THE LIGHT-HARVESTING COMPLEX OF PHOTOSYSTEM II IN BARLEY. STRUCTURE AND CHLOROPHYLL ORGANIZATION

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The isolated light-harvesting complex of photosystem II (LHCII) in barley had a chlorophyll (Chl) *a/b* ratio of 1.37 (mol/mol) and contained an average of 13.1 Chl molecules per 25 kD polypeptide chain, corresponding to 7.6 molecules of Chl *a* and 5.5 molecules of Chl *b*. Tryptic digestion of native LHCII yielded a 16 kD fragment which contained 12 of these 13 Chl molecules. The 16 kD fragment started with the serine 52 of the mature polypeptide, as determined by N-terminal amino acid sequence analysis and comparison with the amino acid sequence deduced from the nucleotide sequence of a corresponding gene in wheat (LAMPPA et al. 1985). Circular dichroism measurements of LHCII in 1% SDS or at extremes of pH, showed that there were at least two populations of Chl *a* and that Chl *a* was much more sensitive to changes in the aqueous environment than Chl *b*. We present a model for the folding of LHCII with four transmembrane helices, based on the above results and on the application of the rules of CHOU and FASMAN (1978) and PAUL and ROSENBUSCH (1985) for the prediction of secondary structure using the wheat sequence.

I. INTRODUCTION

The light-harvesting complex of photosystem II (LHCII) in maize is composed of at least five polypeptides with slightly different mobilities during SDS-PAGE (5). Analyses of the nuclear

genome in tomato and petunia have shown that the genes coding for polypeptide components of LHCII are present in several copies and organized in five small multigene families (12, 26). The amino acid sequences deduced from the

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Abbreviations: CD = circular dichroism; Chl = chlorophyll; DTT = dithiothreitol; HPLC = high pressure liquid chromatography; LHCII = light-harvesting complex of photosystem II; PAGE = polyacrylamide gel electrophoresis; PSII = photosystem II; PTH = phenylthiohydantoin; RC = reaction center complex; SDS = sodium dodecyl sulfate; Tricine = N(tris(hydroxymethyl)methyl)glycine.

nucleotide sequences of these different genes were more than 90% identical, and the differences were concentrated in the N-terminus. It is thus very likely that the amino acid sequences of the component polypeptides of LHCII in barley also differ mainly at their N-termini.

LHCII is accessible to antibodies from both sides of the thylakoid membrane (2) and the N-terminal part of the major (i.e., most abundant) polypeptide from LHCII has been shown to be involved in membrane stacking (21). Circular dichroism and polarized infrared spectroscopy have indicated that LHCII has a mainly α -helical conformation (23). Analysis of two-dimensional crystals has revealed that oligomeric LHCII has a three-fold symmetry axis and three or four transmembrane helices per monomer (16, 19). Excitation energy transfer between Chl molecules occurs with almost 100% efficiency within the complex and the pigments are protected against contact with the aqueous environment by the folding of the polypeptide chain (29). Circular dichroism spectra in the visible range suggested that the Chl *b* molecules in LHCII isolated by preparative SDS-PAGE were arranged round a three-fold symmetry axis (28, 30).

Despite this information, it is not known which parts of the polypeptide chain are in contact with the water phase and which are buried in the membrane, or which residues are involved in pigment co-ordination. In this investigation, circular dichroism spectra of trypsinized LHCII and LHCII exposed to SDS or extremes of pH were used to study the organization of the pigments. We present a model of LHCII based on proteolytic digestion patterns and sequencing data, using the methods of CHOU and FASMAN (10) and PAUL and ROSENBUSCH (24) for the prediction of secondary structure.

2. MATERIALS AND METHODS

2.1. Biological preparations

Wild type barley seedlings (*Hordeum vulgare* cv. Svalöfs Bonus) were grown for 7 days in vermiculite under 16 h light and 8 h dark cycles. Thylakoids for SDS-PAGE were prepared as described by MACHOLD et al. (20).

2.2. Isolation of Chl-protein complexes

Grana membranes were isolated using a modification of the Triton X-100 method of BERTHOLD et al. (7) as described by MØLLER and HØJ (22). The membranes were solubilized using dodecyl- β ,D-maltoside and fractionated into water-soluble polypeptides derived from the oxygen-evolving site, LHCII, and the reaction center complex of photosystem II (RC) by centrifugation on 10-30% sucrose gradients containing 10 mM-Tricine, pH 8.0 and 0.025% Triton X-100 (9, 14).

