaxanthin; WT, wild type; Zea, zeaxanthin.

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† The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1 and S2.

‡ The abbreviations used are: PS, photosystem; CD, circular dichroism; Chl, chlorophyll; DCCD, N,N′-dicyclohexylcarbodiimide; DM, dodecyl $b$-mal-

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istic of LHClII because 90% of thylakoid Neo is bound to LHClIb trimers (16). Neo has the lowest efficiency of energy transfer to Chl in all carotenoids (17), but it is a good scavenger for singlet O$_2^*$, which is very important for photoprotection in PSII (18). It has been observed with recombinant LHClIb that Neo is subject to reversible dissociation under different high ambient temperatures despite its high affinity to LHClIb (19). In *Cucurbita reflexa*, a plant that does not synthesize Neo, the N1 binding position could be replaced by 9-cis Vio (20). The isomerism of all-trans Vio to 9-cis Vio, which replaces 9-cis Neo in the N1-position, could easily be induced under elevated temperatures in native LHClIb, isolated from winter rye leaves (21). It suggests that a 9-cis-5,6-epoxy-3-hydroxy carotenoid is required for this position.

Monomeric LHClIb is organized as three transmembrane (TM) α-helices (helices A, B, C) and one amphipilic α-helix (helix D) and an amphipathic short 3$_{10}$-helix (helix E) at the luminal side. Between helices E and C, the structure folds into two antiparallel strands, which are stabilized by an interstrand ionic pair (Asp-111–His-120) and other H-bonds (14). It is proposed that the luminal region is not only a connector between the TM helices B and C of LHClIb, but also a switch that regulates the multiple functions of LHClIb (22). The aim of this work was to investigate the structural and functional significances of the antiparallel strands structure between the TM helices B/C. For this purpose, three amino acids (Val-119, His-120, and Ser-123) in the region have been exchanged to Phe, Leu, and Gly, respectively. The changes in pigment stoichiometry, characteristics of optical spectra, capacity for non-radiative dissipation, the characteristics of macro structure in a low detergent medium, and the characteristics of low pH-induced fluorescence lowering of different LHClIb species have been investigated, and the possible functional mechanisms of the motif are discussed in this article.

**EXPERIMENTAL PROCEDURES**

Construction of the Lhcb1 Gene—Lhcb1 cDNA was synthesized using random primers and a RT-PCR kit (Invitrogen, Carlsbad, CA) from total RNA isolated from 14-day-old pea (*Pisum sativum* L.) seedlings. The Lhcb1 gene was amplified from 1 µl of cDNA in a 20-µl PCR reaction mixture containing 0.2 µm forward primer 5′-C(A/G)ATGGC(T/C)GCTTCATC- (A/C)ATG-3′, and the reverse primer 5′-CC(C/T)TCTTCA-CAAAAAACAAATAGAC-3′ and 0.5 µl of (200 units/µl) Taq DNA polymerase (Promega). Forty cycles of the PCR program (30 s at 94 °C, 30 s at 60 °C, and 120 s at 72 °C) were run, followed by a final extension step at 72 °C for 10 min. The PCR product was cloned into the pMD-18T vector (Takara, Shiga, Japan) and transformed into *Escherichia coli* strain DH5α. The gene was checked by sequencing and aligned with GenBank™ Lhcb1 sequences. The gene entry number is AY845253.

Two restriction sites (NdeI and Xhol) were introduced to the 5′ and 3′ termini of Lhcb1, respectively, via PCR with the forward primers 5′-ACATATGAGGAAAGTCTGCTACCCAC-3′ and the reverse primer 5′-ACTCGAGATTTTCTGGAGAAGTTTGG-3′. The NdeI-Xhol fragments were inserted into the pET-23 (a) expression vector (Novagen) and transferred into *E. coli* strain BL21. By introducing the restriction site Xhol, an additional sequence CTCGAG was added to the reading frame in the 3′-end, which resulted in adding two extra amino acids Leu and Glu to Lhc1. All the genes were checked by sequencing and aligning with GenBank™ Lhcb1 sequences.

