ELSEVIER

Biochimica et Biophysica Acta 1231 (1995) 265–274

BBA Biochimica et Biophysica Acta

Three-dimensional crystallization of the light-harvesting complex from *Mantoniella squamata* (Prasinophyceae) requires an adequate purification procedure

Claudia Welte *, Ralf Nickel, Aloysius Wild

Institute of General Botany, Johannes Gutenberg University, D-55099 Mainz, Germany

Received 12 September 1994; accepted 9 May 1995

Abstract

We present a new purification procedure for the light-harvesting complex of *Mantoniella squamata* whereupon three-dimensional crystallization succeeded. Previous purification methods were based on density centrifugations as the only separating principle. We have extended this preparation procedure by applying anion-exchange and molecular-sieve chromatography techniques. Purity and stability of the complex were proved by denaturing and non-denaturing polyacrylamide-gel electrophoresis, and spectroscopic measurements. With respect to contaminating lipids the purified pigment-protein complex was examined by thin-layer chromatography and the aggregation and/or oligomeric states were investigated by gel filtration, fluorescence emission, and electron microscopy. Molecular-mass determination by gel filtration gave evidence for a trimeric organization of the purified native complex. Our purification procedure resulted in mixed protein-detergent micelles, small and homogeneous, the ideal material to start crystallization trials. We found involuntary aggregation to be easily detected by additional fluorescence emission at red-shifted wavelength (700 nm) as compared to the main emission at 680 nm. Electron-microscopic images proved this emission at 700 nm to be really due to aggregation, which could be prevented if sufficient detergent were present in the samples during the whole purification procedure. The ultimate proof that our efforts have been successful was the appearance of hexagonal crystals.

Keywords: Photosynthesis; Light-harvesting complex; Three-dimensional crystallization; Membrane protein; (*M. squamata*)

1. Introduction

Integral membrane proteins have fundamental functional importance in cellular events such as transport of ions across membranes, cell–cell recognition, signal transduction and energy conversion. The elucidation of molecular mechanisms underlying these processes requires the knowledge of structural details. Structural information can especially be obtained by electron-or X-ray-diffraction studies of two- [1–3] or three-dimensional crystals [4,5]. The success of three-dimensional crystallization of any membrane protein depends predominantly on an appropriate purification procedure yielding great amounts of native homogenous preparations. In this particular case homogeneity was defined as the protein being free of contaminating other proteins and/or lipids. In addition, the purified molecules should be found in one dispersion state [6]. For a long time the optimal approach to such a membrane-protein purification procedure was difficult because, in contrast to globular proteins, membrane proteins are not soluble in aqueous solutions. Only after considering the hydrophobic nature of the integral membrane proteins and therefore adding detergents to the purification-buffer systems, was progress made [7,8]. Especially for the identification of membrane proteins involved in photosynthesis a great step forward could be achieved. The bacterial photosynthetic reaction center was one of the few membrane proteins whose structure could be determined at high resolution [9,10]. Additional bacterial reaction centers have been characterized [11,12]. Furthermore, the structure of the Photosystem I complex of a cyanobacterium has been determined at 6 Å [13] and recently the successful

Abbreviations: LHC, light-harvesting complex; LHC II, light-harvesting complex of Photosystem II; DM, dodecyl maltoside; PAGE, polyacrylamide-gel electrophoresis; SDS, sodium-dodecyl sulfate; PG, phosphatidyl glycerol; DGDG, digalactosyl glyceride; PEG, poly(ethylene glycol); OG, octyl glucoside; Chl, chlorophyll

* Corresponding author. Fax: +49 6131 393787.

SSDI 0005-2728(95)00085-2

three-dimensional crystallization of Photosystem II from spinach was reported [14,15].

One of the most intriguing and yet not satisfyingly elucidated questions of photosynthesis is still the process of absorption of solar energy and the transduction through inner antenna to the reaction centers. The most prominent antenna in higher plants is the major light-harvesting complex of Photosystem II (LHC II), comprising about half of the total chlorophyll in the thylakoid membrane. Despite extensive reconstitution studies [16], the interactions between pigments and the protein are not completely understood. Recently, impressive electron-diffraction data of highly ordered two-dimensional crystals have been reported by Kühlbrandt et al. [17], which provided a three-dimensional map at 3.4 Å. From these data it could be calculated that one LHC II monomer binds 12–13 chlorophylls, two carotenoids and consists of three membrane-spanning α-helices. Structural analysis by electron diffraction, electron microscopy and image processing has resulted in additional conformation data of other light-harvesting systems from several photoautotrophic organisms [18]. Although three-dimensional crystals of the LHC from pea could already be obtained several years ago [19], well-ordered crystals of sufficient size, allowing a higher resolution at the atomic level, are not yet available.

We have chosen the LHC of *Mantoniella squamata* for our attempt to further investigate light-harvesting systems. *Mantoniella squamata*, a green flagellate, is regarded as a primitive organism [20] because of the existence of three different chlorophyll species (a, b and c) within the light-harvesting complex [21]. In contrast to higher plants [22,23], *M. squamata* possesses only one light-harvesting complex, delivering the radiant energy to both photosystems without any preference [20]. Therefore, we considered it important to obtain more information about the pigment–protein interactions, which might give us insight into the process of evolution of the light-harvesting systems.

2. Material and methods

2.1. Culture and growth conditions

M. squamata (Plymouth No. LB 1965/5) was cultivated in an artificial sea-water medium according to Müller [24]. The algal cells were grown under low-light conditions (2 W m^{-2}, 16 h/d) at a temperature of 20°C. The cells were harvested (centrifugation, 2000 × g, 10 min) after 20 days at the late logarithmic growth phase. The procedures were described in detail previously [25].

2.2. Isolation of the light-harvesting complex

All steps were carried out at 4°C or on ice unless specified. Furthermore, it was necessary to protect the material from light exposure.

The isolation in its main features was based on the method described previously by Herold et al. [26]. The collected algal material was resuspended in a 20 mM Bis-Tris buffer (pH 6.5), containing 2 mM $MgCl_2$, 10 mM KCl, 1 mM Na_2-EDTA (isolation buffer). The cells were disrupted by glass-bead homogenization (MSK Braun, Melsungen, Germany) and unbroken cells as well as larger fragments were removed by filtration. The cell homogenate was frozen immediately in liquid nitrogen and thawed for three times. Soluble proteins were removed by centrifugation (30000 × g, 15 min) and solubilization was accomplished with n-dodecyl β-D-maltoside (DM, Sigma). For this purpose the pellet was resuspended in isolation buffer containing 50 mg detergent per mg of total chlorophyll. The chlorophyll content was determined as described by Ziegler and Egle [27]. The suspension was stirred for 60 min at room temperature (24°C) and loaded on a discontinuous (six steps, from 10 to 35%) sucrose gradient. The centrifugation was carried out in a swing-out rotor (TST 28/38, Kontron) at 100000 × g for 16 h at 4°C. The third and largest fraction representing the LHC was collected and directly subjected to further purification.

