Random Mutations Directed to Transmembrane and Loop Domains of the Light-Harvesting Chlorophyll *a*/*b* Protein: Impact on Pigment Binding[†]

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ABSTRACT: The major light-harvesting complex of photosystem II (LHCII) can be reconstituted in vitro by folding its bacterially expressed apoprotein, Lhcb, in detergent solution in the presence of chlorophylls and carotenoids. To compare the impact of α-helical transmembrane domains and hydrophilic loop domains of the apoprotein on complex formation and stability, we introduced random mutations into a segment of the protein comprising the stromal loop, the third (C-proximal) transmembrane helix, and part of the amphipathic helix in the C-terminal domain. The mutant versions of Lhcb were screened for the loss of their ability to form stable LHCII upon reconstitution in vitro. Most steps during the screening, including expression of the recombinant protein, its reconstitution with pigments, and the assay for complex formation by measuring energy transfer from chlorophyll *b* to chlorophyll *a*, were performed as one-vessel reactions on 96-well microtiter plates. This enabled us to screen several hundred mutant Lhcb versions. Mutants that had lost their ability to form stable LHCII carried between one and four amino acid exchanges. Among the single-point mutations, several were at positions in the C-proximal transmembrane helix, including an amino acid that is thought to be directly involved in chlorophyll binding. However, we also found four point mutations in the stromal loop domain that, in our assay, completely abolished the formation of stable LHCII. These data show that the stromal loop domain has a significant impact on LHCII formation and/or stability in vitro.

The major light-harvesting complex of photosystem II (LHCII),[1] the most abundant pigmented protein in plants, is a useful model for studying the folding of membrane proteins in vitro (*1*). The native structure of LHCII is known in near-atomic detail (*2*), and the apoprotein can be refolded in vitro in the presence of pigments (*3*, *4*) and can be expressed in bacteria and, thus, is easily mutated (*5*, *6*). According to a current model, membrane proteins typically fold in two stages (*7*). First, the transmembrane α-helices form as independently folding units, and in the second step, these α-helices then associate with one another via van der Waals interactions, lipophobic effects (the entropy gain due to the replacement of lipid molecules on the helix surface), or interhelix ion pairs forming in the hydrophobic environment (*8*). This scenario leaves little room for participation in the folding process of the loop domains in membrane proteins. Consistently, it has been shown for bacteriorhodopsin (bR), one of the few other membrane−protein complexes that can be reconstituted in vitro, that cuts in loop domains do not interfere with successful folding (*9*). Moreover, bR has been reconstituted from recombinant fragments of its apoprotein opsin, containing two to five transmembrane α-helices. All possible combinations of these fragments that complement each other to the full seven transmembrane domains of opsin refold and, with retinal added, yield functional bR, demonstrating that a covalent connection of the α-helices in the loop domains is not required (*10*).

The near-atomic structure of LHCII provides some information about helix−helix interactions in the molecule. The first (N-proximal) and third (C-proximal) transmembrane α-helices are intertwined to form a supercoil with a stretch of eight amino acids each at a helix center-to-center distance of only 8.5 Å. Moreover, the two helices are linked by ion pairs, E65−R182 and R70−E180 (*2*). The middle helix does not seem to make direct contact with the other two helices but is connected with them through chlorophyll (chl) molecules situated between them. Many of the chls that are visible in the molecular structure of LHCII are ligated to amino acid side chains in the transmembrane helices. Also, the two carotenoids that are visible in the structure closely interact with the first and third transmembrane helices.

Much less is known about the significance of the stromal and lumenal loops with respect to the structure and function of LHCII. To obtain information about the significance of loop domains for the refolding of Lhcb into stable pigmented LHCII, we performed a mutational analysis in recombinant LHCII. To be able to compare the significance of mutations in a loop domain with a transmembrane helix in Lhcb, we used a controlled-fidelity polymerase chain reaction (PCR) (*11*, *12*) to introduce random mutations in an Lhcb segment comprising the stromal loop and the C-proximal transmembrane helix. The resulting mutants were screened for the

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[1] Abbreviations: bR, bacteriorhodopsin; chl, chlorophyll; IPTG, isopropyl thio-β-D-galactoside; Lhcb, light-harvesting chlorophyll *a*/*b* protein of photosystem II; LHCII, major light-harvesting complex of photosystem II; MTP, microtiter plate; OG, octyl glucoside; PCR, polymerase chain reaction.

