



Novel type of red-shifted chlorophyll *a* antenna complex from *Chromera velia*: II. Biochemistry and spectroscopy



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ABSTRACT

A novel chlorophyll *a* containing pigment–protein complex expressed by cells of *Chromera velia* adapted to growth under red/far-red illumination [1]. Purification of the complex was achieved by means of anion-exchange chromatography and gel-filtration. The antenna is shown to be an aggregate of ~20 kDa proteins of the light–harvesting complex (LHC) family, unstable in the isolated form. The complex possesses an absorption maximum at 705 nm at room temperature in addition to the main chlorophyll *a* maximum at 677 nm producing the major emission band at 714 nm at room temperature. The far-red absorption is shown to be the property of the isolated aggregate in the intact form and lost upon dissociation. The purified complex was further characterized by circular dichroism spectroscopy and fluorescence spectroscopy. This work thus identified the third different class of antenna complex in *C. velia* after the recently described FCP-like and LHCr-like antennas. Possible candidates for red antennas are identified in other taxonomic groups, such as eustigmatophytes and the relevance of the present results to other known examples of red-shifted antenna from other organisms is discussed. This work appears to be the first successful isolation of a chlorophyll *a*-based far-red antenna complex absorbing above 700 nm unrelated to LHCI.

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1. Introduction

The pigment–protein complexes involved in the photosynthetic energy conversion fall into two functional classes: the reaction centers (RCs) that carry out the photoactivated charge separation reaction and the light–harvesting complexes (antennas) that capture radiation and deliver the energy to the reaction centers in an efficient and regulated manner. Large numbers of chlorophyll (Chl) molecules are bound within the light–harvesting complexes relative to the reaction centers in order to increase the absorption cross-section, moreover, other pigment classes, carotenoids (tetraterpenoids) and phycobilins (open-ring tetrapyrrols) accompany chlorophylls (cyclic tetrapyrrols) in different groups of light–harvesting complexes in order to expand the useful range of wavelengths beyond the blue and red regions absorbed by chlorophylls.

Generally, the energy transfer in a photosynthetic system proceeds downhill along an energy gradient towards a pool of pigment

isoenergetic with the excited state of the reaction center [2]. This energy corresponds to wavelengths of about 700 nm and 680 nm for photosystem I (PSI) and photosystem II (PSII), respectively. However, it has been known that light–harvesting systems connected to both photosystems contain chlorophyll molecules absorbing at longer wavelengths than the respective primary donors of the RCs. Hence, the transfer of excitation energy from these low-energy forms to the reaction center is a thermally activated process. The nature of these red-shifted forms of chlorophylls has been more thoroughly investigated in the case of PSI of plants and cyanobacteria, where both the peripheral antenna (LHCI in plants) and the light–harvesting domains of the core complex have been shown to contain the low-energy pigments [3].

As for the red-shifted chlorophylls associated with the PSII antenna systems, the situation appears less clear. Antenna systems providing excitation to PSII while absorbing above 690 nm were found in green alga *Ostreobium* (Bryopsidales). In the case, the far-red chlorophylls were located in a variant of Lhca1 proteins, normally a component of the PSI light–harvesting machinery [4,5]. Recently, red-shifted Chl *a*, was described in association with a form of the LHCI subunit in the moss *Physcomitrella patens* [6]. Among cyanobacteria, *Acaryochloris marina* can be thought of as an organism relying solely on red-shifted chlorophylls, although in this case the spectral shift is achieved by replacing Chl *a* with chlorophyll *d* as a major light–harvesting pigment [7]. In diatoms and brown algae, the production of a far-red (>700 nm) absorbing

Abbreviations: CD, circular dichroism; CLH, *Chromera* light–harvesting [complex]; FCP, fucoxanthin–chlorophyll protein; NPQ, non-photochemical quenching of fluorescence; Red-CLHc, red-shifted *Chromera* light–harvesting [complex]; XLH, *Xanthomonas* light–harvesting [complex]; PS, photosystem

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and emitting antenna in adaptation of changing light quality has been studied since at least 1960s [8–15]. However, to our knowledge, no study reporting purification of the heterokont red-shifted antenna and its characterization has been published. The relatively best understood appears to be the antenna complex from the pennate diatom *Phaeodactylum tricorutum*. In this case a partial isolation was presented [16]. In this organism, the chromatic adaptation was shown to be accompanied by a formation of a protein complex exhibiting fluorescence emission around 710 nm at room temperature. The complex was unstable and dissociated rapidly after cell breakage, however, the authors managed to perform sucrose gradient centrifugation showing the complex fractionating towards the lower part of the band containing the light-harvesting complexes indicating that it is a larger complex. Moreover, these authors suggested that the 710 nm emitter might co-purify with PSII.

In the accompanying paper [1] it is shown that upon transition from blue to red cultivation light, *Chromera* (*C.*) *velia* undergoes a chromatic adaptation that shares many similarities with diatoms. The chromatic adaptation manifests itself by a pronounced change in the emission spectrum of the cells, with a maximum of fluorescence emission shifting from 686 nm to 714 nm with a concomitant appearance of an absorption band around 705 nm. The far-red emitter appeared to be an aggregate of ~17 kDa protein, most probably without direct association with either photosystems, although it was shown that *in vivo* the far-red excitation was capable of driving photosystem II photochemistry in the red-light adapted cultures.

C. velia is a species associated closely with corals [17,18] that is a type of environment known to host other organisms shown to possess red-shifted antenna complexes connected to the PSII. Consequently, the presence of such antenna system in *C. velia* is not surprising. In addition a recent molecular phylogenetic study demonstrated that *C. velia* has a large number of genes coding for light-harvesting complexes [19] belonging to the light-harvesting (LHC) family, suggesting a possibility for large plasticity. Although majority of the genes appeared as a sister branch to a group comprising fucoxanthin-containing LHC (FCP, LHCF) from diatoms and LHC complexes from dinoflagellates, other complexes with less clear affiliation were also identified.

