The Folding State of the Lumenal Loop Determines the Thermal Stability of Light-Harvesting Chlorophyll *a*/*b* Protein†

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ABSTRACT: The major light-harvesting protein of photosystem II (LHCIIb) is the most abundant chlorophyll-binding protein in the thylakoid membrane. It contains three membrane-spanning α helices; the first and third one closely interact with each other to form a super helix, and all three helices bind most of the pigment cofactors. The protein loop domains connecting the α helices also play an important role in stabilizing the LHCIIb structure. Single amino acid exchanges in either loop were found to be sufficient to significantly destabilize the complex assembled in vitro [Heinemann, B., and Paulsen, H. (1999) *Biochemistry 38*, 14088−14093. Mick, V., Eggert, K., Heinemann, B., Geister, S., and Paulsen, H (2004) *Biochemistry 43*, 5467−5473]. This work presents an analysis of such point mutations in the lumenal loop with regard to the extent and nature of their effect on LHCIIb stability to obtain detailed information on the contribution of this loop to stabilizing the complex. Most of the mutant proteins yielded pigment−protein complexes if their reconstitution and/or isolation was performed under mild conditions; however, the yields were significantly different. Several mutations in the vicinity of W97 in the N-proximal section of the loop gave low reconstitution yields even under very mild conditions. This confirms our earlier notion that W97 may be of particular relevance in stabilizing LHCIIb. The same amino acid exchanges accelerated thermal complex dissociation in the absence of lithium dodecyl sulfate (LDS) and raised the accessibility of the lumenal loop to protease; both effects were well correlated with the reduction in reconstitution yields. We conclude that a detachment of the lumenal loop is a possible first step in the dissociation of LHCIIb. Dramatically reduced complex yields in the presence but not in the absence of LDS were observed for some but not all mutants, particularly those near the C-proximal end of the loop. We conclude that complex stabilities in the absence and in the presence of LDS do not correlate and most likely are determined by different structural characteristics, at least in LHCIIb but maybe also in other membrane proteins.

The structure of intrinsic membrane proteins is largely dictated by the hydrophobic environment in the lipid bilayer, which is a more favorable environment than the aqueous phase for the hydrophobic amino acid side chains in the transmembrane protein domains. The lipid environment strongly stabilizes secondary structure in these domains, β sheets or, in most cases, α helices, since these shield the strongly polar peptide bonds from the hydrophobic lipid phase (*1*). As proposed in the two-stage model of Popot and Engelman (*2*, *3*), these α helices can be viewed as independently forming structural units within the polypeptide that then, in a second step during membrane protein folding, associate. The final arrangement of helices, characteristic for each polytopic membrane protein, is stabilized by helix−helix interactions such as knobs-into-holes packing (*4*) and electrostatic interactions, often favored by sequence motifs (*5*, *6*). In a recent extension of the two-stage model, Engelman et al. (*7*) proposed that helix association is followed by further events such as the folding of the loop domains and cofactor binding. They propose that the structure of loop domains that fold into the lipid bilayer may be stabilized by forming hydrogen bonds with transmembrane protein sections.

In general, less is known about the folding and structure determination of membrane proteins compared to water-soluble proteins (*8*, *9*). This is mainly due to the much smaller number of membrane proteins whose atomic structure is known and that can be folded in vitro. One such protein is the major light-harvesting chlorophyll *a*/*b* protein (LHCIIb)[1] of the plant photosynthetic apparatus, probably the most abundant membrane protein on Earth. The near atomic structure of LHCIIb was elucidated by electron crystallography (*10*) and has recently been refined by X-ray crystallography (*11*). The LHCIIb apoprotein, either native (*12*) or recombinant (*13*), spontaneously folds and assembles

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[1] Abbreviations: bR, bacteriorhodopsin; DTT, dithiothreitol; LDS, lithium dodecyl sulfate; LHCIIb, major light-harvesting antenna protein of photosystem II, containing the apoproteins Lhcb1−3; Lhcb1, apoprotein of LHCIIb; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PSII, photosystem II; SDS, sodium dodecyl sulfate.

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with pigments, 14 chlorophylls and at least 3 carotenoids, in detergent solution to yield structurally authentic LHCIIb (*14*). Xanthophylls, in particular lutein, and chlorophyll *b* are required for stable complexes to form (*12*, *15*, *16*), whereas chlorophyll *a* can be omitted (*17*). Reconstitution of recombinant LHCIIb in vitro allows mutational analyses of LHCIIb assembly to be performed to identify structural elements essential for proper folding, pigment binding, and stabilization of the native structure.

LHCIIb contains three transmembrane α helices, two of which intertwine to form a left-handed super helix that is stabilized, among other interactions, by two ion pairs that at the same time coordinate chlorophyll cofactors. The two closely interacting helices, together with the third membrane-spanning helix, bind most of the photosynthetic pigments. The role of the loop domains, connecting these helices, for stabilizing the pigment−protein complex is less clear. Therefore, we challenged the structural significance of the stromal loop by introducing mutations into random positions of the recombinant protein. To our surprise, we found several single amino acid exchanges that completely abolished stable pigment−protein complex formation under the in vitro conditions used (*18*). A more extensive mutational analysis of the lumenal loop revealed that single amino acid exchanges in at least one-third of its positions prevented the protein from folding and assembling with pigments (*19*). These amino acid exchanges were evenly spread over the entire length of the loop, but their nature seemed far from random: Whereas all three acidic and roughly half of the polar residues and glycines have been hit, none of the three basic and only one out of sixteen hydrophobic amino acids were among the mutations selected for complex destabilization. This prompted us to study the consequences for LHCIIb stability of individual amino acid exchanges in more detail, expecting to obtain information on which interactions or structural components of the lumenal loop domain are so essential for stabilizing the monomeric pigment−protein complex.

MATERIALS AND METHODS

Lhcb1 Mutants. Mutants of *lhcb*1*2 gene "AB80" from pea (*20*) bearing single amino acid exchanges were obtained by random mutagenesis and selection of nonreconstituting mutants as described in ref *19*. The exchanged amino acids were located in the lumenal loop domain of LHCIIb, between positions V90 and Q122 (*10*), and among the four C-proximal amino acids of helix 1. A His_6 tag was added to the C-terminus of W97R by exchanging the 3′ DNA fragment of the gene via *Eco*RI and *Bst*EII (New England Biolabs, Frankfurt am Main, Germany) restriction with the expression plasmid C3.2h (*21*), coding for the Lhcb1 sequence tagged by a stretch of six histidine residues. Proteins were overexpressed as described in ref *13*.

