N-Proximal Sequence Motif in Light-Harvesting Chlorophyll *a*/*b*-Binding Protein Is Essential for the Trimerization of Light-Harvesting Chlorophyll *a*/*b* Complex†

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ABSTRACT: The major light-harvesting complex (LHCII) of photosystem II can be reconstituted in its native, trimeric form starting from its apoprotein light-harvesting chlorophyll *a*/*b*-binding protein (LHCP), pigments, and thylakoid lipids. In this paper we identify segments in the LHCP polypeptide that are essential for the formation of stable LHCII trimers by analyzing N- and C-terminal deletion mutants of LHCP and mutants carrying point-specific amino acid exchanges. C-Terminal deletions that do not abolish pigment binding to LHCP do not affect trimerization either. By contrast, on the N-terminus of LHCP, where as many as 61 amino acids can be deleted without significant effects on pigment binding, only 15 amino acids are dispensible for LHCII trimer formation . This indicates that structural elements between amino acids 16 and 61 are involved in the stabilization of LHCII trimers but not monomers. Closer inspection of this protein domain in a more detailed mutation analysis revealed that amino acids W16 and/or Y17 as well as R21 are essential for the formation of LHCII trimers. These amino acids are conserved in virtually all known sequences of LHCII apoproteins but only in some of the minor chlorophyll *a*/*b* complexes. Possible functions of the crucial residues are discussed.

In the photosynthetic apparatus in higher plants, both photosystem I and photosystem II (PSI and PSII, respectively)[1] contain a number of peripheral, chlorophyll *a*/*b*-containing light-harvesting complexes. The most prominent one among these is the major light-harvesting chlorophyll *a*/*b* complex of PS II, LHCII, which probably is the most abundant membrane protein complex on earth. The crystal structure of LHCII has recently been analyzed at 3.4-Å resolution (Kühlbrandt et al., 1994), making this complex an attractive target for attempts to understand the light-harvesting function in photosynthesis.

Among all the chlorophyll *a*/*b*-containing complexes of PSII, LHCII is most likely the only one that forms trimers in the thylakoid membrane (Jansson, 1994). Pulse-labeling experiments during the greening process in pea leaves demonstrate that LHCII monomers are accumulated first and then assembled into trimers (Dreyfuss & Thornber, 1994a). However, little is known so far about which structural features of monomeric LHCII promote trimerization and which molecular interactions stabilize the trimeric complexes.

Lipids, particularly phosphatidylglycerol (PG) lipids, are thought to be involved in the stabilization of LHCII trimers (Rémy et al., 1984; Trémolières et al., 1994). When trimeric LHCII is treated with phospholipase, it dissociates into monomers (Rémy et al., 1982; Nuβberger et al., 1993). The same is true when the first 49 or 51 amino acids on the N-terminus of the LHCII apoprotein, LHCP, are removed by limited proteolysis but not if only eight N-terminal amino acids are split off (Nuβberger et al., 1993). Interestingly, removal of 50 or so amino acids from the N-terminus of LHCP also releases PG from the complex (Nuβberger et al., 1993) which is otherwise tightly bound to trimeric LHCII, even in the presence of strong detergents (Trémolières et al., 1981). These findings suggest that the N-terminal domain of LHCP is important for trimerization and that PG binding to this protein region is functionally correlated with the formation of LHCII trimers.

Monomeric LHCII can be reconstituted *in vitro* from LHCP and pigments in the presence of detergents (Plumley & Schmidt, 1987; Paulsen et al., 1990). Reconstitutions *in vitro* with mutated versions of LHCP revealed that the entire hydrophilic domain on the N-terminus and part of the hydrophilic domain on the C-terminus of LHCP are dispensible for the formation of stable pigment−LHCP complexes (Cammarata & Schmidt, 1992; Paulsen & Hobe, 1992). Recently, we succeeded in reconstituting LHCII in the trimeric form (Hobe et al., 1994). This opened the possibility to investigate whether the N- and C-proximal protein domains, which are not needed for pigment binding, might be essential for the oligomerization of the complex. In this work, we show that LHCII loses its capability to trimerize when more than 15 amino acids are removed from the N-terminus of LHCP. Moreover, we identify a number of amino acids in the N-terminal protein domain which appear to be essential for trimer formation of LHCII.