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complete loss of their ability to reconstitute with pigments in vitro to form stable LHCII. Among the selected clones, we found several that carried single-amino acid exchanges in the stromal loop. To our knowledge, this is the first time that single amino acids in loop domains have been shown to be essential for the successful refolding of a chl *a*/*b* binding protein.

MATERIALS AND METHODS

Bacterial Strains, Pigments, and Enzymes. The bacterial hosts that were used were *Escherichia coli* strains JM101 from New England Biolabs (Schwalbach, Germany) and SCS110 from Stratagene (Heidelberg, Germany). All enzymes and dNTPs were obtained from New England Biolabs, Amersham Pharmacia Biotech (Freiburg, Germany), and Boehringer-Mannheim (Mannheim, Germany). Lhcb was overexpressed in bacteria (*4*), harboring an expression construct of the *lhcb*1 gene "AB80" (*13*) from pea. A modified version of the original construct was used in this work, which encoded the authentic N-terminal sequence ^{1}MRKSATTKKVASS^{13} (*14*). Total pigment extract was isolated from pea leaves (*4*). The chl concentrations were determined spectroscopically according to the method of ref *15*. In all reconstitution experiments, aliquots of this crude extract were used, containing chl *a* and chl *b* in a 3:1 ratio. Octyl glucoside (OG) and isopropyl thio-β-D-galactoside (IPTG) were obtained from Biomol (Hamburg, Germany).

Creating a Mutant Library. The introduction of random mutations in *lhcb*1 followed the PCR-based method of Vartanian et al. (*11*) and Shafikhani et al. (*12*), reducing the fidelity of the Taq DNA polymerase in the PCR by addition of Mn ions and by biased dNTP concentrations (*16*, *17*). PCR primers 5′-TTCCCCGGTGACTACGG-3′ and 5′-CGGGAACAAAGTTGGTGGC-3′ which flank the sequence between R141[2] and A210 were purchased from MWG-Biotech (Munich, Germany) and used without further purification. The mixtures for PCRs contained PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 5 mM $MgCl_2$], 100 pmol of each primer, 1 μmol of template DNA, 0.25 mM dATP and dCTP, 1.25 mM dGTP and dTTP, and 2.5 units of Taq DNA polymerase (Oncor Appligene, Heidelberg, Germany). To check the influence of Mn^{2+} on the PCR yield, two parallel reactions were always performed with and without 0.5 mM Mn^{2+}. The PCR mixtures were overlaid with mineral oil, and submitted to 50 cycles of 30 s at 95 °C, 30 s at 52 °C, and 120 s at 72 °C, followed by 10 min at 72 °C in a Mastercycler (Eppendorf, Hamburg, Germany). The extended elongation time was used to make up for the reduced rate of DNA elongation by the polymerase under lower-fidelity conditions.

The mutated PCR product was cloned between the *Bst*EII and *Bcl*I restriction sites into Lhcb, substituting the sequence between R141 and A210 (Figure 1). DNA sequencing was performed, using the T7 sequencing kit with ^{35}S-labeled dATPαS (Amersham Pharmacia Biotech). For sequencing, the primer 5′-CTT GCC ATA TGG GCC ACT CAG GTT ATC TTG-3′ was used, annealing 35 bp upstream of the mutated insert.

