Biochimica et Biophysica Acta, 590 (1980) 309—323

BBA 47831

THE *P*-700-CHLOROPHYL *a*-PROTEIN COMPLEX AND TWO MAJOR LIGHT-HARVESTING COMPLEXES OF *ACROCARPIA PANICULATA* AND OTHER BROWN SEAWEEDS

J. BARRETT [a] and JAN M. ANDERSON [b]

[a] *School of Biological Sciences, Macquarie University, North Ryde, N.S.W. 2013 and*
[b] *CSIRO, Division of Plant Industry, P.O. Box 1600, Canberra City, A.C.T. 2601 (Australia)*

(Received September 10th, 1979)

Key words: Brown algae; Chlorophyll-protein complex; Fucoxanthin; Photosynthesis; Thylakoid membrane

Summary

Acrocarpia paniculata thylakoids were fragmented with Triton X-100 and the pigment-protein complexes so released were isolated by sucrose density gradient centrifugation. Three main chlorophyll-carotenoid-protein complexes with distinct pigment compositions were isolated.

(1) A *P*-700-chlorophyll *a*-protein complex, with a ratio of 1 *P*-700: 38 chlorophyll *a*: 4 β-carotene molecules, had similar absorption and fluorescence characteristics to the chlorophyll-protein complex 1 isolated with Triton X-100 from higher plants, green algae and *Ecklonia radiata.*

(2) An orange-brown complex had a chlorophyll *a* : c_2 : fucoxanthin molar ratio of 2 : 1 : 2. This complex had no chlorophyll c_1 and contained most of the fucoxanthin present in the chloroplasts. This pigment complex is postulated to be the main light-harvesting complex of brown seaweeds.

(3) A green complex had a chlorophyll *a* : c_1 : c_2 : violaxanthin molar ratio of 8 : 1 : 1 : 1. This also is a light-harvesting complex.

The absorption and fluorescence spectral characteristics and other physical properties were consistent with the pigments of these three major complexes being bound to protein. Differential extraction of brown algal thylakoids with Triton X-100 showed that a chlorophyll c_2-fucoxanthin-protein complex was a minor pigment complex of these thylakoids.

Abbreviations: Chl, chlorophyll: Tricine, *N*-tris-(hydroxymethyl) methylglycine; SDS, sodium dodecyl sulphate.

Introduction

Marine algae contribute considerably to global photosynthesis [1]. The greater part of this marine photosynthesis occurs in temperate zones and is effected by the benthic brown algae, *Phaeophyta*, and a variety of brown and golden phytoplankton [2,3]. All of these differ from terrestrial plants and green and red algae in having chlorophyll *c*, instead of chlorophyll *b* or phycobilins, as an adjunct to chlorophyll *a* in the light-harvesting pigment-protein complexes of their respective thylakoids [4]. Furthermore, in the brown seaweeds and in several classes of phytoplankton the carotenoid, fucoxanthin, as well as chlorophyll *c*, contributes significantly to the harvesting of the predominating blue-green light which filters through seawater to these algae [5,6].

Chlorophyll *c* differs from chlorophylls *a* and *b* in that it is a magnesium-porphyrin and the phytylated propionic side chain of Chls *a* and *b* is replaced by an unesterified acrylic side chain [7]; thus this tetrapyrrole is much less liphophilic than either Chls *a* or *b*. Two different Chls *c* occur in different relative amounts in brown algae [8,9]: Chl c_1 has one vinyl side chain, whereas Chl c_2 has two vinyl side chains [7]. The absorption spectra in organic solvents of Chls c_1 and c_2 are of the magnesium-porphyrin type, exhibiting low absorption in the red region at a shorter wavelength (approx. 635 nm) than that of Chl *a* (660 nm) and Chl *b* (645 nm) but a greater absorption in the blue region of the spectrum [10]. Absorption and fluorescence action spectra of Chls *a* and *c* complexed to protein in chloroplasts of *Laminaria digitata* [11], are in accord with the differences between the spectra of free Chl *a* and Chl *c* in organic solvents, but exhibit red shifts to be expected from the complexing of the chlorophylls to protein [12].

Recent evidence from polyacrylamide gel electrophoresis indicates that most of the chlorophyll and some carotenoid are complexed to proteins in higher plant thylakoids [13—15]. Data from Resonance Raman spectroscopy also are consistent with chlorophylls being bound to protein in such complexes [16].

This paper is concerned with the isolation and partial characterisation of the major intrinsic pigment-protein complexes of *Acrocarpia paniculata* [17] thylakoids. These are a *P*-700-chlorophyll *a*-β-carotene-protein complex and two major chlorophylls *a* + *c*-carotenoid-protein complexes. Brief descriptions of the major chlorophyll-protein complexes of *Ecklonia radiata* [18] and *A. paniculata* [19] have been previously reported.

Materials and Methods

Fronds of *A. paniculata* [17] were gathered from rock shelves about 1 m below low tide level at Guerilla Bay, New South Wales. Other seaweeds were collected from different water levels according to their habitat. All seaweeds were transported the 180 km to Canberra in chilled seawater with constant aeration. Seaweeds were either used immediately for critical experiments, or stored in frequently changed and aerated seawater for 2—3 days after collection.

Chloroplast isolation. Thalli were cut from stems with a razor blade and the tissue washed with chilled seawater; 50-g amounts were macerated in 200 ml

isolation medium containing 1 M sorbitol and 2% (w/v) poly(vinylpyrrolidine) (M_r 10 000) in filtered seawater in a Waring blendor at 4°C for 1 min at top speed. The homogenate was further disrupted in the 400 ml vessel of a Sorval Omnimixer at top speed for 1 min. The homogenate was filtered through a pad consisting of two layers each of cotton gauze, Miracloth and cotton gauze. The filtered homogenate was centrifuged at 750 × *g* for 10 min and the pellet resuspended in isolation medium using a glass and Teflon homogeniser. The suspension was centrifuged at 120 × *g* for 10 min, the pellet discarded, and the supernatant (200 ml) was centrifuged at 750 × *g* for 10 min to sediment the chloroplasts. These were washed four times with seawater containing 1 M sorbitol, reducing the initial volume of solution (200 ml) by half each time.

