Reconstitution of Light-Harvesting Complexes from *Chlorella fusca* (Chlorophyceae) and *Mantoniella squamata* (Prasinophyceae)

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Dedicated to Professor W. Rüdiger (München) on the occasion of his 60th anniversary

Algae, Reconstitution, Light-Harvesting Complex, Photosynthesis, Pigment-Protein Interaction

Reconstitution experiments of light-harvesting complexes were performed with the green alga *Chlorella fusca* and the chlorophyll *c*-containing prasinophyte *Mantoniella squamata* using a modified method according to Plumley and Schmidt [Proc. Natl. Acad. Sci. U.S.A. **84,** 146–150 (1987)]. Changing the pigment supply quantitatively or qualitatively in the reconstitution mixture homologous and heterologous reconstitutes were obtained. In contrast to higher plants, light-harvesting polypeptides from green algae are able to bind the chlorophylls as well as the xanthophylls in different stoichiometries. Heterologous reconstitutes of *M. squamata* polypeptides give further evidence for a rather high flexibility of pigment recognition and binding. This is the first report of successful reconstitution of a chlorophyll *c*-binding protein. Contrary to chlorophyll *c*-less light-harvesting complexes, the reconstitution of *M. squamata* is strongly pH-controlled. In summary, the results give evidence for a high specificity of porphyrin ring recognition and variability in xanthophyll binding capacity. Therefore, it is suggested that at least in algal light-harvesting proteins chlorophyll organization may be determined by other mechanisms than xanthophyll binding.

Introduction

The photosystem II antenna complex LHC II is the most abundant pigment-protein complex, accounting for approximately half of the chlorophyll (Chl) and half of the protein of the thylakoid membrane [1]. Detailed knowledge exists concerning the polypeptide composition [2], the distinct pigment stoichiometry of the protein subunits [3] and the amino acid sequences deduced from genes of the nuclear multigenomic *cab*-family from different plants [4]. (For review see: [5–7].) The molecular structure, especially the organization and functional orientation of the non-covalently bound chromophores are currently under intensive investigation.

Methodically, there are two major approaches to obtain structural information about the molecular organization of pigment-protein complexes. First, the two- or three-dimensional crystallization and characterization by electron microscopy or X-ray diffraction studies. Electron diffraction studies of two-dimensional crystals and image-analysis of isolated LHC II revealed a trimeric subunit organization [8]. Electron diffraction of LHC II to 3.2 Å resolution [9] provided the three-dimensional determination at 6 Å resolution of two-dimensional LHC II crystals and resolved 15 porphyrin rings within the three membrane-spanning α-helices [10]. Unfortunately, the orientation of xanthophylls and the location of pigment-protein interactions in LHC II on the molecular level is not detectable at this resolution and three-dimensional crystals are not yet available. Nevertheless, histidines, glutamines and asparagines in or near presumed membrane-spanning regions are supposed to be pigment ligands [11, 6].

The second approach is the performance of *in vitro* reconstitution experiments. Since Plumley and Schmidt, 1987 [12] established a method for *in vitro* reconstitution of antenna complexes, results have been obtained concerning the molecular requirement for functional pigment-protein interaction by varying the pigment amount or pigment ratios [12–15] or modifying the LHCP by deletion mutagenesis [16, 17]. These recent reconstitution studies revealed that most of the N-terminal and part of the C-terminal hydrophilic regions are dis-

Abbreviations: Chl, chlorophyll; LHC, major light-harvesting complex(es); LHC II, light-harvesting complex(es) of photosystem II; LHCP, light-harvesting Chl-binding protein(s), PS, photosystem; SDS, Sodiumdodecylsulphate; PAGE, Polyacrylamidegelelectrophoresis; HPLC, high pressure liquid chromatography.

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pensible for pigment binding [17]. Amino acids 50–57 and 204–212, encompassing one of three conserved histidine residues of a *Pea* chlorophyll *a*/*b* binding protein were essential for reconstitution of a functional antenna complex [16].

Until now, there is only poor knowledge of the mechanisms of pigment binding, possible sites of protein-pigment interaction and/or pigment-pigment interaction in LHC. The question remains open, whether the chlorophyll binding by the polypeptides is similar to that obtained from the well investigated and highly resolved structure of bacterial reaction centers [18] or synergetic rather than based on individual pigment binding sites provided by the protein [17]. Furthermore, nothing is known about the binding characteristics of the xanthophylls, except the fact that these molecules are constitutive for functional LHC II [12].

Algae differ in pigment and polypeptide composition of their membrane-intrinsic LHC in comparison to LHC II of higher plants [19]. Since it is uncertain, if specific PS I or PS II antenna complexes are present in various alga species [20, 21], we use the term LHC II exclusively for higher plant light-harvesting complexes of PS II. The major antenna complexes of algae are termed LHC. For light-harvesting chlorophyll binding proteins the abbreviation LHCP is used [17].

A very interesting question concerns the binding properties of Chl *c*. Although Chl *c* replaces Chl *b* in Chl *a*/*c* antenna complexes of several families of algae [19], these chlorophylls are structurally extremely different. Chl *c* molecules have a *trans*-acrylic rather than a propionic side chain in position 17 with an acidic carboxyl group which is not esterified to phytol or another ester group [22]. *In vitro* LHC reconstitutes with Chl *c* have not yet been described. Another aspect concerns the observation that in algal LHC the pigment stoichiometry is highly dynamic. In a comparative analysis of marine Chlorophyta LHC Chl *a*/*b* ratios in a range from 0.64 up to 0.95 has been described [23]. Moreover, since there is no indication in higher plants that the pigmentation of the LHC can be modified by external factors [24] variability of the pigment distribution of algal LHC *in vivo* has been reported; the xanthophyll as well as the Chl *a*/*b* ratios depend on environmental parameters such as light conditions [25, 26] or the ontogenetic stage [27].

Our investigation aims to answer the following questions: (i) Does algal LHC show *in vitro* similar flexible pigment binding properties as induced by external factors *in vivo*? (ii) What are the binding characteristics of Chl *c*? (iii) How specific is the pigment recognition of algal LHCP?

