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Short Communication

Antheraxanthin, a Light Harvesting Carotenoid Found in a Chromophyte Alga[1]

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ABSTRACT

The pigments of the chromophyte freshwater alga, *Chrysophaera magna* Belcher were analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) to reveal the presence of chlorophylls *a* and *c*, β-carotene, fucoxanthin, and antheraxanthin. The presence of antheraxanthin was verified by comparison of TLC R_F values, HPLC retention times, and absorption features to those of authentic, synthetic antheraxanthin. Antheraxanthin accounted for about 15% of the total carotenoid content of *C. magna*. The molar ratio of the major carotenoids was antheraxanthin:fucoxanthin:β-carotene, 1:2.3:3.3. The whole-cell absorption spectrum revealed a broad band between 470 and 520 nanometers which was attributed to fucoxanthin and antheraxanthin *in vivo*. Upon extraction in hydrocarbon, this broad absorption region was lost. The *in vivo* fluorescence excitation spectrum for 680 nm emission revealed the energy transfer activities and light harvesting roles of chlorophylls *a* and *c*, and fucoxanthin. In addition, an excitation band was resolved at 487 nanometers which could be attributed only to antheraxanthin. Comparison of whole-cell fluorescence excitation spectra of *C. magna* with the diatom *Phaeodactylum tricornutum*, which possesses fucoxanthin but not antheraxanthin, supports the assignment of the 487 nm band to antheraxanthin. This is the first report of a photosynthetic light harvesting function of the xanthophyll, antheraxanthin. This carotenoid broadens the absorption cross-section for photosynthesis in *C. magna* and extends light harvesting into the green portion of the spectrum.

The role of accessory pigments in supporting photosynthetic O_2 evolution in algae was established by the work of Dutton and Manning (8) and Haxo and Blinks (15; see ref. 20). Subsequent studies by a number of investigators (reviewed in refs. 4 and 20) revealed that, in addition to Chl *b* and *c*, at least three xanthophyll carotenoids efficiently transfer absorbed light energy to Chl *a* to support photosynthesis. These include peridinin in dinoflagellates, fucoxanthin in diatoms and brown algae, and siphonaxanthin and siphonein in certain macrophytic green algae (4, 20). Other carotenoids present in thylakoids are not identified in photosynthetic action spectra (20, 26), nor *in vivo* fluorescence excitation spectra for Chl *a* emission (1, 12) and are thought to function primarily in photoprotection (7).

Photosynthetic accessory pigments function to broaden the absorption cross-section of Chl *a* and to allow for utilization of spectral regions that are poorly or not absorbed by Chl *a*. This latter role is of prime importance in aquatic species, as dissolved and particulate (living and nonliving) matter and water itself greatly influence the optical properties of water columns (16, 19). Even in clear, oligotrophic bodies of water, most of the red wavelengths are absorbed within the top few centimeters. Consequently, the Soret absorption bands of the Chl (400–440 nm) become far more important in driving photosynthesis than the red absorption region (650–690 nm). Further, dissolved substances in the water can very effectively filter the blue part of the spectrum, therefore leaving a 'green window' for light absorption. This effect is particularly pronounced at depth in the water column (19).

The optical properties of natural bodies of water probably provided the selective pressures for the evolution of light harvesting pigments, such as certain xanthophyll carotenoids and the phycobiliproteins, that can effectively utilize light energy between 450 and 630 nm. Indeed, in all but the Chlorophyta, pigments with significant or major absorption in the green portion of the spectrum are not only common but are often more abundant than Chl *a* (2, 11, 16, 23).

The present investigation sought to determine whether xanthophyll carotenoids other than those commonly known to function in light harvesting could be shown to be important in photosynthetic energy transfer. Using *in vivo* fluorescence excitation spectroscopy, pigment analyses, and *in vivo* absorption spectroscopy, we demonstrate the photosynthetic light-harvesting function of the xanthophyll, antheraxanthin, in the freshwater, coccoid chromophyte alga, *Chrysosphaera magna* Belcher. This carotenoid, in addition to fucoxanthin, Chl *a*, and Chl *c*, constitutes the light-harvesting system of this algal species.