Reconstitution by Detergent Exchange. Monomeric pigment−protein complexes were reconstituted by changing the detergent from dodecyl sulfate to mainly octyl glycoside as reported in ref *22*. The protein concentration was 0.4 μg/μL, and total pigment extract from pea leaves (*13*) was used with a final chlorophyll concentration of 1 μg/μL during reconstitution (determined according to ref *23*). The pigment extract contained chlorophyll *a* and chlorophyll *b* in a 3:1 ratio and xanthophylls from pea leaves, consisting of lutein, neoxanthin, and violaxanthin at a 3:1:1 weight ratio, with a xanthophyll:chlorophyll molar ratio of 0.2 as was determined by HPLC analysis (*24*). Additional xanthophylls with the same composition were supplied only where indicated to a final concentration of 0.4 μg/μL in the reconstitution mixture. Reconstitution buffer contained 100 mM Tris−HCl (pH 9), 5 mM ϵ-aminocapronic acid, 1 mM benzamidine, 12.5% sucrose, and 2% lithium dodecyl sulfate. DTT was added after boiling to a final concentration of 1 or 10 mM as indicated.

Reconstitution by Subsequent Freeze−Thaw cycles. A second method for reconstituting monomeric LHCIIb consisted of three subsequent cycles of freezing and thawing as described in ref *12*. The concentration of Lhcb1 and chlorophylls as well as buffer composition was the same as for detergent-exchange reconstitution. The reconstitution mixture always contained xanthophylls of the composition mentioned above at 0.4 μg/μL and 10 mM DTT.

Partially Denaturing Gel Electrophoresis. Partially denaturing LDS−PAGE (*25*) was performed as described previously (*13*) on discontinuous gels with a 1 cm stacking gel (4.5% polyacrylamide) and a 10 cm resolving gel (15% polyacrylamide), containing 10% and 5% glycerol, respectively. Deriphat−PAGE (*26*) was performed on the same gels and under the same conditions as LDS−PAGE, using 12 mM Tris, 0.15% (w/v) Deriphat 160 (Henkel, Düsseldorf, Germany), and 48 mM glycine as the running buffer. Coomassie-stained bands of pigment−protein complexes and apoprotein were digitally photographed with a VersaDoc imaging system, model 3000 (BioRad Laboratories Inc.), and the intensity of the stained protein bands was measured as the peak height with Quantity One 4.2.3 software (BioRad, München, Germany). The relative intensities of pigment−protein bands were calculated as percentages of the sum of the intensities of pigment−protein and nonpigmented protein bands. Relative intensities obtained on one gel were normalized to that of wild-type Lhcb1.

Dissociation Kinetics at 37 °C. The dissociation of monomeric LHCIIb at 37 °C was measured by observing the decrease in the extent of intracomplex energy transfer from chlorophyll *b* to chlorophyll *a*, monitored by the decrease of chlorophyll *b*-stimulated chlorophyll *a* fluorescence emission. Measurements were performed as described in ref *27* in a fluorimeter (Fluoromax 3, Jobin Yvon Spex Instruments S.A. Inc., France) thermostated to 37 °C, with excitation and emission at 470 and 680 nm, respectively. Complexes were reconstituted by detergent exchange as described above, except the reconstitution buffer contained no benzamidine and ϵ-aminocaproic acid. Following reconstitution, mixtures were centrifuged for 20 min at 20000*g* and 4 °C. The reconstitution mixture was diluted 60-fold with ice-cold buffer (0.1 M Tris (pH 7), 1% (w/v) OG, and 10% glycerol), kept on ice for 5 min, and then transferred to the preheated cuvette (37 °C). Decay of chlorophyll *a* fluorescence was fitted to first-order kinetics (Table Curve 2D, SPSS Inc., Chicago, IL), and time constants were calculated as the reciprocals of apparent rate constants.

Trypsin Digestion. Monomeric complexes were reconstituted by detergent exchange as described above (reconstitution buffer without benzamidine and ϵ-aminocapronic acid), and 10 mM Hepes/KOH, pH 8, and 0.1 mg/mL trypsin

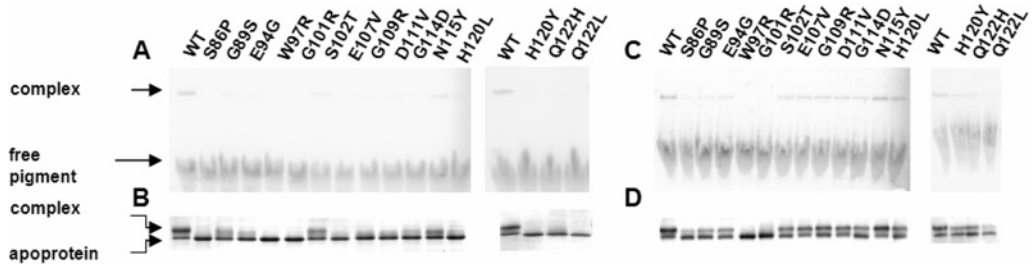

FIGURE 1: Reconstitution of the mutant Lhcb1 version by changing the detergent (in the presence of 10 mM DTT and 0.4 μg/μL xanthophylls) and analysis by partially denaturing LDS−PAGE (A, B) or Deriphat−PAGE (C, D) on 15% polyacrylamide gel. WT = wild-type Lhcb1.

(Roche Applied Science, Mannheim, Germany) were added. The mixture was incubated at 25 °C for time periods as indicated, and the reaction was stopped by adding 0.5 mM PMSF.

Denaturing SDS−PAGE. Denatured proteins were separated according to their molecular weight using the gel system of Schägger et al. (*28*). Gels contained 16.5% acrylamide/bisacrylamide and had a cross-linking ratio of 3%. No SDS was added to the gels. Unstained sample buffer (*28*) was added to the protein samples; the mixtures were boiled for 2 min, and then applied to the gel.

