ISOLATION OF A F694–CHLOROPHYLL *a*–PROTEIN COMPLEX WITH LOW FLUORESCENCE YIELD FROM BROWN ALGAE

J. BARRETT† and S. W. THORNE*

School of Biological Sciences, Macquarie University, North Ryde, NSW 2013 and **CSIRO, Division of Plant Industry, PO Box 1600, Canberra City, ACT 2601, Australia*

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1. Introduction

The isolation of intrinsic proteins from membranes has been accomplished largely by the use of neutral detergents [1]. Steroidal anionic detergents also have been used for the solubilization of cytochrome oxidase [2,3] cytochromes *b* [4] and erythrocyte glycoprotein [5]. Digitonin, which is non-ionic has been used for preparations of PSI and PSII macrocomplexes [6–8]. For the isolation and analysis of the chlorophyll–protein complexes, Triton X-100 and sodium dodecylsulphate have been extensively employed [9]. However, the former detergent may cause a blue-shift in the chl–protein spectra and the latter agent can cause excessive dissociation of chl from their complexes with polypeptides.

The P700 chl *a*–protein complex and two major light-harvesting complexes have been isolated from brown algae by Triton X-100 [10]. Selective partial extraction of *Acrocarpia paniculata* thylakoids by Triton X-100 in low concentration also revealed the presence of a separate chl c_2–protein of small M_r and a far-red spectral form of chl *a*. We have attempted to release selectively intrinsic proteins from the photosynthetic membranes of brown algae by sequential use of steroidal and alkyl detergents. The isolation of several new pigment–protein complexes has been reported in [11]. This paper described the isolation of a large chl *a*–protein complex which has absorbance and fluorescence properties consistent with it being a PSII reaction centre complex.

Abbreviations: chl, chlorophyll; PSI and PSII, photosystem I and II; Tricine, *n-tris*-(hydroxymethyl)methyl glycine; LDAO, lauryldimethylamine oxide; Hepes, 4-(2 hydroxyethyl)-1-piperazine–ethane sulphonic acid

† Visitor to Plant Industry, CSIRO, Canberra, to which address correspondence should be sent

2. Materials and methods

Fronds of *Acrocarpia paniculata* were collected from submerged plants at low-tide off a rock-reef at Guerilla Bay, NSW *Padina commersonii* was collected from channels between rock-clefts. Both the seaweeds were kept in cold, aerated seawater until used, usually within 24–48 h. Ruptured chloroplasts were prepared from these fronds as in [10]. The molar ratio of chl *a*/chl *c* of these chloroplasts was 4.0–4.8.

For selective solubilization of the chl–protein complexes, the ruptured chloroplasts (containing 8 mg total chl *a* + *c*) were successively extracted with 1% cholate (pH 8.0 and 9.0), 1% deoxycholate (pH 8.0 and 9.0) and finally with 1% LDAO (pH 8.0 and 9.0): all were in 100 mM Tricine buffer (pH 8.0). The weight ratio of cholate to chl *a* + *c* (w/w) was 60:1 at the start. Extraction was at 4°C with stirring for 8 h, and the suspension was then centrifuged at 15 000 × *g* for 10 min. The supernatants (40–50 ml) were concentrated to 1 ml using Centriflo CF 25 cones, and the concentrate was applied to a tube of a sucrose density gradient prepared as in [12]. Centrifugation was carried out in a Beckman SW 41 rotor at 2°C at 273 000 × *g* for 60 h.

Absorption spectra were obtained using a Cary 14 spectrophotometer. Fluorescence emission and excitation at 77° K were recorded as in [13]. Chl *a* and chl *c* were assayed in 80% acetone [14]. Chl *a*, chl c_1 and chl c_2 molar ratios were estimated in ethanol by fluorescence spectroscopy [12].

For electrophoresis gels, agarose C (Pharmacia)

was used in 1% (w/v) concentration at a thickness of 2 mm on glass plates (10 × 20 cm). Gels were prepared in Tris–glycine buffer (pH 8.3 or 9.3), with conductivity of 450 $\mu\Omega$. When pH 7.0 was required Hepes was added to this buffer. Digitonin or potassium cholate were included in the gel at a level of 0.05% (w/v); the running buffer (as for the gels) contained the detergent at 0.025% (w/v). Electrophoresis was carried out on a Pharmacia flat-bed apparatus at 4°C, at a current of 2–3 mA. for 12 h. Gels were stained at 20°C with Coomassie blue R (0.2%, w/v).

