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Polypeptide sequence of the chlorophyll *a/b/c*-binding protein of the prasinophycean alga *Mantoniella squamata*

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Abstract

The primary structure of the Chl*a/b/c*-binding protein from *Mantoniella squamata* is determined. This is the first report that protein sequencing reveals one modified amino acid resulting in a LHCP-specific TFA-cleavage site. The comparison of the sequence of *Mantoniella* with other Chl*a/b*- and Chl*a/c*-binding proteins shows that the modified amino acid is located in a region which is highly conserved in all these proteins. The alignment also reveals that the LHCP of *Mantoniella* is related to the Chl*a/b*-binding proteins. Finally, possible Chl-binding regions are discussed.

Abbreviations: a.m.u. – atomic mass unit; LHC - light-harvesting complex; LHC II – major LHC of Photosystem II; LHCP – light-harvesting chlorophyll-binding protein; LSIMS – liquid secondary ion mass spectrometry; TFA – trifluoroacetic acid

Introduction

The majority of the photosynthetic pigments in the thylakoid membrane of higher plants and algae are located in the light-harvesting complexes of the photosystems (Thornber et al. 1990). It is generally accepted that the chromophores are non-covalently bound to the protein, but detailed knowledge about the pigment–protein-interaction is still lacking. Models based on sequence analysis and topological studies of the LHC II polypeptide of higher plants (Karlin-Neumann et al. 1985; Bürgi et al. 1987) have suggested the existence of three transmembrane helices. These models are supported by the three-dimensional structure of the LHC II at 6 Å resolution (Kühlbrandt and Wang 1991). According to Kühlbrandt and Wang the chlorophylls are arranged in two levels near the upper and lower surfaces of the membrane and orientated almost perpendicularly to the membrane plane. The known residues for chlorophyll binding in bacterial complexes are histidine, glutamine and asparagine (Zuber 1985; Michel et al. 1986; Chitnis and Thornber 1988), but they do not occur in sufficient numbers in the transmembrane segments of the LHCP of higher plants. This might suggest that the chlorophylls are liganded by other amino acid residues as well or they are orientated by water molecules and hydrogen bonds (Kühlbrandt and Wang 1991). The carotenoids are also essential components of the LHC (Plumley and Schmidt 1987; Herrin et al. 1992), but nothing is known about their interaction with the polypeptide.

Unlike the LHC of higher plants, the LHC of algae exhibit a great variation in pigment composition and in the number of bound pigments (Wilhelm 1990; Hiller et al. 1990). Comparative analyses of the primary structure of the LHCP with the associated pigments will reveal further insight into the pigment–protein-interaction. In this context the prasinophycean alga *Mantoniella squamata* is of special interest, because it is regarded as a primitive representative of the chloro-

phyta (Van den Hoek et al. 1988). Its isolated LHC (Wilhelm et al. 1986; Fawley et al. 1986) shows an unusual pigment composition, apart from Chl *a* and Chl *b* it additionally binds Chl *c* (Mg 2,4-divinyl pheoporphyrin a_5 monomethyl ester) (Jeffrey 1989). The LHC further contains prasinoxanthin as its major carotenoid, which is also involved in energy transfer to Chl. Moreover, *Mantoniella* is able to change the pigment composition of the LHC in response to luminous intensity during growth (Wilhelm et al. 1990). Recent reconstitution experiments with the LHC have provided evidence for a rather high flexibility of pigment recognition and binding (Meyer and Wilhelm 1993). In that study it has been shown that the reconstituted LHC is able to assemble pigments which are not an integral part of the LHC in vivo. Furthermore, no immunological relationship can be detected between the LHC of *Mantoniella* and brown algae (Passaquet et al. 1991), green algae and higher plants, except for closely related prasinophycean algae (Fawley et al. 1986).

Summing up, the LHC of *Mantoniella squamata* differs from known LHC in various aspects. Up to now research has focused more on the role of pigments than on the protein structure. It is the aim of this study to examine the polypeptide as a possible cause of those unusual features of the LHC.

