Single Amino Acids in the Lumenal Loop Domain Influence the Stability of the Major Light-Harvesting Chlorophyll *a*/*b* Complex†

Vera Mick,‡ Kristina Eggert,‡ Bo Heinemann,§ Sonja Geister,|| and Harald Paulsen*,‡

Institut für Allgemeine Botanik, Johannes Gutenberg-Universität Mainz, D-55099 Mainz, Germany, Revotar Biopharmaceuticals AG, Neudorfstrasse 24a, 16761 Hennigsdorf, Germany, and Physikalische Biochemie, Universität Potsdam, 14415 Potsdam, Germany

Received December 8, 2003; Revised Manuscript Received March 9, 2004

ABSTRACT: The major light-harvesting complex of photosystem II (LHCIIb) is one of the most abundant integral membrane proteins. It greatly enhances the efficiency of photosynthesis in green plants by binding a large number of accessory pigments that absorb light energy and conduct it toward the photosynthetic reaction centers. Most of these pigments are associated with the three transmembrane and one amphiphilic α helices of the protein. Less is known about the significance of the loop domains connecting the α helices for pigment binding. Therefore, we randomly exchanged single amino acids in the lumenal loop domain of the bacterially expressed apoprotein Lhcb1 and then reconstituted the mutant protein with pigments in vitro. The resulting collection of mutated recombinant LHCIIb versions was screened by using a 96-well-format plate-based procedure described previously [Heinemann, B., and Paulsen, H. (1999) *Biochemistry 38*, 14088−14093], enabling us to test several thousand mutants for their ability to form stable pigment−protein complexes in vitro. At least one-third of the positions in the loop domain turned out to be sensitive targets; i.e., their exchange abolished formation of LHCIIb in vitro. This confirms our earlier notion that the LHCIIb loop domains contribute more specifically to complex formation and/or stabilization than by merely connecting the α helices. Among the target sites, glycines and hydrophilic amino acids are more prominently represented than hydrophobic ones. Specifically, the exchange of any of the three acidic amino acids in the lumenal loop abolishes reconstitution of stable pigment−protein complexes, suggesting that ionic interactions with other protein domains are important for correct protein folding or complex stabilization. One hydrophobic amino acid, tryptophan in position 97, has been hit repeatedly in independent mutation experiments. From the LHCIIb structure and previous mutational analyses, we propose a stabilizing interaction between this amino acid and F195 near the C-proximal end of the third transmembrane helix.

The major light-harvesting antenna of photosystem II (LHCIIb)[1] is one of the few integral membrane proteins that can be produced in its recombinant form and then refolded in vitro (*1*, *2*). This makes it an attractive model for studying the folding and stability of membrane proteins. LHCIIb belongs to the family of chlorophyll (chl) *a*/*b* proteins and contains chlorophyll *a* and *b* molecules and carotenoids in a structure that efficiently harvests light and transfers its energy to the photosynthetic reaction centers. Electron crystallographic analysis of LHCIIb (*3*) revealed three transmembrane helical protein domains and a fourth amphiphilic helix located on the lumenal membrane surface. These helices ligate most of the chlorophylls and the carotenoids. The first (N-proximal) and the third (C-proximal) transmembrane helices intertwine in a supercoil and are linked to each other by two ion bridges (*3*). The middle helix makes no direct contact with the other ones but supposedly interacts with them through chlorophyll molecules.

Such helix−helix interactions have been proposed to be a major driving force during folding and assembly of helical membrane proteins. The "two-stage model" of Popot and Engelman (*4*, *5*) views the transmembrane α helices as initially indepently folding units that then in a second step converge and assemble. Consistent with this model, the transmembrane helices of bacteriorhodopsin form spontaneously in vitro, even in the absence of the retinal chromophore (*6*). The helices then assemble correctly even when they are no longer covalently linked due to cuts in the loop domains (*7*−*9*). An only limited importance of the protein loops is further suggested by the observation that bacteriorhodopsin tolerates various changes in its loop regions (*10*, *11*), including the replacement by structureless GGS-based pep-

† This work has been funded in part by the Deutsche Forschungsgemeinschaft (Pa 324/5-3) and Bundesministerium für Bildung und Forschung (Center of Multifunctional Materials and Miniaturized Devices).

* To whom correspondence should be addressed. E-mail: paulsen@mail.uni-mainz.de. Telephone: 49-6131-3924633. Fax: 49-6131-392-3787.

‡ Johannes Gutenberg-Universität Mainz.

§ Revotar Biopharmaceuticals AG.

|| Universität Potsdam.

[1] Abbreviations: bR, bacteriorhodopsin; chl *a*/*b*, chlorophyll *a*/*b*; DTT, dithiothreitol; F_{680}/F_{660}, ratio of fluorescence emission intensities at 680 nm to that at 660 nm; LDS, lithium dodecyl sulfate; Lhcb, light-harvesting chlorophyll *a*/*b* protein of photosystem II; LHCIIb, major light-harvesting complex of photosystem II, containing apoproteins Lhcb1−3; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

10.1021/bi036198s CCC: $27.50
Published on Web 04/20/2004

tide linkers (*12*), although in some cases the construct stabilities were reduced compared to that of the wild type.