In this area of special interest, it is useful to perform a comparative analysis of two very different Chl *b* containing LHC. The LHC of *C. fusca* binds beside Chl *a* and Chl *b*, lutein, violaxanthin, α-trishydroxycarotene and neoxanthin, a pigmentation almost similar to that of higher plants. *M. squamata* LHC contains next to Chl *a* and Chl *b* also Chl *c** (Mg 2,4-divinylphaeoporphyrin a_5 monomethyl ester) and as xanthophyll components prasinoxanthin, violaxanthin and neoxanthin [28]. The 30 kDa and 28 kDa polypeptides of *C. fusca* are associated with 18 pigment molecules (8 Chl *a*, 7 Chl *b*, 2–3 xanthophylls) but the 23 kDa protein [29], equivalent to the two 22 kDa and 24 kDa subunits [30] of *M. squamata*, binds 6 Chl *a*, 7 Chl *b*, 1 Chl *c* and 5 xanthophylls [unpublished data]. Therefore, the xanthophyll/chlorophyll ratio seems to be twice as high in *M. squamata* as in *C. fusca* [29]. The LHC of *M. squamata* shows no cross-reaction with antibodies against LHC from other green algae and higher plants, and *vice versa* [31]. These features distinguish the *Mantoniella* type LHC from any other known light-harvesting complex [32]. Furthermore, there is evidence that the pigment pattern of *M. squamata* changes its composition in response to light intensity during growth. High light conditions provide an additional pigment [33] whose identity is under investigation.

Materials and Methods

Plant material

The algal cells were grown under continuous low-light conditions (3 W/m^2) in a growth thermostat (Kniese, Marburg, F.R.G.) for 10 days at 16 ± 1 °C. *Chlorella fusca* (strain C 1.1.10, Shihirira *et* Krauss) was cultured in liquid medium according to [34] with 0.3% CO_2 supplement to the bubbling air source. *Mantoniella squamata* (Plymouth No. LB 1965/5; culture collection, Marine Lab., Plymouth, U.K.) was grown without CO_2 supplement in an artificial sea-water medium according to [35]. The cells were harvested by mild centrifugation

(2000 × *g*, 15 min) at the end of the logarithmic growth phase. The cultures were unialgal but not axenic.

Pigment and thylakoid isolation

The pigments required for the reconstitution procedure (Chl *a*, Chl *b*, Chl c_2, lutein, prasinoxanthin, peridinin and α/β carotene) were isolated from *Chlorella fusca, Mantoniella squamata* and *Amphidinium klebsii* using a preparative HPLC technique (elution gradient see HPLC analysis). The pigments were identified due to the retention time on the column and by spectroscopic analysis. The fractions were collected and absorption spectra recorded using a Hitachi U-2000 spectrophotometer. The peak ratio respective the % III/II peak ratio was determined [36]. The pigment fractions were phase separated by ethyl acetate, washed twice and vacuum-dried at room temperature. Quantification was carried out by absorption in organic solvent and calculated by means of the specific extinction coefficient [37–39]. Thylakoid membranes were prepared as given in [40]. The reconstitution procedure using non-delipidated but heat-denatured thylakoids according to [12] was modified as follows: The thylakoids were solubilized in a buffer containing 100 mM TRIS (HCl), 5 mM 6-aminocaproic acid, 1 mM benzamidine, 12.5% sucrose (wt/vol) and 2% SDS (wt/vol). The pH of the solubilization buffer was adjusted appropriately for the different polypeptides and pigments under investigation (see Results). The chlorophyll content of the thylakoid solution was adjusted to 0.4 mg/ml. After 1 min solubilization at room temperature the thylakoids were centrifuged 10 min at 5000 × *g* to remove starch and remaining cell wall fragments and used directly for reconstitution.

Reconstitution procedure

15 µg pure chlorophylls and/or 10 µg xanthophylls were dissolved in 3 µl diethylether and the same amount of ethanol, transferred to 10 µl of a 100 mM TRIS (HCl) medium (pH adjusted dependent on the complexes under investigation) containing 30% sucrose (wt/vol) and 2% SDS (wt/vol) and successively injected to 30 µl of the thylakoid solution. After addition of 3 µl 1 M dithiothreitol the mixture was vortexed and 1 min at 100 °C heat-denatured. Reconstitution could be achieved by treating the samples with three cycles of freezing (6–12 h) at −22 °C and thawing 10 min at room temperature. In cases of successful reconstitution, the procedure was carried out again in order to obtain a sufficient amount of reconstituted product for comprehensive characterization using 180 µl of the thylakoid solution and 90 µg chlorophylls and/or 60 µg xanthophylls deluted in 2 × 3 µl diethylether/ethanol and 60 µl transfer medium. If two different pigments were used for reconstitution, the weight ratios were 1 : 1 in case of chlorophylls and 3 : 2 for chlorophylls and xanthophylls. The molar pigment ratios of the reconstitution mixture are given in Table I. Routinely, a negative control was performed separating the reconstitution mixture directly after heat denaturation.

Separation of the pigment-protein complexes

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under non-denaturating conditions was performed according to [41]. Analysis of the green slab gels was carried out using a Shimadzu dual-wavelength flying spot scanner CS-9000.

Characterization of the light-harvesting complexes

The native control LHC and the reconstituted products were cut from the gels, homogenized and eluted in 0.05 M TRIS-borate buffer (pH 8.2) by centrifugation (10 min, 6000 × *g*). The eluted complexes were stable over several weeks when stored at −22 °C. Polypeptide composition was analyzed by SDS-PAGE under denaturing conditions according to [42]. The gels were silver-stained by a modified method of [43]. Absorption spectroscopy was performed using a Shimadzu MPS-2000 spectrophotometer. Fluorescence excitation and emission spectra at 77 K were recorded using a Hitachi F-3000 spectrofluorometer as described in [28].

HPLC analysis

The pigments of control and reconstituted LHC were isolated by ethyl acetate and NaCl to produce phase separation. 20 µl of the organic solution was injected to a Nucleosil 120-C_{18}-5 µ column (125 × 8 × 4 mm) on LATEK-P-402 HPLC system and detected at 440 nm by a LATEK spectrophotometer VISI–DUO (Latek, Eppelheim, F.R.G.). The system was controlled by Axxi-Chrom 747

chromatograph datasystem software (Axxiom, Calabasa, U.S.A.). Methanol/water gradients were used as follow: eluent A MeOH/H_2O 60:40 (v/v), 0.5% (v/v) triethylamine, eluent B MeOH (0 min 60% B, 20 min 100% B, 30 min 100% B; pressure at start 130 bar; flow 1.5 ml/min). The method given in [28] was used for calculating the pigment stoichiometry.