Crude chloroplasts were resuspended in 50 mM tricine (pH 8.0) containing 100 mM KCl and 10 mM EDTA (200 ml/100 g wet tissue) in a glass homogeniser using 6—8 passes of the Teflon plunger. The resultant suspension was centrifuged at 750 × *g* for 10 min. This procedure, designed to remove extrinsic proteins and mucilage from the thylakoids, was repeated until the supernatant was clear. Chloroplasts which were not used immediately were stored in liquid N_2 in 1 M sorbitol/seawater to avoid aggregation.

Triton X-100 fragmentation of thylakoids. Chloroplasts were osmotically shocked by suspending aliquots (1—2 mg Chls *a* + *c*) in 100 ml of 5 mM Tricine (pH 8.0) containing 10 mM KCl for 10 min at 4°C. The suspension was then centrifuged at 15 000 × *g* for 10 min. The thylakoids were fragmented by incubation in 1% (w/v) Triton X-100(Rohm and Hass) in 50 mM Tricine (pH 8.0) using a Triton X-100/Chl *a* + *c* weight ratio of 50, for 30 min at 4°C with stirring. The extract was centrifuged at 15 000 × *g*. The supernatant contained 80—90% of the total Chl. Extraction of the pellet at a Triton X-100/Chl *a* + *c* ratio of 40 released the remaining chlorophyll-proteins.

Sucrose density gradients (11 ml per tube) were made in 50 mM Tricine (pH 8.0) (Fig. 1). 1 ml of Triton X-100 extract was applied to each tube. When necessary, Triton X-100 extracts were concentrated by centrifugation in Centriflo cones (CF 25A Amicon) at 1000 × *g*. Centrifugation was carried out in a Beckman SW 41 rotor at 2°C at 273 000 × *g* for 48—60 h. Fractions were sampled either by collection of drops, or when bands were discrete, by the use of a fine needle.

Differential extraction of chloroplasts with Triton X-100. Acrocarpia chloroplasts (fresh or stored for several months at 77 K) were osmotically shocked as above and then extracted with 0.5% Triton X-100 in 50 mM Tricine (pH 8.0) at a Triton X-100/Chl *a* + *c* ratio of 50, with stirring at 4°C for 20 min. The suspension was centrifuged at 15 000 × *g* for 10 min and the peller obtained was extracted with two-thirds the initial amount of the 0.5% Triton X-100 buffer. This procedure was repeated four times, and the six extracts obtained were subjected to sucrose density gradient centrifugation at 273 000 × *g* for 48 h at 2°C as above.

Detemination of pigments. Chlorophylls *a* and $c_1 + c_2$ were quantitatively estimated after extraction into chilled 80% acetone by measuring the absorbance of the extracts at 663 and 630 nm on a Cary 14R spectrophotometer, using the equations of Jeffrey and Humphrey [20]. Fluorescence emission and

excitation spectra at 77 K were recorded in 60% glycerol in 50 mM Tricine (pH 8.0) using a fluorescence spectrophotometer incorporating automatic correction for the photomultiplier and monochromator responses and variation in output of the light source [21]. A sensitive fluorometric method was developed to measure the molar ratios of Chl *a*/Chl $c_1 + c_2$ and Chl c_1/Chl c_2 by fluorescence spectroscopy at 77 K of 95% ethanol extracts of the pigment complexes. This followed the same procedure used for the determination of Chl *a*/Chl *b* ratios of higher plants [22]. Relative fluorescence emission values (ϕ) for Chl c_1 and Chl c_2 previously determined by S.W. Thorne (in Ref. 10) were used.

The carotenoids and Chls c_1 and c_2 were identified by spectra, and by thin-layer chromatography. The pigments of various fractions in 80% acetone were taken into diethyl ether at 4°C using cold 5% aqueous NaCl to create two phases. The ether phase was washed twice with cold 5% NaCl. Chlorophylls *a*, c_1 and c_2 were separated by poly(ethylene) thin-layer chromatography [10]; the pigments were eluted immediately with acetone and concentrations calculated as above [20]. For carotenoid chromatography, the chlorophylls were saponified at 4°C for 60 min in methanolic KOH (60% aqueous KOH/CH_3OH, 1 : 10 v/v). The carotenoids were extracted into diethyl ether and the ether phase was washed several times with glass-distilled water. The water was removed by freezing and the ethereal extract was concentrated under N_2. Chromatography was carried out on Silica gel G plates (Merck) with benzene acetone (3 : 1, v/v) as developing solvent. The separated carotenoids were immediately eluted from the silicagel using diethyl ether for β-carotene and ethanol for the xanthophylls. Carotenoids were identified by their R_F values and absorption maxima [23]. The observed λ_{max} were for β-carotene in diethyl ether, 477, 449 and 428 nm; fucoxanthin in ethanol, 470, 448 and 420 nm; violaxanthin in ethanol, 469, 439 and 416 nm.

The concentration of the carotenoids was also estimated spectrally after partition of the pigments in a two phase mixture. The fraction (0.3 ml) was shaken with CH_3OH/H_2O (9/1, v/v) (2.7 ml) and light petroleum, 60—80°C fraction (1.5 ml). Xanthophylls and Chl $c_1 + c_2$ remained in the methanol phase, while Chl *a* and β-carotene went into the lighter phase. Absorption spectra of both phases were recorded and the carotenoid concentrations calculated by the equations of Kirk [24].

P-700 concentration in thylakoid fractions was assayed by two methods, using a millimolar extinction coefficient of 64 [25]: (a) from the ferricyanide-oxidised minus ascorbate-reduced difference spectrum of samples in 50 mM Tricine (pH 8.0) [26]; (b) from the light-induced absorbance change at 698 nm of samples in 50 mM Tricine buffer (pH 8.0) containing 3 mM sodium ascorbate and 166 μM methyl viologen [27].

Photosystem 2 activity was assayed in a Chance Aminco dual wavelength spectrophotometer fitted with a side-illumination attachment; reduction of dichlorophenolindophenol was measured at 590 nm, using either water or diphenylcarbazide as the electron donor [28].