MATERIALS AND METHODS

Axenic cultures of *Chyrsosphaera magna* were obtained from the British Culture Centre of Algae and Protozoa (No. 911/1), and were maintained in DY III medium (21) at 15°C under a 14/10, L/D light regime with light levels of about 150 to 200 μE m^{-2} s^{-1} PAR[2], provided by cool-white fluorescent lamps. *Phaeodactylum tricornutum* (UTEX 646) was grown as described by Friedman and Alberte (9) and was used for comparison. Cells were harvested by filtration (Whatman GF/F) or centrifugation for pigment extractions, while cells examined for *in vivo* spectral properties were diluted into fresh medium before analysis.

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[2] Abbreviations: PAR, photosynthetically active radiation; UTEX, University of Texas Culture Collection.

Pigments were extracted following the recommended precautions of Jensen (17). Cells were exhaustively extracted with ice-cold absolute methanol. The methanolic extracts were freed of insoluble material by centrifugation, and mixed first with an equal volume of peroxide-free diethyl ether and then with 10% (w/v) NaCl. The partitioned ether layer was concentrated under dry N_2 gas, and stored under N_2 in darkness at −20°C.

Pigment extracts were chromatographed on thin layer plates of cellulose (Eastman No. 13255), Silica Gel 60 (Brinkman No. SG60-5538/7), and Silica Gel G (Brinkman No. SIL G-100 U.V. 254), and on a C_{18} reverse-phase Porisil (IBM) HPLC column. Pigments were developed by TLC in 3:1 (v/v) light petroleum ether (b.p. 60–80°C):chloroform, 30 or 40% acetone in hexane and R_F values determined. Pigments obtained from TLC, whole extracts and standards were chromatographed by HPLC using a linear gradient of 70% (v/v) methanol:water (solvent A) and 100% ethyl acetate (solvent B) as described by Friedman and Alberte (9). The TLC zones were eluted with either neat diethyl ether or acetone for spectral examinations. Authentic, synthetic antheraxanthin (all-*trans*) was used as a standard. Relative pigment concentrations were obtained after TLC or HPLC using known extinction coefficients (13, 17, 27) or based on relative absorption at 450 nm.

Pigment absorption features were determined on either a Beckman model 34 or an Aminco DW-2 Dual Beam spectrophotometer. Whole cell absorption features were determined on the Aminco DW-2 using an end-on photomultiplier set-up with opal glass to reduce light scatter. Cell suspensions possessing A_{max} = 0.04 at 680 nm were used for *in vivo* spectrofluorometry to avoid self-absorption and fluorescence quenching. Energy-corrected excitation spectra and quantum-corrected emission spectra were obtained on an Aminco SPF-500 spectrofluorometer (10, 11). Emission spectra (1 nm bandpass) were obtained with excitation at 430 nm (± 10 nm) while excitation spectra (1 nm bandpass) were obtained for the 680 (± 5 nm) emission.

RESULTS

Pigment Composition. TLC of total pigment extracts of *C. magna* sp. revealed the presence of six pigmented zones. When the spectral properties and R_F values of these zones were compared to published values (17, 27), Chl *a* and *c*, and the carotenoids, β-carotene, fucoxanthin, and antheraxanthin, were identified. A trace of pheophytin *a* was also detected (< 0.1%). Antheraxanthin was resolved usually as two adjacent TLC zones with slightly different R_F values but identical spectral properties. Based on relative absorption at 450 nm, antheraxanthin accounted for about 8% of the total pigment, and for about 12 to 15% of the total carotenoid. Using extinction coefficients, antheraxanthin accounted for about 15% of the total carotenoid, fucoxanthin for about 35% and β-carotene for about 50%. The molar ratio of the carotenoids was antheraxanthin:fucoxanthin:β-carotene, 1:2.3:3.3.