The folding and assembly of LHCIIb in vitro do not fit quite as nicely with the two-stage model. In this protein, α helix formation is in part dependent on the presence of pigments (*13*). Attempts to assemble LHCIIb from fragments of its apoprotein have not been successful; rather, deletions from the protein from the N- or C-terminus by more than 60 or 11 amino acids, respectively, completely abolished formation of the pigment−protein complex, suggesting its highly synergetic stabilization (*14*−*16*). In an earlier contribution, we compared the significance of transmembrane helix and loop domains for the folding and stability of LHCIIb in vitro by randomly introducing single-amino acid exchanges and then isolating those protein mutants that could no longer form stable pigment−protein complexes. When the mutations were targeted to the stromal loop and the third transmembrane helix simultaneously, we observed approximately the same number of "lethal" hits abolishing LHCIIb reconstitution in either domain, suggesting that the loop is about as significant for successful LHCIIb assembly as the α helix (*17*). We identified eight amino acid positions in the stromal loop domain whose exchange caused the failure of LHCIIb reconstitution in vitro.

To obtain a more complete picture of the participation of loop regions in the assembly and stabilization of LHCIIb, we performed a random mutational analysis targeted to the lumenal loop, connecting the first and second transmembrane helices. In approximately one-third of the amino acid positions, we have found point mutations that prevented refolding of the apoprotein and/or stable pigment binding, demonstrating the significance of this protein loop domain for the assembly of stable LHCIIb.

MATERIALS AND METHODS

Site-Specific Mutagenesis. An expression construct of the *lhcb*1*2 gene "AB80" from pea (*18*) was used, encoding the mature form of Lhcb1 except amino acids 3 and 4 are lacking [^{1}MRATTKKVASS^{11} (*15*)]. This construct was modified to introduce a unique *Sac*I restriction site near the C-proximal end of helix 1 and a unique *Sph*I endonuclease site near the N-terminus of helix 2. To introduce the *Sph*I site, directed mutagenesis was performed with sense primer 5′-GCT CAA AGC ATG CTT GCC ATA TGG G-3′, introducing the L125M amino acid substitution. This substitution had, however, no effect on reconstitution ability (not shown). The *Sac*I restriction site was introduced by utilizing sense primer 5′-CCC AGA GCT CTT GTC TCG C-3′, introducing no amino acid substitution. In both cases, antisense primer 5′-TGG ATC AAA GCT TCC ACC TGG GTA AAG TGG-3′ was used. The PCR product was used as a "mega antisense primer" in a second PCR according to ref *19* with sense primer 5′-ATT TGC TTT GTG AGC GG-3′. The final PCR product was cloned into the wild-type plasmid, using the *Eco*RI and *Bst*EII restriction sites. All primers were purchased from MWG-Biotech (Munich, Germany) and used without further purification. Enzymes and dNTPs were obtained from New England Biolabs (Frankfurt am Main, Germany), Amersham Pharmacia Biotech (Freiburg, Germany), and Roche (Mannheim, Germany). All constructs were verified by sequencing (Genterprise AG, Mainz, Germany).

Creating a Mutant Library. A mutant library of Lhcb1 was generated as described previously (*17*). The *lhcb1* fragment defined by sense primer 5′-CGG TGA CTA CGG TTG GG-3′ and antisense primer 5′-TGG ATC AAA GCT TCC ACC TGG GTA AAG TGG-3′ was amplified by using reduced-fidelity PCR (*20*−*22*), generating random mutations throughout the sequence, and ligated into the *Sac*I and *Sph*I restriction sites of the wild-type *lhcb1* gene. The cloned fragment encoded amino acids between L85 and S123 (numbering according to ref *3*).

Screening of Lhcb1 Mutants. A one-vessel procedure for the expression of the Lhcb1 mutants and their reconstitution with pigments on 96-well plates was developed in our previous study (*17*). As described there in detail, energy transfer from chl *b* to chl *a* was assessed by exciting chl *b* at 480 nm and detecting chl *a* (680 nm) and chl *b* (660 nm) emission, using a Fluoroscan Ascent apparatus (Labsystems, Helsinki, Finland) operated with Ascent version 2.1. A low F_{680}/F_{660} ratio was taken as an indication of an unsuccessful reconstitution.

Screening by Partially Denaturing Gels. From each 96-well plate, the 10 mutant proteins exhibiting the lowest F_{680}/F_{660} ratio were expressed in larger amounts, using the method described in ref *2*. Reconstitution was performed by changing the detergent from dodecyl sulfate to octyl glycoside as reported previously (*23*). The protein concentration was 0.4 μg/μL, and the total pigment extract from pea leaves (*2*) was used with a final chlorophyll concentration of 1 μg/μL during reconstitution, determined according to ref *24*. The pigment extract contained chl *a* and chl *b* in a 3:1 ratio and xanthophylls (lutein, neoxanthin, and violaxanthin at a 3:1:1 weight ratio) in a molar ratio of 0.2 compared to chlorophylls as determined by HPLC analysis (*25*). Reconstitution buffer contained 100 mM Tris-HCl (pH 9), 5 mM ϵ-aminocaproic acid, 1 mM benzamidine, 12.5% (w/v) sucrose, and 2% (w/v) lithium dodecyl sulfate. DTT was added after boiling to a final concentration of 1 mM.