Results

Chloroplast isolation and fragmentation with Triton X-100

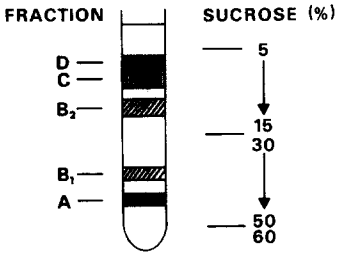
A. paniculata was a brown seaweed available throughout the year. It was

relatively free of mucus and had filamentous thalli from which chloroplasts could be obtained more readily than those seaweeds which have lamellar thalli. The yield of chloroplasts was 6—8 mg total Chl $a + c$ per 50 g wet weight of fronds. The molar ratio of Chl $a/c_1 + c_2$ of 3.5—4.0 was higher than that of *E. radiata* chloroplasts [18]. The molar ratio of Chl c_2/Chl c_1 was 3.4. Electron microscopy showed that the chloroplasts had intact envelope membranes, although the apposition of the pallisaded sets of three double thylakoid membranes was perturbed. Chloroplasts were also prepared from seaweeds from five orders of *Phaeophyta: E. radiata, Phyllospora comosa, Sargassum* sp., *Scytothamnus australis, Hormosira banksii, Padina commersonii, Cladostephus spongiosus, Colopomenia sinuosa,* and *Pocockiella variegatus.* The molar ratios of Chls *a/c* were either similar to that of *A. paniculata* or somewhat higher, according to habitat or season. The most variable results were obtained with *Phyllosporum* where the Chl *a/c* molar ratio was sometimes as high as 9.

When freshly isolated, washed chloroplasts were fragmented with 1% Triton X-100 (Triton/Chl ratio of 50) about 90% of the pigment was released in one extraction, and the rest was released with a second extraction leaving a mucilaginous deposit in the centrifuge tube. When chloroplasts had been stored at 77 K for several months, multiple extractions were necessary to completely fragment the thylakoids with 1% Triton X-100. The fractionation of Triton X-100 extracts of *Acrocarpia* thylakoids by sucrose-density gradient centrifugation resulted in the appearance of several discrete pigment zones (Fig. 1): a narrow apple-green band near the bottom of the tube (fraction A), two orange-brown zones of different buoyancy (fractions B_1 and B_2), a main green band (fraction C) contiguous with fraction B_2, and a minor yellow-green zone (fraction D). Fraction B_1 was present in *Acrocarpia* but was diminished or absent in *Ecklonia, Sargassum* and *Phyllospora.*

Characterisation of chlorophyll-protein complexes

The absorption spectrum of fraction A from *Acrocarpia* (Fig. 2) had absorption maxima at 674 nm and 438 nm due to Chl *a*, but no absorption attributable to Chls c_1 or c_2. The slight asymmetry on the red side of the Soret peak is due to a small amount of β-carotene. Thin-layer chromatography of the extracted pigments showed that only Chl *a* and β-carotene were present. The absence of Chls c_1 or c_2 was confirmed by fluorescence emission spectroscopy at 77 K of ethanol extracts. The molar ratio of Chl *a*/β-carotene was 10 (Table I). Fraction A contained *P*-700; assay of the amount of *P*-700 present, by either the chemical or light-induced oxidation method, showed one molecule of *P*-700 for 38 antennae Chl *a* molecules for the *Acrocarpia* complex. About 18% of the total chlorophyll of *Acrocarpia* thylakoids was present in the *P*-700-Chl *a*-protein complex. The main fluorescence emission peak of the Acrocarpia *P*-700-Chl *a*-protein complex at 77 K was at 680 nm, with a weak satellite peak at 728 nm. The fluorescence yield was extremely low (Fig. 3) (cf. Ref. 21). The excitation spectrum with a peak at 436 nm due to Chl *a*, and some excitation at 490 nm due to β-carotene, shows that β-carotene is transferring its energy to Chl *a*. All of the *P*-700-Chl *a*-protein complexes isolated from the seaweeds examined had spectral characteristics and Chl/*P*-700 ratios similar to those of the *Acrocarpia* complex.



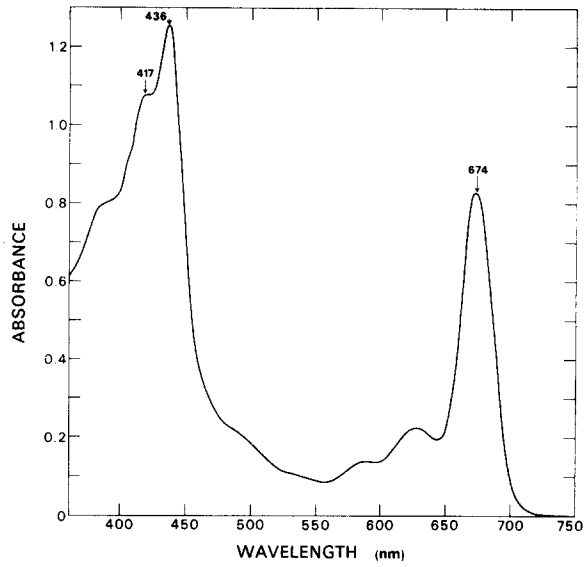


Fig. 1. Scheme of the separation of Chlorophyll-proteins in the 1% Triton X-100 extract of *Acrocarpia* thylakoids in sucrose density gradients. Sucrose: 60% (1 ml), 30—50% (5 ml), 5—15% (5 ml) and Triton X-100 extract (1 ml), all in 50 mM Tricine (pH 8.0). Centrifugation in a Spinco SW 41 rotor, at 275 000 × *g* for 48 h at 2°C.

Fig. 2. Absorption spectrum of *Acrocarpia* fraction A (*P*-700-Chl *a*-protein complex) in 50 mM Tricine (pH 8.0).

Both fractions B and C were enriched in Chls *c* compared to chloroplasts. The absorption spectrum of fraction B (Fig. 4) shows enhanced absorption at 460—465 nm and 634—638 nm due to Chl c_2 and in the 500—550 nm region due to fucoxanthin.