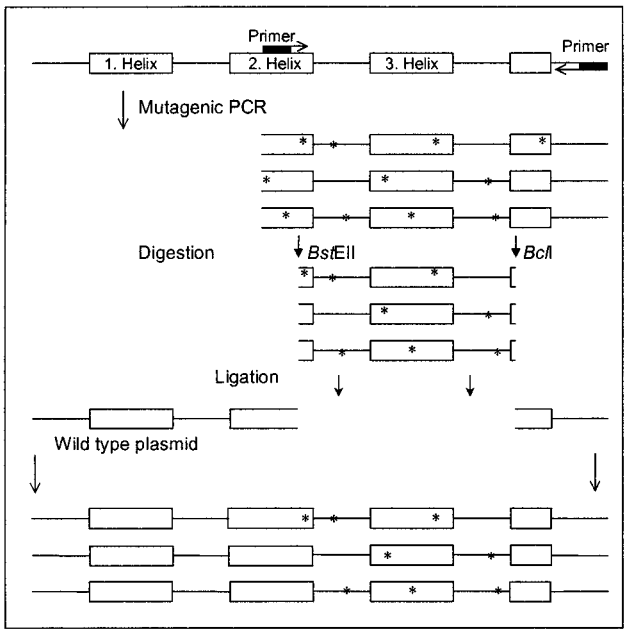

FIGURE 1: Introduction of random mutations into specific domains of Lhcb. By including Mn^{2+} and biased dNTP concentrations in the PCR mixture, we introduced a number of random mutations into the PCR product between the two primers. By cloning the PCR product into the wild-type plasmid, we generated a library of mutants all carrying different mutations. The Lhcb segment mutated in this work is indicated. Asterisks represent random mutations.

Overexpression and Reconstitution in Microtiter Plates (MTP). Single clones were picked and grown in 200 μL of LB-Amp medium [1% tryptone, 0.5% yeast extract, and 1% NaCl (pH 7.5) with 100 μg/mL ampicillin] per well in a 96-well MTP (Sarstedt, Nümbrecht, Germany), overnight at 37 °C with slight agitation. A replica plate was made by inoculating 50 μL of LB-Amp medium with 5 μL of the overnight culture, using a MTP replicator (Integra Bioscience, Fernwald, Germany). After growth for 1 h, 10 μL of IPTG (10 mM) was added to each well of the replica plate, and the mutants were incubated at 37 °C overnight without agitation. For sedimentation of the cells, the MTPs were centrifuged at 1800 G for 10 min. The supernatants were removed carefully with a water aspirator pump, and the cells were lysed with freshly prepared lysis buffer [50 mM Tris-HCl (pH 8.0), 25% (w/v) sucrose, 1 mM ethylenediaminetetraacetic acid, and 4 mg/mL lysozyme]. The MTP was shaken slowly at room temperature for 10 min, and 10 μL of 0.5% (w/v) lithium dodecyl sulfate was added to each well, followed by shaking for an additional 10 min. When the cell suspensions had cleared, 25 μL of 2× reconstitution buffer [200 mM sodium borate (pH 9.0), 25% (w/v) sucrose, and 10 mM dithioerythritol (*1*)] was added to each well and the plate was shaken slowly at room temperature for an additional 10 min. Total pigment extract corresponding to 800 μg of chl was dissolved in 50 μL of ethanol. This ethanolic pigment solution was added to 2 mL of reconstitution buffer (see above), containing 4% (w/v) OG, under vortexing. Twenty microliters of the pigment solution was added to each well of the MTP, and the plate was slightly agitated and left for 5 min for the refolding to take place.

[2] Point-specific mutations are termed A#B, with A being the original amino acid, # its position in the Lhcb, numbered according to ref *2*, and B the amino acid present after the exchange. Throughout this work, the one-letter amino acid code is used.

Reconstitution Measurements. The MTP containing the reconstitution mixtures was centrifuged for 5 min at 4 °C and 1800*g*, and energy transfer from chl *b* to chl *a* was measured directly in the MTP by exciting chl *b* at 480 nm and detecting chl *a* emission at 680 nm, using a Fluoroscan Ascent apparatus (Labsystems, Helsinki, Finland) operated with Ascent software version 2.1. The excitation wavelength of chl *b* was deliberately chosen on the long-wavelength side of the chl *b* emission spectrum to minimize coexcitation of chl *a*.