Fucoxanthin identified from *C. magna* possessed the same absorption features and HPLC retention time as that purified from *Phaeodactylum tricornutum* (9). The relative amount of fucoxanthin in *C. magna* is lower than that typically found in chrysophytes (27) and the diatoms, *P. tricornutum* (9), and *Skeletonema costatum* (11), where it accounts for 60 to 80% of the total carotenoid.

Identification of antheraxanthin by R_F and absorption features was verified by cochromatography on TLC and HPLC with an authentic (all-*trans*) standard. The standard and TLC-isolated antheraxanthin had identical retention times (±0.04 min) on HPLC (Fig. 1). The R_F values of TLC-purified antheraxanthin in five different chromatography systems are compared to the authentic standard in Table I. Agreement between these data and absorption features of the TLC isolates (maxima at 424, 447, and 475 nm in 100% acetone; 424, 447, and 478 nm in diethyl ether; and 425, 448, and 475 nm in 100% ethanol) and the authentic standard demonstrates that the identification of antheraxanthin was reliable. The absorption features and R_F values obtained for isolated antheraxanthin are in full agreement with those of Withers *et al.* (27) for antheraxanthin from a single strain of the chrysophycean-like alga, *Olisthodiscus luteus* Carter.

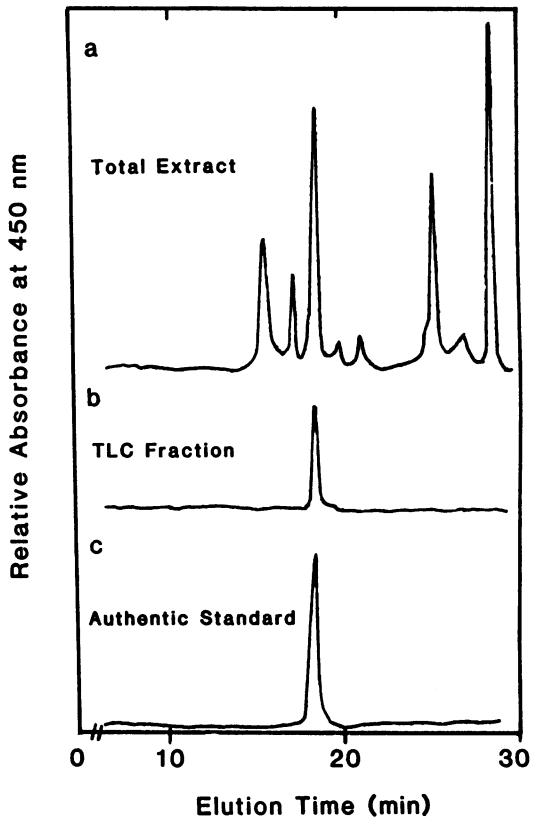

FIG. 1. Comparison of HPLC separations of whole-cell acetone extract of *Chrysosphaera magan* (panel a), antheraxanthin isolated by TLC (see "Materials and Methods") (panel b), and authentic antheraxanthin standard. The relative absorption at 450 nm is plotted versus retention time.

Table I. *Summary of R_F Values Obtained for Chrysosphaera magna Antheraxanthin and Authentic Antheraxanthin Standard (All Trans Isomer) in Different TLC Systems*

TLC System	Antheraxanthin R_F Value	
	Standard	*C. magna*
1. 3:1 Petroleum ether:chloroform (Cellulose)	0.37	0.37
2. 30% Acetone in hexane (Silica Gel 60)	0.33	0.33
	0.36[a]	0.36[a]
3. 40% Acetone in hexane (Silica Gel 60)	0.60	0.57
	0.65[a]	0.60[a]
		0.65[a]
4. 30% Acetone in hexane (Silica Gel G)	0.33	0.27
		0.33[a]
5. 40% Acetone in hexane (Silica Gel G)	0.61	0.64
	0.68	0.71

[a] Trace.

