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Arrangement of the light-harvesting chlorophyll *a/b* protein complex in the thylakoid membrane

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The transverse orientation of the light-harvesting chlorophyll *a/b* protein complex of Photosystem II (LHC II) in the thylakoid membrane of pea was investigated using surface radioiodination with Iodo-Gen™. The labelling effects on LHC II of four different membrane preparations were compared. One preparation was oriented right-side-out (intact thylakoids); two of them had an inside-out orientation exposing the lumenal surface (inside-out vesicles; PS II particles) and one had both sides of the membrane exposed (mechanically damaged thylakoids). It was found that LHC II could be iodinated only in membrane preparations with an exposed lumenal surface. Isolated apoproteins were chemically cleaved. Fragments analysis revealed a tyrosine residue located eight amino acids from the C-terminus as the single iodination site. It is concluded that the C-terminus of LHC II points towards the lumenal side of the thylakoid. Differences in the labelling behaviour of the LHC apoproteins could be assigned to a heterogeneity in the C-terminal region in which the tyrosine residue is replaced by phenylalanine.

Introduction

The light-harvesting chlorophyll *a/b* protein complex of Photosystem II (LHC II) is the predominant integral protein complex of the thylakoid membranes from green algae and higher plants. It is composed of several immunologically related polypeptides [1,2]. Nuclear genes or cDNA complementary to the mRNA encoding a precursor to the major polypeptide in pea [3,4] and petunia [5,6] and *Lemna* [7,8] have been isolated and sequenced. Both the hydropathy plot of the amino-acid sequence [6,7] and infrared dichroism data [9] predicted three or four transmembrane regions of the polypeptide chain. The amino-terminus is located in the stroma [10]. In view of the current interest in the structure of chlorophyll-protein complexes, we investigated the orientation of the C-terminus by means of surface labelling.

Studies of the transverse arrangement of proteins across the thylakoid membrane are greatly facilitated by the availability of sealed inside-out vesicles and of PS II particles exposing the former lumenal surface to the medium [11–13]. However, experimental approaches for studying the conformation of the LHC II employing proteolytic digestion gave few data, since only an amino-terminal segment of the protein seemed to be susceptible to proteinases [1,10]. The use of topologically selective chemical labeling was therefore indispensable. According to the sequence data for pea LHC II [3,4], one of the eight tyrosine residues is

Abbreviations: Iodo-Gen, 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril; LHC II, light-harvesting complex of Photosystem II; PS II, Photosystem II; SDS, sodium dodecylsulphate.

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situated in the C-terminal hydrophilic segment of the major apoprotein of LHC II eight amino-acids away from the C-terminus. We therefore made use of a technique that labels surface-exposed tyrosines.

In the present work radioiodination of tyrosines was carried out with Iodo-Gen™. This method has been reported to be gentle and topologically specific [14,15] and to yield high specific incorporation during surface labelling of thylakoids [16].

Materials and Methods

Membrane preparations

Right-side-out thylakoids were prepared from 21-day-old pea seedlings (*Pisum sativum* var. carnosa vs. sugar snap) as described in Ref. 17, modified according to Ref. 18.

Broken thylakoids were obtained by subjecting thylakoids to one cycle of freeze-thaw in low-osmotic- and low-ionic-strength media (5 mM NaCl/1 mM EDTA/5 mM Tricine-NaOH (pH 7.8)). This treatment leads to mechanical damage of the membrane with a concomitant release of plastocyanin from the thylakoid lumen [19].

PS II particles were prepared as described in Ref. 12.

Thylakoid vesicles of opposite sidedness were obtained as described in Ref. 20 with the following modifications. The thylakoids were disintegrated by passage through a French pressure cell press (American Instrument Company, U.S.A.) at a pressure of 23 MPa and a flow rate of 5 ml · min^{-1}. The concentration of both polymers (dextran and poly(ethylene glycol)) during the phase partitioning was 5.4%.