An outstanding feature of the pigment composition of fractions B_1 and B_2 was the absence of Chl c_1. That only Chls *a* and c_2 were present was confirmed by poly(ethylene) thin-layer chromatography and by a sensitive fluorometric method which could have detected the presence of one part of Chl c_1 in 100 parts of Chl c_2. The molar ratio of total Chls $a + c_2$/fucoxanthin was 1.5 Table I). Fractions B_1 and B_2 were identical in their pigment components and the proportions of these.

The absorption spectrum of fraction C (Fig. 4) compared to that of chloroplasts showed increased absorption at 460 and 634 nm due to Chls $c_1 + c_2$, and

TABLE I

MOLAR RATIOS OF PIGMENTS IN THE CHLOROPHYLL-PROTEIN COMPLEXES OF *A. PANICULATA*

Complex	Chl *a*	*P*-700	Chl c_1	Chl c_2	Fucoxanthin	Violaxanthin	β-Carotene
Fraction							
A	38	1	0	0	0	0	4
B_1 and B_2	2	0	0	1	2	trace	0
C	4	0	0.5	0.5	0.05	0.5	trace

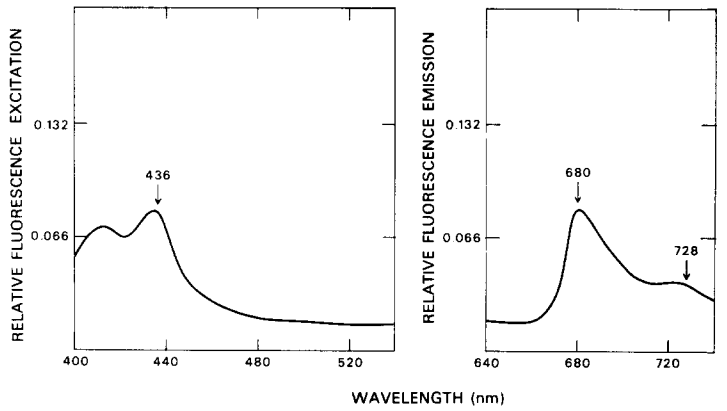


Fig. 3. Fluorescence excitation and emission spectra of *Acrocarpia* fraction A (*P*-700-Chl *a*-protein complex) at 77 K. The emission wavelength for the excitation spectrum was 684 nm and the excitation wavelength for the emission spectrum was 436 nm. The absorbance of the sample was 0.1 at 674 nm.

there was no enhanced absorption in the 500—550 nm region due to fucoxanthin. A marked shoulder at 481 nm in the spectrum is due to violaxanthin, the principal carotenoid present. The absorbance ratio of the Soret peak/Red peak was 2.25 which was much higher than for the *P*-700-Chl *a*-protein com-

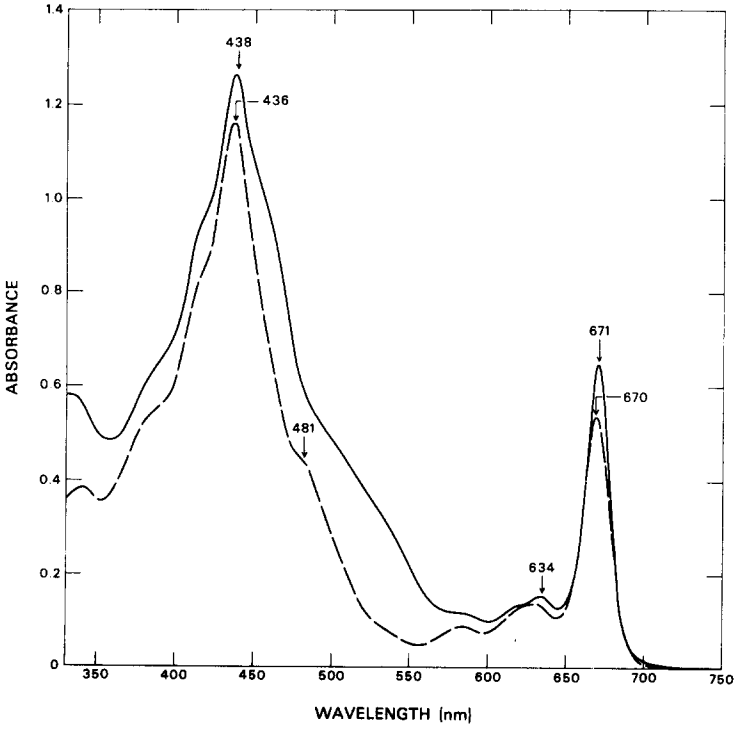


Fig. 4. Comparison of the absorption spectra of the light-harvesting complexes of *Acrocarpia:* (a) ——, Fraction B_1 (Chl *a* + c_2-fucoxanthin-protein complex); (b) - - - - -, fraction C (Chl *a* + c_1 + c_2-violoxanthin-protein complex) in 50 mM Tricine buffer (pH 8.0).

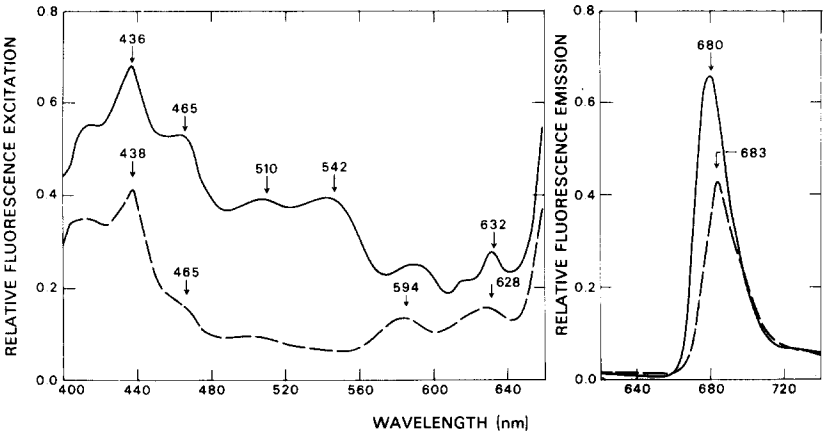


Fig. 5. Fluorescence excitation and emission spectra of the light-harvesting complexes of *Acrocarpia* at 77 77 K. (a) ————, Fraction B_1 (Chl *a* + c_2-fucoxanthin-protein complex); (b) - - - - - -, fraction C (Chl *a* + c_1 + c_2-violaxanthin-protein complex): The emission wavelength for excitation spectra was 681 nm and the ecitation wavelength for the emission spectra was 440 nm. Absorbance was 0.1 at 671 nm.

