Biochimica et Biophysica Acta 806 (1985) 107–115
Elsevier

BBA 41681

The chlorophyll-protein complexes of *Prochloron* sp. (Prochlorophyta)

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(Received May 3rd, 1984)
(Revised manuscript received September 20th, 1984)

Key words: Chlorophyll-protein complex; Chlorophyll *b*; P-700; Photosystem I; Light-harvesting complex; (*Prochloron*, Prochlorophyta)

Chlorophyll-protein complexes have been isolated from *Prochloron* sp. by SDS-polyacrylamide gel electrophoresis and SDS-sucrose-gradient centrifugation. Complexes associated with Photosystem I have significant amounts of chlorophyll *b* and a principle polypeptide of 70 kDa. The largest Photosystem I complex had an M_r of more than 300 000 kDa, a chlorophyll *a*/*b* ratio of 3.8 and a chlorophyll/P-700 ratio of approx. 100. Complexes enriched in chlorophyll *b* showed reduced electrophoretic mobility compared to spinach LHCP3, a higher Chl *a*/*b* ratio (approx. 2.4) and had a principle polypeptide of 34 kDa. Neither the 34 kDa or any other polypeptide showed cross-reactivity with antibodies to spinach light-harvesting chlorophyll *a*/*b* protein in a Western blot test.

Introduction

The Prochlorophyta, with the single genus *Prochloron,* is currently the sole group of prokaryotic oxygenic autotrophs which possess Chl *b* [1,2]. The presence of Chl *b*, together with appressed thylakoids, sets this group apart from the cyanobacteria and places it as potentially the direct descendant of the line which gave rise to the green algae and higher plants. Other features may be similarly interpreted but could equally support an independent acquisition of Chl *b* [3–7].

The ultrastructure of *Prochloron* has been well documented [8–11]. Much less attention has been paid to the Chl-protein complexes of *Prochloron* and particularly to the protein(s) carrying Chl *b*. A single earlier report has suggested that *Prochloron* contains light-harvesting Chl *a*/*b* protein (LHCP) with electrophoretic and spectral properties similar to LHCP of green algae and higher plants [3]. The present study indicates significant differences between the Chl *a*-*b* protein(s) of one *Prochloron* sp. and the light-harvesting chlorophyll *a* and *b* proteins of green algae and higher plants and, furthermore, indicates differences between the respective P-700-Chl *a* proteins. A preliminary report of this work has been presented elsewhere [12].

Materials and Methods

Prochloron was obtained from colonies of the ascidian *Diplosoma virens* growing under dead stagshorn coral on the reef flat at Heron and One Tree reefs (Capricornia region, Great Barrier Reef). Material was transported to the laboratory as frozen cells, frozen crude thylakoid preparations, or in living ascidian colonies. Algal cells were pressed out of cut ascidian colonies into buffered sea water (0.1 Tris-acetate, pH 9.2), washed once in 0.1 M Tris-acetate (pH 9.2) and then broken by

Abbreviations: Chl, chlorophyll; CP1, chlorophyll-protein complex I; LHCP, light-harvesting chlorophyll *a*/*b* protein; PS I, II, Photosystem I, II.

single passage through Yeda ($1 \cdot 10^9$ Pa) or French ($2.7 \cdot 10^9$ Pa) pressure cell. After filtering through two layers of Miracloth, cells and cell debris were removed by centrifugation at $500 \times g$ for 10 min. Thylakoids were obtained by centrifugation at $2500 \times g$ for 15 min. and thylakoid fragments by subsequent centrifugation at $10\,000 \times g$ and $20\,000 \times g$ for 15 min. Thylakoids and thylakoid fragments were washed in 0.1 M Tris-acetate before use. Variations in the mode of transportation, breakage of cells or type of thylakoid preparation did not affect the results obtained in this study.

Separation of chlorophyll-protein complexes

Washed thylakoids were solubilised in SDS (SDS/chlorophyll, 15 : 1; w/w) centrifuged at $40\,000 \times g$ for 15 min and aliquots (approx. 6 μg Chl) of the supernatant applied to cylindrical (0.5 × 10 cm) 6% polyacrylamide gels after addition of sucrose to 5% w/v. The gels were run at 2 mA/gel until the sample entered the gel and then at 5 mA/gel for 15 min. 0.1 M Tris-acetate buffer (pH 9.2) was used throughout.