Western Blot. Proteins separated by SDS−PAGE were electrophoretically transferred to a PVDF membrane (Fluorotrans transfer membrane, PALL GmbH, Dreieich, Germany) and probed with a polyclonal anti-Lhcb1 antiserum (produced by Eurogentec, Herstal, Belgium) according to standard procedures. Bound secondary antibodies fused to alkaline phosphatase were detected by using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as a substrate.

RESULTS

In our previous study (*19*) we have described several mutants bearing single amino acid exchanges in the lumenal loop domain that have been selected from a random library due to their lower stability. Indeed, no pigment−protein complexes could be detected after partially denaturing LDS−PAGE. However, this analysis did not give us information on whether the protein simply does not fold at all to form monomeric LHCIIb or whether the complexes formed were not stable enough to survive the partially denaturing polyacrylamide gel. Therefore, we analyzed the mutants by using various procedures of different stringencies with regard to complex stability.

Partially Denaturing Gel Electrophoresis. As a first step, we repeated the reconstitution experiments under conditions known to increase the yield of recombinant LHCIIb with impaired complex stability: We raised the concentration of the reductant DTT in the reconstitution mixture and added extra xanthophyll to the total pigment extract. As an alternative to LDS−PAGE, we used a less stringent electrophoretic system with the mild detergent Deriphat in the running buffer (*26*). Figure 1 shows that under these conditions in fact some but not all mutant Lhcb1 versions showed green pigment−protein bands on partially denaturing LDS gels, and all of them with the exception of W97R formed green bands in the Deriphat−PAGE (Figure 1C,D). However, the intensity of the pigment−protein bands in Figure 1A,C and, consistently, the ratio of pigmented protein to nonpigmented protein visible upon Coomassie staining (Figure 1B,D) varied between different mutants. This indicates a more gradual decrease in complex stabilities of at least some mutant Lhcb1 versions, although all mutants exhibit significantly decreased stabilities compared to wild-type recombinant LHCIIb, according to the LDS−PAGE (Figure 1A,B).

In addition to detergent-exchange reconstitution (Figure 1) mutants were reconstituted by subsequent freeze−thaw cycles, a more stringent reconstitution method, known to prevent the formation of more labile mutant LHCIIb (*22*) (gels not shown). The combination of the two different reconstitution methods and the two electrophoretic systems resulted in four conditions of varying stringency. To compare reconstitution yields independently of slight variations of total protein amounts loaded on the gels, yields were expressed as the density of Coomassie-stained pigment−protein bands relative to the density of pigmented plus nonpigmented protein bands (Figure 2). The calculated values have been normalized to the relative intensities of pigment−protein complex bands obtained for the wild type. For the wild type the highest yield of LHCIIb (70%) was obtained with freeze−thaw reconstitution, followed by Deriphat−PAGE. Using other combinations of reconstitution methods and gel electrophoresis conditions, about 60% of the protein was reconstituted with pigments. As the pigment content of the complex bands contributes to their density, the calculated values are to be regarded as upper estimates of the respective percentage of proteins organized in pigment−protein complexes.

Under the mildest condition, i.e., detergent-exchange reconstitution followed by Deriphat−PAGE (Figure 2A, upper gray line), reconstitutions with all of the Lhcb1 mutants except W97R and G101R gave 60−90% of the yield obtained with the wild-type protein. Under the other conditions, yields were lower, in many cases considerably lower, compared to those of the wild type. These other three reconstitution conditions gave similar results with regard to yields of reconstituted pigment−protein complexes. Therefore, in the following we will refer to the former procedure as a "mild condition" and to the other three procedures as a "harsh condition".

To assess whether complex yields obtained by the mild and harsh reconstitution conditions were correlated among the various Lhcb1 mutants, relative intensities obtained in Deriphat− and LDS−PAGE following detergent-exchange reconstitution were plotted on the *x* and *y*axes, respectively (Figure 3). The bulk of mutants reached 60−90% of the wild-type's intensity on Deriphat gels (mild condition) but only between 0% and 70% on LDS gels (harsh condition). According to their position in the diagram, four groups of

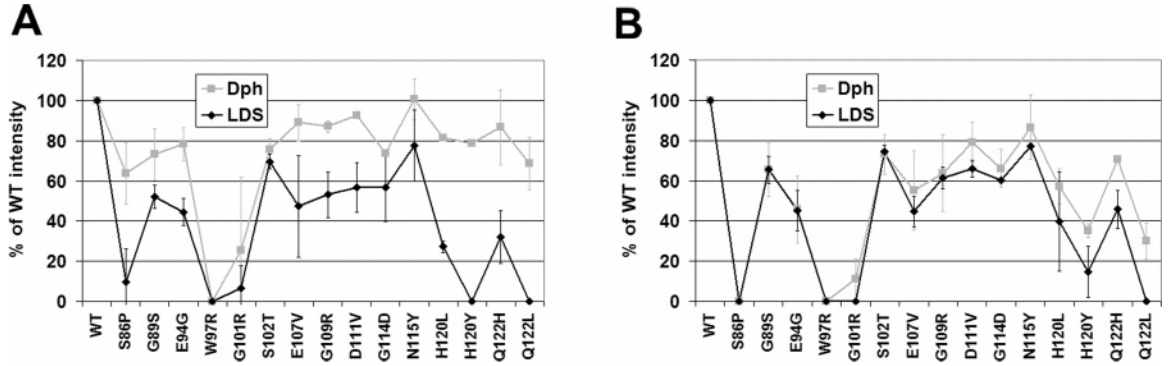

FIGURE 2: Intensity of green pigment−protein bands in percent of the WT complex band intensity. LHCIIb mutants were reconstituted by changing the detergent (A) or by subsequent freeze−thaw cycles (B) in the presence of 0.4 μg/μL xanthophyll and 10 mM DTT. Partially denaturing gels were run with LDS (LDS) or Deriphat (Dph) buffer as indicated in the legends. Mean values are calculated from intensities obtained on 2−3 gels, and error bars reflect the standard deviations. Mutants are named according to their amino acid exchange; the numbering corresponds to that of Kühlbrandt et al. (*10*), and the letters before and after the numbers denote the original and the substituting amino acids, respectively. WT = Lhcb1.