Materials and methods

Mantoniella squamata (Strain no. LB 1965/5, Culture collection, Marine Lab., Plymouth, UK) was grown according to Wilhelm et al. (1986). The isolation and purification of the LHC was carried out as described by Herold et al. (1991). The pigments were removed by extraction with 80% (v/v) acetone at −20 °C and the LHCP was collected by centrifugation at 4 °C. The cysteine residues of the LHCP were reduced by DTT and alkylated by iodoacetamide.

The LHCP was digested with endoproteinase Lys-C (EC 3.4.21.50, Wako), endoproteinase Glu-C (EC 3.4.21.19, Boehringer Mannheim) or trypsin (EC 3.4.21.4, Sigma) in 0.1 M NH_4HCO_3, pH 8.1 for 6–20 h at 20 °C or, for Glu-C, at 37 °C. The protein-enzyme ratio was 200:1 for Lys-C, 20:1 for Glu-C and 50:1-20:1 for trypsin. In addition, the LHCP was cleaved between Asp and Pro with 75% (v/v) formic acid at 37 °C. A LHCP-specific cleavage occurred in anhydrous TFA at 37 °C for 15 min. The cleavages were terminated by freezing and lyophilizing.

Peptides were separated either by HPLC or by PAGE. The reverse-phase HPLC was performed on a Nucleosil C_4 column (1000 Å, 4.6 × 50 mm, Macherey–Nagel) using a gradient between water with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B). The gradient ran from 0% B to 60% B within 60 min, reached 95% B after 72 min and returned to initial equilibration conditions after 5 min. The flow rate was 0.5 cm^3/min. The column effluent was monitored at 220 nm and 280 nm. The collected fractions were lyophilised. SDS–PAGE was performed either according to Laemmli (1970) with double-concentrated electrode buffer or using the separating gel and the electrode buffer as described by Schägger and von Jagow (1987). Gels were run in the BioRad Minigel apparatus at room temperature.

For amino acid analysis the samples were hydrolyzed in the vapors of 6 M HCl at 110 °C for 24 h. The hydrolysates were analysed on a Biotronic LC 3000 amino-acid analyser with post column ninhydrin reaction.

The peptides separated by SDS–PAGE were transferred to polyvinylidine fluoride membrane (Immobilon, Millipore) by electroblotting, stained with amido black, cut out and sequenced. Lyophilised peptides were transferred to the membrane in 25 mm^3 neat TFA. Sequence analysis was performed on a Knauer Protein Sequencer 810. The phenylthiohydantoin amino acids were identified according to Frank (1989).

Mass spectra were accumulated using a triple quadrupole mass spectrometer (model 710, Finnigan-MAT, San Jose, CA, USA) equipped with either an electrospray ionisation source (Analytica of Branford, Branford, CO, USA) or a LSIMS probe. Spectra were accumulated in profile mode scanning from 400–2000 a.m.u in 4 s. Collision-induced dissociation experiments were carried out scanning in centroid mode using a collision energy of 20 e V using 2.5 mTorr of Argon.

Sequence alignments were performed using the sequence analysis software package (7.1) of the Genetic Computer Group (University of Wisconsin).

Results

Figure 1 shows the polypeptide pattern of the isolated and purified LHCP from *Mantoniella squamata*. Two polypeptides could be distinguished, one polypeptide with an apparent molecular weight of 23.5 kDa and a second one of 25 kDa. Even though the two polypeptides had not been separated for the cleavage proce-

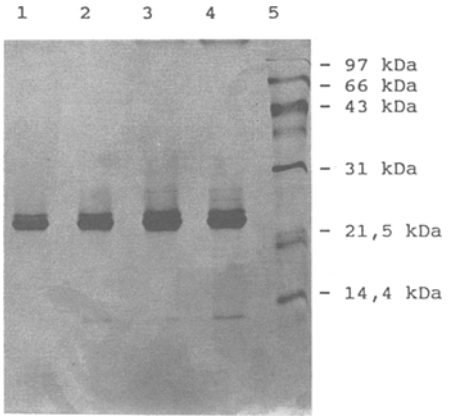


Fig. 1. Polypeptide composition of the LHCP from *Mantoniella squamata* separated by Laemmli SDS–PAGE. The gel was silver-stained using a modified procedure by Heukeshoven and Dernick (1988). Lane 1, purified LHCP; lanes 2,3,4, isolated LHCP; lane 5, molecular mass markers.