Partially denaturing LDS−PAGE (*26*) was performed as described previously (*2*) on discontinuous gels with a 1 cm stacking gel [4.5% (w/v) polyacrylamide] and a 10 cm resolving gel (12%), containing 10 and 5% (v/v) glycerol, respectively.

RESULTS

Random Mutagenesis. In our previous work (*17*), we had simultaneously introduced random mutations in a transmembrane helix and loop domain of Lhcb1 to assess the sensitivity of protein folding and complex stability toward amino acid exchanges in these two types of domains. In this study, we focused on the lumenal loop domain of the protein, aiming to obtain a more complete picture of its sensitive amino acid positions.

The target domain for random mutagenesis comprised the entire lumenal loop as well as five amino acids of the first and one of the second transmembrane helix. This corresponds to a *Sac*I−*Sph*I fragment of the Lhcb1 expression plasmid. Following the mutation step, this fragment was religated into the nonmodified expression plasmid to confine the mutagenesis to the target protein section. Random mutagenesis was performed by low-fidelity PCR using biased nucleotide concentrations and Mn^{2+} ions (*17*, *20*). The PCR conditions

Table 1: Mutation Matrix for Mutagenic PCR

	to G	to A	to T	to C
from G		5	1	0
from A	4		16	1
from T	6	13		8
from C	1	2	2	

were optimized to create a maximum number of single-amino acid exchanges in the target domain requiring one or two nucleic acid exchanges due to the ambiguity of the genetic code. To this end, two consecutive rounds of PCRs were performed with the 100-fold diluted product of the first reaction serving as a template for the second one (*22*). Under the conditions used in this and our previous work (*17*), the mutation rate was estimated to be ca. 0.6 mutation/100 bp per PCR round, consistent with the mutation rate of 0.49% reported by Shafikhani et al. (*22*).

In our previous work, we had found that there was a bias toward exchanges of A and T, whereas all four bases were incorporated with approximately the same probability (*17*). In the study presented here, we again see a strong bias toward mutations in A (30%) and T (50%) positions over G (10%) and C (10%) positions, but the incorporated nucleotides were also somewhat biased toward A (34%) and T (32%) compared to G (19%) and C (15%), rendering the transversion of A to T or vice versa the most frequently observed base exchange (Table 1). This trend has been described before for similar mutageneses with DNA from *Bacillus subtilis* (*22*).

Screening of the Mutant Library. As described previously (*17*), a 96-well plate procedure was used to screen mutated proteins, allowing analysis of several thousand mutants at a reasonable effort. Single Lhcb1 expression clones originating from the random mutagenesis reactions were grown in the troughs of 96-well plates. All subsequent steps were performed as single-vessel reactions on the same plate. These steps included growing the bacteria, induction of Lhcb1 expression, lysis of the bacteria, enrichment and solubilization of Lhcb1 inclusion bodies, reconstitution of the protein with chlorophylls and carotenoids to yield monomeric recombinant LHCIIb, and screening for those proteins in which mutations had affected their ability to form stable pigment−protein complexes. This screen was done by fluorescence emission measurements (not shown), taking advantage of the fact that in reconstituted LHCIIb the excitation energy of chl *b* is efficiently transferred to chl *a*. Therefore, upon excitation of chl *b*, significant emission of chl *a* at 680 nm, rather than chl *b* at 660 nm, indicated successful LHCIIb formation. From each 96-well plate, we routinely picked 10 clones exhibiting the lowest F_{680}/F_{660} ratios and subjected these to a second level of screening.

Of all the selected clones, proteins were expressed on a larger scale and subjected to low-stringency detergent-exchange reconstitution (*23*). The reconstitution products were then analyzed by partially denaturing polyacrylamide electrophoresis (not shown). Similar results were obtained for reconstitutions that were carried out at pH 7 instead of pH 9 in the standard procedure. A total of 4300 mutants were analyzed; of these, 140 gave shortened or no expression products, presumably due to the mutagenic introduction of stop codons, and were disregarded. Thirty-three full-length

Table 2: Induced Amino Acid Substitutions

single mutations (15)[a]	double mutations (10)	triple mutations (3)	quadruple mutations (1)
S86P	L85M/S102F	S86P/E107K/L113M	K91I/E94G/F98Y/L118S
G89S	V90D/W97G	V90A/K91I/I104F	
E94G (2)[b]	V90G/E94V	F98L/Q103L/H120L	
W97R (3)[b]	K91I/W97R		
G101R	W97C/A121G		
S102T	F98Y/Q122L		
E107V	S102P/S123G		
G109R	F105L/L113M		
D111V	L113S/N115K		
G114D	V119A/Q122L		
N115Y			
H120L			
H120Y			
Q122H			
Q122L (2)[b]			

[a] Number of mutants found. [b] Number of mutants bearing the same amino acid substitution identified in independent experiments.