The proportion of chlorophyll in each chlorophyll-protein complex was estimated from densitometer scans at 670 and 650 nm of the gels as previously described [13]. Alternatively, chlorophyll-protein complexes were separated by sucrose density centrifugation essentially as described by Argyroudi-Akoyonoglou and Thomou [14], except that 0.1 M Tris-acetate pH 9.2 was used throughout. The gradient was centrifuged at $238\,000 \times g$ for 16 h in a Beckman SW-41 rotor at 4°C, and analysed at 670 nm by displacement with 60% sucrose through a 3 mm pathlength flow cell in a Gilford 2400 spectrophotometer. The peaks from the gradient scans were cut out and weighted for determination of the relative Chl *a* distribution.

Separation of polypeptides

Peptides were separated by the method of Laemmli [15]. Molecular weights were determined by coelectrophoresis of the following proteins: phosphorylase *a* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The 'Western' blot test for cross-reactivity between antibodies for spinach LHCP and *Prochloron* polypeptides was as described by Andersson et al. [16].

Determination of pigments

Concentrations of Chl *a* and Chl *b* were calculated from absorbance, at 663 and 645 nm, of chloroplast extracts in acetone/H_2O (4 : 1, v/v), using the equations of Arnon [17]. P-700 concentration in fractions was assayed, using a millimolar extinction coefficient of 64 [18] from the light-induced absorbance change at 700 nm (730 nm reference wavelength) measured on an Aminco dual-wavelength spectrophotometer [19]. Samples contained 1 mM sodium ascorbate and 1 mM DCMU.

Absorption and fluorescence (emission and excitation) spectra

Absorption spectra were recorded at room temperature using a Pye Unicam SP8-200 spectrophotometer. Fluorescence emission and excitation spectra were recorded on samples, either at room temperature diluted in buffer, or at 77 K in 66% (v/v) glycerol on a Perkin-Elmer MPF-44B Fluorescence spectrophotometer in the ratio mode but not further corrected. All fluorescence measurements were made at less than 0.05 *A* at the red absorbance maximum of Chl *a*.

Results

In *Prochloron* cells from *Diplosoma virens* the Chl *a*/*b* ratio was found to vary in different collections from about 3.0 to 6.0. Variation in the Chl *a*/*b* ratio could not, however, be correlated with season, submergence, depth or substratum to which the host was attached. Unless otherwise stated, the results presented refer to material collected between November and May with a Chl *a*/*b* ratio of 3.0–3.4.

Fluorescence emission and excitation spectra for whole *Prochloron* cells at 77 K are shown in Fig. 1. The emission spectrum for excitation at both 435 and 475 nm shows two peaks – at 685 nm and 695 nm. This spectrum resembles those found for green algae (cf. Ref. 20), rather than those for higher plants. However, in green algae, cyanobacteria and red algae, the position and relative magnitude of the long-wavelength fluores-