Results

Reconstitution products

In Table I the various pigments and the molar pigment ratios of the reconstitution mixture are listed which lead to successful reconstitution of LHC. No reconstitution resulted without chromophore supplement. Homologous as well as hetero-

Table I. *In vitro* reconstituted LHC obtained from a mixture of heat-denatured thylakoids containing an excess of various pigments. Reconstitution was performed using non-delipidated heat-denatured thylakoids under addition of various quantified pigments. Therefore, total proteins and pigments of the thylakoids and additionally given pigments were available for LHC reconstitution. Thylakoids were quantified by chlorophyll determination. The molar pigment ratios of the reconstitution mixtures were calculated by HPLC analysis of thylakoids. Reconstitution required i) the provided excess of pigments and ii) an altered pigment stoichiometry in the reconstitution mixture (molar pigment ratio) in comparison to the thylakoids. No LHC reconstitution could be obtained without chromophore supplement and by adding different amounts of pigments in thylakoidal *in vivo* stoichiometry. Furthermore, changing the molar pigment ratios of Chl *a*/Chl *b*/car 1:0.1:0.07 by adding 90 µg Chl *a* to *C. fusca* thylakoids or Chl *a*/Chl *b*/Chl *c*/car 1:0.2:0.03:0.2 in case of *M. squamata* did not yield reconstitution products under all pH conditions tested. *C. fusca* LHC was reconstitutable independent of the adjusted pH. Compare the decline of the yield when reconstitution was performed with Chl *b* supply under different pH conditions. A pH of 10.3 provided the best possible yield (Chl *a* content [% of control LHC] detected by densitometric analysis) of reconstituted *C. fusca* LHC. In contrast, reconstitution of *M. squamata* LHC required the distinct pH listed. Homologous (HO) and heterologous (HE) reconstituted LHC of both algae could be obtained.

Pigments added	Amount [µg]	Molar pigment ratios Chl *a*/Chl *b*/car	Yield [%]	pH
Chlorella fusca		Thylakoids containing 72 µg total chlorophylls (Chl *a*/Chl *b*/car 1:0.3:0.2)		
Chl *b*	90	1:2 :0.2	47.2	10.3 HO
Chl *b*	90	1:2 :0.2	38.7	8.0 HO
Chl *b*	90	1:2 :0.2	34.6	6.0 HO
Lutein	60	1:0.3:1.9	34.0	10.3 HO
Chl *a* + Chl *b*	90 + 90	1:0.7:0.2	25.1	10.3 HO
Chl *a* + lutein	90 + 60	1:0.1:0.7	39.0	10.3 HO
Chl *b* + lutein	90 + 60	1:2 :1.9	42.3	10.3 HO
Prasinoxanthin	60	1:0.3:1.8	33.6	10.3 HE
Peridinin	60	1:0.3:1.8	35.0	10.3 HE
α/β Carotene	60	1:0.3:1.9	33.6	10.3 HE
Pigments added	Amount [µg]	Molar pigment ratios Chl *a*/Chl *b*/Chl *c*/car	Yield [%]	pH
Mantoniella squamata		Thylakoids containing 75 µg total chlorophylls (Chl *a*/Chl *b*/Chl *c*/car 1:0.7:0.1:0.6)		
Chl *b*	90	1:2.8:0.1:0.6	9.0	8.5 HO
Prasinoxanthin	60	1:0.7:0.1:2.6	4.6	9.5 HO
Chl c_2	90	1:0.7:3.4:0.6	5.7	8.5 HE
Lutein	60	1:0.7:0.1:2.8	5.9	7.5 HE

logous reconstitution products of *C. fusca* and *M. squamata* could be obtained. It has to be emphasized that the total thylakoidal protein and pigment content and the chromophores given in excess, were available in the reconstitution mixture. In homologous complexes, reconstitution was achieved by adding an excess of those pigments which are also present under *in vivo* conditions (inherent chromophores), whereas heterologous products are defined as complexes reconstituted with pigments absent in the native LHC. Although *M. squamata* LHC contains Chl *c**, the complex reconstituted with Chl c_2, is defined as a heterologous complex. Since both Chl *c* species are derived of the chlorophyllide *a* line, Chl *c** has a propionic acid at position 7 of the ring IV, whereas in Chl c_2 an acrylic acid side chain is substituted in this position and a vinyl residue is bound in position 8 [44].

Due to the solubilization of the thylakoids a complete separation of the monomeric form of LHC [41] (control) from the core complex of PS II was achieved by SDS-PAGE under non-denaturating conditions. No dimeric or trimeric LHC was detectable using the solubilization system described (Fig. 1 and 2).

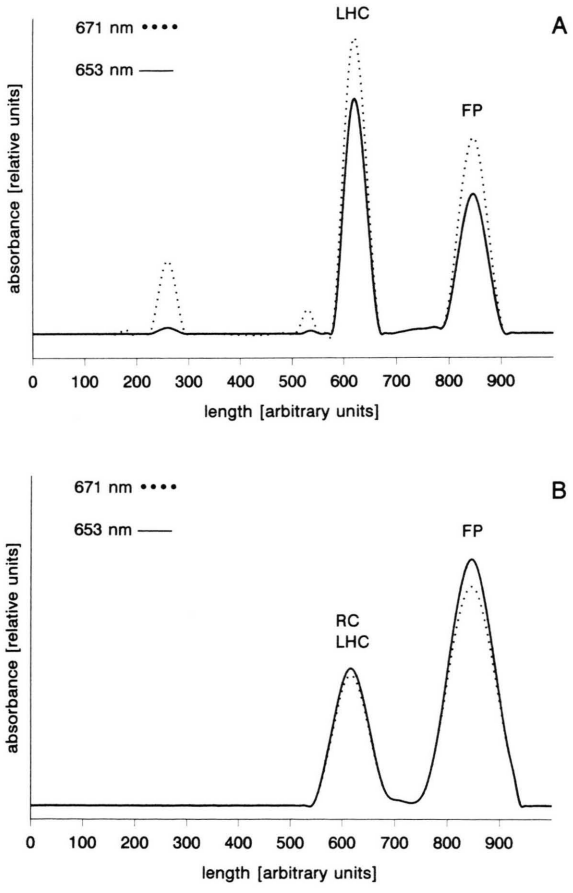

Fig. 1. Densitometric scans of gels after SDS-PAGE under non-denaturing conditions. Separation of solubilized untreated thylakoids, pH 10.3 from *C. fusca*, control LHC (A). Separation of a reconstitution mixture with *C. fusca* thylakoids and Chl *b* supplement, pH 10.3 treated by freeze/thaw cycles (B) RC LHC, reconstituted LHC. Detection wavelengths: 671 nm and 653 nm, baseline 700 nm. Electrophoresis of the reconstitution mixture directly after heat denaturation yielded exclusively a free pigment band (FP).