3. Results and discussion

Most brown algae chloroplasts were susceptible to extraction by detergents under the conditions specified, but in some instances the chloroplasts were less easily solubilized. We have therefore termed the chloroplasts, respectively, detergent-susceptible and detergent-resistant. With detergent-susceptible chloroplasts, the 1% cholate, pH 8.0 and 9.0, extracts together accounted for 8.0% of the total chl *a* and 12% of the total chl *c* of the chloroplasts. Fig.1 shows the result of a sucrose gradient analysis of extracts from these chloroplasts. An olive-green band was found towards the bottom of the tube containing the 1% cholate (pH 8.0) extract and was present to a lesser extent in the tube containing the extract at pH 9.0. This band was very faint or absent from the deoxycholate extracts analyses. The latter has instead buoyant-bands rich in chl c_2–protein and fucoxanthin–protein [11]. The olive-green band (fig.1a) of the cholate extract had no visible fluorescence when irradiated with near UV light, whereas the other pigmented bands emitted strong red fluorescence. The absorption spectrum (fig.2) of the olive-green band had a relatively broad red peak with its A_{max} at 673–674 nm, and had a low ratio of $A_{\text{Soret max}}/A_{\text{red max}}$ (1.5–1.7) with a distinct splitting of the Soret band, A_{max} were at 416 nm and 438 nm. The absorbance spectrum indicates that there was some fucoxanthin–protein (A_{max} 500–540 nm) and chl c_2–protein (A_{max} 450–465 nm and 638 nm) present in this fraction.

The molar ratios of chl *a*/chl *c* of the olive-green band determined in several experiments varied from 8/1–12/1. Fluorescence analysis of ethanolic extracts showed that chl *a* and chl c_2, but not chl c_1, to be present in the olive-green complex. The presence of fucoxanthin in this fraction was confirmed by extracting the pigments into cyclohexane and removing the chlorophylls from this extract with cold 20% w/v HCl. The spectrum of the carotenoid remaining was recorded in CS_2 (A_{max} 508, 478, 450). The fluorescence emission of the olive-green complex was extremely weak, with a fluorescence quantum efficiency of between 0.005 to 0.01 at 77 K. A single peak, at 694 nm, was seen in the emission spectrum (fig.3) in the many samples of this fraction examined. The fluorescence exitation spectrum (fig.3) showed

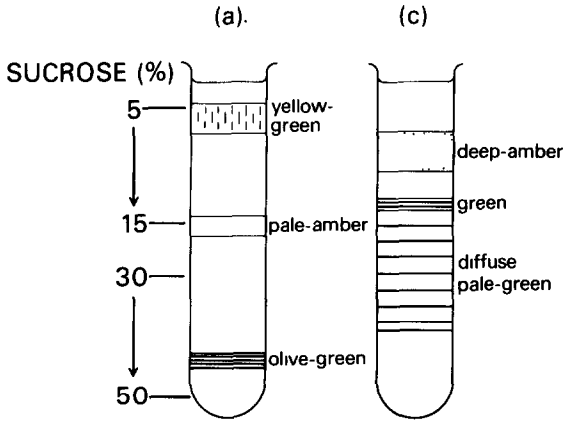

Fig.1. Scheme of separation by sucrose gradient centrifugation of chl–protein complexes isolated from detergent-susceptible chloroplasts by sequential extraction. Sucrose gradients and centrifugation: see text. (a) 1% cholate, pH 8.0; (b) as in (a) pH 9.0; (c) 1% deoxycholate, pH 8.0; (d) as in (c) pH 9.0. Only extracts at pH 8.0 shown.

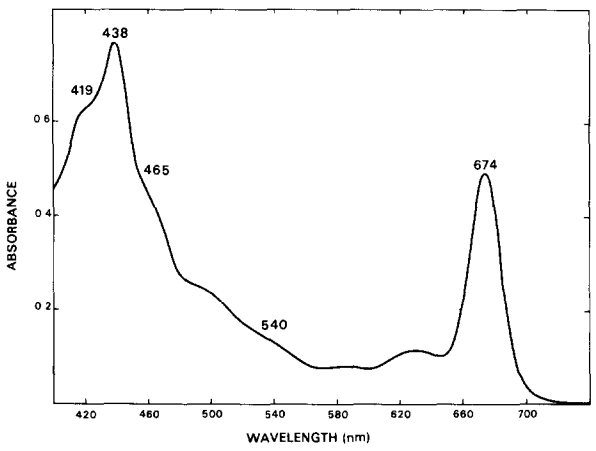

Fig.2. Absorption spectra of heavy olive-green band from 1% cholate extracts (fig.1a).