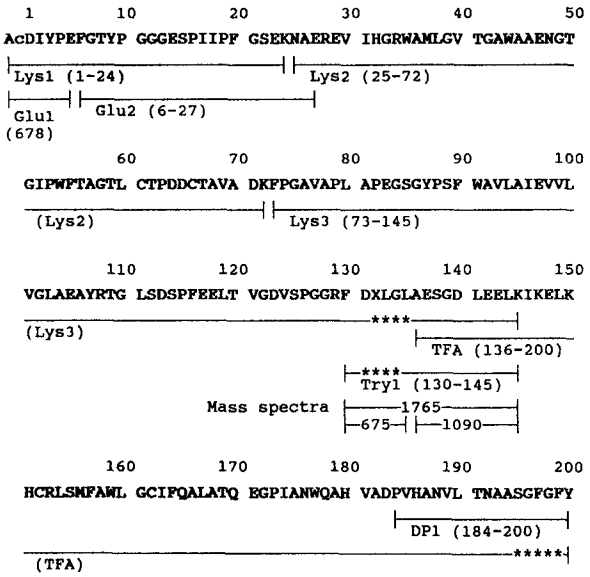


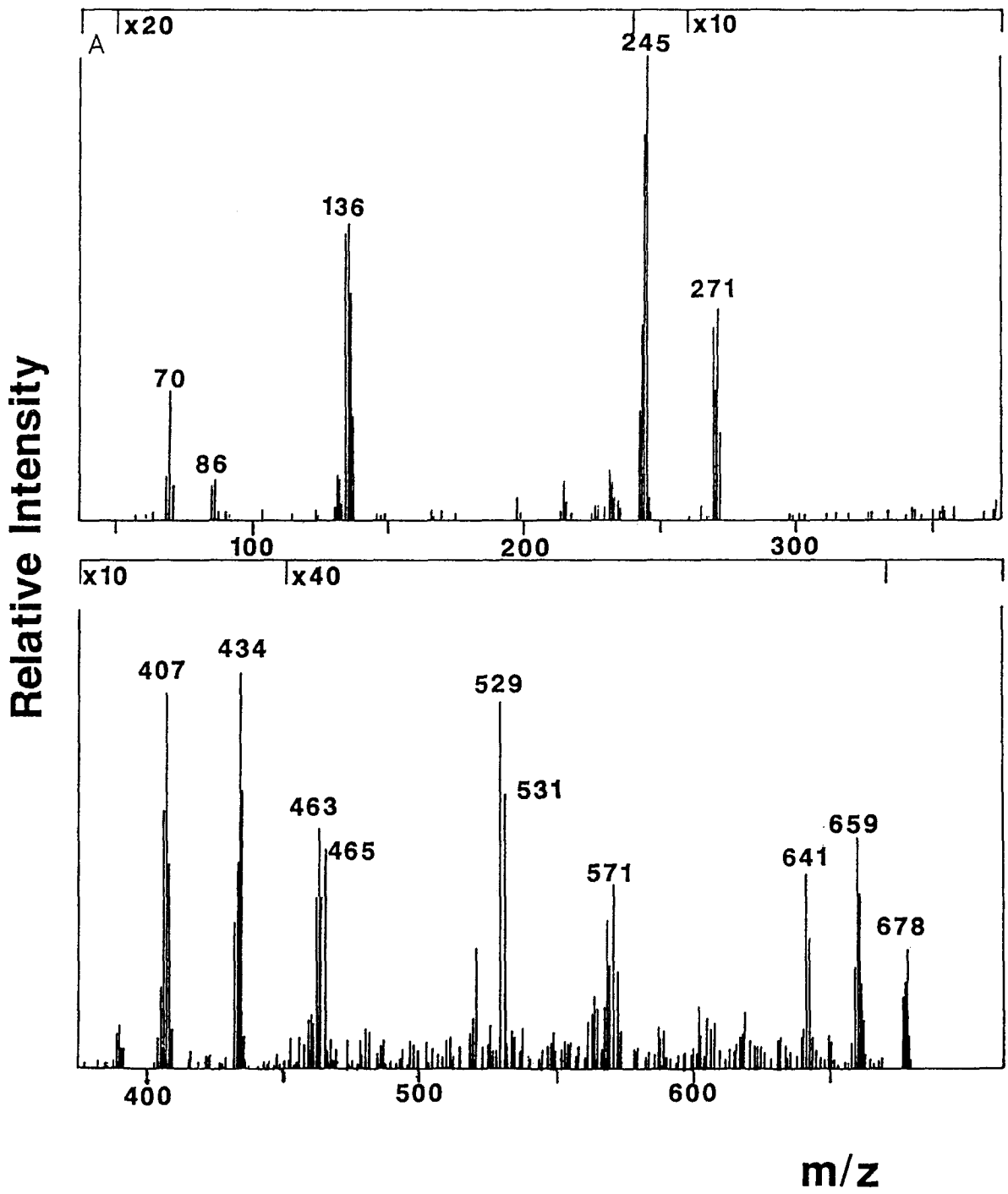
Fig. 2. Amino acid sequence of LHCP from *Mantoniella squamata* obtained by a combination of Edman degradation and mass spectrometry. The fragments are named according to the cleavage method: Lys – endopeptidase Lys-C, Glu – endopeptidase Glu-C, Try – trypsin, TFA – TFA-cleavage, DPc – DP-cleavage, * – not detected. For mass spectra the mass of the $(M+H)^+$ ions of the peptides is given.