2.3. Electrophoresis

Samples were completely denatured by boiling in 1% SDS, 5 mM-DTT, 5 mM- NaHCO_3 . SDS-PAGE was performed in gels containing 6 M-urea as described by PICCIONI et al. (25).

Non-denaturing SDS-PAGE of native samples was carried out using 1 mm thick slab gels containing 10, 12.5 or 15% polyacrylamide. Lowering the SDS concentration in the gel from 0.1 to 0.03% and changing the acrylamide: bisacrylamide ratio from 30 : 0.8 to 33 : 1.1 decreased the degree of dissociation of the Chl-protein complexes during electrophoresis. The gels were run at 4 °C using buffer system II (20). Proteins were visualized by staining with Coomassie Brilliant Blue R250 or the alkaline silver stain described by WRAY et al. (32).

2.4. Proteolytic treatment

Isolated LHCII (0.5 mg Chl $\cdot$ ml⁻¹) was incubated at 4 °C with trypsin, (Type III), chymotrypsin (Type II, both from Sigma), *Staphylococcus aureus* protease V8, subtilisin A, pronase E or protease K. The protease concentration varied between 20 $\mu\text{g} \cdot \text{ml}^{-1}$ and 1 mg $\cdot$ ml⁻¹. The samples were incubated for 24 h when low amounts of protease were used, or 2.5 h when the protease concentration was high. LHCII was then separated from the protease by centrifugation on 10-30% sucrose gradients containing 10 mM-Tricine, pH 8.0 and 0.025% Triton X-100 at 80.000 $\times$ g for 24 h or more.

2.5. Amino acid sequence determination

After proteolytic treatment, degraded LHCII was purified by sucrose density gradient centrifugation as described above. The sample was boiled in 1% SDS, 5 mM-DTT to dissociate the complex into its component peptides, which were then purified by gel-filtration on an AcA 34 (LKB Ultrogel) column equilibrated in 0.05% SDS, 100 mM-NaHCO₃, pH 9.0, 0.5 mM-DTT.

Alternatively, the green Chl-protein band was excised from unstained gels after non-denaturing SDS-PAGE (section 2.3), the slices crushed and the protein eluted by twice incubating with five volumes of distilled water. The extract was lyophilized, redissolved in a small volume of distilled water and purified by gel-filtration as described above. The peptide fractions were lyophilized, and then redissolved in a small volume of distilled water prior to amino acid sequence analysis using an Applied Biosystems 470A gas phase sequencer and the program O2NRUN. PTH-amino acids generated by the sequencer were identified by HPLC on a 4.6×125 mm column of Spherisorb S5-ODS 2 (Phase Sep), using Waters equipment with 254 and 313 nm detectors.

2.6. Spectroscopic measurements

Absorption spectra were recorded on an Aminco DW2a spectrophotometer. Circular dichroism spectra in the visible range were recorded at room temperature using a Roussel-Jouen Dichrograph III equipped with a 1 cm quartz cell. For measurements between 260 and 200 nm a Cary 61 spectropolarimeter and a quartz cell with 0.5 cm pathlength was used. The optical density of the samples was adjusted to a value of about 1 at 670 nm for spectra between 350 and 750 nm and to about 0.3 for measurements in the ultraviolet range.

2.7. Chlorophyll content

The chlorophyll concentration was determined spectroscopically in 80% acetone as described by ARNON (3). Protein was quantitated by amino acid analysis after 24 h hydrolysis in 6 N-HCl at 108 °C in evacuated, sealed tubes. Amino acid analyses were performed on a Durrum D-500 amino acid analyzer.

Figure 1. CD spectrum of purified LHCII in its native state, and after denaturation by 10% Triton X-100.