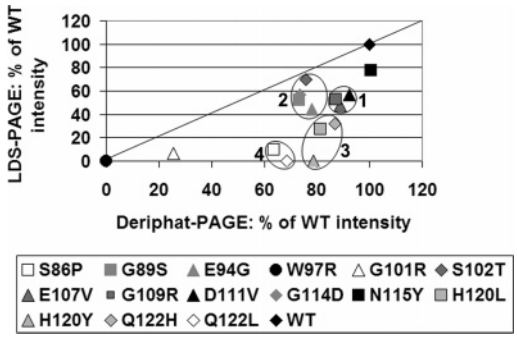

FIGURE 3: Intensities of green pigment−protein bands obtained on Deriphat and LDS gels after detergent change reconstitution (Figure 2A) plotted on the *x* and *y* axes, respectively. Mutants exhibiting similar intensities are marked by ellipses with group numbers referred to in the text and used in Figure 5. The black line gives the positions where the yields in either procedure would be identical.

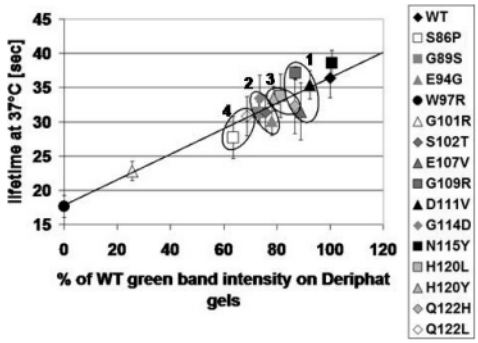

FIGURE 4: Time constants (s) of LHCIIb dissociation at 37 °C measured as the decay of sensitized chl *a* fluorescence upon chl *b* excitation (*y* axis) plotted against the intensities of green pigment−protein bands obtained after detergent change reconstitution followed by Deriphat−PAGE (Figure 2A). For kinetic measurements mutants were reconstituted by changing the detergent with total pigment extract and in the presence of 1 mM DTT. Mean values have been calculated from 3−4 measurements. Stability groups are marked by ellipses according to the ones in Figure 3.

mutants were tentatively defined as indicated in Figure 3. The grouping is tentative in that it is not meant to describe significantly different properties of individual mutants but helps to refer to mutants behaving differently under different experimental regimes. The mutants W97R and G101R are not included in these groups because they yielded little or no LHCIIb in either system. N115Y was not grouped either, because this mutant showed higher yields compared to the other mutants in the Deriphat system (100%) as well as in the LDS system (80%). Mutants of group 1 gave the highest yields in both the Deriphat (80−90% of the wild-type intensity) and the LDS (about 50%) systems. In group 2, yields are as in group 1 on LDS but lower (70−80%) on Deriphat gels. Mutants of groups 3 and 4 both yielded little pigmented protein (less than 40% of the wild-type yield) when isolated by LDS−PAGE but showed higher if different yields in the Deriphat system (80−90% and 60−70%, respectively). As becomes obvious with these examples, the two regimes apparently sort the mutants according to different characteristics, as the ranking of the mutants with respect to relative complex yields is different under these two conditions.

Complex yields were independent of the reconstitution method when the complexes were isolated on LDS gels (Figure 2). On Deriphat gels, however, complex yields depended on the reconstitution method. We therefore could not exclude that, in this case, the observed reconstitution yields were determined by the efficiency of the reconstitution step in the first place rather than by different stabilities of the mutants. But as the lifetime measurements revealed (see below), results of the low-stringency isolation are related to the thermal stabilities of the complexes.

Dissociation Kinetics of LHCIIb Mutants at 37 °C. Principally, pigment−protein complex yields in reconstitution experiments are determined by the protein folding and pigment binding efficiencies on one hand and by the stability of complexes formed during the isolation procedure on the other hand. The two regimes compared in Figure 3 used the same reconstitution but different isolation procedures, indicating that different yields reflect different stabilities of the reconstituted complexes. For a more direct assessment of complex stabilities, dissociation kinetics at 37 °C were performed (Figure 4). The disappearance of intracomplex energy transfer from chlorophyll *b* to chlorophyll *a* during thermal dissociation was monitored. The decrease in sensitized chl *a* fluorescence could be fitted with a single-exponential decay, yielding apparent time constants that give a measure of the thermal stability of the complexes. All mutants, even W97R, exhibited detectable energy transfer from chlorophyll *b* to chlorophyll *a* as compared to control

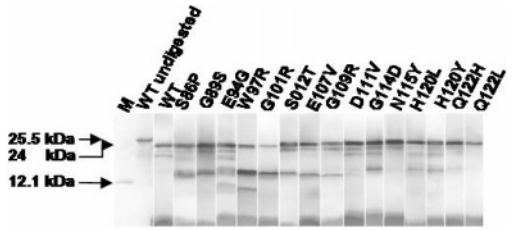

FIGURE 5: Following detergent change reconstitution with total pigment extract and 1 mM DTT, complexes were digested with 0.1 μg/μL trypsin for 25 min at 25 °C. Samples were separated by a denaturing SDS−PAGE and probed with anti-Lhcb1 antibody on a Western blot. A 0.75 μg sample of undigested wild-type Lhcb1 (WT) and 0.2 μg of a truncated Lhcb1 protein with a molecular mass of 12.1 kDa were used as markers (M). The protein amount in the digested samples corresponded to 3 μg of undigested protein.

measurements with the pigments in the absence of protein (not shown). The decrease of chlorophyll *a* fluorescence was accompanied by an increase of chlorophyll *b* fluorescence (not shown).

We found that the calculated lifetimes correlated quite well with the relative green band intensities obtained after detergent-exchange reconstitution followed by Deriphat−PAGE (Figure 4). Stability groups defined in Figure 3 are shown in Figure 4 as well. Mutants of stability groups 1−4 were not clearly separated regarding their lifetimes; most mutants showed lifetimes in the range of 30−34 s. In accordance with their behavior in gel electrophoresis, the most unstable mutants W97R and G101R dissociated significantly faster than any of the other mutants or the wild type. Clearly, the lifetimes of mutant LHCIIb versions at 37 °C do not correlate with their complex yields in the LDS−PAGE system (not shown). In this system, mutants of, e.g., group 2 gave LHCIIb yields as high as those of group 1 whereas the dissociation kinetics were significantly different. Consequently, thermal dissociation times and complex yields in the Deriphat system rank the mutant complexes according to apparently the same stability properties, whereas complex yields in the LDS system do not.