**Mutagenesis**—Site-directed mutagenesis to Lhcb1 was carried out with a MutanBEST site-directed mutagenesis kit (Takara Tokyo, Japan). The mutagenesis primers designed for the mutations were as follows: S123G, 5′-TGCTTTGTGCACTGAACC-3′; H120L, 5′-TGCTTTGTGCAAGAACC-3′; V119F, 5′-AGCTTTGTTTATGAC-3′. In the mutant expression clone, the sequences of the entire section, which had been amplified by PCR during the mutagenesis procedure, were verified by DNA sequencing. The apoproteins of different Lhcb1 species were overexpressed and isolated using the method described in Ref. 23.

**Preparation of Recombinant LHClIb**—The Lhcb1 apoproteins were reconstituted with total thylakoid pigments according to the method described in Paulsen et al. (23). The trimeric LHClIb were reconstituted in a Ni$^{2+}$-chelating Sepharose fast flow column (chelating Sepharose fast flow: Amersham Biosciences, Uppsala Sweden; column: 0.8 cm × 4 cm, Bio-Rad) according to the method of Rogl et al. (24).

The reconstituted trimeric complexes were separated by sucrose density gradients centrifugation in a 12.5-ml centrifuge tube containing 0.1–1.0 µm sucrose density gradient, 2.0 mM dodecyl β-D-maltoside (DM) (Sigma), and 5 mM phosphate buffer (pH 7.5), and centrifuged at 230,000 × g and 4 °C for 18 h. The bands corresponding to trimers were collected.

**Pigment Analysis**—Sucrose gradient bands containing trimeric LHClIb were analyzed for their pigment stoichiometry with Waters 600 high performance liquid chromatography (HPLC) (Waters). The pigments were extracted with 2-butyl alcohol according to the method described in Ref. 25. The 2-butyl alcohol extract was applied to an RP-C18 HPLC column (Merck, Germany) and separated with a gradient from 70 to 100% acetone, at a rate of 1 ml/min. Pigments were quantified by comparing the integrated peak areas to calibrated peaks of known pigment quantity.

**Absorption Spectra**—Samples were diluted with the dilution buffer to ~10 µg/ml Chl. The absorption spectra were recorded using a Shimadzu UV-VIS 2550 spectrophotometer (Shimadzu, Kyoto, Japan) at room temperature. The wavelength step was 0.5 nm, the scan rate was 100 nm/min, and the optical pathlength was 1 cm.

**Circular Dichroism Spectra**—CD spectra were recorded in a Jasco 810 spectropolarimeter (Jasco, Tokyo, Japan) at 10 °C. The concentration of the samples was adjusted to OD = 1 at the Q$_s$ transition of Chl a. The spectra were measured from 350 to 750 nm at a scan rate of 100 nm/min. The measurements were repeated four times and averaged.

**Low Temperature Fluorescence Emission Spectra**—The low temperature fluorescence emission spectra were recorded with a Hitachi F-4500 spectrofluorometer (Hitachi, Japan). The samples were diluted to 50 ng/ml Chl with 80% (v/v) glycerol in 50 mM HEPES/NaOH (pH 7.5) and then frozen in a sample tube by immersing the sample in liquid nitrogen. The fluorescence emission spectra were recorded from 600 to 780 nm, with the...
excitation wavelengths set to 480 nm or 436 nm, and slit widths set to 5 nm for excitation and 2.5 nm for emission.

Chlorophyll Fluorescence Yield—Changing of Chl fluorescence yield upon the acidification of the medium was monitored with a Walz PAM 101 fluorometer (Walz, Effeltrich, Germany) with the method described in Ref. 26, with the following modification: concentration of samples was adjusted to 0.5 μg/ml Chl in a medium containing 0.2 mM DM, 5 mM phosphate buffer (pH 7.5) before the measurement. Acidification of the medium was achieved by adding HCl so that the pH of the medium reached the desired values (pH 6.0, 5.5, 5.0, and 4.5), and the Chl fluorescence was monitored continuously with the fluorometer. 30 s after the acidification; the N,N’-dicyclohexylcarbodiimide (DCCD) (Sigma) was injected to the medium, so that the final concentration of DCCD was 0.02 mM. The changes of Chl fluorescence yield were further monitored. The whole measuring procedure was carried out with stirring at 20 °C.

