

Dicyclohexylcarbodiimide-binding proteins related to the short circuit of the proton-pumping activity of photosystem II Identified as light-harvesting chlorophyll-a/b-binding proteins

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In photosynthesis of higher plants, photosystem II drives electron transfer from the water-oxidizing manganese centre at the luminal side to bound plastoquinone at the stromal side of the thylakoid membrane. Proton release into the lumen and proton uptake from the stroma, i.e. net proton pumping, follows as consequence of vectorial electron transport. The proton pumping activity can be short circuited by covalent modification with *N,N'*-dicyclohexylcarbodiimide ($cHxN$)₂C of certain proteins in the 20–28-kDa range. After modification, protons from water oxidation are no longer released into the thylakoid lumen, but instead transferred through the photosystem complex to protonate the photoreduced bound quinone at the other side of the membrane [Jahns, P., Polle, A. & Junge, W. (1988) *EMBO J.* 7, 589–594]. Here we identify the pertinent ($cHxN$)₂C-binding proteins by amino acid sequence analysis and localize ($cHxN$)₂C-binding sites within their primary structure. The proteins that are associated with the proton short circuit are light-harvesting chlorophyll-a/b-binding proteins. Our results imply that in addition to acting as antennae they may serve another function: the funneling into the thylakoid lumen of protons, which are liberated in the water-oxidizing Mn centre.

In green plants, photosynthetic electron transport from water to NADP⁺ is mediated by three membrane-spanning enzyme complexes: photosystem II, cytochrome *b*₆*f* and photosystem I. Photosystem II oxidizes water at the luminal side and reduces bound plastoquinone at the stromal side of the membrane (Babcock, 1987). These redox reactions are accompanied by proton release into the lumen and proton uptake from the stroma (Junge, 1982). The oxidation of water occurs at a Mn centre, which is most probably located on the luminal side of the membrane, and within the large photosystem II complex, which is composed of at least 15 polypeptides (Murata and Miyao, 1987). The polypeptides involved in Mn binding and water oxidation have not yet been identified (Rutherford, 1989).

Protons which are produced during water oxidation are rapidly released into the thylakoid lumen. Within 100 µs to milliseconds after a short flash of light (Förster and Junge, 1985) they are detectable by absorption changes of neutral red (Junge et al., 1979). In dark-adapted thylakoids the four-period pattern of proton release in response to a series of short light flashes, 1:0:1:2 (Fowler, 1977; Saphon and Crofts, 1977; Förster and Junge, 1985), reflects the four consecutive transitions between the redox states of the Mn centre, S₀–S₁, S₁–S₂, S₂–S₃ and S₃–S₄ (Kok et al., 1970). Recently, this pattern of proton release has been debated. Lavergne and

Rappaport (1990) have reported that only a fraction of photosystem II centres follows this proton-release pattern in thylakoid membranes. In photosystem-II-enriched membrane fragments and in isolated photosystem II reaction centres (Wacker et al., 1990; Lübbbers and Junge, 1990) the pattern was less pronounced. It is conceivable that the nature of the electrostatic compensation by protons around the Mn centre is modulated by its protein environment.

Proton release from the Mn centre into the lumen can be blocked after covalent modification of thylakoids with ($cHxN$)₂C (Jahns et al., 1988; Jahns and Junge, 1989), without any concomitant effect on the oxygen-evolving capacity. The seemingly unreleased protons are instead transferred across the thylakoid membrane to the reduced bound quinone (Jahns and Junge, 1989). In other words, ($cHxN$)₂C induces an intraprotein short circuit of the proton-pumping activity in photosystem II. ($cHxN$)₂C binds to several membrane proteins of thylakoids. Aside from the proteolipid subunit of the H⁺-ATPase, [¹⁴C]($cHxN$)₂C labels at least five polypeptides in the 20–28-kDa range. Under conditions where incubation with ($cHxN$)₂C elicits the proton short circuit, selective labeling of two polypeptides is observed, one at 20 kDa the other at 24 kDa (Jahns et al., 1988). One might speculate that the ($cHxN$)₂C-binding proteins are involved in proton release from the manganese centre into the lumen, with the ($cHxN$)₂C-binding amino acids located near the manganese centre.

