Mutant Trimers of Light-Harvesting Complex II Exhibit Altered Pigment Content and Spectroscopic Features†

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ABSTRACT: Mutants of plant light-harvesting complex II (LHC-II) were produced by refolding the complex in vitro from bacterially expressed apoprotein and purified pigments by a method which yields native-like LHC-II in a single step. Amino acid residues known from the structure of the complex [Kühlbrandt, W., et al. (1994) *Nature 367,* 614−621] to bind chlorophyll (Chl) were replaced with nonbinding residues by site-directed mutagenesis. Recombinant monomeric and trimeric pigment−protein complexes were separated by density gradient centrifugation, and their pigment composition was determined. Six out of nine mutants formed trimers with Chl *a*:Chl *b* ratios and Chl contents which suggested they were lacking one Chl *a* or *b* per polypeptide. In this way, the identities of Chls *a*1, *a*2, *a*3, *b*5, and *b*6 were confirmed as Chl *a* or *b*, respectively, whereas Chl *b*3 in the structure was found to be a Chl *a*. Absorption and fluorescence emission spectra of the mutant lacking Chl *a*2 indicated a central role for this Chl in energy transfer to the reaction center.

Solar energy for plant photosynthesis is collected by the light-harvesting pigment−protein complexes (LHCs)[1] in the chloroplast thylakoid membrane. LHC-II, the most abundant of these complexes, is associated with photosystem II and alone accounts for roughly one-third of the total thylakoid membrane protein. It forms stable trimers in the membrane and in detergent solution. Light energy absorbed by LHC-II is transmitted to the reaction center via a series of similar but less abundant LHCs (*1*). Unlike the reaction center proteins which contain only chlorophyll (Chl) *a*, the plant LHCs also bind various amounts of Chl *b* and are therefore often referred to as Chl *a*/*b* binding proteins (*2*, *3*). LHC-II is by far the best-characterized Chl *a*/*b* binding protein. The structure of the complex from pea has been determined at 3.4 Å resolution (*8*), and a wide variety of biochemical and spectroscopic data are available.

LHC-II isolated from thylakoid membranes contains several isoforms of the polypeptide. In pea LHC-II, the major polypeptide has 232 amino acids and binds a total of 12−15 Chls *a* and *b* per monomer (*4*). The Chl *a*:Chl *b* ratio of the complex is 1.2−1.4, depending on isolation conditions and, to some extent, on the method of Chl determination (*3*−*6*). The LHC-II monomer contains three or four carotenoids, including two luteins and one neoxanthin, and others in substoichiometric amounts (*7*). The LHC-II structure (*8*) shows 12 Chls, 5 of which have been assigned to Chl *b* and 7 to Chl *a*, on the basis of their proximity to the two carotenoids in the center of the monomer which were assigned to luteins. However, as the structural difference between Chl *a* and Chl *b* was not visible at 3.4 Å resolution, this preliminary assignment still awaits confirmation. Amino acid residues responsible for Chl binding were identified for 9 of the 12 Chls.

A minimum requirement for understanding the ultrafast energy transfer between the pigment molecules in LHC-II (*9*, *10*) is the unambiguous assignment of the identified Chls to Chl *a* or Chl *b*. Two recent studies have suggested models for light energy transfer in LHC-II based largely on time-resolved chlorophyll spectroscopy, which broadly confirmed the structure-based assignments while questioning the identity of two (*11*) or four individual Chls (*12*).

Our approach to identifying the Chls is to modify their binding sites in the polypeptide by point mutation and then to examine the mutant complexes for missing Chls. In this way, it should be possible not only to decide which Chl is *a* or *b* but also, in combination with spectroscopic measurements, to attribute spectral features to individual Chls. The best system for expression and assembly of recombinant LHC-II would obviously be transgenic plants. Indeed, modified pea LHC-II genes introduced into transgenic tobacco were expressed and formed native complexes (*13*). It proved difficult however to separate recombinant LHC-II from the endogenous complex. Moreover, site-directed mutagenesis of Chl ligands resulted in expression levels that were far too low for biochemical and structural studies.

A viable alternative for producing LHC-II mutants is in vitro refolding. LHC-II is one of the few membrane protein complexes that has been refolded from its components. Refolding has been achieved not only with the naturally occurring mix of polypeptides (*14*) but also with a bacterially expressed single polypeptide (*15*). Refolded monomers can be induced to form trimers by the addition of specific lipids. Two-dimensional crystals of trimers produced in this way are indistinguishable from those of LHC-II isolated from

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[1] Abbreviations: Ala, alanine; Arg, arginine; Chl, chlorophyll; Glu, glutamate; HPLC, high-performance liquid chromatography; LHC, light-harvesting complex; Phe, phenylalanine; wt, wild-type.

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thylakoid membranes, indicating that the structures of native and recombinant complex are virtually identical (*16*). Low-temperature absorption spectroscopy confirmed that all spectral features characteristic of native LHC-II were also present in the refolded complex (*17*). A number of earlier mutagenesis studies of LHC-II have focused mainly on the effect of deletions and point mutations on the ability of the complex to refold and form trimers, rather than on Chl binding (*18*−*22*).

Recently, we developed a method for refolding His-tagged LHC-II into trimers in a single step on a Ni^{2+} chelating column (*23*). In the study presented here, we use this method to generate a series of mutants of LHC-II. In these mutants, particular amino acid side chains that are known from the structure to provide ligands for Chl binding were replaced by others that are unable to bind Chl. The stability of these mutants and their ability to form trimers were assessed by density gradient centrifugation. Spectra of the gradient fractions were recorded, and their Chl content and Chl *a*:Chl *b* ratios were determined by HPLC. Recombinant complexes can also be assembled into trimers by insertion of the polypeptide into isolated thylakoid membranes (*24*), although not in the quantities required for spectroscopy and pigment analysis. The accompanying paper (*25*) shows that the results obtained in this way with a similar set of mutants are fully consistent with ours.