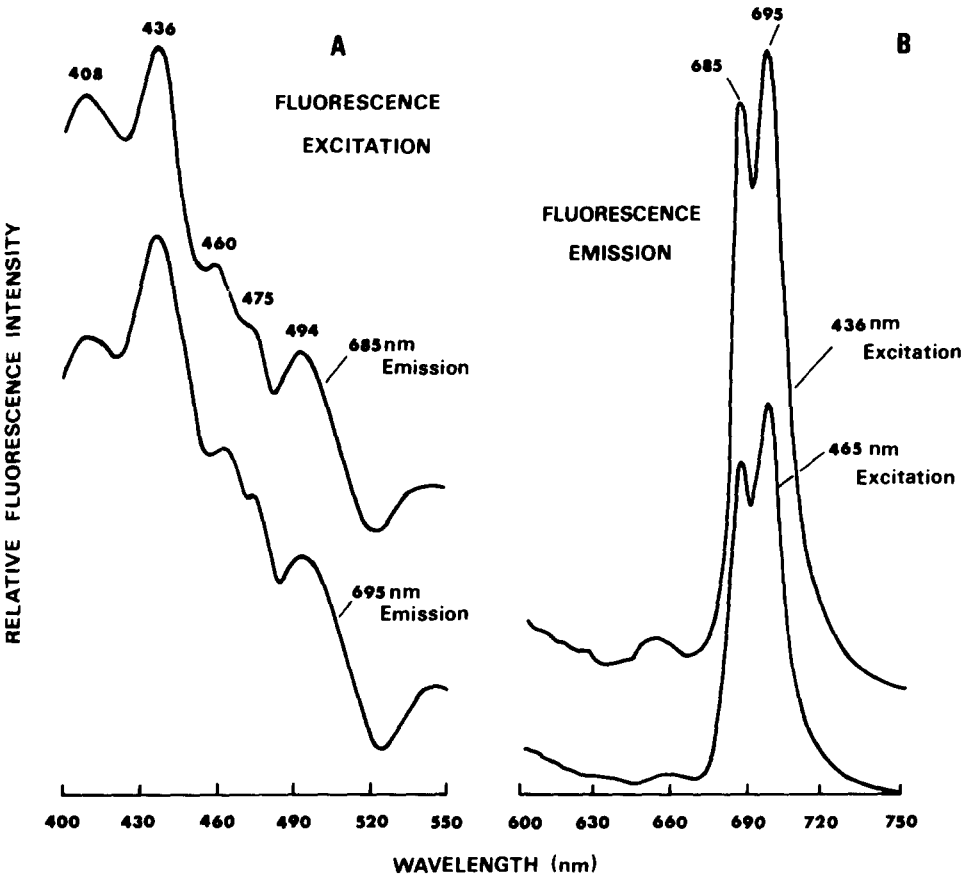


Fig. 1. Fluorescence excitation and emission at 77 K of *Prochloron* cells. The emission from the 465 nm excitation was obtained with the same instrument setting used for the 435 nm excitation.

cence emission shows great variability [21–23]. The excitation spectrum of both F_{685} and F_{695} is very similar and shows contributions from Chl *a*, Chl *b* and carotenoids. Thylakoids gave similar results, except that a small shoulder was sometimes noted at 710 nm.

Thylakoid membranes were solubilised in SDS and the Chl-protein complexes separated by SDS polyacrylamide gel electrophoresis. The gel patterns obtained varied with extraction conditions; some representative gels are shown in Figs. 2 and 3. Chl *b* was found to be present in all bands and no band showed the low ratio of 1.2 of Chl *a* to Chl *b* characteristic of LHCP from green plants. In comparison with spinach thylakoids, the Chl-protein complexes of *Prochloron* thylakoids showed a number of differences (Fig. 2). A high M_r species (CP1*) was present and the CP1 and CP1a were greatly reduced. (The nomenclature used in this paper is based on Anderson et al. [24]). No band corresponding to LHCP3 was present and the fastest-moving complex (designated as Chl *a-b*) corresponded to CPa of spinach thylakoids. Separation of complexes from thylakoids with a Chl *a/b* ratio of 6.0 showed the fastest migrating complex could be resolved into two with different Chl *a/b* ratios (results not shown). The band designated Chl *a-b** was cut from the gel and reelectrophoresed, giving rise to the original Chl *a-b** complex, some Chl *a-b* complex and some free pigment (Fig. 3).

It will be noted from Figs. 2 and 3 that the relative proportions of bands CP1* and CP1 were variable. These bands are assigned to Photosystem I on the bases of their relative mobility, low fluorescence under ultraviolet light and long wave-