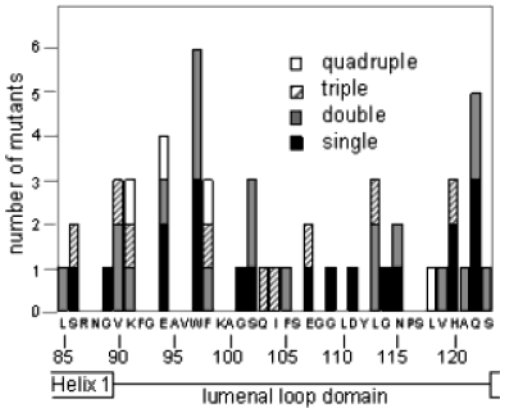

FIGURE 1: For each amino acid in the target domain, the number of hits in independent single or multiple mutations is given. Amino acids and borders of helix and loop domains are numbered according to that in ref *3*.

Table 3: Character of Amino Acids Hit as Single Mutations

	hydrophobic	polar	acidic	basic	glycine
no. of occurrences in sequence	16	11	3	3	6
no. of single hits	1	5	3	0	4
% hits	6	45	100	0	67

Lhcb1 mutants showed no reconstitution in three independent experiments. The DNA encoding the target domains of these mutants was sequenced to identify the amino acid exchanges; the result is shown in Table 2. Figure 1 shows where the amino acid exchanges are located in the target domain. In addition to 15 different single-amino acid exchanges, 10 double, 3 triple, and 1 quadruple mutations were found. In the case of multiple hits, it is impossible in most cases to tell which amino acid exchange is responsible for the inhibition of pigment−protein complex formation; therefore, only the single-amino acid exchanges were further analyzed. Table 3 summarizes the frequency at which different types of amino acids have been hit by single-amino acid exchanges. The relative frequency is high for acidic and polar amino acids and glycine and low for hydrophobic and basic amino acids.

DISCUSSION

Distribution of Single-Amino Acid Exchanges Influencing the Stability of LHCIIb. Single-amino acid exchanges reducing the stability of LHCIIb are found nearly equally distributed over the entire mutated sequence with no obvious hot-spot regions (Figure 1). Approximately 30% of the residues in the loop domain have been hit as single mutations, confirming our earlier finding that the loop domains are sensitive toward amino acid exchanges that reduce complex stability. In the electron crystallographic analysis of the LHCIIb structure (*3*), the section between amino acids A100 and P116 was not resolved, indicating a higher mobility or otherwise less defined structure. We have no indication of a lower sensitivity of this loop section toward mutations and conclude that it contributes as much to LHCIIb formation or stability as the rest of the lumenal loop domain.

In the study by Zhou et al. (*27*), the edges of transmembrane helices turned out to be sensitive targets for enhancing the stability of diacylglycerol kinase, an integral membrane protein of *Escherichia coli*, rendering these regions potential targets for destabilizing exchanges as well. Two of the four amino acids defining the edges of the two transmembrane helices have been hit in single mutations in this study (Figure 1); however, the neighboring amino acids have not, and when the density of hits over the entire loop is considered, no marked sensitivity is seen at the transitions between helix and loop structures.

The loop domains of Lhc proteins show a much lower degree of sequence conservation than the transmembrane helices (*28*), particularly when different members of the Lhca and Lhcb protein families are compared. Only the apoproteins of the major trimeric LHCIIb, Lhcb1 and Lhcb2 and, to a lesser extent, Lhcb3, exhibit more extensive similarity in their loop regions. When the loop regions of individual Lhcb proteins are compared between different higher-plant species, they are almost identical (*29*, *30*). The only segment of the lumenal loop that is conserved throughout the Chl *a*/*b* family is the $W_{97}FXAG_{101}$ motif (*31*).

The conserved $W_{97}FXAG_{101}$ motif is thought to bind the pigment cluster of chl a_6, chl a_7, and one lutein headgroup (*32*). Thus, mutations in this region may have an impact on pigment binding. In our mutational analysis, the W and G of the WFXAG motif have been hit (for W_{97}, see below) whereas F and A are not found among the sensitive positions, or they show up in only multiple mutations. If the WFXAG motif is particularly significant for pigment binding or complex stabilization, then our analysis suggests that, with regard to the stability of monomeric pigment−protein complexes of Lhcb1, the rest of the lumenal loop is about as important.

Limitations of the Mutational Analysis. It should be kept in mind that our mutations could not replace each amino acid with any other amino acid. Our mutational rate only allowed for single nucleotide exchanges within each codon. Because of the degeneracy of the genetic code, five to eight of the nine possible nucleotide exchanges (six on average) led to a different amino acid. However, this is only a partial limitation, as glycine, possessing a highly degenerate code, nevertheless turned out to be a particularly frequent target (see below). The bias in nucleotide exchange (Table 1) further limited the probability of certain amino acid exchanges. G and C positions are less frequently mutated than A and T positions; consequently, codons containing G and C exclusively are 4-fold less likely to be altered than A- and T-containing codons. Again, this is a partial limitation only, as G114 with a purely G- and C-containing codon is among the singular hits in our study.

We have not been able to carry our mutational analysis into saturation, although we have analyzed a much larger number of mutants in this study than in our previous one (*17*). Saturation would be reached when all single-amino acid exchanges appear in more than one independent round of mutation and selection. In the study presented here, this is only true for four of 13 single hits. We would have needed many more rounds of mutation to repeat all single hits, due to the unexpectedly high number of single-amino acid exchanges in the lumenal loop domain that abolish formation of the pigment−protein complex under our reconstitution conditions. Therefore, the observed 30% of sensitive amino acid positions must be considered a lower estimate; it is highly likely that a larger number of random mutations would reveal an even larger percentage of amino acid positions that have an impact on LHCIIb formation or stability.

