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Comparative biochemical, functional and ultrastructural studies of photosystem particles from a Cryptophycea: *Cryptomonas rufescens*; isolation of an active phycoerythrin particle

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Key words: Photosystem I; Photosystem II; Light-harvesting chlorophyll a/c_2; Phycoerythrin-Photosystem II complex; Electron microscopy; (*C. rufescens*)

Three subchloroplast fractions and free phycoerythrin have been separated from *Cryptomonas rufescens* by action of digitonin and ultracentrifugation on sucrose density gradient. They were characterized as Photosystem (PS) I, PS-II and light-harvesting complexes. Besides chlorophyll (Chl) *a* and c_2, high-performance liquid chromatography exposed the presence of noticeable amounts of *cis*-alloxanthin, in addition of the previously reported carotenoids alloxanthin, crocoxanthin and α-carotene. The heavy band (fraction 1), which presented absorption and fluorescence characteristics of PS I complex appeared up to three times enriched in P-700 (Chl *a* to P-700 ratio: 150). These PS I vesicles exhibited more efficient far-red absorption and contained high amounts of α-carotene, a low alloxanthin complement, few chlorophyll c_2 and a specific enrichment in a pigment presumed to be chlorophyll reaction center I. The low-temperature absorption of both other fractions, previously related to PS II, exhibited a very low contribution of far-red absorbing chlorophyll *a* forms. Their carotenoid contents were highly modified in a large depletion of α-carotene, contrasting with the increment of xanthophyll amounts. Besides its high chlorophyll c_2 content, fraction 2 exhibited an enhanced alloxanthin content. This fraction, according to its photosynthetical activities and polypeptide composition, was interpreted as the major chlorophyllous light-harvesting complex (Chl a/c_2 complex) connected to some PS II reaction centers. By contrast, the third recovered fraction was an active phycoerythrin-xanthophyll-PS II complex greatly enriched in PS II reaction centres and poor in light-harvesting chlorophyll. Phycoerythrin, the major light-harvesting pigment of this fraction, efficiently transferred energy to PS II reaction centers. Although many features were comparable in the two PS II fractions, the photosynthetical activities revealed an opposite repartition of PS II reaction centres and light-harvesting antennae. Negatively stained preparations of fraction 3 confirmed that thylakoid vesicles were present and associated with small geometrical units corresponding to phycoerythrin. High-molecular-mass polypeptides (97 and 87 kDa) were detected in this fraction and were tentatively proposed as linkers between phycoerythrin units and thylakoid membrane. The lightest fraction 4 contained only free phycoerythrin. A functional model describing the organization of Cryptophyceae thylakoid membrane is proposed.

Abbreviations: Chl *a* (c_2), chlorophyll *a* (c_2); DCIP, 2,6-dichlorophenolindophenol; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulphonic acid; HPLC, high-performance (pressure) liquid chromatography; LDS, lithium dodecyl sulfate; LDAO, lauryldimethylamine *N*-oxide; LHC I (II), light-harvesting complex of Photosystem I (II); P-700, Photosystem I reaction centers; PS I (II), Photosystem I (II).

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Introduction

The accessory pigments of Cryptophyceae, chlorophyll c_2 and phycobiliproteins are efficient light-harvesting pigments for photosynthesis [1,2]. By contrast with Cyanobacteria and Rhodophyceae, phycoerythrin is located in the intrathylakoidal space, while Chl c_2-protein complex is bound to the thylakoid. A specific Chl a/c_2-protein complex related to PS II has been isolated from *Chroomonas* [3] and *Cryptomonas rufescens* [4] by LDS-polyacrylamide gel electrophoresis and digitonin-sucrose density gradient. Up to now, there have been few studies on the PS I particles [5] and no data on purified phycoerythrin-PS II particles. In the present work, we succeeded in isolating three fractions whose polypeptide composition, pigment content (revealed by HPLC separation), morphological aspects (observed by negative-staining preparations) and photosynthetical activities allowed us to identify them as a PS I complex highly enriched in P-700 and two PS II complexes: respectively, a Chl a/c_2 complex and a particular phycoerythrin-PS II complex. These results will be discussed and compared with those obtained with other Chromophyceae and higher plants.

Materials and Methods

Cultures conditions. Cryptomonas rufescens were grown on S_2T_2 medium at room temperature as previously described [6].

Isolation of subchloroplast fractions

Cryptomonas cells were washed twice in 50 mM Hepes-Na buffer (pH 7.4) with 10 mM $MgCl_2$ and 2 mM EDTA. The cell suspension was passed through a French pressure cell at 10.4 MPa. These membrane fragments were treated with 2% digitonin, giving a detergent/Chl ratio of 60 : 1 (w/w). The mixture was incubated for 50 min in the dark at room temperature with gentle stirring. The brown homogenate was layered on a sucrose step gradient (5–40% sucrose) (w/w) in 50 mM Hepes-Na buffer (pH 7.4), then centrifuged 17 h at 80 000 × *g*

Spectroscopic measurements

Absorption spectra were recorded at room temperature using a DW2 Aminco-Chance spectrophotometer and fluorescence spectra were performed as previously described [7].

P700 concentration was assayed by measuring photooxidation detected with a DW2 Aminco-Change spectrophotometer in the dual-beam mode [8] at 697 nm (reference at 730 nm). For this purpose, 10 mM sodium ascorbate was added to 1 ml of fractions collected from sucrose gradient or 1 ml of preparations of broken cells suspended in 50 mM Hepes-Na buffer (pH 7.4)

DCIP reduction was performed on fractions collected from sucrose gradient, or on broken cells suspended in Hepes-Na buffer (pH 7.4), as described by Berkaloff and Duval [9].

Low-temperature absorption spectra

The 77 K absorption spectra were realized with a home-made device already described [10]. Deconvolution of the absorption spectra into Gaussian components was achieved according to the method of French et al. [11].

Estimation of protein content

Total proteins as well as membrane proteins have been estimated with the Bio-Rad protein assay kit I with bovine plasma γ-globulin as standard. Membrane proteins from the various fractions were purified by washing twice with Hepes-Na Buffer, and centrifuged 3 h at 246 000 × *g*.

Pigment analysis, HPLC methods

Homogenization of the membrane particles with a Potter-Elvehjem grinder in a mixture of ethanol petroleum diethyl ether acetone (60 : 20 : 10 : 10, v/v), allowed extraction of pigments. HPLC separation of the pigments was performed on a reverse-phase Du Pont Zorbax octadecyl silica (ODS) column, the mobile phase consisting of a gradient (0.1%–40%) of dichloromethane in a mixture of acetonitrile methanol (70 : 30, v/v) and containing 0.5% triethylamine to improve chlorophyll c_2 recovery. A Hewlett-Packard 1040A diode-array spectrophotometer allowed pigment absorption spectra to be obtained, without one need to stop solvent flow. Determination of the pigment amounts was obtained after external calibration of the DPU multichannel integrator associ-

ated to the detector with available standards. The extinction coefficients used were those determined by Ziegler and Egle [12] for chlorophyll *a* and by Jeffrey and Humphrey [13] for chlorophyll c_2. The $E^{1\%}_{1cm}$ of zeaxanthin [14] was used for alloxanthin and *cis*-alloxanthin, that of α-cryptoxanthin [15] for crocoxanthin, and α-carotene was calibrated with the extinction coefficient cited by Britton [14]. The extinction coefficient used for phycoerythrin was previously published [6].