Accessibility to Trypsin Digestion. The lumenal loop domain contains two potential trypsin cleavage sites (K91 and K99), and a third one is found at the N-proximal end of helix 1 (R87). In native or recombinant wild-type LHCIIb, these potential cleavage sites are largely protected from trypsin cleavage, as the apoprotein is only N-terminally truncated by trypsin, resulting in a digestion product of about 24 kDa. On the other hand, unfolded Lhcb1 is completely digested by trypsin (*22*).

When the trypsin digestion products of the various mutant LHCIIb versions were compared (Figure 5), we saw, in addition to the 24 kDa degradation product, various amounts of a fragment of 14−15 kDa that was hardly visible in the wild type. This fragment is quite prominent in the most unstable mutants W97R and G101R, where, at the same time, the digestion product predominant in the wild type at 24 kDa is significantly reduced. In the mutants E94G and W97R a third Lhcb1 fragment with a molecular weight <12.1 kDa is visible after 25 min of digestion (Figure 5), caused by secondary cleavage of the 14−15 kDa fragment (see below).

Since all of the mutations are localized in the lumenal loop, one may assume that this domain is rendered more trypsin-sensitive in the mutant proteins. Cleavage in the lumenal loop would result in fragments of 9.4 kDa (N-terminal) and 14.6 kDa (C-terminal) in size. To test whether the 14−15 kDa fragment in Figure 5 corresponds to the expected C-terminal fragment, we extended the mutant W97R by a C-terminal His_6 tag. If the 14−15 kDa fragment includes the C-terminus, it would be expected to exhibit a somewhat larger apparent size in the mutant because of the His_6 extension. Figure 6 shows that in fact this is the case, for both the 14−15 kDa fragment and the <12.1 kDa fragment, which consequently also contains the C-terminus. Upon extended digestion, the His_6-labeled W97R complex also produces bands that apparently have lost the His_6 extension, as they comigrate with the digestion products of unmodified W97R. This is in accordance with our previous observations that extended trypsin digestion can remove a C-terminal His tag from LHCII (unpublished results).

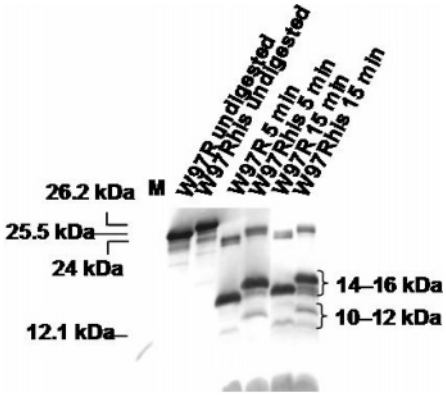

FIGURE 6: W97R and a W97R mutant with a C-terminal His tag (W97Rhis) were reconstituted and digested as described in Figure 5. A Western blot with samples taken after different time spans of digestion and alternating loadings of W97R and W97Rhis is shown. A 0.54 μg sample of undigested Lhcb1 (WT) and 0.2 μg of a truncated Lhcb1 protein with a molecular mass of 12.1 kDa were used as markers (M). The protein amount in the digested samples corresponded to 2.1 μg of undigested protein. WT = wild-type Lhcb1. M = 12.1 kDa truncated Lhcb1.

The putative N-terminal fragment resulting from a trypsin cut in the lumenal loop could not be detected (Figure 6), possibly due to further cleavage. The nearest basic amino acid following the lumenal loop domain is R142. Cleavage at this position would result in a 10 kDa C-terminal fragment, probably identical with the <12.1 kDa fragment visible in Figure 5 (panel W97R). The fact that the C-terminal fragment of the protein survives even when most of the nearly full length protein has disappeared indicates that the primary cut in the lumenal loop is not immediately followed by complex dissociation, as this is known to render the entire length of the protein trypsin-sensitive. We conclude that the appearance of the 14.6 kDa fragment indicates an enhanced accessibility of the lumenal loop in the mutant LHCIIb versions.

To assess the accessibility of the lumenal loop, the relative amounts of the 14−15 kDa product compared to the 24 kDa product were quantified (Figure 7). Except for the mutants with exchanges of Q122, higher efficiencies of protease cleavage in the lumenal loop led to reduced lifetimes at 37 °C (Figure 7), suggesting that the enhanced accessibility of the lumenal loop is connected with and possibly the reason for the instability of the mutants.

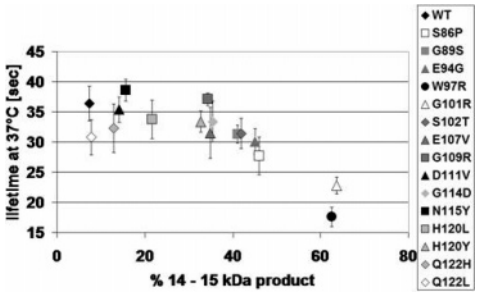

FIGURE 7: Correlation between complex lifetimes at 37 °C and the amount of the 14−15 kDa tryptic digestion product. The intensities of the 24 kDa signal and the 14−15 kDa signal (plus the 10 kDa signal in the case of W9R) after a 25 min trypsin digestion (Figure 5) were added to 100%, and the relative intensity of the 14−15 kDa signal was plotted on the secondary *y* axis, with the lifetime at 37 °C (Figure 4) on the primary *y* axis. The mutants were listed according to the position of the amino acid exchange.

DISCUSSION

Different Structural Properties of the Lumenal Loop Affect the Stability of LHCIIb in the Presence or Absence of LDS. The destabilized mutants studied in this work have been selected from an Lhcb1 library with random amino acid exchanges in the lumenal loop domain (*19*). Mutants that did not yield pigment−protein complexes under the conditions applied were isolated and sequenced. Destabilizing amino acid exchanges thus identified were equally distributed throughout the entire target domain, but exchanges of polar, especially acidic, amino acids and glycines were observed at a higher frequency than exchanges of hydrophobic or basic amino acids (*19*).