Thermal Stability Analysis—The thermal stability of reconstituted LHCII was measured by observing the decrease of energy transfer from complex-bound Chl b to Chl a upon gradual dissociation of the complexes at 37 °C. The samples were diluted to 10 μg/ml Chl with dilution buffer (12.5% (w/v) sucrose, 2.0 mM DM, and 5 mM phosphate buffer (pH 7.5)). The decay of Chl a fluorescence emission (excitation and emission wavelengths of 480 and 680 nm with slit widths of 5 and 2.5 nm, respectively) was measured in a Hitachi F-4500 spectrofluorometer thermostatted at 37 °C. Measurements were taken for 18 min at 1-s intervals. The course of fluorescence decay was fitted into first-order kinetics (Y = Y0 + A.exp (−kX)). The lifetimes of the complexes at the elevated temperature (37 °C) were calculated based on the rate constant (k). The quality of the fitting was evaluated using residual R² criteria. The R² criterion was set to 0.95 for a good fitting.

Photobleaching—The concentration of the samples was adjusted with the dilution buffer so that the absorption at the maximum in the Qy region was 0.75. The complexes were then illuminated with white light (4000 μE m⁻² s⁻¹) with stirring. After each time interval, the cuvette was removed from the light source, and the absorption spectrum was recorded with a Shimadzu UV-VIS 2550 spectrophotometer (Shimadzu, Kyoto, Japan) in the range of 600–750 nm (27).

Characteristics of Aggregation—LHCIIb aggregation was induced according to the method described in Ref. 28. The LHCIIb samples were diluted with detergent-free buffer so that the DM concentration was 0.049 mM. The sample was then centrifuged at 20,000 × g for 20 min at 4 °C. The pellet containing LHCIIb aggregate was resuspended in 5 mM phosphate buffer (pH 7.5) containing 0.049 mM DM. Fluorescence emission spectra were measured at 77 K as described above.

RESULTS

Pigment Stoichiometries—The three amino acids, Val-119, His-120, and Ser-123, in the antiparallel strands in the luminal loop domain between the TM helices B/C were exchanged to Phe, Leu, and Gly, respectively. All the mutants yielded stable reconstituted monomeric and trimeric complexes, as is shown with partially denaturing polyacrylamide gel electrophoresis (PAGE) (Fig. 1). The mobility of different mutant complexes was similar as that of the wild type (WT) LHCIIb, both in sucrose density gradient (data not shown) and in PAGE.

The pigment stoichiometry of the WT and the mutant LHCIIb were calculated based on the pigment: (2 Luts) ratio as described in Ref. 29 (Table 1). From Table 1, we can see that the reconstituted WT LHCIIb bound 7.61 Chl a, 7.20 Chl b, and 0.98 Neo. Compared with the WT LHCIIb, the three mutants bound about 0.55 less Chl b and 0.09 (V119F), 0.19 (S123G), and 0.27 (H120L) less Neo per monomer, respectively. It is shown from the pigment stoichiometry of different LHCIIb species that the mutagenesis in the luminal loop not only reduced the Neo binding, but also disturbed the Chl b occupancy.

Absorption Spectra—To obtain information on the influence of mutagenesis on the transition energy of the chromophores in LHCIIb, absorption spectra of the recombinant LHCIIb were measured at room temperature (Fig. 2). The absorption maximum in the Qy region was at 674 nm (Qy transition of Chl a). The Qy transition of Chl b appeared at 650 nm as a shoulder. It is clearly shown, that the Qy transition of Chl b at 650 nm reduced strongly in all mutant LHCIIbs. Comparing the absorption spectra of the three mutants with those measured with the recombinant WT LHCIIb lacking Neo (30, 31), it can be seen that the mutant LHCIIb showed a similar decrease at 650 nm to the recombinant WT LHCIIb lacking Neo.

The mutagenesis strongly influenced the absorption spectra in the Soret region. From the difference spectra (Fig. 2B), it is shown that all mutants possess a pronounced decrease in the absorption at 488 nm, which was ascribed to the 0–0 absorption transition of Neo (32). Comparing the absorption spectra with the pigment stoichiometry measured with HPLC, it is apparent that the decrease in the absorbance at 488 nm is related mostly to the amount of Neo, because the amplitude at 488 nm in the difference spectra correlates well with the loss of Neo binding in different mutants.