Negative-staining technique

Fractions removed from the sucrose gradient were directly placed on carbon films on electron microscope grids and stained according to the method of Dilworth and Gantt [16].

Gel electrophoreses

Polyacrylamide gel electrophoreses were performed on slab gel according to the method of Laemmli [17], with a 5% stacking gel and a 10–22% resolving gel. Fractions collected on sucrose gradient were recentrifuged at 200 000 × *g* for 5 h, and the pellet was suspended in 0.2 M Tris-HCl buffer (pH 8.8) with 3% LDS, 2% sucrose and 100 mM β-mercaptoethanol. Gels were stained with Coomassie blue. The molecular masses of polypeptides were obtained by co-electrophoresis of protein markers (Pharmacia electrophoresis calibration kit): phosphorylase (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)

Results

The absorption spectrum of whole cells with a phycoerythrin to Chl ratio of 3.6 for 10^6 cells is presented in Fig. 1. Centrifugation on discontinuous sucrose density gradient of digitonin-treated thylakoids gave three membrane fractions (Fig. 2). The heavy fraction 1, bright-green colored, was isolated in the 30% sucrose layer, and fraction 2, yellow-green, in the 15% sucrose layer. As shown by absorption spectra at room temperature (Fig. 1), these fractions contained only chlorophylls and

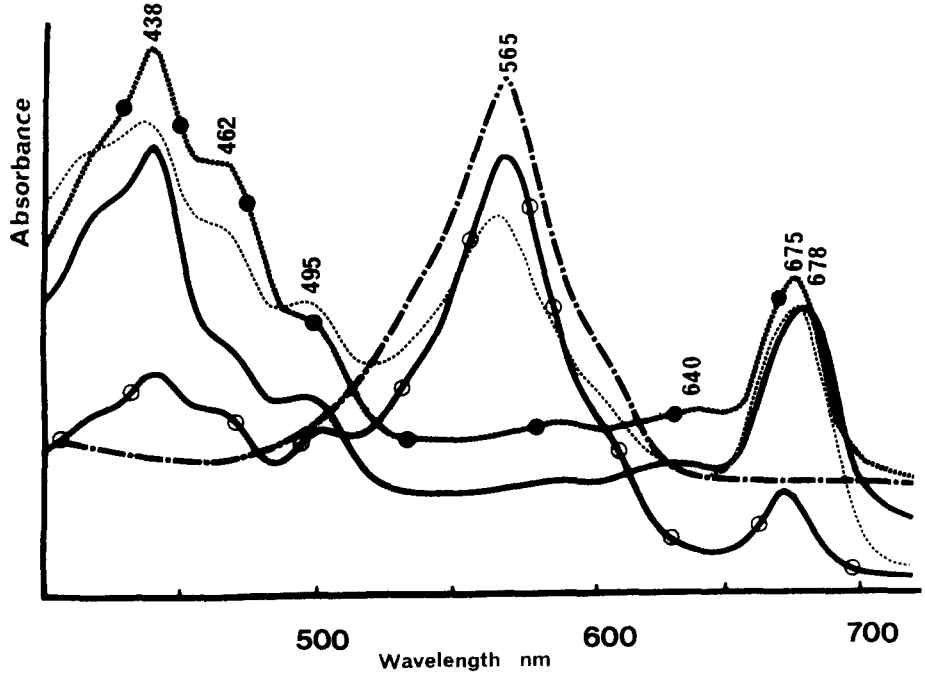

Fig. 1. Absorption spectra of whole cells and fractions separated by sucrose density gradient. - - - - - -, Whole cells; ———, fraction 1; ●———●, fraction 2; ○———○, fraction 3; – · – · –, fraction 4.

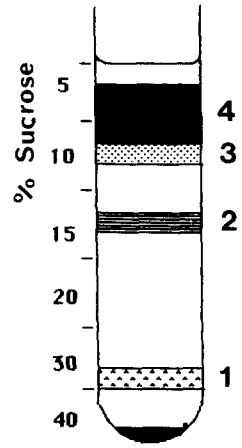

Fig. 2. Separation of subchloroplast fractions from *Cryptomonas rufescens*. After fragmentation by a French pressure and solubilization by digitonin of the thylakoid membranes, the mixture were put on a sucrose-density gradient and centri - fuged.

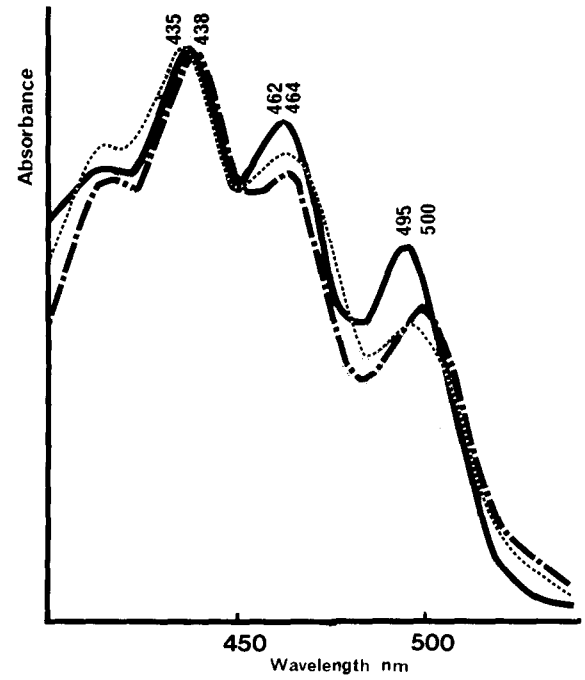

Fig. 3. Absorption spectra at 77 K of subchloroplast fractions separated by sucrose density gradient, ———. Fraction 1; ·–·–·, fraction 2; - - - - - -, fraction 3.

carotenoids. The absorption spectrum of the light fraction 3, recovered in the 10% sucrose layer, exhibiting the 565 nm peak of phycoerythrin, is similar to that of whole cells (Fig. 1). The last upper pink fraction 4, which represents about 50% of the total proteins (Table I), is typically phycoerythrin, with spectral characteristics of type III Cryptophyceae phycoerythrin [18].

Fig. 3 reveals differences among 77 K absorption spectra of the three chlorophyllous fractions in the blue range: the relative heights of the various peaks were distinct and their positions were shifted to longer wavelengths in fraction 3. Wide variations were observed in the shapes of the red absorption bands (Fig. 4): fraction 1 exhibited two peaks at 672 and 676 nm, whereas fractions 2 and 3 had the same absorption maximum at 670 nm (Fig. 4). The faint Chl c_2 absorbance at 639 nm could only be observed in fraction 2. The main red absorption band in spectra of the thylakoid membrane could be reasonably well matched by the sums of eight Gaussian components (Figs. 5–7).