3. RESULTS

3.1. Chlorophyll content of purified LHCII

LHCII was isolated by sucrose density gradient centrifugation of PSII membranes solubilized with dodecyl- β ,D-maltoside as described previously (14). Native preparations had strong CD signals in the red that are attributed to Chl *a* (688 nm) and Chl *b* (657 nm), respectively (Figure 1). The ratio of these signals was the same as the spectroscopically determined Chl *a*/Chl *b* ratio of 1.40 (w/w). Denatured LHCII had much weaker CD signals which were due to the intrinsic dichroism of the pigments and the peaks were shifted to shorter wavelengths.

The number of Chl molecules bound per polypeptide was determined using amino acid analysis to quantitate the protein. Colorimetric assays were found to give unreliable estimates of the concentration of Chl-proteins. We found 13.1 ± 2.4 Chl molecules per 25 kD polypeptide (molecular weight of the mature polypeptide calculated from the amino acid sequence deduced from the nucleotide sequence of a corresponding gene in wheat (18)). Assuming that there are five component polypeptides of LHCII and all bind Chl, this gives about 65 Chl molecules per oligomer. The preparation had a molar Chl *a*/*b* ratio of 1.37 ± 0.05 and 5.5 ± 1.0

Figure 2. SDS-PAGE of protease-treated LHCII under (a) non-denaturing conditions and (b) after boiling in SDS and run under denaturing conditions in the presence of 6 M-urea. For (a) LHCII was added to the gel without removing the proteases, whereas for (b) protease-treated LHCII was purified by sucrose gradient centrifugation before SDS-PAGE.

A) Lane 1: LHCII control, Lane 2: LHCII incubated with trypsin, Lane 3: LHCII incubated with chymotrypsin, Lane 4: LHCII incubated with subtilisin, Lane 5: molecular weight markers, Lane 6: wild type thylakoid membranes.

B) Lane 1: LHCII control, Lane 2: LHCII incubated with trypsin, Lane 3: LHCII incubated with protease K, Lane 4: LHCII incubated with Staph. aureus protease V8, Lane 5: LHCII incubated with pronase E, Lane 6: LHCII incubated with chymotrypsin, Lane 7: LHCII incubated with subtilisin, Lane 8: molecular weight markers, Lane 9: wild type thylakoid membranes.

molecules Chl *b* and 7.6 ± 1.4 molecules Chl *a* per 25 kD polypeptide.

3.2. Proteolytic digestion and the domain structure of LHCII

When purified LHCII was treated with proteases, non-denaturing SDS-PAGE showed green bands with a slightly higher mobility than the control (Figure 2a). The difference in mobility was minimal, regardless of which protease had been used. Using high concentrations of protease or incubation times up to 48 h at 4 °C did not give rise to smaller green fragments, and the amount of Chl-protein was unchanged as judged by non-denaturing SDS-PAGE.

When LHCII was digested with trypsin or chymotrypsin prior to SDS-PAGE under dena-

turing conditions, only one fragment of 16 kD was visible, indicating that the rest of the polypeptide chain had been degraded to peptides of less than 2-3 kD (Figure 2b). This 16 kD fragment was not further digested when very large amounts of trypsin or chymotrypsin were used, suggesting that it forms a tightly folded domain.

Treatment with low amounts of subtilisin A yielded a fragment with the same mobility during SDS-PAGE under denaturing conditions. Exposure to high concentrations of subtilisin A, pronase E or protease K degraded LHCII into peptides that were too small to be detected on SDS-polyacrylamide gels (Figure 2b). There was, nevertheless, no apparent change in the amount or mobility of the green protein band during non-denaturing SDS-PAGE beyond that

induced by trypsin-treatment, showing that LHCII was held together by non-covalent interactions even though the polypeptide backbone had been cleaved in several places.

Higher oligomers of LHCII were observed after non-denaturing SDS-PAGE in the digested material as well as the control. After protease treatment, the mobility of the oligomers was also slightly changed, indicating that the oligomers were not composed of undigested material. Since most of the LHCII dissociated into the monomeric form even under non-denaturing electrophoretic conditions, we used gel-filtration to study the effect of proteolytic digestion on the structure of LHCII. An AcA 34 column was equilibrated in 0.05% SDS, 100 mM-NaHCO₃, pH 9.0, 0.5 mM-DTT and calibrated separately with blue dextran 2000, thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), aldolase (158 kD), bovine serum albumin (67 kD) and ovalbumin (43 kD) as standards. Non-denatured LHCII migrated as a complex with an apparent molecular weight of 275-300 kD both before and after proteolysis, thus demonstrating that protease-treatment did not remove peptides that were important for the stability of the oligomers. Raising the SDS concentration to 0.1% did not change the elution point of oligomeric LHCII.