A complete biochemical and biophysical characterization of two major types of light-harvesting complexes isolated from cells grown in a natural illumination was presented recently [20]. One of these complexes was identified in a complex with PSI and shown to be homologous to PSI associated antenna of red algae (LHCr). The other light-harvesting complex did not form aggregates with either photosystem and its sequence exhibited homology to the FCP proteins. However, its structure as studied by electron microscopy and its spectroscopic properties indicated a strong similarity to antenna complexes of xanthophyte *Xanthonema debile* [21], hence it was labeled CLH (*Chromera* light-harvesting) complex. This complex, lacking Chl *c* contained iso-fucoxanthin-like pigment as the major carotenoid species.

In this study, a description of the complete purification procedure of a novel *C. velia* antenna complex is presented. This protein complex containing red-shifted chlorophyll *a* is expressed during transition of the culture from blue (or natural daylight) to red illumination and is responsible for the marked spectral changes that accompany the chromatic adaptation. The most pronounced of these being the shift of the emission maximum of the whole cells from 686 nm to ~714 nm. Furthermore, connection of the complex to PSII was demonstrated by the oxygen evolution and fluorescence induction kinetics driven by the far-red illumination. This to our knowledge represents a first successful complete isolation and characterization of a red-shifted antenna system other than LHCI. Hence we believe that the present pair of publications constitutes an important step in the understanding of processes of photo acclimation in photosynthetic members of the group Chromalveolata, which includes important species of marine and freshwater phytoplankton.

2. Material and methods

2.1. Culture and growth conditions

Cells of *C. velia* were grown in 5 L Erlenmeyer flasks at 28 °C in modified f/2 saline medium for diatoms [22] and bubbled with filtered air. The culture was irradiated by incandescent light provided by 60 W tungsten filament light bulb with an intensity of 15 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, the light regime was set to 15 hour light–9 hour dark cycle. Thylakoid membrane preparation, solubilization and separation of protein complexes on sucrose gradient were done as described in [20].

2.2. Chromatographic procedures

Purification procedures were based on those described earlier for the isolation of antenna complexes from diatoms, brown algae and *C. velia* [20,23–25]. All steps were performed in the following buffer solution (Buffer A): 50 mM Tris–HCl pH 7.5, 2 mM KCl, and 0.03% *n*-Dodecyl β -*D*-maltoside.

Gel-filtration was performed using Superdex 200 GL 10/300 gel-filtration column (GE Healthcare) at a flow rate of 1 mL/min using Econ 4020 FPLC system (Econ, Czech Republic) using Buffer A. The analysis was performed in the dark. The buffer was supplied from an ice-cooled storage flask.

Anion-exchange chromatography was performed on a 2 mL column packed with DEAE Sepharose CL-6B at 1 mL/min using Econo Pump EP-1 (Bio-Rad, Germany) using a locally made gradient mixer. Before applying the zone 2 from the sucrose density gradient to the anion-exchange chromatography column (DEAE Sepharose CL6, resin volume 2 mL) equilibrated with Buffer A, the samples were washed with the same buffer on an Amicon membrane filter (10 kDa cut-off) to remove sucrose. The whole procedure was performed in a cold room at 4 °C. The samples were eluted with a gradient of NaCl in Buffer A (for details see Fig. 2). Prior to the gradient start, the column was washed extensively with Buffer A without adding NaCl. The eluent at this stage did not contain any proteins. The chromatography was performed at 4 °C in the dark.

2.3. Spectroscopy

2.3.1. Absorption

Room temperature absorption spectra were recorded with a UV300 spectrophotometer (Spectronic Unicam, UK). Spectra of whole cells were recorded using Shimadzu UV-2600 spectrometer equipped with ISR-2600Plus integrating sphere (Shimadzu, Japan).

2.3.2. Fluorescence

Fluorescence emission and excitation spectra were recorded on the Spex Fluorolog-2 spectrofluorometer (Jobin Yvon, USA) with slit width of 2 nm. Room temperature spectra of whole cells were measured in the perpendicular geometry. Samples were stirred to prevent cell sedimentation. The concentration of the sample was adjusted to absorbance <0.1 to avoid distortion of spectra by reabsorption. Measurements of protein samples at 77 K were done in a reflection mode using locally made holders immersed in liquid nitrogen in a Dewar vessel.

2.3.3. Circular dichroism

Circular dichroism (CD) spectra were recorded with a Jasco J-715 spectropolarimeter using bandpass of 2 nm. Samples in quartz cuvettes were placed into a water-cooled sample holder and maintained at 4 °C for the duration of the measurement to prevent fast dissociation of aggregated state of the pigment–protein complexes.

2.4. Pigment composition

Analysis of pigment composition was performed according to the procedure described in [20], using reverse phase Sunfire™ C8 column (4.6×250 mm, $5 \mu\text{m}$, silica-based, end-capped, Waters, USA) methanol, acetonitrile water, and hexane gradient elution. The photosynthetic pigment molar ratios were estimated from areas under the chromatograph peaks. The molar extinction coefficients were (in $\text{dm}^3 \text{mmol}^{-1} \text{cm}^{-1}$) 71.4 for Chl *a* at 665 nm [26], 144 for violaxanthin at 439 nm, 134 for zeaxanthin at 472 nm, and 139 for β -carotene at 453 nm [27]. As the extinction coefficient for the isomer of iso-fucoanthin hasn't been yet determined, we used the coefficient of fucoxanthin, 109 at 453 nm [27].

2.5. Protein composition analyses

Protein composition was determined by SDS-denaturing gel electrophoresis (SDS-PAGE) on 12% precast gel (C.B.S. Scientific) using Coomassie Brilliant Blue or silver staining. Apparent molecular weights were estimated by co-electrophoresis of a low molecular weight protein standard (Fermentas). Antenna complexes present in zone 2 from sucrose gradient centrifugation were separated by colorless native polyacrylamide gel electrophoresis (CN-PAGE) according to [28] using 4.5–14% linear gradient of acrylamide. During electrophoresis, protein complexes were visible without staining.

MS/MS analysis was performed on a NanoAcquity UPLC (Waters) online coupled to an ESI Q-TOF Premier Mass spectrometer (Waters) as described in [20].

