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The major light-harvesting pigment protein of *Acaryochloris marina*

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Abstract The major light-harvesting protein complex containing chlorophyll (Chl) *d* was isolated from *Acaryochloris marina* thylakoid membranes. Isolation was achieved by detergent solubilisation followed by separation on 6–40% sucrose gradients using ultracentrifugation. The best Chl *d* yield (70%) used 0.3% dodecyl maltoside, 0.15% octyl glucoside, 0.05% zwittergent 3-14 with the detergent:total Chl *d* ratio around 10:1 (w/w). Characterisation of the light-harvesting pigment protein complex (lhc) involved non-denaturing electrophoresis, SDS–PAGE, absorbance and fluorescence spectroscopy. The main polypeptide in the lhc was shown to be ca. 34 kDa and to contain Chl *d* and Chl *a*, indicating that the *Acaryochloris* lhc is similar to that of prochlorophytes. The Chl *a* level varied with the culture conditions, which is consistent with previous findings.

Key words: Chlorophyll *d*; Light-harvesting pigment proteins; Chl *a*/*b* protein; Photosynthesis; *Acaryochloris marina*

1. Introduction

Cyanobacteria (or Cyanophyceae) have been recognised since the 19th century and the Cyanobacteria were defined initially as photosynthetic oxygenic organisms having chlorophyll (Chl) *a* and phycobiliproteins. However, recent discoveries have necessitated broadening of this definition with regard to the pigment composition. First, in the early 1970s *Prochloron* [1] was discovered lacking phycobiliproteins and possessing Chl *b*, in addition to Chl *a*. This was followed by the discovery of two other organisms with similar pigmentation, *Prochlorothrix hollandica* and *Prochlorococcus marinus*. These three organisms have been placed informally into a group called the prochlorophytes and attempts have been made to place these organisms either into a separate phylum, the Prochlorophyta [1], or into an order of Cyanobacteria, the Prochlorales [2].

In 1996, *Acaryochloris marina* was discovered [3], which possessed mainly Chl *d*, a small amount of Chl *a* and phycobiliproteins. The discovery of an organism where Chl *d* is the predominant pigment [4–6] has raised many interesting questions concerning the role of this Chl, not only in its biochemistry, but also the past evolutionary history of the organism and its present ecological niche. A more recent pigment composition analysis of *A. marina* has shown pheophytin *a* and the epimer of Chl *d*, Chl *d*′, to be present [7], but the precise arrangement of photosynthetic pigments remains to be resolved. Prior to the discovery of *A. marina*, Chl *d* had not been regarded as being present in any photosynthetic organism [8].

Unpublished evidence indicates that *A. marina* falls with the cyanobacterial radiation (Miyashita, personal communication). In the prochlorophytes, it has been discovered that the Chl *b*, along with some of the Chl *a*, is borne mainly on a single, characteristic light-harvesting protein (pcb). This protein is quite different from the protein involved in binding Chl *a*+*b* in green algae and higher plants and possibly arose from the photosystem II (PSII) inner antennae proteins, CP43 and CP47 [9,10]; both groups of Chl-binding proteins involved, isiA and pcb, have been shown to be homologous to CP43 and CP47 and to have roles in light-harvesting [11].

There is no previous publication on the nature of the light-harvesting Chl *d*-binding protein. Here we describe the isolation and purification of the light-harvesting complex (lhc) from *A. marina* and present evidence that it is similar to the pcb protein of prochlorophytes.