In the present work, less stringent procedures of reconstitution and isolation were used that allowed reconstitution of most of the mutants with pigments, albeit at significantly different complex yields (Figure 1). This opened the possibility to study the contribution of the lumenal loop to LHCIIb stability in detail by individually analyzing the impact of the single amino acid exchanges in the various mutants. To compare the various mutants with regard to their stabilities, we used two different levels of stringency, a harsh and a mild condition. The harsh condition, represented by three different procedures all giving approximately the same results, generally led to lower complex yields in the case of the mutants than the mild condition. Each condition, whether harsh or mild, contains a reconstitution step in which Lhcb1−pigment complexes are formed, and a subsequent isolation step in which the complexes are separated from noncomplexed material and in which more stable complexes are selected according to the stringency applied. We assume that differences among mutant proteins with respect to their folding and pigment binding behavior will mostly affect the efficiency of the reconstitution step, whereas differences in the stabilities of the mutant protein−pigment complexes will lead to higher or lower yields in the complex isolation step. Since the mild condition and one of the procedures represented by the harsh condition both employed the same reconstitution step (detergent exchange from LDS to OG) but different isolation protocols for the reconstituted complexes (mild electrophoresis on a Deriphat gel on one hand and stringent electrophoresis in the presence of LDS on the other hand), the observed differences between the harsh and mild conditions in complex yields cannot be due to different folding and pigment binding efficiencies but are most likely due to the different stabilities of the recombinant complexes under the different conditions. For the mild condition, we have shown directly that complex yields correlate with complex lifetimes in thermal dissociation kinetics (Figure 4). The mild and harsh regimes mainly differ in their employment of LDS. The mild condition includes a detergent-exchange step from the strongly denaturing anionic detergent LDS to a nonionic detergent during reconstitution, so it gets rid of most of the LDS during the reconstitution step and further avoids LDS during complex isolation, whereas all three procedures described as a harsh condition use at least 0.1% (w/v) LDS in the reconstitution or isolation part or both. We therefore conclude that the presence of dodecyl sulfate at 0.1% (w/v) or higher either in the reconstitution mixture or during gel-electrophoretical complex isolation decreased complex stabilities and were mainly responsible for reducing complex yields in the harsh as compared to the mild reconstitution condition. However, the reconstitution yields of the various LHCIIb mutants are quite differently affected by the mild and harsh conditions. Consequently, the destabilization by LDS is far from uniform when the various Lhcb1 mutants are compared with each other. Some mutant pigment−protein complexes are only slightly less stable in the presence than in the absence of LDS (e.g., group 2 in Figure 3), whereas other mutants, although reasonably stable under the mild condition, yield hardly any pigment−protein complexes under the harsh regime (e.g., group 4 in Figure 3). We conclude that the lumenal loop makes different contributions to LHCIIb stability in either the presence or absence of LDS and that these contributions to complex stability are differentially affected by the various mutations in this protein domain.

Thermal Destabilization Correlates with Local Unfolding of the Lumenal Loop Domain. The average lifetime in thermal dissociation experiments of mutant LHCIIb correlates not only with complex yields obtained under mild conditions but also with the appearance of a 14−15 kDa peptide upon tryptic digestion of the mutant pigment−protein complexes (Figure 7). Those mutants that have a particularly short average lifetime tend to exhibit a higher amount of the 14−15 kDa peptide, with the exception of Q122H and Q122L. The 14−15 kDa degradation product contains the C-terminus of the polypeptide chain (Figure 6) and presumably is the result of proteolytic cleavage in the lumenal loop domain. The expected N-terminal fragment of 9.4 kDa was not observed, possibly due to further cleavage or weaker binding of the polyclonal antiserum to the N-terminal part of the Lhcb1 protein. The occurrence of a stable degradation product indicates a locally restricted trypsin sensitivity. Proteolysis demands a substrate-like conformation of the polypeptide chain. Adoption of this cleavable conformation requires the accessibility and flexibility of at least 12 amino acids that surround the scissile peptide bond (*29*). In LHCIIb, only the N-terminal protein segment is susceptible to proteases such as trypsin or thermolysin (*22*) whereas the loop domains are not, indicating a limited accessibility and/or flexibility of the latter.

The relative amounts of the 14−15 kDa product are therefore likely to reflect the tendency of part of the lumenal loop domain to unfold, or at least detach from the remaining pigment−protein complex. This may be comparable to local

unfolding events near the surface of soluble proteins that can start their thermal unfolding (*30*−*33*). Since the accessibility of the lumenal loop toward protease correlates with the rate constant of complex dissociation, the local unfolding of the lumenal loop is likely to be one of the first steps during if not the initiation of the thermal denaturation of LHCIIb.

Mutants G101R and G109R deviate from the correlation between apparent rate of thermal dissociation and trypsin sensitivity of the lumenal loop (Figure 7). Both show higher protease sensitivities than would be expected from their lifetimes. The simple reason for this may be the introduction of an additional trypsin cleavage site in the lumenal loop domain. The two mutants with exchanges of Q122 both show unexpectedly low amounts of the 14−15 kDa protease digestion product (Figure 7). The position of Q122 immediately adjacent to helix C suggests that its exchange may induce structural effects such as a repositioning of helix C without causing the exposure of the lumenal loop.

There is no noticeable relation between the thermally destabilizing effect of amino acid exchanges and their chemical nature. The largest effect on thermal stability is introduced by the exchange W97R. As has been discussed by Mick et al. (*19*), W97 may be involved in an aromatic interaction with F195 (see Figure 2 in ref *19*). This interaction is in fact consistent with the recently published crystal structure of LHCIIb (*11*), but its significance for the overall structure still needs to be tested by replacing W97 with a nonpolar residue. If this interaction exists, it would explain the strong effect of mutating W97, since this amino acid would then be a connector of all three transmembrane helices in LHCIIb, the first and second one via the lumenal loop and the third one by direct interaction with F195.