All the membrane preparations were ultimately suspended in iodination buffer (50 mM sucrose/10 mM NaCl/5 mM sodium phosphate (pH 7.5)). For PS II particles this buffer was supplemented with 5 mM $MgCl_2$. The chlorophyll concentration was determined in 80% acetone [21].

Radioiodination

To a glass scintillation vial, coated with Iodo-Gen (Pierce) [15], 3 ml of the membrane suspension (1.5 mg Chl/ml) and 3 mCi of $Na^{125}I$ (11 mCi/μg I^-) were added. The reaction was allowed to proceed for 3 min at 4°C then the mixture was transferred into 20 ml of 200 mM NaI. The membranes were sedimented in a clinical centrifuge, washed several times in 150 mM NaI and finally resuspended in 10 mM Tricine-NaOH (pH 7.8).

LHC II analysis

LHC II was isolated from radiolabelled membranes as described in Ref. 17, with the modification of Ref. 18. The protein was delipidated in 80% acetone and cleaved at Asp-Pro sites by partial acid hydrolysis in 75% formic acid for 72 h at 37°C. Subsequent molecular sieve chromatography was performed on Bio-Gel P 30 (−400 mesh, 1.8 × 70 cm, 2 ml/h) in 50% formic acid. Radioactivity was measured in a gamma counter MR 480 (Kontron). N-terminal amino-acid sequence determination was performed by automated Edman degradation on a Beckman 890 C sequencer essentially as in Ref. 22 and on the Applied Biosystems 470A sequencer according to the manufacturers' operator's manual. SDS-polyacrylamide gel electrophoresis was done on a 12.5% gel according to [23]; the dried gels were subjected to autoradiography on Fuji X-ray film. High-voltage paper electrophoresis (Whatman No. 1 paper, 50 V/cm) was run at pH 2 (formic acid/acetic acid/water, 1 : 4 : 45 (v/v)) for 1 h.

Results and Discussion

Four different membrane preparations were surface-labelled. One of them had the right-side-out orientation (intact thylakoids); two of them had an inside-out orientation (PS II particles, inside-out vesicles) and one had both sides of the membrane exposed (mechanically damaged thylakoids). Fig. 1 shows tracks of SDS polyacrylamide gels stained with Coomassie blue as well as autoradiograms of the radioiodinated membranes. Pea LHC II migrates as two predominant bands with apparent molecular masses of 27 (major component) and 25 kDa. Little label is found in the region of the apoproteins of LHC II in the labelling pattern of intact thylakoids (Fig. 1, lane 2). In contrast, the three membrane preparations with an accessible inner thylakoid surface show a considerable label incorporation in this

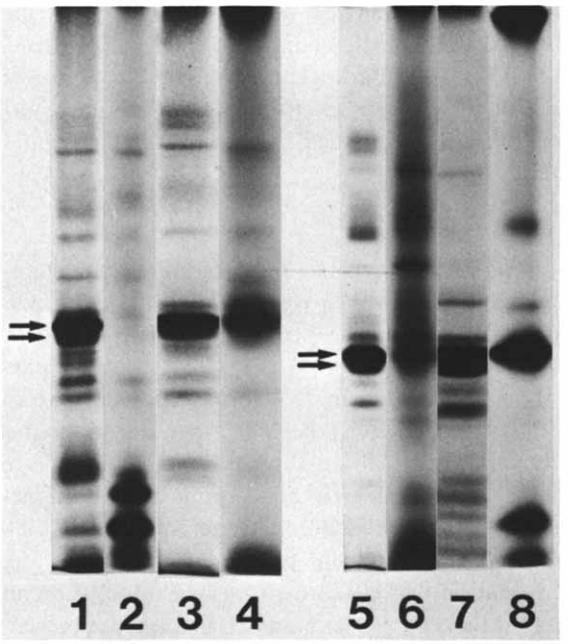

Fig. 1. SDS-polyacrylamide gel electrophoresis of ^{125}I-labeled membranes. The gels were stained for protein (lanes 1, 3, 5 and 7) and subjected to autoradiography (lanes 2, 4, 6 and 8). Intact thylakoids (1 + 2), broken thylakoids (3 + 4), inside-out vesicles (5 + 6), PS II particles (7 + 8). The arrows indicate the major 27 kDa and the minor 25 kDa LHC II apoproteins of pea.