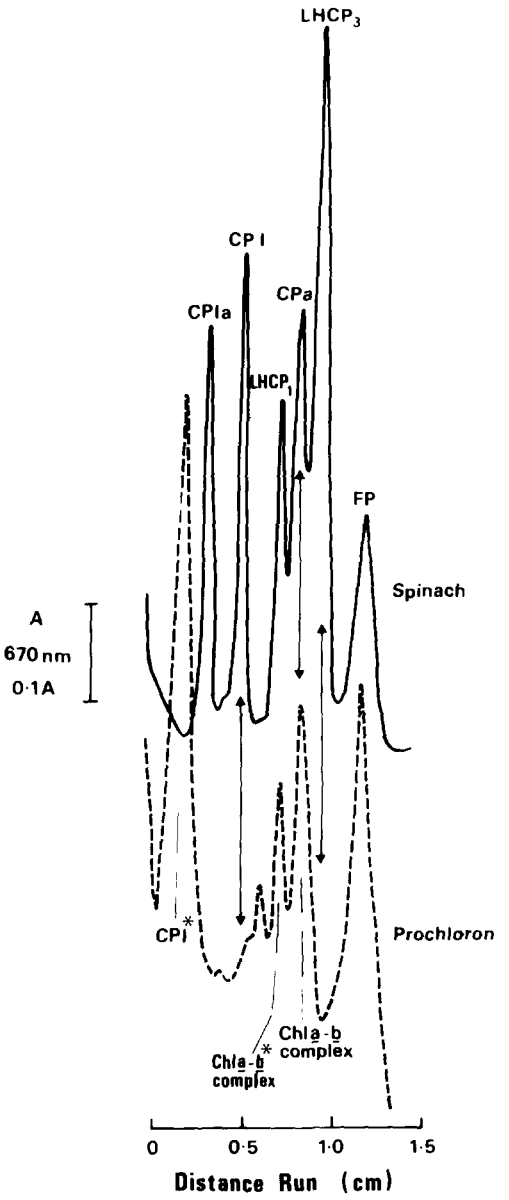


Fig. 2. A densitometer tracing at 670 nm of the chlorophyll-protein complexes of Spinach and *Prochloron* separated by SDS-polyacrylamide gel electrophoresis.

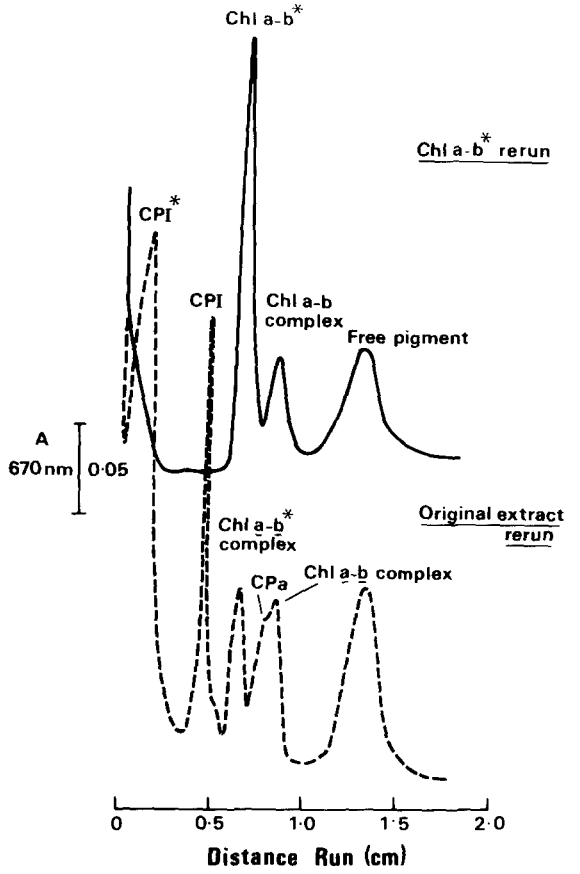


Fig. 3. Densitometer tracing at 670 nm of a Chl *a-b** band cut from the gel and re-electrophoresed on an SDS-polyacrylamide gel together with a sample of the original SDS solubilised thylakoids.

length absorbance at 674 nm. Although bands CP1* and CP1 contained Chl *b*, they were enriched in Chl *a* compared to thylakoids. As shown in Fig. 4, the bands Chl *a-b** and Chl *a-b* were enriched in Chl *b*, but not to the same extent as LHCP1–3 of spinach: characteristic ratios of Chl *a*/*b* were 2.2–2.4 (spectra for Chl *a-b* are not presented because of its comigration with CPa). Fluorescence spectra in Fig. 5 show that Chl *b* was efficiently coupled to Chl *a* (emission at 682 nm) in the Chl *a-b** complex and that energy transfer could be disrupted by heating samples to 60°C. when the majority of fluorescence excited by 465 nm light was emitted at 655 nm.

Larger quantities of the Chl-proteins were separated using the method of Argyroudi-Akoyunoglou and Thomou [14]. *Prochloroon* thylakoids were solubilised in SDS (SDS/Chl, 15 : 1) and centrifuged on a SDS-sucrose gradient (A, 10–25%; B, 5–25%). Three principal bands containing chlorophyll were apparent (Fig. 6). Each gradient was subfractionated; fractions were analysed (Table I)

for Chl-protein content (A and B), Chl *a/b* ratio (B) and P-700 content (B). The initial major zone contained mainly Chl *a-b**, Chl *a-b* and free pigment. The bottom zone contained mainly CP1* and was characterised by an absorbance maximum of 676 nm, fluorescence emission maxima at 688 and 710 nm, and was enriched 4-fold in P-700.