MATERIALS AND METHODS

Construction of Mutants. Both C-terminal deletion mutants (Y224Z, T226Z)[2] have been described before (Paulsen & Kuttkat, 1993).

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[1] Abbreviations: Chl, chlorophyll; PG, phosphatidylglycerol; LHCII, light-harvesting complex of photosystem II; LHCI, light-harvesting complex of photosystem I; LHCP, light-harvesting chlorophyll *a*/*b*-binding protein; PSI, photosystem I; PSII, photosystem II; LM, lauryl maltoside.

[2] Point-specific mutants are termed by A#B, with A being the original amino acid(s), # being its (their) position(s) in LHCP, and B the amino acid(s) present after the exchange; Z designates a stop signal in the place of the original amino acid.

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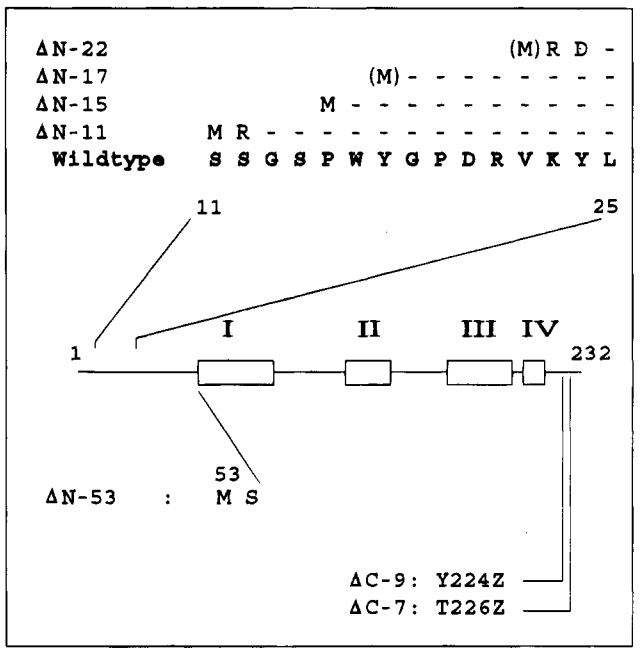

FIGURE 1: Terminal deletion mutants of LHCP. The figure represents the primary structure of LHCP. The position and length of α-helical stretches I, II, III, and IV (boxes) refer to α-helical stretches B, C, A, and D, respectively, in Kühlbrandt et al. (1994). Amino acids are numbered with respect to the mature apoprotein. Dashes represent unchanged amino acids as compared to the wild type.

The N-terminal deletion mutants are named with respect to the position of their first amino acid in the sequence of the mature LHCP. The numbering of amino acids in LHCP starts one residue downstream from the leading methionine, which was shown to be posttranslationally removed in spinach (Michel et al., 1991).

N-Terminal deletion mutants ΔN-11, ΔN-15, and ΔN-53 were constructed by exonucleolytic digestion and religation as described in Paulsen and Hobe (1992).

The N-terminal deletion mutant ΔN-22 was constructed by exchanging the N-terminal part of pLHCP (amino acids 1−177) with a fragment comprising amino acids 60−177. This fragment was isolated as a 350-nucleotides *Sca*I/*Bst*EII fragment from the AB80 gene from pea (Cashmore, 1984) and religated into an LHCP expression vector prepared as follows. The pLHCP expression plasmid [LHCP-2 in Paulsen et al. (1990)] was opened with *Bam*HI and trimmed to blunt ends by mung bean nuclease digestion. The fragment coding for amino acids 1−177 of pLHCP was removed by a *Bst*EII digestion. The remaining vector was then ligated with the *Sca*I/*Bst*EII fragment. This leads to a N-terminal sequence of MRDLG, where MRD in position 22−24 of mature LHCP replaces VKY of the wild-type sequence (see Figure 1).

