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The unusual light-harvesting complex of *Mantoniella squamata*: supramolecular composition and assembly [1]

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The supramolecular properties of the chlorophyll *a*/*b*/*c* light-harvesting protein complex of *Mantoniella squamata* were analyzed. The complex is built up of at least two subunits of M_r 20000 and 22000, which are encoded in the nucleus as precursor proteins of M_r 27000. The chlorophyll *a*/*b*/*c* light-harvesting complex is the dominating protein of the thylakoids and is fractured with the protoplasmic membrane face as a 7.5 nm particle. These particles form paracrystalline arrays with a purported hexagonal arrangement in native thylakoids and form similar arrays when reconstituted in liposomes. The light-harvesting complexes are supposed to be trimers with a trigonal arrangement of the subunits. Preliminary amino acid sequence data show that the chlorophyll *a*/*b*/*c* light-harvesting complex of *M. squamata* is more related to the chlorophyll *a*/*b* complex of higher plants than to the light-harvesting complexes of chromophytan algae.

Introduction

An efficient conversion of solar energy into chemical energy during photosynthesis requires an effective absorption of photons to drive electron transport. Therefore, plants have developed specialized light harvesting systems which deliver the absorbed energy to the reaction centres. [1,2]. These light-harvesting systems optimize photosynthesis and adapt the photosynthetic apparatus to the different light environments and long- and short-term changes in light intensity and light wavelength [3].

Higher plants and green algae contain chlorophyll (Chl) *a*/*b* proteins as light-harvesting systems. The major light-harvesting complex, LHCII, makes up to 50% of the total protein and Chl mass [4]. It is a trimer built up of three identical or closely related polypeptides of M_r 25000 [5,6].

Aquatic organisms have developed light-harvesting complexes especially absorbing light in the 'green gap' of Chl [7]. Cyanobacteria and red algae contain biliproteins as major pigments in addition to Chl *a* [8,9]. Cryptomonads contain biliproteins and Chl a/c LHCs; in the heterogeneous group of the chromophytes, containing brown algae, diatoms, dinoflagellates and chrysophytes, Chl *a*/*c* LHCs and special carotenoids have evolved [10]. The importance of the chromophytes becomes evident, considering that 50% or more of the total annual photosynthetic carbon fixation (10^{11} tons) is produced in aquatic environments [11]. Therefore, the characterization of the photosynthetic apparatus of chromophytes is desirable.

Light-harvesting complexes and Photosystem I and II have been isolated and partly characterized by spectral properties and polypeptide composition from brown algae [12,13], diatoms [14,15], dinophytes [16], chrysophytes [17], and cryptophytes [18,19]. The biosynthesis of Chl *a*/*c* light-harvesting polypeptides of the diatom *Phaeodactylum tricornutum* has been investigated by in vitro translation/immunoprecipitation experiments, demonstrating that these proteins are nuclear encoded and translated on cytoplasmic ribosomes [20,21]. Recently, three cDNAs encoding precursors of these fucoxanthin-Chl proteins (fcp) of this alga have been isolated and sequenced [22]. Although these polypeptides show homology to conserved parts of the polypeptide chains of Chl *a*/*b* LHCs of higher plants and green algae, some striking differences were detected, especially in the C-terminal region as well as in the leader sequence and in the mode of import into the

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Abbreviations: Chl, chlorophyll; LH, LHC, light-harvesting, light-harvesting complex; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; SDS, sodium dodecylsulfate.

[1] This study is dedicated to Professor Dr. W. Rüdiger on the occasion of his 60th birthday.

chloroplast [22,23]. These differences and the limited homologies have influenced the contradictory immunological experiments. Antisera raised against Chl *a/b* light-harvesting polypeptides did not or only weakly crossreact with Chl *a/c* light-harvesting proteins of diatoms [24–26]. The immunological crossreactions between chromophytan LHCs are also very heterogeneous, indicating larger differences in the light-harvesting polypeptides of these algal groups compared to higher plant systems [13]. Therefore, it is of interest to investigate the relationship between Chl *a/b* and Chl *a/c* light-harvesting systems.

We chose to study the green flagellate *M. squamata* which is placed with the Prasinophyceae, a group of primitive green algae. Its light-harvesting complex contains Chl *a*, *b* and *c* [27]. Thus, this type of alga can be considered to be derived from an ancestor for classes of Chlorophyta and perhaps Chromophyta. Immunological investigations revealed that antibodies raised against its light-harvesting polypeptide crossreacted with the light-harvesting protein of the related *Micromonas pusilla* but did not show any immunological relation to light-harvesting polypeptides of any other green algae or land plants tested [28].

The aim of the present study was to expand the knowledge on the structure, substructure and subcellular localization of this complex and to provide insights on the biogenesis of the light-harvesting polypeptides within this alga.

Materials and Methods

Cultures and growth conditions. Mantoniella squamata was grown at 15°C in 2 liter Erlenmeyer flasks in Müller medium as described earlier [29, 30]. The cultures were gased moderately with 2% CO_2 in air. The light intensity was 3.5 W/m^2 and a light:dark regime of 16:8 h was applied.

Isolation and characterization of the light-harvesting complex. The purification of the Chl *a/b/c* LHC largely followed the protocol developed for the isolation of the Chl *a/c* LHC of the cryptophyte *Cryptomonas maculata* [19].