In experiment A, Chl *a-b** was most abundant in the tailing shoulder samples of the initial major zone with CP1 comprising most of the middle peak. Experiment B was run with a lower starting percentage of sucrose in the gradient in the hope of resolving Chl *a-b* and Chl *a-b**. This was achieved (perhaps fortuitously), Chl *a-b** now for-

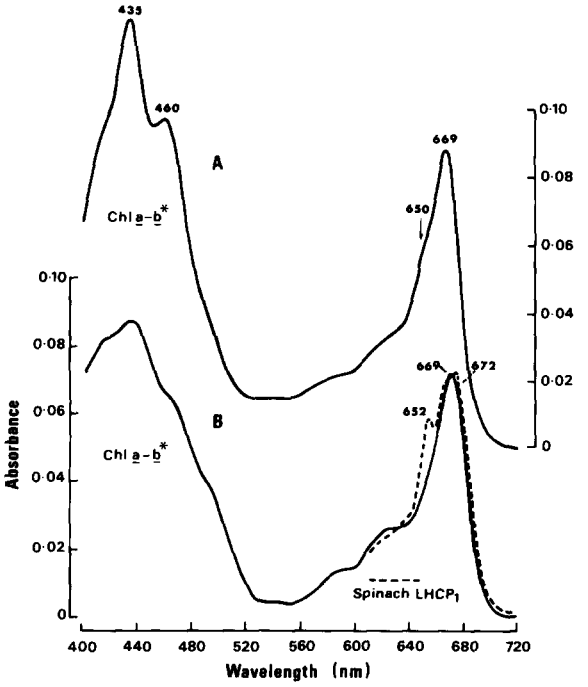


Fig. 4. Absorbance spectra of Chl *a-b** complex recorded in gel slices at room temperature after separation by SDS-polyacrylamide gel electrophoresis. Upper spectrum (A), complex (Chl *a/b* ratio of 2.2) from thylakoids having Chl *a*: *b* of 3.0. Lower spectrum (B), complex (Chl *a/b* ratio fo 2.4) from thylakoids having Chl *a/b* of 6.0. The absorbance spectrum of spinach LHCP, is also shown.

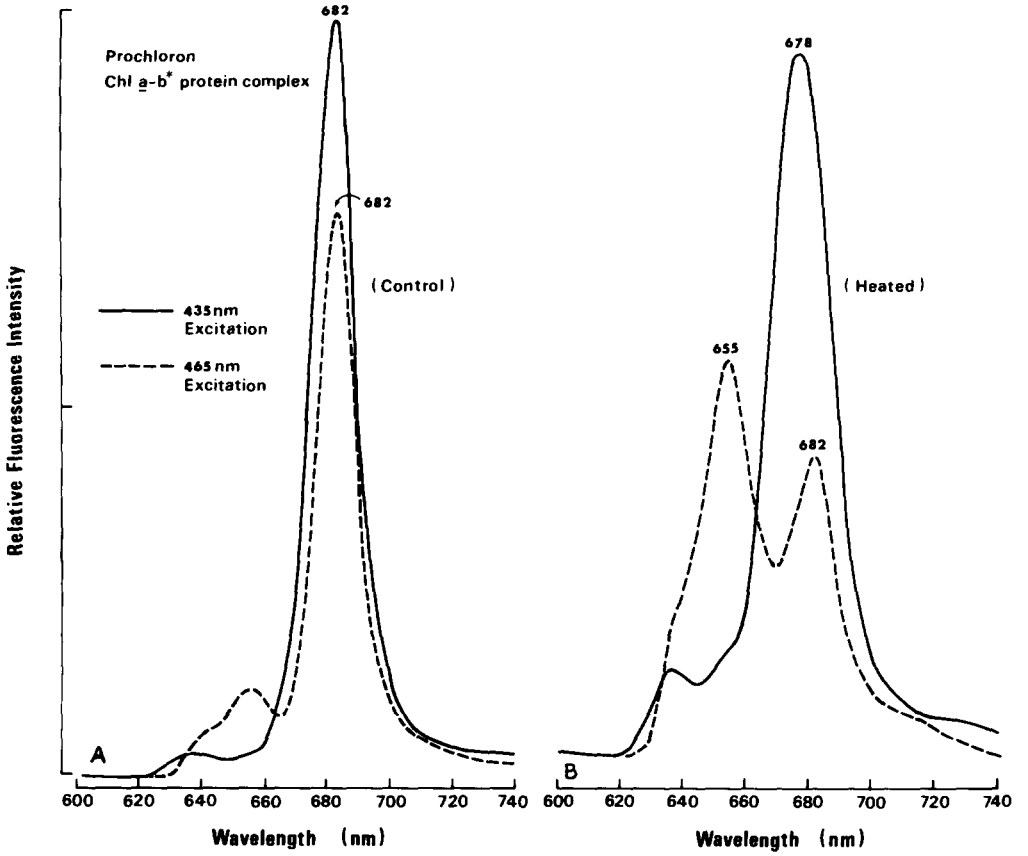
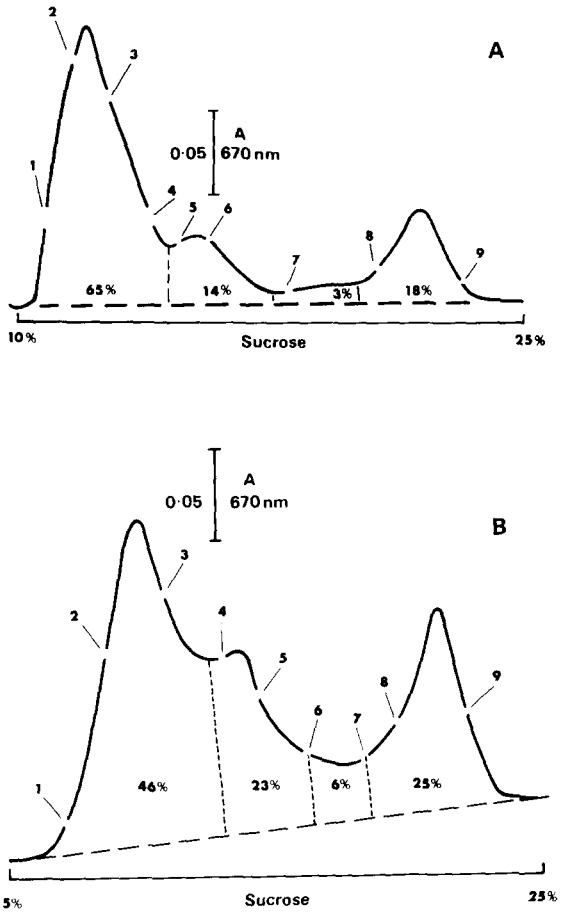