Protein sequences were further analyzed for similarity to known eukaryotic antenna of the LHC family as described in detail in the Supplementary data. The sequences were aligned to a dataset of LHC-homologues and the alignment results processed to obtain a phylogenetic tree using the neighbor-joining algorithm. The sequences were chosen so as to obtain the maximum coverage of groups of organisms known to possess the red antennae. The full list of gene products used for the analysis is also given in the Supplementary data file, Table 2.

Theoretical isoelectric point of the proteins was obtained using an online computation tool [29].

3. Results

3.1. Isolation of the far-red antenna proteins

Cultivation of cells was performed using low-intensity incandescent illumination (predominantly red/far-red emitter). These cultivation conditions were chosen based on the high intensity of the far-red

emission in such cultures as shown in the Supplementary data Fig. 1. For comparison, spectra of culture grown in normal sunlight are also presented. Insets show the spectra of the incident radiation expressed in photons/ m^2 .

The isolation of the far-red absorbing species was done by the same approach as described earlier for isolation of the antenna complexes from the “daylight” culture of *C. velia* [20], starting with the solubilization of the thylakoid membranes with *n*-dodecyl β -D-maltoside followed by the sucrose density gradient centrifugation. The gradient zonation, which corresponded to the cited work, is schematically shown in Fig. 1a for convenience. The antenna-containing zone (zone 2) was found to contain the far-red emitter and thus chosen for further analysis. In agreement with earlier observations on the daylight cultures described in the above mentioned paper, photosystems were found in the sucrose gradient zones 3 and 4, well separated from zone 2. Hence, the possibility of the longwave emission arising from photosystem I could safely be excluded. This was also confirmed by further analyses, see below.

Fig. 1b presents the native electrophoresis of the antenna zone from the sucrose gradient showing that the zone split into two distinct bands. The lower, brown band (Fig. 1b, 1), was assigned to the FCP-like antenna complex [20] based on its single fluorescence emission peak at about 688 nm at 77 K and SDS-PAGE electrophoresis (not shown).

The upper, green band (2) exhibited a pronounced emission maximum at 717 nm with a smaller, broad peak around 686 nm. The position of the green band above the CLH-band on the native gel suggests that the species responsible for the far-red emitting band forms in its native state aggregates larger than oligomers of the CLH. Moreover, when the upper part and lower part of the gradient zone 2 were sampled separately, the lower part appeared to contain higher amount of the red-emitting species, compared to the upper part, in agreement with the native electrophoresis result (not shown). These results showed that the red-emitting complex of *C. velia*, unlike the diatom far-red antenna [16], survived the cell breakage and solubilization of thylakoid membranes in amount permitting its further purification. Two different purification approaches were tested, anion-exchange chromatography and gel-filtration [23–25], in order to obtain the far-red antenna samples in quantity sufficient for further biophysical and biochemical characterization. From now on, we will apply the notation Red-CLHc for the red-shifted complex.

3.1.1. Anion-exchange chromatography

Results of this purification step are presented in Fig. 2, panel a, spectra of selected chromatographic fractions in panel b and their respective SDS-PAGE gels in panel c. Initially, a small amount of protein containing

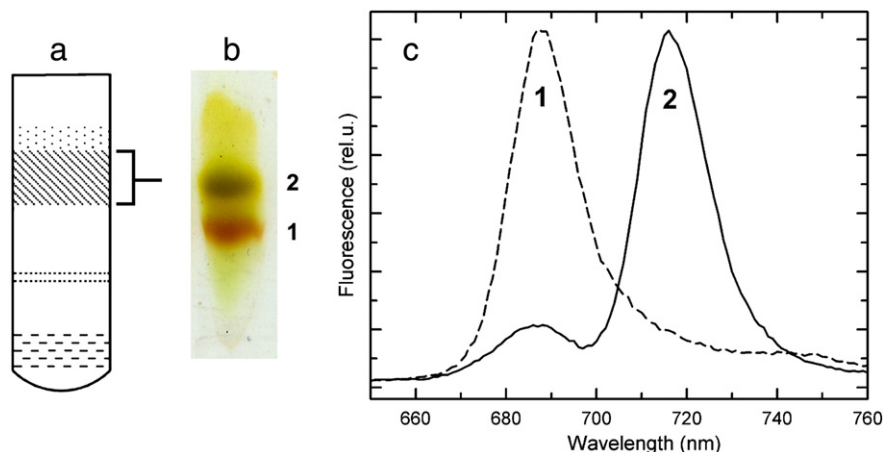


Fig. 1. (a) Schematic drawing of the zones of the sucrose gradient obtained from the thylakoid membranes of *C. velia*; (b) native electrophoresis of the second zone; (c) the 77 K emission spectra of the bands 1 and 2 in the native electrophoresis.

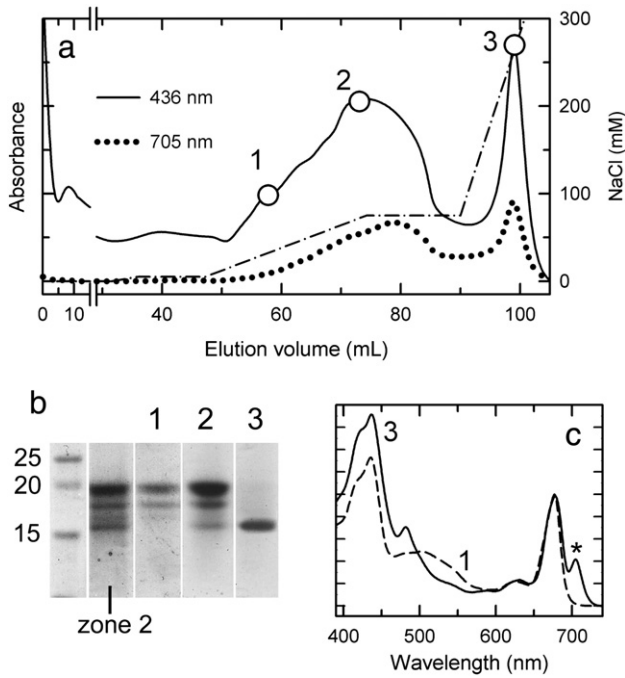


Fig. 2. (a) Results of the anion-exchange chromatography on DEAE Sepharose CL6B of the antenna-containing zone from the sucrose gradient from cells of *C. velia*. Solid line – absorbance at 436 nm; dotted line – absorbance at 705 nm, dash-dotted line – NaCl concentration (scale on the right side of the graphs); (b) Coomassie-stained SDS-PAGE gel of the fractions; (c) absorption spectra of fractions 1 (solid line) and 3 (dashed line) denoted by circles in the left panel. Spectra of the intermediate fraction 2 corresponded to a linear combination of spectra 1 and 2, in agreement with (b) hence were omitted for clarity. The asterisk highlights the red-shifted absorption band of fraction 3.