dures no sequence heterogeneity could be detected. The reason for the divergent molecular weights of the polypeptides is still unknown.

The amino acid sequence of the LHCP from *Mantoniella squamata* and the sequencing strategy is shown in Fig. 2. The LHCP consists of 200 amino acids and has a molecular weight of 21260 Da. The N-terminal aspartic acid is acetylated, which was shown by collision-activated dissociation mass spectra of the Lysl-fragment (1–24) and of the Glul-peptide (1–5, Fig. 3A). The overlapping sequence between the Lys1- and the Lys2-fragment (25–72) was found in the peptide Glu2 (6–27). The order of Lys2 and Lys3 was derived from an incomplete Lys-C digest, which produced the required Lys2+3-fragment (25–145). In order to examine the carboxy-terminal region of the LHCP the TFA-fragment (136–200) was sequenced which resulted in the detection of a DP-cleavage site. The DPc-fragment (184–200) was sequenced by Edman degradation and analyzed for its amino acid composition, which led to consistent results.

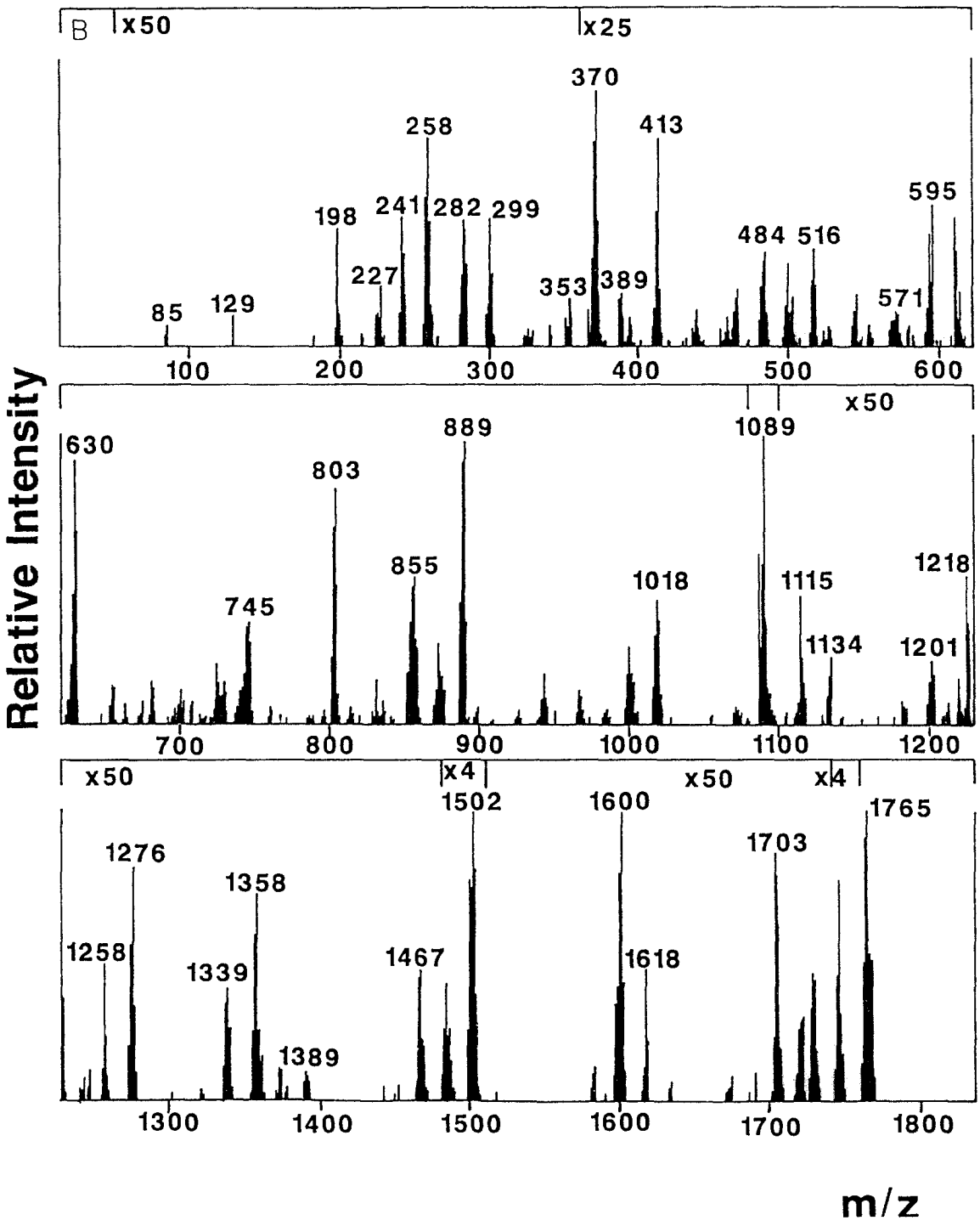
Due to the acetylated N-terminus no sequence information was expected from the Edman degradation of the uncleaved LHCP, but surprisingly this resulted in a sequence starting with alanine (136). This indicated that a cleavage must have happened during the first Edman cycle. Treatment of the LHCP with anhydrous TFA at 37 °C for 15 min also led to this specific TFA-break in the LHCP, which was confirmed by SDS–PAGE. When