Excitation and emission measurements were both performed from above the MTP to increase the measured signal. Energy transfer between chl *b* and chl *a* will cause the emission of the energy donor, chl *b* (F_{660}), to decrease and the emission of the acceptor, chl *a* (F_{680}), to increase upon excitation of chl *b*. Therefore, the F_{680}/F_{660} ratio is expected to increase significantly when energy transfer takes place and thus was taken to be a fluorescence indicator of energy transfer. The fluorescence signal of energy transfer was dependent on the pigment concentration; chl concentrations were uniform within one experimental series but varied slightly between different pigment mixtures for reconstitution. Therefore, the correlation between the fluorescence signal of energy transfer and the actual reconstitution taking place had to be calibrated for each experimental series. To do this, 10 reconstitution mixtures from each series, representing F_{680}/F_{660} ratios from the entire observed range, were applied to a partially denaturing polyacrylamide gel to test whether recombinant LHCII had formed. The minimum value of the fluorescence signal that correlated with reconstituted complex formation was then taken as a criterion with which to estimate pigment binding in the other mutants on the MTP. From all mutants exhibiting a fluorescence signal of energy transfer that was lower than this threshold value, Lhcb was purified and submitted to a standard reconstitution procedure (*18*). From mutant Lhcb clones exhibiting wild-type length and no ability to reconstitute, the 212 bp mutated inserts were sequenced.

RESULTS

Random Mutagenesis in a Segment of Lhcb. The domain of interest for this study comprises the sequence between R141 and A210, including the C-terminus of the second transmembrane helix, the entire third helix, the N-terminal part of the amphipathic fourth helix, and the loop domains between the helices. This domain is flanked by the *Bst*EII and *Bcl*I endonuclease restriction sites that are unique in the expression plasmid. A library of Lhcb mutants was created as shown in Figure 1. By means of mutagenic PCR, random mutations were induced in the amplified DNA segment. The PCR product was digested with endonucleases and cloned into the wild-type plasmid, replacing the wild-type sequence between the two restriction sites. The plasmid library was then transformed into bacterial cells.

The number of induced amino acid exchanges should preferably be one per insert, but because of the ambiguity of the genetic code, we aimed at two to three base exchanges per insert. The observed mutation rates induced by adding Mn^{2+} and biased nucleotide concentrations vary among reports (*11*, *16*, *19*, *20*), maybe because they are somewhat template-specific (*21*). However, the mutations obtained are mostly transitions and transversions with few or no nucleotide deletions or insertions. With an increase in the Mn^{2+} concentration during the PCR, the mutation rate rises, but as the yield of the PCR product is becoming smaller with the increase in Mn^{2+} concentration (*16*), we did not raise Mn^{2+} concentrations above 0.5 mM. To introduce the desired number of mutations (see below), the dilution method of Shafikhani and co-workers (*12*) was employed, where the product of one PCR round was 100-fold diluted and used as the template in the next round under the same conditions, each round adding to the number of mutations.

Table 1: Mutation Matrix for Mutagenic PCR[a]

	to G	to A	to T	to C
from G		10	8	1
from A	16		21	3
from T	7	10		24
from C	0	3	3	

[a] All 106 nucleotide mutations observed by sequencing are listed, showing the distribution of transitions and transversions. There is a bias for exchanging A and T nucleotides, whereas C is less frequently mutated.

We based our experimental procedure on the mutation rate reported by Shafikhani et al. (*12*) of 0.49% in the presence of 0.5 mM Mn^{2+}, thus predicting three rounds of PCR for introducing approximately three mutations per 212 bp insert. The mutation rates we observed appear to be consistent with this estimate. Analysis of the mutations that were obtained showed that the random mutagenesis procedure was not entirely random, and that the types of mutations induced were biased toward exchanges of A and T, whereas C was more rarely mutated (Table 1), in agreement with refs *11* and *12*.