iso-fucoxanthin was eluted even at 5 mM NaCl, amount of this fraction varied among samples. Fractions eluting at salt concentration 30–60 mM, (1) were enriched in the heavier (~20 kDa) proteins with pronounced absorption in the 480–550 nm region, corresponding to the CLH antenna complex described earlier [20]. As the salt concentration increased above 60 mM, a smaller, approximately 17 kDa protein became prominent, the presence of which coincided with the appearance of the absorption band around 700 nm and relative increase of the peak at 480 nm corresponding to carotenoid violaxanthin. Thus, one can conclude that the 17 kDa protein is the antenna complex responsible for the far-red absorption. The fact that this protein eluted at high-salt concentrations from the anion-exchange column indicates that it is strongly charged at the pH > 7 (see below). The same analysis was performed on a daylight-adapted culture, the result is shown in the Supplementary data Fig. 2. Much lower proportion of the “high-salt” fractions compared to CLH was observed in this case.

The amplitude of the far-red absorption band decreased in time, even when the fractions were stored in the dark at 4 °C, indicating that the isolated complex was unstable and possibly damaged by the above described purification procedure. Also, the initial amplitude of the absorption band around 700 nm relative to the Q_y varied among the high-salt fractions from different isolations which suggests that the complex was sensitive to the handling of the zone 2 (e.g. freezing and thawing) before it had been applied to the DEAE column. In order to avoid the effect of salt on stability of the far-red antenna, gel-filtration was introduced as an alternative purification approach.

3.1.2. Gel-filtration

The antenna zone collected from the sucrose density gradient was loaded on the Superdex 200 GL 10/300 column and eluted with the Buffer A (see Material and methods). Fractions were collected based on their absorption spectra. Results of the analysis are presented in Fig. 3. Panel a of the figure shows the elution profiles detected at

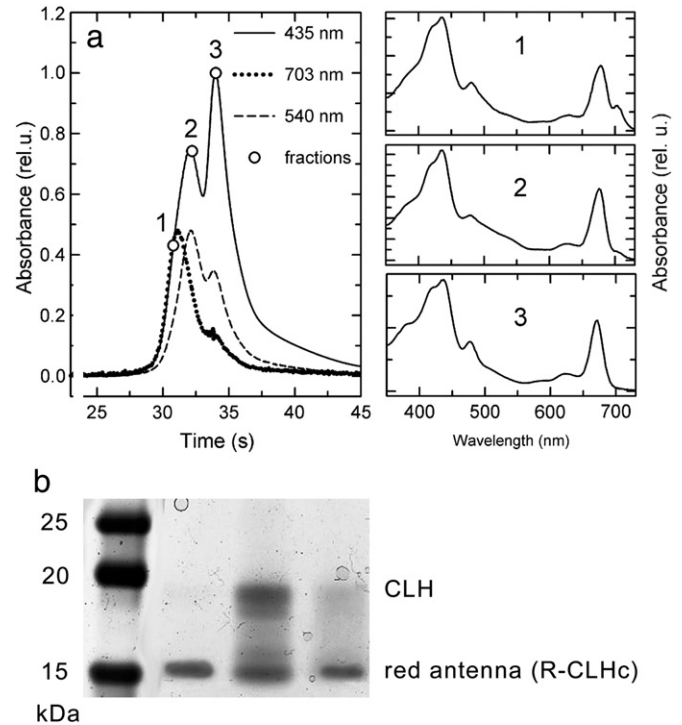


Fig. 3. Results of the gel-filtration of the antenna-containing zone from the sucrose gradient from cells of *C. velia* grown under incandescent light. (a) Left: elution profiles at wavelengths given in the graph legend; (a) right: absorption spectra of fractions denoted by circles in the left panel; (b) silver-stained SDS-PAGE gel of fractions 1–3.

435 nm, 540 nm and 703 nm with times of fraction collection denoted with circles. Whole absorption spectra of the fractions are shown on the right side. Panel b shows the SDS-PAGE gel of the same fractions. The gel-filtration data showed that the initial fraction contained a ~17 kDa protein with a pronounced absorption band around 705 nm, apparently corresponding to the high-salt fractions from the anion-exchange chromatography. This fraction was followed by a mixture of proteins enriched in CLH while still containing a tail of the far-red antenna peak. The third fraction appeared to contain the same protein as fraction 1, however, the 700 nm band was not present in the absorption spectrum and the Q_y band was narrower and shifted by about 6 nm to the shorter wavelengths as compared to the fraction 1 (from 677 nm to 672 nm). This fraction is probably composed of monomeric subunits of the far-red antenna. This interpretation is strongly supported by the circular dichroism spectra presented in Fig. 5.