The molecular weight determined by gel-filtration is higher than that calculated from the apparent molecular weights of the five component polypeptides of maize LHCII (143 kD) (6). The addition of 6 acyl lipid molecules (27) and 13 Chl molecules per subunit, gives 225 kD. The reason for this discrepancy is not clear, but it is conceivable that barley LHCII could contain 6 or 7 homologous polypeptides.

3.3. N-terminal amino acid sequence of the 16 kD fragment

To determine which part of the polypeptide chain was contained in the 16 kD tryptic fragment, we determined its N-terminal amino acid sequence (four separate analyses) and aligned it with the amino acid sequences that have been deduced from the nucleotide sequences of the corresponding genes.

Trypsin-treated LHCII was purified either by

Figure 3. N-terminal amino acid sequence analysis of the 16 kD tryptic fragment from LHCII. Comparison with the amino acid sequence deduced from the nucleotide sequence of the corresponding gene in wheat (18) showed that this fragment started with amino acid 52 of the mature protein.

sucrose density gradient centrifugation or by eluting the protein from unstained gel slices after denaturing SDS-PAGE. In both cases the protein was separated from buffer components and small peptides by gel-filtration on an AcA 34 column equilibrated in 0.05% SDS, 100 mM-NaHCO₃, pH 9.0, 0.5 mM-DTT. Samples prepared by gradient centrifugation had a lower background during sequence analysis than those eluted from gels. The N-terminal amino acid sequence (16 residues) of the 16 kD fragment (Figure 3) was identical to the amino acid sequence deduced from the nucleotide sequence of the corresponding gene from wheat (18), beginning with amino acid 52 of the mature polypeptide, or 14 amino acids in front of the first transmembrane helix predicted by KARLIN-NEUMANN et al. (15).

At each sequencing step there were several minor PTH-amino acid peaks in addition to the main peak. This may be due to contamination by small peptides that were not completely removed by gel-filtration. Another possibility is that the trypsin was contaminated with traces of other proteases, or that the high amount of trypsin used to ensure complete digestion (1 mg · ml⁻¹) and the low incubation temperature may have caused self-digestion and lower specificity of the trypsin. Trypsin normally cleaves on the C-terminal side of lysine and arginine residues. The fact that the amino acid immediately before serine 52 (the first amino acid of the 16 kD fragment) is leucine (18) suggests that a certain amount of chymotrypsin-like digestion occurred.

Figure 4. CD spectrum of purified LHCII before and after trypsin-treatment.

3.4. Changes in the pigment organization in trypsinized LHCII

Circular dichroism spectra of trypsin-treated LHCII indicated that the organization of Chl *a* was disturbed, as shown by a 56% decrease of the signal at 688 nm (Figure 4). Moreover, there was a change in the ratio of the signals at 476 and 493 nm and a general loss of detail at lower wavelengths. In contrast, the organization of Chl *b* appeared to be unchanged, since there was no decrease in the intensity of the signal at 657 nm. These results suggest that some of the Chl *a* and xanthophyll molecules are bound close to the outer surface of LHCII, and hence the thylakoid membrane, and that Chl *b* is organized in the core of LHCII.

After proteolytic treatment the molar Chl *a/b* ratio had decreased from 1.37 ± 0.05 to 1.25 ± 0.04 , and the protein/Chl ratio (w/w) had decreased from 2.20 ± 0.46 to 1.50 ± 0.51 . The number of Chl *b* molecules (after protease-treat-

ment) was calculated to be 5.3 ± 2 per 16 kD polypeptide chain (which is the same molar ratio as the untreated sample), and there were 6.6 molecules of Chl *a*, indicating that one molecule of Chl *a* per polypeptide was lost.