2. Materials and methods

A. marina was grown at 28°C under illumination at 10–30 μE m^{-2} s^{-1} and continuous aeration in ESS medium, which contained ES additives [12] in sterilised seawater. Cells were spun at 7000×*g* (rotor GS-3, Sorvall) and washed twice in 0.1 M Tris–acetic acid pH 9.0, 2 mM EDTA, 1 mM phenylmethanesulphonyl fluoride (PMSF) in filtered seawater. Cells were broken by passing twice through a pre-chilled French pressure cell at 100 MPa. Unbroken cells were removed by centrifuging at 5000×*g* for 10 min (Beckman JA 17 rotor). The supernatant was centrifuged at 35 000×*g* for 15 min to collect the large thylakoid membrane fragments (LTM). The LTM pellet was washed once with 10 mM Tris–acetic acid (pH 9.0), 2 mM EDTA, 1 mM PMSF and resuspended in the same buffer to a Chl *d* concentration of 0.5 mg ml^{-1}. The LTM supernatant was loaded onto a 30% sucrose cushion and centrifuged for 30 min at 80 000×*g* in an SW40Ti rotor. The resultant green band was centrifuged for 1 h at 150 000×*g* to pellet the small thylakoid membrane fragments (STM).

For the isolation of pigment–protein complexes, the membranes were solubilised for 30–60 min in the dark at 4°C using various detergent regimes. The detergent:Chl *d* ratio was kept in the range of 10–20:1 (w/w). The dissolved complexes were separated on sucrose gradients (6–40%) containing 0.05% dodecyl maltoside (doDM) and 10 mM Tris–acetic avid (pH 9.0) at 150 000×*g* for 16 h at 4°C (Beckman XL 90, SW40Ti rotor). The green pigment-containing fractions were collected with a syringe. Room temperature absorption spectra were performed using a Varian CARY 1 spectrophotometer. Chl *d*

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Abbreviations: Chl, chlorophyll; doDM, dodecyl maltoside; lhc, light-harvesting complex; OG, octyl glucoside; PMSF, phenylmethanesulphonyl fluoride; PSI, photosystem I; PSII, photosystem II; ZW14, zwittergent 3-14

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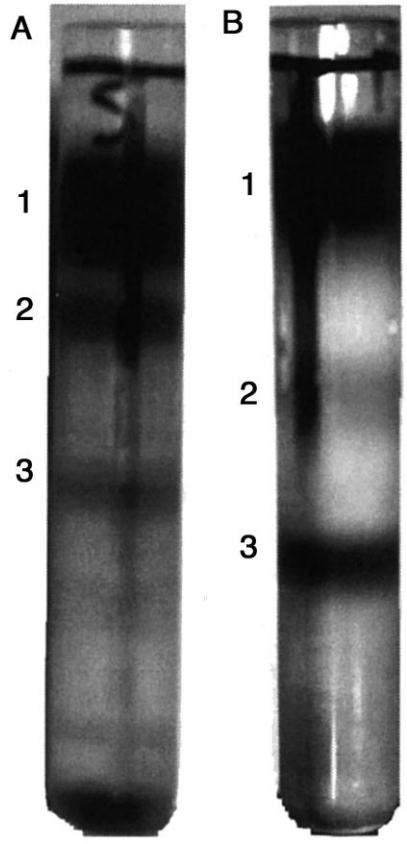

Fig. 1. Sucrose density gradient (6–40%) ultracentrifugation of detergent-solubilised pigment-binding proteins isolated from *A. marina* thylakoid membranes. A: Membranes solubilised with 0.5% SDS. B: Membranes solubilised with 0.3% doDM, 0.15% OG and 0.05% ZW14.

contents were estimated from methanol extracts using the molar extinction coefficient = 98.6 at 696 nm [13]. Fluorescence spectra were performed at room temperature using a Hitachi F-4500 fluorescence spectrophotometer (5 nm slit width for emission and excitation).