region (Fig. 1, lanes 4, 6 and 8). Apparently, the labelled tyrosine(s) in these three preparations must belong to (a) segment(s) exposed to the lumen of the thylakoid. To analyse this (these) segment(s) further, we isolated the LHC II after the labelling experiments, cleaved the protein at Asp-Pro sites by partial acid hydrolysis and fractionated the fragments by gel filtration. Fig. 2a shows the ultraviolet elution diagram of a Asp-Pro fragment separation. The corresponding four radioactivity profiles are shown in Fig. 2b. As expected from the autoradiography in Fig. 1, lane 2, very little label is introduced into the fragments of LHC II derived from the labelling of intact thylakoids. Fractionation of Asp-Pro fragments of LHC II, derived from membranes with an exposed inner thylakoid surface, produced in each of the three preparations a strongly radiolabelled fragment of an apparent molecular weight of 1900 corresponding to peptide E in the ultraviolet elu-

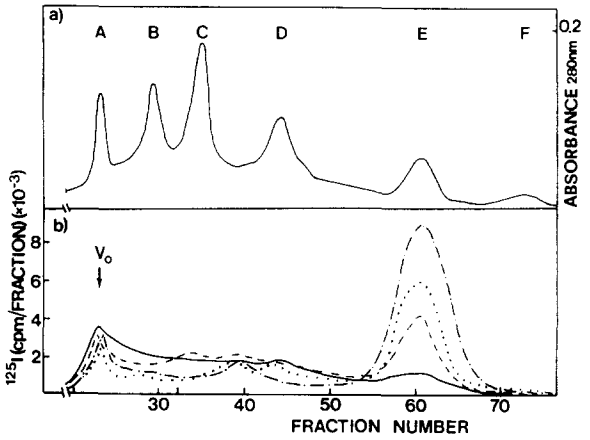

Fig. 2. Gel filtration of partially hydrolysed radioiodinated LHC II on Bio-Gel P-30. (a) Absorbance at 280 nm was monitored. (b) Elution profiles of radioactivity. ———, Intact thylakoids; ·–·–·, PS II particles; ·····, broken thylakoids; — — —, inside-out vesicles.

tion diagram. Edman degradation of this fragment gave the sequence of the C-terminal segment 217–233 of the major apoprotein of LHC II (Fig. 4). All the radiolabel was recovered in cycle nine, corresponding to tyrosine residue 225.

The failure of the seven remaining tyrosines of LHC II to incorporate a detectable amount of label from either side of the membrane, despite their being situated in hydrophilic surroundings, is probably due to a very effective steric hindrance which reduces the accessibility of the hydrophilic segments of the protein. This phenomenon was

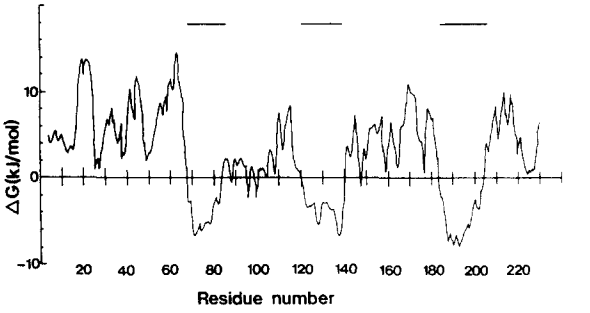

Fig. 3. Hydrophobicity plot for the major LHC II apoprotein of pea. The amino-acid sequence shown in Fig. 4 was used. Mean hydrophobicities using the values in Ref. 26 are plotted for successive seven-residue segments. The three regions of the polypeptide predicted to cross the membrane are indicated by bars.