Fig. 5. Fluorescence emission spectrum at 77 K of Chl *a-b** complex after separation by SDS-polyacrylamide gel electrophoresis. (A) Before heating; (B) after heating.



ming a distinct peak, but with the loss of the peak due to CP1 and a concomitant increase in CP1* in the heaviest band. The proportion of chlorophyll in the upper part of both gradients (principally Chl *a-b* and Chl *a-b**) was very similar as was that in the lower part of both gradients (CP1 + CP1*).

Polypeptide analysis

The polypeptide composition of *Prochloron* thylakoids is very different from that of spinach (Fig. 7), particularly in the possession of intensely staining bands in the region of 30–35 kDa and absence of peptides in the region 25–28 kDa. The latter is the region of LHCP apoproteins obtained from green plants. When Chl *a-b** and Chl *a-b* were cut from nondissociating gels run at 4°C, and rerun on Laemmli gels at room temperature, both showed peptides at 30–35 kDa (with a particularly strong band at 34 kDa) in addition to bands at 40–55 kDa. CP1* only just entered the gel, despite losing most of its Chl, but also gave rise to minor peptides of 22, 20, 17 and 16 kDa.

The polypeptide composition of the SDS-

Fig. 6. Fractionation of SDS-solubilised *Prochloron* thylakoid fragments by sucrose-density gradient centrifugation assayed at 670 nm. (A) 10–25% and (B) 5–25% sucrose.

TABLE I

PROPORTIONS OF CHLOROPHYLL-PROTEIN COMPLEXES, CHLOROPHYLL *a/b* RATIOS AND CHLOROPHYLL/P-700 RATIOS, IN FRACTIONS SEPARATED BY SUCROSE GRADIENT CENTRIFUGATION FROM SDS-SOLUBLISED THYLAKOIDS

Experiment A, 10–25% sucrose gradient; experiment B, 5–25% sucrose. Fractions numbered from the start of sampling as shown in Fig. 6.