For isolation of the LHC, 2 liters of 3–4-week-old cells of *M. squamata* were harvested by centrifugation and resuspended in 40 ml isolation buffer (50 mM Tricine/NaOH, 1 mM DTT, pH 7.5). The cells were broken by passage through a French pressure cell (Colora, Lorch) at 10000 psi (1 psi = 6.895 kPa). Cell debris and starch were removed by centrifugation (2000 × *g*, 10°C, 5 min). The thylakoids were washed twice in isolation buffer to remove peripheral proteins and were sedimented by centrifugation (140000 × *g*, 30 min, 10°C). The membrane pellet was resuspended to give a final Chl concentration of 400 μg/ml. The thylakoids were solubilized by 1% (w/v) Triton X-100 or octyl glucoside (15 min); Triton X-100 was used for most of the isolations. The Chl *a/b/c* LHC was separated by sucrose density centrifugation (5–20% sucrose (w/v), 0.05% Triton X-100 in isolation buffer, 150000 × *g*, 16 h, 10°C, Kontron rotor TST 41.14). Eluted LHC was diluted with isolation buffer and pelleted by centrifugation (16–20 h, 140000 × *g*, Kontron rotor TFT 65, 10°C).

Gel electrophoresis. Pigment protein complexes were subjected to LDS- and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Ref. 31 using separating gradient gels of 10–20% or gels of 17.5%, and 5% stacking gels. The samples were either applied in native condition or denatured by boiling for 2 min.

Analytical procedures. Absorption spectra of cells and isolated complexes were determined at room temperature using a Hitachi U3200 spectrophotometer equipped with an end-on photomultiplier. Fluorescence emission spectra were recorded at room temperature or at 77 K as described [32].

Chlorophyll *c* was determined using the equations of [33]; Chl *a* and Chl *b* were estimated after Kirk [34]. Protein was determined according to Ref. 35.

Immunological procedures. The major polypeptides identified as constituents of the LHC of *M. squamata* were isolated by preparative SDS-PAGE. The polypeptide bands were either stained with Coomassie brillant blue R-250 or visualized by soaking the gels in a cold solution of 3 M KCl for 15 to 30 min at 4°C. In both cases the protein bands were excised from the gels, electroeluted into dialysis tubes and dialysed exhaustively against isolation buffer; protein concentrations were determined.

Preimmune sera were taken from the rabbits before the first immunization. About 50 μg of purified polypeptides in 50 μl distilled water were mixed with 0.2 ml Freund's complete adjuvant and injected into the rabbits. Booster injections followed 1, 2, 8, 15, 22, 29, 36, 43, 50, 57, 64 and 71 days after the first immunization. Ten days after the final immunization the antisera were obtained and their specificity characterized by Western-immunoblotting according to Ref. 36.

Protein sequencing. The Chl *a/b/c* LH polypeptides and fragments were partially sequenced. Polypeptide fragments were obtained by treatment with 70% formic acid according to EMBL protocols [37]. The polypeptides and their fragments were separated on SDS-polyacrylamide gels and blotted on Immobilon P membranes as outlined by the producer (Waters, Millipore). The polypeptide bands were visualized and excised; sequence analysis was carried out in a pulsed-liquid-phase sequenator 477A from Applied Biosystems. PTH-amino acids were analyzed on-line according to the instructions of the manufacturer [38].

RNA isolation, in vitro translation and immunprecipitation. Total RNA and poly(A)$^+$ RNA of *M. squamata*

were isolated and purified using the Stratagene RNA Isolation Kit and Poly(A)Quick mRNA Isolation Kit. In vitro translation was carried out according to Pelham and Jackson [39] for 1 h at 30°C in 50 μl reaction volumes containing 40 μl rabbit reticulocyte lysate (Amersham RPN 90, Amersham, Braunschweig) and 30 μCi [^{35}S]Methionine of > 1000 Ci/mmol (Amersham). After complete translation 2 μl aliquots were withdrawn and spotted onto glass-fiber filters (Whatman GF/C) to determine the rate of incorporation. The remaining samples were used for SDS-PAGE and immunoprecipitation. Indirect immunoprecipitation was performed after [40] modified by Ref. 41 using protein A-Sepharose. The immunoprecipitates were resuspended in Laemmli sample buffer [31], heated for 2 min at 100°C and electrophoresed on 15% gels in comparison with the total in vitro translation products, unlabelled LH polypeptides and molecular weight marker proteins (SDS-70, Sigma, Munich). Gels were stained, destained, impregnated with Amplify (Amersham) for 15 to 30 min, dried and fluorographed at −80°C for 24 h to 28 days.

Electron microscopy. Ultrathin sectioning and immunogold labelling: Cells of *M. squamata* were cryofixed by dipping small droplets of cell suspensions into liquid propane cooled by liquid nitrogen [18]. Water exchange and fixative infiltration were performed in methanol containing 1% glutaraldehyde for 8 h at −90°C, followed by successive 8 h periods at −60°C and −30°C. The cells were washed in acetone and embedded in Lowicryl K4M at 0°C according to producers specifications (Chemische Werke LOWI, Waldkreuzburg). The samples were polymerized for 48 h by UV-radiation in a Balzers freeze-substitution unit FSU 010.

Ultrathin sections were cut with a Reichard-Jung Ultracut ultramicrotome. The sections were immunogold labelled using preimmune sera and antisera in dilutions of 1:2000, 1:4000 and 1:6000 [42]. Finally, the sections were poststained with aqueous uranyl acetate [43] and examined in a Philips 301G electron microscope operated at 80 kV.

Freeze fracture procedure: Purified LHC preparations of *M. squamata* were incorporated into soybean lecithin liposomes [44]. Freeze fracture of proteoliposome preparations and of whole cryofixed cells of *M. squamata* and isolated thylakoids was performed as described earlier [18,19]. Optical diffraction was measured according to Ref. 59.