Circular Dichroism Spectra—Circular dichroism in the visible range reflects the excitonic pigment-pigment interactions, which is a powerful method to study the organizational state of LHCIIb. The CD spectrum of the WT LHCIIb (Fig. 3) exhibits
the negative CD bands at 680 nm and 650 nm, with a shoulder at 648 nm in the Qy region and at 493 nm and 473 nm in the Soret region, the same as for the fully pigmented complexes (19, 33). Interestingly, the three mutants LHCIIb presented similar CD spectra, to varying extent, to those shown by the recombinant LHCIIb lacking part of Neo (19). In the WT LHCIIb, the amplitude of the negative band at 493 nm was maximal in the Soret region, while the amplitude of the band at 473 nm was two-thirds of that of 493 nm. In the mutants V119F, H120L, and S123G, the amplitude of the negative CD band at 493 nm decreased, while that of the CD band at 473 nm increased strongly. The ratio of the amplitude of these two negative CD signals in the Soret region (CD493 nm/CD473 nm: Neo-band) (Table 2) was calculated as described in Ref. 19, so that the change of the CD signal in the Soret region could be compared quantitatively. From Table 2, we can see that compared to the WT LHCIIb, the change of the Neo-band is H120L > V119F > S123G. Comparing the change of the Neo-band (Table 2) with changes of the amount of the bound Neo in the complex (Table 1), it is apparent that the two factors are not correlated.

**Low Temperature Fluorescence Emission Spectra**—Fig. 4 shows the low temperature fluorescence emission spectra of all mutant species. From Fig. 4, it is shown that the fluorescence emission spectra of the different mutants are essentially identical to the WT LHCIIb; all exhibit characteristics of LHCIIb with one emission peak at 679 nm and a satellite peak at 740 nm, with no visible fluorescence emission from Chl b at shorter wavelength. The mutagenesis to the amino acids (Val-119, His-120, and Ser-123) in the lumenal region did not hinder the complete energy transfer from Chl b to Chl a within the complexes.

**Thermal Stability of Different LHCII Species**—To test whether the mutagenesis causes changes in the structural stability of LHCIIb, the thermal stability of different LHCIIb species were investigated by monitoring the decay kinetics of Chl b-sensitized Chl a fluorescence emission at 37 °C, because only within the intact complexes can the excitation energy in Chl b be transferred to Chl a. The lifetime of the decay procedure was taken as a measure of the stability of different LHCIIb species (Table 3). To our surprise, the thermal stabilities of the trimeric form of all the mutants did not change significantly, although
the pigment-pigment interactions, as we observed in CD spectra, did change drastically in different LHCIIb species.

**LHCIIb Photoprotection**—Under strong light conditions, if the overexcited singlet Chls* cannot be dissipated into heat, they may turn into triplet Chls*, which in turn react with \( \cdot O_2^\bullet \) (16). The singlet \( \cdot O_2^\bullet \) is highly reactive and it oxidizes the Chls rapidly. As Neo is a good scavenger for singlet \( \cdot O_2^\bullet \) (16), it is of interest to investigate whether changes in the Neo binding result in changes in the characteristics of photoprotection of LHCIIb under strong light conditions. The photobleaching of LHCIIb was evaluated by monitoring the decrease in the Qy absorption of the Chls bound in the complexes under strong illumination. Fig. 5 presents the time course of the photobleaching of LHCIIb under strong Illuminations. From Fig. 5, it is shown that although the initial rate of photobleaching of different LHCIIb species remained unchanged, the stability of the mutant LHCIIb was reduced to different extents. The observed data were fitted into a first-order exponential decay function \( Y = Y_0 + A \exp( -k \cdot X ) \) and the parameters \( Y_0 \), \( A \), and \( k \) were taken to analyze the photoprotection capacity of different LHCIIb species. The \( Y_0 \) value is the asymptote for the decay function, which indicates the amount of pigment protected from bleaching (30). The parameter \( A \) indicates the amount of pigment photobleached in the reaction, and \( \tau_r \) (\( \tau_r = 0.693/k \)) is the lifetime of the pigment in different LHCIIb species. Table 4 presented these parameters for the different LHCIIb species. In Table 4, it is shown that the photoprotection index of S123G is altered strongly. The \( Y_0 \) value of S123G is reduced significantly compared with the WT LHCIIb, from 0.34 in the WT to about 0.08 in S123G (Table 4). The changes of the resistance to photodamage of H120L and V119F were not as significant as in S123G; the \( Y_0 \) of H120L reduced from 0.34 in WT to 0.21 and remained almost unchanged in V119F. When comparing the \( \tau_r \) of each mutant with the WT LHCIIb, it is shown that the Chl lifetime of the complexes under strong illumination reduces only slightly in all the mutants.