MATERIALS AND METHODS

Genetic Material and Mutagenesis. We used the plasmid C3.2h (kindly provided by K. Kosemund and H. Paulsen, University of Mainz, Mainz, Germany) to express a histidine$_6$-tagged pea LHC-II polypeptide of gene lhcb1*2 (*26*), originally known as cab AB80 (*27*; EMBL database accession name PSCAB80). Mutagenesis was performed by PCR, using mutagenic primers and a double-stranded plasmid as a template, followed by a *Dpn*I digest prior to transformation of supercompetent *Escherichia coli* cells (*28*, *29*). Enzymes were purchased from Stratagene (Quick Change kit). Mutant strains were checked by sequencing the complete lhcb1*2 insert, followed by a second round of selection for expression of mutant LHC-II. Wild-type (wt) and mutant polypeptides were expressed in the form of inclusion bodies which were purified as described previously (*14*). One liter of bacterial culture gave a typical yield of 50−150 mg of polypeptide.

Refolding and Protein Isolation. LHC-II was refolded as described previously (*23*). Chl binding protein was eluted as a green fraction from the Ni^{2+} column, loaded onto a sucrose density gradient [10 to 40% sucrose, 0.1% dodecyl maltoside, and 20 mM Tris-HCl (pH 7.5)], and centrifuged for 20 h in a Kontron TST41 rotor at 40 000 rpm and 4 °C. LHC-II monomers for partially denaturing gel electrophoresis were refolded by the original method of Paulsen et al. (*30*).

Partly Denaturing Electrophoresis. Acrylamide gels (15%) measuring 6 cm × 8 cm × 0.1 cm with 50 μL wells were prepared without SDS and run at 25 mA and 4 °C in the dark (*31*) in a standard Bio-Rad gel apparatus. Samples were loaded in 10% glycerol without SDS. The SDS concentration in the running buffer was 0.1%. Green bands were cut out and mashed by pressing through a syringe with a metal grid glued to its tip. The gel pieces were extracted with 5% SDS and 0.1 M Tris-HCl (pH 7.5) by shaking for 5 min, and then acetone was added to a final concentration of 67%.

HPLC Analysis. Pigment content of gradient and gel fractions was analyzed on a Spherisorb ODS-1 HPLC column (250 mm × 4.6 mm) from Alltech using the method of Gilmore and Yamamoto (*32*). Acetone was added to aqueous samples to a final solvent concentration between 67 and 80% prior to injection. Peaks were quantified by integration of the 440 nm trace and comparison to a calibration curve of purified standards. The concentration of Chl standards was determined photometrically in 80% acetone according to the method of Porra et al. (*33*). For carotenoids, an extinction coefficient $E^{1\%}_{1cm}$ of 2500 at the absorption maximum was used.

Spectroscopy. Room-temperature absorption spectra were recorded on a Perkin-Elmer photometer in a 1 cm cuvette at a spectral bandwidth of 2 nm. Fluorescence emission spectra were measured at room temperature with a Hitachi F-4500 fluorimeter with the excitation wavelength set to 469 nm, on the red side of the Soret Chl *b* absorption band. The excitation slit was set to a 1 nm bandwidth and the emission slit to 2.5 nm. The cuvette measured 1 cm in the direction of the excitation beam and 0.5 cm in the perpendicular direction.

RESULTS

LHC Mutants. Targets for mutagenesis were the nine amino acid residues in the LHC-II sequence which are known from the structure to provide ligands for Chl binding (*8*). All Chl binding residues were replaced by Ala with the following exception. Bacteria transformed with construct Q197A did not express inclusion bodies, and therefore, Q197L was used instead. Chl *a*6 is thought to be ligated by the peptidyl carbonyl group of glycine 78; we therefore replaced this residue with the bulky amino acid phenylalanine so that Chl binding would be sterically hindered. A minor change from leucine to valine in the preceding residue was necessary for introducing a new restriction site for selecting positive mutant clones.

Trimer Formation and Stability of Mutants. Column-refolded LHC-II mutants were analyzed by sucrose density gradient centrifugation which separated trimers from monomers and unbound pigments (Figure 1). Clear trimer bands were obtained reproducibly for mutants 4−9. Mutants 1 (*a*4), 2 (*a*5), and 3 (*a*6) yielded only faint trimer bands in ca. 50% of the experiments. A green monomer band was observed for all mutants. Slightly different positions of the bands may reflect variations in the lipid content of the refolded complex and of the sucrose concentration in the gradient.

Some mutants exhibited an additional band just above the monomer. This was more or less continuous with the monomer band (Figure 1, lanes 0 and 9), but occasionally clearly separated from it (Figure 1, lane 5). The extra band was highly enriched with Chl *a* and probably contained material that was either incompletely or incorrectly folded to begin with, or had decayed during centrifugation. It did not occur reproducibly and was not investigated further.

To assess the stability of refolded LHC-II mutants, the material eluted from the Ni^{2+} affinity column was subjected to partially denaturing gel electrophoresis (Figure 2). Under these stringent conditions, clear trimer bands are found for mutants 4 (*b*5), 5 (*a*1), 6 (*a*3), 7 (*b*3), and 8 (*b*6). Mutant 9 (*a*2) no longer has a trimer band, indicating that this trimer

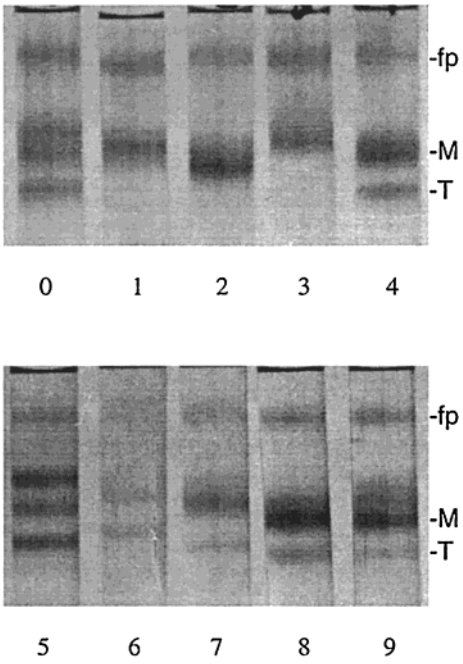

FIGURE 1: Sucrose density gradients of LHC-II refolded on a Ni^{2+} chelating column. For mutant numbers, see Table 1. fp, free pigment; M, monomers; T, trimers.