Results

Isolation and spectral properties of the Chl a / b / c LHC

Three pigment containing bands were separated after sucrose density gradient centrifugation upon solubilization of the thylakoid membranes with 1% Triton X-100 and a Chl:detergent ratio of 1:25. Band 1 at the top of the gradient contained free pigment and almost no protein. Band 2 was identified as the highly purified Chl *a / b / c* LHC, whereas band 3 was enriched in Photosystem I but still contained additional, contaminating proteins, when analyzed by SDS-PAGE.

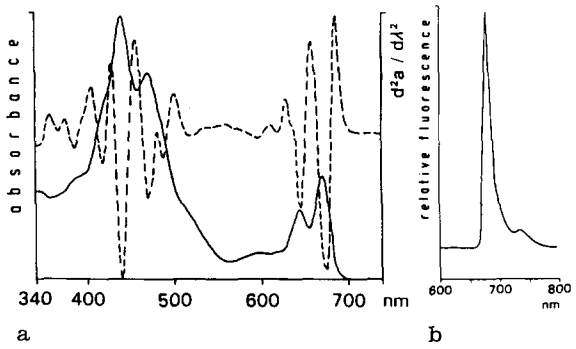

Fig. 1. (a) Absorption spectrum (---) and second derivative (---) of the isolated and solubilized Chl *a / b / c* LHC of *M. squamata*. (b) 77 K fluorescence emission spectrum; excitation wavelength: 460 nm.

Fig. 1a shows the absorption spectrum of the purified Chl *a / b / c* LHC of *M. squamata* isolated by sucrose density gradient centrifugation. The absorption spectrum of the Chl *a / b / c* LHC is characterized by major absorption maxima at 437, 468 and 646 and 673 nm. Two minor maxima at 598 and 620 nm and the shoulders at 486, 520 and 640 nm were determined by the second derivative. The 673 nm absorption is characteristic for Chl *a*. The 646 nm absorption peak is mainly attributed to Chl *b* but has contributions from the Chls *a* and *c*, as do the smaller absorption bands in the red region. The shoulder at 500 nm is due to the absorption by carotenoids. The 77 K fluorescence emission spectrum of the complex (Fig. 1b) shows a main emission maximum at 681 nm with an additional minor emission at 732 nm. No Chl *c* emission peak was detected at 635 nm upon excitation at 460 nm. All of the excitation energy was finally transferred to Chl *a* within the complex. This feature demonstrates the excellent energy transfer and functional integrity of the solubilized and purified LHC.

Polypeptide composition of the Chl a / b / c LHC

The Chl *a / b / c* LHC of *M. squamata* was pelleted by centrifugation and subjected to native and denaturating SDS-PAGE as compiled in Fig. 2. Lane 2b shows the native LHC migrating at the top of the gel as a deep green band with an apparent molecular weight of about 80000. Hardly any free pigments migrated at the front and no other protein bands could be detected after staining with Coomassie Brillant Blue R250.

After denaturation by heating for 2 min at 100°C the native complex was dissociated into two polypeptides with M_rs of 20000 and 22000 (2c). The M_r 20000 polypeptide stained more intensely with Coomassie

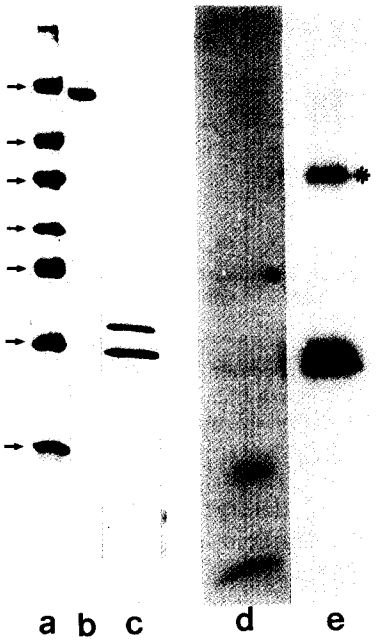

Fig. 2. SDS-PAGE of the purified Chl *a/b/c* LHC. Lanes a–c: SDS gradient PAGE. (a) Reference proteins; (b) native (non-heated) solubilized complex; (c) subunit composition of the heat denatured complex; lanes d–e: SDS-PAGE; (d) formic acid cleavage products of M_r 22000 polypeptide with M_rs of 15000 and 7000; (e) electroeluted and re-electrophoresed Chl *a/b/c* LHC after heat denaturation. Reference proteins marked by arrows: bovine albumin (66000), ovalbumin (45000), glyceraldeyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000), trypsin inhibitor (20100), α-lactalbumin (14200).

brillant blue 250 than the M_r 22000 band. When both polypeptides were re-electrophoresed after electroelution, in addition to the two polypeptides of M_r 20000 and 22000, a third band with a M_r of 45000 was visible, which is interpreted as a dimeric aggregation product of the polypeptides (Fig. 2e).

Amino acid sequence analysis and formic acid cleavage

Heat denatured Chl *a/b/c* LHC of *M. squamata* was subjected to SDS-PAGE and blotted onto Immobilon P membranes. The blots were briefly stained with Coomassie Brillant Blue, destained and the two bands of the LHC subjected to total amino acid analysis and Edman degradation. The total amino acid compositions of the two polypeptides are shown in Table I. Minimal molecular masses of 17478 and 17656 Da were calculated on the basis of the amino acid analysis for the two polypeptide bands obtained by SDS-PAGE. Amino acid sequence analysis of the M_r 22000 polypeptide was not possible because the N-terminus was blocked. Analysis of the M_r 20000 band gave the partial sequence shown in Table II.