The N-terminal deletion mutant ΔN-17 and the mutants WY16,17AV, R21Q, and K23Q were constructed by site-specific mutagenesis (Kunkel et al., 1987) as described in Paulsen and Kuttkat (1993). The sequences of the primers used were as follows: GTC TGG TCC CAT GGT TAA TTT CTC (ΔN-17), GTC TGG TCC GAC CGC TGG GCT TCC (WY16,17AV), GTA CTT AAC CTG GTC TGG (R21Q), and GCC TAA GTA CTG AAC ACG (K23Q). Mutagenesis with the primer for ΔN-17 was performed on a ΔN-15 plasmid; amino acid exchange mutants were constructed starting from a full-length LHCP expression plasmid.

Reconstitution and Trimerization. Preparation of monomeric LHCII and subsequent oligomerization was carried out as in Hobe et al. (1994) with the following modifications. The lipid extract from thylakoids was replaced by dipalmitoyl-L-α-phosphatidyl-DL-glycerol (Sigma, Munich, Germany) which was added (200 μg/mL) to the apoprotein solution before the injection of pigments. All subsequent steps for the preparation of the LHCII suspension were the same as in Hobe et al. (1994). This suspension of reconstituted LHCII was precipitated by centrifugation (10 min, 14000*g*) and solubilized in 0.1% LM for 2 h at 0 °C before analysis on sucrose gradients. The gradients (12.5−25% sucrose and 0.1% LM) were spun for 17 h at 300000*g* and 4 °C in a Kontron RPS-55-T rotor.

Partially denaturing gel electrophoresis was carried out as described earlier (Paulsen & Hobe, 1992).

RESULTS

Fifteen N-Terminal Amino Acids of LHCP Are Not Required for Trimerization. In earlier experiments we found that the entire hydrophilic N-terminal domain of LHCP and part of the hydrophilic C-terminal domain are dispensible for the formation of stable LHCII monomers *in vitro* (Paulsen & Hobe, 1992; Paulsen & Kuttkat, 1993). Here we studied whether these domains or parts thereof are essential for trimer formation of reconstituted LHCII. Therefore, we constructed a series of mutant versions of LHCP carrying N- and C-terminal deletions. Figure 1 shows the constructs.

Most of the N-terminal deletion mutants have been characterized by partial protein sequencing. The exchange of the first one or two amino acids as compared to the original AB80 sequence is due to the construction of the mutants (see Materials and Methods). Not depicted in Figure 1 is the LHCP clone ΔN-2, which is close to the wild-type structure except it is missing amino acids 3 and 4 (Hobe et al., 1994). Former analysis of N-terminal deletion mutants revealed that in some cases but not in others the leading methionine has been deleted in the bacterially expressed protein. Therefore, the starting methionine is shown in parentheses in the mutants ΔN-17 and ΔN-22, which have been characterized by DNA sequencing rather than protein sequencing. All C-terminal deletion mutants have been analyzed by DNA sequencing (Paulsen & Kuttkat, 1993).

All of these LHCP mutants have been reconstituted with pigments and then trimerized *in vitro* (Hobe et al., 1994). Some of the sucrose gradients, used to separate trimeric complexes from the monomeric ones by ultracentrifugation, are shown in Figure 2. It is obvious from Figure 2B that all the C-terminal deletion mutants can still be trimerized. Therefore, the C-terminus of LHCP, as far as it is not involved in pigment binding or stabilization of the monomeric complex, is not involved in trimer formation either.

On the other hand, in the N-terminal deletion series (Figure 2A), only the first mutants in the series, carrying shorter deletions, still form trimers, whereas the mutant ΔN-17 and all the following shorter versions of LHCP do not. Since the mutant ΔN-15 (which still trimerizes) differs from the mutant ΔN-17 by only two additional codons, this confines the "break point" in the deletion series, where trimerization is abolished, to only two amino acids, namely, W16-Y17.