The polypeptide composition of the fractions isolated from sucrose gradients was resolved by 12.5% SDS–PAGE [14] or with the 8–16% iGel (Gradipore). Before samples were applied to the gels they were denatured by incubating at ~55°C for 15 min with 0.3 M Tris–HCl pH 6.8, 2% (w/v) SDS and 50 mM dithiothreitol. Gels were run at 100 V in the Bio-Rad Mini-Protean II electrophoresis system. Low molecular weight markers (Sigma) were used to estimate polypeptide size. Gels were stained with Coomassie brilliant blue R 250 (Sigma). Further separation of the Chl–protein complexes used a modified non-denaturing electrophoresis method [15] where 0.1% SDS replaced 0.2% deriphate and the gel contained 7.5% polyacrylamide. Samples were electrophoresed at 4°C in the dark on a Mini-Protean II (Bio-Rad). The green bands were excised and suspended in the light-path of a fluorescence spectrophotometer to obtain spectral data on the Chl–protein complexes.

3. Results

Conventional methods of detergent extraction, non-denaturing gels and dissociating PAGE were used to separate the Chl-binding protein complexes of *A. marina*. The small size of the cells and a tough cell wall result in poor cell breakage in the French press, with typical breakage efficiency of 20–30%; this improved only marginally by a second passage through the press.

Several detergents, including doDM, octyl glucoside (OG), SDS, digitonin, Triton X-100 and zwittergent-type detergents

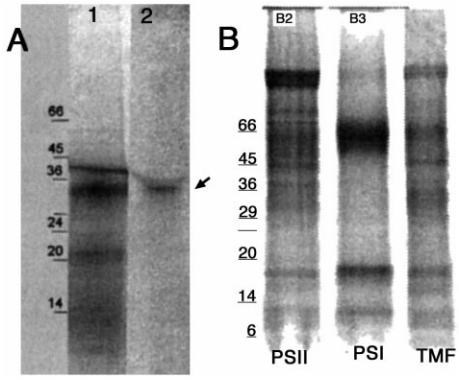

Fig. 2. Polypeptide composition of lhc, PSII and PSI fractions partially purified from *A. marina* using SDS–PAGE. A: lhc fractions from sucrose density ultracentrifugation were separated using 8–16% iGel™ (Gradipore). The arrow shows the main polypeptides of lhc are ca. 34 kDa. Lane 1: generated from solubilised LTM. Lane 2: generated from solubilised STM. B: PSII- and PSI-enriched fractions from sucrose density ultracentrifugation (bands B2 and B3, respectively, from Fig. 1) and thylakoid membrane fragments (TMF) were separated using 12.5% SDS–PAGE.

($n = 8$ to $n = 16$), were used in a first attempt to solubilise and separate the pigment proteins. Thylakoid fragments were solubilised most successfully with a combination of 0.3% doDM, 0.15% OG and 0.05% zwittergent 3-14 (ZW14) for 30 min. A high concentration of SDS produced a similar degree of solubilisation but spectroscopic and polypeptide characteristics were affected. Generally, three discrete bands could be distinguished after a sucrose gradient ultracentrifugation (Fig. 1). The lowest band was bright green and enriched in photosystem I (PSI) components as shown by subsequent SDS–PAGE analysis (Fig. 2B) where the band at ca. 60 kDa dominated, although there were a few bands below 20 kDa, and the poly-

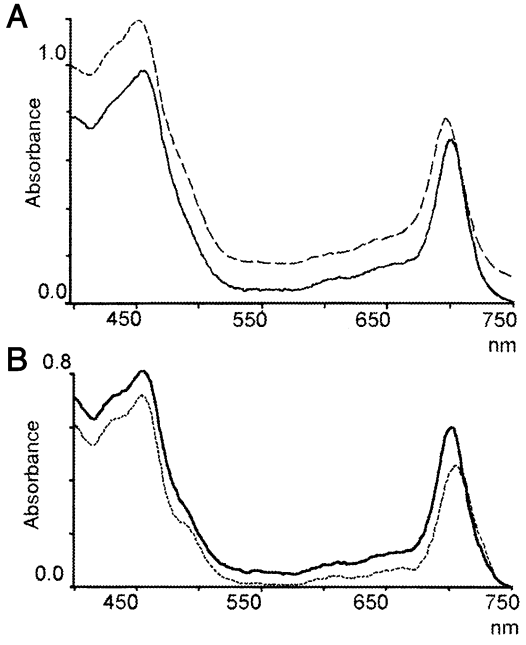

Fig. 3. Absorbance spectra of partially purified pigment-binding proteins from *A. marina* thylakoid membranes. lhc-enriched fraction solubilised using: 0.5% SDS (dashed line); 0.3% doDM, 0.15% OG, 0.05% ZW14 (continuous line). B: PSII- (continuous line) and PSI- (dashed line) enriched fractions solubilised using 0.3% doDM, 0.15% OG, 0.05% ZW14.