Mutations located in the N-proximal half of the lumenal loop domain, up to E107, seemed to trigger local unfolding and to reduce thermal stability to a larger extent than mutations in the C-proximal part (Figure 7). The N-proximal half of the loop contains the newly discovered amphipathic helix E (*11*), which starts with W97. Amino acid exchanges in this part of the protein may alter the structure or orientation of helix E, and these potential alterations are likely to affect the position of W97. The exchange G101R places a hydrophilic amino acid on the hydrophobic surface of the amphipathic helix E and, therefore, is likely to cause a destabilization or rotation of the helix to avoid the exposure of this hydrophilic residue to a hydrophobic environment. Since the strength of aromatic interactions, like the one presumably undergone by W97 and F195, strongly depends on the distance and relative orientation of the participating aromatic systems (*34*), structural changes in and/or repositioning of helix E are likely to weaken this interaction. It should be noted that the mutational analysis described here may help to understand the contribution of the lumenal loop as a whole or parts thereof to LHCIIb stability but will give only limited information on the contribution of individual amino acids in this protein domain. This is because the basis of our analysis is a random selection of amino acid positions that influence complex stability. To fully appreciate the contribution of individual amino acids, a more systematic exchange with various other amino acids in each position would be required.

Differences in LDS-Induced and Thermally Induced Unfolding. By contrast to the thermal stability of LHCIIb, its stability toward LDS apparently is not related to the accessibility of the lumenal loop domain (Figure 3), indicating different functions of the lumenal loop domain in stabilizing LHCIIb toward either thermally induced or LDS-induced denaturation (see above). LDS-induced denaturation may be due to the sequestering of its hydrocarbon chain in hydrophobic pockets of the protein, where, in the case of LHCIIb, the cofactors are bound. The lumenal loop domain may help in shielding these hydrophobic pockets, and this function would then be hampered to different extents by the various amino acid exchanges. Mutations in positions 120 and 122 strongly destabilize LHCIIb in the presence but not in the absence of LDS (Figure 2). These mutations are positioned in two antiparallel β strands in the C-proximal part of the lumenal loop, suggesting that these β strands have some significance for the shielding by the lumenal loop of the hydrophobic interior of the LHCIIb molecule. Another possible explanation is that amino acids positioned near the edges of the lumenal loop domain (S86, H120, Q122) are critical for its shielding function toward LDS, as theses positions are close to the hydrophobic environment of the transmembrane α helices.

As a quantitative measure of membrane protein stability, both the temperature dependence of unfolding in the presence of nonionic detergents and the dependence on SDS concentration of denaturation have been used (*35*−*40*). Our observation suggests that these two procedures may assay different structural contributions to overall stability. On the other hand, Lau and Bowie (*37*) reported that both thermally induced and SDS-induced denaturation of diacylglycerol kinase starts with the unfolding of the cytoplasmic extramembranous protein domain, unlike the situation in the case of LHCIIb, where detachment of the lumenal loop correlates with the heat-induced unfolding in nonionic detergent but not with LDS-induced denaturation. Therefore, the special structural requirements for protein stabilization toward SDS or LDS may be a peculiarity of LHCIIb with its numerous hydrophobic cofactors bound.

Lumenal Loop Domain as a Putative Adaptive Control of Physiological Function. The observation of mutations in the lumenal loop leading to an increased exposure of this domain toward protease attack and at the same time lowering the overall stability of the complex strengthens our previously proposed view of the lumenal loop as a functional switch (*19*). In vivo, the detachment of the lumenal loop may be brought about by interactions with lumenal components or by the acidification of this compartment owing to photosynthetic proton transport. The conformational change of the lumenal loop may then reposition the transmembrane helices and the coordinated pigments such that energy-dissipating centers are generated. Protonation-induced detachment of the lumenal loop would allow access to violaxanthin of the enzyme violaxanthin deepoxidase as a prerequisite for establishing nonphotochemical quenching during light-stress situations (*41*). And finally, an overall destabilization of LHCIIb or a facilitated access to proteases, caused by a partial unfolding of the lumenal loop, may be a prerequisite for LHCIIb degradation as a long-term adaptation to light stress which has been reported to be regulated, at least in part, on the substrate level (*42*). A 16 kDa degradation product of Lhcb2 was observed in thylakoids in which light stress had been induced (*43*). This fragment may correspond

to the 14−15 kDa fragment described in this work and indicate that during LHCIIb degradation by thylakoid proteases the first cleavage occurs in the lumenal loop.