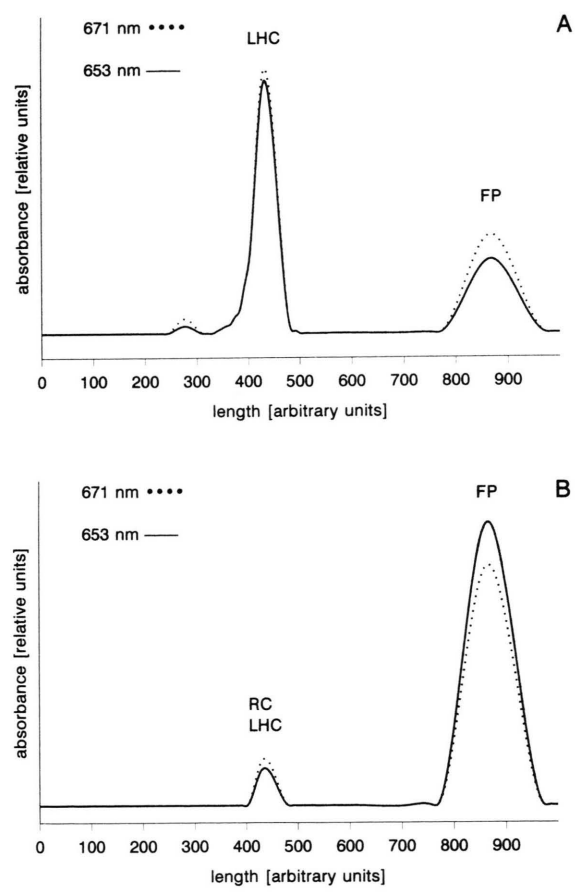

Fig. 2. Densitometric scans of gels after SDS-PAGE under non-denaturing conditions. Separation of solubilized untreated thylakoids, pH 8.5 from *M. squamata*, control LHC (A). Separation of a reconstitution mixture with *M. squamata* thylakoids and Chl *b* supplement, pH 8.5 treated by freeze/thaw cycles (B) RC LHC, reconstituted LHC. Used parameter see Fig. 1.

As revealed by densitometric analysis all reconstituted products (examples are given in Fig. 1 and Fig. 2) exhibited the same R_f values as the native LHC (*C. fusca* 0.86, Fig. 1 B; *M. squamata* 0.72, Fig. 2 B). Due to the excess of pigments a large free pigment band existed together with the reconstituted pigment-proteins. No other pigment-containing complexes were detectable. Only the free pigment band was obtained by electrophoretic separation of the samples directly after heat denaturation.

The reconstitution procedure yielded exclusively LHC from *C. fusca* and *M. squamata*. As verified using SDS-PAGE under denaturing conditions according to [42] polypeptides of the reconstituted complexes exhibited an identical molecular weight to those composing the native LHC. Silver-staining revealed the 28 kDa and 30 kDa subunits of LHC from *C. fusca* and the 23 kDa LHC polypeptides in case of *M. squamata* (data not shown).

Energy transfer of reconstituted LHC was examined by fluorescence excitation and emission spectra at 77 K. The homologous and heterologous reconstituted LHC of *C. fusca* (Fig. 6, Fig. 7) and *M. squamata* (Fig. 10, Fig. 11) revealed efficient energy transfer from the bound chlorophyll and xanthophyll molecules to Chl *a*. The emission maxima (*C. fusca* 682 nm, *M. squamata* 681 nm) partly blue-shifted in heterologous reconstituted LHC. The maximal shift observed was 1.5 nm (Fig. 7 E). Selective excitation of Chl *b* (465 nm, bandpass 3 nm) or Chl *c* (449 nm, bandpass 3 nm) did not alter the fluorescence emission features (spectra not shown). The presentation of spectral data of reconstituted complexes is limited to four examples from *C. fusca* (Fig. 3, 6, 7) and *M. squamata* (Fig. 8, 10, 11).

Characterization of homologous and heterologous reconstituted LHC of C. fusca

Homologously reconstituted LHC of *C. fusca* were achieved by varying the relative amount and stoichiometry of inherent pigments in the reconstitution mixture (Table I). Interestingly, no reconstitution products could be obtained by adding an excess of Chl *a* to thylakoids of *C. fusca* (Table I). Also various amounts of homologous pigments from thylakoids (*in vivo* pigment ratio) did not lead to reconstitution.

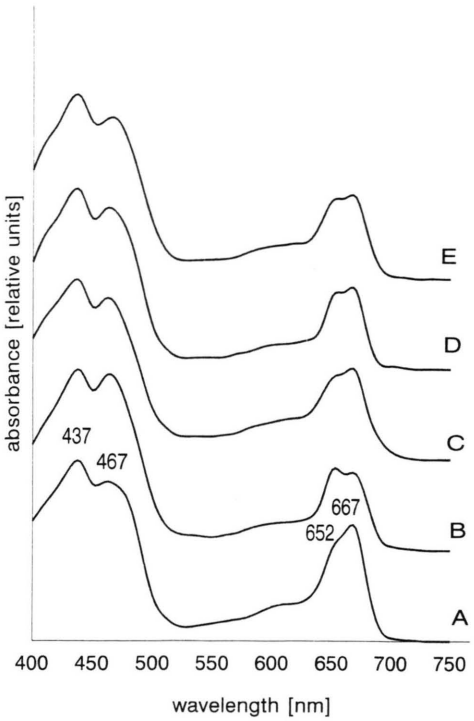

Fig. 3. Absorption spectra of control and reconstituted LHC from *C. fusca*. Control (A). Reconstitution was performed using Chl *b* (B), prasinoxanthin (C), peridinin (D) and α/β carotene (E). Spectra are normalized at the maximum (437 nm).

Fig. 3 shows room temperature absorption spectra of control and reconstituted LHC from *C. fusca*. Excess of Chl *b* (Fig. 3 B) provided an increased absorbance at 652 nm of the reconstitution product. The 467 nm maximum is as high as the 437 nm peak of Chl *a*. This feature indicated a more prominent presence of Chl *b* in this pigment-protein complex. Reconstitution with addition of Chl *b*/lutein leads to a decreased 437/467 ratio while combinations of Chl *a*/Chl *b* and Chl *a*/lutein did not influence the absorption characteristics (spectra not shown).