3.1.3. Pigment content

As seen already in the native electrophoresis bands, Fig. 1, there was a marked difference in color between the two types of antenna, the brown, iso-fucoxanthin containing, CLH and Red-CLHc, likely containing more violaxanthin. Consequently, it could be expected that most cellular antenna-bound iso-fucoxanthin would be contained in CLH and loss of this carotenoid during purification reflects the separation of CLH from Red-CLH. These observations were supported by pigment analyses of various fractions containing the Red-CLHc antenna as well as the FCP-like CLH that are summarized in Table 1. For the Red-CLHc, the violaxanthin:iso-fucoxanthin ratio was close to 2 in the aggregates, decreasing to ~1 in monomers. The sample of aggregates from the anion-exchange had lower carotenoid to Chl *a*, compared to the gel-filtration fraction. As seen from the comparison of chromatograms in Figs. 2 and 3, it appeared that the separation of fractions was lower in gel-filtration than in anion-exchange, causing likely some contamination by CLH in Red-CLHc fractions and vice versa, accounting for higher relative amount of violaxanthin in the former and iso-fucoxanthin-like

Table 1

Pigment content relative to chlorophyll *a* (%) of different fractions of red antenna (Red-CLHc) and CLH preparations, as shown in Figs. 2 (anion-exchange) and 3 (gel-filtration). Numbers in square brackets indicate the labels of fractions in Figs. 2 and 3. Only samples indicated by asterisk (*) exhibited the red-shifted absorption band at ~705 nm.

Complex	Fraction	Chl <i>a</i>	Violaxanthin	Iso-fucoaxanthin-like
Red-CLHc	Anion-exchange [3]*	100	35	16
	Gel-filtration [1], oligomeric*	100	42	27
	Gel-filtration [3], monomeric	100	39	37
CLH	Anion-exchange [2]	100	4	43
	Gel-filtration [2]	100	27	38

carotenoid in the latter. The CLH from anion-exchange possessed very low violaxanthin amount; this observation might be also due to the fact that the violaxanthin is not strongly bound to the complex and might be removed during the purification procedure.

The fact that separation of proteins by the gel-filtration does not require the presence of salt allows testing of stability of the complex at different conditions. The results are presented in Fig. 4. In the left panel, the control sample (circles) represents the fraction 1 collected from the gel-filtration that contains the far-red antenna aggregates. All experiments were performed at room temperature in the dark. The sample was initially dissolved in Buffer A without adding salt; the spectrum of the sample is represented by the solid line in the right panel. As seen in the left panel of Fig. 4 even in the control sample the absorbance band at 700 nm decreased in amplitude by about 40% in 1 h. When 200 mM NaCl was added, the rate of the bleaching of the 700 nm band increased about 2 times, confirming prior observations from the anion-exchange chromatography. Even greater effect was achieved by lowering the pH of the buffer in which the complex was dissolved. In the right panel, the final absorption spectrum is drawn in dashed line. One can see that the disappearance of the 700 nm band is accompanied by narrowing and a blue shift of the Q_y absorption band of Chl *a* and a slight blue shift and decrease of the violaxanthin absorption band around 480 nm. Moreover, the complex was found to be very sensitive to freezing–thawing. One cycle of freezing (in liquid nitrogen) and thawing was enough to lower the absorbance at 700 nm by as much as 50% (data not shown). The stability of the complex increased when the sample was kept at 4 °C all the time, at least for the time needed to record its CD spectra as described below. Nevertheless, long term storage at this temperature did result in the loss of the far-red absorption band.

The stability assay indicates that the complex is held together by electrostatic force. When the charge is compensated for by addition of Na^+ or the partial charge of respective titratable residues removed by lowering of pH, the aggregate comes apart. It is worth noting here that neither the salt concentration nor the pH used in the stability test was out of range of the expected physiological values.

The instability of the aggregates at room temperature as well as their sensitivity to freezing and thawing prevents their efficient storage as well as performing of time consuming measurements in instruments not equipped with temperature control. Finding of means to increase its stability seems necessary in order to allow a more detailed investigation of the energy transfer in this interesting pigment–protein complex.

3.1.4. Protein identification and sequence analysis

The ~17 kDa protein bands of the Red-CLH from the whole thylakoid native electrophoresis (shown in the accompanying paper of Kotabova et al.), anion-exchange (Fig. 2b) and gel-filtration (Fig. 3b) were subjected to trypsin digestion and mass spectrometric analysis. The resulting peptides are listed in the supporting data, Table 1 in Appendix A. Comparison of results of all the samples indicated two proteins of theoretical molecular weight of about 20 kDa and isoelectric points of ~5 and 6, respectively, to be present consistently in all samples possessing the red-shifted Chl *a* absorption (C_velia_1646 and 532 in the Supplementary data Table 1). Hence, we concluded that these represented the minimum necessary condition for the appearance of the red-shifted absorption band. The retrieved sequences were further analyzed with respect to their similarity to known LHC-homologues of other organisms with the aim of identifying candidates for the proteins capable of forming red-shifted antenna complexes in different taxonomical groups. The resulting tree (Supplementary data Fig. 3A) corresponded extremely well to the recently published phylogenetic analysis of the *C. velia* antenna genes [19], that is, four broad groups of LHC-homologues can be defined: i) the antennae of the green lineage; ii) the LHCr/LHCr-like group including the red algae LHCs in the basal position as well as antenna complexes from various other groups, including the *C. velia* LHCr; iii) the family of stress-related Li818/LHCx proteins; iv) the LHCf/FCP family, comprising also the FCP-like antenna of *C. velia*, the CLH. Within this group a well supported cluster was formed, that contained both sequences found in the Red-CLHc. This group is designated “R-CLHc-like”, expanded view of this group is given in Supplementary data Fig. 3B.