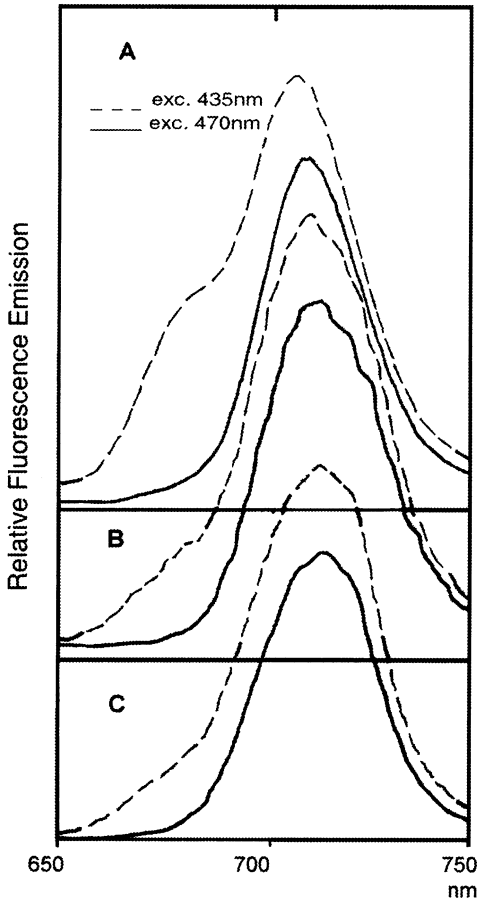

Fig. 4. Fluorescence emission spectra of lhc, PSII and PSI fractions partially purified from *A. marina*. A: lhc; B: PSII; C: PSI.

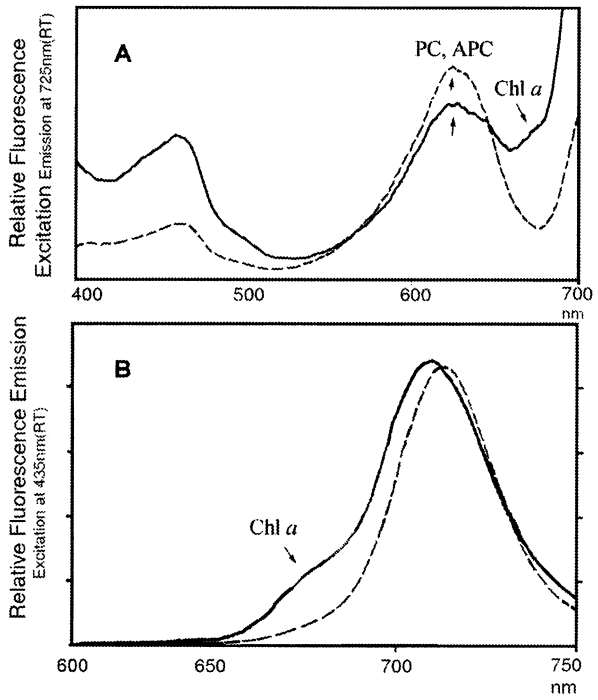

Fig. 5. Pigment characterisation of lhc-enriched fractions of *A. marina* cells grown under different conditions. Fluorescence excitation spectra (emission at 725 nm) of living *A. marina* cells at room temperature. Fluorescence emission spectra (excitation at 435 nm) of lhc-enriched fractions, separated by sucrose density unltracentrifugation, from *A. marina* cells grown under the following conditions: culture 1: cells were grown at >100 μE m^{-2} s^{-1}, at 20°C (continuous line); culture 2: cells were grown at <10–30 μE m^{-2} s^{-1}, at 28°C (dashed line). PC: phycocyanin; APC: allophycocyanin.