3.5. Effects of SDS on pigment organization and oligomeric structure in LHCII

Circular dichroism spectroscopy showed that SDS induced rapid changes in the organization of the pigments. Within 3 min of the addition of SDS to a final concentration of 1%, the intensity of the Chl *a* signal at 688 nm had diminished by more than 50% (Figure 5). There were also dramatic changes in the ratio of the signals at 476 and 493 nm and a general loss of detail at lower wavelengths, similar to those observed after trypsin treatment. In contrast, the Chl *b* signal at 657 nm and the peak at 674 nm were almost unchanged. Upon prolonged exposure to

Figure 5. CD spectrum of purified LHCII before and 3 min after the addition of SDS to a final concentration of 1%.

1% SDS these two signals slowly decreased with the same kinetics (Figure 6) and after 2 h the two signals still had 77 and 74% of their original intensity. After the first rapid decrease, the Chl *a* signal diminished with the same slow rate as the signals at 657 and 674 nm, indicating that there are at least two populations of Chl *a*. After 2 h the signal at 688 nm retained 18% of its original intensity.

The changes observed in the CD spectrum between 460 and 500 nm upon incubation in 1% SDS or trypsinization are similar to the differences between the CD spectrum of oligomeric and monomeric LHCII isolated by non-denaturing SDS-PAGE (4). To test whether these changes were caused by the dissociation of LHCII into monomers, LHCII was incubated in 1% SDS for up to 30 min and then applied to an AcA 34 gel-filtration column equilibrated in 0.1% SDS, 100 mM-NaHCO₃, pH 9.0, 0.5 mM-DTT. The protein eluted as a sharp peak with an apparent molecular weight of 275-300 kD and there was little free Chl or monomer. Thus the

changes in the CD spectrum are not due to the dissociation of oligomeric LHCII into monomers, but to the destabilizing effect of SDS on the Chl organization of LHCII.

Figure 6. Time course of the denaturation of LHCII in 1% SDS measured by the decrease in the intensity of the CD signals in the red region.

Figure 7. CD spectra of the undenatured photosystem II reaction center (RC) and the RC after 2 and 10 min incubation in 1% SDS.

3.6. Denaturation of the reaction center complex of PSII by SDS

A reaction center complex (RC) of photosystem II, composed of Chl_a -protein 2, Chl_a -protein 3, cytochrome b_{559} , a 32 and a 34 kD polypeptide, most of the 33 kD polypeptide from the oxygen-evolving site plus ca 19 lipid molecules was isolated by sucrose density gradient centrifugation of PSII membranes solubilized with dodecyl-maltoside (14). The reaction center complex was much more sensitive to SDS than LHCII. Incubation in 1% SDS rapidly decreased the degree of pigment organization, as shown by the diminishing intensity and the broadening of the CD signals at 673 and 686 nm (Figure 7). After 10 min there was only a small residual CD signal, but the Chl still retained

some organization since the peak had not shifted to the blue and there was still exciton splitting. Correlated with these changes in the CD spectrum, RC lost the ability to transport electrons from the artificial donor diphenyl carbazide to the acceptor dichlorophenol indophenol and was totally inactive after 5 min.

3.7. pH stability of LHCII

LHCII was incubated for 2 h at 4 °C at different pH values before recording the CD spectra. Between pH 3.5 and 10.5 no change in the pigment organization could be detected. At pH 11 the signals at 658 and 688 nm had diminished by 5 and 33%, respectively, and at pH 12 the intensity of the signals had further decreased to 45 and 18% of their original intensity and the negative peak at 657 nm had become much broader (Figures 8 and 9). Samples that had been incubated at pH 12 showed only free pigment during non-denaturing SDS-PAGE in tube gels (not shown), indicating that although the complex retained some degree of pigment organization, it had become very unstable.

At low pH the signal at 688 nm also started decreasing before that at 657 nm, confirming

Figure 8. pH-dependent changes in the pigment organization of LHCII measured by CD spectroscopy. The spectrum of undenatured LHCII at pH 8.0 was taken as the reference.

Figure 9. CD spectrum of LHCII after 2 h incubation at 4 °C at (a) pH 8.0 (control), pH 11.0 and pH 12.0, and at (b) pH 8.0, pH 3.0 and pH 1.0.

Figure 10. Ultraviolet CD spectrum of native LHCII and LHCII incubated for 2 h at 4 °C at pH 2.5.

that Chl *a* is more accessible from the water phase than Chl *b*. After 2 h incubation at pH 3.0 the organization of Chl *b* was apparently unchanged, but the Chl *a* signal at 688 nm had decreased by 32%. Moreover, there was a change in the ratio of the signals at 476 and 492 nm, similar to that observed upon incubation in 1% SDS or proteolysis.