***In Vivo* and Fluorescence Features.** The whole-cell absorption spectrum of *C. magna* (Fig. 2) revealed bands due to Chl *a* (435 and 680 nm) and Chl *c* (465 and 635 nm) in the Soret and red spectral regions. The shoulder around 480 to 490 nm was attrib-

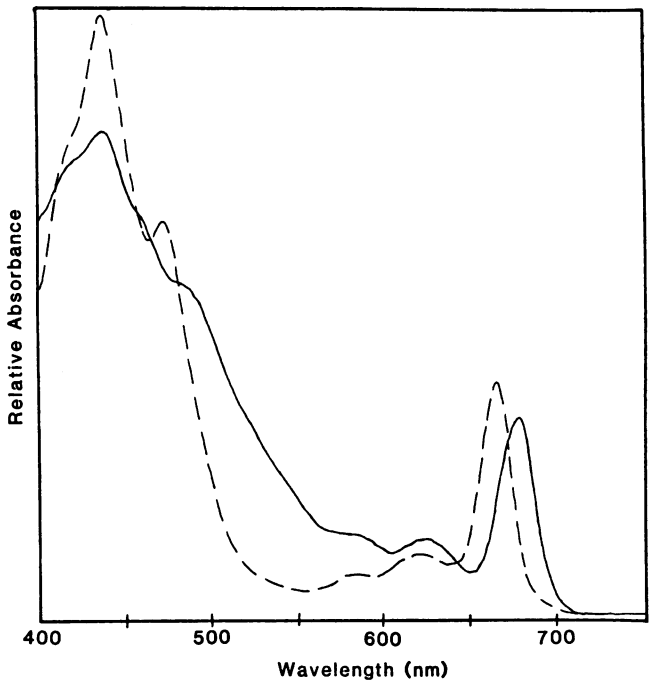

FIG. 2. *In vivo* room temperature absorption spectrum of *Chrsysophaera magna* (A_{max} = 0.04 at 680 nm) (——) compared to the 80% (v/v) acetone extract of whole cells (- - - - -). In addition to the blue-shift of all absorption maxima seen in the *in vivo* spectrum, there is a dramatic loss in absorption between 500 and 600 nm in the acetone extract.

uted to both antheraxanthin and fucoxanthin, and the broad absorption region between 500 to 550 nm was principally due to fucoxanthin (8, 9–12, 24, 25). It is likely that antheraxanthin also contributes absorption in this spectral region (see below). The broad absorption between 470 and 520 nm seen in the *in vivo* spectrum was lost when the cells were extracted into hydrocarbon (Fig. 2). A 'blue-shift' in absorption is common for most pigments when they are extracted from their native membrane environment, but especially dramatic in the case of xanthophylls (7, 26).

Whole-cell fluorescence emission spectra of *C. magna* when excited across the Soret region (430 ± 10 nm), revealed a dominant peak at 681 nm with a small contribution of 725 nm emission (Fig. 3). No fluorescence due to Chl *c* (650 nm) was observed (10). When whole cells were excited at 487 (± 5) nm (see below), there was an increase in the 681 nm fluorescence (Fig. 3).

The fluorescence excitation spectrum for the 680 nm emission of *C. magna* is shown in Figure 4 where it is compared with *P. tricornutum* which possesses fucoxanthin, but not antheraxanthin (13). When the fluorescence excitation spectra for Chl *a* emission were normalized to equal Chl *a* concentrations (Fig. 4), three major distinctions could be made between *C. magna* and *P. tricornutum*. First, the contribution of Chl *c* excitation (465–470 nm) to Chl *a* emission appeared greater in the diatom than the chromophyte. In fact, *C. magna* has only a slightly higher Chl *a*:*c* ratio (5.2 ± 0.3) than *P. tricornutum*. (Chl *a*:*c* = 4.3 ± 0.2). Increased excitation in the 460 to 470 nm spectral region in *P. tricornutum* is likely due to contributions of short wavelength *in vivo* absorption bands of fucoxanthin; this carotenoid accounts for 60 to 80% of the total carotenoid in this diatom (9). Second, an excitation peak at 487 nm is present in *C. magna* and absent from *P. tricornutum*. This peak is attributed to the *in vivo* state of antheraxanthin, as it cannot be accounted for by fucoxanthin because this peak is absent from *P. tricornutum*. Preferential excitation of the 487 nm peak increased the relative

