S31-008

The major light-harvesting protein complex in *Acaryochloris marina*

M Chen, RG Quinnell and AWD Larkum

School of biological sciences, The University of Sydney, NSW 2006,Australia

Keywords: *Acaryochloris marina*, chlorophyll d, Light-harvesting pigment-proteins, Chl a/b binding protein(pcb)

Introduction

Prior to the discovery of *Acaryochloris marina* (Miyashita, 1996), Chl *d* had not been regarded as present in any photosynthetic organism; *A. marina* is the only known organism that has Chl *d* as the predominant pigment. This organism has small amounts of Chl *a* and phycobiliproteins (PBP) in addition to Chl *d*. Many interesting questions have been raised with the discovery of this organism, including: what are the biochemical reactions in which Chl *d* are involved, what is the evolutionary history of Chl *d*-binding proteins and what characteristics define the ecological niche of *A. marina*.

Unpublished evidence indicates that *A. marina*, like the prochlorophytes, fall within the cyanobacterial radiation (Miyashita pers. com). Interestingly, phylogenetic tree reconstructions, based on 16S rRNA sequences, show that the three groups of prochlorophytes occupy different branches within the cyanobacterial radiation (Turner, 1997). 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 that involved in binding Chl *a/b* in green algae and higher plants and probably arose from the PSII inner antennae proteins, CP43 and CP 47 (La Roche 1996; van der Staay 1998). 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 (Park *et al.,* 1999).

Previous work on *A. marina*, focused on the nature of the light-harvesting pigments: Chl *d* and phycobiliproteins. Schiller *et al.* (1997) showed that Chl *d* is the major light-harvesting pigment and efficiently passes on this energy to both PSI and PSII. Hu *et al.* (1998) investigated the role of Chl *d* in PSI and came to the conclusion that Chl *d* is the chlorophyll involved in P740, the equivalent of P700. Marquardt *et al.* (1997) first isolated phycobiliproteins from *A. marina.* Later Hu *et al. (*1999) isolated rods which contained either phycocyanin or phycocyanin plus allophycocyanin and showed that these were attached to unappressed regions and transferred excitation energy to Chl *d* of PSII.

This publication focuses on the nature of the light-harvesting Chl *d*-binding protein and here we describe the isolation and purification of the lhc from *A. marina* and present evidence that it is similar to the pcb protein of prochlorophytes.

Materials and Methods

Acaryochloris marina cells were harvested in their late logarithmic growth phase by centrifugation at 7,000 x g. Harverst cells were washed twice using 0.1 M Tris-acetic acid pH 9.0, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF) in filtered seawater. Cells were broken by passing twice through a pre-chilled French pressure cell (100 MPa). The large thylakoid membrane fragments (LTM) were sedimented by centrifugation at 35,000 x g for 15 min and resuspended in 10 mM Tris-acetic acid (pH 9.0), 2 mM EDTA, 1 mM PMSF.

The LTM supernatant was loaded onto a 30% sucrose cushion and centrifuged (30 min at 80,000 × g). The resultant green band was retained and the small thylakoid membrane fragments (STM) pelleted by centrifugation for 1 h at 150,000 × g.

To solubilise of pigment-protein complexes, the STM were treated for 60 min in the dark with continual stirring at 4°C using 0.5% mixed detergents dodecyl maltoside (doDM): octyl glycoside (OG): zwittergent 3-14 (ZW 14) = 0.3%: 0.15% :0.05%. The dissolved complexes were separated on sucrose linear gradients (6-40%) containing 0.05% doDM and 10 mM Tris-acetic acid (pH 9.0) at 150,000 x g for 16 h at 4°C. Pigment-containing bands were collected with a syringe.

The polypeptide composition of the fractions isolated from sucrose gradients was resolved by 12.5% SDS-PAGE (Laemmli 1970) or 8-16% iGel^{TM}(Gradipore). Further separation of the chlorophyll-protein complexes used a modified non-denaturing electrophoresis method (Peter, 1991), where 0.1% SDS replaced 0.2% deriphat and the gel contained 7.5% polyacrylamide. Prior to loading, samples were solubilised on ice using 0.5% doDM, and then electrophoresed at 4°C in the dark on a Mini Protean II (Biorad). The green bands were excised and suspended in the light path of a fluorescence spectrophotometer (Hitachi F-4500) to obtain spectral data on the pigment-protein complexes. Fluorescence spectra were performed at room temperature using a (5 nm slit width for emission and excitation). Absorption spectra (Varian CARY 1 spectrophotometer) were performed at room temperature.