Photosynthetic reaction centres are surrounded by light-harvesting chlorophyll-a/b-binding proteins (LHC proteins), a large class of proteins with molecular masses of 20–30 kDa (for reviews, see Peter and Thornber, 1988; Green, 1988; Chitnis and Thornber, 1988). They serve as light-harvesting antennae. LHC proteins of both photosystems II and I are

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Abbreviations. CAB, chlorophyll a/b binding; ($cHxN$)₂C, *N,N'*-dicyclohexylcarbodiimide; LHC, light-harvesting chlorophyll a/b binding.

Note. The novel amino acid sequence data published here has been deposited with the EMBL sequence data bank.

structurally and immunologically closely related to each other (Pichersky et al. 1989; Evans and Anderson, 1986). Some of the LHC proteins of photosystem II are involved in the stacking of thylakoid membranes (Bennett et al., 1980; Mullet, 1983). In stacked thylakoids their association with photosystem II or I, and thereby the distribution of light quanta, is regulated by phosphorylation of the N-terminus (Mullet, 1983).

A multigene family coding for LHC proteins has been characterized in different species, for example in pea (Coruzzi et al., 1983; Cashmore, 1984; Polans et al., 1985), tomato (Pichersky et al., 1985, 1987a, b, 1989), petunia (Dunsmuir et al., 1983; Dunsmuir, 1985) and lemma (Karlin-Neumann et al., 1985; Kohorn et al., 1986), as reviewed, for example, in Chitnis and Thornber (1988). Based on the deduced amino acid sequences, Karlin-Neumann et al. (1985) have proposed a model for the folding of LHC II polypeptides in the thylakoid membrane. They suggested three membrane-spanning α -helical regions with the N-terminus at the stromal side and the C-terminus at the luminal side of the membrane. Five different types of LHC proteins (photosystem II type I-II and photosystem I type I-III) have been characterized in tomato. They are encoded by at least 14 different CAB genes (summarized in Pichersky et al., 1989; Pichersky and Green, 1990). Sequence alignments reveal two strongly conserved regions among all CAB-gene-encoded proteins (Pichersky et al., 1989; Pichersky and Green, 1990). According to the model of Karlin-Neumann et al. (1985), the first and third membrane-spanning regions fall into these conserved regions.

For thylakoids from pea chloroplast, we isolated (cHxN)₂C-binding proteins and their (cHxN)₂C-containing CNBr fragments. Partial sequences of these fragments were determined. Except for the proteolipid subunit of the H⁺-ATPase, all proteins labeled by [¹⁴C](cHxN)₂C were LHC proteins. The particular 24-kDa protein was found to be highly similar to the CAB8 gene product from tomato described by Pichersky et al. (1989), the particular 20-kDa protein (pea), although belonging to the same class, could not be yet identified as any particular gene product.

Following the proposed structure of LHC II proteins (Karlin-Neumann et al., 1985) and of CAB-gene-encoded proteins (Pichersky and Green, 1990) the supposed (cHxN)₂C-binding sites were located at the luminal side of the thylakoid membrane. A second function for some of these antennae proteins, proton release from the manganese centre into the lumen, is discussed.

MATERIALS AND METHODS

Preparation

Stacked thylakoids were prepared from pea seedlings as previously described (Polle and Junge, 1986). The plant material was homogenized in 5 mM MgCl₂, 10 mM NaCl, 0.4 M sorbitol and 100 mM tricine/NaOH, pH 7.8, and centrifuged. The pelleted chloroplasts were osmotically shocked in 5 mM MgCl₂, 10 mM NaCl and 10 mM tricine/NaOH, pH 7.8, and the thylakoid membranes were spun down again by centrifugation. This final pellet was resuspended in 5 mM MgCl₂, 10 mM NaCl, 0.4 M sucrose and 10 mM tricine/NaOH, pH 7.8. Unstacked thylakoids were prepared in the same way, with MgCl₂ omitted from all media. The resulting stock solutions containing 1.5–2 mg/ml chlorophyll were stored at –80°C.

(cHxN)₂C incubation

Stacked thylakoids were incubated with (cHxN)₂C (ethanol solution) in a medium containing 5 mM MgCl₂, 10 mM NaCl and 2 mM tricine/NaOH, pH 7.8, at chlorophyll concentrations of 5–10 μ M (final concentration of ethanol < 1%, by vol.). For destacked thylakoids, the same medium without MgCl₂ was used. Following incubation for 10 min at room temperature and under dim light, the suspension was centrifuged for 15 min at 16000 \times g. The resulting pellets were either resuspended directly in electrophoresis sample buffer or used for chloroform/methanol extraction according to Wessel and Flügge (1984). After chloroform/methanol treatment the extract, containing mainly chlorophyll, and the protein pellet were resuspended in sample buffer and used for electrophoresis.