At pH values below 2.5 the signal at 657 nm decreased rapidly, suggesting that the protein was unfolding. This was confirmed by measuring CD spectra between 260 and 200 nm (Figure 10). The decrease in ellipticity at 208 and 222 nm was indicative of a pronounced decrease in the amount of α -helix. However, the amplitude

Figure 11. Polyacrylamide gel (A) before and (B) after staining with Coomassie brilliant blue. Proteins were separated by non-denaturing SDS-PAGE at 2 °C using buffer system II (20). Lane 1: LHCII after trypsin treatment. Lane 2: LHCII control, Lane 3: LHCII after 2 h incubation at pH 1.5, Lane 4: wild type thylakoid membranes.

Figure 12. Denaturation of RC at low pH measured by CD spectroscopy. Spectra were recorded after incubation for 2 h at (a) pH 7.0 (---), pH 3.5 (-----), pH 3.0 (—) and pH 2.5 (·····) and (b) pH 1.0. The latter spectrum is drawn to a different scale.

of the signal at 688 nm did not decrease further, but shifted to 690 nm, and a new peak was observed at 706 nm when the pH was lowered below 2.5. This new signal increased in intensity as the pH was lowered to 0.5. Below pH 1.5, the Chl was completely pheophytinized and there were strong new CD signals at 400, 444 and 468 nm. SDS-PAGE showed that the pigments were no longer associated with the protein (Figure 11). Instead, there was a pheophytin aggregate to which the new CD signals are attributed.

3.8. pH stability of RC

The pigment organization of RC remained unchanged over a wide pH range. The Chl was still bound at pH 12 as judged by non-denaturing tube gel electrophoresis (not shown), but became unstable below pH 4 (Figure 12). At pH 3.5 the negative peak at 686 nm retained 67% of

Figure 13. Ultraviolet CD spectrum of native photo-system II reaction center (RC) and RC incubated for 2 h at 4 °C at pH 2.0.

its original intensity, but at pH 3.0 the Chl was completely disorganized as evidenced by the very broad trough, the blue-shift and the absence of exciton splitting. CD measurements in the UV range (Figure 13) showed a decrease in the ellipticity at 208 and 222 nm at pH 2.0, indicating unfolding of the α -helices and confirming that the protein was denatured.

As found for LHCII, a new signal at 706 nm appeared when the pH was lowered to 2.5 or less. The intensity of the signal increased as the pH decreased. This signal, which had a positive component at 706 nm and a smaller negative component at 690 nm, was attributed to pheophytin aggregates.

4. DISCUSSION

LHCII has been shown to be composed of at least five polypeptides in maize (5). Proteolytic digestion studies indicated that LHCII is an extremely stable complex held together by non-covalent interactions – presumably hydrogen bonding between charged or polar residues buried in the membrane – even when the polypeptide chain was cleaved in so many places that the peptides were too small to be detected after electrophoresis under denaturing condi-

tions on polyacrylamide gels containing 6 M-urea. The same preparation showed only a slight increase in the mobility of the green band during non-denaturing SDS-PAGE.

Despite the fact that intact LHCII can be resolved into five polypeptide bands by SDS-PAGE under denaturing conditions (5), a single fragment with an apparent molecular weight of 16 kD was observed after digestion with trypsin or chymotrypsin. Amino acid sequence analysis and comparison with the amino acid sequence derived from wheat (18) showed that the 16 kD fragment started with residue 52 of the mature polypeptide chain. The amino acid sequences deduced from the nucleotide sequences of the genes coding for LHCII polypeptides in *Lemna gibba* (15), wheat (18), tomato (26) and pea (11) are more than 90% identical, and the first 16 residues of the 16 kD tryptic fragment from barley are identical to those of wheat, pea and tomato. This suggests that several polypeptide components of LHCII in barley could give rise to tryptic fragments with identical N-terminal sequences.