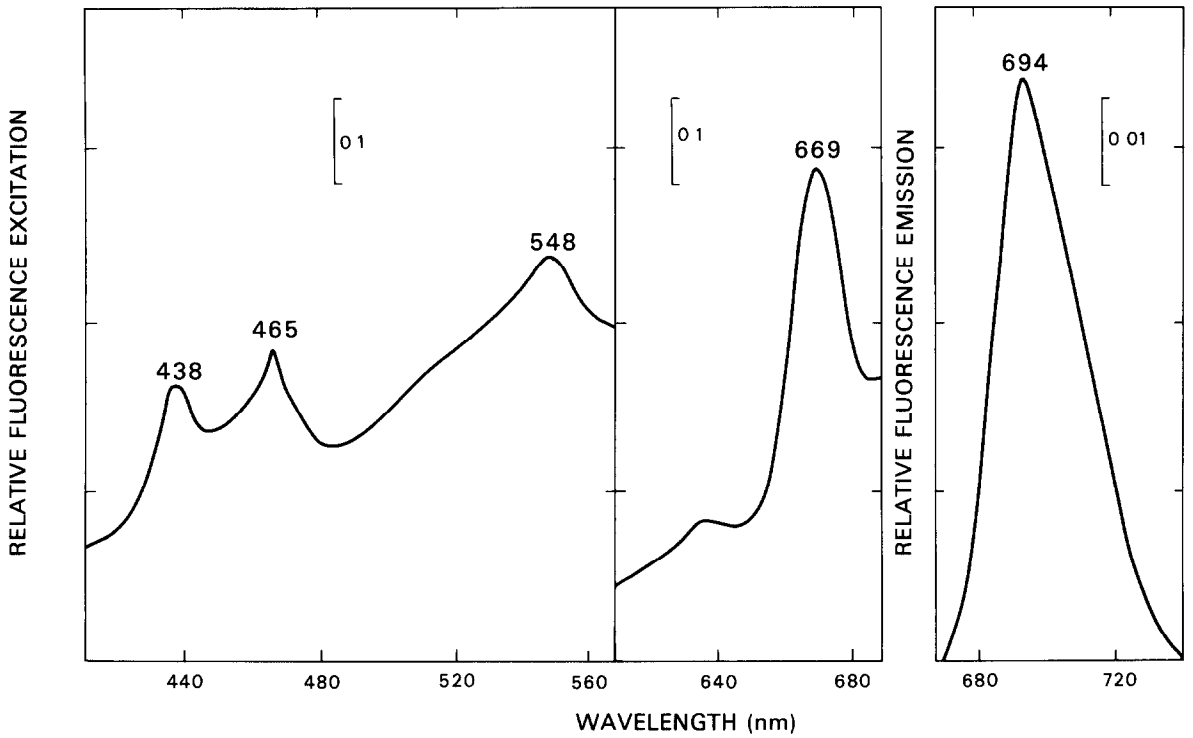

Fig.3. Fluorescence excitation and emission spectra at 77 K of (a) the olive-green fraction from 1% cholate extract (fig.1a). The emission wavelength for the excitation spectra was 694 nm, and the excitation wavelength for the emission spectra was 440 nm. Identical emission maxima were obtained when the excitation wavelength was 460 nm, 525, 540 or 668 nm. A_{673} was 0.1 in 66% glycerol.

that both chl c_2–protein and fucoxanthin–protein contributed to the fluorescence emission at 694 nm, as well as a chl *a*–protein species with a fluorescence excitation peak at 668–669 nm. Photoaction spectra of brown algae chloroplasts have shown that light absorbed by chl *a*–protein and fucoxanthin–protein in situ contributes to PSII [15,16]. The term F694–chl *a*–protein complex will now be used to denote the chl *a*-complex which has the single fluorescence emission peak at 694 nm with very low fluorescence yield.