sequencing the Lys3-fragment (73–145) two amino acids were detected in each of the first cycles; one sequence could be assigned to the beginning of the TFA-peptide (136–145). The subdigestion of the Lys3-fragment (73–145) with trypsin gave three peptides, of which Tryl (130–145) was the one with the TFA-cleavage site. The sequence of the beginning of Tryl (130–135) could only be clearly determined for the first two amino acid residues, after that no further degraded amino acid residue was detectable, even though the second sequence (136–145) starting at the TFA-cleavage site was visible.

In order to prove the existence of a single peptide, mass spectrometry was used (Fig. 3B and C). The mass spectra of the Tryl-fragment showed $(M+H)^+$ ion at m/z 1766.1. After TFA-treatment two smaller peptides were observed, the $(M+H)^+$ ions of which appeared at m/z 1091.4 and m/z 675.5. The molecular weights of the TFA-products added up to exactly the molecular weight of the Tryl-fragment, which indicated that no water molecule was added as is the case in an usual cleavage of a peptide bond. The amino acid analysis of the Tryl-peptide (D/N 2.1; S 0.9; E/Q 3.2; G 2.2; A 1.2; L 2.8; F 1.0; K 1.0 and one unidentified peak) indicated that two amino acid residues were missing and in addition, one peak appeared which might have come from an unknown residue. The collision-activated dissociation mass spectra recorded on the $(M+H)^+$ ions of Tryl-peptide (Fig. 3B) and of 675-peptide (Fig. 3C)