TABLE I

PIGMENT COMPOSITION OF PHOTOSYNTHETICAL ACTIVITIES OF WHOLE CELLS AND SUBCHLOROPLAST FRACTIONS OF *CRYPTOMONAS RUFESCENS*

Samples	Proteins as % of total proteins	Membrane protein as % of total membrane protein	Chl *a*/Chl c_2 (mol/mol)	Chl *a*/P-700 (molar ratio)	PS II activity (μ mol DCIP reduction) [a]
Cells	100	100	9.7	350–400	19
1	5.2	14.3	28.5	150	0
2	5	14.7	4.8	0	3.5
3	20.7	7.5	7.8	0	100
4	48	0	–	0	0

[a] Photosystem II activity (μmol DCIP per mg Chl/h). Reaction mixture: 50 μm DCIP/0.5 mM diphenylcarbazide, 1 ml fraction in Hepes-Na buffer.

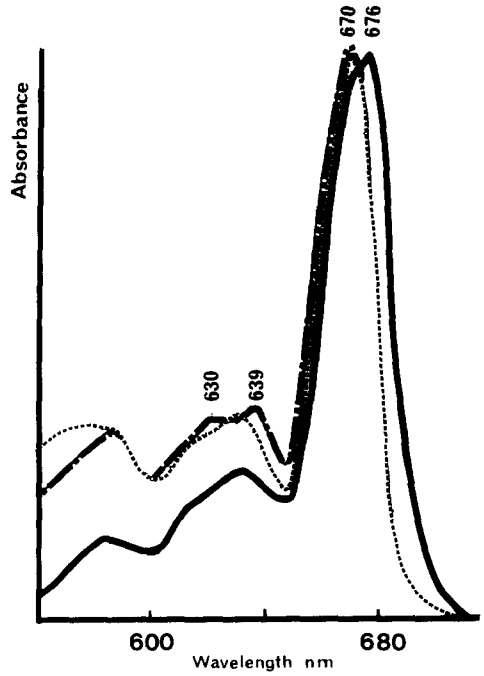

Fig. 4. Absorption spectra at 77 K of subchloroplast fractions separated by sucrose density gradient, ———. Fraction 1; ·–·–·, fraction 2; - - - - - -, fraction 3.

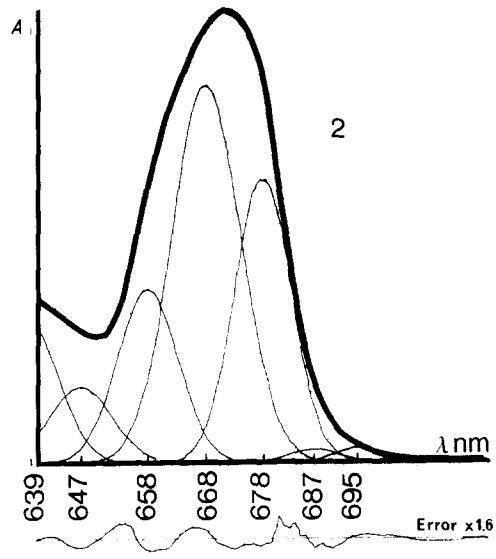

Fig. 6. Gaussian analysis of absorption spectra at 77 K of fraction 2.

Peak wavelengths and half-widths of the Gaussian curves needed to be slightly modified among the various fractions to obtain better fits to chlorophyll absorption spectra. Three forms of Chl *a* allowed deconvolution of the major part of the

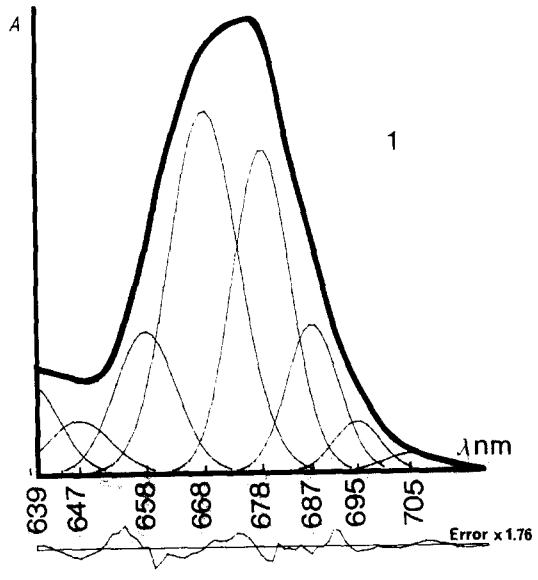

Fig. 5. Gaussian analysis of absorption spectra at 77 K of fraction 1.

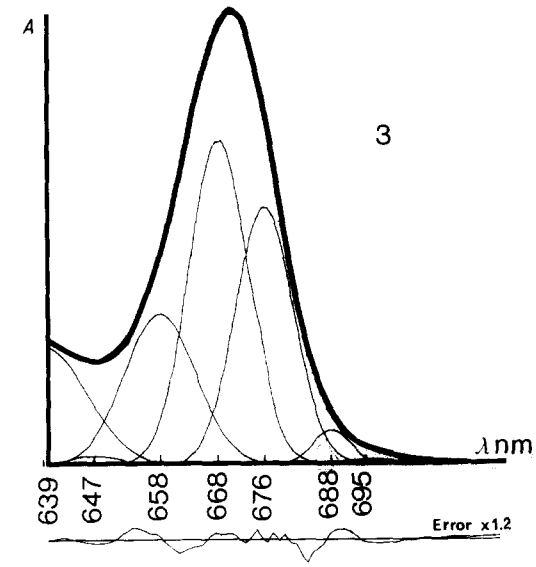

Fig. 7. Gaussian analysis of absorption spectra at 77 K of fraction 3.

spectra from all our fractions: Chl *a* 658, 668 and 678 (Figs. 5–7). Their relative abundances were weakly modified and Chl *a* 668 always predominated. Nevertheless, fraction 1 (Fig. 5) largely differed from the two others by the high amount of two far-red absorbing forms: Chl *a* 696 and 705, so that the three longer wavelength forms were responsible of about 17% of absorption, when they were very reduced in both other fractions.

As shown by Fig. 8, our non-aqueous reverse-phase HPLC method appeared to be suitable for a good separation of the various pigments from a crude extract. Chl c_2 was eluted first and followed by xanthophylls: alloxanthin, *cis*-alloxanthin and crocoxanthin; Fig. 9 shows absorption spectra of *cis*-alloxanthin and alloxanthin. The main Chl *a* elution peak was accompanied by small proportions of other components with different polarity but which had similar spectroscopic parameters. The comparison of the HPLC profiles of the various fractions reproductively revealed the presence in fraction 1 of increased amounts of a tetrapyrrole pigment with a Soret band maximum at 432 nm eluting about 30 s before Chl *a* (Fig. 8). The less polar pigment is α-carotene.