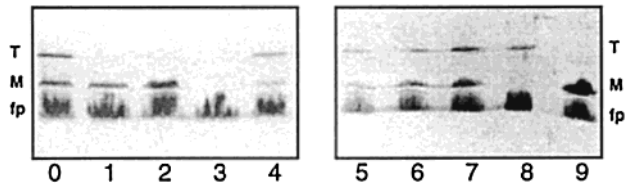

FIGURE 2: Unstained, partially denaturing gel with green bands of wt and mutant LHC-II refolded on a Ni^{2+} chelating column. For mutant numbers, see Table 1. fp, free pigment; M, monomers; T, trimers.

is only stable under the mild conditions prevailing on a sucrose density gradient but not under the harsher conditions of the partially denaturing gel. A similar effect was observed with mutant 3 (*a*6), which gave no green band at all on the gel under standard conditions; however, a green monomeric complex was seen on gels run for a short time only (~30 min, not shown). This material was used for HPLC analysis (see below). Surprisingly, mutant 8 (*b*6) shows a trimer but no monomer band on the gel, indicating that the monomer in this case is less stable, whereas the stability of the trimer seems to be comparable to those of the others.

In the LHC-II structure, there are three Glu−Arg salt bridges which at the same time serve as Chl ligands. Two salt bridges link helices A and B, coordinating Chls *a*1 and *a*4, whereas the third salt bridge links two residues in helix C and coordinates Chl *b*5. Mutants 1 (*a*4), 4 (*b*5), and 5 (*a*1) in which the Glu in each of the three salt bridges had been replaced with Ala were able to assemble into monomers, but trimer formation did not indicate a consistent effect on the stability of the complex. Mutants 4 and 5 lacking the residues coordinating Chl *b*5 and *a*1, respectively, produced trimers, but mutant 1 lacking the residue coordinating Chl *a*4 usually did not.

Chlorophyll Content. The pigment content of column-refolded LHC-II trimer and monomer bands from sucrose density gradients was determined by HPLC since the spectrophotometric Chl assay (*33*) was too inaccurate, due to the low Chl concentration in the samples. At the same time, the HPLC chromatograms enabled us to assess the three carotenoids, lutein, neoxanthin, and violaxanthin. As lutein is known to be present in two copies per monomer (*3*), it was used as an internal standard to quantify pigments relative to protein. To provide a comparison with earlier studies, LHC-II mutants were prepared by the conventional method of detergent precipitation (*30*), purified by partially denaturing gel electrophoresis, and analyzed by HPLC. This procedure yields only monomers.

Table 1: Chl *a*:Chl *b* Ratios of Refolded LHC-II wt and Mutants[a]

	trimers from density gradients	monomers from density gradients	monomers from gel
wt	1.22 ± 0.06 (14)	1.62 ± 0.28 (12)	1.03 ± 0.17 (11)
1, E65A, *a*4	1.40 ± 0.17 (4)	2.07 ± 0.39 (7)	0.90 ± 0.11 (5)
2, H68A, *a*5	1.10 ± 0.08 (3)	1.37 ± 0.09 (7)	0.85 ± 0.04 (5)
3, LG77/78VF, *a*6	2.21 ± 0.27 (3)	2.10 ± 0.09 (7)	2.27 ± 0.22 (3)
4, E139A, *b*5	1.38 ± 0.13 (7)	2.34 ± 0.28 (7)	0.86 ± 0.06 (5)
5, E180A, *a*1	1.11 ± 0.12 (6)	1.30 ± 0.31 (6)	0.93 ± 0.04 (3)
6, Q197L, *a*3	0.96 ± 0.08 (6)	1.02 ± 0.11 (6)	0.58 ± 0.08 (4)
7, H212A, *b*3	1.06 ± 0.05 (6)	1.13 ± 0.08 (6)	0.86 ± 0.07 (4)
8, Q131A, *b*6	1.57 ± 0.14 (6)	1.90 ± 0.28 (6)	2.25 ± 0.16 (4)
9, N183A, *a*2	1.14 ± 0.12 (6)	1.40 ± 0.31 (6)	0.86 ± 0.04 (5)

[a] Number of experiments in parentheses.

As shown also in the accompanying paper, the Chl *a*:Chl *b* ratio of the refolded product depends on the pigment ratio present at the refolding stage. We therefore kept close to a starting Chl *a*:Chl *b* ratio of 1.4 which is typical of native LHC-II prepared by our standard procedure (*34*). In this way, we were able to minimize the variation of the Chl *a*:Chl *b* ratio in different experiments. Table 1 shows the Chl *a*:Chl *b* ratio of wt and mutant complexes. The Chl *a*:Chl *b* ratio is statistically the most relevant parameter, because it does not suffer from eventual variations of the internal lutein standard.

Monomers and trimers from sucrose density gradients are consistent in showing that mutants 2 (*a*5), 5 (*a*1), 6 (*a*3), 7 (*b*3), and 9 (*a*2) contain less Chl *a* than wt. A lower Chl *b* content is found in mutants 1 (*a*4), 3 (*a*6), 4 (*b*5), and 8 (*b*6). For mutants 1 (*a*4), 2 (*a*5), and 3 (*a*6), we occasionally observed faint trimer bands which were sufficiently concentrated for HPLC analysis.

The monomers from partially denaturing gels all exhibit lower Chl *a*:Chl *b* ratios with the exception of mutants 3 (*a*6) and 8 (*b*6). The lower Chl *a*:Chl *b* ratio of the wt sample, as compared to that of the material recovered from the sucrose density gradient, indicates a selective loss of Chl *a* caused by gel electrophoresis. Conversely, most of the column-refolded monomers have lost Chl *b* on the sucrose gradient, causing an increase in the Chl *a*:Chl *b* ratio. The reason for this difference is unknown. Trimers of column-refolded LHC-II are clearly the most intact form of the recombinant complexes and presumably do not lose Chl during gradient centrifugation. We therefore focused on this material for our subsequent analysis.