Fraction		1	2	3	4	5	6	7	8	9
% CP1*	A	0	0	0	0	0	0	66	87	–
	B	–	0	–	–	–	–	–	91	–
% CP1	A	0	0	7	41	86	89	34	11	–
	B	–	0	–	6	–	–	–	4	–
% Chl *a-b**	A	0	2	32	25	7	0	0	0	–
	B	–	6	–	87	–	–	–	–	–
% Chl *a-b*	A	24	46	29	17	0	0	0	0	–
	B	–	52	–	3	–	–	–	–	–
Free Chl	A	76	53	33	17	7	11	0	2	–
	B	–	42	–	4	–	–	–	5	–
Chl *a/b*	B	2.3	3.5	2.8	2.6	2.6	2.7	3.2	3.7	3.8
Chl/P-700	B	> 4000	> 4000	> 4000	2604	773	305	143	87	104

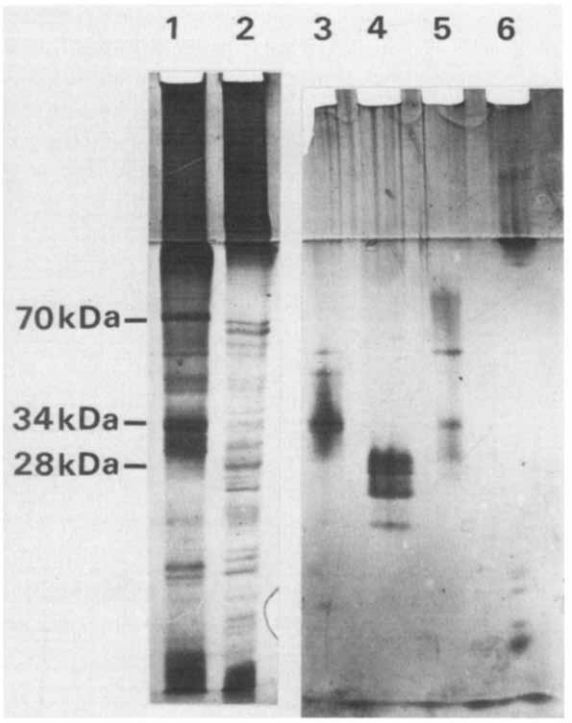


Fig. 7. Polypeptides of *Prochloron* thylakoids and chlorophyll-protein complexes rerun at room temperatures without boiling. The complexes were cut from a tube gel run at 4°C as in Fig. 2. Peptides are stained with silver stain. Lanes: (1) *Prochloron* thylakoids; (2) spinach thylakoids; (3) *Prochloron* Chl *a-b* complex; (4) spinach $LHCP_3$; (5) *Prochloron* Chl *a-b** complex; (6) *Prochloron* CP1*.

sucrose-density gradient fractions is shown in Fig. 8. A band at 34 kDa is present in fractions 1–5 of gradient A and is the predominant species in fractions 2 and 3, which also contain the highest proportions of Chl *a-b** and Chl *a-b* (Table I). A band at 70 kDa is present from fraction A4 onwards and is the principal peptide in fraction A8 which contained 98% of its chlorophyll in CP1* and CP1. Similar results were obtained from gradient B as also shown in Fig. 8. Fraction B2 shows prominent peptides at 46 and 52 kDa in addition to that at 34 kDa. We tentatively attribute both the former to Photosystem II chlorophyll apoproteins. Fraction B4 is dominated by the 34 kDa peptide as expected from its very high Chl *a-b** content. The Chl *a-b** content was 87% of the total Chl in this fraction and it had a Chl *a/b* ratio of 2.6. If all the remaining Chl were Chl *a*, the true

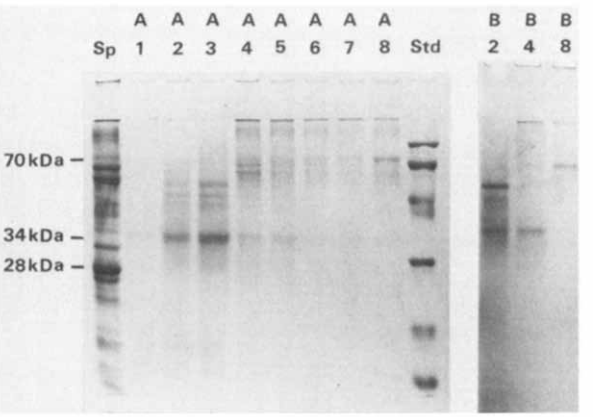


Fig. 8. Polypeptides from the SDS-sucrose-density gradients separated under fully denaturing conditions. Lanes are from left to right: spinach thylakoids; *Prochloron* expt. A 1–8; protein standards; expt. B samples 2, 4 and 8.

Chl *a/b* ratio for Chl *a-b** would be 2.26. Fraction B8 shows only the 70 kDa Photosystem I peptide and no peptide at 34 kDa, despite a considerable Chl *b* content.