Screening of Mutated Recombinant Lhcb for Its Reconstitution with Pigments. To be able to screen a sufficiently large number of mutated versions of Lhcb for their ability to form stable LHCII, we had to simplify the procedure of protein expression and reconstitution such that it could be done with MTPs. An important modification introduced into one of the standard procedures (*18*) was that reconstitution took place in a crude cell lysate, without prior purification of Lhcb, taking advantage of the fact that the only protein present in this suspension that would bind pigments under refolding conditions was Lhcb. Furthermore, complex formation was initiated by simply adding the pigments, dissolved in an OG-containing solution, to the protein solution in lithium dodecyl sulfate (*1*). These modifications allowed us to perform all experimental steps from growing single bacterial clones containing mutant *Lhcb* expression plasmids to reconstitution of LHCII, and its detection by fluorescence measurement, in a one-vessel reaction on the same MTP.

The fluorescence signal of energy transfer does not reliably allow us to distinguish between Lhcb mutants that do not reconstitute and those that form LHCII with a lowered stability. Moreover, the mutants exhibiting no energy transfer between chl *b* and chl *a* include the less interesting ones that carry stop codons in their coding sequence, thus giving rise to truncated proteins. Therefore, we added two more levels of screening. First, from those bacterial clones exhibiting a fluorescence signal of energy transfer below the threshold level, Lhcb was isolated and its size was analyzed on a fully denaturing polyacrylamide gel to select the clones

Table 2: Mutations Abolishing Reconstitution of LHCII[a]

single mutations (9)	double mutations (18)	triple mutations (11)	quadruple mutations (4)
G146E	R142P/F192S V178E/K182E	I143N/S190P/F194C	G146V/E175V/G184S/E207V
D153G	G145C/D153G V178A/E207V	I143T/G193V/Q197H	
L155P	V151M/E180V E180V/V200A	L148P/P170S/G184C	Y156H/L164V/E180V/V196A
P163L	V152G/F192L E180V/T201A	V151E/E180V/M188L	
E180G	V152A/N208H E180V/L206M	G158S/A174T/E180G	P157T/R185S/V196D/I199F
E180V	P163S/G184C E180D/E207K	D162G/L166I/K182M	
K182E	E171V/L176S G184D/F195I	L164V/M191R/F195R	D169V/L176V/L181H/L186S
G184D	E171G/A187P M188L/F192L	D169V/E180D/F189L	
F189L	V178L/E180G F195S/A198D	A172T/V196D/T201P	
		K179E/G202V/G204V	
		F189S/F192L/Q197R	

[a] Mutants that are not able to build stable pigment−protein complexes by the detergent exchange method (*18*) are sorted by the number of mutations in each clone.

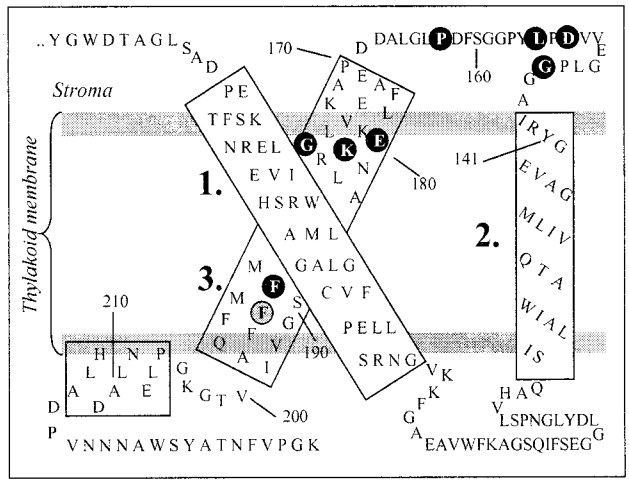

FIGURE 2: Position of single-amino acid exchanges in the LHCII structure (*1*). The black circles represent single mutations, whereas the gray circle represents a double mutant where the second mutation is considered to have no influence on reconstitution. The numbering of amino acids is as in ref *1*. The gray shades illustrate the approximate locations of the membrane surfaces.

expressing full-length Lhcb. Subsequently, all full-length versions of Lhcb were reconstituted using the low-stringency detergent exchange reconstitution technique (*18*) and examined on a partially denaturing gel to exclude those mutants that still formed LHCII with a lower stability.