plex (1.46), and this probably reflects, in part, differences in the binding of chlorophylls to the proteins. Fraction C is contained both Chl c_1 and Chl c_2. The molar ratio of the Chl *a*/Chls c_1 + c_2 was 3.0 and Chls c_1 and c_2 were present in about equal amounts (Table I). The fluorescence emission spectrum of fraction C (Fig. 5) was similar to that of fractions B. Only the Chl *a* has a fluorescence emission peak; there are none for either Chl c_1 or Chl c_2 (636—640 nm) in either fractions B or C. The excitation spectrum of fraction C shows little energy transfer in the 500—560 nm region in the absence of fucoxanthin. The violaxanthin present in fraction C noticeably does not contribute as largely to the excitation spectrum as fucoxanthin does to the excitation spectrum of fraction B (Fig. 5). The fluorescence excitation spectrum of fraction B has a low peak at 463 nm, in addition to those of chlorophyll *a* (440 nm and 420 nm). This excitation peak arises from the Chl c_2-protein component in fraction B. Fraction C has a less pronounced peak at 460 nm due to a Chl c_1 + c_2-protein component.

The absorption spectrum of fraction D which contained about 10% of the total chlorophyll was generally similar to that of fraction C except that the 480 nm shoulder was more distinct. The absorbance ratio of the Soret peak/Red peak of 2.65 was higher than for any other of the pigment-protein complexes. The fluorescence emission yield was also high.

Photochemical activities

Photosystem I activity was detected only in the *P*-700-Chl *a*-protein. In all of *P*-700-Chl *a*-protein complexes isolated from the brown algae, *P*-700 could be detected both by oxidized minus reduced difference spectra and by light-induced absorbance changes. Photosystem II activity was not detected in any of the chlorophyll-protein fractions using either water or diphenylcarbazide as the electron donor. *Acrocarpia* chloroplasts treated with 1% Triton X-100

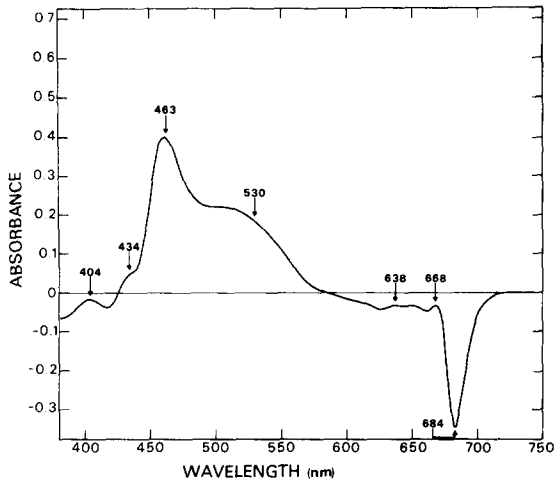


Fig. 6. Difference absorption spectrum of fraction B_1 (Chl *a* + c_2-fucoxanthin-protein complex) vs. fraction A (*P*-700-Chl *a*-protein complex). The absorbance (0.9) of the two fractions was equalized at their red maximum.

photoreduced 40 μmol dichlorophenolindophenol/mg Chl per h with diphenylcarbazide as the electron donor.

Difference Spectra

As the absorption spectra of the chlorophyll-protein complexes are a composite of different chromophoric centres, difference spectroscopy was used to cancel out the contribution of chromophores common to the fractions, revealing spectral components in excess in a particular fraction. Fraction A which has only Chl *a* and β-carotene was used as a reference. The absorption of each sample was equalised at the A_{max} of the red peak.

The difference spectrum (Fig. 6) of fraction B_1 versus fraction A showed a major peak at 463 nm due to Chl c_2 and a hump centred at 520—530 nm due to fucoxanthin. The fraction A side of the difference spectrum showed a distinct peak at 684 nm. The difference spectrum (Fig. 7) of fraction C vs. fraction A exhibited a major peak at 456 nm and a shoulder ascribable to violaxanthin at 480 nm. A peak with A_{max} at 684 nm was present also on the fraction A side of the curve. The excess contribution of the 684 nm component of the difference spectra (Figs. 6 and 7) to the absorbance of the red region of fraction A (Fig. 2) was estimated by cutting out and weighing the area of chart paper under each curve. It was estimated that the 684-nm components of fractions B and C accounted for at least 22% of the total absorbance of fraction A in the red region. These values would be greater if either fractions B or C also contained some 684 nm component.

Differential extraction of thylakoids with Triton X-100

Lowering the concentration of Triton X-100 decreased the extractability of the chlorophyll-protein complexes from thylakoids. Advantage was taken of this to differentially extract these complexes from thylakoids of *Acrocarpia*

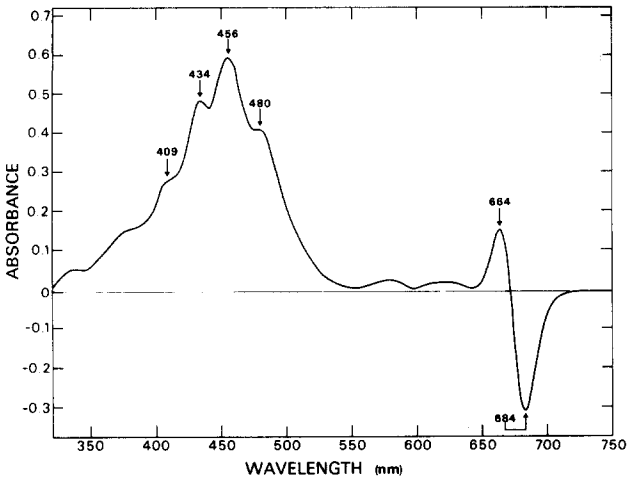


Fig. 7. Difference absorption spectrum of fraction C (Chl *a* + c_1 + c_2-violaxanthin-protein complex) vs. fraction A (*P*-700-Chl *a*-protein complex) isolated from *Acrocarpia*. Each fraction had an absorbance of 0.9 at their red absorption maximum.