Gel electrophoresis

Separation of thylakoid membrane proteins was performed by SDS/PAGE according to Laemmli (1970) with some modifications, as described by Engelbrecht et al. (1986), using 13.5% acrylamide and 4 M urea in the separation gel. Peptide fragments were separated by SDS/PAGE as described by Schägger and von Jagow (1987) with 16.5% acrylamide (3% cross linker) gels.

Autoradiography

[¹⁴C](cHxN)₂C (Amersham, Braunschweig) in toluene solution was dried under a stream of nitrogen and resolved in ethanol, yielding a 4–5-mM stock solution. Incubation of thylakoid membranes with [¹⁴C](cHxN)₂C was performed as described above. Autoradiographs were obtained by exposing a β_{max} X-ray film (Amersham) to the dried gel for 12–60 h at room temperature.

CNBr fragmentation and amino acid sequence analysis

Proteins were cleaved by CNBr treatment as follows. Protein bands were cut from the acrylamide gel and transferred into Eppendorf tubes. Freshly prepared 0.5–1 M CNBr (in 75% formic acid) was added and incubated under permanent vortexing overnight for about 12–16 h. The gel slices were discarded and the CNBr solution, containing the protein fragments, was dried under vacuum. The dried samples were resuspended with electrophoresis sample buffer and fragments separated by SDS/PAGE. Gels were either stained with Coomassie blue or the protein was electroblotted onto poly(vinylidene difluoride) membrane (Immobilon, Millipore), which was recovered for amino acid sequence analysis. Blotting was performed at a current of about 1 A for 3 h in sodium carbonate buffer at pH 9.9. Poly(vinylidene difluoride)-bound protein was stained with Ponceau S or Coomassie blue.

The amino acid sequence analysis was kindly provided by Prof. J. Hoppe and Dr V. Hoppe (Würzburg) using an Applied Biosystems 470A gas-phase sequencer combined with an online phenylthiohydantoin amino acid analyzer 120A (Applied Biosystems).

RESULTS

As shown previously, incubation of stacked thylakoid membranes with (cHxN)₂C short circuits the proton pumping activity of photosystem II (Jahns et al., 1988; Jahns and Junge,