The screening started out from a total library of 3350 clones, obtained from six independent mutation experiments. About 10%, 327 clones, exhibited a fluorescence signal of energy transfer below the threshold, indicating the absence of pigment−protein complex formation. Only about half of these, 161 clones, were full-length and were reconstituted by using the detergent exchange method (*18*). The final screening resulted in 42 mutant versions of Lhcb that were unable to form even relatively unstable LHCII in vitro. The amino acid exchanges identified by sequencing of these 42 clones are listed in Table 2.

Mutations Abolishing Reconstitution. We have detected several clones with single-amino acid exchanges which have lost their ability to reconstitute. Some of the mutations are not surprisingly located in the part of the third transmembrane helix that is intertwined with the first helix, including E180, K182, G184, and F189 (Figure 2). Through previous sequencing of a clone that was mutated in M188 but still able to bind pigments with wild-type stability (not shown), we assume that the mutation F192L in the double mutant (M188L/F192L) is deleterious for reconstitution as well. We have also found several single mutations in the stromal loop domain that render Lhcb unable to reconstitute, including G146, D153, L155, and P163. In addition to the single mutants, we have obtained a number of double, triple, and quadruple mutants. Figure 3 shows how these mutations are distributed over the individual residues in the amino acid sequence. Where several mutations of one kind accumulate in one position, these are independent mutations with different (combinations of) amino acid exchanges.

DISCUSSION

The Stromal Loop Domain Has an Impact on Lhcb Folding and/or Pigment Binding. Eight single-amino acid exchanges are described in this paper that completely abolish the formation of recombinant LHCII under the conditions used in this work. Of these eight point mutations, four are located in the third transmembrane helix and four in the stromal loop of the protein (Figure 2). One more amino acid, F192 in the third helix, is also likely to be essential for LHCII formation since it appears in the nonreconstituting double mutant (M188L/F192L) where the other exchanged amino acid, M188 in mutant M188V, has been shown to have no impact on LHCII formation as long as this is the only exchange (not shown). Of course, we cannot exclude the possibility that this double mutation only cooperatively destroys protein function even though the individual mutations do not, as has been shown in a double mutant of a D2 protein from *Synechocystis* photosystem II, where both mutations were present in a loop domain, 10 residues apart (*22*).

If one takes the amino acid positions into account that appear as single mutations and the one assumed amino acid from the double mutant mentioned above, the density of exchanges in the two protein domains of amino acids that abolish LHCII formation is about the same: five out of 29 amino acids in the third transmembrane helix and four out of 25 in the stromal loop domain. These data clearly show that this loop domain of Lhcb contributes to protein folding and/or stable pigment binding and cannot be viewed merely as a nonspecific linker of two pigment-binding helices. Moreover, our data suggest that LHCII reconstitution is about as sensitive to mutations in the stromal loop domain as it is to mutations in the third helix.

Among the various mutations obtained in this study, only the single-amino acid exchanges allow unambiguous conclusions. For the multiple mutations, we do not know which of