Although Chl *b* might have been nonspecifically associated with these Photosystem I complexes from Fractions A8 and B8, heating them in the presence of SDS resulted in a large (approx. 10 ×) increase in fluorescence at 77 K with the increase coming from Chl *b*, as well as Chl *a*. Before heating there was no separate fluorescence emission from Chl *b*, even when the excitation wavelength was 465 nm. It is to be noted that no fraction showed any significant peptide at 28 kDa, the position of the apoprotein of spinach LHCP3 which showed in all experiments a characteristically greater electrophoretic mobility with its principal polypeptides at 28 and 25 kDa. Furthermore, 'Western' blots of *Prochloron* polypeptides showed no cross-reactivity of the 34 kDa peptide (or any other peptide) with antibodies to spinach LHCP under conditions in which the 25 and 28 kDa polypeptides of spinach and pea thylakoids reacted strongly.

Discussion

The Chl-protein complexes of *Prochloron* show two features of particular note: an atypical Chl *a-b* binding protein, and a Photosystem I complex

(designated CP1*), also containing Chl *b*, with a very high relative molecular mass. In cyanobacteria up to 6 forms of CP1 have been reported ranging in relative molecular mass from 100 to 400 kDa [23,25,26]. The CP1* of *Prochloron* appears to be very similar to the largest of these complexes, rather than to the CP1a of green algae and higher plants. All oligomeric forms of CP1 have in addition to a principal polypeptide of 60–70 kDa, lower-molecular-weight polypeptides in the range 10–24 kDa. Cyanobacteria have three polypeptides of 14, 13 and 10 kDa, whereas the higher plant CP1a contains additional peptides [27,28] in the 20–24 kDa range. Although we have not investigated CP1* extensively, re-electrophoresis of CP1* showed four polypeptides of 22, 20, 27 and 16 kDa, suggesting some features in common with CP1a of higher plants. It may be significant that we found Chl *b* to be associated with all the Photosystem I complexes of *Prochloron*. A light-harvesting complex specificially associated with PSI, and containing Chl *b*, has recently been reported for higher plants [27,28]. This had a Chl *a/b* ratio of 3.7 and several polypeptides of 19–24 kDa relative molecular mass, distinct from those of the LHCP associated with Photosystem II. It was subsequently shown that a single polypeptide of 20 kDa binds Chl *b* in this complex and that this peptide does not cross react with antibodies to the principal Photosystem I polypeptide of 67 kDa or to LHCP II [29].

Those Chl-protein complexes of *Prochloron* which are enriched in Chl *b* with respect to the whole membrane, that is Chl *a-b** and Chl *a-b*, showed marked differences from those of spinach LHCP1–3. These were a higher Chl *a/b* ratio and possession of polypeptides of higher apparent relative molecular mass. Minor LHCP species with similar Chl *a/b* ratios and polypeptides of 29–32 kDa have been reported from several higher plant species and a green alga *Acetabularia cliftoni* [30,31]. It is possible that one of these minor forms of LHCP is similar to part of the Chl *a-b* complexes studied here, since both thylakoids and reelectrophoresis of the Chl *a-b** and Chl *a-b* complexes showed polypeptides in the region of 30 kDa particularly when located by silver staining of the gels. Such polypeptides were not, however, obvious in fractions, separated by sucrose-gradient centrifugation, containing Chl *a-b** and Chl *a-b* complexes in which the 34 kDa polypeptide was predominant. We have obtained very similar results for the *Prochloron* sp. found in the ascidian *Lissoclinum patella* (Hiller and Larkum, unpublished data). Schuster et al. [32] have also reported on a similar polypeptide composition for a Chl *a-b* binding protein from *Prochloron* of *Diplosoma virens* obtained from the Red Sea.

Possession of CP1* links *Prochloron* with the cyanobacteria and this accords with the evidence of the carotenoid and lipid composition [3,6,7,33] and 16 S r-RNA [4], although Van Valen [5] had disputed the diagnostic value of the latter. The major feature linking *Prochloron* with green algae and higher plants has been possession of Chl *b*, LHCP and thylakoid stacking. Our data reveal substantial differences between LHCP1–3 and the Chl *a-b* binding proteins of *Prochloron* and do not exclude a dual evolutionary acquisition of Chl *b* and the binding protein(s).

Acknowledgements

This work was supported by research grants from Macquarie and Sydney Universities. We are grateful for assistance in this work by Ms A. Post, and to Dr. P. Mather and Mr. D. Parry, for help with identification of ascidian host material. 'Western' blot tests were kindly performed by Dr. I. Ryrie.

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