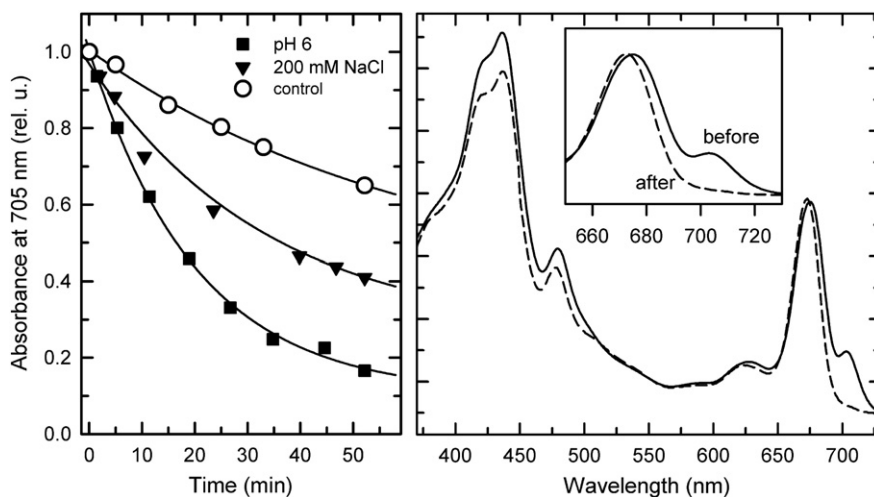


Fig. 4. Stability of the far-red absorbing pigment–protein complex from *C. velia*. The left panel shows normalized absorbance at 705 nm versus time for an untreated sample (control, circles), sample supplied with 200 mM NaCl (triangles) and sample in which the pH was lowered from 7.5 to 6 by addition of diluted HCl. Lines through the data points are exponential fits used solely to simplify orientation in the figure. The right panel shows an example of the initial and final absorption spectra of the sample. The Q_y region is shown in detail in the inset.

3.2. Spectroscopy of the purified far-red antenna

In order to further characterize the structure and function of the 17 kDa far-red absorbing pigment–protein complex, the steady state fluorescence spectroscopy at 77 K and the circular dichroism spectroscopy (at 4 °C) were employed.

3.2.1. Fluorescence spectroscopy

Both the high-salt fraction from anion-exchange chromatography and the fraction 1 (aggregates) from the gel-filtration exhibited a prominent emission band at 717 nm at 77 K (Fig. 5) which was missing from the spectra of monomers obtained both directly from the gel-filtration and by dissociation of aggregates as described in the previous section. The 717 nm emission maximum was accompanied by another peak at 686 nm. The latter was also the only maximum observed in the emission spectra of the monomers, as shown in Fig. 5. The emission spectra presented in Fig. 5 were measured using excitation into violaxanthin (480 nm) to obtain emission of complexes in which at least some the excitonic interaction between different pigment pools and consequently the structure of the complexes was preserved in native form. When the excitation spectrum of the 680 nm band was measured (not shown) it appeared to contain small contribution from iso-fucoxanthin absorbing around 510 and 540 nm in addition to violaxanthin.

Fig. 5 also shows the excitation spectrum of the far-red band of aggregates, measured for emission at 725 nm. It clearly shows that the carotenoid with the major contribution to the energy harvesting in the far-red antenna is violaxanthin as seen from the prominent peak at 485 nm although there was also a small contribution of iso-fucoxanthin as seen in the 500–550 nm region. Moreover, the excitation spectrum shows a clearly resolved split of the Q_y band into two components peaking at 667 nm and 679 nm, respectively.

3.2.2. Circular dichroism

The CD spectra of the different aggregation states of the far-red antenna protein are plotted in Fig. 6. The samples were prepared by the gel-filtration and correspond to the data presented in Fig. 3. The spectra were measured at 4 °C to increase the stability of the samples during measurement. The upper trace, a, represents the putative aggregated state of the far-red antenna pigment–protein complex (fraction 1 from the gel-filtration), the middle trace, b, corresponds to the fraction 3 of the gel-filtration, i.e. assumed monomer. The lower trace, c, is the spectrum of the fraction 1 after incubation at pH 6, i.e. the aggregate

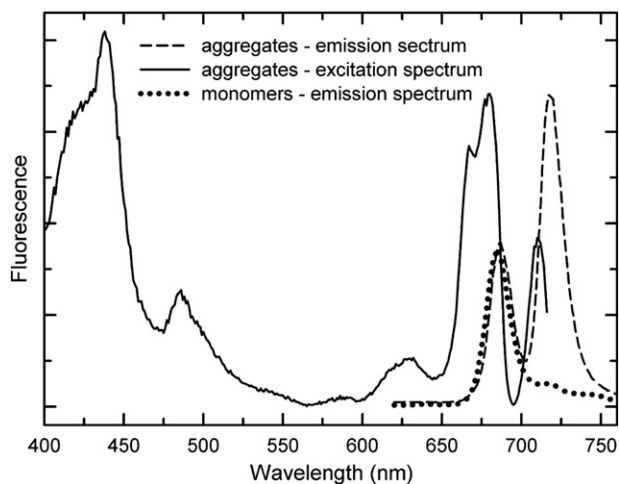


Fig. 5. Fluorescence spectra at 77 K of purified aggregates and monomers of the far-red absorbing pigment–protein complex from *C. velia*. Dashed line – emission spectrum of aggregates; dotted line – emission of monomers. Excitation wavelength 480 nm in both cases. Solid line – excitation spectrum of the far-red emission band of aggregates measured at 725 nm.

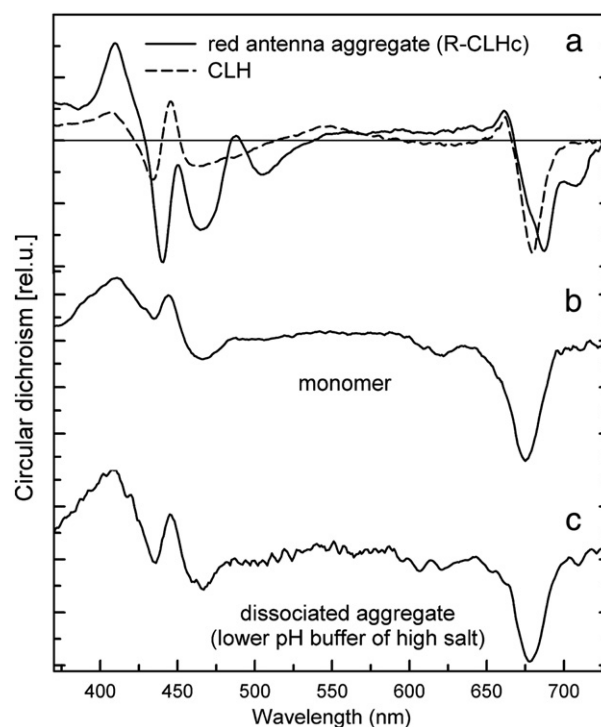


Fig. 6. Circular dichroism spectra of far-red absorbing pigment–protein complex from *C. velia*. (a) CD spectrum of the aggregate (solid line), CD spectrum of the CLH complex is presented for comparison; (b) CD spectrum of monomers (late eluting fraction from gel-filtration); (c) CD spectrum of the dissociated aggregate after prolonged incubation at pH 6.