The pigment composition of whole cells and various subchloroplast fractions expressed as molecules of individual pigments per 1000 molecules of Chl *a* is presented in Table II and their ratios are indicated in Table III. The amount of Chl c_2 appeared to be very low in fraction 1, the Chl c_2/Chl *a* molar ratio of which was about three times lower than in whole cells. This fraction 1 was also largely depleted in xanthophylls, and particularly in alloxanthin. By contrast, fraction 1 was especially enriched in α-carotene as compared to whole cells as well as to other fractions. The main part of Chl c_2 was associated with fraction 2 particles, the Chl c_2 amounts of which, related to Chl *a*, appeared, respectively, to be 2- and 6 fold increased as compared with whole cells and fraction 1 particles (Table II). Alloxanthin was the main xanthophyll in fraction 2, as in all the particles analyzed. In this fraction its amount reached its maximum, approaching 80% of xanthophylls, to the detriment of both *cis*-alloxanthin and crocoxanthin (Table IV). Fraction 2 particles were particularly deficient in α-carotene, their xanthophyll/α-carotene ratio being 9-fold that of fraction 1 (Table III). Fraction 3 particles contained Chl c_2 and were especially enriched in xanthophylls (Table III). Nevertheless, the proportions of the various xanthophylls remained nearly the same as in whole cells (Table IV).

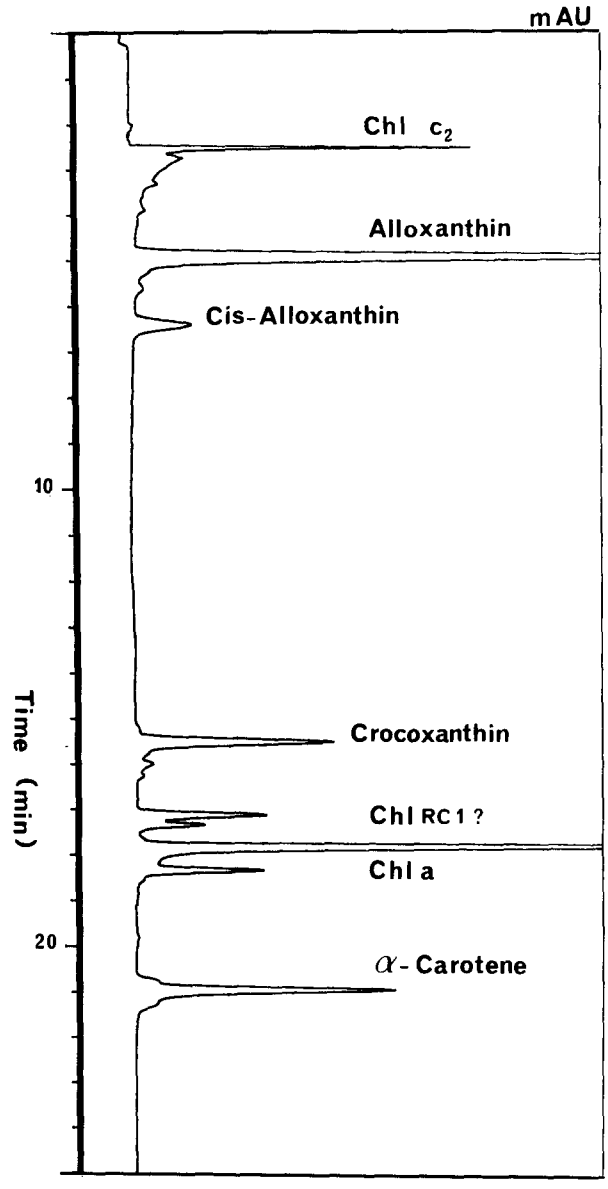

Fig. 8. HPLC chromatogram of fraction 1 monitored at 445 nm.

The main fluorescence peak of fraction 1 was located at 720 nm (Fig. 10). This fraction retained P-700 photoactivity (Table I) with a Chl *a*/P-700 ratio of about 150, while in whole cells the Chl

TABLE II

PIGMENT COMPOSITION OF WHOLE CELLS AND MEMBRANE FRACTIONS (1, 2 AND 3) OBTAINED ON SUCROSE DENSITY GRADIENT

Samples	Pigment (molecules of pigment/1000 molecules of Chl *a*)						
	Chl *a*	Chl c_2	alloxanthin	*cis*-alloxanthin	crocoxanthin	α-carotene	ε-carotenoids
Cells	1000	103	207	13	57	42	319
Fraction 1	1000	35	147	12	53	64	276
Fraction 2	1000	206	343	17	73	15	448
Fraction 3	1000	127	362	25	100	20	507

TABLE III

PIGMENT MOLAR RATIOS OF WHOLE CELLS AND MEMBRANE FRACTIONS (1, 2 AND 3) OBTAINED ON SUCROSE DENSITY GRADIENT

Samples	Pigment ratios					
	Chl *a* / Chl *c*	ε-Ch / ε-carotenoids	Chl *c* / ε-xanthophylls	Chl *a* / α-carotene	ε-xanthophylls / α-carotene	alloxanthin / *cis*-alloxanthin
Cells	9.7	3.45	3.6	23.8	6.6	15.6
Fraction 1	28.5	3.75	4.7	15.6	3.3	12.2
Fraction 2	4.8	2.7	2.3	66.7	28.9	20.2
Fraction 3	7.9	2.2	2	50	24.3	14.5

a/P-700 ratio was 350–400. Thus, fraction 1 appears up to three times enriched in P700. This complex did not exhibited any DCIP reduction in the presence of diphenylcarbazide, which proved the absence of PS II electron transfer activity. The polypeptide patterns of this fraction (Fig. 11) revealed the presence of two heavy polypeptides at 67 and 69 kDa, three polypeptides both close to 47–43 kDa and a lot of low-molecular-mass polypeptides ranging from 24 to 15 kDa (Fig. 11).

TABLE IV

XANTHOPHYLL COMPOSITIONS OF WHOLE CELLS AND MEMBRANE FRACTIONS (1, 2 AND 3) OBTAINED ON SUCROSE DENSITY GRADIENT, AS PERCENT OF TOTAL XANTHOPHYLLS

Samples	Pigment amount (% of xanthophylls)		
	alloxanthin	*cis*-alloxanthin	crocoxanthin
Cells	74.7	4.7	20.6
Fraction 1	69.3	5.7	25
Fraction 2	79.2	3.9	16.9
Fraction 3	74.3	5.1	20.6

The 24-19-18 kDa polypeptides were always important, but no polypeptide could be attributable to phycoerythrin subunits. Considering these data, we can conclude that fraction 1 is very enriched in PS I subunits.