Table 2 shows the ratio of Chl per two luteins for wt, and the change in the Chl *a* and *b* content for each mutant relative to this number. These figures have a higher standard deviation, because they were averaged over several independent folding experiments. They demonstrate though that mutant trimers have retained most of their Chls and have

Table 2: Difference in Chl *a* and *b* Content[a] between Mutants and wt

	trimers from density gradients	monomers from density gradients	momoners from gel
wt	(14)[b]	(12)	(8)
Chl *a*	7.6 ± 0.8	7.8 ± 1.1	5.3 ± 0.34
Chl *b*	6.1 ± 0.4	5.1 ± 1.0	5.7 ± 0.17
1, E65A, *a*4	(4)	(4)	(5)
ΔChl *a*	−0.4 ± 0.43	−1.4 ± 0.20	−0.7 ± 0.12
ΔChl *b*	−0.9 ± 0.46	−2.6 ± 0.19	−0.2 ± 0.33
2, H68A, *a*5	(3)	(4)	(5)
ΔChl *a*	−0.9 ± 0.18	−1.3 ± 0.18	−0.7 ± 0.38
ΔChl *b*	−0.3 ± 0.25	−0.5 ± 0.33	−0.1 ± 0.21
3, LG77/78VF, *a*6	(3)	(4)	(2)
ΔChl *a*	1.8 ± 1.07	0.1 ± 0.52	2.2 ± 0.55
ΔChl *b*	−2.0 ± 0.23	−1.6 ± 0.50	−2.4 ± 0.21
4, E139A, *b*5	(7)	(4)	(5)
ΔChl *a*	−0.5 ± 0.51	−1.1 ± 0.55	−1.2 ± 0.49
ΔChl *b*	−1.1 ± 0.52	−2.6 ± 0.35	−0.8 ± 0.66
5, E180A, *a*1	(6)	(4)	(2)
ΔChl *a*	−0.4 ± 0.44	−0.9 ± 1.19	−0.2 ± 0.09
ΔChl *b*	0.3 ± 0.44	0.7 ± 0.53	0 ± 0.22
6, Q197L, *a*3	(6)	(4)	(3)
ΔChl *a*	−0.7 ± 0.94	−2.2 ± 1.16	−2.0 ± 0.02
ΔChl *b*	0.9 ± 0.35	0.2 ± 0.63	0.2 ± 0.06
7, H212A, *b*3	(6)	(4)	(5)
ΔChl *a*	−0.4 ± 0.53	−1.0 ± 0.40	−0.6 ± 0.34
ΔChl *b*	0.5 ± 0.29	0.6 ± 0.69	−0.1 ± 0.23
8, Q131A, *b*6	(6)	(4)	(7)
ΔChl *a*	0 ± 0.51	−1.2 ± 0.39	1.5 ± 0.88
ΔChl *b*	−1.3 ± 0.51	−2.1 ± 0.28	−2.4 ± 0.45
9, N183A, *a*2	(6)	(4)	(7)
ΔChl *a*	−1.6 ± 0.37	−2.6 ± 0.58	−0.4 ± 0.25
ΔChl *b*	−0.7 ± 0.40	−1.1 ± 0.12	0 ± 0.16

[a] Number of chlorophylls calculated per LHC monomer asuming two luteins per monomer. [b] Number of experiments in parentheses.

not lost significant amounts of Chl unspecifically. An apparent slight increase in one or the other Chl species seen in some instances can be attributed to the limited accuracy of our measurements since it is unlikely that any mutant would actually bind more Chl *a* or *b* than wt.

With these uncertainties taken into account, the figures in Table 2 indicate that trimers of mutants 1 (*a*4), 2 (*a*5), 4 (*b*5), 5 (*a*1), 6 (*a*3), 7 (*b*3), and 8 (*b*6) have lost about one Chl each. From the Chl *a*:Chl *b* ratios in Table 1, we can decide whether the missing Chl is *a* or *b*. For mutants 2 (*a*5), 5 (*a*1), 6 (*a*3), and 7 (*b*3), the figures indicate the absence of a Chl *a*, whereas in mutants 1 (*a*4), 4 (*b*5), and 8 (*b*6), a Chl *b* is lacking. Mutant 9 (*a*2) clearly has lost a Chl *a*, and maybe a Chl *b* as well. Mutant 3 (*a*6) differs more than others from wt in its Chl *a* and *b* content. This means either that one or two Chl binding sites have changed their affinity from *b* to *a* or, if one accepts that the number of bound Chls *a* is unlikely to increase, a drastic loss of up to three Chls *b*. The apparent, abnormally high Chl *a* content would then imply a partial loss of the internal standard, lutein. In any case, mutant 3 (*a*6) should be regarded with caution.

Chlorophyll Assignment. Via comparison of the missing Chl species in each mutant trimer with the original structure-based assignment, our data confirm that E180 (mutant 5), Q197 (mutant 6), and N183 (mutant 9) each coordinate a Chl *a* and that E139 (mutant 4) and Q131 (mutant 8) both coordinate a Chl *b*. The data for mutant 7 indicate that H212 binds a Chl *a* instead of a Chl *b*.

Analysis of mutants 1 (*a*4) and 2 (*a*5) which are impaired in trimer formation tend to confirm that *a*5, coordinated by H68, is a Chl *a* but suggest that *a*4, coordinated by E65, may be a Chl *b*. The pigment content of monomers isolated on density gradients is in line with this. However, as these mutants are less stable, they may suffer from distortions of their structure that may affect other pigment binding sites. Conclusions regarding the identity of the corresponding Chls should therefore be regarded as tentative.

Table 3: Number of Minor Xanthophylls in LHC-II Trimers[a]

	violaxanthin	neoxanthin
refolded wt (5)	0.37 ± 0.12	0.92 ± 0.04
3, LG77/78VF (3)	0.19 ± 0.05	0.05 ± 0.04
4, E139A (7)	0.21 ± 0.06	0.44 ± 0.15
8, Q131A (4)	0.34 ± 0.08	0.54 ± 0.07

[a] Number of experiments in parentheses. The xantophyll content was calculated assuming two luteins per monomer.

Carotenoid Content. Only three mutants were found to differ from wt in relative carotenoid content when analyzing the trimers from density gradients (Table 3). Mutant 3 (*a*6) has lost virtually all the neoxanthin and some of its violaxanthin, whereas mutants 4 (*b*5) and 8 (*b*6) exhibit a decrease in the level of neoxanthin to about half the wt level. Mutant 4 (*b*6) may have lost some violaxanthin as well. Analysis of the other mutant trimers yielded values which were statistically indistinguishable from wt.