**Characteristics of Aggregation**—Aggregation of LHCIIb is accompanied by the appearance of a new fluorescence emission at 700 nm (\( F_{700} \)) in addition to the emission of Chl \( a \) around 680 nm (\( F_{680} \)) in the 77 K Chl \( a \) fluorescence, which increases along with the increase of the size of the aggregate (34). Based on this principle, the degree of the aggregation can be analyzed quantitatively with the ratio \( F_{700}/F_{680} \) (35). In this work, we measured the 77 K fluorescence emission spectra after the LHCIIb aggregation was induced by diluting the DM concentration to 0.049 mM (28). Fig. 6 presents the 77 K fluorescence emission spectra of different LHCIIb species upon excitation at 436 nm. It can be deduced from Fig. 6 that the mutant S123G (\( F_{700}/F_{680} = 0.987 \) in S123G versus 0.892 in WT) markedly aggregates. No noticeable differences were detected to the mutations V119F and H120L compared with the WT (\( F_{700}/F_{680} = 0.898 \) in V119F and 0.893 in H120L versus 0.892 in WT).

**Chlorophyll Fluorescence Yield**—Besides absorbing sufficient solar energy, LHCIIb has another important function in dissipating excessive excited energy, so that the PSII is protected.

### TABLE 3

<table>
<thead>
<tr>
<th>Lhc1b</th>
<th>( \tau (s) )</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>132.13 ± 5.84*</td>
</tr>
<tr>
<td>S123G</td>
<td>133.71 ± 10.39</td>
</tr>
<tr>
<td>H120L</td>
<td>134.17 ± 13.25</td>
</tr>
<tr>
<td>V119F</td>
<td>134.67 ± 7.87</td>
</tr>
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</table>

* All values are given as the average of 3–5 individual measurements ± standard deviation.

### TABLE 4

<table>
<thead>
<tr>
<th>Lhc1b</th>
<th>( Y_0^a )</th>
<th>( A^b )</th>
<th>( \tau_r^c (\text{min}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.34 ± 0.08</td>
<td>0.58 ± 0.16</td>
<td>15.06 ± 1.8</td>
</tr>
<tr>
<td>S123G</td>
<td>0.08 ± 0.03</td>
<td>0.84 ± 0.22</td>
<td>14.06 ± 1.7</td>
</tr>
<tr>
<td>H120L</td>
<td>0.21 ± 0.03</td>
<td>0.70 ± 0.19</td>
<td>13.11 ± 1.1</td>
</tr>
<tr>
<td>V119F</td>
<td>0.33 ± 0.05</td>
<td>0.59 ± 0.15</td>
<td>13.85 ± 0.9</td>
</tr>
</tbody>
</table>

* \( Y_0 \), asymptote.  
* \( A \), amplitude.  
* \( \tau_r \) (\( \tau_r = 0.693/k \)), half value time of the decay.

FIGURE 5. Photobleaching of WT and mutant LHCIIb. The decay curves show the total Qy absorption relative to the initial value (the initial value was set to 1). The points are the average of five individual experiments. Solid line, WT; dotted line, S123G; dashed line, H120L; dash dot line, V119F.

FIGURE 6. 77 K fluorescence emission spectra of WT and mutant LHCIIb. The samples were dissolved in a media containing 0.049 mM DM. Solid line, WT; dotted line, S123G; dashed line, H120L; dash dot line, V119F.
A Functionally Significant Motif in LHCIIb