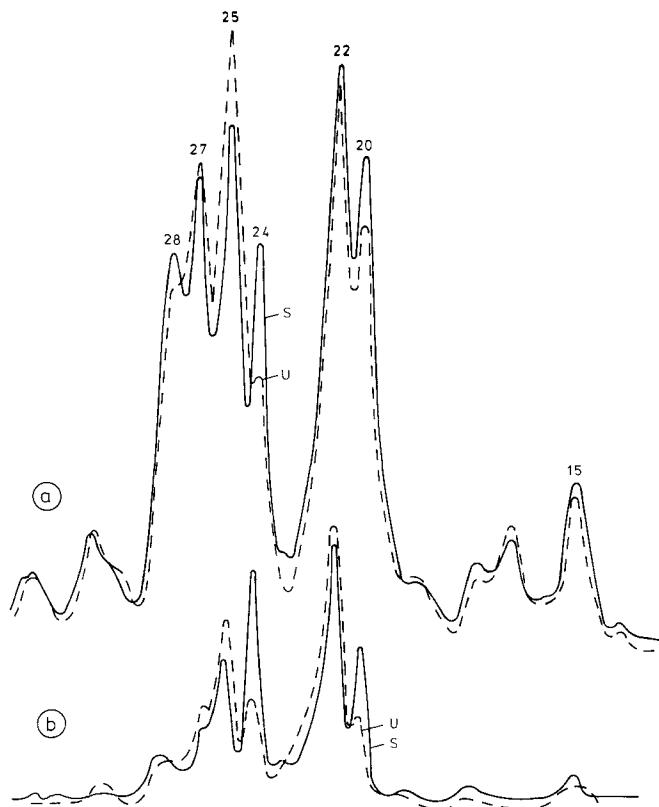


Fig. 1. Differential labeling with $(\text{cHxN})_2\text{C}$ of stacked and unstacked thylakoids: the influence of denaturation in the presence of unreacted $(\text{cHxN})_2\text{C}$. Densitograms of autoradiographs of $[^{14}\text{C}](\text{cHxN})_2\text{C}$ -labeled thylakoid membrane proteins separated by SDS/PAGE. Solid lines (S), stacked thylakoids; dotted lines (U), unstacked thylakoids. Molecular masses are indicated in kDa. After $[^{14}\text{C}](\text{cHxN})_2\text{C}$ incubation and subsequent centrifugation, samples were either treated with chloroform/methanol as described in (Wessel and Flügge, 1984) prior to addition of electrophoresis sample buffer (b), or sample buffer was added without further treatment (a)

1989). On the other hand, incubation of unstacked thylakoids with $(\text{cHxN})_2\text{C}$ shows no influence on the protonic reactions in photosystem II. This behaviour is independent of the stacking conditions during flash spectrophotometric measurements of the proton-pumping activity. $[^{14}\text{C}](\text{cHxN})_2\text{C}$ binds to several proteins in the 20–30-kDa range. Enhanced binding of $[^{14}\text{C}](\text{cHxN})_2\text{C}$ during incubation of stacked thylakoids is only observed for two particular proteins with apparent molecular mass of 20 kDa and 24 kDa (Jahns et al., 1988).

We investigated $(\text{cHxN})_2\text{C}$ binding to proteins of stacked and unstacked thylakoids under various conditions. The labeling pattern, and thus the preferential labeling of the 20-kDa and 24-kDa protein in stacked thylakoids was not always reproducible. This was attributed to some further $(\text{cHxN})_2\text{C}$ binding after addition of the denaturing electrophoresis sample buffer (including SDS, urea and 2-mercaptoethanol) to thylakoid membranes, which contained unreacted $[^{14}\text{C}](\text{cHxN})_2\text{C}$. Different post-incubation times prior to electrophoresis runs had caused the variation of results. The unwanted labeling was prevented if unreacted $(\text{cHxN})_2\text{C}$ was removed by chloroform/methanol treatment (according to Wessel and Flügge, 1984) prior to denaturation. Fig. 1 shows the differences in $(\text{cHxN})_2\text{C}$ binding with and without chloroform/methanol treatment. If unwanted $(\text{cHxN})_2\text{C}$

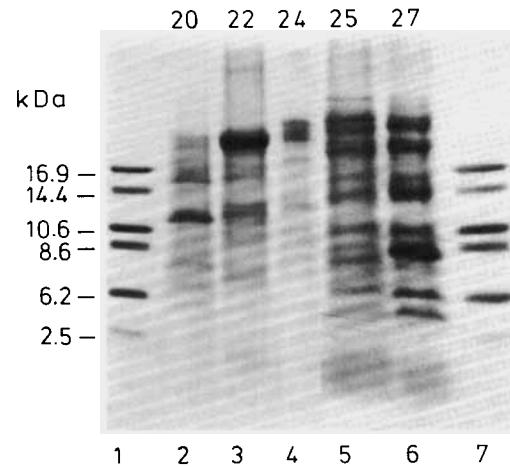


Fig. 2. CNBr -fragmentation patterns of $(\text{cHxN})_2\text{C}$ -binding proteins. Coomassie-blue-stained gels were prepared as described by Schägger and von Jagow (1987). Total acrylamide concentration 16.5% with 3% crosslinker, without urea. Lanes 1 and 7, molecular mass standards, molecular masses as indicated; lane 2–6, fragments of CNBr -cleaved proteins, molecular mass of the respective original protein is indicated at the top

binding to proteins was reduced (see Fig. 1 b), the selective binding of $(\text{cHxN})_2\text{C}$ to the 20-kDa and 24-kDa proteins in the stacked configuration evolved more clearly. Furthermore, labeling of other proteins than those in the 20–30-kDa range was nearly reduced to the background level.

From these and our earlier results (Jahns et al., 1988) it is conceivable that covalent binding of $(\text{cHxN})_2\text{C}$ to the 20-kDa and/or 24-kDa proteins may have caused the proton short circuit in photosystem II. However, one had still to consider that binding of $(\text{cHxN})_2\text{C}$ to other proteins (22 kDa, 25 kDa and 27 kDa) could also be involved, perhaps if those were labeled at different sites depending on the stacked and unstacked configuration of the membranes. Therefore we investigated all $(\text{cHxN})_2\text{C}$ -binding proteins in the 20–30-kDa range.