Spectra. Room-temperature absorption spectra were recorded for wt and the five mutant trimers which yielded sufficient material. The wild-type recombinant complex shows the main Chl *a* Q_y absorption band at 674 nm well separated from the Chl *b* peak at 652 nm which is characteristic of native LHC-II. The mutant spectra were consistent with the results of the pigment analysis. Figure 3A shows spectra of mutants 4 (*b*5) and 8 (*b*6) in which the Chl *b* peak is merely a shoulder, confirming that both complexes have lost Chl *b*.

Figure 3B shows the absorption spectra of LHC-II mutants deficient in Chl *a* normalized to the Chl *b* peak which in these mutants is separated from the Chl *a* peak as clearly as in wt. For mutant 6 (*a*3), the most obvious difference is a broad decrease in the main Chl *a* absorption peak extending from 660 to 680 nm. By contrast, the spectrum of mutant 7 (*b*3) exhibits a particularly deep dip between the Chl *a* and Chl *b* peaks, suggesting that Chl *b*3 (now reassigned to Chl *a*) has only a small red shift. The major difference for mutant 9 (*a*2) is located on the long-wavelength side of the Chl *a* peak, indicating that Chl *a*2 which is likely to be missing in this mutant has the highest red shift in LHC-II. Table 2 suggests that mutant 9 (*a*2) may lack a Chl *b* as well which would, however, not affect the red side of the Chl *a* absorption peak.

The fluorescence spectrum of mutant 9 (*a*2) is in agreement with the red shift of Chl *a*2. The entire emission peak has shifted by 4 nm toward higher energy (Figure 4), whereas the emission peaks of all other mutant trimers were shifted only marginally toward shorter wavelengths. This means that Chl *a*2 must have the lowest energy level in the complex.

DISCUSSION

The purpose of this study was twofold. On one hand, we aimed to identify individual Chls in LHC-II experimentally as Chl *a* or Chl *b*. We chose a biochemical approach, first

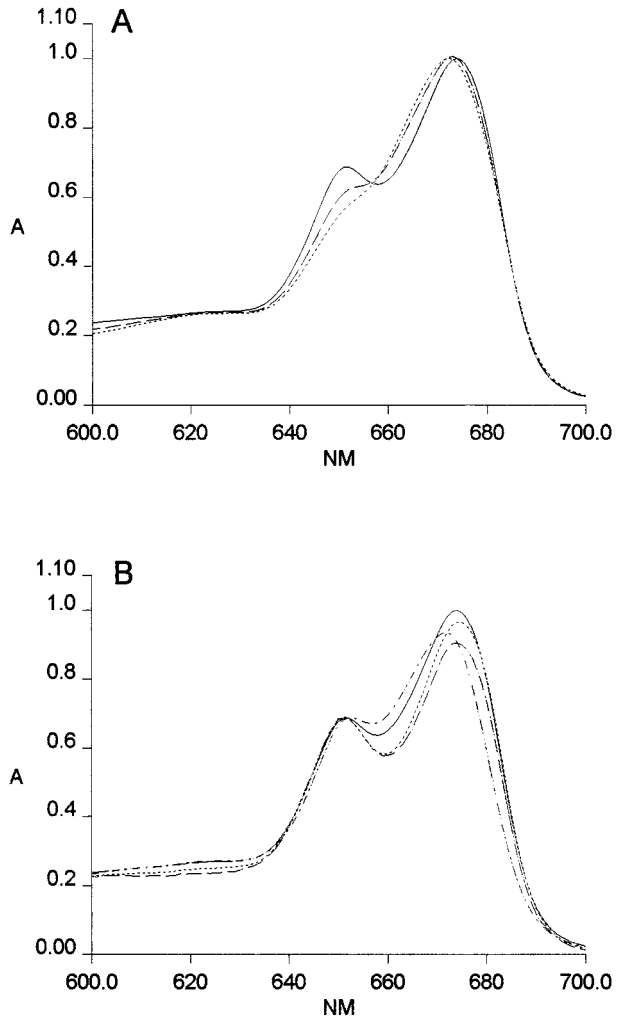

FIGURE 3: Absorption spectra of mutant LHC-II trimers. (A) Spectra of mutants lacking Chl *b*, normalized to the Chl *a* absorption maximum at 674 nm: (—) wt, (– – –) mutant 4 (Chl *b*5), and (•••) mutant 8 (Chl *b*6). (B) Spectra of mutants lacking Chl *a*, normalized to the Chl *b* absorption maximum at 652 nm: (—) wt, (– – –) mutant 6 (Chl *a*3), (•••) mutant 7 (Chl *b*3, reassigned to Chl *a*), and (–•–) mutant 9 (Chl *a*2).

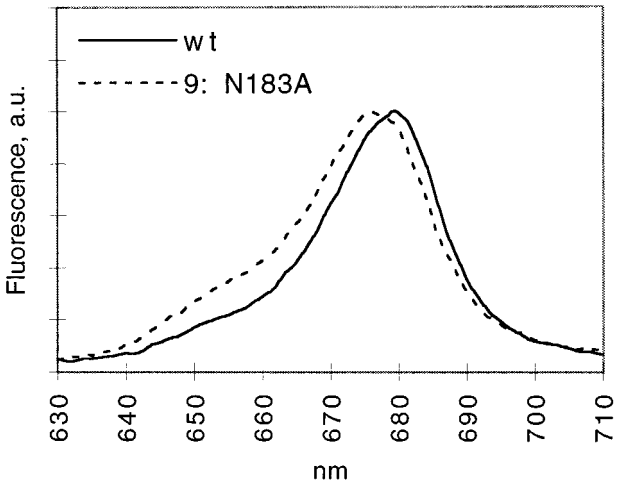

FIGURE 4: Normalized fluorescence emission spectrum of mutant 9 and wt. The excitation wavelength was 469 nm.

removing particular Chl ligands from the apoprotein by site-directed mutagenesis and then refolding the complex in vitro from its components. The other aim was to identify stable LHC-II mutants that are able to form trimers for future biophysical and structural characterization.