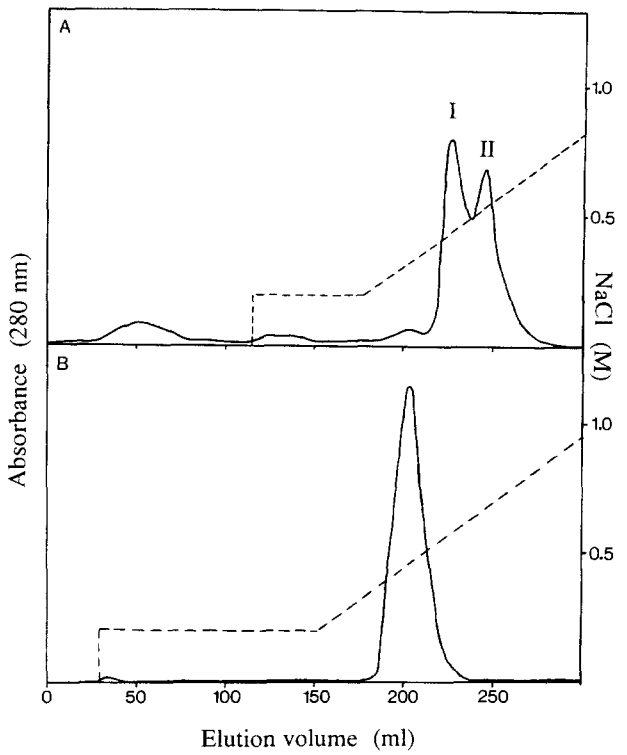

Fig. 1. Anion-exchange chromatography. (A) Elution profile of the first anion-exchange chromatography (Q-Sepharose FF, Pharmacia). Elution of the LHC was performed with a continuous NaCl gradient (0.2–1 M) in 20 mM BTS, 10 mM KCl, 2 mM $MgCl_2$ and 0.5 mM DM (pH 6.5) as indicated (—-). The elution of proteins was monitored by absorption at 280 nm. (B) Elution profile representing the fractionation of the LHC by a second anion-exchange chromatography, developed under the same conditions as described under (A).

2.3. Spectroscopic investigations

Absorption spectroscopy was performed at room temperature using a Shimadzu MPS-2000 spectrophotometer. Further investigations have been made with low-temperature fluorescence spectroscopy at 77 K. Excitation and emission spectra were recorded with a Hitachi F-3000 fluorometer. The total chlorophyll content was adjusted to approx. 0.5 μg/ml.

2.4. Polyacrylamide-gel electrophoresis (PAGE)

Polypeptide composition was analyzed by polyacrylamide-gel electrophoresis under denaturing conditions. Prior to electrophoresis, the pigments were removed by the addition of acetone. The precipitated proteins were redissolved in denaturing buffer according to Laemmli [28] and sonicated for 2 min. After electrophoresis in 18% polyacrylamide gels the polypeptides were silver-stained with the technique described by Heukeshoven and Dernick [29]. Apparent molecular weights were determined using protein standards (protein test mixtures 4 and 5, Serva).

Non-denaturing polyacrylamide-gel electrophoresis was accomplished as described in detail by Dunahay and Staehelin [30]. The pigment-protein complexes were subjected to separation in 10% polyacrylamide gels without further treatment.

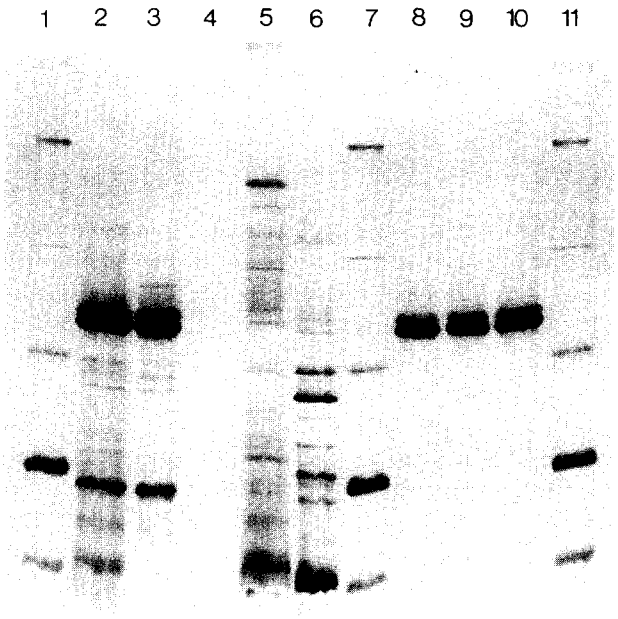

Fig. 2. SDS-PAGE. Polypeptide composition at different stages of the purification. Lanes 1, 7, 11 representing the same protein standards outlined in Fig. 4. Lane 2, polypeptide composition of the cell homogenate; lane 3, LHC isolated with sucrose density centrifugation; lanes 4–8, different fractions from the first anion-exchange chromatography: 4, free pigments; 5, fraction eluted at 200 mM NaCl; 6, fraction eluted at 330 mM NaCl; and 8, LHC (both peaks pooled). Lane 9, LHC eluted from the second anion-exchange chromatography; lane 10, LHC eluted from the gel filtration.