As this study progressed some batches of chloroplasts were found to be more resistant to solubilization by detergents. In these instances, even if the cholate to chls *a* + *c* (w/w) ratio was raised 4-fold to 240: 1 little solubilization of the P694–chl *a*–protein complex occurred. Instead the bulk of this complex was shown by absorption and fluorescence spectroscopy to be in the 1% deoxycholate (pH 8.0) extracts of the chloroplasts, and to a lesser extent in the 1% deoxycholate (pH 9.0) extracts. Little, if any, was in the LDAO (pH 8.0) extracts, and none in the LDAO extracts at pH 9.0. With LDAO (pH 9.0) bands containing chl *a* + c_2–fucoxanthin–protein complex [12] were obtained on sucrose gradient analysis of the extract. The difference in susceptibility of the chloroplasts to the detergents may be related to a seasonal variation in the polysaccharide content of the seaweeds. The chloroplasts from these two algae contain sugars characteristic of glycoproteins (J. B., Dudman, unpublished). On sucrose density gradient centrifugation of the deoxycholate extracts from these resistant chloroplasts the F694–chl *a*–protein was found to be in a zone extending through the lower half of the sucrose gradient (fig.4). This more diffuse fraction had a yellowish-green hue when it was concentrated. The absorption spectrum showed the presence of small amounts of chl c_2–protein, but not the fucoxanthin–protein. Occasional fractions had no chl c_2–protein either. The fluorescence excitation spectrum showed that a chl *a* species with an excitation maximum at 669 nm was the principal contributor to the fluorescence emission at 694 nm. Fig.5 shows excitation spectra in the blue–green region of

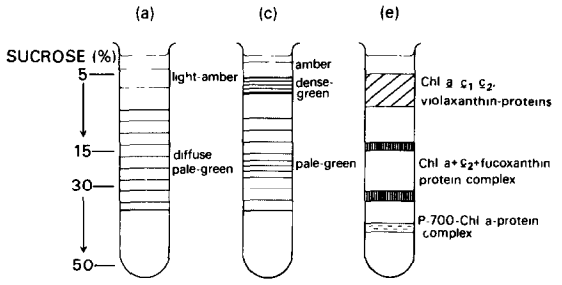

Fig.4. Scheme of separation by sucrose density gradients of chl–protein complexes isolated from detergent-resistant chloroplasts by sequential extraction. Sucrose gradients: see text. (a) 1% cholate, pH 8.0; (b) as in (a) pH 9.0; (c) 1% deoxycholate, pH 8.0; (d) as in (c) pH 9.0; (e and f) LDAO, pH 8.0 and 9.0. Only extracts at pH 8.0 are depicted.

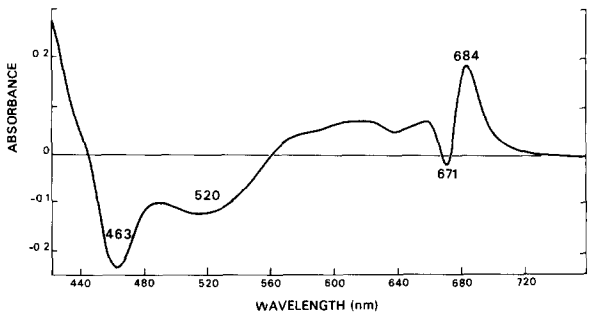

Fig.6. Difference absorption spectrum of F694–chl *a*–protein complex from the 1% cholate (pH 8.0) versus a chl *a*–protein obtained by sucrose gradient centrifugation of a 1% LDAO (pH 9.0) sequential extract of chloroplasts. Each fraction had an absorbance of 0.9 at their red absorption maximum.