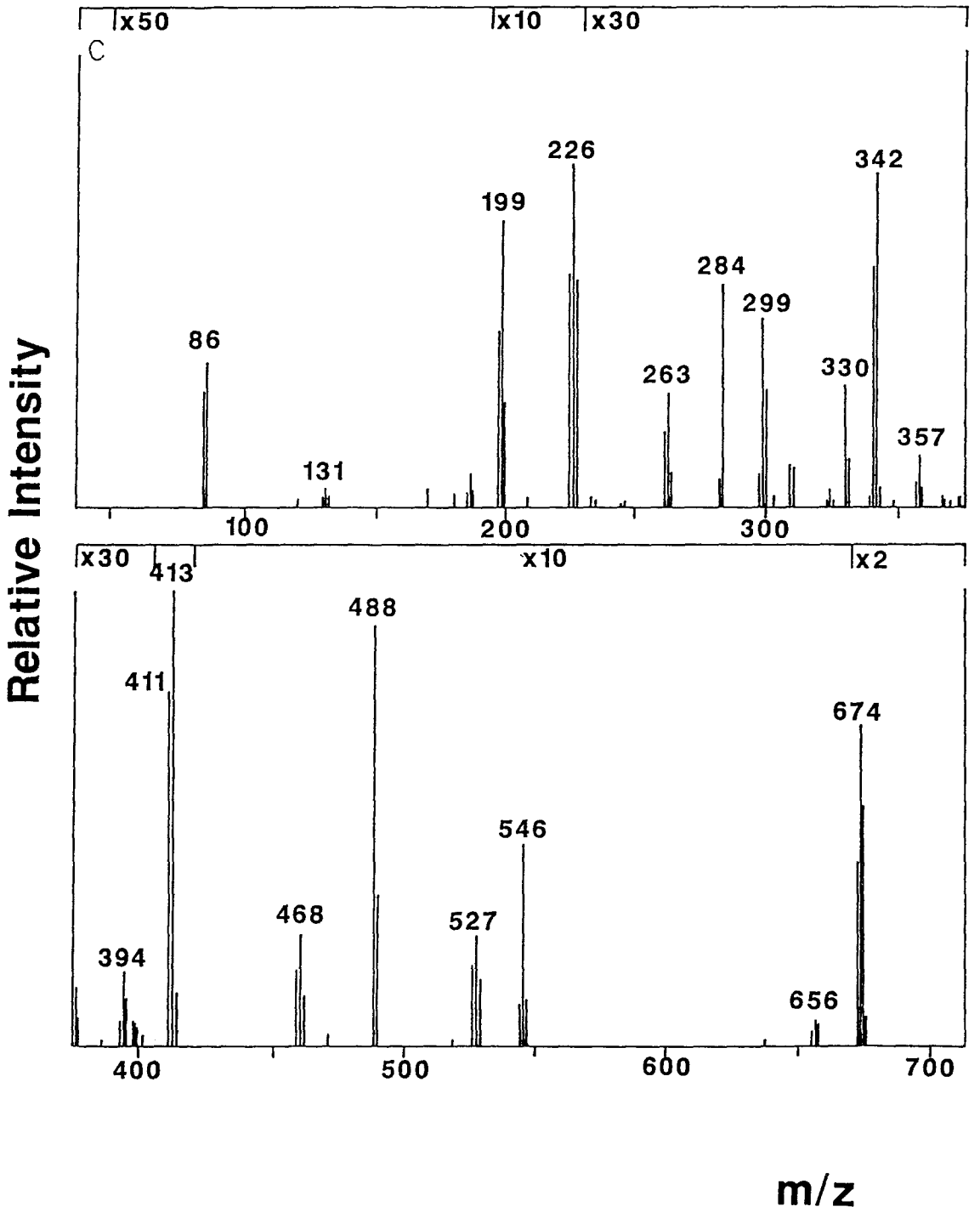
b_n		158	271	434	531	678
	Ac	D	I	Y	P	E
y_n		678	521	408	245	148

Fig. 3. Collision-induced dissociation mass spectra of $(M+H)^+$ ions of Glu1-peptide (A), Try1-peptide (B) and the 675-peptide (C, 130–136). Fragment ions of type b and y are shown above and below the sequence.



b_n	148	263	392	505	562	675	746	875	962	1019	1134	1247	1376	1505	1618	1746
	F	D	X	L	G	L	A	E	S	G	D	L	E	E	L	K
y_n	1764	1617	1502	1374	1261	1204	1090	1019	890	803	746	631	518	389	260	147

Fig. 3. Continued.



b_n	129	244	373	486	543	656
	F	D	X	L	G	L
y_n	674	527	412	283	170	113

Fig. 3. Continued.