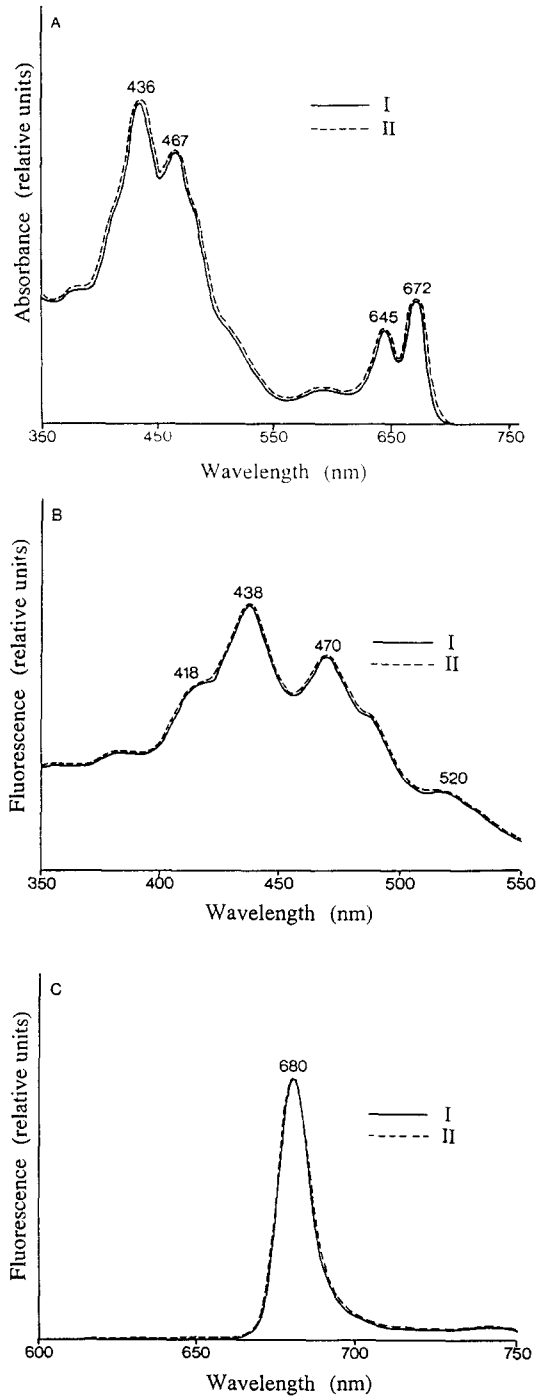

Fig. 3. Spectroscopic characterization of the LHC purified by anion-exchange chromatography. Comparison of the two main peaks (I and II) eluted from the first anion-exchange chromatography. (A) Absorption spectra at room temperature. (B) Fluorescence excitation spectra at 77 K. Emission wavelength 680 nm. (C) Fluorescence emission spectra at 77 K. Excitation wavelength 475 nm. All samples had been adjusted to an equal chlorophyll *a* concentration. The spectra stand representative for all following measurements where no alteration was obvious.

2.5. Lipid analysis

Identification of lipids remaining associated with the purified protein was carried out by thin-layer chromatog-

raphy on precoated silica-gel 60 plates (Merck, Germany). A small volume of concentrated protein solution was either applied without further treatment on the silica gel (see also Nußberger et al. [31]) or lipids were extracted with a mixture of chloroform/methanol 2:1 and 0.7% aqueous NaCl solution as reported elsewhere [32]. The lipids were separated with a solvent system including chloroform/methanol/acetate/water (85:25:15:3) and were visualized by iodine vapor. The relative amounts of the lipids were estimated by comparing the intensity of the lipid spots with those of lipid standards of known concentration. As standards phosphatidylglycerol (PG, Sigma) and digalactosyl glyceride (DGDG, Sigma), two characteristic lipids in thylakoid membranes, were chosen.

2.6. Electron microscopy

Preparation of negatively stained specimens was performed by the single-droplet procedure according to Harris et al. [33]. Carbon-support films were initially glow-discharged for 20 s to render them hydrophilic and adsorptive for the protein. LHC sample (10–20 μl, 0.1 mg/ml) was allowed to adsorb on the carbon film for a few seconds. Salts present in the buffer solution had to be removed by two washing steps with bidistilled water. Grids were finally negatively stained with 2% aqueous uranyl acetate solution. Excess stain as well as all the other liquids before was removed by touching the grid on the edge of a filter paper. The grids were then allowed to dry at room temperature. Electron microscopy was carried out using a Zeiss EM 900 transmission-electron microscope. Micrographs were recorded with a Kodak EM film type 4489. Photographic prints were made on Agfa Brovira speed paper grade 3.

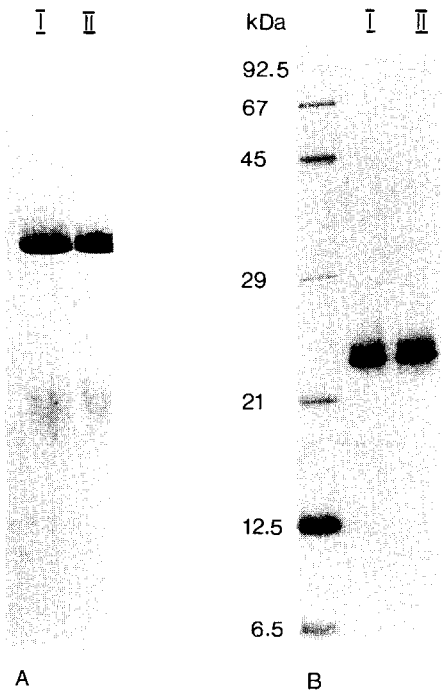

Fig. 4. Electrophoretic characterization of the two fractions (I and II) eluted from the first anion-exchange chromatography on native and denaturing PAGE. (A) Mobility of fraction I and II in native electrophoresis. 30 μg of total chlorophyll have been applied. (B) Polypeptide composition of the LHC under denaturing conditions. 0.5 μg of total chlorophyll have been applied. Lane 2 representing fraction I, lane 3: fraction II eluted from the anion-exchange chromatography, lane 1: protein standards (test mixture 4 and 5, Serva).

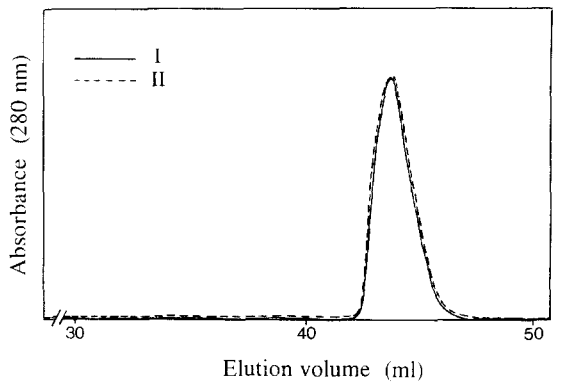

Fig. 5. Gel-filtration chromatography. Gel-filtration chromatography (Sephacryl 300, Pharmacia) indicated the same mobility for both fractions (I and II) eluted from the first anion-exchange chromatography. The gel filtration performed with LHC after the second anion-exchange chromatography gave the same results. The LHC was eluted with a flow rate of 0.1 ml/min in 20 mM BTS, 0.05% NaN_3, 0.5 mM DM and 100 mM NaCl (pH 6.5). The elution of the proteins was monitored by absorption at 280 nm.

3. Results

3.1. Isolation and purification of the LHC

The light-harvesting complex from *M. squamata* was solubilized by the treatment of the isolated thylakoids with the non-ionic detergent DM. For the quantitative isolation of the LHC a concentration of 50 mg DM per mg total chlorophyll was sufficient. Separation from other membrane complexes was achieved by sucrose-density centrifugation. Four pigmented bands were visualized and specified according to Herold et al. [26]. The third and predominant fraction representing the LHC was collected and subjected to further purification including different chromatographic techniques.