Destabilizing Amino Acid Exchanges. The amino acid exchanges were not clustered in any section of the lumenal loop domain; on the other hand, the distribution of hit amino acids was far from random with regard to the character of the exchanged amino acids. With the exception of one hydrophobic residue, only polar, acidic, and glycine amino acids were hit in single mutations.

The acidic residues, all of which were exchanged, appeared to be a particularly sensitive target in this mutational study (Table 3). All three acidic residues were substituted with small apolar residues (G and V), although in every case substitution with the other acidic residue could have been reached by two different single-nucleotide exchanges. Of course, we cannot exclude the possibility that the significance of these mutations is in the decrease in size or polarity. However, the astonishingly different mutation frequency of acidic versus basic amino acids (three of three vs zero of three) suggests that it is the loss of negative charge that abolishes the ability of the protein to form stable pigment complexes. By contrast, the loss of positive charge does not appear to have an impact. If we look at the charges introduced by the amino acid exchanges, we see the opposite trend: three mutants that no longer reconstitute carry an additional positive charge but only one carries an extra negative charge.

If there is in fact a correlation between added positive net charge in the lumenal loop and LHCIIb destabilization, this may have a regulatory function. When thylakoids are highly energized, the lumen pH drops to values of ~5. The low lumen pH then activates violaxanthin deepoxidase, triggering the formation of zeaxanthin in the xanthophyll cycle. This in turn dissipates excess excitation energy that resides in the light-harvesting system and helps to avoid photooxidative damage due to overexcitation (*33*−*37*). Regardless of whether the deepoxidase gains access to an LHCIIb-bound violaxanthin or the pigment needs to dissociate from the complex before being converted (*38*, *39*), a relatively loose structure of the pigment−protein complex is needed on its lumenal surface before violaxanthin deepoxidation is to take place. This could be brought about by a pH-regulated

structural reorientation of the lumenal loop. In fully energized thylakoids, at least part of the D and E side chains in the lumenal loop of LHCIIb become protonated and, thus, lose their negative charge. This may lead to a partial detachment of the lumenal loop from the rest of LHCIIb if the acidic side chains at neutral pH form salt bridges with basic amino acids. According to the LHCIIb crystal structure, an interaction is possible between E94 and K203 in the loop between helix 3 and the amphiphilic helix. In fact, for bR the cross bracing of loop domains via salt bridges has been suggested to be a prerequisite for properly positioning the adjacent transmembrane helices (*40*). The position of E107 and D111 in the LHCIIb crystal structure is not well-resolved (*3*), but independent of their exact position, the nearest basic amino acid is K91, located in the lumenal loop domain. K91 was hit in three multiple mutation events but never as a single mutation, suggesting but not proving its participation in the stabilization of LHCIIb. Besides salt bridges, acidic amino acids may be involved in H-bond interactions with polar amino acids, and these interactions may be regulated by pH as well. It would be conceivable that the lack of such interactions of the lumenal loop domain also lowers the overall stability of LHCIIb or interferes with the folding of its apoprotein. Therefore, we propose that mutations decreasing the net negative charge of the lumenal loop mimic the situation in energized thylakoids where negatively charged side chains become protonated causing the loop to adopt a less compact structure and that this explains the failure of these mutants to form stable LHCIIb in vitro. This conjecture can be tested by more structurally conservative exchanges of D and E residues in the stromal loop with N and Q, respectively. The prediction is that these also lead to LHCIIb destabilization.

The notion of negative charges potentially playing a significant role in the lumenal loop domain is supported by sequence data of Lhcb1−3 proteins in *Arabidopsis* (30). Although differences between Lhcb1- and -2 and Lhcb3 include charged amino acids, the net charge of the lumenal loop domain in Lhcb3 is −1, as in Lhcb1 and -2, and the acidic amino acids of Lhcb1 and -2 are conserved in Lhcb3.

The hydrophobic amino acids, constituting ~40% of the amino acids in the target domain, are clearly underrepresented by only one residue (W97) among the amino acids hit in single-mutation events (Table 3). In our previous study (*17*), two deleterious exchanges of hydrophobic amino acids were observed in the stromal loop domain, one L to P exchange and one P to L exchange. In either case, the hydrophobicity was conserved but P was involved, an amino acid known to introduce kinks into the polypeptide backbone and, thus, likely to change the overall structure of a domain. We conclude that hydrophobic amino acids, although abundant in LHCIIb loop domains, do not significantly add to complex stability.