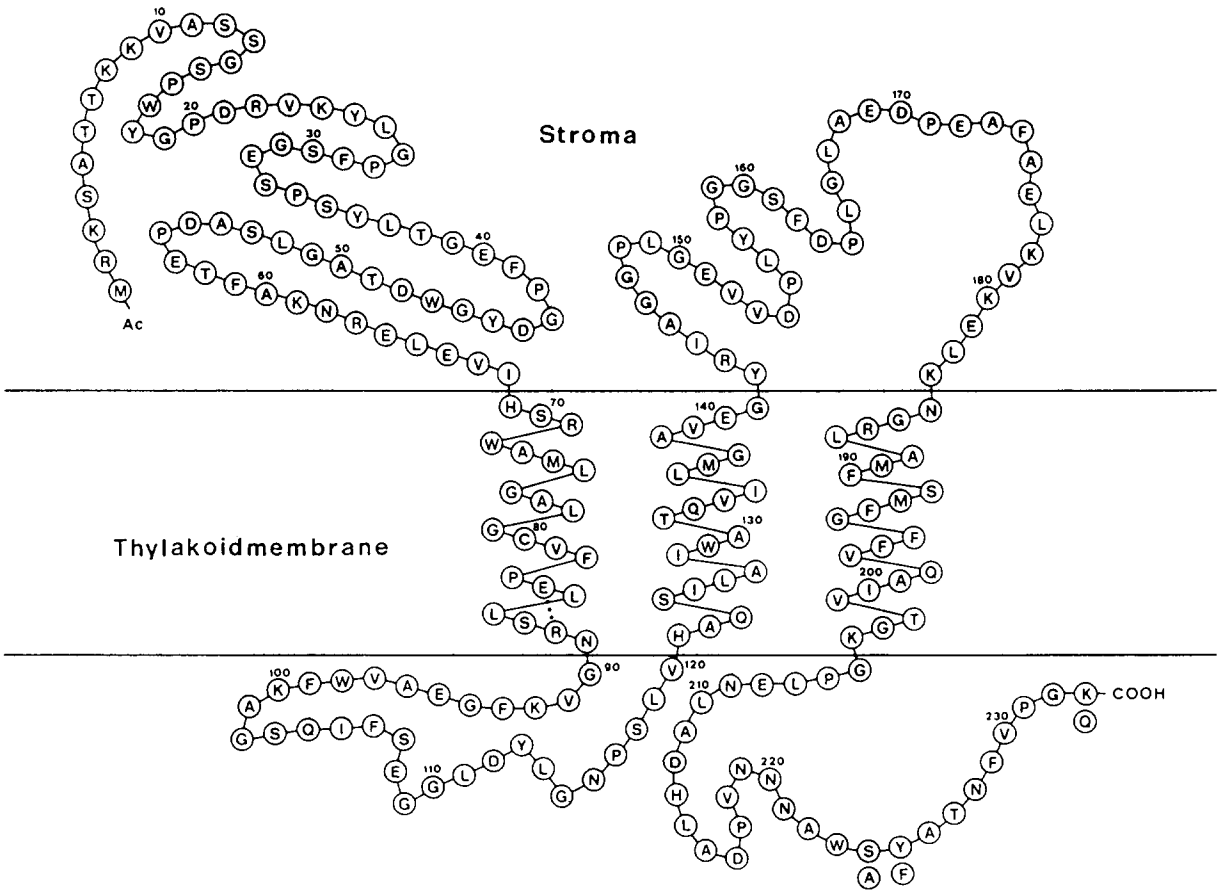

Fig. 4. A model for the organization of LHC II with respect to the thylakoid membrane bilayer. The amino-terminus is exposed to the stroma, the carboxyl-terminus to the thylakoid lumen. The amino-acid sequence derived from Edman degradation with the exception of three segments marked in Fig. 5 for which the DNA derived sequence in Ref. 3 was used. Sites of high turn probability [25] are stressed by U-turns. The dotted line marks a possible intrahelical salt bridge between Glu-84 and Arg-88. The side-chain groups of the other charged amino acids within the three transmembrane helices are expected to remain outside the hydrophobic region of the membrane [27]. At the positions 224, 225 and 233, the corresponding amino acids of a minor protein fraction are shown. Details are in the text.

also observed in proteinase digestion studies, which revealed a pronounced resistance of LHC II towards proteolysis [1].