The pigment-protein complex was precipitated with 25% (w/v) poly(ethylene glycol) (PEG 4000, Merck) in isolation buffer and pelleted by centrifugation (30000 × g, 20 min). The pellet was redissolved and further solubilized with n-octyl β-glucoside (OG, Sigma) at a concentration of 2.5% (w/v) in the same buffer. Incubation with 60 mg per mg chlorophyll ($a + b$) was allowed for 60 min at room temperature (24°C). Unsolubilized material was removed by centrifugation (30000 × g, 20 min) and the dark-green supernatant was applied immediately to a 2.5 ×

10 cm anion-exchange column (Q-Sepharose FF, Pharmacia). The buffer system used for pre-equilibration of the column was 20 mM Bis-Tris (pH 6.5) containing 2 mM $MgCl_2$, 10 mM KCl and 0.5 mM DM (equilibration buffer). Having its isoelectric point in the range of 4.5 [20,26], the LHC is negatively charged at pH 6.5 and bound strongly to the anion-exchange residues. To avoid random aggregation of the LHC due to non-specific hydrophobic interactions, sufficient detergent (0.5 mM DM) had to be present during the whole purification procedure. Therefore, all following treatments included the same amount of DM. After elution of the free pigments by washing the column with equilibration buffer, several contaminating proteins were already separated with 200 mM NaCl in the equilibration buffer. Bound proteins were further eluted with a linear gradient of NaCl (200 mM–1 M NaCl) in the same buffer at a flow rate of one ml/min. Elution of proteins was monitored by their pronounced

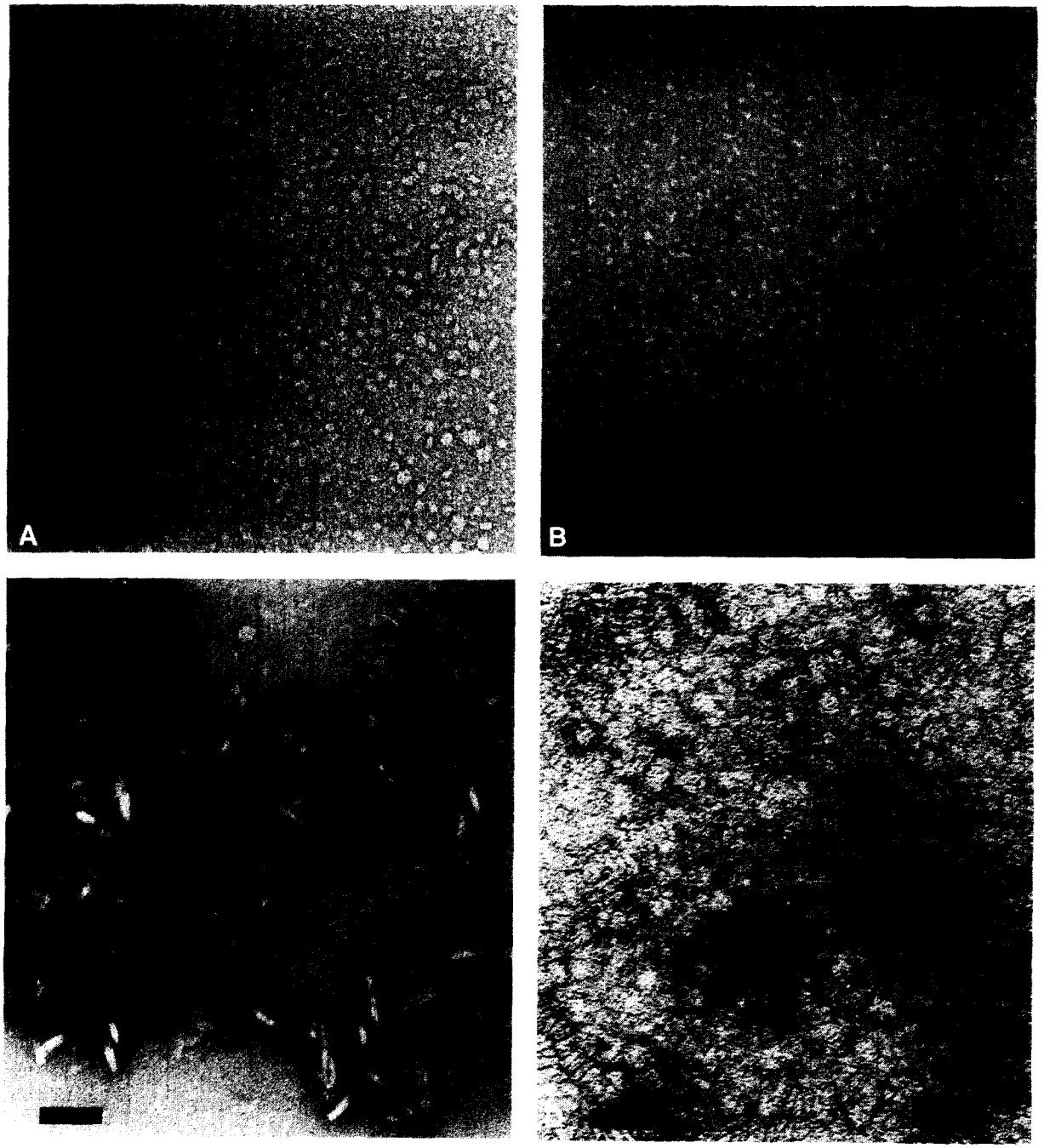

Fig. 6. Electron-microscopic images of the purified LHC, negatively stained with 2% (w/v) uranyl acetate. (A) LHC eluted from the gel filtration in 0.5 mM DM. (B) LHC after detergent exchange in 1% (w/v) OG. (C) LHC, dialyzed against detergent free buffer. (D) Crystal fragments (arrowheads) of the LHC. Crystals, obtained as described in the legend of Fig. 8 were dissolved in crystallization buffer. String-like aggregates at side view were visuable. Scale bars in (A), (B) and (C) indicate 150 nm; scale bar in (D) indicates 90 nm.

absorption at 280 nm. All peaks shown in the elution profile (Fig. 1A) were analyzed for polypeptide composition (Fig. 2).

Spectroscopic characterization revealed identical absorption and fluorescence features of the two main peaks (I and II), which are characteristic for the light-harvesting complex (Fig. 3). In addition, the same chlorophyll *a/b* ratios were determined. Native and denaturing gels gave further evidence for the identity of the material from both fractions (Fig. 4). Last of all, protein of the two main fractions eluted at the same time from the gel-filtration column (Fig. 5). Because of all this evidence for an identity of peak I and II, fractions containing LHC were pooled and concentrated to 3 ml in a stirred ultrafiltration cell using a 10 kDa exclusion membrane (Omega Cell, Filtron). The concentrated LHC solution was desalted (Pd 10 DG, Bio-Rad) and applied for a second time to anion-exchange chromatography. For this purpose the column was prepared and developed in the same manner as for the first run, except that the washing of the column was directly performed with 200 mM NaCl. The LHC eluted in one peak at a concentration of NaCl of about 420 mM (Fig. 1B).