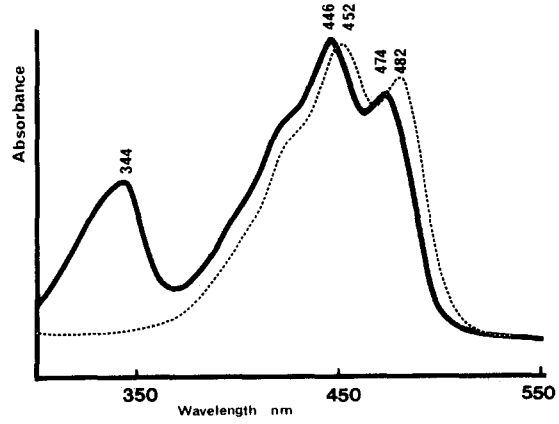

Fig. 9. Absorption spectra of *cis*-alloxanthin (———) and alloxanthin (- - - - -) of fraction 2 in the elution medium acetonitrile methanol dichloromethane (56 : 24 : 20, v/v).

In fraction 2, the main fluorescence peak was at 686 nm, corresponding to Chl *a* short wavelengths (Fig. 10). The PS II activity (DCIP reduction in the presence of diphenylcarbazide) was very low for this fraction when compared to whole cells (Table I). These results indicated that this fraction was enriched in pigment antenna and poor in reaction centres. Furthermore, this fraction did not contain PS I complex, since no P-700 photo-oxidation was ever detected. Fig. 11 shows that four major polypeptides (47 to 39 kDa) were present together with 24 to 19 and 69 kDa polypeptides. These characteristics, associated with pigment composition, (Table II) allowed us to identify this fraction as a light-harvesting chlorophyll a/c_2-PS II complex.

The fluorescence emission maxima of fraction 3 was observed near 686 nm, as in fraction 2, but the 625 nm peak attributed to phycoerythrin was always higher (Fig. 10). Using 485 nm excitation light, the ration F_{625}/F_{686} of fluorescence emissions at 625 nm and 686 nm was 1.8 (Fig. 12).

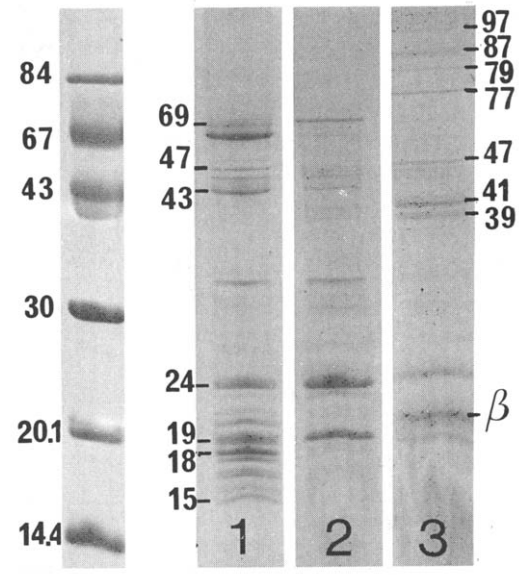

Fig. 11. Polypeptide profiles of subchloroplast fractions of *Cryptomonas rufescens* analyzed on 10–22% gradient LDS-polyacrylamide gels (molecular masses of protein markers from Pharmacia kit: phosphorylase, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa); β, β-subunit of phycoerythrin.

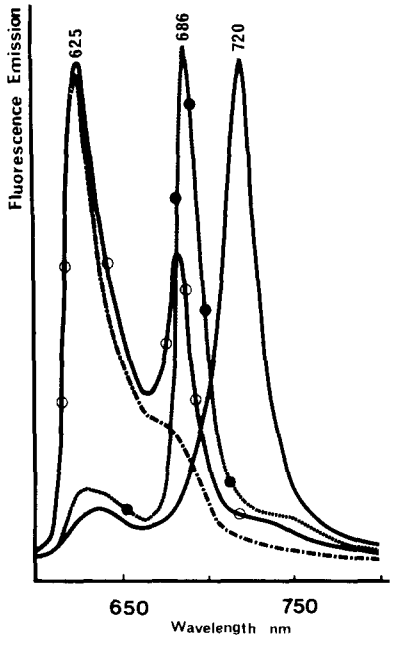

Fig. 10. Fluorescence emission spectra at 77 K of fractions separated by sucrose density gradient; spectra were obtained with a 485±50 nm excitation light. ———, Fraction 1; ●———●, fraction 2; ○———○ fraction 3; ·–·–·, fraction 4.

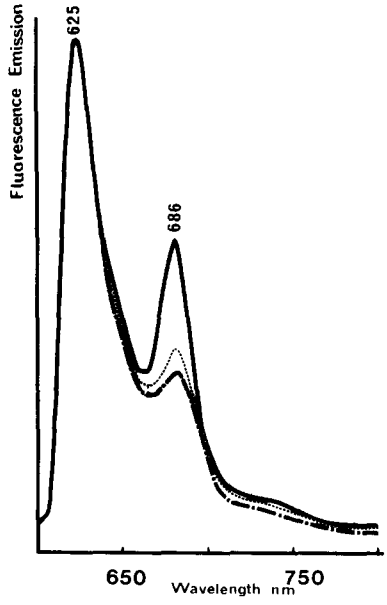

Fig. 12. Fluorescence emission spectra at 77K of fraction 3 in 50 mM Hepes-Na buffer. ———, 485±50 nm excitation light of intact fraction; - - - - - -, 550±5 nm excitation light of intact fraction; ·–·–·, 550±5 nm excitation light of dissociated fraction in lower buffer concentration (5 mM Hepes-Na buffer).