In order to obtain additional sequence information, the M_r 22000 polypeptide was isolated by preparative SDS-PAGE and subjected to formic acid treatment. The cleavage products were separated by analytical

TABLE I

Amino acid composition of light-harvesting chlorophyll proteins from M. squamata in comparison to those of Phaeodactylum tricornutum [51], Amphidinium carteri [14] and Pisum sativum [61]

Amino acid	*Mantoniella*		*Phaeodactylum*	*Amphidinium*	*Pisum*
	20000	22000			
D/N	9.1	7.0	9.9	11.8	8.6
T	11.8	13.0	5.4	3.1	3.4
S	9.3	10.4	7.7	8.6	7.7
E/Q	15.9	14.1	10.8	5.9	7.7
P	12.4	11.8	4.7	2.4	6.9
G	27.5	28.9	11.4	8.3	12.5
A	22.5	18.8	10.0	18.7	9.4
V	8.7	8.7	6.2	6.6	6.9
C	2.6	8.1	0.2	0.0	0.4
M	1.4	1.0	1.1	4.5	2.2
I	5.6	7.0	5.4	4.5	3.0
L	13.0	13.3	8.6	5.5	9.9
Y	3.7	3.6	2.6	3.8	3.4
F	9.9	10.8	5.7	3.8	6.0
K	5.1	4.7	3.8	10.4	5.2
H	4.0	2.4	1.6	2.1	1.3
R	7.4	6.4	4.3	0.0	3.0
W	n.d.	n.d.	n.d.	n.d.	2.6

TABLE II

Homology of M. squamata LH polypeptides with Chl a / b polypeptides from soybean [63] and fucoxanthin-Chl-protein (FCP2) from P. tricornutum [22]

Identical amino acids are capitalized, colons indicate identical amino acids, dots conservative substitutions and spaces lack of conservation.

Mantoniella	X A - E S G D L E E L K I K E L K V X R L
	. . . . . : . : : : . : : : : . : :
	200
Soybean	A d - d p e a L a E L K v K E L K n g R L
	: : : : . . : . : . : . : : .
	53
Phaeodactylum	A d G d q e k f d r L r y v E i K h g R i

SDS-PAGE and blotted on Immobilon P membranes for sequence analysis. Formic acid cleaved the polypeptide into two distinct fragments with M_r values of about 7000 and 15000 (Fig. 2d). A weak third band corresponding to an M_r of 30000 is most probably a dimer of the M_r 15000 polypeptide because both cleavage products were very similar in their amino acid composition (data not shown). The M_r 15000 and M_r 30000 bands were blocked and could not be analysed. The M_r 7000 band had the same N-terminal amino acid sequence as already determined for the M_r 20000 subunit. This result is an indication that the partial sequence obtained for the M_r 22000 polypeptide is also an internal sequence due to cleavage during preparation.

In vitro translation and immunprecipitation

Approximately 1 mg total RNA were isolated from 2 l of exponentially growing algal cells. The purified total RNA of *M. squamata* had a 260:280 nm ratio of about 2, indicating no major contamination by DNA or proteins. Selection of the total RNA on oligo dT-cellulose columns yielded about 3 μg RNA in the poly(A)$^+$ fraction which was almost completely free of rRNA and tRNA when analysed by denaturing agarose gel electrophoresis. Both total RNA and poly(A)$^+$ RNA were translated in vitro with reticulocyte lysate and the products were electrophoresed and fluorographed. Figs. 3a and 3d show the translation products of both preparations in the range of M_r 5000 up to 100000. In vitro translation of the poly(A)$^-$ RNA, which was tested simultaneously, resulted in the synthesis of only a few proteins (not shown).

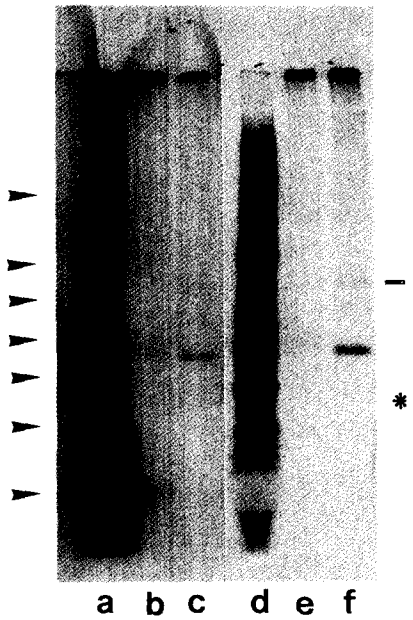

Fig. 3. In vitro translation /immunoprecipitation products of *M. squamata* total RNA (a–c) and poly(A)$^+$ RNA (d–f). Protein synthesis from total RNA (a) and poly(A)$^+$ RNA (d); Immunoprecipitation of total RNA (b) and poly(A)$^+$ RNA (e) translation products with preimmune serum. Immunoprecipitation of total RNA (c) and poly(A)$^+$ RNA (f) with anti-LHC; the bar indicates aggregated subunits with a M_r of 54000; the asterisk marks the position of the native subunits at M_r 22000. Arrowheads indicate reference proteins as in Fig. 2a.

Indirect immunprecipitation with protein A sepharose resulted in a highly specific precipitation reaction for both total RNA as well as poly(A)$^+$ RNA (Fig. 3c, f). A single protein species with an M_r of about 27000 and a weaker signal of about 50000 were detected. The M_r 50000 band might represent a dimer of the M_r 27000 band, as was the case when electroeluted polypeptides of the Chl *a/b/c* LHC were reelectrophoresed (Fig. 2e). The M_r 27000 polypeptide was about 5000 to 7000 larger in molecular weight compared to the native subunits when run on the same gel system. The difference in molecular weight may be attributed to an amino terminal transit peptide which is cleaved from the precursor molecule during import into the chloroplast. The precipitation signal was much stronger in the translation reaction with poly(A)$^+$ RNA than in the translation reaction with total RNA. No precipitation signal could be observed with preimmune serum (Fig. 3b, e) or when the translation reaction of poly(A)$^-$ RNA was used in those immunprecipitation experiments. This is a clear indication that the polypeptides of the Chl *a/b/c* LHC are encoded by nuclear DNA.