Quantitative HPLC analysis of the pigment composition verified the conclusions resulting from absorption spectroscopy. Fig. 4 and 5 show the relative pigment distribution of control and reconstituted LHC calculated in mol pigment per 1000 mol Chl *a*. The *C. fusca* complex reconstituted with an excess of Chl *b* (Fig. 4 B) contained one third more Chl *b* molecules (Chl a/b = 0.9) as the control (Fig. 4 A) (Chl a/b = 1.4). Obviously, this

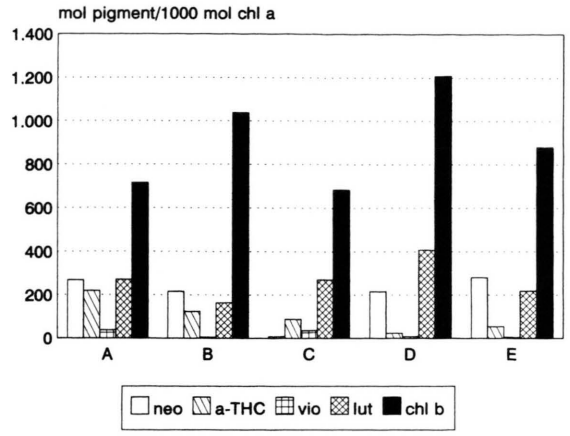

Fig. 4. Quantitative HPLC analysis of control and homologous reconstituted LHC from *C. fusca*. Control (A), Chl *b* (B), lutein (C), Chl *b*/lutein (D) and Chl *a*/Chl *b* (E).

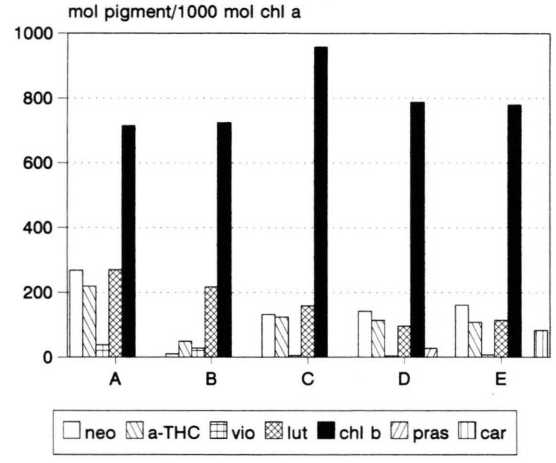

Fig. 5. Quantitative HPLC analysis of control and homologous and heterologous reconstituted LHC from *C. fusca*. Control (A), Chl *a*/lutein (B), peridinin (C), prasinoxanthin (D) and α/β carotene (E). Note the lack of peridinin in the heterologous reconstituted *C. fusca* complex (E).

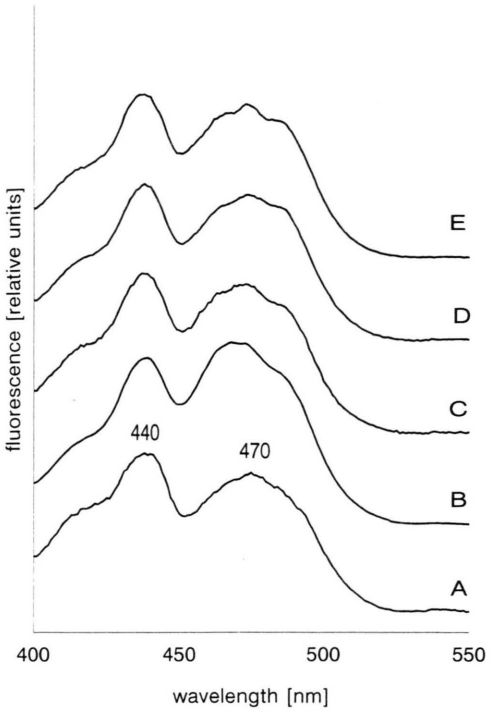

Fig. 6. Fluorescence excitation spectra at 77 K of control and reconstituted LHC from *C. fusca*. Emission wavelength 680 nm; emission bandpass 10 nm; excitation bandpass 3 nm. Spectra are normalized at the maximum (440 nm). Legend see Fig. 3. Note the enhanced transfer from Chl *b* (B) of the Chl *b* reconstituted complex. Compare also Fig. 3 B and Fig. 4 B.

pigment-protein complex possessed an increased energy transfer of the chromophores emitting in the range of 470 nm (Fig. 6 B). The 77 K fluorescence emission spectrum (Fig. 7 B) shows no Chl *b* emission. Therefore, we conclude that a relatively enlarged amount of Chl *b* is functionally arranged by the polypeptides contributing to energy transfer. Enrichment of the reconstitution mixture with Chl *b* and lutein led to an increase of each pigment given in excess (Fig. 4 D); the fluorescence excitation and emission features (spectra not shown) are similar to those of the Chl *b* reconstituted LHC (Fig. 6 B and 7 B). A relative stabilization of the chromophore pattern was observed by addition of Chl *a* and Chl *b* (Fig. 4 E). Also lutein supplement provided a stable pigment distribution regarding Chl *b* and lutein but decreased the relative amount of neoxanthin (Fig. 4 C). The same effect was visible in the complex reconstituted with Chl *a* and lutein (Fig. 5 B).

Heterologous *C. fusca* LHC were obtained by changing the carotenoid supply for reconstitution, quantitatively and qualitatively (Table I). In contrast to reconstitution using LHC inherent chlorophyll species in different stoichiometry, no carotenoid so far tested prevents reconstitution like an excess of Chl *a*.

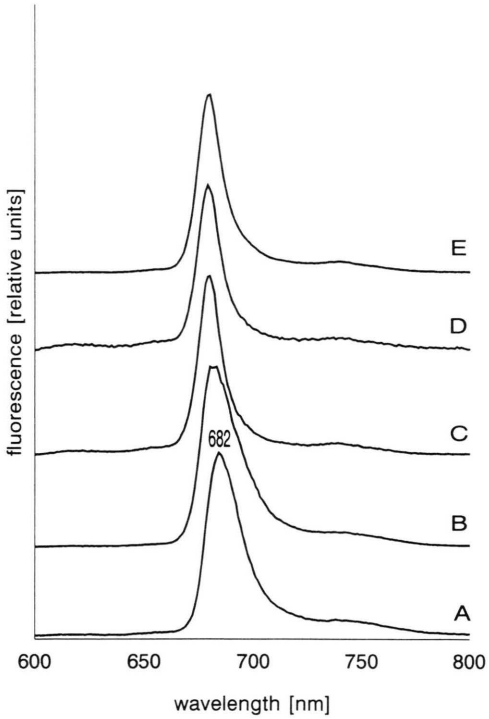

Fig. 7. Fluorescence emission spectra at 77 K of control and reconstituted LHC from *C. fusca*. Excitation wavelength 475 nm; emission bandpass 3 nm; excitation bandpass 10 nm. Spectra are normalized at the Chl *a* emission maximum. Legend see Fig. 3.