The only hydrophobic residue, W97, that in our mutational study is shown to be important for LHCIIb stability stands out in that it has been hit in three independent mutagenesis events. This W is conserved in most chl *a*/*b* proteins (*31*). In membrane proteins, tryptophans are frequently found near the membrane surfaces and seem to prefer the hydrated interface rather than the hydrophobic core of the bilayer (*41*). For the photosynthetic reaction center of purple bacteria, Schiffer et al. (*42*) reported that tryptophan residues can link

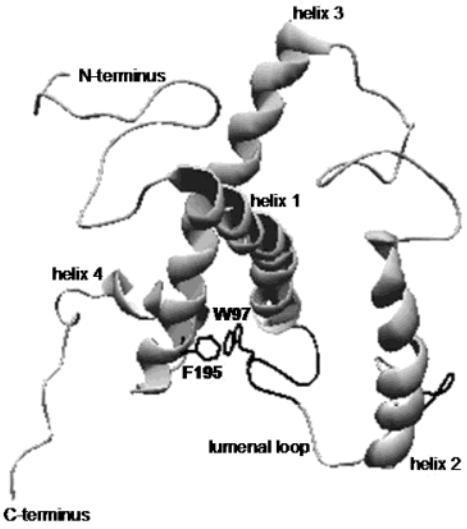

FIGURE 2: Relative positions of W97 and F195 in the three-dimensional backbone structure of LHCIIb (W. Kühlbrandt, personal communication). Side chains of W97 and F195 and the structurally resolved parts of the lumenal loop domain are highlighted in black. The three-dimensional structure was displayed by Deep View/Swiss Pdb Viewer version 3.7 (www.expasy.org/spdbv).

distant chain segments. Structural data (*3*) indicate a linking function for W97 as well, as the lumenal loop appears to be pulled into the membrane by this residue that comes into the proximity of F195 at the distal end of helix 3 (Figure 2). The distance between the centers of the aromatic rings could not be determined exactly but is likely to be in the range of 4−6 Å, allowing aromatic interaction which is also favored by the edge-to-face orientation of the residues (*43*). This interaction, providing a linkage of the lumenal loop and helix 3, may be essential for LHCIIb stability. F195 is conserved in most chl *a*/*b* proteins as well (*31*), and it was hit three times in our previous study, although in multiple mutants exclusively (*17*). Interestingly, in progressing exo deletions from the C-terminus of recombinant LHCIIb apoprotein, it was a W, too, in position 222 whose loss caused destabilization of the complex (*15*). Point mutation of W222 to H or G, although it did not affect pigment binding, abolished formation of trimeric complexes, whereas replacement with F restored trimerization (*15*). In the study presented here, W97 has been replaced with R in three single mutants. According to the point presented above, the loss of pigment−protein complex formation in this case can also be due to introduction of an extra positive charge. To confirm the significance of W97, we need to exchange it with noncharged amino acids.

Roughly half of the polar amino acids have been hit as single mutations, making them a more sensitive target for destabilizing mutations than hydrophobic amino acids. There is no apparent preference for polar residues with certain functional groups such as hydroxyl or amide groups. A striking example for the dependence of LHCIIb stability on the correct polar amino acids in its lumenal loop is the apparently rather conservative S102T exchange. The repositioning of the hydroxyl group may be sufficient to abolish stable LHCIIb formation.

Glycines also turned out to be rather frequent targets for destabilizing mutations, as four of six glycine residues have been hit in single-mutation events (Table 3). Glycine, as the

smallest amino acid, can be counted among neither the polar nor the hydrophobic amino acids; it is known to provide local flexibility to polypeptides. Two of the G residues have been replaced with amino acids carrying positive charges, which in these cases may have affected LHCIIb formation (see above).

Taken together, these observations confirm and extend our previous notion that the loop domains in LHCIIb have a more structurally specific impact on the formation and/or stabilization of LHCIIb than merely serving as passive connectors of the α-helical transmembrane domains. If the latter was the case, we would not expect single-amino acid exchanges in every third position to disable the protein to form a stable complex with pigments. Probably even more than one-third of the amino acid positions are sensitive ones because our mutation analysis was not extended into saturation. The larger number of destabilizing amino acid exchanges analyzed in this work compared to our previous one (*17*) allows us to draw some conclusions about the nature of intramolecular interactions undergone by the lumenal loop. The highest frequency of mutational hits abolishing LHCIIb reconstitution was among hydrophilic amino acids, specifically acidic ones, pointing to stabilizing electrostatic or H-bond interactions. These interactions may not only be important for LHCIIb stability but also play a role in regulatory functions of LHCIIb such as the xanthophyll cycle in which the lumenal loop may be involved. Possibilities for such interactions like the ones between E107 or D111 and K91 will be tested by site-specific exchanges in these positions and then testing the impact of these mutations on complex stability or functional properties such as conversion of violaxanthin to zeaxanthin.

Since we characterized our mutants simply by measuring the yield of the pigment−protein complex in the reconstitution assay, we cannot tell whether non-reconstituting mutants failed to form pigment−protein complexes at all, due to inhibition of the folding process, or whether the mutants folded in a wild-type-like process, but produced insufficiently stable complexes. Studies with bR constructs with single-loop domains replaced with linkers of GGS repeats indicated a participation of some of the loop domains in both the folding and stability of bR (*12*, *40*). Time-resolved measurements of pigment−protein complex assembly with the protein mutants will be needed to test whether structural alterations in the lumenal loop domain of LHCIIb also affect protein folding. The question of whether some of the mutants assemble into complexes with reduced stabilities will be the subject of further studies.