peptide composition resembled that of the *Acaryochloris* PSI complex (cf. Fig. 2B, lane B3) [4]. The middle band (Fig. 1, A2 and B2) was probably an enriched PSII fraction contaminated with PSI complex, as shown from the polypeptide pattern in PAGE analysis (Fig. 2B, lane B2). The major polypeptides were 91/99, 60, 47, 43 along with bands at 30–38 kDa. The large 91/99 kDa polypeptides were the linker polypeptides, whose major function is associated with the core of the phycobiliprotein complex. The upper band (Fig. 1, A1 and B1), which was dark blue–green, contained over 35% of the total of Chl *d* and Chl *d*-binding protein complex.

This top dark blue–green band has been shown from a variety of analyses to contain light-harvesting Chl *d*-binding protein complex(es) (lhc). The absorption spectrum of the lhc band is shown in Fig. 3, where this band is compared with the PSI- and PSII-enriched fractions. The spectra are very similar since Chl *d* is the dominant pigment in all of them. However, the red band of the lhc-enriched fraction was slightly blue-shifted compared to the others.

The polypeptide analysis of the lhc-enriched fraction is shown in Fig. 2A. The fraction generated from LTM (lane 1) was contaminated with phycobiliproteins with subunits at around 20 kDa [16]. However, STM fragments yielded a single band with a molecular mass of 34–35 kDa.

Fluorescence emission spectra of the three sucrose density bands are shown in Fig. 4. A major band was present in all fractions, which is attributed to Chl *d* with an emission maximum between 705 and 725 nm. There was a shoulder in the lhc fraction when it was excited at 435 nm; this is attributed to Chl *a*. Smaller shoulders occur in the PSI and PSII fractions. The contribution of this Chl *a* shoulder varied between cultures and clearly depended on the growth conditions, especially the cell density and light environment at the time of harvest. Fig. 5 shows relative fluorescence excitation and emis-

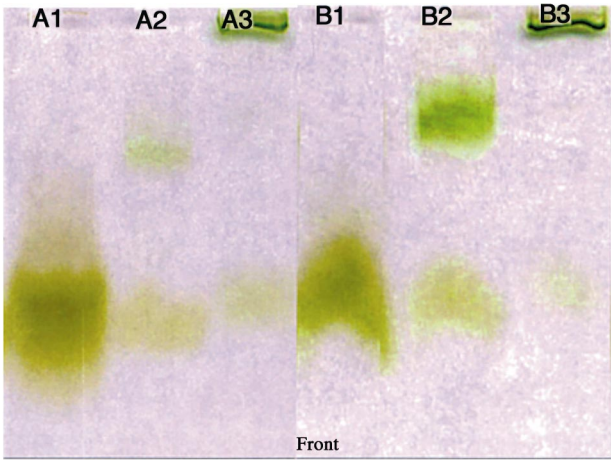

Fig. 6. Non-denaturing gel electrophoresis of lhc, PSII and PSI fractions partially purified from *A. marina*. lhc fractions (from sucrose density gradient bands A1 and B1, see Fig. 1); PSII-enriched fractions (from sucrose density gradient bands A2 and B2, see Fig. 1); PSI-enriched fractions (from sucrose density gradient bands A3 and B3, see Fig. 1).