Lowering the pH of the medium to 4.5 caused a decrease in the fluorescence yield of the WT LHCIIb up to 2%, which was identical in the mutants H120L and V119F. Interestingly, S123G showed a clear fluorescence lowering (14.5%) in response to the acidification under the same conditions. Fig. 7B shows the results of a titration experiment of fluorescence lowering as a function of pH, namely from pH 6.0 to pH 4.5, for different LHCIIb mutants. It is clearly shown that the fluorescence lowering in S123G increased strongly in parallel with the decrease of the pH value (the fluorescence lowering changed from 3.3% at pH 6.0 to 14.5% at pH 4.5), while the fluorescence lowering of the other three species showed only a slight change as the pH value in the medium decreased from pH 6.0 to pH 4.5 (from in WT: 1.3% at pH 6.0 to 2.4% at pH 4.5; in H120L: 1.2% at pH 6.0 to 2.1% at pH 4.5; and in V119F: 1.4% at pH 6.0 to 2.2% at pH 4.5). Closer inspection (Fig. 7B, inset) reveals that the decrease of the fluorescence in H120L is less intensive than the WT LHCIIb when the pH of the medium changed from 6.0 to 5.5, while that of V119F remained unchanged in this pH range. Only when the pH value of medium dropped below 5.5 did the fluorescence lowering of H120L and V119F parallel the change of medium pH in a similar manner to the WT LHCIIb.

Because the reagent DCCD inhibits low pH-induced fluorescence quenching via its covalent binding to the carboxyl side chains in LHCCI (8, 39), it was used to check whether the fluorescence lowering was related to protonation of side chains in the complexes. In Fig. 7A, it is shown that DCCD reversed the fluorescence lowering in S123G completely. In contrast, the recoveries of fluorescence yields in WT, H120L, and V119F were negligible after the injection of DCCD. The later caused a slight fluorescence recovery, a similar extent to the fluorescence lowering of H120L and V119F parallel the change of medium pH in a similar manner to the WT LHCIIb.

From photodamage under strong light conditions (36), one of the signals of excessive light on the thylakoid lumen is the acidification of thylakoid lumen (8). Because the antiparallel strands in LHCIIb are located in the luminal side, it is of significance to investigate whether the mutants are sensitive to change of pH of the medium. For this purpose, the kinetics of fluorescence yield of different LHCIIb species as the function of pH value of the medium. Inset, the amplified view of the WT LHCIIb and the mutant H120L and V119F. The experiment was executed with the same procedure as the kinetics of Chl fluorescence yield. The pH value of the medium ranged from pH 6.0 to 4.5, F0, the fluorescence yield before adding HCl; Fh, the fluorescence yield 30 s after adding HCl. Solid line, WT; dotted line, S123G; dashed line, H120L; dash dot line, V119F.

In Fig. 7A, it is shown that the WT LHCIIb has only a slight decrease in fluorescence upon acidification of the medium. However, all the mutants showed a significant decrease in fluorescence yield, with the exception of S123G, which showed a similar decrease to the WT LHCIIb when the pH of the medium changed from 6.0 to 5.5, while that of V119F remained unchanged in this pH range. Only when the pH value of medium dropped below 5.5 did the fluorescence lowering of H120L and V119F parallel the change of medium pH in a similar manner to the WT LHCIIb.

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DISCUSSION

LHCIIb provides a unique opportunity for studies of the roles of individual amino acids or particular structural features of the protein because LHCIIb is one of only a handful of membrane proteins that can be refolded in vitro (23, 40, 41), and still maintain characteristics that are similar to the native ones in vivo as demonstrated by detailed spectroscopic and biochemical analyses (33, 42), and for which a high-resolution structure is known (5, 14).

In this work, site-directed mutagenesis has been employed to study the structural and functional significance of the antiparallel strands in the luminal loop between TM helices B/C of LHCIIb. Three amino acids (Val-119, His-120, and Ser-123) in the region have been exchanged to Phe, Leu, and Gly, respectively. Our results demonstrate that the characteristics of these amino acid residues (Val-119, His-120, and Ser-123) are not indispensable for refolding and maintenance of the thermal stability of the pigment protein complexes, because the mutations can bind pigments in the in vitro reconstitution system and refold correctly into functional LHCIIb complexes with similar thermal stability (Table 3) and a-helix content in the complex (Supplemental Table 1) to the WT LHCIIb.
A Functionally Significant Motif in LHCIIb

The Ionic Pair in the Antiparallel Strands Is Very Important for Pigment Conformation of the Neo Domain—The mutagenesis on sites Val-119, His-120, and Ser-123 leads to a minor change in LHCIIb pigment stoichiometry, which is accompanied by conformational change of the Neo domain in the complexes (Fig. 3).