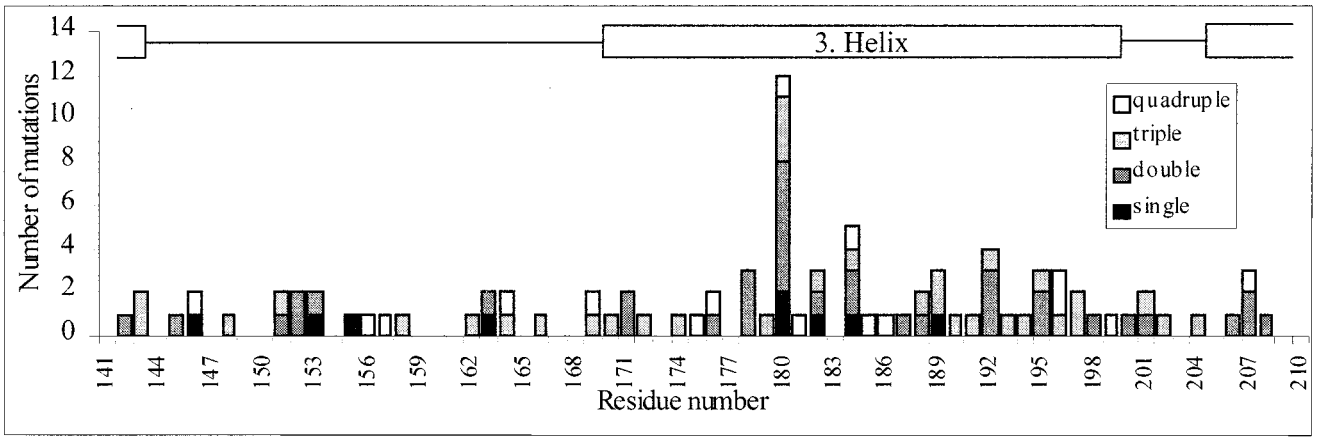

FIGURE 3: Distribution of mutations between individual amino acids. Each amino acid is shown with the actual number of hits, and the type of mutant in which these are found. Residues are numbered as described in the legend of Figure 2. The structure elements of LHCII are displayed above the sequence, with boxes representing the second, third, and fourth helices.

the amino acid exchanges is responsible for the loss of reconstitution or whether only the combination of these exchanges has an impact on formation of stable LHCII. The distribution of the mutated residues is fairly even along the sequence (Figure 3), whereas the number of mutations, including those that accumulate in the same positions, is somewhat shifted toward the third helix. In this helix, residues V178, E180, G184, and F192 are more frequently mutated. Position V178 which does not appear among the single mutations but is hit three times in double mutations may also be a sensitive position with regard to LHCII formation.

We cannot make any statement about the importance for LHCII formation of those amino acid positions that do not appear in single mutants or in any of the mutants. It is likely that there are more positions where a single-amino acid exchange prevents reconstitution. To be sure to identify those, we would have to increase the number of single mutations into saturation, i.e., such that the same positions appear several-fold in independent mutagenesis experiments. The only amino acid that has been exchanged in two different single mutation events is E180 (see below). A more systematic mutational analysis addressing all amino acids can be achieved by other techniques such as the exchange of all amino acids in the domain of interest with alanine (*23*). On the other hand, the random mutagenesis approach has an advantage in that a large number of mutations is obtained very rapidly so that functionally important protein domains that are more sensitive to mutations than others are easily identified. Moreover, at least in some cases, the identity of the newly introduced amino acid(s) as compared to the exchanged one(s) in mutated positions may give a clue about which property of the original amino acid is important for function, particularly when several exchanges have been obtained in a single position. It should be kept in mind, however, that the number of possible amino acid exchanges in one position, reached by single-base exchanges only, has a maximum of nine and usually is limited to five to seven because of the degenerate code. Also when the bias in nucleotide exchanges is taken into account, making some exchanges less likely than others, it is clear that even with a saturating number of single mutations one would not be able to identify all possible mutations in LHCII that abolish stable complex formation.

Mutations in the Third Transmembrane Helix. Glutamate in position 180 sticks out in this analysis by showing the highest number of hits in both single and double mutations (Figure 3). E180 is seen in the LHCII crystal structure to form an ionic bridge with R70 in the first transmembrane helix; this interaction is thought to stabilize the arrangement of these two helices relative to each other (*2*). It is conceivable that the exchange of E180 with any other amino acid that does not carry a negative charge would interrupt this interaction and, thus, render the complex unstable. Of the nine possible single-nucleotide exchanges in the codon GAA, five result in codons for amino acids without a negative charge, one results in an E codon, two result in D codons, and one results in a stop codon. In the two single mutations observed in position 180, E is exchanged with G and V. It is possible that the number of accessible amino acid exchanges in all the other positions that would prevent the protein from folding and/or stable pigment binding is lower which would explain why E180 exchanges appear more often in nonreconstituting mutants.