and *Padina*. Sucrose density gradient centrifugation analysis of six successive extractions of *Acrocarpia* chloroplasts with 0.5% Triton X-100 in 20 mM Tricine (pH 8.0) showed the orange-brown complex was mostly in the first extract and located in the centrifuge tube in the same position as fraction B_1 (Fig. 1); the remainder came out in the second extract. The complex, having Chl *a* and Chls c_1 + c_2 located similarly to fraction C (Fig. 1), was distributed

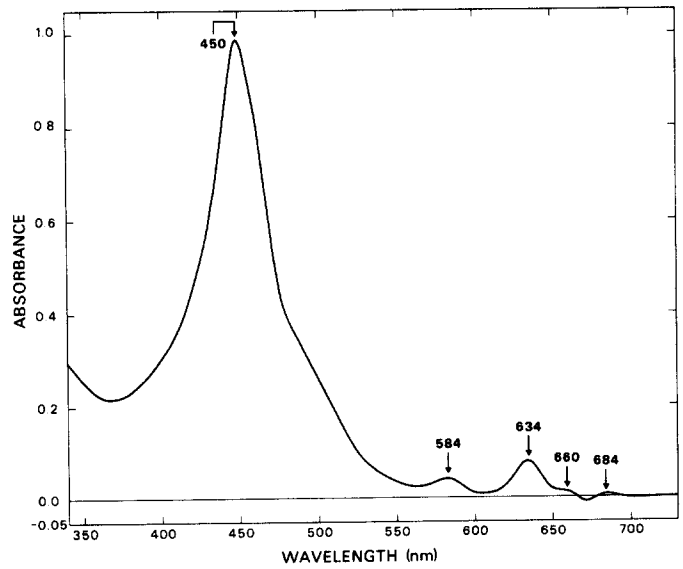


Fig. 8. Difference absorption spectrum of the first extract versus the second extract from the differential solubilization of *Acrocarpia* thylakoids with Triton X-100, as detailed in Materials and Methods. Each extract had an absorbance of 1.0 at 672 nm.

through the six extracts. A buoyant zone containing Chls $a + c_1 + c_2$-carotenoid-protein complexes was also present in the sixth tube. The *P*-700-Chl *a*-protein complex was not extracted until the Triton X-100 concentration was increased. A difference spectrum (Fig. 8) of the first extract versus the second extract, equalized at A_{max} (672 nm), from this experiment shows peaks on the extract first side of the spectrum at 634, 584, and 540 nm, due to Chl c_2 complexed to a protein. Weak absorption peaks at 684 nm and 660 nm were also present.

The occurrence of a minor Chl c_2-protein complex as a component of *Acrocarpia* thylakoids was established when osmotically shocked chloroplasts (1 mg Chls $a + c$) were repeatedly treated with 0.05% Triton X-100 (20 ml) in 50 mM Tricine (pH 8.0), using a Teflon glass homogeniser. The first three or four extracts were colourless, but concentration of succeeding straw-coloured extracts by means of low-porosity Centriflo cones yielded a deep-amber solution. The absolute absorption spectrum of this fraction was very similar to that of the difference spectrum (Fig. 8) having a main absorption peak at 450 nm and minor peaks at 586 and 638 nm, and an even weaker peak at 682 nm. Chl c_2 and fucoxanthin were the only pigments present in this fraction.

Discussion

We have reported the isolation of chlorophyll-carotenoid-protein complexes from a brown seaweed having laminar thalli, *E. radiata* [18]. Difficulties encountered in the rupturing of cells and the presence of copious mucilage resulted in low yield and hindered dispersion of the thylakoid membranes and the resolution of the various chlorophyll-protein complexes. The use of a seaweed with filamentous thalli and improvements in the isolation procedure, such as maintaining a high salt and sorbitol concentration prior to the osmotic shock, has improved the yield of chloroplasts and the separation of the various chlorophyll-protein complexes.

The chloroplasts from brown seaweeds are more resistant to disintegration by Triton X-100 than those of higher plants. That relatively stronger binding forces are present in thylakoids of brown seaweeds is suggested by the retention of the stacking pattern of the thylakoid membranes, after the thylakoids had been extensively extracted with 1% Triton X-100 (Barrett, J. and Goodchild, D.J., unpublished results).

Fraction A from *Acrocarpia*, and the other brown seaweeds examined, has absorption spectral characteristics almost identical with those of the *P*-700-chlorophyll *a*-protein complex isolated from *E. radiata* [18] and with those obtained from higher plants [13,29]. This displacement of the α-peak of the Chl *a* 4.5 nm to the red compared to the light-harvesting chlorophyll *a*-proteins of this study and of higher plants [14] is characteristic. One *P*-700 is associated with 38 Chl *a* molecules; a similar stoicheiometry is found for the *P*-700-Chl *a*-protein complexes obtained by Triton X-100 from thylakoids of higher plants [15,30]. Retention of the geometry of the chlorophyll molecules essential for efficient energy transfer within the isolated *Acrocarpia* *P*-700-Chl *a*-protein complex is shown by the oxidation of *P*-700 by light. β-Carotene is the only carotenoid present as is the case with higher plant *P*-700-Chl *a*-protein com-

plexes [15,30] and *E. radiata* [18]. The very low fluorescence yield and the position of the main fluorescence emission at 680—684 nm, are in accord with the properties of *P*-700-Chl *a*-proteins (Chl/*P*-700 of 40) isolated by Triton X-100 from higher plants [15,30]. However, the fluorescence properties of these Triton X-100 *P*-700-Chl *a*-proteins are not identical to those we have observed for the complex in brown algae thalli (cf. Ref. 31). The characteristic long-wavelength fluorescence emission at 735 nm which has been associated with Photosystem I [30] is lacking. This is present in spinach *P*-700 chlorophyll *a*-protein complex isolated by SDS-polyacrylamide gel electrophoresis [13] and in digitonin Photosystem I particles [32].