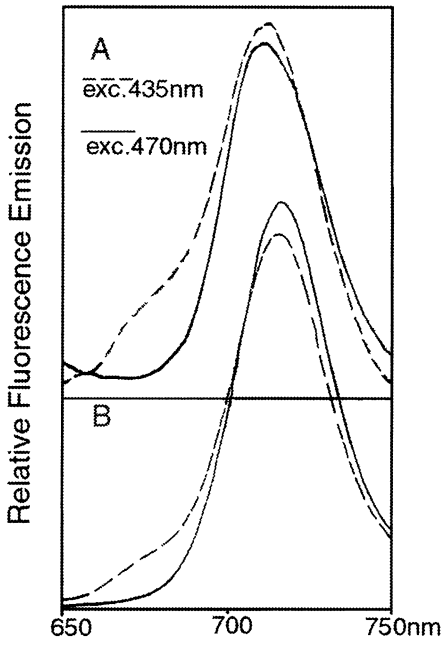

Fig. 7. Fluorescence emission spectra of lhc and PSII proteins isolated from *A. marina*. A: lhc band excised from non-denaturing gel. B: PSII band excised from non-denaturing gel. Dashed line, excitation at 435 nm; continuous line, excitation at 470 nm.

sion spectra for two *A. marina* culture conditions: culture 1 was grown under >100 μE m^{-2} s^{-1} at 20°C, while culture 2 was grown at 10–30 μE m^{-2} s^{-1} at 28°C. The Chl *a* increased under strong light intensity, but the phycobiliprotein content increased under low light intensity with Chl *a* decreasing.

The nature of the lhc in the top sucrose density band was further analysed using non-denaturing gels (Fig. 6, A1 and B1). A single green band was obtained for the two detergent separations (A1 and B1) and was distinct from the green band obtained from the PSII-enriched sucrose density band (Fig. 1). The lhc band from the non-denaturing gel was subjected to SDS–PAGE and yielded a major polypeptide of 34–35 kDa (data not shown). The lhc band generated from a non-denaturing gel had a fluorescence emission centred at 720 nm (Fig. 7) with a shoulder at 665 nm under excitation by 435 nm light. The PSII fraction from the non-denaturing gel yielded polypeptides of 33–38, 43 and 47 kDa on SDS–PAGE analysis (data not shown) and had a fluorescence emission band centred at 725 nm, with only a slight shoulder at 665 nm stimulated by excitation at 435 nm (Fig. 7).

4. Discussion

This work strongly supports the presence of an lhc in *A. marina* with a polypeptide of approximate molecular mass 34 kDa and with Chl *d* as the predominant pigment. The evidence is based on the separation of sucrose density bands enriched in a protein with a polypeptide of this mass and giving rise to a distinct band on non-denaturing gels, also with a polypeptide of similar mass. Apart from this evidence we have employed a number of other detergents with varying results (results not shown), which have lead to the same conclusion.

At present we know little about the arrangement of the lhc of *A. marina* in the thylakoid membranes. Marquardt et al. have recently presented evidence on the fine structure of cells of *A. marina* [17]. There are up to 12 concentrically arranged thylakoids, which are largely appressed. Phycobiliproteins are attached to unappressed regions of the thylakoids in the stromal space [17]. Several features of the thylakoid arrangement and structure are unique amongst photosynthetic organisms and therefore it will be interesting to find out the arrangement of the lhc. Since light energy absorbed by light-harvesting Chl *d* is passed on to both PSI and PSII the lhc is presumably connected in some way to both photosystems. Mimuro et al. present evidence for uphill excitation migration in *A. marina* [5]. This will certainly be necessary if Chl *a* is the active Chl in P680 and it raises the question of how excitation energy migrates in the lhc if, as our results indicate, it binds both Chl *a* and Chl *d*. All the evidence indicates that the lhc of *A. marina* is similar to that of prochlorophytes and quite distinct from the 22–28 kDa CAB/CAC light-harvesting protein of the plastids of algae and higher plants [18]. Further support for this hypothesis comes from the fact that a pcb degenerate probe to N-terminal sequence and a region conserved of pcb from *Prochlorococcus* sp. [10] has produced a PCR product from DNA of *A. marina* with a sequence homologous to pcb (Chen, Hiller and Larkum, unpublished).

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