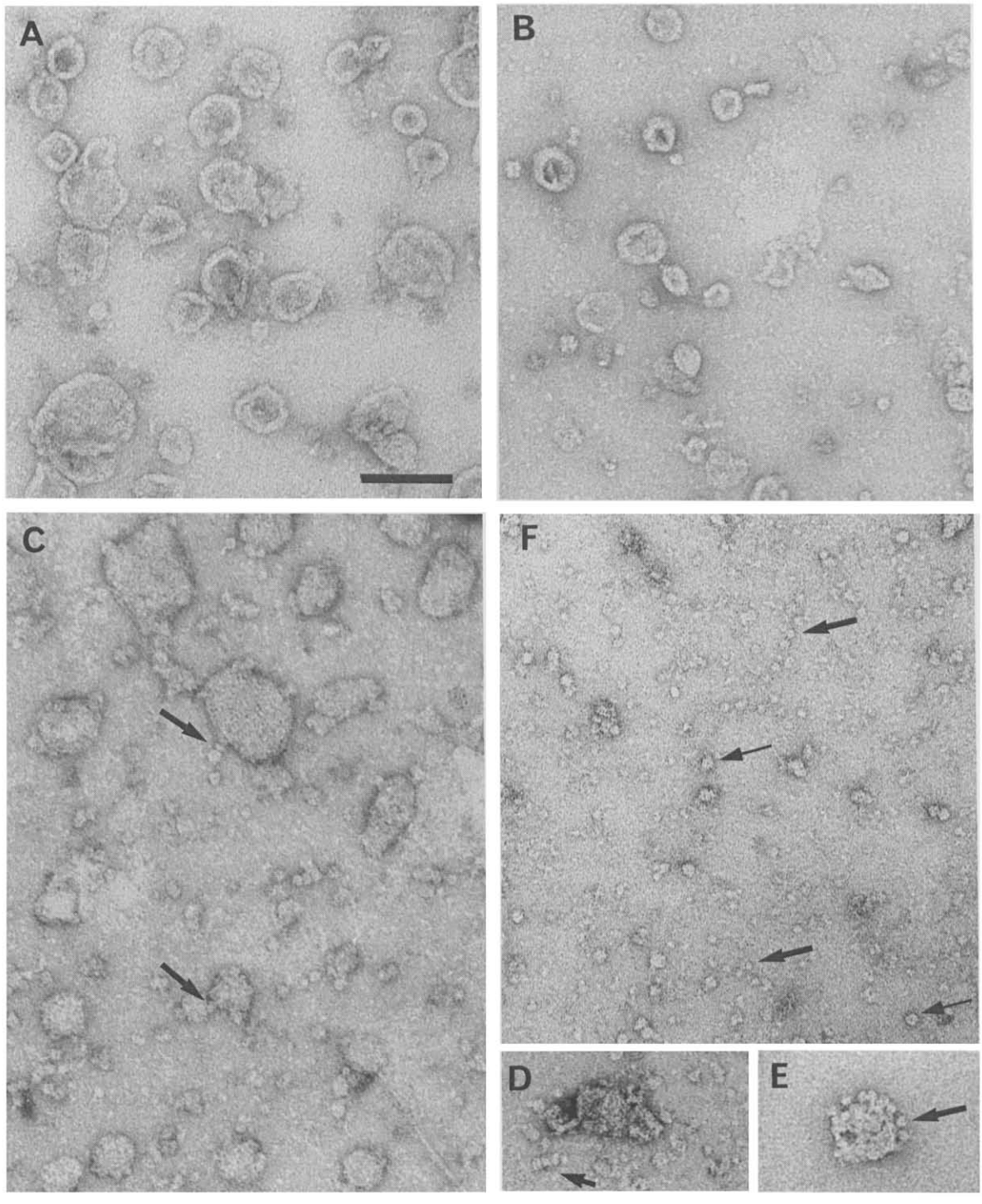

Fig. 13. Negatively stained preparations of the different fractions obtained by sucrose density gradient. (A) Thylakoid vesicles of fraction 1, (B) membrane fragments of fraction 2 (the vesicles are smaller than in fraction 1), (C) fraction 3 (thylakoid vesicles with phycoerythrin units attached to the thylakoid membranes (arrows)), (D) fraction 3 (phycoerythrin particles stacked to form rods of four or three units (arrow)), (E) fraction 3 (thylakoid vesicles surrounded by phycoerythrin units (arrow)), (F) fraction 4 (isolated phycoerythrin units (thick arrows) and particle with central hole stained (thin arrows)). The bar correspond to 0.1 μm; $\times$149000.

This ratio increased either with green excitation (550 mn) (F_{625}/F_{686}, 2.7) or when the pigments were disconnected by lower buffer concentration, (5 mM Hepes-Na buffer) whatever could be the excitation light (F_{625}/F_{686}, 3.2 with green light). In the latter case, taking into account that the 625 nm fluorescence is due to phycoerythrin, and that a low F_{625}/F_{686} ratio indicates an efficient energy transfer between phycoerythrin and chlorophylls, these observations suggested that fraction 3 contained highly coupled PS II centre-phycoerythrin antennae. In addition, we found that fraction 3 was depleted of P700. These conclusions were confirmed by measuring PS II photosynthetic electron transfer activities, which were evidenced through reduction of DCIP in the presence of diphenylcarbazide (Table I); this fraction appeared then 5- and 30-times enriched compared, respectively, to whole cells or fraction 2. The polypeptide composition of fraction 3 appeared more complex (Fig. 11). If some polypeptides were common with those of fraction 2 (three polypeptides from 47 to 39 kDa and the two 24 and 19 kDa) four specific high-molecular-mass polypeptides (100 to 97, 87 to 85, 79 to 77 and 77 to 75 kDa) were also characterized. The 20.5 kDa polypeptide appeared pink colored when gels were not stained with Coomassie blue. This was related to the phycoerythrin β-subunit [18].

The fraction 4 was depleted of P-700 and PS II activity and contained only phycoerythrin (Fig. 1).

Negative staining of membranes of each fractions were observed (Fig. 13). Fraction 1 (thylakoid membranes) appeared as closed vesicular structures of about 70–130 nm diameter (Fig. 13(A)). In fraction 2, the vesicles were smaller: 25–60 nm diameter (Fig. 13(B)). In both cases, no particles were attached to membranes, but some polyedric particles were found dispersed among vesicles. The lightest fraction 3 (Fig. 13(C)) gave vesicles slightly smaller than those of fraction 2. They were covered with the polyedric units already observed in fraction 2. These units of about 10–12 nm in diameter and 6 nm thick seemed to be stuck to the membranes and sometimes associated as groups or rods of three or four units (Fig. 13(D,E)). These polyedric structures were also obtained in fraction 4, which contained only phycoerythrin (Fig. 13(F)). They can, therefore, represent the molecular arrangement of phycoerythrin, isolated as in fraction 4, or associated to thylakoid vesicles as in fraction 3. Besides these 10-nm structures, we found in fractions 3 and 4 bigger geometrical units (10–15 nm) with a central strained spot which looked like the pictures of the ribulose-bisphosphate carboxylase enzyme molecular preparations from *Synechococcus* [19].

Discussion

Our results reveal that the detergent treatments generally used to solubilize membranes affect the photosystem preparations differently. Hence, we have tested different detergents and buffers in order to obtain at the same time the best photosystem separation, together with a high photosynthetical activity. Thylakoids fragmented by digitonin in Hepes-Na buffer gave clearly separated fractions with their own functional activity. Among a large range of digitonin/chlorophyll ratios tested, the best results were obtained with a 60 (w/w) ratio. However, the separation was never perfect, and contamination of fractions by free phycoerythrin could not be avoided. In these experimental conditions, 36% of membrane proteins were recovered in the three membrane fractions obtained. If the phycoerythrin-containing fraction 3 reached up to 10.5% of total proteins, it represented only 7.5% of the membrane proteins. The similar relative proportions of membrane proteins associated with each fraction offered the possibility of valuable physiological analyses. Lauryldimethylamine oxide in sucrose phosphate citrate magnesium medium (SPCM buffer) was also used, according to the technique of Clement-Metral and Gantt [20]. By contrast to the results obtained with *Porphyridium*, the separation of the two photosystems was badly achieved, and no functional activity could be detected in the fractions. Triton, a more drastic detergent, did not give good results for *Cryptomonas*, the phycoerythrin complex being always dissociated. Digitonin was successfully used in the present work, allowing clearly separation of different submembrane fractions.