dissociated into monomers. One of the most conspicuous features of the spectrum of the aggregate is the negative band in the far-red region peaking at ~708 nm that clearly corresponds to the far-red peak in the absorption spectrum. This feature was missing in spectra of the monomers indicating its origin in the inter-subunit interactions within the aggregate. Further negative maxima in the spectrum were observed at 688 nm, 505 nm, 465 nm and 440 nm. Pronounced positive maxima were located at 662 nm, 488 nm, 450 nm and 410 nm. Overall, the spectrum in the Q_y region of Chl *a* absorption was highly asymmetrical, suggesting complex excitonic interactions. Most of these were lost upon dissociation of the aggregates into monomers as evidenced by the change of the shape of the spectrum into a single negative band peaking at 680 nm, accompanied with a smaller negative peak around 620 nm. Interestingly, these two spectral features along with the disappearance of the positive band at 662 nm caused the spectrum to become very similar to the light-harvesting complexes of xanthophytes *Xanthonema* and *Pleurochloris* [20,31]. The dissociation was further accompanied by the loss of the structure in the carotenoid absorption region (440 to 500 nm), indicating that the binding of violaxanthin was also affected and possibly the carotenoid was lost altogether from the protein complex (in agreement with the pigment analyses described above).

A CD spectrum of the CLH complex of *C. velia* was presented recently [20] showing a high degree of similarity with spectra of XLH complexes from *X. debile*, but rather surprisingly very low similarity to the FCP antenna of diatoms or the LHClI and LHClI of higher plants (e.g. [24,32–34]). The spectrum of the CLH complex of *C. velia* is shown in Fig. 6 (dashed line in panel a). Obviously, the spectra share several qualitative features, namely the presence of the positive peak at 662 nm and the “peak-trough-peak” feature in the 410–440 nm region. On the other hand, the positive peak in the CLH spectrum at 550 nm, which was observed also in xanthophyte (albeit shifted to shorter wavelengths) and dinoflagellate [35] antenna complexes, is missing in the far-red antenna suggesting a difference in the carotenoid binding. Surprisingly, the presence of two negative peaks in the 440–500 nm region bear similarity to

spectra of both LHCI (see [36]) and LHCI [33] from higher plants, although in case of LHCI, the peaks are present also in the monomeric complexes [36] whereas in the far-red antenna of *C. velia* they appear to be the property of the aggregated form only.

4. Discussion

Analyses presented in the preceding sections indicate that the molecule carrying the red-shifted Chl *a* in the *C. velia* cells grown under red/far-red illumination is an oligomeric complex of proteins homologous to LHC, hereby denoted Red-CLHc, for Red-Chromera Light-Harvesting complex. The complex could be easily extracted from the thylakoid membrane while maintaining its spectroscopic properties. However, the low stability *in vitro* suggested that inter-complex interactions with other components of the photosynthetic membrane might be necessary to maintain its structure *in vivo*. The complex co-purified in the sucrose gradient with the other major type of *C. velia* antenna, the CLH, from which it could be separated using native electrophoresis, anion-exchange chromatography and gel-filtration. The data from the sucrose gradient centrifugation as well as gel-filtration suggest that the assemblies of the far-red complex are slightly larger than the oligomers of the CLH. Importantly, the Red-CLHc was not found to form specific complexes with either photosystem.

The absorption spectrum of Red-CLHc in the Q_y region peaked at 677 nm with a shoulder around 667 nm. At 77 K these features split into two distinct bands. A prominent band was present at 705 nm. The absorption spectra of whole cells were mostly determined by the absorptive properties of the Red-CLHc complex. Hence energetics of the thylakoid membrane of the red-light adapted culture is such that the lowest energy state of the antenna is isoenergetic with the PSI primary donor, which we observed around 705 nm using flash photolysis on isolated PSI-LHCr complexes (not shown) while the energy transfer to PSII relies on thermal activation. The amplitude of the 705 nm transition in protein varied between 25 and 40% of the main Q_y band in various preparations, likely indicating varying degree of integrity of the complex. At the same time the samples showed low F688/F714 ratios of the emission bands. This is at first approximation consistent with population of the antenna energy levels following the Boltzmann distribution, where the relative intensity of emission bands in fluorescence spectrum follows from the probability for an exciton to reside in a particular pigment pool, i.e. red (677 nm) and far-red (705 nm) in our (simplified) case. While the 677 nm is favored by the apparently larger number of pigment molecules present (larger absorbance), the far-red state will be more prominent in the emission spectrum due to its lower energy; see [37] for discussion of the red-shifted antenna states. The Stokes shift of the spectral bands appeared to be less than 10 nm far-red transitions, much smaller than what was found for the far-red Chl *a* forms in Lhca and PSI cores [38].

Unlike the CLH complex, which employs iso-fucoanthin as the main carotenoid, the red antenna was shown to contain mainly violaxanthin, which was consistent with the presence of the absorption band around 477 nm. Analysis of the excitation spectra indicated that energy absorbed by violaxanthin was transferred to the 705 nm band suggesting that this carotenoid plays the light-harvesting function. Moreover, as shown in the accompanying paper, no difference was found in the emission spectra excited mainly into violaxanthin (490 nm) or iso-fucoanthin (540 nm). This observation could be interpreted in terms of unrestricted energy migration between the Red-CLHc and CLH complexes within the thylakoid membrane.