as well as of their methylated and acetylated derivatives (data not shown) provided the sequence FDXL-GLAESGDLEELK. The amino acid X has a residue weight of 129, which might be in accordance with glutamic acid, but there were some inconsistencies. First, the result of the Edman sequencing where no residues were detectable after FD (130, 131) which would be a very unusual behaviour for a glutamic acid residue at this position. Second, glutamic acid should be excluded because the spectra of the methylated Tryl-peptide indicated only one carboxyl group which resulted from aspartic acid (131). Possibly, the amino acid X is the proline derivative 3,4-dihydroxyproline, which corresponds with the molecular weight and the fragmentation im MS/MS spectra.

Discussion

Recently five *1hc*-genes of the LHCP of *Mantoniella squamata* were deposited in the EMBL data base (accession number: X69524, X69525, X73539 and X73540 from Rhiel and Mörschel (1993) and Z22782 from Jiao and Fawley). Two genes, X73539 and X73540, are completely identical to the protein isolated from the photosynthetic apparatus of the alga. The other genes are also very similar, they contain between one and three amino acid exchanges. According to the gene and protein sequences, the transit peptide is cleaved between alanine and aspartic acid and the N-terminal aspartic acid becomes acetylated. Up to now, only one N-terminal acetylation from a higher plant has been identified (Michel et al. 1991). Looking at the modified amino acid X, the two corresponding genes have proline at its position. These findings confirm the hypothesis that X is a posttranslationally modified proline, presumably 3,4-dihydroxyproline.

The N-terminal region of the LHCP of *Mantoniella squamata* is definitely shorter than the N-terminus of other Chl*b*-binding proteins (Fig. 4). In higher plants and green algae the N-terminal segment of LHCP is necessary for grana formation (Mullet 1983). The reduced length of this part is in good agreement with the observation that in *Mantoniella* true partition regions do not occur (Krämer et al. 1988).

The LHCP sequence of *Mantoniella squamata* is aligned with the deduced amino acid sequences of *1hc* genes of a higher plant (Cashmore 1984), Chl*b*-containing algae (Imbault et al. 1988; Long et al. 1989; La Roche et al. 1990; Larouche et al. 1991) and Chl*a*/*c* algae (Grossman et al. 1990; Hiller et al. 1993) in