On the other hand, if E180 is such a sensitive target, one would also expect to isolate a similar number of mutants with exchanges of R185, the other ion bridge-forming amino acid in the third transmembrane helix. Its codon, AGA, can be transformed by single-nucleotide exchanges into five codons for amino acids without a positive charge, three codons for positively charged amino acids, including two that also encode R, and one for a stop codon. However, position 185 appears only in one quadruple mutant in the analysis where R is exchanged with S. The lack of hits in position R185 in this mutational analysis suggests that the E65−R185 ion pair is less important for stabilizing LHCII than the R70−E180 ion pair. This notion is corroborated by the finding that mutants E65A (H. Rogl and W. Kühlbrandt, personal communication), as well as the double mutant E65Q/H68L (C. Yang, K. Kosemund, C. Cornet, and H. Paulsen, unpublished results) in which the E85−R185 ion pair is also disturbed, still form relatively stable recombinant LHCII monomers. On the other hand, H. Rogl and W. Kühlbrandt (personal communication) observed that the mutant E180A, affecting the R70−E180 ion pair, can also be reconstituted with pigments, although possibly at a stability lower than that of the mutant E65A.

The negative effect of the G184D mutation on LHCII formation may be explained by the introduction of a noncompensated charge into the membrane-immersed part of the protein helix which may be sufficient to destabilize the protein structure. Support comes from the observation that the single mutation G184S does not influence complex formation (not shown). F189 is, along with E180, K182, and G184, in the third transmembrane domain, identical or conservatively substituted in all members of the chl *a*/*b* protein family (*24*). F189 is located in the vicinity of one of the carotenoid molecules seen in the structure, and this interaction may be weakened when F189 is exchanged with L so that the pigment−protein complex is no longer sufficiently stabilized. The exchange of F192 with L which presumably is responsible for the failure of the double mutant (M188L/F192L) to reconstitute (see above) also affects a position that is conserved in most chl *a*/*b* proteins and, according to the structure, is in contact with the same lutein molecule.

Mutations in the Stromal Loop Domain. Among the mutated positions found in the stromal loop domain, only P163 is conserved in all chl *a*/*b* proteins (*24*). Exchange of P163 with L renders the protein unable to assemble with pigments in vitro. Prolines often form kinks in polypeptide backbones; such a kink is possibly needed for proper positioning of the loop. In position 155, the reverse exchange of L with P abolishes reconstitution of the protein. The stromal loop is unusual in that it contains five prolines. It may be surprising that the addition of yet another one has such a profound effect on folding and/or pigment binding. However, all of these prolines in their correct positions may be needed for correctly folding the entire loop domain. There is no obvious explanation for the impairment of complex formation of the remaining two mutants, D153G and G146E. The fact that charges are involved in both exchanges may indicate that the distribution of charges in the stromal loop domain has an impact on Lhcb folding and/or pigment binding.

A number of studies in *Synechocystis* have focused on the importance of loop domains for protein function of the chl-binding proteins CP47, D1, and D2 of photosystem II. These studies include deletions and point mutations in loop c (*25*) and loop e (*26*−*28*) of CP47, a lumenal domain of D2 (*22*), and a stromal (*29*) and a lumenal (*30*) domain of D1. Several of these mutations rendered the organisms nonphotosynthetic. However, in studies in vivo, it is difficult to determine whether mutations affect protein folding and stability or whether they impair the interaction of the mutated protein with other proteins of the photosynthetic apparatus.

Experiments in which random mutagenesis in Lhcb is limited to single loop domains are being carried out. The shorter target peptide in these analyses will allow us to extend the number of mutation events into saturation. Moreover, we will extend the analysis to reversions of a primary mutation that abolishes reconstitution, by a secondary mutation, possibly in a distant domain, that renders the protein capable again of assembling into LHCII. From these analyses, we expect to obtain further information about the significance of single amino acids in the protein loop domains in the folding and pigment binding of Lhcb.

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