Curve analysis of absorption spectra of chloroplasts and chlorophyll-protein complexes, using Gaussian-shaped components has shown that there are several forms of chlorophyll *a* in vivo [33,34]. The absorption spectrum of *P*-700-Chl *a*-protein of *Euglena* sp. and higher plants contained six spectral forms of Chl *a* [33], including a component with A_{max} (684 nm) which accounted for 15—18% of the Chl *a*. Normal chloroplasts of higher plants were shown by Hiller et al. [35] to contain a component absorbing at 684 nm using difference spectra of chloroplasts from normal and lincomycin-treated greening barley and peas. The 684 nm component revealed in the difference spectra of *Acrocarpia* *P*-700-Chl *a*-protein complex contributed at least 22% to the absorption of the red peak, close to the value calculated by deconvolution analysis for *Euglena* [33]. Since about 2.5% of the Chl *a* of the *Acrocarpia* complex is in *P*-700, some 75% of the Chl *a* therefore contributes to the absorption of the other wavelength components.

The other two major pigment complexes together contain about 70% of the Chl *a*, and most of Chls c_1 and c_2. They are termed light-harvesting complexes as they have fluorescence emission which overlaps with the absorption of the reaction centre complexes of Photosystem I and II and they have no detectable Photosystem I or II photochemical activity. Significantly each complex contains a distinct pigment composition (Table I); fractions B_1 and B_2 contain a Chl *a* + c_2-fucoxanthin-protein complex, and fraction C includes a Chl *a* + c_1 + c_2-violaxanthin-protein complex. The fucoxanthin complex has no Chl c_1, while the other complex has Chl c_1 in equal amounts to Chl c_2. The significance of the distribution of the two Chls *c* is not apparent as Chl c_1 differs from Chl c_2 only by having an ethyl instead of a vinyl side chain, with little difference in their absorption spectra.

Another distinction between the two light-harvesting complexes is in the carotenoid present. Significantly, most of the chloroplast fucoxanthin is found in the orange complex. The chlorophyll/fucoxanthin molar ratio is greater than found in a metal-pigment-protein complex (Chl *a*, 2; Chl *c*, 1; fucoxanthin, 1) isolated from a diatom, *Phaeodactylum tricornutum* by Holdsworth and Arshad [36]. Fraction B is enriched in violaxanthin but it has traces of other carotenoids (Table I), and it has less carotenoid on a chlorophyll basis than Fraction C. Fucoxanthin absorbs further into the green region of the spectrum than the other carotenoids or Chls *c* [5,6,19], especially fitting it to be a chromophore in the light-harvesting complexes of marine algae. Goedheer [37] found efficient energy transfer for both fucoxanthin and Chl c_2 in *Laminaria* [11,37]. Tanada [38] calculated that the light absorbed by fucoxanthin was utilized

with the same efficiency as that of Chls *a* and *c* in *Navicula minima*. The amount of chlorophyll associated with the two complexes was not the same in the seaweeds examined. In *Acrocarpia*, *Padina* and *Ecklonia*, the complexes had about equal amounts of chlorophyll, but in *Sargassum* sp. and *P. comosa* the yield of the fucoxanthin complex was only 1/5th that of the violaxanthin complex on a chlorophyll basis. The variable yield of the fucoxanthin complex, as well as it containing the main light-harvesting carotenoid, fucoxanthin, suggests that this complex has an analogous role to that of the light-harvesting Chl *a*/*b*-protein complex of higher plants [19].

Notably, with both light-harvesting complexes all the fluorescence is emitted by Chl *a* and none is emitted by Chls *c* at $c = 636$ nm (Fig. 5). This similarity of the fluorescence excitation spectra to the absorption spectra in the blue-green region (Fig. 4) of both complexes indicates that Chl *a*, Chls *c* and the cartenoids are in a highly ordered molecular arrangement. Further, there is efficient energy transfer from the carotenoids, Chls c_1 and c_2 and from the shorter wavelength forms of Chl *a* to the longer wavelength form of Chl *a*. These features demonstrate that the molecular organization of the pigments of both light-harvesting complexes has not altered to any extent during isolation, and are also consistent with the pigments being bound to protein. Indeed the behaviour of these complexes on SDS polyacrylamide gel electrophoresis in the presence of Triton X-100 or of cholate showed that all the pigments are associated with protein.

The *P*-700-Chl *a*-protein complex was the most firmly embedded complex in the thylakoid of all examined seaweeds, withstanding removal from the membrane unless high concentrations of Triton X-100 or steroidal detergents were used, particularly when chloroplasts had been stored at 77 K. This is in direct contrast to the behaviour of the *P*-700-Chl *a*-protein of higher plants where differential extraction with Triton X-100 showed that it was most easily removed from the thylakoids [39]. The Chl $a + c_1 + c_2$-violaxanthin complex was more tightly embedded in the thylakoids than the Chl $a + c_2$-fucoxanthin-protein complex.

A *P*-700-Chl *a*-protein complex and two major light-harvesting complexes have been isolated from several species of brown seaweeds, but other minor chlorophyll-carotenoid protein complexes also occur in the thylakoids. The difference spectrum (Fig. 8) from the differential extraction of *Acrocarpia* thylakoids with 0.5% Triton X-100 revealed the existence of a Chl *c* component with Soret peak maximum at 450 nm. This was confirmed by the isolation of a Chl c_2-fucoxanthin-protein complex having the same absorption maxima as in Fig. 8, and of low molecular weight [40]. A Chl *a*-protein and a yellow carotenoid-protein of low molecular weights have now been isolated from *Acrocarpia* chloroplasts. Both minor components are present in fraction D [40].

The occurrence of Chls *c*, instead of Chl *b* as a photoaccessory pigment in the brown algae raises some interesting questions. Chlorophyll *c* has an acrylic acid side chain instead of phytylated propionic acid; consequently stabilization of the Chl *c*-containing protein complex by extensive interactions of a long alkyl chain with hydrophobic amino acids of the polypeptide skeleton as found in the bacteriochlorophyll-protein complex from a green photosynthetic bacterium [41] is not possible. As found for protohaem in cytochromes *b* and

haemoglobins [42,43], it is probable that the Chls c_1 and c_2 are bound to protein through a strong π-electron donor, e.g., histidine liganding to the central metal. This structure would be consistent with the spectra of Chl *c* complexed to pyridine [10]. The stability of these Chl *c*-containing complexes implies that the Chl *c* is in a hydrophobic pocket of the protein. Efficient transfer of energy from the Chls *c* and the carotenoids requires close proximity to the receptor Chl *a*. Possible molecular topography for the peridinin-Chl *a*-protein complex of *Glenodinium* sp. has been proposed [44,45]. Since fucoxanthin and peridinin are both allenic xanthophylls there may be some similarity of molecular arrangement in the light-harvesting units of the brown seaweeds and these marine dinoflagellates.