Amino Acids W16 and/or Y17 and R21 but Not K23 Are Essential for Trimerization. The results from the N-terminal deletion series point to the two aromatic amino acids W16

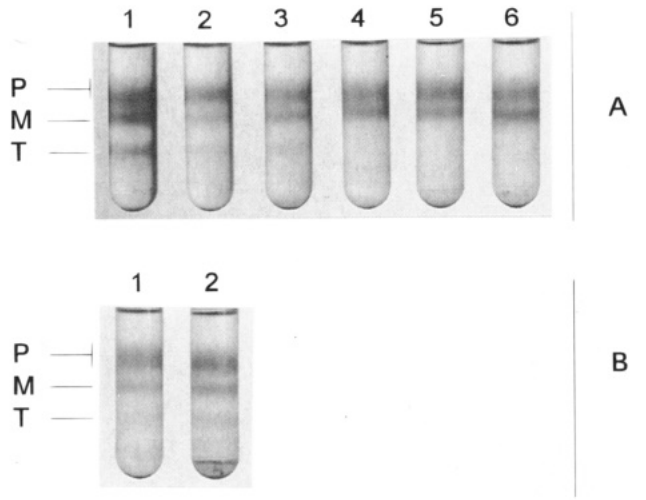

FIGURE 2: Sucrose gradient centrifugation of reconstituted LHCII containing N- and C-terminally deleted LHCP mutants. LHCP deletion mutants were reconstituted and assayed for trimerization under identical conditions (see Materials and Methods). Solubilized suspensions of reconstituted LHCII were loaded onto linear sucrose gradients (12.5−25% sucrose and 0.1% LM) and spun for 17 h (300000*g*) at 4 °C. (A) N-Terminal deletion mutants. 1 = ΔN-2; 2 = ΔN-11; 3 = ΔN-15; 4 = ΔN-17; 5 = ΔN-22; 6 = ΔN-53. (B) C-Terminal deletion mutants. 1 = ΔC-9; 2 = ΔC-7. P = unbound pigment, M = LHCII monomers, T = LHCII trimers.

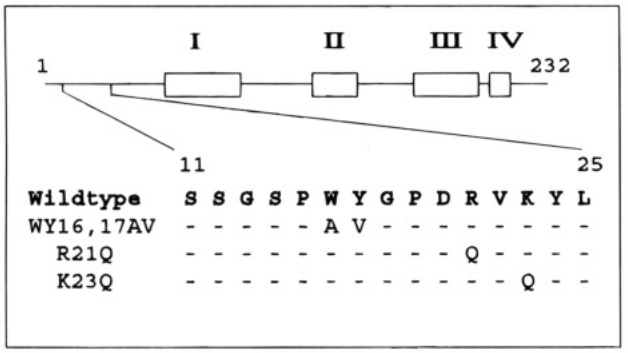

FIGURE 3: Internal mutations of LHCP. The scheme and symbols are as described for Figure 1.

and Y17 as being essential for LHCII trimer formation. However, an alternative explanation for the mutant ΔN-17 failing to trimerize would be that in going from mutant ΔN-15 to ΔN-17 the N-terminal protein sequence simply falls below a critical length that is necessary for the oligomerization of the complexes. In order to distinguish between these possibilities, we exchanged the amino acids WY in position 16 and 17 with the nonaromatic amino acids A and V in a full-length LHCP (Figure 3). The sequences of all site-specific exchange mutants were verified by DNA sequencing.

As shown in Figure 4, a full-length LHCP with mutations in positions 16 and 17, the mutant WY16,17AV, does not yield LHCII trimers. This confirms that W16 and/or Y17 are indeed involved in trimer formation, probably by undergoing some stabilizing interaction(s) with other components of the trimeric complex.

As mentioned in the introduction, other groups have found indications for PG interacting with the N-terminal domain of LHCP in LHCII trimers. If PG binding indeed contributes to the stabilization of LHCII trimers, one would expect the deletion of a PG binding site in the LHCP sequence to destabilize LHCII trimers or to prevent trimer formation altogether. Therefore, we looked for possible PG binding

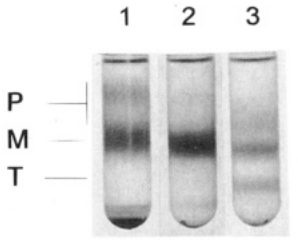

FIGURE 4: Sucrose gradient centrifugation of reconstituted LHCII containing site-specifically mutagenized LHCP. Trimerization assays were performed as described for Figure 2. 1 = WY16,17AV; 2 = R21Q; 3 = K23Q. P = unbound pigment, M = LHCII monomers, T = LHCII trimers.