Determination of the Chl content of LHCII showed that it binds an average of 13.1 ± 2.4 Chl molecules per 25 kD polypeptide or 5.5 ± 1.0 molecules Chl *b* and 7.6 ± 1.4 molecules Chl *a*. Rounding to the nearest integer gives 6 molecules of Chl *b* and 8 molecules of Chl *a* per 25 kD and a Chl *a/b* ratio of 1.33. Alternatively, there might be 5 molecules of Chl *b* and 7 molecules of Chl *a* with a Chl *a/b* ratio of 1.40. On the basis of our data it was not possible to decide between these possibilities. It has been assumed in these calculations that all of the component polypeptides of LHCII bind Chl equally. Spectroscopic data have indicated that Chl *b* molecules in LHCII monomers are oriented around a three-fold symmetry axis (13, 28, 30). This means that the Chl *b* molecules are either organized in multiples of three, or that there are three Chl *b* molecules around a three-fold symmetry axis plus two or three separately organized Chl *b* molecules. The latter hypothesis is supported by the existence of a slight shoulder on the Chl *b* CD signal in the red.

Trypsin-treated material had a lower Chl *a/b* ratio (1.25 ± 0.04 vs 1.37 ± 0.05), and the protein/Chl ratio (w/w) had changed from 2.20

± 0.46 to 1.50 ± 0.51 , indicating the loss of one molecule of Chl *a* per polypeptide. Circular dichroism spectra in the visible range indicated that the organization of Chl *a* and xanthophylls had been affected, but there was no change in the organization of Chl *b*. This means that with the exception of one Chl *a* molecule, all of the Chl is bound to the 16 kD tryptic fragment of LHCII.

When LHCII was incubated in 1% SDS or at extremes of pH the organization of Chl *a* and xanthophylls was disturbed before that of Chl *b*. Since these treatments did not cause the dissociation of LHCII into its subunits, this means that part of the Chl *a* and xanthophyll molecules are organized in that part of the protein that is normally exposed to the outer surface of the thylakoid membrane, so these pigment molecules could mediate excitation transfer between adjacent membranes.

Assigning CD signals to specific pigment species has been difficult since interactions between pigments and between pigments and the polypeptide chain induce exciton splitting and shifts in the peak wavelengths. Thus the peak at 674 nm has been alternatively attributed to Chl *a* (8) or to exciton splitting due to interactions between Chl *b* trimers (13, 28, 30). The latter model was based on spectra of monomeric LHCII isolated by preparative SDS-PAGE, where the CD signals due to Chl *a* were nearly non-existent. The different resistance to SDS and the kinetics of disappearance of the signals at 688, 674 and 657 nm indicate that the peak at 674 nm is indeed mainly due to exciton splitting between Chl *b* molecules, but one cannot exclude a possible contribution from Chl *a*.

Analysis of two-dimensional crystals of LHCII preparations with the electron microscope indicated that each polypeptide subunit contained three or four transmembrane helices (16). KARLIN-NEUMANN et al. (15) proposed a model for the folding of an LHCII polypeptide with three transmembrane helices. This model was based on hydropathy plots and left large parts of the polypeptide chain exposed to the water phase, and thus potentially accessible to proteolytic digestion. In contrast, we found that the 16 kD fragment was not degraded to smaller fragments by high concentrations of trypsin or

Figure 14. Tentative model for the folding of the 16 kD tryptic fragment of LHCII, based on the sequence deduced for wheat. Aspartic acid and glutamic acid residues are coloured yellow, lysine and arginine are coloured blue.

chymotrypsin, indicating that it forms a compact domain. No other fragments could be detected, suggesting that the N- and C-terminal parts of the polypeptide chain had been degraded to peptides of less than 2-3 kD.

Since the 16 kD fragment contained all but one of the Chl molecules bound per 25 kD LHCII polypeptide, we thought it of interest to draw a tentative model of the folding of the 16 kD fragment. Knowing from spectroscopic measurements that LHCII had a high content of alpha-helical structure (14, 23) we assumed that

the polypeptide chain crossed the membrane in helical conformation, as is the case for bacteriorhodopsin or the bacterial reaction center complex, and that each membrane-spanning segment contained 24 amino acid residues. Turns were predicted by the method of PAUL and ROSENBUSCH (24) which is specifically designed for use with membrane proteins. Since residues in turn positions are assumed to be at the surface of the protein and in contact with the water phase, we also applied the rules of CHOU and FASMAN (10) for the prediction of beta-turns.