Acknowledgements

We are particularly indebted to Dr. S.W. Thorne, CSIRO for fluorescence spectroscopy and discussions, and to Mrs. Shelley Barrett for technical assistance. We thank Dr. N.K. Boardman for his initiating interest and continued discussions. One of us (J.B.) wishes to thank the Australian Research Grants Committee for financial support.

References

1 Morris, I. (1974) Sci. Prog. Oxf. 61, 99—122
2 Mann, K.H. (1973) Science 182, 975—981
3 Bunt, J.S. (1975) in Primary Productivity of the Biosphere (Leith, H. and Whittaker, R.H., eds.), pp. 169—184, Springer-Verlag, New York
4 Halldal, P. (1970) in Photobiology of Microorganisms (Halldal, P., ed.), pp. 17—55, Wiley, Interscience, London
5 Kirk, J.T.O. (1979) Aust. J. Mar. Freshwater Res. 30, 81—91
6 Jerlov, N.G. (1968) Optical Oceanography, pp. 167—171, Elsevier, London
7 Dougherty, R.C., Strain, H.H., Svec, W.A., Uphaus, R.A. and Katz, J.J. (1970) J. Am. Chem. Soc. 92, 2826—2833
8 Jeffrey, S.W. (1976) J. Phycol. 12, 349—354
9 Jeffrey, S.W. (1969) Biochim. Biophys. Acta 177, 456—467
10 Jeffrey, S.W. (1972) Biochim. Biophys. Acta 279, 15—33
11 Goedheer, J.C. (1970) Photosynthetica 4, 97—106
12 Brown, J.S. (1977) Photochem. Photobiol. 26, 319—326
13 Anderson, J.M., Waldron, J.C. and Thorne, S.W. (1978) FEBS Lett. 92, 227—233
14 Henriques, F. and Park, R.B. (1978) Biochem. Biophys. Res. Commun. 81, 1113—1118
15 Thornber, J.P., Markwell, J.P. and Reinman, S. (1979) Photochem. Photobiol. 29, 1205—1216
16 Lutz, M. (1977) Biochim. Biophys. Acta 460, 408—430
17 Womersley, H.B.S. (1964) Aust. J. Bot. 12, 53—110
18 Barrett, J. and Anderson, J.M. (1977) Plant Sci. Lett. 9, 275—283
19 Anderson, J.M. and Barrett, J. (1979) in Ciba Foundation Symposium, Vol. 61, pp. 81—104, Excerpta Medica, Amsterdam
20 Jeffrey, S.W. and Humprey, G.F. (1975) Biochem. Physiol. Pflanz. 167, 191—194
21 Boardman, N.K. and Thorne, S.W. (1968) Biochim. Biophys. Acta 153, 448—458
22 Boardman, N.K. and Thorne, S.W. (1971) Biochim. Biophys. Acta 253, 222—231
23 Jeffrey, S.W. (1968) Biochim. Biophys. Acta 162, 271—285
24 Kirk, J.T.O. (1977) Plant Sci. Lett. 9, 378—380
25 Hiyama, T. and Ke, B. (1972) Biochim. Biophys. Acta 267, 160—171
26 Marsho, T.V. and Kok, B. (1971) Methods Enzymol. 23, 515—522
27 Anderson, J.M., Woo, K.C. and Boardman, N.K. (1971) Biochim. Biophys. Acta 245, 398—408
28 Vernon, L.P. and Shaw, E.R. (1969) Biochem. Biophys. Res Commun. 36, 878—884
29 Shiozawa, J.A., Alberte, R.S. and Thornber, J.P. (1974) Arch. Biochem. Biophys. 165, 388—397
30 Boardman, N.K., Anderson, J.M. and Goodchild, D.J. (1978) in Current Topics in Bioenergetics (Sandi, D.R. and Vernon, L.P., eds.), Vol. 8, pp. 35—108, Academic Press, New York

31 Goedheer, J.C. (1972) Annu. Rev. Plant Physiol. 23, 87—112
32 Satoh, K. and Butler, W.L. (1978) Plant Physiol. 61, 373—379
33 French, C.S., Brown, J.S. and Lawrence, M.C. (1972) Plant Physiol. 49, 421—429
34 Brown, J.S., Alberte, R.S. and Thornber, J.P. (1974) Proc. Congr. Photosynth. 3rd Rehovot, 1974 (Avron M., ed.), pp. 1951—1962, Elsevier, Amsterdam
35 Hiller, R.G., Pilger, T.B.G. and Genge, S. (1977) Biochim. Biophys. Acta 460, 431—444
36 Holdsworth, E.S. and Arshad, J.H. (1977) Arch. Biochem. Biophys. 183, 361—373
37 Goedheer, J.C. (1969) in Progress in Photosynthesis Research (Metzner, H., ed.), Vol. 2, pp. 811—817, Tubingen
38 Tanada, T. (1951) Am. J. Bot. 38, 276—283
39 Briantais, J.M. (1969) Physiol. Vég. 7, 135—180
40 Barrett, J. and Thorne, S.W. (1979) Proc. Aust. Biochem. Soc. 12, 95
41 Fenna, R.E. and Matthews, B.W. (1976) Brookhaven Symp. Biol. 28, 170—182
42 Lemberg, R. and Barrett, J. (1973) Cytochromes, pp. 58—121, Academic Press, London
43 Antonini, E. and Brunori, M. (1971) Hemoglobin and Myoglobin in their Reactions with Ligands, North Holland, Amsterdam
44 Song, P.-S., Koka, P., Prézelin, B.B. and Haxo, F.T. (1976) Biochemistry 15, 4422—4427
45 Song, P.-S. (1978) Trends Biochem. Sci. 3, 25—27