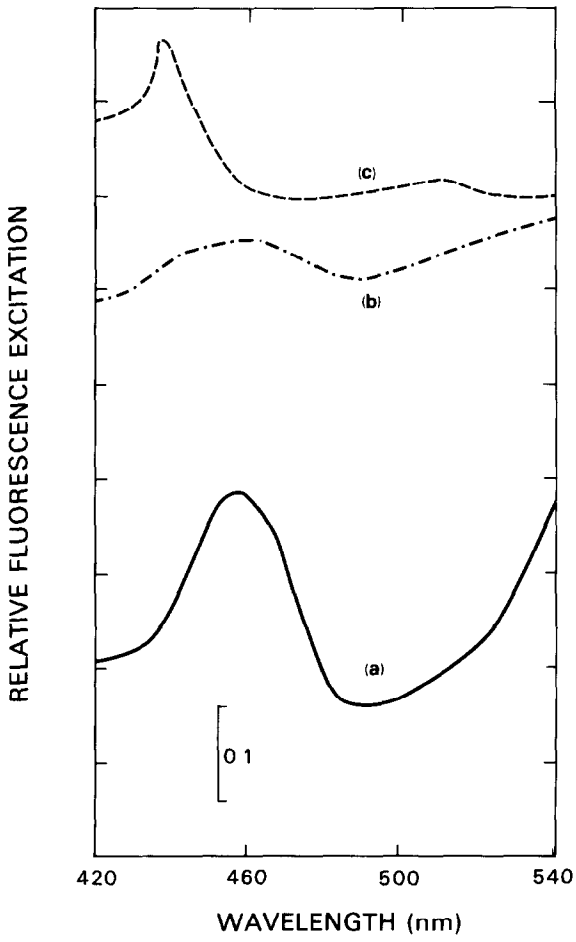

Fig.5. Fluorescence excitation spectra at 77 K of different fractions from the pale-green zones of the sucrose gradient analysis of 1% deoxycholate extracts (fig.4c). The emission wavelength was 694 nm for these spectra.

various fractions from the pale-green zone obtained on sucrose density centrifugation of 1% deoxycholate extracts (fig.4c).

Absorption difference spectroscopy has revealed the existence of multiple spectral species of pigment–proteins in the light-harvesting complexes of brown algae and an excess of a 684 nm form of chl *a* in the P700–chl *a*–protein complex of these algae [12]. A difference spectrum (fig.6) of the F694–chl *a*–protein complex (A_{682}/A_{674} = 0.75) versus an LDAO (pH 9.0) sucrose gradient centrifugation fraction which has a A_{682}/A_{671} of 0.30 showed that the F694–chl *a*–protein complex has an excess of a 682 nm species of chl *a*–protein over any present in the LDAO fraction. The excess was estimated to be ~12% of the asorbance represented by the red-peak of the absorption spectrum of the F694–chl *a*–protein complex. A high A_{682}/A_{674} ratio is a good indicator of the presence of the F694–chl *a* species in fractions obtained from the cholate or deoxycholate extracts.

From the foregoing evidence of the absorption difference spectroscopy and the fluorescence spectroscopy on both types of chloroplasts we conclude that the weak fluorescence emission peak at 694 nm is due to the excitation of a chl *a* species which has an A_{max} at 682 nm, and that this chl *a* species is excited mainly by antennae chl *a* which has an A_{max} at 668–670 nm. The light-harvesting of the core chl *a* is increased by association with the F695-complex of chl c_2–protein and fucoxanthin–protein.

Attempts to electrophorese the F694–chl *a*–complex extracted by cholate from chloroplasts were not

successful due to exclusion at acrylamide concentrations (4–6%) which allow P700–chl *a*–protein complex to penetrate the gel. In agarose gels over pH 7.0–9.0, however, the complex moved as a single zone slightly towards the cathode when using digitonin, but slightly towards the anode when cholate was used, whereas all the light-harvesting complexes moved markedly towards the anode with either detergent. In all instances, electrophoresis caused the F694–chl *a*–protein complex to emit strong red fluorescence, suggesting that perturbation of the structure of the complex had occurred or migration of a stabilizing component from the complex.

The heavy F694–chl *a*–protein complex had no detectable PS 2 activity as assayed by dichloroindophenol reduction using diphenylcarbazide as an electron donor [17]. Both *Acrocarpia paniculata*, with fibrillar thalli, and *Padina commersonii* which has thin laminar thalli yielded the same type of F694–chl *a*–protein complexes.

Higher plant chloroplasts in vivo have two fluorescence emission bands at 77 K at 685 and 695 nm which have been assigned to PSII and one at 735 nm which has been assigned to PSI [18,19]. However, isolated chl–protein complexes from PSII and from light-harvesting complexes have bands at 685 and 681 nm [7,20,21]. No specific complex has yet shown a single emission band at 694 nm as observed for this F694–chl *a*–protein complex. Further all other complexes have a higher fluorescence yield.

The pattern of the fluorescence emission peak of this new chl *a*–protein complex, together with its extremely low fluorescence yield (even less than that of isolated P700–chl *a*–protein complex) suggests strongly that this fraction contains the PSII reaction centre of brown algae. The high A_{438}/A_{418} ratio is also indicative of the F694–chl *a*–protein being a reaction centre complex. Furthermore, the very close association of both the chl c_2–protein and the fucoxanthin–protein (both known PS 2 light-harvesting pigments) with the F694–chl *a*–protein complex supports the contention that this is a PSII reaction centre complex. We estimate that the F694–chl *a* component of the complex accounts for ~1% of the total chl *a* of the chloroplast.

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