Both methods indicated identical turn positions when they were applied to the amino acid sequence deduced from the nucleotide sequence of the corresponding gene in wheat (18). Comparison with sequences from other species (pea, petunia, tomato and *Lemna gibba*) showed that with one exception, the deduced amino acid sequences were identical at the predicted turn positions and that this amino acid substitution did not change the result of the prediction. By this method we obtained a model with four transmembrane helices, where both the N- and C-terminal parts of the polypeptide chain are exposed to the water phase at the outer surface of the membrane (Figure 14). This is in agreement with the results of proteolytic digestion studies by ANDERSSON et al. (2) who showed that LHCII was not digested by trypsin in inside-out thylakoid membrane vesicles, but that trypsin-treatment of right-side out vesicles caused a slight increase in the mobility of LHCII. ANDERSON and GOODCHILD (1) have recently obtained evidence that the N- and C-termini are both located on the stroma surface, using an antibody raised against a synthetic peptide based on the deduced C-terminal sequence.

The first predicted transmembrane helix starts with phenylalanine 58, immediately after a predicted turn, and eight residues before the beginning of the first helix predicted by KARLIN-NEUMAN et al. (15). This introduces two glutamic acids and one arginine into the hydrophobic core of the membrane, but since the acidic and basic amino acids are three residues apart, they might form a salt bridge, which would neutralize the charges. In this way, the potential tryptic cleavage sites lysine 60 and arginine 62 could be protected. Alternatively, the charged residues buried in the interior of the membrane might form salt bridges between helices and thereby help to stabilize the complex. The next predicted turn (Figure 14) starts with alanine 86, followed by the second transmembrane helix, beginning with valine 90. The hydrophathy of this segment is compatible with transmembrane localization. Again there are a lysine and a glutamic acid three residues apart whose charges may be neutralized by salt bridges. The next predicted turn starts with glycine 114. This is followed by the third transmembrane helix starting with leucine 118,

which is identical to the second helix predicted by KARLIN-NEUMAN et al. (15). This segment contains no charged amino acids.

The next part of the sequence is rich in proline and glycine and contains several potential turn sites. We did not attempt to make any detailed prediction for this part of the polypeptide chain. The fourth predicted transmembrane helix starts with lysine 177 and is shifted by nine residues relative to the one predicted by KARLIN-NEUMAN et al. (15). The projecting helices and loops in the lumen may correspond to the projections seen by KÜHLBRANDT (16) upon analysis of two-dimensional crystals in the electron microscope. The apparent molecular weight of the fragment would be compatible with tryptic cleavage at lysine 203, which is buried in the membrane in the model of KARLIN-NEUMAN et al. (15). The electrophoretic mobility of undigested LHCII agrees well with the molecular weight calculated from the deduced amino sequence. However, the tryptic fragment might migrate abnormally during SDS-PAGE, and we cannot exclude that the C-terminal cleavage occurred at some other residue. It should be noted that there are several potential cleavage sites for trypsin and chymotrypsin exposed to the water phase in our model. Their protection against proteolytic digestion must be due to the tightly folded and stable tertiary structure of the polypeptide.

There has been much speculation about how the polypeptide and the Chl molecules interact to achieve the precise organization of the pigment molecules that is important for the function of the Chl-proteins. Histidine residues have been proposed to bond to the central Mg ion in the porphyrin ring, but the 16 kD tryptic fragment of LHCII contains only two histidine residues. The seven glutamine and asparagine residues in a bacteriochlorophyll *c* binding polypeptide from chlorosomes of a green photosynthetic bacterium have been proposed to bind the seven bacteriochlorophyll *c* molecules (31), but again, there are no striking coincidences of this kind in LHCII. One possibility to determine where the Chl molecules are bound would be to bind them covalently to the polypeptide chain by means of a chemical crosslinking reagent, to digest the protein to small peptides and to

determine the position of the modified residues by amino acid sequence analysis. Another method, which is more time-consuming but yields more information about the precise organization of the pigments, is to produce three-dimensional crystals of Chl-proteins and to analyze them by X-ray diffraction. KÜHLBRANDT (17) has recently reported important progress in this respect in that two crystal forms of LHCII have now been prepared.

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