Circular dichroism spectra measurements indicate that the mutants V119F and H120L show similar CD spectra to the complexes lacking Neo (19). Comparing the decrease in the amplitude of the Neo CD band (CD_{473 nm}/CD_{493 nm}) (Table 2) with the amount of missing Neo caused by mutagenesis (Table 1), we can see that there is no correlation between the loss of Neo binding and the increment of the value of the CD_{473 nm}/CD_{493 nm} signal in mutant LHCIIb, as was observed with the WT LHCIIb lacking part of Neo binding (19). Although V119F lost only 0.09 Neo, it presents a more pronounced conformational change than S123G, which lost about 0.17 Neo. The diversity in the phenomena with the WT LHCIIb binding different amount of Neo and those with mutant LHCIIb implies that the mutagenesis made to the antiparallel strands structure in the lumenal loop not only influences the binding of Neo in the region, but also changes the pigment conformation which increases the change in the Soret region in the CD spectra.

As is revealed by the crystal structure at near atomic resolution (14) the Neo locates in the Chl b-rich region, and associates, via an H-bond, with Tyr-112 that locates in the antiparallel strands. The basis for the antiparallel strands is an ionic pair structure between His-120 and Asp-111. The position of Tyr-112 and hence that of the molecule Neo is readily influenced by the mutagenesis on His-120 and Val-119, because they are in close interaction with Tyr-112 in the antiparallel strands region. The salt bridge between His-120 and Asp-111 is lost upon exchanging His-120 to Leu. Exchanging Val-119 to Phe has introduced one phenol ring in the region, which brings a strong electrostatic effect into the antiparallel region. All these changes may alter the position of Tyr-112, which, in turn, changes the Neo conformation. The CD spectral measurements confirm stronger changes to the mutations H120L and V119F than to S123G. This observation suggests that the ionic pair structure is very important for adjusting the position of Tyr-112 and hence the orientation of Neo.

The Mutagenesis in the Antiparallel Strands Structure Influences the Electronic Transition and S\textsubscript{2} Excitation State of Chls—Loss of some pigment binding in the mutants is accompanied by the reduction in the Q\textsubscript{b} transition of Chl \textit{b} at 650 nm and the 0–0 transition of Neo at 488 nm (43, 44) (Fig. 2). The changes in the absorption spectra coincide with those observed in the Neo-deficit LHCIIb (16, 31). The decrease in the absorbance at 650 nm in the Neo-deficit LHCIIb has been ascribed to the change of orientation of some Chl \textit{b} molecules, which alters the Neo-Chl \textit{b} interaction (30, 31). In our experiments, the decrease in the absorbance at both 650 nm in the Q\textsubscript{y} region and 488 nm in the Soret region can partly result from the reduction in the Chl \textit{b} or Neo binding, because the amplitude of both the 650 nm and 488 nm bands in the different spectra correlate well with the loss of Chl \textit{b} and the Neo in each mutant. Because Neo and Chl \textit{b} are in close interaction in LHCIIb (30), it cannot be excluded that any change of the absorption spectra is attributed partly to the change in the transition energy of the chromophore as a consequence of altered pigment interaction.

Change in the electronic transition of Chls results in reduced energy transfer efficiency, which includes the reductions not only in the fluorescence quantum efficiency of Chl \textit{a} (Supplemental Fig. S1), but also in the translation of Neo to the Chl \textit{a} fluorescence emission (Supplemental Fig. S2). This observation is in agreement with the earlier work that reduced Neo binding in LHCIIb decreases the fluorescence quantum efficiency (19, 27) and the trimeric LHCIIb always quenches more slowly than the monomeric species (45), which bind less Neo (43, 46). Neo is functionally significant in energy transfer in LHCIIb in different ways. First of all, it transfers directly its S\textsubscript{2} energy to Chl \textit{b} (47, 48). Secondly, it influences the energy transfer efficiency from Car to Chls, because the Arabidopsis mutant npq2, which is lacking Neo, shows a longer Car \textit{s} lifetime and less energy transfer from S\textsubscript{2} state of Car to Chls, compared with WT plants (49). Our results demonstrate that the antiparallel strands structure in the luminal loop plays an important role in regulating the interactions between Neo and Chl \textit{b} and the Chl \textit{S}\textsubscript{2} excitation state.