Partial sequences of the $(\text{cHxN})_2\text{C}$ -binding proteins at 20 kDa, 22 kDa, 24 kDa, 25 kDa and 27 kDa were determined by the following procedure. Protein bands were excised from acrylamide gels and fragmented in the gel with CNBr . The fragments were separated by a further SDS/PAGE and blotted on poly(vinylidene difluoride) membrane. The smallest fragments, which contained the $(\text{cHxN})_2\text{C}$ -binding site [obvious from parallel experiments with $[^{14}\text{C}](\text{cHxN})_2\text{C}$ -labeled proteins] were then used for sequence analysis. It was attempted to identify the $(\text{cHxN})_2\text{C}$ -binding proteins and also to locate the $(\text{cHxN})_2\text{C}$ -binding amino acids within the primary structure.

Fig. 2 shows the fragmentation pattern of the $(\text{cHxN})_2\text{C}$ -binding proteins produced by CNBr treatment. The proteins were cleaved to various degrees depending on the accessibility to CNBr of the denatured proteins in the gel slices. The degree of CNBr cleavage and thus the fragmentation pattern was highly reproducible for all proteins. The same fragmentation pattern was obtained independent of its origin from untreated or $(\text{cHxN})_2\text{C}$ -labeled proteins and independent of extraction from stacked and unstacked thylakoids (not shown).

The amino acid sequence was analyzed for the smallest fragments which were labeled with $[^{14}\text{C}](\text{cHxN})_2\text{C}$ in control experiments. This ensured that we obtained a sequence from

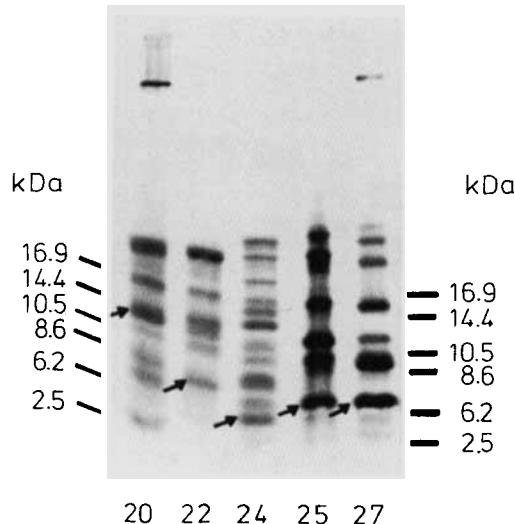


Fig. 3. $(cHxN)_2C$ -labeling patterns of CNBr-fragmented proteins. Autoradiographs from gels prepared as described in Fig. 2. Molecular masses as indicated on both sides were derived from Coomassie-blue-stained gels using the same standards as those shown in Fig. 2. Molecular mass of the respective original protein is as indicated at the bottom. The arrows in the figure indicate the respective fragments which were subsequently used for amino acid analysis.

the $(cHxN)_2C$ -binding protein and not from an unlabeled comigrating protein. Fig. 3 shows autoradiographs of $[^{14}C](cHxN)_2C$ -labeled and CNBr-fragmented proteins. The fragments marked with an arrow were used for sequence analysis. From fragmentation of the 22-kDa, 25-kDa, and 27-kDa protein, we obtained $[^{14}C](cHxN)_2C$ -labeled fragments of about 6 kDa molecular mass as smallest, from the 20-kDa and 24-kDa proteins of about 3–4 kDa molecular mass. In case of the 20-kDa protein, the 10-kDa fragment had to be analyzed instead of the smaller ones, because of the low yield and its inefficient binding to poly(vinylidene difluoride) membrane. The sequences of the chosen fragments are shown in Fig. 4 (upper sequence). Sequence analysis revealed a single unambiguous sequence for each fragment. This proved the purity of the $(cHxN)_2C$ -binding fragments. We found similarity between the sequences determined for proteins from pea, and published sequences derived from CAB genes from tomato (see lower sequence in Fig. 4), which code for LHC proteins. The nomenclature of CAB genes is given in the right of Fig. 4. Pichersky et al. (1989) described five different CAB-gene types in tomato, two coding for LHC proteins supposedly associated with photosystem II [photosystem II type I (Pichersky et al., 1985) and type II (Pichersky et al., 1987a)] and three coding for LHC protein supposedly associated with photosystem I [photosystem I type I (Pichersky et al., 1987b), type II (Pichersky et al., 1988) and type III (Pichersky et al., 1989)]. For the $(cHxN)_2C$ -labeled fragments of the 25-kDa and 27-kDa proteins (pea) we determined identical sequences which were highly similar (more than 85%) to photosystem II type LHC proteins (tomato). We could not attribute the amino acid sequence to any particular gene out of ten possible genes in tomato (Pichersky et al., 1989), because of the high degree of similarity in the shown region between all of them. The respective sequence shown in Fig. 4 was derived from the CAB3C gene (Pichersky et al., 1985). Nearly the same degree of similarity existed with LHC proteins from pea chloroplasts which are encoded by the pAB96 cDNA clone (Coruzzi et al., 1983) and the AB80 gene (Cashmore, 1984). This sequence is presented below the CAB3C sequence in Fig. 4.