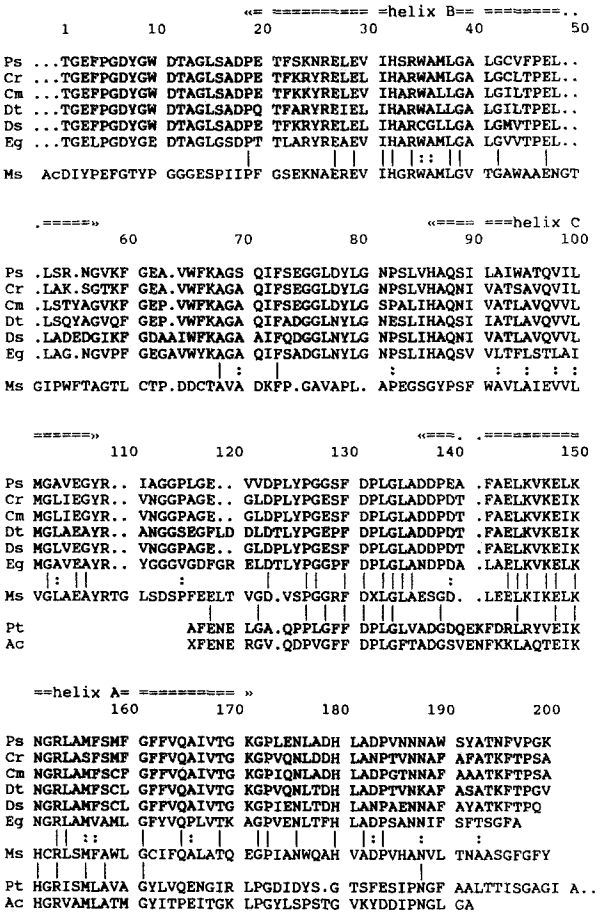


Fig. 4. Sequence alignment between the LHCP of *Mantoniella squamata* (Ms) and Chl*a*/*b*- and Chl*a*/*c*-binding proteins. Ps – *Pisum sativum* (Cashmore 1984), Cr – *Chlamydomonas reinhardtii* (Imbault et al, 1988), Cm – *Chlamydomonas moewusii* (Larouche et al. 1991), Dt – *Dunaliella tertiolecta* (La Roche et al. 1990), Ds – *Dunaliella salina* (Long et al. 1989), Eg – *Euglena gracilis* (Muchhal and Schwartzbach 1992), Pt – *Phaeodactylum tricornutum* (Grossman et al. 1990), Ac – *Amphidinium carterae* (Hiller et al. 1993). The helices are arranged according to Kühlbrandt and Wang (1991) and Allen (1992). Lines mark amino acid identity with the *Mantoniella* sequence, colons indicate 4 or 5 identical amino acid in the Chl*b*-containing plants with *Mantoniella* LHCP. Dots represent gaps introduced into the sequences to maximize homology.

Fig. 4. The comparision shows conserved segments in the transmembrane regions of the Chl*b*-containing plants, especially in the helices B and A. Identical amino acids are also found in the neighbouring regions of helix A. The shorter N-terminal region of the LHCP of *Mantoniella* exhibits sequence divergence. From this alignment it can be assumed that the LHCP of *Mantoniella* is arranged in a similar way as the LHCP of higher plant (Karlin-Neumann et al. 1985; Bürgi et al. 1987; Kühlbrandt and Wang 1991), i.e. with three

hydrophobic domains, the N-terminal segment being on the stromal side and the C-terminus on the lumenal side of the thylakoid membrane. It has been shown for higher plants that three transmembrane regions are required for insertion into the thylakoid membrane (Auchincloss et al. 1992) and also for the formation of a stable pigment–protein complex (Paulsen and Hobe 1992). This seems to apply to *Mantoniella* LHCP, too. Examing the sequences as a whole, a high degree of homology between pea, *Chlorophyceae* and *Euglena* (>70%) becomes clearly recognizable, while the number of identical amino acids between the LHCP of *Mantoniella* and Chl*b*-binding proteins is significant smaller (30–35%). Nevertheless, it is obvious that the LHCP of *Mantoniella* belongs to the Chl*b*-binding proteins.

The alignment of Chl*a*/*c* sequences with Chl*a*/*b* sequences is under debate. Green et al. (1992) compared the first transmembrane region to helix B of Chl*a*/*b*-binding proteins while Grossman et al. (1990) aligned it to helix A. The helices B and A have correspondingly conserved residues, which can also be seen in *Mantoniella*, suggesting that these two membrane-spanning regions may be the result of an internal gene duplication (Green et al. 1991). Comparing the Chl*a*/*c* algal sequences with *Mantoniella* according to Grossman et al. (1990), identical amino acids in the helix A and in its preceding region became obvious, including the sequence motif FDPLG being conserved in most LHCPs analyzed so far (Green et al. 1992; Hiller et al. 1993). As this study wants to point out it is exactly this motif which contains the modified amino acid X in *Mantoniella*. Current analyses of the posttranslationally modified proline should lead to its clear identification. Further studies will concentrate on the question if this posttranslational modification may be involved in the flexibility of pigment recognition and pigment binding of the LHCP (Meyer and Wilhelm 1993).

Looking at the presumed Chl-binding sites, two of the three histidines in Chl*b*-containing plants are conserved in *Mantoniella*, one in helix A (Grossman et al. 1990) and the second one in the C-terminal region (180). It is remarkable that no histidine, asparagine or glutamine can be found in helix C. Reconstitution experiments with mutant pea LHCP (Paulsen and Hobe 1992; Cammarata and Schmidt 1992; Paulsen and Kuttkat 1993) have identified segments of the LHCP playing an important role in the formation of stable pigment–protein-complexes. It has been shown that these segments are a hydrophilic domain at the beginning of helix B and a hydrophilic domain between helix A and the C-terminus, which are also conserved regions in *Mantoniella*.

The present results provide evidence that the LHCP of *Mantoniella* is phylogenetically related to Chl*a*/*b*-binding proteins. The LHCP also possesses an unusual modified amino acid in a highly conserved sequence motif which is found in all Chl-binding proteins.

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