The pigment analysis reported in this paper was carried out by reverse-phase HPLC. The method we have developed was primarily for the separation of higher plant pigments [21]. Its origi-

nality consists in the total lack of water in the mobile phase, as in the method of Nelis and De Leenheer [22]. It has the advantage of giving a good separation of *cis-trans* isomers, and thus allowed us to determine unambiguously the presence of *cis*-alloxanthin in this algal strain. Nevertheless, a difficulty was encountered concerning Chl c_2 interaction with the stationary phase, resulting in very large trailing peaks. In such non-aqueous systems, a lowered ionization of the Chl c_2 molecule was obtained with the addition of 0.5% triethylamine, currently used in normal-phase chromatography [23]. We could then obtain a good recovery of Chl c_2. The rapidity of extraction and analysis procedures preventing the extracts against photodestruction, allied to the great resolution capacity of the detection, allowed accurate identification of the pigments contained in the various membrane fractions. Furthermore, the calibration of the detector response for the individual pigments at their own absorption maximum permitted their quick and precise quantitation.

HPLC analysis confirms that the main pigments in the Cryptophyceae are Chl *a* and Chl c_2 which represent about 80% of pigment molecules, as in other Chromophytes [24]. With the presence of α-carotene [25], like Chlorophyceae, [15] *Cryptomonas rufescens* differs from others Chromophyceae, which contain only β-carotene [26]. Its xanthophyll content is composed of two major pigments: alloxanthin and crocoxanthin [27]. The diacetylenic alloxanthin is the principal xanthophyll and seems to be more specific to the Cryptophyceae than the monoacetylenic crocoxanthin, which has been reported in Cyanobacteria and Rhodophyceae [26]. Besides these pigments, we have shown noticeable amounts of *cis*-alloxanthin which could not be detected earlier by the conventional thin-layer chromatography [28].

The low-temperature absorption spectrum of fraction 1 exhibits more efficient absorption at long wavelengths. Its deconvolution into Gaussian components needs large amounts of far-red absorbing Chl *a* forms, as currently observed in PS I fractions from others Chromophytes [29,30] and higher plants [10]. Low amounts of Chl c_2 could be measured, as noticed in PS I from a *Chroomonas* strain [3] and contrarily to *Fucus* PS I preparations [29], in which it was not detected. These particles are depleted is carotenoids with respect to chlorophylls; and among terpenoids, the proportion of non-hydroxylated carotenoids is enhanced, the xanthophyll/α-carotene ratio being half that of whole cells. This pigment composition compares well with those of PS I vesicles from Chromophytes [29,31], with α-carotene mainly restricted to the PS I core. However, a discrepancy appears with a subchloroplast fraction of *Fucus*, in which the PS I core complex was shown to contain high amounts of violaxanthin [32,33]. Recently, a chemically new chlorophyll, designated chlorophyll RC1, has been isolated from spinach chloroplasts and from *Scenedesmus* [34]. Its properties suggest close correlation with the reaction center of PS I, if not its identity with the chromophore of P-700 itself [35]. The main arguments of this hypothesis come from the enrichment in chlorophyll RCI of PS I particles obtained from several organisms, including *Cryptomonas maculata* [36], and from the identity of its molar ratio of P-700. Based on the data presented by these authors, including its structure as a 13^2-hydroxy-20-chloro-chlorophyll *a*, the elution time of this pigment in reverse-phase would be predicted to be slightly shorter than that of Chl *a*. The reverse-phase HPLC developed by Henry et al. [37] did not allow them to separate chlorophyll reaction center 1 from Chl *a* in membrane fractions from higher plants. The specific increase in our fraction 1 of a tetrapyrrole pigment eluting about 30 s before Chl *a* (Fig. 8) suggests that it could correspond to chlorophyll RC1, the absorption properties of which are comparable [34,36]. Investigations into its precise identification are in progress.

The fraction 1 was highly enriched in photooxidizable P-700 chlorophyll as compared to whole cells. These results agree with those obtained using different detergents on several other Chromophyceae [5,33,38]. Such PS I complexes can be analyzed by electrophoresis. They revealed either three chlorophyll complexes for *Cryptomonas* [4] or only one for *Chroomonas* [3]. These complexes probably represent different aggregation states of P-700 chlorophyll. In higher plants, such PS I chlorophyll-protein complexes are divided into a core complex (1) associated with one or two 60–70 kDa apoprotein(s) and a specific

light-harvesting Chl *a/b* complex (LHC I) [39]. Two polypeptides of similar molecular weight range have been obtained with Chromophyceae preparations [29,31,40]. The higher plant PS I contains also chlorophyll *b* and three polypeptides (20–24 kDa range) which are associated with the specific antenna, as found also in *Cryptomonas*. By comparison with green plants, one can assume that such 18–24 kDa polypeptides and Chl c_2 are also localized in the PS I antenna. In that view, the *Cryptomonas* PS I complex organization appears structurally and functionally very similar to those of other photosynthetic plant cells.

Fractions 2 and 3, with respect to their spectral absorption and fluorescence characteristics, can be related to the light-harvesting and PS II subunits. The physiological characteristics of fraction 2 proves that it corresponds to a PS II Chl a/c_2-light-harvesting complex [3–5]. It is mainly composed of short wavelength-absorbing Chl *a* forms, as revealed by low-temperature absorption curve analysis. Chl c_2 is very abundant in this fraction, the Chl a/c_2 ratio of which is 6-fold lower than in the PS I-enriched particles. Fraction 2 is very rich in carotenoids, especially alloxanthin, but the amount of α-carotene is very low. This composition of the Chl a/c_2 light-harvesting complex of *Cryptomonas* is very similar to the PS II fucoxanthin Chl *a*/Chl *c* complex from other Chromophytes [29,31,38]. Nevertheless, it differs from the Chl a/c_2 protein complex from a *Chroomonas*, in which a thin-layer chromatography separation could only reveal the presence of a single xanthophyll [3]. Energy transfer from Chl c_2 to Chl a is tightly coupled in fraction 2, as little 640 nm fluorescence emission from Chl c_2 is detected. However, after treatment by detergent, there is always a shift of the maximal fluorescence emission from 690 to 686 nm. As shown by photosynthetical activity, this active fraction contains mostly light-harvesting pigments, with very few reaction centers. This *Cryptomonas* PS II chlorophyllous complex looks like the light-harvesting Chl a/c_2 complex isolated from other Cryptophyceae [3,5], but differs by its functional activity and cannot be equivalent to active PS II fractions of *Fucus* [41]. Analyses of polypeptide composition support also the presence of PS II reaction centers among light-harvesting complexes in this fraction. The 47–39 kDa polypeptides which can be related to PS II core complex have approximately the same molecular mass as apoproteins (43–47 kDa) associated with the PS II core in higher plants [39]. The lighter 19–24 kDa polypeptides, also obtained from *Cryptomonas maculata* membranes [40], can be compared to the Chl *a/b* light-harvesting polypeptides from higher plants [39]. Structural analyses by electron microscope of negatively stained *Cryptomonas* fractions 1 and 2 look like the PS I and PS II vesicles obtained by digitonin treatment of spinach chloroplast [42]. Unfortunately, using this technique, it was not possible to differentiate unstacked and stacked parts of the thylakoid membranes.