Chl-Binding Mutants. Since LHC-II can be refolded with polypeptide expressed in *E. coli*, experiments with genetically engineered protein are readily performed. Earlier work with progressively N- and C-terminally truncated LHC-II polypeptides revealed a sharp transition from normally folding complexes with wt pigment composition to a complete inability of the protein to assemble (*17*, *18*). N- and C-terminal deletions were therefore not a viable approach for producing stable mutants lacking individual Chls. As shown in this and the accompanying paper (*25*), stable LHC-II mutants with altered pigment content can be made by changing only selected Chl-binding residues in the polypeptide. In this study, we restricted ourselves to the mutagenesis of the nine amino acid residues which are known from the structure to act as Chl ligands.

Specificity of Chl Binding. The specificity of binding sites for Chl *a* or *b* does not appear to be absolute. Early refolding experiments carried out with roughly equal amounts of Chl *a* and *b* yielded complexes with a Chl *a*:Chl *b* ratio much lower than that of native LHC-II (*14*). Later, it was found that the Chl *a*:Chl *b* ratio of LHC-II from *Chlorella fusca* can change upon addition of Chl *a* or *b* to isolated thylakoids (*35*). It has been shown that under certain conditions LHC-II monomers can fold in vitro with only Chl *b* and carotenoids present, although the same does not hold for Chl *a* (*36*, *37*). Recently, a detailed spectroscopic investigation was performed on complexes with low Chl *a*:Chl *b* ratios (*38*). In these complexes, it was not possible to assign the loss of certain spectral Chl *a* bands to the rise of corresponding Chl *b* bands upon lowering the Chl *a*:Chl *b* ratio. Clearly, some of the binding sites in these artificial complexes are charged with the wrong type of chlorophyll, but it is not known which sites are actually occupied, or how closely these products resemble the native LHC-II. On the other hand, in vivo the binding sites must have a strong preference for one or the other Chl species; otherwise, it would be difficult for the plant to assemble a functional complex with a constant Chl *a*:Chl *b* ratio in the photosynthetic membrane. It seems clear that, although recombinant complexes with unusual pigment compositions can be produced under artificial conditions, they form less easily and are less thermodynamically stable than those with normal pigment content. We therefore assume that in the in vivo folded complex there is no mixed occupancy of Chl binding sites, but they all contain either Chl *a* or *b*; at least this is a useful working hypothesis for modeling energy transfer. The analysis of mutated reconstituted complexes then serves as a guideline for the site assignments, as in our experiments a particular mutant always exhibits a preferential loss of Chl *a* or *b*. It should be kept in mind however that in these complexes some of the binding sites may be occupied by the "wrong" chlorophyll species. A definite answer to this question can only be provided by a high-resolution structure of the complex or by analysis of mutants which have been folded in vivo preferentially in a plant where all other LHC-II genes have been knocked out.

After establishing conditions that yield wt recombinant complex which closely resembled native LHC-II in terms of the Chl *a*:Chl *b* ratio, spectral characteristics, and the ability to form trimers (*23*), we proceeded to use this system

for producing mutants in which individual Chl binding residues had been removed. In our analysis of pigment content, we focused on the trimer fractions, since they were not prone to unspecific pigment loss and would form from monomers that resemble the native conformation most closely. Since some of the Chl binding sites can apparently exhibit lowered specificity toward Chl *a* or *b* under certain conditions, it was obviously important to always refold with the same mix of pigments. In this way, it was possible to compare the wt complex to mutants and to detect meaningful differences in the Chl *a*:Chl *b* ratio and pigment content indicating which type of Chl was missing in each case. To confirm that the change in the Chl *a*:Chl *b* ratio is not the effect of a global disruption of the complex but corresponds to the loss of one specific chlorophyll molecule at a particular site, we quantified the amount of Chl *a* and *b* with lutein as an internal standard.

Detecting a difference of one chlorophyll molecule among 12 in LHC-II requires a measuring accuracy of at least 5% for both chlorophyll content and the internal standard. We can only achieve this accuracy when the two measurements come from the same HPLC trace, as it is the case for chlorophylls and carotenoids. Lutein has been shown to be present in two copies per monomer on the basis of the Chl content of native LHC-II (*39*). The lutein content of reconstituted LCH-II remains unaltered also under stress conditions with hardly any Chl *a* present (*38*). If our mutant complexes had lost one lutein molecule, this would result in an overestimate of the chlorophyll content by a factor of 2. As seen from Table 2, we do not observe large increases in chlorophyll content except in mutant 3 (*a*6) which is the least stable as discussed. Small increases in the levels of one type of Chl or the other are due to the standard deviation of the measurement.

The best argument in favor of lutein as an internal standard comes from the fact that the lutein:neoxanthin:violaxanthin ratio remains constant in wt and the majority of the mutants. It seems highly unlikely that a mutant would lose all three carotenoids in equal proportions. Even mutants 3 (*a*6), 4 (*b*5), and 8 (*b*6) which lose their neoxanthin give a reasonable Chl content on the basis of two luteins per monomer. The only alternative for measuring the numbers of Chl per monomer would be an independent determination of Chl and polypeptide which is much less accurate.

Chl Assignments. The original assignment of Chl *a* or *b* in LHC-II was based on distances between chlorophylls and the two central carotenoids in the electron crystallographic structure (*8*). In the absence of higher-resolution data, this assignment is still largely hypothetical and needs to be confirmed experimentally. In the investigation described here, this has been partly achieved by molecular, biological, and biochemical techniques. From six mutants which formed stable trimers, five confirmed the structure-based assignment of *a*1, *a*2, and *a*3 to Chl *a* and of *b*5 and *b*6 to Chl *b*, while one (7, H212A) indicated that *b*3 should be assigned to Chl *a*. Mutants 1 (E65A), 2 (H68A), and 3 (LG77/78VF) did not form trimers readily which suggests that conclusions pertaining to the identities of Chl *a*4, *a*5, and *a*6 should be regarded as preliminary. The binding sites of Chls *b*1, *b*2, and *a*7 were not assessed since their side chain ligands have not yet been identified.