Results and Discussion

Conventional methods of detergent extraction, non-denaturing gels and SDS-PAGE were used to separate the light-harvesting complexes of *A. marina.* Several detergents, including doDM, OG, SDS, digitonin, triton X-100 and zwittergent-type detergents (n=8 to n=16) were trialed to achieve solubilisation and separation of *A. marina* pigment-proteins. Thylakoid fragments were solubilised most successfully with a combination of doDM, OG and ZW14 in the ratio of 6:3:1 at a concentration of 0.5% total detergent. Generally, three discrete bands could be distinguished on a sucrose gradient (Fig 1).

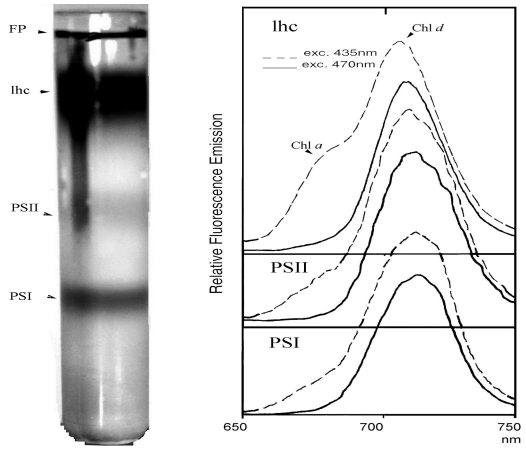

Fig.1 Isolation of pigment-protein complexes. **a**. (left) 6-40% sucrose gradient separation of solubilised thylakoid membranes - 0.5% total detergent (doDM:OG: ZW14 (6:3:1). **b**. (right) The fluorescence excitation spectra of the separated pigment-protein complexes.

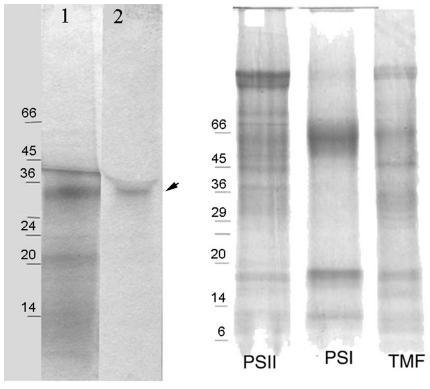

Fig 2 Electrophoresis of pigment-proteins. **a** (left) 8-16% iGel for lhc fractions. Lane 1 lhc from solubilised TLM. Lane 2 lhc from solubilised STM. **b** (right) 12.5% SDS-PAGE of the fractions collected from sucrose gradient. TMF: thylakoid membrane fragments

The upper band from sucrose gradient was dark blue-green and contained over 35% to 60% of the total Chl *d* and was identified, from a variety of analyses, as the light-harvesting Chl *d*-binding protein complex that has a major polypeptide at 34-35 kDa (Fig 2A). The lhc enriched fraction generated from solubilised LTM was contaminated with PBP (Fig 2 lane 1). The molecular masses for the basic subunits of PBP were around 20 kDa (Hu 1999). STM have been purified from PBP (Fig 2 lane 2) by sucrose density centrifugation as described in "Materials and Methods". The room temperature fluorescence excitation spectra of the lhc fractions indicated the presence of phycocyanin and allophycocyanin with peaks in the range 540 nm to 625 nm in the lhc-enriched fraction with 670 nm emission (Fig 3A). In some cases, the polypeptide pattern of the lhc-enriched fraction showed a broad band (or a group of separated bands) at 28 - 36 kDa. These could indicate degradation products in the 34-35kDa range.

The absorption spectra of the solubilised bands from sucrose gradient indicate that all fractions from sucrose gradient show a predominance of chl *d* (data not shown). Fluorescence emission spectra of three discrete fractions are shown in Fig 1b**.** The shoulder indicated in this spectrum is the result of excitation at 435 nm and therefore is attributed to Chl *a*.

The lhc fraction was identified by green gel electrophoresis. Fig 4a shows that there is a distinct green band generated from non-denaturing electrophoresis of the lhc fraction and this lhc green band, when subjected to SDS-PAGE, 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 4b) with a shoulder at 665 nm under excitation by 435 nm.