Freeze-fracture analysis of thylakoids

The Chl *a/b/c* LHC is a distinct complex, which is cleaved with the protoplasmic fracture-face (PF-face) of the thylakoids, as is the case with the Chl *a/b* LHC of higher plants. In normal freeze-fracture preparations, the fracture plane cleaves the thylakoids along the inner hydrophobic space between the two membrane leaflets exposing the exoplasmic and protoplasmic fracture faces together with their characteristic particle patterns. These cleavage patterns are also evident in thylakoids of *M. squamata* as shown previously [30]. However, as an additional unusual feature, the fracture plane exposes directly the exoplasmic surface (ES) together with the protoplasmic fracture faces (PF). Thus, the particle pattern within the membrane and their surface projections are visualized. Fig. 4 shows the exoplasmic surfaces and protoplasmic fracture faces of appressed thylakoid regions as a stratification pattern. The distance from one ES face to the ES face of the following membrane within the thylakoid stacks is about 12.5 nm to 16.0 nm, which is equivalent to the 13 nm to 19 nm width of a whole thylakoid.

The exoplasmic membrane surface (ES) is densely covered by protein complexes which are arranged in a regular, most likely hexagonal, pattern in the form of a membrane crystal as shown in Fig. 4. Measurements of

the lattice repeat distances gave values of about 9.6 nm (a) and 8.8 nm (b), which are in good agreement with the determination by optical diffraction (a: 9.2 to 9.6 nm; b: 9.0 nm).

The protoplasmic fracture-face is characterized by very densely packed particles smaller than 10 nm, which, as surface projections, give rise to the particle pattern on the ES-face. The PF-face generates the impression that the particles are arranged in an irregular pattern, even though some regular arrangements are observed. However, a possible regular arrangement of the particles may be obscured by the dense packing and the fact that not all individual particles are resolved. On the other hand, regular and random arrangements may coexist within the membrane. These could be characterized by different fracture planes.

Reconstitution of the Chl a / b / c LHC in liposomes

The Chl *a / b / c* LHC was concentrated by centrifugation and reconstituted into soybean lecithin liposomes to analyze its supramolecular structure. Reconstitution was achieved by mixing preformed liposomes with the purified detergent solubilized LHC with subsequent removal of the detergent. The efficient incorporation of the complexes was shown by sedimentation of the liposomes. The supernatant contained hardly any Chl, while the liposome pellets were deep green in color. The freeze fracture preparations (Fig. 5a) show a random distribution of the Chl *a / b / c* LHC on both the concave and convex fracture faces of the proteoliposomes. The size of the incorporated complexes was about 7.5 nm (7.2 nm to 7.8 nm) on both fracture faces.

In most liposomes the incorporated Chl *a / b / c* complexes were aggregated and clustered in particle arrays (Fig 5b, c, d). Smooth transitions are observed between irregular clustering (Fig. 5b) at the margins and regular particle arrays, especially in the centre of these aggregation patches (Fig. 5c). The particles in random orientation within the patches have similar sizes to isolated particles. The regular, well arranged arrays show a dense packing of the protein complexes in a purported hexagonal pattern with lattice repeats of