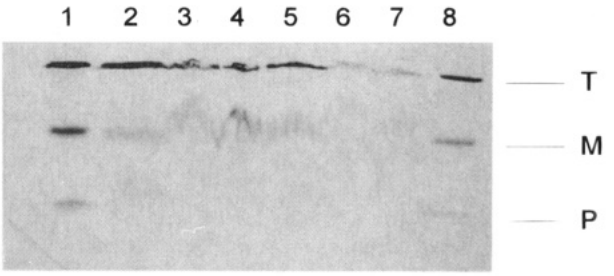

FIGURE 5: Partially denaturing gel electrophoresis of LHCII trimers isolated from sucrose gradients. Trimeric bands from all positive LHCP mutants were removed from sucrose gradients, diluted 10-fold, concentrated in Centricon tubes (molecular mass cutoff 10 kDa; Amicon) and loaded onto the gel after addition of 8% glycerol and 1% octyl glucoside. Lanes 1 and 8: Partially dissociated native LHCII isolated from pea. 2 = ΔN-2; 3 = ΔN-11; 4 = ΔN-15; 5 = ΔC-7; 6 = ΔC-9; 7 = K23Q. T = LHCII trimers, M = LHCII monomers, P = unbound pigment.

sites in the LHCP domain which turned out to be essential for LHCII trimerization in the N-terminal deletion series described above. Such PG binding sites would be expected to include stretches of hydrophobic amino acids, interacting with the hydrophobic parts of the fatty acids, and positively charged amino acids, undergoing ionic interaction with the phosphate group in PG.

There are two positively charged amino acids in the N-terminal protein domain which seems to be essential for trimer formation: R21 and K23. We exchanged either of these amino acids with the also rather hydrophilic but neutral amino acid Q (mutants R21Q and K23Q, see Figure 3). As shown in Figure 4, the mutation of R21 but not of K23 abolishes the reconstitution of trimeric LHCII from the respective LHCP derivative. Mutant R21Q sometimes but not reproducibly yielded a very faint trimeric band in sucrose gradients. This suggests the formation of oligomers of considerably lower stability than the ones with wild-type LHCP.

Trimeric LHCII Containing Mutant LHCP Is Indistinguishable from Native LHCII in Partially Denaturing Gel Electrophoresis. The assignment of the fastest sedimenting band in the sucrose gradients as reconstituted LHCII trimer has been verified earlier (Hobe et al., 1994). To confirm this assignment for the trimerization products containing mutant LHCP, we isolated the trimeric bands from sucrose gradients and loaded the samples on a partially denaturing ("green") gel. Figure 5 shows that the trimeric band of all mutants exhibits the same pattern of bands on a "green" gel. Upon gel electrophoresis, mutant trimers partially dissociate into monomers to about the same extent as native LHCII (not shown; native LHCII samples in lanes 1 and 8 have been partially dissociated). The close similarity between

mutant and native trimers regarding their sedimentation velocity in sucrose gradients and their behavior in partially denaturing gel electrophoresis suggests that these complexes share a similar structure with about the same stabilities. From some of the mutant LHCII trimers we also took CD spectra in the visible region in order to further verify their authentic structure by characterizing pigment−pigment interactions. The spectra (not shown) were indistinguishable from those of native LHCII trimers and reconstituted LHCII trimers containing nonmodified LHCP (Hobe et al., 1994).

DISCUSSION

The Sequence WYGPDR in Position 17−22 of LHCP Contains Amino Acids Essential for LHCII Trimer Formation. In this paper, we present clear evidence that the sequence WYGPDR, 16 amino acid positions from the N-terminus of LHCP, is involved in the formation of LHCII trimers. The deletion of 15 N-terminal amino acids in LHCP does not affect the capability of reconstituted LHCII monomers to trimerize. However, the deletion of the sequence WYGPDR or part thereof completely abolishes trimer formation. Within this sequence, W and/or Y and R appear to be essential. For ease of discussion, the amino acid sequence element WYXXXR will be termed "trimerization motif".