After repeated concentration of the LHC fractions, the pigment-protein complex was subjected immediately to molecular-sieve chromatography (Sephacryl S-300 HR, Pharmacia). The buffer used was 20 mM Bis-Tris (pH 6.5) containing 0.5 mM DM, 100 mM NaCl and, to avoid growth of microorganismns, 0.05% NaN_3. The flow rate was adjusted to 0.1 ml/min and 2 ml fractions were collected. Only one peak was visualized by its absorption at 280 nm similar to those shown in Fig. 5.

3.2. Characterization of the purified LHC

The chlorophyll *a/b* ratio, varying slightly between different purifications, was determined to be in the range of 0.87–0.9, suggesting a high purity of the complex. The absorption spectrum of the purified complex was identical to those of the LHC fractions (I and II) obtained by the first anion-exchange chromatography with typical maxima at 436 and 672 nm (Chl *a*) and 467 and 645 nm (Chl *b*).

After such an extensive purification procedure, the question arose whether the pigment-protein complex remains functionally intact as light-absorbing and energy-transferring system. In order to analyze the intactness of the purified LHC low-temperature fluorescence spectra were recorded. Excitation spectra (Fig. 3B) indicated that the pigments involved in light absorption efficiently contributed to the energy transfer to chlorophyll *a*. As judged by fluorescence-emission studies, where only chlorophyll *a* emission at 680 nm was detectable, this energy transfer was not disrupted during the purification process (Fig. 3C). Absorption as well as fluorescence spectra seemed to be rather similar at each stage of the purification procedure.

The increase in purity during the purification procedures could be demonstrated by SDS-PAGE (Fig. 2). Contaminating, unspecific polypeptides were still present in the sucrose-density fraction, whereas already the first anion-exchange chromatography resulted in the apparent pure LHC. There is evidence that the LHC of *M. squamata* consists of two different polypeptides with molecular masses of 23.5 and 25 kDa, respectively. Similar results were reported earlier [34].

As revealed by lipid analysis, almost all lipids have been removed by the chromatography procedures. We were not able to detect any differences between the two peaks eluted from the first anion-exchange column with regard to lipid contamination. We have, however, evidence for the presence of minor amounts of lipids in the purified protein. By thin-layer chromatography we found several minor spots, two of them comigrating with PG and DGDG standards. These residual lipids might be necessary for the maintenance of the organization of the LHC. Because of the limited resolution of the method it is not possible to obtain more detailed information.

The purified LHC exists in one oligomeric form as judged by gel-filtration chromatography (Fig. 5). After calibration of the gel-filtration column with six different marker polypeptides, the molecular mass of this oligomer in detergent solution could be determined to be 170 kDa. This is in agreement with a trimeric organization of the LHC. The trimer appears to be biochemical and physical stable, as can be concluded from the results of exposure to a range of temperatures and pH values, as well as long-term storage. Native gel electrophoresis confirmed that the LHC consists in one oligomeric state.

To obtain more information concerning the homogeneity of the LHC suggested by gel filtration and native gel electrophoresis, the LHC preparations were analyzed by electron microscopy. As visible in the negative contrast, our purification procedure resulted in mixed protein-detergent micelles of mostly the same size (Fig. 6A), the ideal material to start crystallization trials.

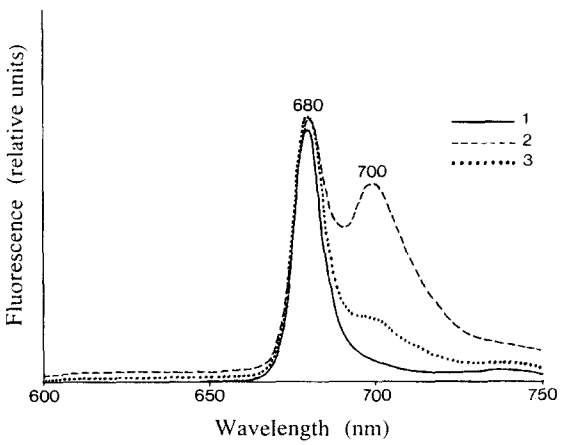

Fig. 7. Fluorescence-emission spectra for aggregated (2) and disaggregated (1) LHC at 77 K. Detergent induced disappearance of the long wavelength emission (3).

For crystallization experiments we chose the detergent OG. The necessary exchange of the detergent DM for OG was achieved using the method described by Guthrie et al. [35]. Detergent exchange was performed by anion-exchange chromatography using Q-Sepharose FF again as the material of choice. The purified LHC was loaded without further concentration on the 1×5 cm packed column. Detergent exchange occurred while washing the column with at least 4 volumes of 20 mM Bis-Tris, 0.05% NaN_3 (pH 6.5) containing OG or any other detergent which was examined in the crystallization experiments. The complex was eluted in one step with 600 mM NaCl in the same buffer. Neither fluorescence nor absorption spectra revealed any alteration in spectroscopic features of the LHC during detergent exchange and were similar to those shown in Fig. 3. As indicated by electron-microscopic images, the size of the protein-detergent micelles was reduced in the case of OG compared to DM (Fig. 6B).