The absorption spectra of heterologous reconstituted *C. fusca* LHC (Fig. 3C–3E) are similar to that of the control (Fig. 3A). As presented in Fig. 5D and 5E, LHC polypeptides of *C. fusca* assemble the non-inherent xanthophyll prasinoxanthin and a mixture of α/β carotene. In both cases the Chl *b* contribution was stable and the other xanthophylls reduced. The LHC reconstitute with prasinoxanthin showed no significant absorbance in the region from 500 nm to 540 nm (see Fig. 3C). Nevertheless, the very small relative amount of prasinoxanthin (6.8% of total carotenoids) detected by HPLC was somehow connected to the polypeptides. The apparent heterologous complex reconstituted with peridinin did not assemble this xanthophyll. No peridinin was detectable by HPLC analysis, however, an increase of the relative Chl *b* amount was measured (Fig. 5C). The fluorescence excitation spectrum of the heterologous *C. fusca* complex reconstituted with prasinoxanthin showed no contribution to energy transfer of this xanthophyll in the range of 500 nm to 540 nm (Fig. 6C) as expected, since prasinoxanthin was not detectable by absorption spectroscopy (Fig. 3C).

Characterization of homologous and heterologous reconstituted LHC of M. squamata

The LHC of *M. squamata* was reconstitutable by changing the pigment supply in the reconstitution mixture, quantitatively and qualitatively (Table I). The native LHC of *M. squamata* (Fig. 8A) absorbs light in the range from 500 nm to 540 nm due to xanthophyll prasinoxanthin. Clearly, there are no differences of the control spectrum (Fig. 8A) to the spectra of reconstituted products (Fig. 8B–E). Nevertheless, detailed analysis of the spectra resulted in an increased 437/673 ratio (2.8) and 467/645 (3.2) ratio of the homologous complexes reconstituted with prasinoxanthin (Fig. 8E) and heterologous with Chl c_2 (Fig. 8C) in comparison to the control (2.2 respec-

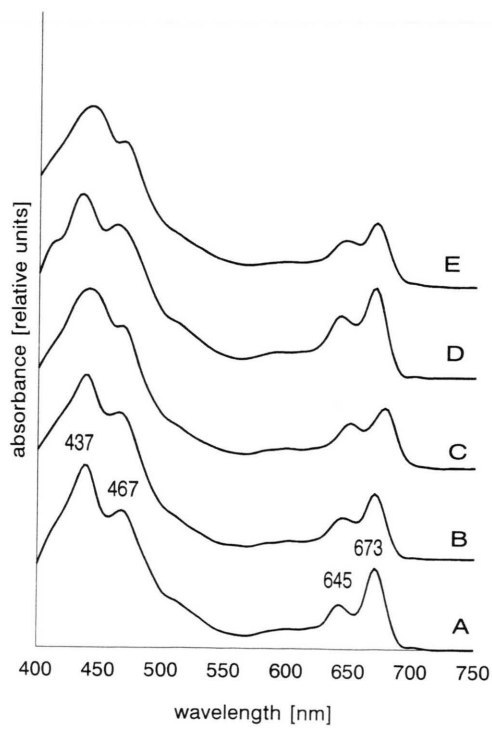

Fig. 8. Absorption spectra of control and reconstituted LHC from *M. squamata*. Control (A), Chl *b* (B), Chl c_2 (C), lutein (D) and prasinoxanthin (E). Spectra are normalized at the maximum (437 nm).

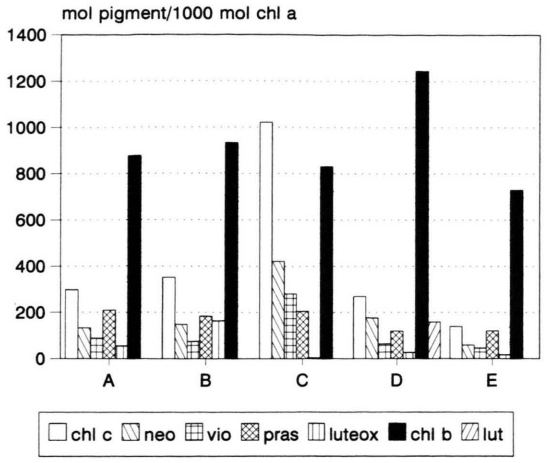

Fig. 9. Quantitative HPLC analysis of control and reconstituted LHC from *M. squamata*. Legend see Fig. 8.

tive 2.8). This observations may indicate that these reconstituted complexes contain relative more blue light absorbing pigment species underlying the spectrum in the blue region, and therefore, increasing the 437 nm and 467 nm peaks. In contrast to *C. fusca* the complex reconstituted with Chl *b* of *M. squamata* (Fig. 9B) showed a comparable pigment composition as the native control LHC (Fig. 9A). A slight decrease of all pigments per 1000 Chl *a* was detected in the homologous prasinoxanthin reconstitute (Fig. 9E).

In contrast to the heterologous reconstituted LHC of *C. fusca* the LHCP of *M. squamata* assemble a high amount of non-inherent chromophores. Lutein, when applied in excess, contributed to 29% of total xanthophylls in the reconstituted *M. squamata* LHC; also an enhanced Chl *b* contribution was detected (Fig. 9D). Supplement of Chl c_2 resulted in a drastic increase of the relative Chl *c* content and a parallel increase of neoxanthin and violaxanthin (Fig. 9C). As revealed by 77 K fluorescence spectroscopy these Chl *c* molecules are functionally arranged providing efficient energy transfer (Fig. 10C), Fig. 11C).