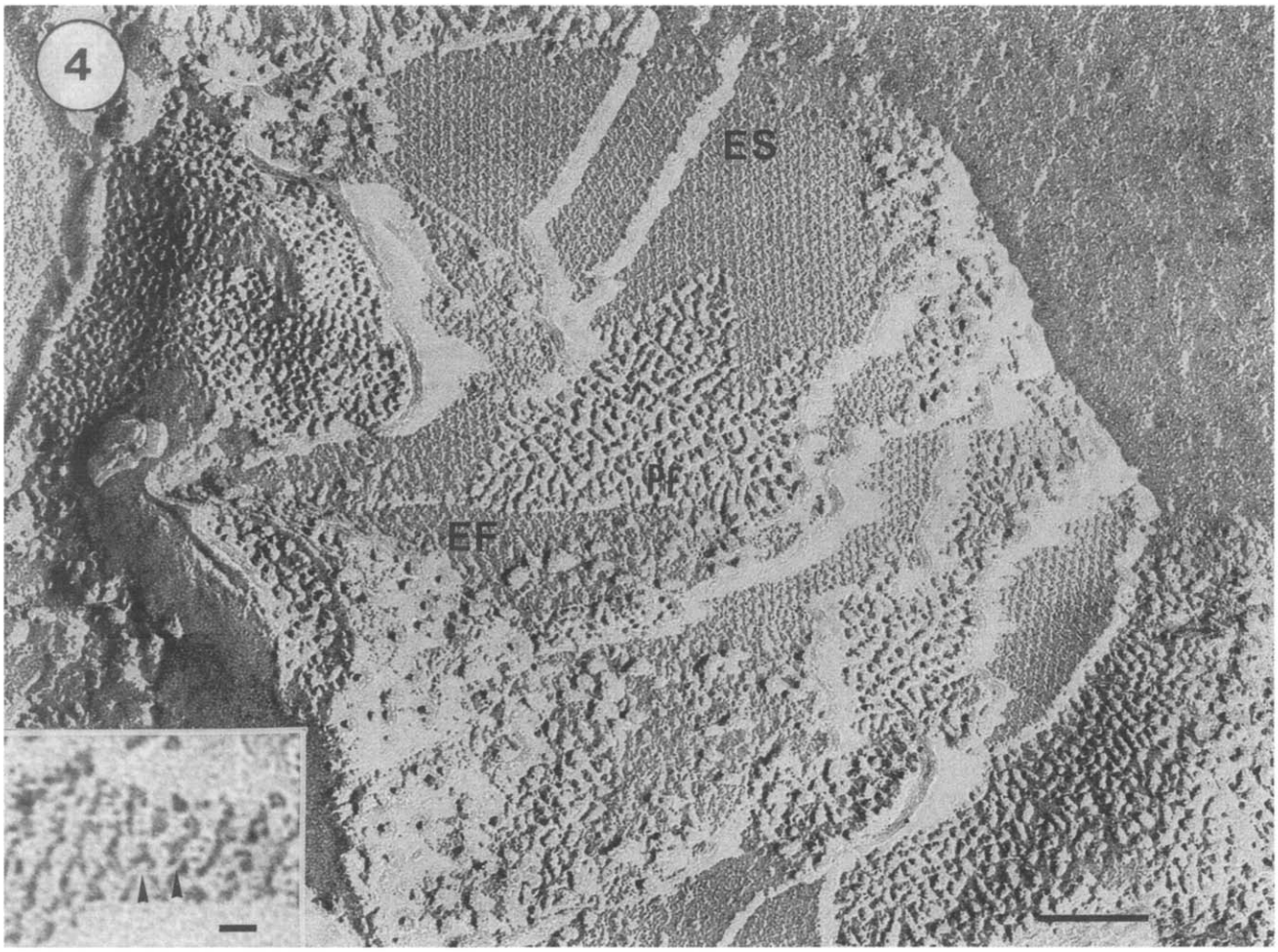

Fig. 4. Freeze-fractured thylakoids of *M. squamata*. The fracture crosses several stacked thylakoids. The ES-face is characterized by a para-crystalline arrangement of the freeze-fracture particles. Rowed PF-particles are marked by arrow. The inset shows the structural elements of the lattice (arrowhead). Exoplasmic (EF), protoplasmic (PF) fracture faces; exoplasmic surface (ES). Bar: 100 nm; Bar of inset: 10 nm.

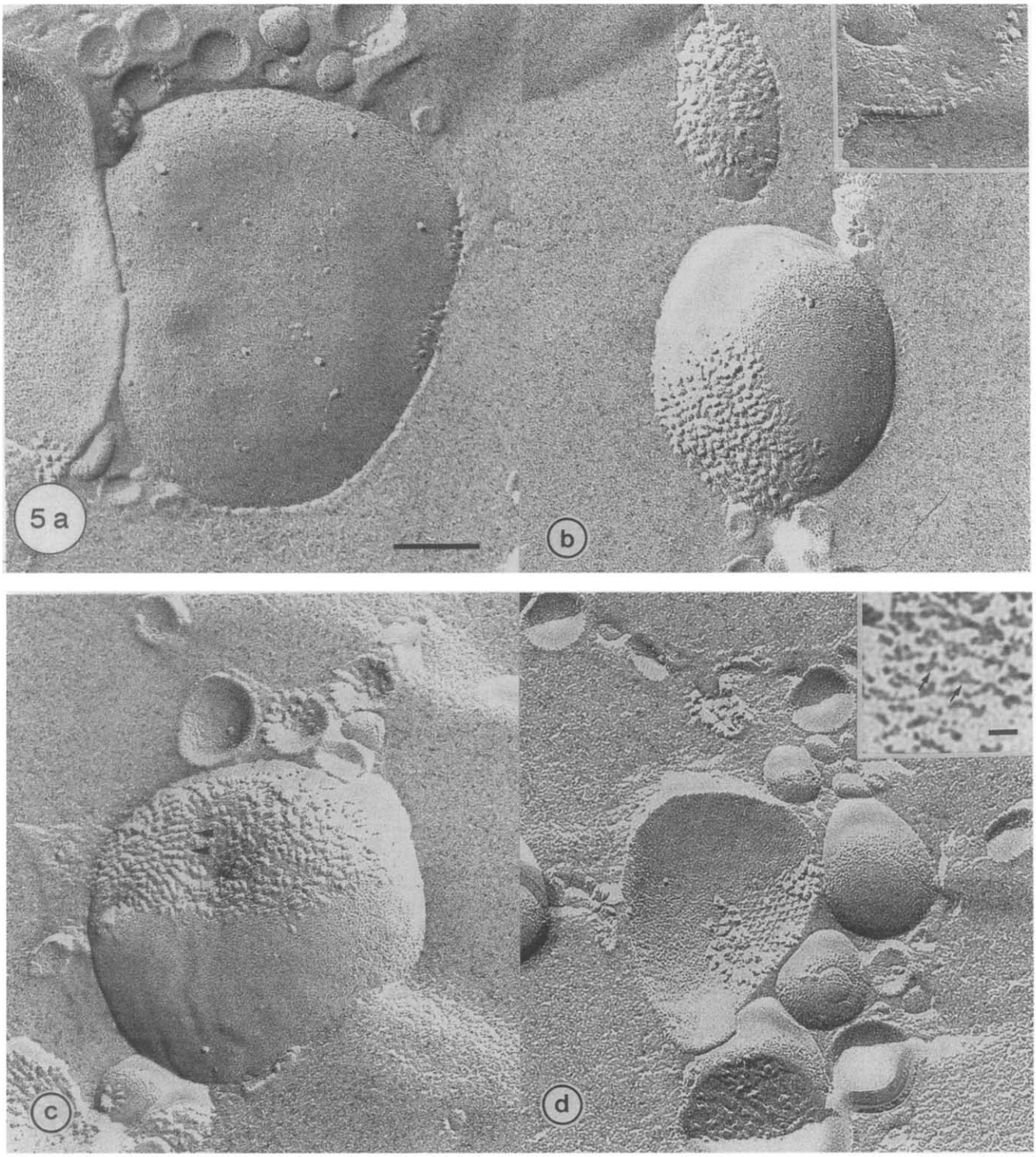

Fig. 5. Proteoliposomes containing purified Chl *a*/*b*/*c* LHC. Liposome with isolated LHC particles (a); most of the LHC particles are aggregated within the lipid layer of the liposomes (b, c). Some ordered arrays are visible (arrowheads). Inset (b) shows a fracture pependicular to the lipid membrane with LHC particles in profile view. Para-crystalline arrangement of the reconstituted LHC particles (d). The inset shows the purported hexagonal pattern and the elements of the para-crystalline lattice (arrows). Bar: 100 nm; Bar of inset d: 10 nm.