The labelling behaviour of LHC II (Figs. 1, 2) demonstrates that tyrosine 225 is accessible only if the lumenal side of the thylakoid membrane is exposed during surface labelling experiments. And because the eight amino acids from tyrosine 225 to the C-terminus are not enough to span a membrane, the labelling behaviour also demonstrates that the C-terminus of LHC II is situated on the lumenal surface of the thylakoid membrane. This finding, along with the well-established orientation of the NH_2-terminus [10] and the existence of three or four membrane spanning segments (Fig. 3) [6,9] leads to the model shown in Fig. 4. A similar model was proposed in Ref. 7 based on DNA analysis in *Lemna*.

Quite in contrast to the results shown above, the smaller (25 kDa) LHC II apoprotein, which is also the minor one, seemed not to be susceptible to iodination with Iodo-Gen. The autoradiograms in Fig. 1 showed very little if any label in the corresponding band. This agrees with our finding that the C-terminal Asp-Pro-fragment E (Fig. 2) is heterogeneous with regard to three amino acids.

(Recall that the different apoproteins of LHC II were not separated before the fragmentation.) High-voltage paper electrophoresis of this fragment leads to a minor component (20% of the total peptide material) yielding alanine, phenylalanine and glutamine instead of serine 224, tyrosine 225 and the C-terminal lysine 233 (Figs. 4 and 5). If one assumes that the minor component in Asp-Pro fragment E belongs to the minor 25 kDa apoprotein, then the absence of label in this protein must be due to the lack of tyrosine-225 and not to the different accessibility of the C-terminal segment. We also sequenced the rest of the Asp-Pro fragments (Fig. 2), fragments derived from cleavages at Asn-Gly sites and at tryptophan residues and the N-terminus of isolated LHC II after trypsin [10] or clostripain treatment, but we were not able to detect any other heterogeneity in the protein material with our methods (data not shown).

By comparing the established partial protein sequences with those in Refs. 3 and 4, derived from DNA and cDNA analysis respectively, a specific heterogeneity is marked in the highly homologous polypeptide chains (Fig. 5). Obviously there exists a multiple gene family for LHC II apoproteins in pea, as is true for petunia [5,6] and *Lemna* [8], at least two of the genes being expressed although in different amounts. It has been previously suggested that the different immunologically related LHC II apoproteins [1,2] are in fact a mixture of closely related polypeptides arising from the different members of a LHC II gene family [3,5]. Our data confirm this thesis. But it is still unclear if the genes are expressed differentially during development or varying environmental conditions.

Unfortunately, we could not sequence the N-terminal part of the apoproteins, as they have an acetylated N-terminus [24]. The reason for the large difference in the apparent molecular weight (1500) between the larger and the smaller form of LHC II therefore remains unknown.

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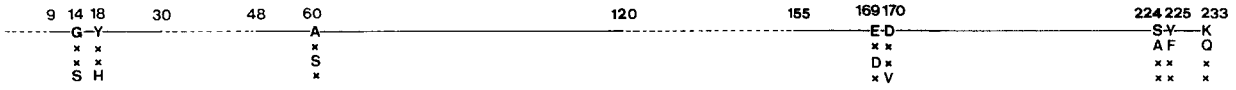

Fig. 5. Differences between four protein sequences of LHC II from pea. First line: sequence of the major protein component (polypeptide sequencing). Second line: sequence of the minor protein component (polypeptide sequencing). Third line: DNA derived sequence from Ref. 3. Fourth line: cDNA derived sequence from Ref. 4. The number of the amino acids refers to Fig. 4. A cross denotes identical amino acids as compared to the first line. Broken lines indicate LHC II segments which have not been sequenced by automated Edman degradation. The corresponding limits are indicated.

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