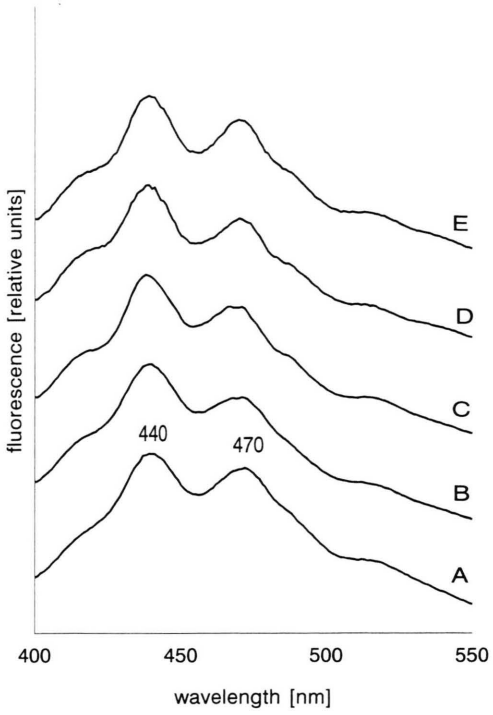

Fig. 10. Fluorescence excitation spectra at 77 K of control and reconstituted LHC from *M. squamata*. Used parameter see Fig. 6. Legend see Fig. 8.

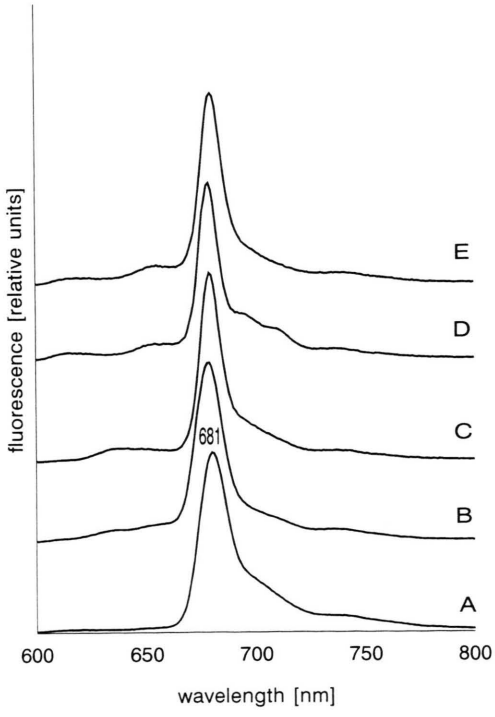

Fig. 11. Fluorescence emission spectra at 77 K of control and reconstituted LHC from *M. squamata*. Used parameter see Fig. 7. Legend see Fig. 8.

pH dependence of successful reconstitution

The most striking difference between the reconstitution behavior of the LHC from *C. fusca* and *M. squamata* is a strong pH control in case of *M. squamata* LHCP. Table I gives the pH values of the mixture leading to successful reconstitution. In case of *C. fusca* the pH 10.3 for the best possible yield (calculated by densitometric analysis: Chl *a* content in percent of control LHC) of reconstitution products is indicated. Reconstitution can also be obtained, by adjusting the pH of 6.0 or 8.0, but the yield of reconstituted LHC decreased, comparatively (Table I). Prevention of reconstitution by varying the pigment stoichiometry of the reconstitution mixture by addition of Chl *a* was observed under all pH conditions tested (Table I). It is clear, that polypeptides from *C. fusca* reassembled the inherent and non-inherent chromophores independently of the pH.

In contrast, reconstitution of *M. squamata* LHC is strongly pH-controlled (Table I). The pH conditions were tested in the pH range from 6.0 to 12.0 in steps of 0.5 pH units. The pH optimum depended on the different pigment species added to the thylakoid solution. Interestingly, using Chl *b* (homologous) or Chl c_2 (heterologous) for reconstitution, both required pH 8.5 although the structure and hydrophobicity of these chromophores are extremely different. Furthermore, no reconstitution products could be achieved by adding Chl *a* to *M. squamata* thylakoids under all pH conditions tested.

Discussion

In this paper we have demonstrated *in vitro* reconstitution of LHC from *C. fusca* (Chlorophyceae) and *M. squamata* (Prasinophyceae). Whereas the polypeptides of the reconstituted LHC were identical of those composing the native LHC, the pigment distribution altered, depending on the different chromophore supplement, as revealed by absorption spectroscopy and quantitative pigment analysis. Heterologous reconstituted LHC as well as changes in pigment stoichiometry of reconstitution products has not yet been observed. These data lead to a new insight in the pigment binding ability of LHC.

Our results from reconstitution of *C. fusca* LHC were partly similar to data obtained from experiments with higher plant LHC II. *C. fusca* LHC was reconstitutable with an excess of various pigments (Table I). A pH of 10.3 in the reconstitution mixture enhanced the yield of reconstituted products comparatively to pH 6 or pH 8. This phenomenon was also observed by [12] using delipidated thylakoids of spinach. The authors suggest that a high pH promotes solubilization of acetone precipitated apoproteins. Since we used SDS-solubilized heat-treated thylakoids an enhanced pigment binding affinity of the polypeptides in an unprotonated state *in vitro* seems to be reasonable, too. Concerning the Chl *a*/*b* ratio and the xanthophyll composition, the native LHC from *C. fusca* accords with the values from higher plants [19, 28]. Comparative analysis of *Chlamydomonas reinhardtii* PS II antenna complexes with those of higher plants supports the notion that both systems are very similar [45]. Highly conserved *cab* gene regions [4] and similarities in hydrophobicity profiles of *cab* protein amino acid sequences [46] of higher plants and green algae are known. Therefore, similar responses to pH changes in reconstitution experiments can be expected and reflect similarities in the molecular requirement and the best suitable protein environment of functional pigment assembly *in vitro*.

In contrast to similarities of LHC of *C. fusca* and higher plants in reconstitution behavior, quantitative HPLC analysis verified changes of the pigment composition in reconstituted *C. fusca* LHC. Enhanced supply of Chl *b* resulted in a complex containing one third more Chl *b* molecules than the control (Fig. 4A, Fig. 4B). These molecules efficiently transfer energy to Chl *a* (Fig. 6B, Fig. 7B). No changes of the Chl *a*/*b* ratio was observed in reconstituted LHC II of *Pea* by varying the Chl *a*/*b* ratio in the mixture [14]. Similarity in pigment composition and relative pigment stoichiometry of pigments in native and reconstituted CP2 complexes were demonstrated by others [12, 16]. Methodological difficulties as discussed by [16] *e.g.* the possible loss of Chl *a* molecules performing SDS-PAGE and, therefore, a relative enhanced Chl *b* portion in reconstituted LHC cannot be neglected, but since a stabilization of the chromophore pattern was obtained by supplement of Chl *a* and Chl *b* (Fig. 4E) a possible loss would affect all complexes treated that way.