L X P E V F T S I G I I N V P F I . . L L . K . . . L . T .	20 kDa (pea) CAB-7 (tomato)
L A V P G I L V P E A L G L G N X V I H .	22 kDa (pea) CAB-6 (tomato)
L A I L G Y F I Q G L V T G V G P Y Q N L L D A	24 kDa (pea) CAB-8 (tomato)
L G A L G X X X P E L L X X N G C V F A R C V F S R . .	25 kDa (pea) CAB-3 (tomato) pAB96/AB80 (pea)
L G A L G C V F P E L L S G N G V K F G Y A A R E R E .	27 kDa (pea) CAB-3 (tomato) pAB96/AB80 (pea)

Fig. 4. Partial amino acid sequences of CNBr fragments of $(cHxN)_2$ C-binding proteins from pea (upper sequence). The respective second lines give sequences derived from CAB genes from tomato as described in Pichersky et al. (1989). Identical amino acid residues are indicated by dots. For the fragment from the 25-kDa and 27-kDa protein the third line shows a sequence derived from the cDNA clone pAB96 (Coruzzi et al., 1983) and the AB80 gene (Cashmore, 1984) of pea

The fragment obtained from cleavage of the 22-kDa protein (pea) was highly similar (16 out of 18 residues) to the photosystem I type I LHC proteins (tomato) encoded by the CAB6A or AB6B genes (Pichersky et al., 1987b; Hoffman et al., 1987).

The fragment of the 24-kDa protein (pea) was highly similar (22 out of 23 residues) to the protein encoded by the CAB8 gene (Pichersky et al., 1989) from tomato and corresponded to the photosystem I type III LHC proteins. Analysis of another fragment of the 24-kDa protein obtained by enzymatic digest with V8 protease revealed a partial sequence of eight amino acids, that was identical to a stretch in the CAB8 gene product (not shown).

The sequence of the fragment from the 20-kDa protein (pea) showed about 60% similarity to a protein from tomato encoded by the CAB7 gene (photosystem I type II; Pichersky et al., 1988). Due to of the comparatively lower degree of similarity it was conceivable that the 20-kDa protein might be a product of a still-uncharacterized CAB gene. For the 20-kDa protein we determined the N-terminal sequence (10 amino acids, not shown). Although we found no similarity to any known sequences, the clear cut sequence proved the purity of the 20-kDa protein band. Polyclonal antibodies, raised against the 20-kDa protein cross-reacted with the other LHC proteins (Jahns and Steinemann, unpublished results), indicating that the 20-kDa protein itself is most probably an LHC protein. On the other hand, polyclonal antibodies against a hydrophobic 20-kDa protein described by Liveanu et al. (1986) and against the 20-kDa apoprotein of CP24 (Spangfort et al., 1990) showed no cross-reactivity with our 20-kDa protein (not shown).