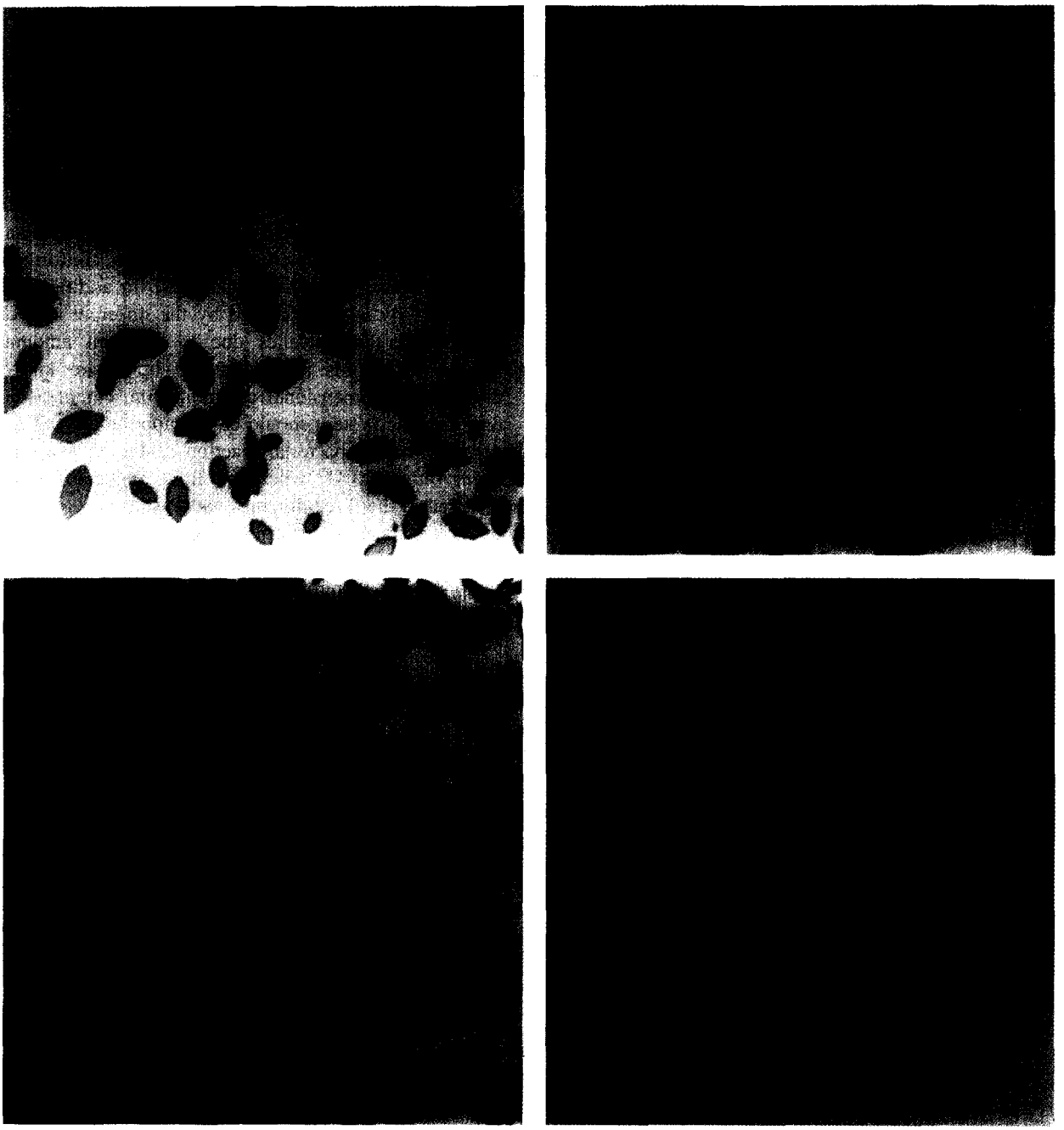

Fig. 8. A few examples of crystal forms found for the LHC of *Montoniella squamata*. The approximately size of these hexagonal or square-like crystals is 10–50 μm. The crystals formed at a chlorophyll concentration of 4–5 mg/ml and a precipitant starting concentration of 2% PEG/500 mM NaCl. Crystallization was achieved at pH 6.5 by vapor diffusion against 20% (w/v) PEG 4000 and 1 M NaCl at 20°C. Besides 0.97% OG, 0.5% 1,2,3-heptantriol seems to be necessary for crystal formation. In 3–4 weeks, crystal growth started at the edges of the drop.

Prior to crystallization experiments the purified LHC was finally concentrated by ultrafiltration, accomplished as mentioned above. The concentrated solution was relieved from amorphous or randomly aggregated material, whichever might arise from traces of denatured protein in the solution. Involuntary unspecific aggregation could be recognized with negative-contrast electron microscopy (Fig. 6C). Fluorescence-emission spectroscopy provides another, more rapid method to detect aggregates of the LHC. LHC samples dialyzed for several days against buffer, without any detergent, showed an additional emission at approx. 700 nm beside the main chlorophyll *a* emission at 680 nm (Fig. 7, (2)). This long-wavelength emission appeared already in protein samples diluted with detergent-free buffer. In contrast, adding increasing amounts of detergent to the protein samples led to a stepwise decrease of the emission at 700 nm until finally the original spectra were obtained again (Fig. 7, (3,1)). Moreover, each time the long-wavelength emission was noticed, aggregates could be detected in the negative contrast. However, additional emission at 700 nm might indicate starting crystallization as well (Fig. 6D).

Crystallization experiments performed with this material were accomplished by vapor diffusion with the hanging-drop procedure described in detail by McPherson [36]. Specification of the crystallization conditions are given in the legend of Fig. 8.

By the above described procedure we obtained high amounts of LHC-protein of a purity apparently sufficient for crystallization. Within 3–4 weeks crystals of various shape appeared (Fig. 8).

4. Discussion

In the present study we introduced a new method for purification of the LHC from *M. squamata* whereupon three-dimensional crystallization succeeded. The most useful detergent for the solubilization of the LHC was DM. Thereby, the solubilization could be achieved under conditions mild enough to obtain the LHC in the native state as confirmed by different spectroscopic measurements and non-denaturing electrophoresis. Separation of the LHC from other pigment-protein complexes by sucrose-density centrifugation could be shown to be suitable as a first enrichment step of the LHC. Detergent concentrations far below 80 mg per mg of total chlorophyll were still sufficient for the separation of the LHC. Therefore, we reduced the detergent to chlorophyll ratio to avoid superfluous losses of the photosynthetic pigments. Moreover, among different isolation procedures for pigment-protein complexes sucrose-density centrifugation is undoubtedly one of the most gentle. Unfortunately, traces of contaminating proteins were still present after this procedures. These contaminating proteins could be removed by an additional density centrifugation step. After these two density centrifugation steps, there was apparently no longer any contamination by other proteins. However, crystallization never occurred.

Although purity is without any doubt one of the main requirements in successful crystallization, it is not a sufficient criterion if it stands alone. Other Sources of microheterogeneity of the LHC should be taken into account. For crystallization, the homogeneity of the protein solution to be crystallized should be as high as possible. This means, for proper incorporation of the proteins into the crystal lattice, all individual molecules should share the same physical and chemical state. Modified molecules do not fit in the lattice or cannot interact with other molecules and therefore will serve as inhibitors of crystal growth. The elimination of microheterogeneity in the protein environment is difficult to achieve due to the uncountable reasons being possibly responsible for it. For example, during the purification procedure the protein could undergo proteolytic modification and/or coexist in different aggregation or oligomeric states, being conformational unstable [37]. Those heterogeneities at the conformational level are very difficult to detect [38]. In addition, microheterogeneity is often caused by the different size of the protein-detergent micelles due to different amounts of incorporated lipids [1,5]. Because lipids are known to disturb three-dimensional crystallization, complete delipidation of the protein should be presupposed [39]. This was possible for the bacterial reaction center of *Rhodobacter sphaeroides* [40,41] where no lipid molecules could be detected in the three-dimensional crystals.

As we were not certain about the amounts of lipids present in our LHC preparations, we examined the LHC sample for lipids remaining at the protein. Having detected high contents of different lipids within the LHC preparation we decided to apply further purification principles such as anion-exchange chromatography to remove lipid-induced microheterogeneity. Anion-exchange chromatography is a straightforward method to yield structurally identical LHC II [31] and is at the same time well known to be very suitable for the elimination of excess lipids due to their property to interact with the column material [42,43]. Further advantages of this method include the short preparation time and the high reproducibility of the method [44]. To achieve crystallization, chromatographical methods for the purification of pigment-protein complexes have been applied in an increasing number [45–50]. So we replaced the second density centrifugation step by repeated solubilization followed by anion-exchange and molecular-sieve chromatography. Sugars and poly(ethylene glycol) persisting in the solution do not interfere with the column material [51], and therefore it was not necessary to dialyze the LHC-fraction collected from the sucrose-density gradient. The LHC was solubilized additionally with OG. OG possesses a smaller hydrophilic headgroup than DM and could better displace lipids associated with the protein.