Little is known so far about proteins or other molecules interacting with the lumenal surface of LHCIIb. These probably include violaxanthin epoxidase (see above), but there may be more, so far unidentified components of the lumen that bind to LHCIIb. Clearly, our approach cannot be expected to identify such interactions at the lumenal loop of the Lhcb1 complex since we screened our random mutants only for their ability to form stable pigment−protein complexes and not for other functions. However, our observation that hydrophilic and in particular acidic amino acids are essential for Lhcb1 folding and/or pigment binding indicates that interactions of the loop domain with other sections of the protein are involved in these processes. If charges are involved in stabilizing interactions of the lumenal loop, this would open up the possibility, as outlined above, of a pH-dependent destabilization, facilitating violaxanthin conversion or antenna degradation at an acidic pH. Testing this hypothesis in vivo will require transgenic plants having their endogenous Lhcb1 and -2 suppressed by antisense RNA (*44*) and expressing one of the mutant Lhcb1 versions instead.

REFERENCES

1. Plumley, F. G., and Schmidt, G. W. (1987) Reconstitution of chlorophyll *a/b* light-harvesting complexes: Xanthophyll-dependent assembly and energy transfer, *Proc. Natl. Acad. Sci. U.S.A. 84*, 146−150.
2. Paulsen, H., Rümler, U., and Rüdiger, W. (1990) Reconstitution of pigment-containing complexes from light-harvesting chlorophyll *a*/*b*-binding protein overexpressed in *Escherichia coli*, *Planta 181*, 204−211.
3. Kühlbrandt, W., Wang, D. N., and Fujiyoshi, Y. (1994) Atomic model of plant light-harvesting complex by electron crystallography, *Nature 367*, 614−621.
4. Popot, J. L., and Engelman, D. M. (1990) Membrane protein folding and oligomerization: The two-stage model, *Biochemistry 29*, 4031−4037.
5. Popot, J. L., and Engelman, D. M. (2000) Helical membrane protein folding, stability, and evolution, *Annu. Rev. Biochem. 69*, 881−922.
6. Booth, P. J., Flitsch, S. L., Stern, L. J., Greenhalgh, D. H., Kim, P. S., and Khorana, H. G. (1995) Intermediates in the folding of the membrane protein bacteriorhodopsin, *Struct. Biol. 2*, 139−143.
7. Liao, M. J., London, E., and Khorana, H. G. (1983) Regeneration of the native bacteriorhodopsin structure from two chymotryptic fragments, *J. Biol. Chem. 258*, 9949−9955.
8. Kahn, T. W., and Engelman, D. M. (1992) Bacteriorhodopsin can be refolded from two independently stable transmembrane helices and the complementary five-helix fragment, *Biochemistry 31*, 6144−6151.
9. Marti, T. (1998) Refolding of bacteriorhodopsin from expressed polypeptide fragments, *J. Biol. Chem. 273*, 9312−9322.
10. Gilles-Gonzalez, M. A., Engelman, D. M., and Khorana, H. G. (1991) Structure−function studies of bacteriorhodopsin XV, *J. Biol. Chem. 266*, 8545−8550.
11. Teufel, M., Pompejus, M., Humbel, B., Friedrich, K., and Fritz, H. J. (1993) Properties of bacteriorhodopsin derivatives constructed by insertion of an exogenous epitope into extra-membrane loops, *EMBO J. 12*, 3399−3408.
12. Kim, J.-M., Booth, P. J., and Allen, S. J. (2001) Structure and function in bacteriorhodopsin: The role of the interhelical loops in the folding and stability of bacteriorhodopsin, *J. Mol. Biol. 308*, 409−422.
13. Horn, R., and Paulsen, H. (2002) Folding in vitro of light-harvesting chlorophyll *a*/*b* protein is coupled with pigment binding, *J. Mol. Biol. 318*, 547−556.
14. Cammarata, K. V., and Schmidt, G. W. (1992) In vitro reconstitution of a light-harvesting gene product: Deletion mutagenesis and analysis of pigment binding, *Biochemistry 31*, 2779−2789.
15. Paulsen, H., and Hobe, S. (1992) Pigment-binding properties of mutant light-harvesting chlorophyll-*a*/*b*-binding protein, *Eur. J. Biochem. 205*, 71−76.
16. Paulsen, H., and Kuttkat, A. (1993) Pigment complexes of light-harvesting chlorophyll *a*/*b* binding protein are stabilized by a segment in the carboxyterminal hydrophilic domain of the protein, *Photochem. Photobiol. 57*, 139−142.
17. Heinemann, B., and Paulsen, H. (1999) Random mutations directed to transmembrane and loop domains of the light-harvesting chlorophyll *a*/*b* protein: Impact on pigment binding, *Biochemistry 38*, 14088−14093.
18. Cashmore, A. R. (1984) Structure and expression of a pea nuclear gene encoding a chlorophyll *a*/*b*-binding polypeptide, *Proc. Natl. Acad. Sci. U.S.A. 81*, 2960−2964.
19. Chen, B., and Przybyla, A. E. (1994) An efficient site-directed mutagenesis method based on PCR, *BioTechniques 17*, 657−659.
20. Cadwell, C. R., and Joyce, G. F. (1992) Randomization of genes by PCR mutagenesis, *PCR Methods Appl. 2*, 28−33.