about 10.0 nm (a) and 8.6 nm (b) and comparable values of 8.5 to 8.8 nm (a) and 7.9 to 8.1 nm (b) determined by optical diffraction (Fig. 5d). The structures of the crystalline lattice (Fig. 5d, inset) are similar to those observed in thylakoids of *M. squamata* (Fig. 4, inset) and for the chlorophyll *a*/*b* LHC [5]. When

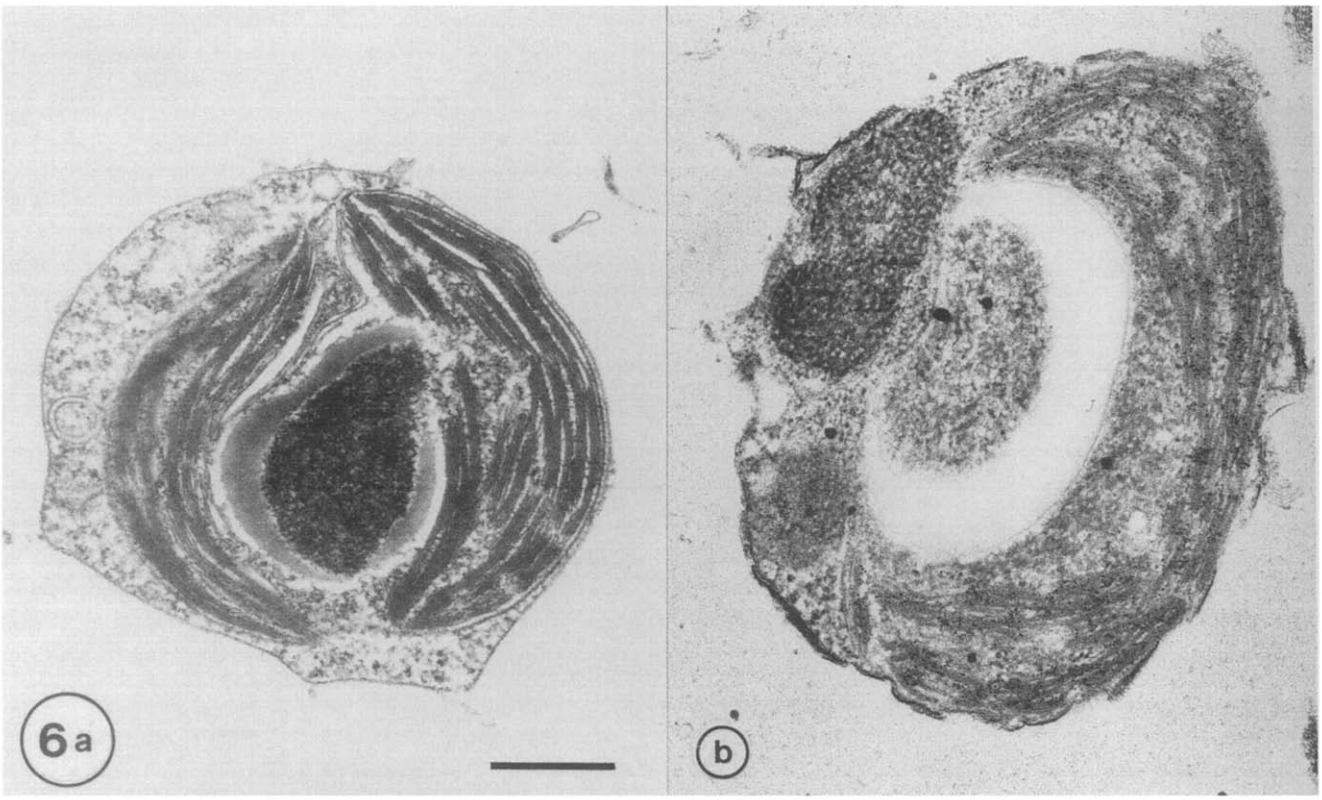

Fig. 6. Electron micrograph of *M. squamata* (Panel a), showing the chloroplast with the pyrenoid, surrounded by starch, and the stacked thylakoids. Immunogold-labeling of the Chl *a / b / c* LHC (Panel b). The label decorates exclusively the thylakoids. Most of the LHC is localized in stacked thylakoids. Bar: 500 nm.

proteoliposomes containing aggregated particle patches were fractured perpendicular to the lipid layer, the height of the complexes could be calculated from the profile views of the particles. Values of 6 to 7 nm were measured for the height of the Chl *a / b / c* LHC of *M. squamata* (Fig. 5b, inset).