To our surprise, in the first anion-exchange chromatog-

raphy the LHC fractionated into two distinct peaks containing spectroscopically identical material. Different phosphorylation states of the LHC described by Kühlbrandt and Barber [52] to be the reason for two peaks of the same pigment-protein complex in the anion-exchange chromatography could be excluded. In a second anion-exchange step the peak stoichiometry changed. This should not be the case for different phosphorylation states. Further on, short-term regulation by phosphorylation of the LHC due to state I–state II transitions have never been found for *M. squamata* [53]. Another possible source for heterogeneity might be the difference in the aggregation state between the two fractions as found to be the case for the light-absorbing system of *Rhodospirillum molischianum* [54]. In our purification procedure, different oligomeric states of the LHC could also be excluded because the LHC from both anion-exchange fractions eluted from the gel filtration in one single peak which means all molecules of the sample share the same size. This is supported by results of native-gel electrophoresis, where the material of the two fractions showed the same mobility. We were convinced that the two peaks have their origin in different amounts of lipids remaining associated with the protein. Germeroth et al. [47] explained the appearance of two peaks during the purification of the B 800-850 LHC of *Rhodospirillum molischianum* found by Kleinekofort et al. [54] to be caused by different amount of phospholipids. However, we were not able to make a distinction between peak I and peak II with regard to lipid quality and quantity due to the limitations of thin-layer chromatography. With our knowledge of lipids interacting with column materials we anticipate further removal of lipids by a second anion-exchange run. From this second run the LHC eluted in one peak and indeed we found decreased lipid contents.

The question arises whether the not further specified residual lipids associated with the LHC of *M. squamata* have any function. The LHC II of higher plants lost 70–90% of the thylakoid lipids during purification [31] while DGDG and PG, two important lipids of the thylakoid membrane [55] could still be visualized. These lipids cannot be removed with non-ionic detergents and might therefore be essential for the stability of the complex. They seem to stabilize the trimers which are a prerequisite for crystallization [31]. Complete delipidation achieved by phospholipase treatment led to disassembly of the native trimeric complex into monomers which lost their ability to crystallize. Furthermore, Krupa et al. [56] could show that especially PG with the fatty acid residue 16:1 supports trimerization. We suggest that the lipids remaining with the LHC of *M. squamata* might have a similar function for the stability of this pigment-protein complex.

As judged by gel filtration, the molecular mass for the LHC of *M. squamata* was determined to be 170 kDa. Assuming that 20–25% of the examined molecular mass is due to the incorporated detergent [57], the calculated molecular mass of approx. 125–135 kDa fits very well with a trimeric model. Further evidence for trimeric organization comes from the comparable mobility of the LHC of *M. squamata* and the LHC of spinach which is known to exist as a trimer (data not shown). A trimeric organization for the LHC of *M. squamata* would also fit well the results of Rhiel et al. [58], who found LHC particles in freeze-fractured thylakoids to be equivalent in size to LHC II of higher plants. Unlike the situation for the LHC of pea [31], detergent treatment with increased concentrations of OG for several days did not result in disaggregation of the complex. Also, long-term storage for several months at 4°C did not destroy the complex. Therefore, the supposed trimer is the only oligomeric state. This is also supported by the fact that the LHC eluted in one peak from the gel-filtration column.

In addition, further investigations concerning the aggregation state have been performed using fluorescence-emission spectroscopy supplemented by electron microscopy. The emission spectra of the purified protein eluted from the gel-filtration column showed only chlorophyll *a* emission at 682 nm when excited at 475 nm. Hence, the accessory chlorophylls were not disconnected during the purification procedure. Several conditions, such as long-time storage of the sample, detergent removal by dialysis or simple diluting the solution with detergent-free buffer as well as cation addition to the solution led to additional emission at approx. 700 nm. On the other hand, this long-wavelength emission disappeared upon reexposure of the dialyzed LHC with increasing concentrations of detergent. Our conclusion that this additional peak at long wavelength was due to the unspecific aggregation of the light-harvesting complex is in agreement with similar observations by Mullet and Arntzen [59] and other authors [60,61]. Further insight into the aggregation state was achieved by transmission-electron microscopy. As mentioned previously [6], negative staining is a good method to investigate the aggregation state of isolated membrane proteins. Therefore, it should be applied in early steps of three-dimensional crystallization.

Our purification procedure resulted in mixed protein-detergent micelles most of them small and homogeneous. The final success of crystallization of the LHC is the best indication for the suitability of the purification procedure presented in this work.

Acknowledgements

We thank Dr. J.R. Harris (Institute of Zoology, Mainz) for his assistance with negative-contrast electron microscopy and Dr. M. Richter (Institute of General Botany, Mainz) for reading the manuscript. This reasearch was supported by the Landesgraduiertenförderung Rheinland Pfalz.