As pointed out above, the loss of one chlorophyll molecule is close to the limit of our analysis. We therefore also included data of monomeric complexes from the sucrose density gradient and monomers formed by a different folding protocol and isolated by partially denaturing gel electrophoresis. Whereas the density gradient complexes have a tendency to unspecifically lose Chl *b*, an unspecific Chl *a* loss is typical for LHC-II isolated from gels. Although we always compare mutants to the identically treated wild type, some effects may not be detected in these complexes. In fact, when comparing the rise and decrease in the Chl *a*:Chl *b* ratio, we found that all gradient-purified monomers and trimers exhibit the same behavior. Seven out of nine of the gel purified monomers are in line with the trimer data. It is clear that these complexes have lost some Chl *a* unspecifically, and this probably obscures the effect of the mutation in some cases.

The accompanying paper (*25*) also investigates the identity of Chls coordinated by particular side chains, using constructs targeting the binding sites for Chl *a*3, *a*5, *b*3, and *b*6. It is particulary instructive to compare our results from trimers with the sucrose density gradients in ref *25*, as these complexes represent the most intact species.

Both studies show in agreement that upon mutation of residue H68 (*a*5), Q197 (*a*3), and H212 (*b*3) Chl *a* is lost preferentially, whereas the mutation of residue Q131 (*b*6) leads to a reduction in Chl *b* content. It is interesting to note that a replacement with hydrophobic or bulky residues has a more pronounced effect on chlorophyll content; hydrophilic replacements of the Chl binding side chain may still allow partial occupancy of the targeted site, whereas bulky or hydrophobic residues may also affect neighboring Chl binding pockets. An example of how neighboring binding sites can influence each other is mutant 6 (*a*3) which gave relatively low yields, indicating low thermodynamic stability; with regard to the absorption spectrum, we see not only a reduction in the magnitude of the main Chl *a* absorption peak but also a decrease in the 660 nm spectral region which we assigned to the neighboring Chl *b*3 (now reassigned to Chl *a*).

By modeling the energy transfer between Chls in LHC-II, Trinkunas et al. (*12*) found a better fit to their spectroscopic data when two of the central Chls *a* (*a*1 and *a*2) in the monomer were replaced by Chls *b*, and Chls *b*1 and *b*2 by Chl *a*. The model was challenged by Gradinaru et al. (*11*), who proposed a different modification also based on energy transfer studies in which Chl *b*5 was assigned to a Chl *a* and Chl *a*6 to a Chl *b*, with all others remaining unchanged. By comparison, our results indicate that *b*5 is indeed a Chl *b*, and that the assignments of *a*1 and *a*2 to Chl *a* are correct. The spectroscopic models depend not only on the unambiguous assignment of all 12 Chls to Chl *a* or *b* but also on the precise distances between them which are currently known only to within 1−2 Å, and in particular on the orientations of their transition dipole moments which at present are entirely unknown.

Location of Neoxanthin. Just as the assignment of Chls, the identity of the central pair of carotenoids in the current LHC-II structure is based on plausibility, because the expected structural differences between the various carotenoids are exceedingly small. If the two carotenoids seen in the center of the monomer are assumed to be lutein, as

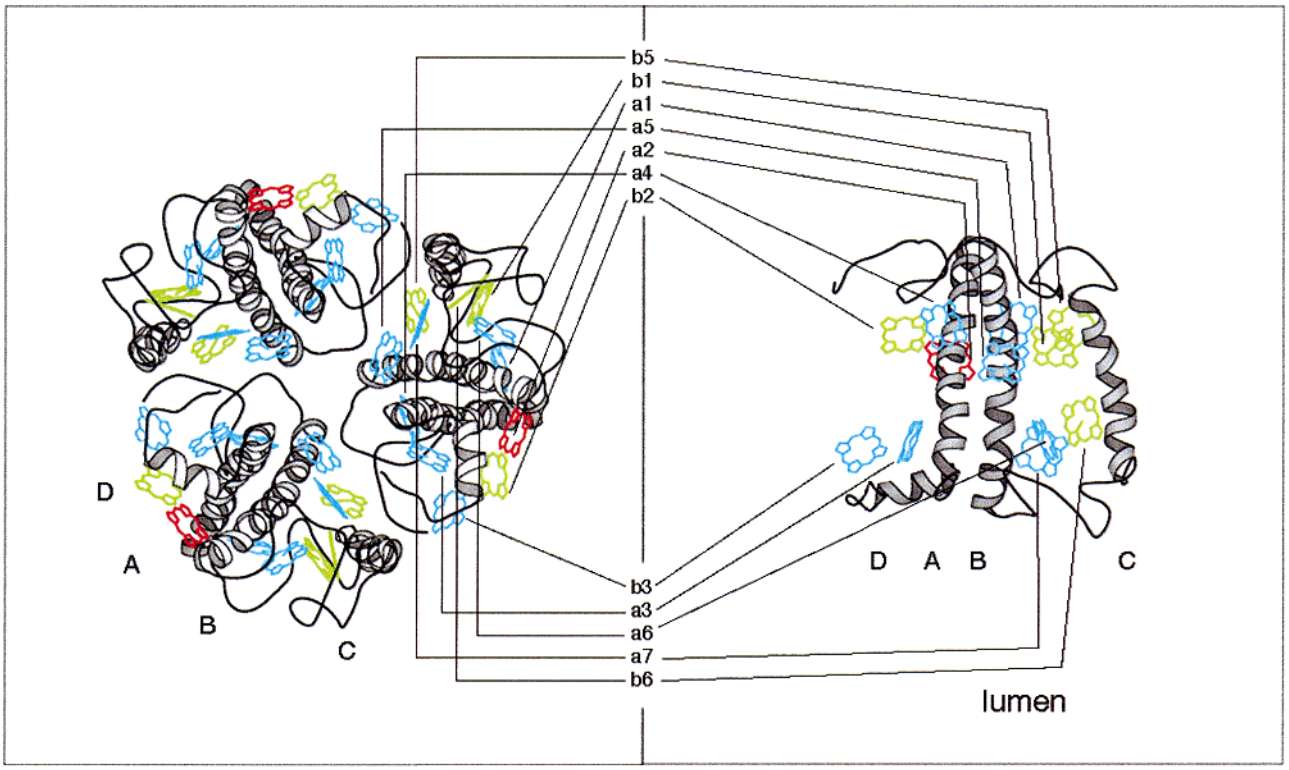

FIGURE 5: LHC molecule as a trimer viewed from the lumenal membrane surface (left) and as a monomer viewed from the side (right). Chl *b* molecules are green and Chl *a* molecules blue; Chl *a*2 is red. The helices (A−D) and chlorophyll molecules are labeled as in ref *8*. The figure was generated from the electron crystallographic data (*8*) using the program Molscript.