DISCUSSION

Based on standard prediction methods Karlin-Neumann et al. (1985) have proposed a tertiary structure of LHC II

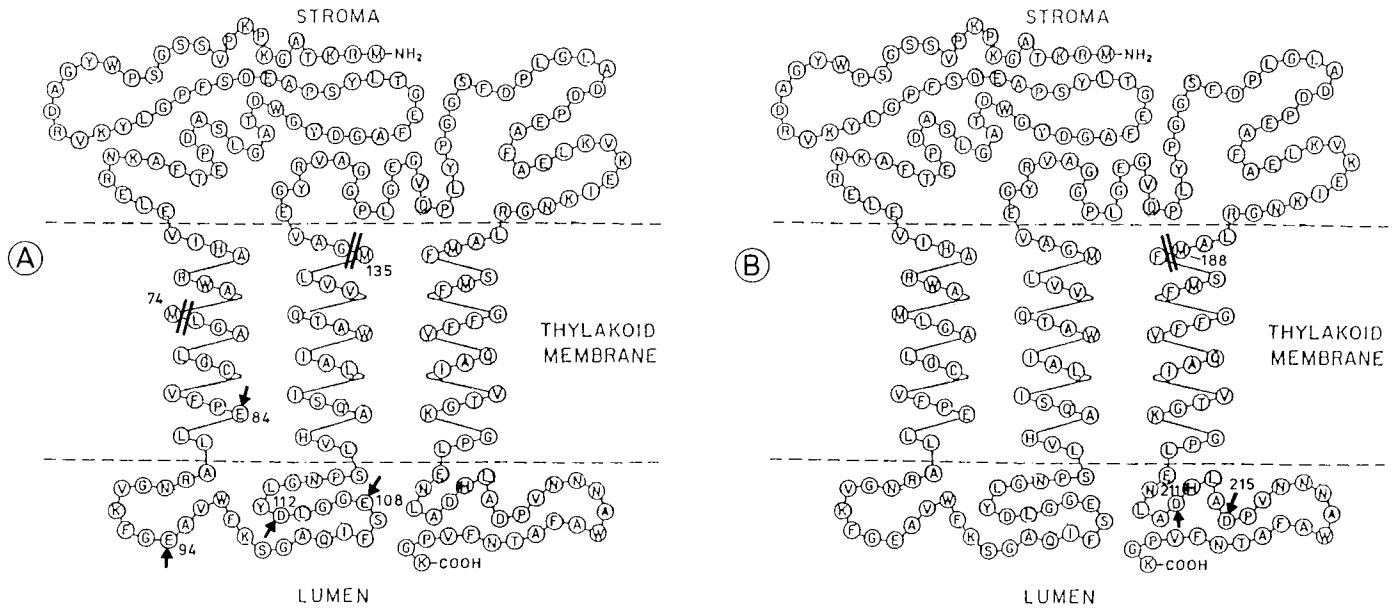


Fig. 5. *Folding model for LHC II proteins proposed by Karlin-Neumann et al. (1985), adapted from Chitnis and Thornber (1988) with slight modifications.* The numbering starts with the N-terminal methionine of the mature protein. (A) Indicated are CNBr cleavage sites (Met74 and Met135) and possible $(\text{cHxN})_2\text{C}$ -binding sites (arrows) derived from sequence analysis of the 6-kDa CNBr fragments of the 22-kDa, 25-kDa and 27-kDa proteins (see also Fig. 3). (B) Cleavage site (Met188) and possible $(\text{cHxN})_2\text{C}$ -binding sites (arrows) derived from sequence analysis of the 4-kDa fragment obtained by CNBr fragmentation of the 24-kDa protein are indicated

proteins in the thylakoid membrane, which is shown in Fig. 5. A very similar model was proposed by Pichersky and Green (1990) for all CAB-gene-encoded proteins in tomato. For the sake of simplicity we discussed our data in terms of the model of Karlin-Neumann et al. (1985). This seems fair because the relevant domains (mainly the first and third transmembrane helix) of all LHC proteins (photosystems I types II) are highly conserved (Pichersky et al., 1989; Pichersky and Green, 1990). The numbering of the amino acid residues used in the following refers to the model shown in Fig. 5. Due to possible additions and omissions in the primary structure of any particular protein, it only serves illustrative purposes. LHC-I-type proteins show a total similarity of about 30% compared with LHC II proteins. This similarity is mainly attributable to the above-mentioned highly conserved regions. The three membrane-spanning domains are probably common to all members of this class of protein.

The 25-kDa and 27-kDa proteins revealed similar cleavage patterns on CNBr treatment (see Fig. 2). Covalently bound [^{14}C](cHxN)₂C was found in corresponding fragments (see Fig. 3). Sequence analysis of the smallest labeled fragments (molecular mass about 6 kDa) revealed very high similarity between the respective cleavage products. They corresponded to the stretch between Met74 and Met135 in the model of Fig. 5.