References

[1] Kühlbrandt, W. (1992) Q. Rev. Biophys. 25 (1), 1–49.
[2] Jap, B.K., Zulauf, M., Scheybani, T., Hefti, A., Baumeister, W., Aebi, U. and Engel, A. (1992) Ultramicroscopy 46, 45–84.
[3] Boekema, E.J. (1990) Electron Microsc. Rev. 3, 87–96.
[4] Garavito, R.M. and Picot, D. (1991) J. Crystal Growth 110, 89–95.
[5] Kühlbrandt, W. (1988) Q. Rev. Biophys. 21 (4), 429–477.
[6] Boekema, E.J. (1991) Micron Microsc. Acta 22 (4), 361–369.
[7] Michel, H. and Oesterhelt, D. (1980) Proc. Natl. Acad. Sci. USA 77, 1283–1285.
[8] Garavito, R.M. and Rosenbusch, J.P. (1980) J. Cell Biol. 86, 327–329.
[9] Deisenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, H. (1985) Nature 318, 618–624.
[10] Deisenhofer, J. and Michel, H. (1989) Science 245, 1463–1473.
[11] Allen, J.P. and Feher, G. (1991) in Crystallization of Membrane Proteins (Michel, H., ed.), pp. 137–153, CRC Press, Boca Raton.
[12] Kerfeld, C.A., Thornber, J.P. and Yeates, T.O. (1993) Protein Sci. 2, 1352–1355.
[13] Krauss, N., Hinrichs, W., Witt, I., Fromme, P., Pritzkow, W., Dauter, Z., Betzel, C., Wilson, K.S., Witt, H.T. and Saenger, W. (1993) Nature 361, 326–331.
[14] Adir, N., Okamura, M.Y. and Feher, G. (1992) in Research in Photosynthesis (Murata, N., ed.), Vol. 2, pp. 195–198, Kluwer, Dordrecht.
[15] Fotinou, C., Kokkinidis, M., Fritzsch, G., Haase, W., Michel, H. and Ghanotakis, D.F. (1993) Photosynth. Res. 37, 41–48.
[16] Paulsen, H. and Kuttkat, A. (1993) Photochem. Photobiol. 57 (1), 139–142.
[17] Kühlbrandt, W., Wang, D.N. and Fujiyoshi, Y. (1994) Nature 367, 614–621.
[18] Cogdell R.J. and Hawthornthwaite, A.M. (1993) in The Photosynthetic Reaction Center (Deisenhofer, J. and Norris, J.R., eds.), Vol. 1, pp. 23–42, Academic Press, New York.
[19] Kühlbrandt, W. (1987) J. Mol. Biol. 194, 757–762.
[20] Schmitt, A., Herold, A., Welte, C., Wild, A. and Wilhelm, C. (1993) Photochem. Photobiol. 57 (1), 132–138.
[21] Wilhelm, C. (1987) Biochim. Biophys. Acta 892, 23–29.
[22] Thornber, J.P., Peter, G.F., Morishige, D.T., Gomez, S., Anandan, S., Welty, B.A., Lee, A., Kerfeld, C., Takeuchi, T. and Preiss, S. (1993) Biochem. Soc. Trans. 21, 15–18.
[23] Marquardt, J. and Bassi, R. (1993) Planta 191, 265–273.
[24] Müller, D. (1962) Bot. Mar. 314, 140–155.
[25] Wilhelm, C., Lenartz-Weiler, I., Wiedemann, I. and Wild, A. (1986) Phycologia 25, 304–312.
[26] Herold, A., Schmitt, A., Wilhelm, C. and Wild, A. (1991) Photosynthetica 25 (4), 645–653.
[27] Ziegler, R. and Egle, K. (1965) Beitr. Biol. Pflanzen 41, 11–37.
[28] Laemmli, U.K. (1970) Nature 227, 680–685.
[29] Heukeshoven, J. and Dernick, R. (1985) Electrophoresis 9, 103–112.
[30] Dunahay, T.G., Staehelin, L.A. (1986) Plant Physiol. 80, 429–434.
[31] Nußberger, S., Dörr, K., Wang, D.N. and Kühlbrandt, W. (1993) J. Mol. Biol. 234, 347–356.
[32] Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 912–917.
[33] Harris, J.R., Gebauer, W. and Markl, J. (1993) J. Struct. Biol. 111, 96–104.
[34] Lee, C.M., Osterbauer N., Jiao, S. and Fawley, M.W. (1992) J. Phycol. 28, 309–311.
[35] Guthrie, N., MacDermott, G., Cogdell, R.J., Freer, A.A., Isaacs, N.W., Hawthornthwaite, A.M., Halloren, E. and Lindsay, J.G. (1992) J. Mol. Biol. 224, 527–528.
[36] McPherson, A. (1982) Preparation and Analysis of Protein Crystals, p. 96, John Wiley & Sons, New York.
[37] McPherson, A. (1991) in Crystallization of Membrane Proteins (Michel, H., ed.), pp 1–51, CRC Press, Boca Raton.
[38] Giege, R., Dock, A.C., Kern, D., Lorber, B., Thierry, J.C. and Moras, D. (1986) J. Crystal Growth 76, 554–561.
[39] Garavito, R.M., Jenkins, J.A., Jansonius, J.N., Karlsson, R. and Rosenbusch, J.P. (1983) J. Mol. Biol. 164, 313–327.
[40] Allen, J.P., Feher, G., Yeates, T.O., Komiya, H. and Rees, D.C. (1987) Proc. Natl. Acad. Sci. USA 84, 6162–6166.
[41] Chang, C.H., El-Kabbani, O., Tiede, D., Norris, J. and Schiffer, M. (1991) Biochemistry 30, 5352–5360.
[42] Garavito, R.M. and Picot, D. (1991) J. Crystal Growth 110, 89–95.
[43] Reiss-Husson, F. (1992) in Crystallization of Nucleic Acids and Proteins (Ducruix, A. and Giege, R., eds.), pp. 175–193, Oxford University Press, New York.
[44] Lin, S.-X., Sailofski, B., Lapointe J. and Zhou, M. (1992) J. Crystal Growth 122, 242–245.
[45] Dekker, J.P., Boekema, E.J., Witt, H.T. and Rögner, M. (1988) Biochim. Biophys. Acta 936, 307–318.
[46] Nunn, R.S., Artymiuk, P.J., Baker, P.J., Rice, D.W. and Hunter, C.N. (1992) J. Mol. Biol. 228, 1259–1262.
[47] Germeroth, L., Lottspeich, F., Robert, B. and Michel, H. (1993) Biochemistry 32, 5615–5621.
[48] Ghosh, R., Hoenger, A., Hardmeyer, A., Mihailescu, D., Bachofen R., Engel, A. and Rosenbusch, J.P. (1993) J. Mol. Biol. 231, 501–504.
[49] Buchanan, S.K., Fritzsch, G., Ermler, U. and Michel, H. (1993) J. Mol. Biol. 230, 1311–1314.
[50] Tsiotis, G., Nitschke, W., Haase, W. and Michel, H. (1993) Photosynth. Res. 35, 285–297.
[51] Scopes, R.K. (1987) Protein Purification –Principles and Practice, Springer, Berlin.
[52] Kühlbrandt, W. and Barber, J. (1988) Biochim. Biophys. Acta 934, 118–122.
[53] Wilhelm, C., Krämer, P. and Lenartz-Weiler, I. (1989) Photosynth. Res. 20, 221–233.
[54] Kleinekofort, W., Germeroth, C., Van den Broek, J.A., Schubert, D. and Michel, H. (1992) Biochim. Biophys. Acta 1140, 102–104.
[55] Murphy, D.J. (1993) in Plant Biochemistry and Molecular Biology (Lea, P.J. and Leegood, R.C., eds.), pp. 113–128, John Wiley, New York.
[56] Krupa, Z., Williams, J.P., Khan, M.U. and Huner, N.P.A. (1992) Plant Physiol. 100, 931–938.
[57] Dekker, J.P., Betts, S.D., Yocum, C.F. and Boekema, E.J. (1990) Biochemistry 29, 3220–3225.
[58] Rhiel, E., Lange, W. and Mörschel, E. (1993) Biochim. Biophys. Acta 1143, 163–172.
[59] Mullet, J.E. and Arntzen, C.J. (1980) Biochim. Biophys. Acta 589, 100–117.
[60] Ruban, A.V. and Horton, P. (1992) Biochim. Biophys Acta 1102, 30–38.
[61] Mullineaux, C.W. (1993) Biochim. Biophys. Acta 1143, 235–238.