Protease digestion experiments performed on isolated native LHCII trimers by Nuβberger et al. (1993) revealed the involvement of amino acids 9−48 in the stabilization of trimeric LHCII. The mutants characterized in the present paper allow a rather precise localization of the involved sequence down to single amino acids. The notion of the sequence WYGPDR being functionally important is corroborated by the fact that it marks the beginning of the N-proximal sequence homology between LHCII apoproteins from different organisms. The same motif is found even in some but not all other Chl *a*/*b* antenna proteins. Figure 6 shows that, within these sequences similar to the trimerization motif, W is strongly conserved while Y and R exhibit different degrees of variability (see below). In LHCI apoproteins, a W is also highly conserved near the N-terminus (Jansson, 1994), but none of the other elements of the trimerization motif are conserved.

The dependency of the oligomerization of LHCII on the trimerization motif suggests that this N-terminal protein domain constitutes a direct or indirect (see below) contact site between monomers. However, it is possible that further sequence elements elsewhere in LHCP are also important; it even appears likely that there are further contact sites between the monomers.

Is the Trimerization Motif Part of a Phosphatidylglycerol Binding Site? The molecular interactions which the trimerization motif is undergoing in order to fulfill its structural function we can only hypothesize about at this point. Unfortunately, the LHCII crystal structure obtained by Kühlbrandt et al. (1994) does not contain information about the protein segment upstream of position 26 (see below). The structure with which the trimerization motif interacts could be located either within the same monomer or in an adjacent monomer within the trimeric complex. The first case would imply that the motif is indirectly involved in trimerization by stabilizing some conformation within one monomer that then is capable of trimerizing.

As a possible intermolecular contact made by the trimerization motif, the hydrophobic, aromatic residues W and Y may interact with hydrophobic features in a second monomer such as (one or more) hydrophobic amino acids or a Chl that is situated close enough to the interface between monomers. Also, stacking interactions of three tryptophans and/or tyrosines within a trimer can be considered as an energetically favorable structural feature which would promote trimer formation.

Pea ab80	(Lhcb1):	12	SGSPWYGPDRVKYLGP	(a)
Pea ab96	(Lhcb1):	7	-S---H----------	(b)
Tomato	(Lhcb1):	12	-S----------	(c)
Rice	(Lhcb1):	13	-------A---L----	(d)
S. pine	(Lhcb1):	13	-T--------L----	(e)
Pea	(Lhcb2):	9	PE-I------P-----	(f)
Tomato	(Lhcb2):	9	PQ-I---E--P-----	(g)
Rice	(Lhcb2):	9	PQ-I------P-----	(h)
Black pine	(Lhcb2):	9	PE-I------P-----	(i)
Pea	(Lhcb3):	1	GNDL----------	(j)
Tomato	(Lhcb3):	1	-NDL----------	(k)
Barley	(Lhcb3):	1	GNDL----------	(l)
Arabidopsis	(Lhcb4):	22	DRPL--PGAISPDWLD	(m)
Barley	(Lhcb4):	22	DRPL-FPGAQAPEYLD	(n)
Tomato	(Lhcb5):	24	ELAK------RIF-PE	(o)
Barley	(Lhcb5):	28	ELAK------RI--PN	(p)
S. pine	(Lhcb5):	29	ELAK------RIF-PE	(q)
Tomato	(Lhcb6):	3	AKKS-IPAV-GGGNLV	(r)
Tomato	(Lhcb6):	5	PKKS-IPAVKSGGNLV	(s)
Spinach	(Lhcb6):	5	PKKS-IPAVKGGGNLV	(t)

FIGURE 6: Comparison of derived N-proximal polypeptide sequences in Chl *a*/*b* proteins of PSII. Numbers indicate the position of the first shown amino acid in the sequence of the respective mature apoprotein. References: (a) Cashmore, 1984; (b) Coruzzi et al., 1983; (c) Pichersky et al., 1985; (d) Matsuoka, 1990; (e) Jansson & Gustafsson, 1990; (f) Falconet et al., 1991; (g) Pichersky et al., 1987; (h) Matsuoka, 1990; (i) Kojioma et al., 1992; (j) Schwartz et al., 1991; (k) Falconet et al., 1993; (l) Brandt et al., 1992; (m) Green & Pichersky, 1993; (n) Morishige & Thornber, 1992; (o) Pichersky et al., 1991; (p) Sorensen et al., 1992; (q) Jansson, 1994; (r) Schwartz & Pichersky, 1990; (s) Schwartz & Pichersky, 1990; (t) Spangfort et al., 1990.