REFERENCES

1. White, S. H. (2003) Translocons, thermodynamics, and the folding of membrane proteins—Minireview, *FEBS Lett. 555*, 116−121.
2. Popot, J. L. and Engelman, D. M. (1990) Membrane protein folding and oligomerization: The two-stage model, *Biochemistry 29*, 4031−4037.
3. Popot, J. L. and Engelman, D. M. (2000) Helical membrane protein folding, stability, and evolution, *Annu. Rev. Biochem. 69*, 881−922.
4. Langosch, D. and Heringa, J. (1998) Interaction of transmembrane helices by a knobs-into-holes packing characteristic of soluble coiled coils, *Proteins: Struct., Funct., Genet. 31*, 150−159.
5. Curran, A. R. and Engelman, D. M. (2003) Sequence motifs, polar interactions and conformational changes in helical membrane proteins, *Curr. Opin. Struct. Biol. 13*, 412−417.
6. Melnyk, R. A., Kim, S., Curran, A. R., Engelman, D. M., Bowie, J. U., and Deber, C. M. (2004) The affinity of GXXXG motifs in transmembrane helix-helix interactions is modulated by long-range communication, *J. Biol. Chem. 279*, 16591−16597.
7. Engelman, D. M., Chen, Y., Chin, C.-N., Curran, A. R., Dixon, A. M., Dupuy, A. D., Lee, A. S., Lehnert, U., Matthews, E. E., Reshetnyak, Y. K., Senes, A., and Popot, J.-L. (2003) Membrane protein folding: beyond the two stage model—Minireview, *FEBS Lett. 555*, 122−125.
8. Booth, P. J. (2003) The trials and tribulations of membrane protein folding in vitro, *Biochim. Biophys. Acta 1610*, 51−56.
9. Hong, H. and Tamm, L. K. (2004) Elastic coupling of integral membrane protein stability to lipid bilayer forces, *Proc. Natl. Acad. Sci. U.S.A. 101*, 4065−4070.
10. Kühlbrandt, W., Wang, D. N., and Fujiyoshi, Y. (1994) Atomic model of plant light-harvesting complex by electron crystallography, *Nature 367*, 614−621.
11. Liu, Z., Yan, H., Wang, K., Kuang, T., Zhang, J., Gul, L., An, X., and Chang, W. (2004) Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution, *Nature 428*, 287.
12. 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.
13. Paulsen, H., Rümler, U., and Rüdiger, W. (1990) Reconstitution of pigment-containing complexes from light-harvesting chlorophyll *a/ b*-binding protein overexpressed in *E. coli*, *Planta 181*, 204−211.
14. Hobe, S., Prytulla, S., Kühlbrandt, W., and Paulsen, H. (1994) Trimerization and crystallization of reconstituted light-harvesting chlorophyll *a/b* complex, *EMBO J. 13*, 3423−3429.
15. Cammarata, K. V. and Schmidt, G. W. (1992) In-vitro reconstitution of a light-harvesting gene product—deletion mutagenesis and analyses of pigment binding, *Biochemistry 31*, 2779−2789.
16. Paulsen, H. and Hobe, S. (1992) Pigment-binding properties of mutant light-harvesting chlorophyll *a/b*-binding protein, *Eur. J. Biochem. 205*, 71−76.
17. Hobe, S., Fey, H., Rogl, H., and Paulsen, H. (2003) Determination of relative chlorophyll binding affinities in the major light-harvesting chlorophyll *a/b* complex, *J. Biol. Chem. 278*, 5912−5919.
18. Heinemann, B. and Paulsen, H. (1999) Random mutations directed to trans-membrane and loop domains of light-harvesting chlorophyll *a/b* protein: Impact on pigment binding, *Biochemistry 38*, 14088−14093.
19. Mick, V., Eggert, K., Heinemann, B., Geister, S., and Paulsen, H. (2004) Single amino acids in the lumenal loop domain influence the stability of the major light-harvesting chlorophyll *a/b* complex, *Biochemistry 43*, 5467−5473.
20. Cashmore, A. R. (1984) Structure and expression of a pea nuclear gene encoding a light-harvesting chlorophyll a/b-binding polypeptide, *Proc. Natl. Acad. Sci. U.S.A. 81*, 2960−2964.
21. Kosemund, K., Geiger, I., and Paulsen, H. (2000) Insertion of light-harvesting chlorophyll *a/b* protein into the thylakoid—Topographical studies, *Eur. J. Biochem. 267*, 1138−1145.
22. 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.
23. 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: Verfication of the concentration of chlorophyll standards by atomic absorption spectroscopy, *Biochim. Biophys. Acta 975*, 384−394.
24. Hobe, S., Niemeier, H., Bender, A., and Paulsen, H. (2000) Carotenoid binding sites in LHCIIb − Relative affinities towards major xanthophylls of higher plants, *Eur. J. Biochem. 267*, 616−624.
25. 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.
26. Peter, G. F. and Thornber, J. P. (1991) Electrophoretic procedures for fractionation of photosystems I and II pigment proteins of higher plants and for determination of their subunit composition, in *Methods in Plant Biochemistry* (Rogers, L. G., Ed.) Vol. 5, pp 195−210, Academic Press, New York.
27. Yang, C. H., Kosemund, K., Cornet, C., and Paulsen, H. (1999) Exchange of pigment-binding amino acids in light-harvesting chlorophyll *a/b* protein, *Biochemistry 38*, 16205−16213.
28. Schägger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, *Anal. Biochem. 166*, 368−379.
29. Hubbard, S. J., Eisenmenger, F., and Thornton, J. M. (1994) Modeling studies of the change in conformation required for cleavage of limited proteolytic sites, *Protein Sci. 3*, 757−768.
30. Gilbert, W. A., Lord, R. C., Petsko, G. A., and Thamann, J. (1982) Laser Raman spectroscopy of biomolecules, *J. Raman Spectrosc. 12*, 173−179.
31. Vriend, G., Berendsen, H. J. C., van den Burg, B., and Venema, G. (1998) Early steps in the unfolding of thermolysin-like proteases, *J. Biol. Chem. 273*, 35074−35077.
32. Hori, T. and Moriyama, H. (2000) The initial step of the thermal unfolding of 3-isopropylmalate dehydrogenase detected by the temperature-jump Laue method, *Protein Eng. 13*, 527−533.
33. Van den Burg, B. and Eijsink, V. G. H. (2002) Selection of mutations for increased protein stability, *Curr. Opin. Biotechnol. 13*, 333−337.
34. 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.
35. Gilles-Gonzalez, M. A., Engelman, D. M., and Khorana, H. G. (1991) Structure−function studies of bacteriorhodopsin XV, *J. Biol. Chem. 266*, 8545−8550.
36. 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.
37. Lau, F. W. and Bowie, J. U. (1997) A method for assessing the stability of a membrane protein, *Biochemistry 36*, 5884−5892.
38. Lau, F. W., Nauli, S., Zhou, Y., and Bowie, J. U. (1999) Changing single side chains can greatly enhance the resistance of a membrane protein to irreversible inactivation, *J. Mol. Biol. 290*, 559−564.
39. London, E. and Khorana, H. G. (1982) Denaturation and renaturation of bacteriorhodopsin in detergents and lipid-detergent mixtures, *J. Biol. Chem. 257*, 7003−7011.
40. Zhou, Y. and Bowie, J. U. (2000) Building a thermostable membrane protein, *J. Biol. Chem. 275*, 6975−6979.
41. Niyogi, K. K. (1999) Photoprotection revisited: Genetic and molecular approaches, *Annu. Rev. Plant Physiol. 50*, 333−359.
42. Yang, D. H., Webster, J., Adam, Z., Lindahl, M., and Andersson, B. (1998) Induction of acclimative proteolysis of the light-harvesting chlorophyll *a/b* protein of photosystem II in response to elevated light intensities, *Plant Physiol. 118*, 827−834.
43. Lindahl, M., Yang, D. H., and Andersson, B. (1995) Regulatory proteolysis of the major light-harvesting chlorophyll *a/b* protein of photosystem II by a light-induced membrane-associated enzymic system, *Eur. J. Biochem. 231*, 503−509.

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