Fraction 3, with chlorophyll pigments and phycoerythrin, is the most complete preparation. The Chl c_2 content is lower than in fraction 2 and the proportion of carotenoids reaches its maximum, with high amounts of *cis*-alloxanthin and crocoxanthin. Like in higher plants, the xanthophylls are largely associated with PS II and light-harvesting complexes. The phycoerythrin is essentially localized in the light-harvesting complex, tightly coupled to chlorophyll centers. As shown by fluorescence emission, transfer of excitation energy between phycoerythrin and chlorophyll is highly efficient. Moreover, the high coupling yield of this phycoerythrin-PS II complex is also attested by its physiological activity. Nevertheless, a small part of phycoerythrin is always uncoupled, as detected by the fluorescence emission peak at 625 nm. This active phycoerythrin-PS II complex can be compared to the active O_2-evolving phycobilisome particles obtained from *Porphyridium* [20]. Many features are rather similar in both fractions 2 and 3 (chlorophylls, xanthophylls and polypeptide range), but these fractions differ by their main pigment antenna and by their photosynthetical activities, with an opposite repartition of reaction centers and light-harvesting antennae. Such data reveal that our fractions are well resolved, and it is noticeable that for the first time a phycoerythrin-xanthophyll-PS II complex with photosynthetic activity has been isolated from a Cryptophycea.

In negatively stained electron microscope preparations, the phycoerythrin-PS II complex appears as vesicles covered by small polyedric par-

ticles, dispersed or closely contiguous. The size of each phycoerythrin unit is very similar to that of one single disc of phycoerythrin rod of phycobilisome of *Porphyridium* or *Synechocystis* 6701 [43,44]. These geometrical units can therefore be assimilated to phycoerythrin discs, and correspond to the densely stained material observed in thylakoid lumen on ultrathin sections [45]. Although these phycoerythrin-thylakoid complexes look like isolated phycobilisome-thylakoid complex obtained from *Porphyridium* [16], they must yet be considered as 'inside-out' vesicles, as for PS II preparations from higher plant thylakoids [46]. However, we cannot exclude that this aspect might also correspond to a random association, and consequently to an artificial reassociation. Nevertheless, this phycoerythrin PS II complex has kept its photochemical activity, and therefore this organization might represent the in vivo state of the complex. Further freeze-fracture studies will complete these observations.

The lack of chlorophylls and the absence of any detectable electron transfer activities in fraction 4 allows us to conclude that it contains only free phycoerythrin.

Conclusions

It is now well known that in phycobilisomes of Rhodophyceae and Cyanobacteria the energy is driven between the different phycobiliproteins by both resonance and chromophore interactions [47]. In Cryptophyceae, there is no phycobilisomes and only one type of phycobiliprotein in each species [18], but various forms of the same pigment [48,49] can exist in the same species. Hiller and Martin [48] suggested that in Cryptophyceae energy transfer may occur between the different forms of the phycobiliprotein. The observation of small phycoerythrin units closely associated to each other in the thylakoid lumen is compatible with this assumption. Furthermore, the phycoerythrin discs are in relation with thylakoid membranes; this association allows transfer of excitation energy collected by phycobiliproteins to the terminal intrinsic acceptor, as for Rhodophyceae and Cyanobacteria [43,44,47]. In these groups, specific high-molecular-weight polypeptides link phycobilisome to thylakoid membrane [50], and we have observed that the phycoerythrin-thylakoid complexes of *Cryptomonas* also contain high-molecular-weight polypeptides. We assume that they may have the same linker function between some phycoerythrin discs and thylakoid membranes. In conclusion, the collected energy might then be transferred from different forms of phycoerythrin to chlorophyll, through particular phycoerythrin units closely linked to the membranes by high-molecular-weight polypeptides. This organization is in agreement with the previous scheme deduced from fluorescence studies [1], and we can propose a functional model for the thylakoid organization of Cryptophyceae with two distinct light-harvesting PS II complexes (Fig. 14) (we have adopted the recent nomenclature system for chlorophyll-protein complexes) [39]. Fraction 1, composed of

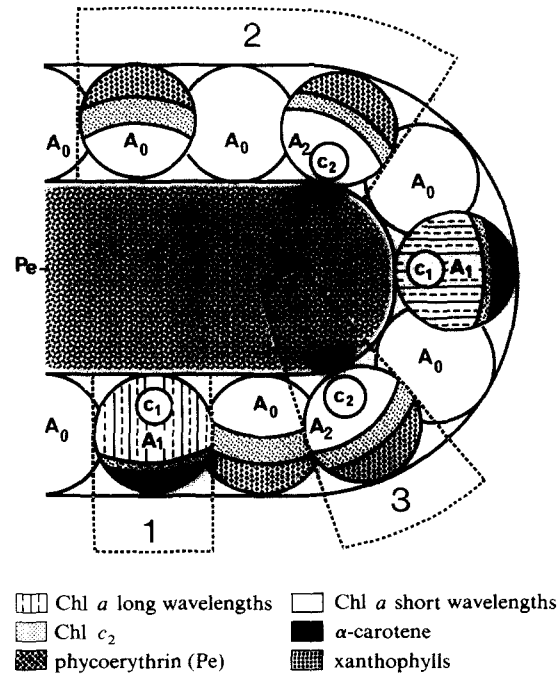

Fig. 14. Schematic interpretation of photosynthetical apparatus of *Cryptomonas rufescens* according to pigment composition and functional activity of the different fractions (1, 2 and 3) separated by sucrose density gradient. PS I, reaction centers (c_1) and light-harvesting antenna A_1 (LHC I); PS II, Reaction centers (c_2), light-harvesting antenna A_2 (LHC II) and chlorophyll a/c_2 light-harvesting antenna, A_0, between A_1 and A_2. This scheme does not take into account the photosystem repartition between stacked and unstacked parts of thylakoids.

long wavelength-absorbing Chl *a* forms and α-carotene, represents PS I. It can be divided into a core complex (core complex 1) and a specific light-harvesting complex A_1 (LHC I) which contains Chl *a*, Chl c_2 and α-carotene. PS I reaction centers can receive excitation energy from the chlorophyll antenna A_0 composed of Chl *a*, Chl c_2 and xanthophylls, probably through A_1, the specific PS I antenna (LHC I). The light-harvesting complex A_0 can also transfer excitation energy to PS II reaction centers (core complex II), perhaps through Chl c_2 and xanthophylls associated in a specific PS II antenna A_2 (LHC II). These elements, A_0, A_2 and core complex II, are found in the membrane vesicles and might correspond to the photochemically active fraction 2. Phycoerythrin, the major light-harvesting pigment of the second antenna unit, is exclusively connected to PS II [1] and is found in fraction 3. The close association between phycoerythrin units and thylakoid vesicles allows an efficient energy transfer between these pigments. This unit particularly enriched in PS II reaction centres contains also Chl c_2 and xanthophylls, perhaps associated with the specific PS II antenna A_2 (LHC II). Although the carotenoids transfer excitation energy [2], their specific localization as well as the Chl c_2 participation have not yet been clearly determined. This scheme will be probably slightly modified further, considering the different repartition of the two photosystems in stacked and unstacked parts of thylakoids, as suggested by freeze-fracture studies [51,52].

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