seems reasonable, our data do provide a hint about the location of neoxanthin. This is the only other carotenoid which is present in LHC-II in stoichiometric amounts. As Table 3 indicates, neoxanthin levels are reduced in mutants 4 and 8 lacking Chl *b*5 and *b*6, respectively. When bands cut out from partially denaturing gels are analyzed, the effect is even more pronounced, resulting in the complete absence of neoxanthin. Mutant 3 (*a*6) is unusual in its almost complete lack of neoxanthin in sucrose density gradient bands; although this mutant seems to have the most perturbed structure, it is interesting to note that all the targeted Chls are in the same region of the complex close to helix C. The presence of a carotenoid in this region may explain a report of energy transfer from carotenoid to Chl *b* (*9*), while others have only found energy transfer to Chl *a* (*40*).

There are several mutants in different organisms defective in the synthesis of certain carotenoids (*9*, *41*−*43*). These mutants suggest that organisms which exhibit defective neoxanthin synthesis still assemble LHC-II as judged from the Chl *a*:Chl *b* ratio or as seen in isolated LHC-II complexes (*9*); however, an isolation of LHC-II from mutants lacking lutein has not been demonstrated. Our results agree with those findings, as some mutants lose neoxanthin, whereas the lutein content seems to be a stable parameter which allows us to use it as a standard for protein concentration.

Trimer Stability. Six of the nine mutants we studied formed trimers readily, although there were considerable differences in stability. Mutants 1 (*a*4), 2 (*a*5), and 3 (*a*6) assembled only low amounts of trimers. This could mean either that the chlorophylls concerned are directly involved in trimer contacts or that the overall structure of the monomer is perturbed to such an extent that the trimer cannot assemble properly. A direct effect of the missing Chl *a*5 on trimer formation seems to be likely for mutant 2, as the Chl is in fact exposed on the trimer interface (Figure 5). On the other hand, Chl *b*5 seems to be similarly exposed, without an apparent effect on trimer formation. The target in mutant 1 which also exhibits deficient trimer formation is Chl *a*4. This is not exposed at the interface, but its absence may affect nearby Chl *a*5. As noted above, mutant 3 (*a*6) has a more severely perturbed structure, and it is therefore not surprising that it cannot assemble into trimers easily. Overall, it is interesting to note that so far all the mutated residues which affect trimer formation are located in helix B. This may point to a special structural or kinetic role of this helix in trimer assembly.

The accompanying paper (*25*) reports that the equivalents of our mutants 6 (*a*3), 7 (*b*3), and 8 (*b*6) form trimers upon insertion into isolated thylakoids, whereas the equivalent of our mutant 2 (*a*5) did not, in complete agreement with our results. The thermal stability of the monomer was highest for the equivalent of our mutant 7 (*b*3), and lowest for the equivalents of our mutants 2 (*a*5) and 8 (*b*6). Again, this is in agreement with our findings.

Interpretation of Spectra. Although the observed room-temperature spectra are not simply a linear combination of the 12 or more different components, comparison of the mutant spectra with the wt spectrum reveals some striking features that can be attributed to particular Chls. Mutant 7 which lacks the ligand for *b*3 (now likely to be a Chl *a*) shows a much deeper dip in its absorption spectrum between the main Chl *a* and Chl *b* peaks than wt. Indeed, the low-temperature absorption spectrum of LHC-II has a peak in this region (*44*). Its position indicates an unusually small red shift for a protein-bound Chl *a*, suggesting a relatively apolar environment. This fits with the position of *b*3 at the

periphery of the LHC trimer, where it is surrounded mostly by lipid and has little contact with the polypeptide. By comparison, the intensity of the main Chl *a* peak in mutant 6 (*a*3) is significantly reduced, suggesting a Chl *a* with a more pronounced red shift, in line with the more polar environment of Chl *a*3 in the interior of the protein. The decrease in intensity around 660 nm may stem from some effect on the neighboring Chl *b*3 (reassigned to Chl *a*) which could be attributed to the altered environment or even to a partial loss of this Chl.

The most interesting spectra are those of mutant 9 which lacks Chl *a*2 and possibly a Chl *b*. The decrease in the absorption on the red side of the Chl *a* peak indicates the absence of a Chl *a* with a particularly large red shift. Correspondingly, the fluorescence spectrum has an emission peak with a considerably larger blue shift than the wt spectrum, suggesting that the missing Chl emits on the far red side of the spectrum. We conclude that Chl *a*2 is associated with the lowest energy level in the complex. This chlorophyll is located at the periphery of the LHC-II trimer (Figure 5) and therefore is perfectly poised for a role in energy transmission to neighboring LHCs or to the reaction center. Indeed, this function has been proposed previously (*45*) purely on the grounds of its peripheral position in the trimer which predisposes it for a role in energy transfer.

In summary, we show that specific chlorophyll binding sites in LHC-II can be knocked out by site-directed mutagenesis. In vitro folding of six of the mutants on a Ni^{2+} affinity column followed by density gradient centrifugation yields stable trimers with well-defined biochemical and spectroscopic properties. Pigment analysis of these mutants suggests that they each lack no more than one or two Chls. In five out of six cases, the missing chlorophyll is consistent with our earlier structure-based assignment of Chls *a* or *b*. In one case, our results suggest that the identity of the Chl should be switched from Chl *b* to Chl *a*. Three mutants do not form stable trimers. Absorption and fluorescence spectra of one mutant provide compelling evidence that Chl *a*2 is critical for energy transmission to the reaction center. In the accompanying study (*25*), some of the same Chl binding sites were investigated independently but replaced with different amino acid residues. Close agreement with our results shows that the observed effects are specific for particular Chl binding sites. Clearly, some of the mutants described here warrant detailed investigation by crystallography and more sophisticated spectroscopic techniques.

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