Another striking fact is the inhibitory effect of Chl *a*. No reconstitution products could be

achieved adding an excess of Chl *a* to thylakoids of *C. fusca* under all conditions tested. A decline in the yield of reconstituted *Pea* LHC by altering the Chl *a*/*b* ratio from 1 to 3 or 1/3 was reported by [14]. Our data reveal, in agreement with [12], a stabilizing effect of Chl *b* supplement on reconstituted LHC. Investigations of the turnover of LHC apoproteins in rice mutants showed the importance of Chl *b* for stabilizing LHC since the apoproteins inserted into membrane were still unstable unless they were associated with Chl *b* [47]. In contrast to the destabilizing role of an excess of Chl *a* on reconstituted algal LHC, Chl *a* structural stabilizes the P-700 chlorophyll-protein complex of the cyanobacterium *Synechococcus* [48]. Experiments concerning the formation of Chl-proteins during greening revealed a higher affinity of apoproteins of the P-700-chlorophyll *a* protein complex and the chlorophyll *a*-protein complex of PS II for Chl *a* than that of LHC II apoproteins [49]. Also, supplement of phaeophytin *a* or phaeophytin *b* prevents reconstitution of spinach LHC II [12] and *C. fusca* LHC (data not shown).

The essential role of xanthophylls has been pointed out for the formation of stable antenna complexes [12, 14]. Moreover, the requirement of carotenoids has been described for the accumulation of functional pigment-protein complexes *in vivo* [50–53]. LHC polypeptides of *C. fusca* assemble to a relative low amount the non-inherent xanthophyll prasinoxanthin and a mixture of α/β carotene, whereas peridinin provides reconstitution, but was not an integrated part of the complex (Fig. 5). We conclude that peridinin forced reconstitution but cannot be arranged by the polypeptides of *C. fusca*. Exchange of xanthophylls with β carotene did not lead to reassembly of LHC II as well as rearranging the epoxide bonds of xanthophylls with HCl, indicating the integrity of these molecules is critical [12]. Interestingly, prasinoxanthin was bound, but only to 6.8% of total xanthophylls, by *C. fusca* polypeptides. Nevertheless, the assembly of xanthophylls, naturally lacking in LHC, as well as changes in the pigment composition, reflect a higher flexibility in pigment-protein interaction, as generally assumed.

In contrast to the heterologously reconstituted LHC complexes of *C. fusca* the LHC of *M. squamata* arranged lutein to 29% of total xanthophylls (Fig. 9D). The inhibitory effect of Chl *a* was also observed analyzing reconstitution experiments with *M. squamata* under all conditions tested (see Results). We have demonstrated for the first time successful reconstitution of a Chl *c*-containing algal LHC (Table I). Furthermore, we achieved functionally arranged heterologous reconstituted LHC of *M. squamata* by supply of Chl c_2 or lutein (Fig. 10, Fig. 11). A drastic change in the pigment distribution was observed in the heterologous Chl c_2 reconstitute of *M. squamata* (Fig. 9C). Since no Chl *c* or Chl *b* fluorescence was detected (Fig. 11C) these non-inherent chlorophylls were functionally assembled providing efficient energy transfer.

Our data confirm observations *in vivo* that algal LHC possess a high potential variability to alter the pigment composition in response to environmental factors [25, 26]. Furthermore, our results imply that both the stoichiometry of chlorophylls and the defined structure of the porphyrin rings are responsible for correct chlorophyll-protein interaction in algal antenna systems. It has been suggested that water molecules and H-binding of the ring hydrogens may direct the chlorophyll organization in LHC II [10]. If one assumes that protein chlorophyll interaction in LHC occurs as in reaction centers of photosynthetic bacteria [18] the structure of the chlorophyll porphyrin ring is decisive for functional arrangement. Our observations of the high specificity of chlorophyll porphyrin ring recognition by the LHCP confirm this fact. We observed that the exchange of the methyl residue of Chl *a* at position 7 by the formyl group of Chl *b* promotes reconstitution. However, if the methyl group at position 7 is preserved but the ethyl group at position 8 is exchanged by a vinyl group as it is in the case in Chl c_2 as well as in Chl *c**, reconstitution is as successful as in the case of Chl *b*.

Reconstitution experiments of higher plant LHC II provided no indication of a segment of LHCP binding a subset of LHC II pigments and the conclusion was given that the stabilization of LHC is highly synergetic rather than based on individual pigment-binding sites provided by the protein [17]. Self-organization and specific interactions of bacteriochlorophylls in micelles [54] and spontaneous assembly of protein-free bacteriochlorophyll *c* molecules exhibiting pigment-pigment interaction and the chlorosome-like ordered

structures [55] underline the possibility that not only distinct pigment-protein interactions but also pigment-pigment interactions are responsible for stability and functional arrangement of the chromophores in antenna complexes.

The xanthophyll pattern of reconstituted algal LHC is extraordinarily dynamic. Variability of xanthophyll distribution in algal antenna systems has been observed *in vivo*, as a response to external factors [25, 26]. The binding mechanism and respective possible binding sites of xanthophylls by the proteins are unknown. Nevertheless, our data clearly indicate that xanthophyll binding has other properties than chlorophyll assembly. No carotene tested prevents reconstitution of LHC. Either the supplied xanthophyll was bound by the polypeptides or ignored, like peridinin by *C. fusca* LHCP. The observed variability of the xanthophyll pattern contradicts high specifity of xanthophyll recognition by the proteins. From our data a possible binding mechanism cannot be deduced. Further experiments are necessary *e.g.* using double or triple bond xanthophylls to answer questions concerning xanthophyll binding properties of LHC more precisely.

The most striking difference of reconstitution behavior between *M. squamata* LHC and *C. fusca* or higher plant LHC is the strict pH dependence of pigment binding by *M. squamata* LHCP. The distinct pH depends on the chromophores given in excess (Table I). The observed pH dependence reflects different protonation states of the polypeptides and not of the chromophores, when comparing the results obtained from *M. squamata* and *C. fusca*. We are far from understanding why the protonation state of the proteins is significant for pigment binding of *M. squamata* LHCP. But determination of the primary structure of the polypeptides will reveal further information.

As long as we do not know the exact pigment-protein ratio in reconstituted LHC, we are not able to determine whether the reconstituted products are loaded with the same or even higher amount of pigments as present *in vivo*. Furthermore, it is not clear whether a correlation exists between the pH effect and the high xanthophyll binding capacity [29] of the *M. squamata* LHCP. To verify this question quantitative experiments are currently in progress. Altogether, our data reveal peculiarities of membrane-intrinsic algal LHC. The features, which are lacking in higher plant antenna complexes provide particular possibilities to study the mechanisms of pigment-protein interaction.

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