Likewise, the positively charged R21 could be essential for LHCII trimerization because it forms a stabilizing ion pair between LHCII monomers. Alternatively, the positive charge may be part of a phosphatidylglycerol binding site. Reports in the literature suggest that phosphatidylglycerol interacts with the N-terminal domain of LHCP in trimeric LHCII and that this interaction is essential for the stability of the trimeric complexes (Nuβberger et al., 1993). In fact, trimeric LHCII can be reconstituted *in vitro* only in the presence of a thylakoid lipid extract or pure dipalmitoylphosphatidylglycerol (Hobe et al., 1994). Therefore, a possible interpretation of our data is that the deletions or alterations of or within the trimerization motif which abolish LHCII trimerization actually destroy a lipid binding site in LHCII and thus destabilize the trimeric form. We are presently testing this possibility by measuring PG binding to the various LHCP mutants.

Structural Implications for the Organization of the PSII Antenna. In contrast to the major LHCII (Lhcb1 and Lhcb2), which is organized in a trimeric state in the thylakoid (Kühlbrandt, 1994), the minor antenna complexes Lhcb4 (CP29), Lhcb5 (CP26), and Lhcb6 (CP24) are generally viewed as monomeric complexes (Peter & Thornber, 1991; Holzenburg et al., 1993; Dunahay & Staehelin, 1986; Bassi et al., 1987), although they have also been reported to be trimeric (Dainese & Bassi, 1991). The alignment of N-proximal amino acid sequences of several Lhcb proteins from

different organisms (Figure 6) reveals that the trimerization motif is conserved to different degrees among the minor Chl *a/b* proteins. Lhcb4 misses a positively charged amino acid and thus resembles the mutant R21Q in our work, which failed to trimerize; this notion is consistent with Lhcb4 being monomeric. On the other hand, the entire motif is nicely conserved in Lhcb5, and Lhcb6 still contains W in the first position and R or K in the last position of the conserved motif. As long as no unequivocal information about the oligomeric state of the minor Chl *a/b* complexes in the thylakoid is available, we are left with two alternative explanations: (i) Lhcb5 and Lhcb6 form oligomers, possibly mixed trimers with Lhcb1 and 2 (see below); however, these are too instable to be isolated after solubilizing the thylakoid. (ii) If the minor Chl *a/b* proteins in fact are monomeric, this means that the formation of trimers is not solely dependent on the presence or absence of the trimerization motif. Conversely, LHCI has been reported to be oligomeric in the thylakoid (Dreyfuss & Thornber, 1994b), although its N-proximal sequence homologous to the trimerization motif resembles the one of Lhcb4 and thus is not expected to promote trimer formation; therefore, oligomerization of LHCI seems to depend on other structural elements.

Interestingly, the conservation of the trimerization motif in the minor antenna proteins does reflect their respective positions between the core complex of PSII and the pool of major LHCII. Lhcb3, which is being discussed as a constituent of LHCII (Harrison & Melis, 1992), and Lhcb5, which has also been found in an LHCII fraction from thylakoids (Bassi & Dainese, 1992), both share the entire sequence WYGPDR with the major LHCP. In the case of Lhcb6, which has also been isolated in close association with LHCII (Barbato et al., 1989), at least part of the motif is preserved. On the other hand, Lhcb4, which does not have a "functional" trimerization motif (see above), does not seem to be in contact with LHCII at all but located between the core complex of PSII (Camm & Green, 1989) and Lhcb6 (Bassi & Dainese, 1992). This correlation suggests that the trimerization motif plays a role not only in stabilizing LHCII trimers but also in contacts between LHCII and the minor Chl *a/b* complexes in its immediate neighborhood. Whether such contacts take the form of relatively unstable heterotrimers or other interactions which somehow resemble interactions within the LHCII trimer cannot be decided at this point. We are presently studying reconstituted minor Chl *a/b* complexes with regard to their oligomerization behavior and their interactions *in vitro* with other complexes of the photosynthetic apparatus.

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