Subcellular localization of the Chl a / b / c LHC

The Chl *a / b / c* LHC was localized by immunogold labeling within the thylakoid system. The antisera raised against the LHC subunits were tested prior to labeling experiments by Western-immunoblotting and in vitro translation/immunprecipitation experiments (Fig. 3). While the preimmune serum did not recognize any protein band in Western-blots, the antiserum recognized both the native LHC band as well as its component SDS-denaturated polypeptides. Fig. 6 shows electron micrographs of ultrathin sections of *M. squamata* and the dense and specific labeling of the thylakoid membranes by anti-LHC antibody (Fig. 6b) in comparison to an unlabelled OsO_4 stained cell (Fig. 6a); no label was found in the chloroplast stroma and the cytoplasm. Most of the label was observed on the stacked regions of the thylakoids. However, a small portion of the label was also consistently found on unstacked thylakoid regions. The preimmune serum did not recognize any antigen on the thin sections of *M. squamata* (not shown).

Discussion

M. squamata, a member of the Prasinophyceae, an ancient group belonging to the Chlorophyta, has a LHC, which simultaneously contains the Chls *a*, *b* and *c* [27]. A LHC harboring three different Chl species is a unique feature and up to now only detected in *M. squamata* and *Micromonas pusilla*. From their pigment composition [45,46], one would suggest a biochemical and structural relationship between the Chl *a / b* LHCs of higher plants and the Chl *a / c* complexes of the chromophytes.

The Chl *a / b / c* LHC of *M. squamata* is resolved in two polypeptides of M_rs 20 000 and 22 000, which contain identical sequence stretches. They are supposed to be the products of at least two related genes, because they differ slightly in their amino acid compositions (Table I). Fawley et al. [28] found similar values as presented here in SDS-PAGE. Thus the M_r of the LH polypeptides from *M. squamata* is significantly smaller than that of the Chl *a / b* polypeptides of higher plants which are in the range of 25 000 to 30 000 [6]. Based on these results, Fawley et al. [28] proposed that the LHC

of *M. squamata* is very different from that of higher plants and green algae and perhaps had an independent genetic origin. However, the M_r of the Chl *a/b/c* LHC polypeptides is similar to the Chl *a/c* polypeptides of the LHCs of chromophytes and cryptomonads, which are all in the range of 17000 to 22000 [14,19,47,48].

The LH polypeptides are arranged in a native complex with an apparent molecular weight of 80000. Molecular weights in the size range of 65000–100000 were determined for Chl *a/b* complexes [49]. Rhiel et al. [19] determined an M_r of 80000 for the native LHC of the cryptomonad *Cryptomonas maculata*; a lower M_r of about 50000 was determined for the Chl *a/c* LHC of the marine dinoflagellate *Glenodinium* sp. in an early study of Boczar et al. [50]. On the other hand, Chl *a/c* LHCs with high M_r of 240000 were isolated by Passaquet et al. [13] from the brown algae *Dictyota dichotoma*, *Laminaria saccharina*, *Pelvetia canaliculata* and *Pylayella littoralis* and for the diatom *P. tricornutum* by Gugliemelli [51]. Corresponding to the interpretation of these authors and of Katoh et al. [52], these aggregates represent oligomeric species of LHCs which are not fully solubilized by gentle detergent treatment. In general, the apparent molecular weights of the Chl *a/b/c* LHC of *M. squamata* and those of chromophytes and cryptophytes are of similar size order and smaller than those of green plants. The molecular weight of about 80000, including an equivalent of about 14 Chl molecules of M_r 900, fits directly to a trimeric structure of the LHC aggregates of *M. squamata*. This is equivalent to the trimeric nature of the LHCII analyzed by electrophoretic procedures [53].

This interpretation is supported by the structural studies, which showed an average diameter of 7.5 nm for the LHC of *M. squamata* on the PF-thylakoid faces [30] and purified and reconstituted in liposomes. This size is equivalent to higher plant LHCII. It is accepted that the 8.0 nm PF-particles of higher plant thylakoids correspond to LHCII particles [54]. Isolated and reconstituted LHCII particles have a similar size [55,56]. Crystallized LHCII aggregates isolated after similar procedures exhibit clearly the trimeric nature of the native aggregates [5,56,57,58], composed of three polypeptides with molecular weights of approximately 25000.

The para-crystalline arrays of *M. squamata* LHC shadowed with carbon-platinum show a purported hexagonal pattern with a lattice repeat distance of about 9 nm. The 'trigons' visible in these arrays are merely parts of the lattice and thus are likely the contribution of more than one particle. However, a hexagonal lattice would be a strong indication for a trigonal (or hexagonal) shape of the particles comparable to those of LHCII of higher plants [59,60], but at the moment there is no proof for this. The apparent discrepancy between the lattice repeat of about 9 nm and the 7.5 nm size of the particles that are supposed to make up the lattice, is explained by the intercalation of lipid molecules between the particles of the lattice.

The similarity of *M. squamata* LHC and LHCII of higher plants and the LHCs of chromophytan algae is reflected by the amino acid composition (Table I) [13,14,51,61] and especially by the partial amino acid sequence data (Table II). Upon comparison with sequences determined from various LHC species from higher plants, a 50% homology is calculated for the region sequenced. The partial sequence is located about 70 amino acids before the C-terminus. This site corresponds to the hydrophilic region preceding membrane spanning helix III of LHCII [62], a domain in which the sequence stretch FDPLGL is highly conserved. However, in the mature fcp proteins of the diatom *P. tricornutum* [22], this sequence corresponds to a domain preceding helix I; helices II and III are not related to the equivalent helices of LHCII. Based upon the size similarities of the polypeptides, the sequences of *M. squamata* LH polypeptides would be predicted to be more similar to the fucoxanthin/Chl LH polypeptides of the diatom *P. tricornutum*; according to the position of the partial amino acid determined, the LH polypeptides of *M. squamata* are more related to the Chl *a/b* LH polypeptides of higher plants. Further studies, especially the cloning and sequencing of the genes encoding the Chl *a/b/c* LHC polypeptides of *M. squamata* are needed to gain information on the phylogenetic relationship.

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