21. Vartanian, J.-P., Henry, M., and Wain-Hobson, S. (1996) Hypermutagenic PCR involving all four transitions and a sizeable proportion of transversions, *Nucleic Acids Res. 24*, 2627−2631.
22. Shafikhani, S., Siegel, R. A., Ferrari, E., and Schellenberger, V. (1997) Generation of large libraries of random mutants in *Bacillus subtilis* by PCR-based plasmid multimerization, *BioTechniques 23*, 304−310.
23. Paulsen, H., Finkenzeller, B., and Kühlein, N. (1993) Pigments induce folding of light-harvesting chlorophyll *a*/*b*-binding protein, *Eur. J. Biochem. 215*, 809−816.
24. Porra, R. J., Thompson, W. A., and Kriedemann, P. E. (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: Verification of the concentration of chlorophyll standards by atomic absorption spectroscopy, *Biochim. Biophys. Acta 975*, 384−394.
25. Hobe, S., Niemeier, H., Bender, A., and Paulsen, H. (2000) Caotenoid binding sites in LHCIIb, *Eur. J. Biochem. 267*, 616−624.
26. Delepelaire, P., and Chua, N. H. (1981) Electrophoretic purification of chlorophyll *a*/*b* protein complexes from *Chlamydomonas reinhardtii* and spinach and analysis of their polypeptides, *J. Biol. Chem. 256*, 9300−9307.
27. Zhou, Y., and Bowie, J. U. (2000) Building a thermostable membrane protein, *J. Biol. Chem. 275*, 6975−6979.
28. Green, B. R., and Durnford, D. G. (1996) The chlorophyll-carotenoid proteins of oxygenic photosynthesis, *Annu. Rev. Plant Physiol. 47*, 685−714.
29. Jansson, S. (1994) Review: The light-harvesting chlorophyll *a*/*b*-binding proteins, *Biochim. Biophys. Acta 1184*, 1−19.
30. Jansson, S. (1999) A guide to the Lhc genes and their relatives in *Arabidopsis*, *Trends Plant Sci. 4*, 236−240.
31. Green, B. R., and Pichersky, E. (1994) Hypothesis for the evolution of three-helix Chl *a*/*b* and Chl *a*/*c* light-harvesting antenna proteins from two-helix and four-helix ancestors, *Photosynth. Res. 39*, 149−162.
32. Green, B. R., and Kühlbrandt, W. (1995) Sequence conservation of light-harvesting and stress-response proteins in relation to the three-dimensional molecular structure of LHCII, *Photosynth. Res. 44*, 139−148.
33. Horton, P., Ruban, A. V., and Walters, R. G. (1994) Regulation of light harvesting in green plants: Indication by nonphotochemical quenching of chlorophyll fluorescence, *Plant Physiol. 106*, 415−420.
34. Horton, P., Ruban, A. V., and Walters, R. G. (1996) Regulation of light harvesting in green plants, *Annu. Rev. Plant Physiol. 47*, 655−684.
35. Niyogi, K. K. (1999) Photoprotection revisited: Genetic and molecular approaches, *Annu. Rev. Plant Physiol. 50*, 333−359.
36. Müller, P., Li, X. P., and Niyogi, K. K. (2001) Non-photochemical quenching. A response to excess light energy, *Plant Physiol. 125*, 1558−1566.
37. Morosinotto, T., Caffarri, S., Dall'Osto, L., and Bassi, R. (2003) Mechanistic aspects of the xanthophyll dynamics in higher plant thylakoids, *Physiol. Plant. 119*, 347−354.
38. Jahns, P., Wehner, A., Paulsen, H., and Hobe, S. (2001) De-epoxidation of violaxanthin after reconstitution into different carotenoid binding sites of light-harvesting complex II, *J. Biol. Chem. 276*, 22154−22159.
39. Morosinotto, T., Baronio, R., and Bassi, R. (2002) Dynamics of chromophore binding to Lhc proteins in vivo and in vitro during operation of the xanthophyll cycle, *J. Biol. Chem. 277*, 36913−36920.
40. Allen, S. J., Kim, J. M., Khorana, H. G., Lu, H., and Booth, P. J. (2001) Structure and function in bacteriorhodopsin: The effect of the interhelical loops on the protein folding kinetics, *J. Mol. Biol. 308*, 423−435.
41. Yau, W. M., Wimley, W. C., Gawrisch, K., and White, S. H. (1998) The preference of tryptophan for membrane interfaces, *Biochemistry 37*, 14713−14718.
42. Schiffer, M., Chang, C. H., and Stevens, F. J. (1992) The functions of tryptophan residues in membrane proteins, *Protein Eng. 5*, 213−214.
43. Burley, S. K., and Petsko, G. A. (1986) Dimerization energetics of benzene and aromatic amino acid side chains, *J. Am. Chem. Soc. 108*, 7995−8001.
44. Ruban, A. V., Wentworth, M., Yakushevska, A. E., Andersson, J., Lee, P. J., Keegstra, W., Dekker, J. P., Boekama, E. J., Jansson, S., and Horton, P. (2003) Plants lacking the main light-harvesting complex retain photosystem II macro-organization, *Nature 421*, 648−652.

BI036198S