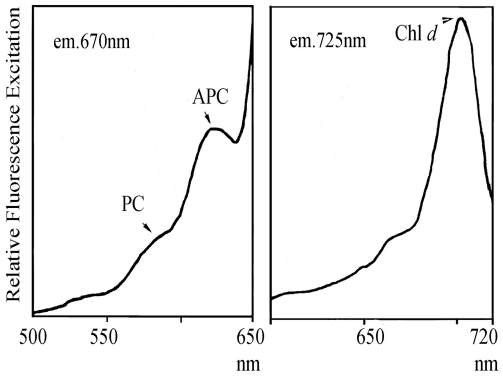

Fig 3 Fluorescence excitation spectra of the lhc fraction. **a.** (left) emission at 670 nm; **b.** (right) emission at 725 nm.

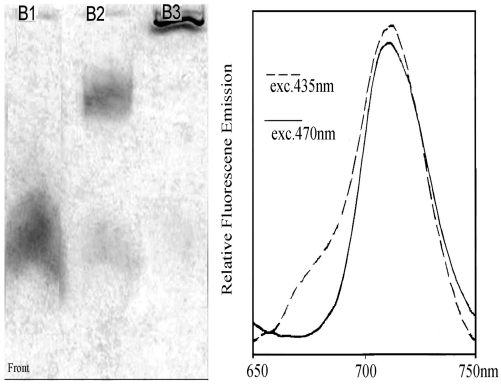

Fig 4a (left) Separation by non-denature gel electrophoresis of the fractions from sucrose gradient centrifugation. B1 lhc fraction; B2 PSII enriched fraction; B3 PSI fraction . **4b**. (right) Fluorescence emission spectra of the gel slice from lhc by excitation at 435 nm for Chl *a* and 470 nm for Chl *d*

Our results strongly support the notion that the lhc of *A. marina* is comprised of polypeptides of approx. mass 34 kDa. This mass is similar to the mass of prochlorophytes (pcb) and quite distinct from the 22-28 kDa CAB/CAC light-harvesting proteins of the plastids of algae and higher plants (Green, 1996). Further support for this hypothesis comes from the fact that a pcb degenerate probe to the N-terminal sequence of a conserved region of pcb from *Prochlorococcus*.sp (La Roche, 1996) yields a PCR product with a sequence homologous to pcb (Chen, Hiller and Larkum, unpublished) against DNA of *A. marina*. 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.,* (2000) present evidence for uphill excitation migration in *A. marina*. This will certainly be necessary if Chl *a* is the active chlorophyll in P680. The question of how excitation energy migrates in the lhc remains to be confirmed but as our results indicate that it binds both Chl *a* and Chl *d*.

Acknowledgements:

We thank the Australian Research Council for supporting this project.

References

Green BR, Durnford DG (1996) *Annual Review of Plant Physiology and Plant Molecular Biology* **47,** 685-714.

Hu Q, Marquardt J, Iwasaki I, Miyashita H, Kurano N, Morschel E, Miyachi S (1999) *Biochimica et Biophysica Acta* **1412,** 250-261

Hu Q, Miyashita H, Iwasaki I, Kurano N, Miyachi S, Iwaki M, Itoh S (1998) *Proceedings of the National Academy of Sciences of the USA* **95,** 13319-13323

Laemmli UK (1970) *Nature* **227,** 680-685

La Roche J, Vanderstaay GWM, Partensky F, Ducret A, Aebersold R, Li R, Golden SS, Hiller RG, Wrench PM, Larkum AWD, Green BR (1996) *Proceedings of the National Academy of Sciences of the USA* **93,** 15244-15248

Marquardt J, Senger H, Miyashita H, Miyachi S, Morschel E (1997) *FEBS Letters* **410,** 428-432

Mimuro M, Hirayama K, Uezono K, Miyashita H, Miyachi S (2000) *Biochimica et Biophysica Acta* **1456,** 27-34

Miyashita H, Ikemoto H, Kurano N, Adachi K, Chihara M, Miyachi S (1996) *Nature* **383,** 402

Park, Y. I., Sandstrom S., Gustafsson P., Oquist G. (1999) *Molecular Microbiology* 32: 123-129.

Peter GF, Thornber JP (1991) *The Journal of Biological Chemistry* **266,** 16745-16754

Schiller H, Senger H, Miyashita H, Miyachi S, Dau H (1997) *FEBS Letters* **410,** 433-436

Turner S (1997*) Plant Systematics and Evolution* (Suppl. 11), 13-52

van der Staay GWM, Yurkova N, Green BR (1998) *Plant Molecular Biology* **36,** 709-716