Cleavage of the 22-kDa protein resulted in a different fragmentation pattern compared with the 25-kDa and 27-kDa proteins, but the radioactive label was found in fragments with similar molecular masses. The 22-kDa protein was similar to photosystem-I-associated LHC proteins (type I, encoded by CAB6 genes). However, the analyzed $(\text{cHxN})_2\text{C}$ -binding 6-kDa fragment corresponded again to the same segment between Met74 and Met135 as for the 25/27-kDa proteins. These data placed the $(\text{cHxN})_2\text{C}$ -binding sites in the 22-kDa, 25-kDa and 27-kDa proteins into the region from the beginning of the first transmembrane helix to the end of the second. Under our membrane incubation conditions $(\text{cHxN})_2\text{C}$ binds

mainly to carboxy groups in the hydrophobic environment (Azzi et al., 1984). Therefore, the four amino acids (marked with arrows in Fig. 5A) at the luminal site of the first transmembrane helix were likely candidates for $(\text{cHxN})_2\text{C}$ binding. Glu84 was the only one out of the four which was conserved in all three proteins. Its localization (according to Fig. 5) in hydrophobic environment further supported that it bound $(\text{cHxN})_2\text{C}$.

The analyzed fragment of the 24-kDa protein contained the last 41 amino acids at the carboxy-terminus of the CAB8 gene product. This fragment originated from CNBr cleavage of the 24-kDa protein at the carboxy side of a methionine residue, which corresponded to Met188 in Fig. 5B. The $(\text{cHxN})_2\text{C}$ -binding amino acids might be the two aspartic acids (Asp211 or Asp215 in the model used). Please note that Glu207 of the model is replaced by Gln in the CAB8 gene product. This would again place the $(\text{cHxN})_2\text{C}$ -binding sites at the lumen side of the thylakoid membrane.

For the 20-kDa-protein we could not predict with confidence the binding site of $(\text{cHxN})_2\text{C}$. The fragment was too large (about 100 amino acids) and the similarity with known sequences from tomato was considerably lower as for the other proteins.

Following alternative models with two membrane-spanning helices (Spangfort et al., 1990) or four membrane-spanning helices (Anderson and Goodchild, 1987), the proposed $(\text{cHxN})_2\text{C}$ -binding site in the 22-kDa, 25-kDa and 27-kDa proteins would be unchanged, while the binding site in the 24-kDa protein would be placed at the stromal side of the membrane.

Conclusions

$(\text{cHxN})_2\text{C}$ binding to LHC proteins is probably responsible for the proton short circuit in photosystem II. Thus, some of these proteins, in addition to serving as light-harvesting antennae, may have an additional function. They may channel

protons which are produced in the catalytic Mn centre of water oxidation into the thylakoid lumen. While this secondary function is essential for transmembrane proton pumping by photosystem II (finally to drive ATP synthesis), it does not seem important for O₂ evolution. First, the proton short circuit induced by (cHxN)₂C does not impair the rate of oxygen evolution under continuous light (Jahns et al., 1988). Furthermore, isolated reaction centre preparations evolve oxygen at high rates without containing LHC proteins (Ghanotakis et al., 1987).

As the 20-kDa and/or 24-kDa proteins were both selectively labeled under stacked conditions, we have suggested that they are involved in the short circuit. In apparent contradiction to this proposal, both proteins are supposedly associated with photosystem I (Pichersky et al., 1989). This may imply that (a) the two proteins are not involved in the proton short circuit, but that some of the other [¹⁴C](cHxN)₂C-labeled LHC proteins are, or (b) they are associated not only with photosystem I, but also with photosystem II. Pichersky et al. (1989) found that, after membrane solubilization, a fraction of the CAB8 gene product (< 10%) comigrates in SDS gels with a LHC-II-containing protein band. We obtained similar results in preliminary experiments with antibodies raised against the 20-kDa and 24-kDa protein and in labeling studies with [¹⁴C](cHxN)₂C. Both, the 20-kDa and the 24-kDa protein, however, were also present in photosystem II membrane preparations (Jahns and Steinemann, unpublished results). Quantitative studies are in progress.

In conclusion, the function of the LHC proteins is to supply light energy to the reaction center, but, at least in photosystem II, they may be involved also in proton release from the manganese centre into the thylakoid lumen.

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