The Antiparallel Strands Are Important in Regulating Fluorescence Yield—Plants are under a continuously changing environment and often face excessive light stress, which interferes with electron transport in thylakoid membrane. As a consequence, it causes the acidification of the lumenal region, which induces a series of physiological processes leading to NPQ. Under strong light conditions, the pH in the lumenal region can drop as low as pH 4.5 (37, 38). It has been observed earlier that, unlike the monomeric LHCII (LHCIIa, LHCIIe), trimeric LHCIIb shows only slight fluorescence lowering upon acidification of the lumenal region and LHCIIb does not bind covalently to DCCD (11). LHCIIb plays an important role in NPQ through the xanthophyll cycle carotenoid-controlled conformational change induced within a few minutes (45, 50). Our results (Fig. 7) support the earlier observation that fluorescent yield decreases slightly in the WT LHCIIb upon the acidification of the medium, even at the extremely low pH value of 4.5, which can only be reversed slightly by treatment with DCCD. The behavior of mutants H120L and V119F (which have showed even less fluorescence lowering and less recovering by DCCD) implies that breaking the salt bridge in the antiparallel strands does not alter the characteristic of LHCIIb in binding DCCD.

In contrast, S123G shows pronounced reduction in Chl fluorescence yield, which increases remarkably in parallel with the decreased pH of the medium. Apart from this, the most important result is that the acid-induced fluorescence lowering in S123G can be fully reversed by adding DCCD, while that in the WT LHCIIb cannot. It is possible that substituting Ser-123 with Gly changes the hydrophilic environment of Glu in the lumenal region, because DCCD binds to negatively charged residues only in the hydrophobic region.

Considering the changes of other properties brought about by exchanging Ser-123 to Gly, that S123G aggregates more readily upon lowering DM concentration in the medium (Fig. 6), and is more susceptible to photodamage (Table 4), it is rea-
reasonable to suppose that the changes of the conformation at the edge of the TM helix C in S123G have resulted in the alteration not only in the trimer-trimer interaction under high protein density (low detergent condition), but also in the energy coupling among the binding pigment in the complexes, that is, high photodamage and fluorescence quenching as well.

Analysis of the possible secondary structures of S123G with the software Deep View/Swiss Pdb Viewer version 3.7 (Fig. 8) reveals that Ser-123, a non-charged polar amino acid, which forms a H-bond partner with the formyl group of Chl\textsubscript{b}605 and some water molecules in the lumenal site, plays important roles in electrostatic interaction of the polypeptide in the region and the orientation of Chl\textsubscript{b}605. Substituting Ser-123 with Gly does not break the H-bond connection to Chl\textsubscript{b}605, but eliminates the H-bond with surrounding water molecules and increases the hydrophobicity of the structure, which leads to a high tendency for the complexes to aggregate (Fig. 6). This effect follows the same principle as that of Mg\textsuperscript{2+} induction of LHCIIb aggregation (51).

Analyzing the pigment scaffold in the region surrounding Ser-123, we can see that the Chl\textsubscript{b}605, which is connected to Ser-123, is in close interact with the Chl\textsubscript{b} cluster and further with Lut 2. It is possible that the mutagenesis of Ser-123 leads to the change of the conformation of Lut 2, which causes the changes in Chl fluorescence yield as presented by Wentworth et al. (13).

Chl \textit{b}605 locates also in the trimer-trimer interface and is in strong dipole interaction with Chl \textit{a}614 of an adjacent trimer (14). The coordination of Chl \textit{b}605-Chl \textit{a}614 can be changed, as a consequence of protonation of carboxyl side chain LHCIIb, to a new fluorescence quenching center (Chl \textit{b}605-Chl \textit{a}614), which regulates NPQ under different light intensities (4). From this point of view, it is reasonable to assume that the hydrophilic character of Ser-123 at the edge of the TM helix C may play a key role in regulating conformation of the loop domain, the trimer-trimer interaction under high protein density, and regulating pigment conformation influencing the fluorescence quenching and heat dissipation of the complexes.

In conclusion, the antiparallel strands structure in the lumenal region of LHCIIb plays important roles by adjusting changes of fluorescence emission of LHCIIb in response to changes of external conditions in three respects: 1) by adjusting the conformation of Neo, which in turn, affects the fluorescence efficiency in LHCIIb; 2) by adjusting LHCIIb aggregation under low detergent conditions; and 3) by regulating acid-induced fluorescence quenching.

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REFERENCES