In the accompanying paper it was shown that red-adapted *C. velia* cells exhibit NPQ enhanced in the far-red region. Earlier results also indicated that *C. velia* possesses a highly efficient xanthophyll cycle based on violaxanthin de-epoxidation [39]. This, along with the results showing Red-CLHc as the major violaxanthin-containing complex indeed suggests it as a possible site of NPQ. As seen in Supplementary data Fig. 2 even in some daylight-grown cultures there appeared to be produced

some amount of the high-salt, Red-CLHc containing fractions, a fact not noted in the previous publication [20]. Hence, it is possible that this complex might serve various functions in different ecological types of *C. velia* and this function might involve excitation quenching. However, as seen in previous sections, Red-CLHc forms the major antenna complex in cultures of *C. velia* grown under predominantly far-red illumination and its primary function is thus undoubtedly light-harvesting rather than energy-dissipating in this case.

In this context one should consider a recent work of Lavaud and Lepetit, [40], who showed a positive correlation between NPQ and fluorescence at 711 (F710 or F711) nm in diatoms, however, unlike Fujita and Ohki [16], seem to have based their study only on the low temperature spectra which slightly complicates analysis of results. The far-red emission bands are commonly attributed to PSI. However, such conclusion is not generally justified. It does apply to plants where the far-red emission is dominated by the contribution of the LHCI complexes above 720 nm. These form major contribution to fluorescence spectrum at 77 K but are significant even at room temperature (both in isolated PSI-LHCI [41], as well as whole leaves [42]).

However, the situation in other eukaryotes, e.g. green algae and diatoms [43,44] is quite different. Here, the PSI far-red emission originates in the core of PSI only and is somewhat less red shifted (~710–715 nm). On the level of whole cells, these red forms contribute negligibly to absorption and consequently, their influence on the shape of the emission spectra is also reduced, compared to plants, as noted e.g. in [44], where even at 77 K only the purified PSI complex but not thylakoids, exhibited pronounced maximum above 700 nm. More importantly, at room temperature, the fluorescence yield of PSI is very low and contribution of PSI to emission at room temperature is completely negligible in isolated antenna-PSI fractions [45].

This is in marked contrast to the situation of red-light *C. velia* where the whole cell emission even at room temperature was dominated by the far-red emission, accompanied by a pronounced absorption band around 705 nm. We have shown here that the species responsible is the antenna complex. This is in agreement with the results from diatoms of Fujita and Ohki [16]. A “practical” result of these observations is that measurements at room temperature are better suited for identification of red antenna complexes in algae and their separation from PSI.

Although there are many similarities between the red shifted antenna complexes of *C. velia* and pennate diatoms, our analyses did not indicate close relation between the proteins forming the *C. velia* red antenna and any light-harvesting complexes of diatoms, including the Lhc15, shown recently to be upregulated under red-light [46] and consequently a promising candidate for the red antenna.

Another group in which red-shifted antenna complexes have been observed so far are prasinophytes, where the respective pigment-protein complex protein was shown to belong to the LHCA family [4] while our result showed that the red antenna in *C. velia* lineage is not related to the complexes associated with the PSI.

The group of protein closest to Red-CHLc contained several sequences from the eustigmatophyte *Nannochloropsis* as well as a sequence from *Vaucheria*, a xanthophyte. Given that the red-shifted antenna was observed in *Vischeria*, also a eustigmatophyte [30], eustigmatophytes appear to be a promising candidate group to search for further examples of the red-shifted antenna.

Unfortunately, none of the proteins placed in the Red-CLHc-like group has been studied in detail yet. It thus remains to be seen whether these proteins correspond to antenna complexes possessing red-shifted Chl *a* species at all. In our opinion, it could be expected that each red antenna represents independent occasion of adaptation to environmental pressure that is the depletion of the incident radiation in the Chl *a* Q_y , with different groups recruiting different types of antenna complexes for this purpose. As the homologs of LHCI, including FCP are generally known to assemble into aggregates, it is possible that the capacity to form the structures in which the pigment interactions give rise to the red-shifted absorption bands is a more general property of the FCP/

LHCf-like pigment–protein complexes. In order to predict possible specific structural motifs responsible for the formation of complexes with quaternary structure that allows for the pigment–protein interactions leading to the chlorophyll *a* transitions with energies below 700 nm more red-shifted proteins must be isolated and characterized. To conclude this section, a brief demonstration of utility of the red-shifted light-harvesting complexes in the conditions of filtering of the photosynthetically active radiation by Chl *a* containing phototrophs is offered. We calculated (as in [47]) except the cell spectra were normalized to area in the region 350–750 nm) the theoretical spectrum of radiation available underwater at 1 meter depth using the standard reference solar spectrum at ground level (AM1.5 global-ASTM G173, [48]), converted to photons/m², and absorption spectrum of water, taken from [49]. The shading effect was modeled using spectrum of the PCP antenna complex from the dinoflagellate *Symbiodinium*, characterized recently [50]. *Symbiodinium* was chosen based on the fact that it is a common coral endosymbiont. The absorption of PCP was set to 0.5 at Q_y maximum. The computation indicated as much as 30% increase in the absorption of the red-adapted form compared to the blue light (day-light) adapted culture, when integrating over the whole spectrum between 350 nm and 750 nm. This advantage disappeared completely when the shading by PCP was not considered and the spectrum of the incidental radiation was calculated using the absorption of water only. Then the blue light culture could capture about 5% more photons. For the reader's convenience the computed irradiation spectrum is provided in the Supplementary data Fig. 5 along with the spectra of other species of organisms adapted to utilization of far-red illumination (*Acaryochloris*, *Ostreobium* [4], and *Phaeodactylum*).

4.1. Concluding remarks

Biochemical and spectroscopic properties of the far-red antenna form *C. velia* rule out the possibility that the far-red emission and absorption in the organism are associated with the PSI associated antenna complexes or PSI core. Instead, the complex is shown to be formed by aggregation of proteins related more closely to the FCP/Lhcf family of light-harvesting complexes. The complex analyzed in this work represents the third major type of light-harvesting antenna found in *C. velia*. Despite its relatively recent discovery *C. velia* thus appears to be a promising candidate for an organism in which a full integration of information ranging from biophysical to genomic can be achieved towards complete mechanistic understanding of regulation of light-harvesting.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbabbio.2014.01.011>.

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