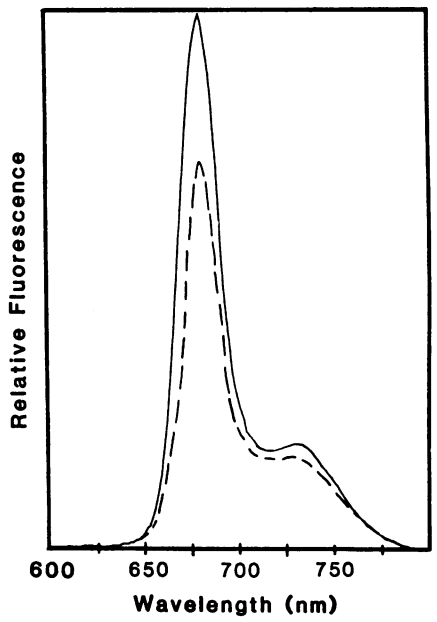

FIG. 3. Corrected room temperature fluorescence emission spectra (± 1 nm bandpass) of whole cells of *Chrysosphaera magna* resulting from excitation at 487 nm (±5 nm, bandpass) (——) compared with excitation at 430 nm (±10 nm, bandpass) (- - - - -). Emission maximum = 681 nm.

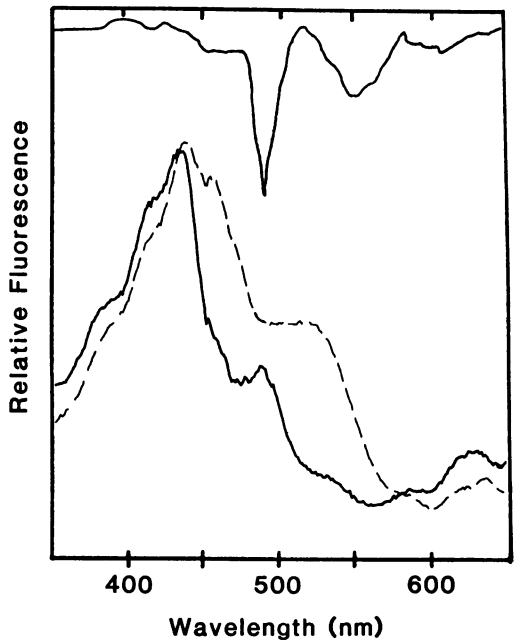

FIG. 4. Corrected, room temperature whole-cell fluorescence excitation spectra of *Chrysosphaera magna* (——) and the diatom *Phaeodactylum tricornutum* (- - - - -). Excitation spectra (±1 nm, bandpass) were obtained for 680 nm (±5 nm, bandpass) and samples of the two species were normalized to equal Chl concentration (A_{max} = 0.04 at 680 nm). The fluorescence excitation difference spectrum for the corrected excitation spectra shows a large difference at 487 nm, attributed to antheraxanthin.

yield of Chl *a* emission (Fig. 3). Third, the dominant broad excitation between 490 and 540 nm seen in *P. tricornutum*, ascribed to fucoxanthin (8–11), was not apparent in *C. magna*. The shoulder around 530 nm observed in the excitation sepctrum of *C. magna* was due to fucoxanthin. The fluorescence excitation difference spectrum of *P. tricornutum* and *C. magna* (Fig. 4) confirms the features attributable to Chl *c* and fucoxanthin

excitation, and shows a major deflection at 487 nm corresponding to the *in vivo* absorption band of antheraxanthin. Collectively, these data demonstrate that Chl *a*, Chl *c*, fucoxanthin, and antheraxanthin are coupled in energy transfer to Chl *a* in *C. magna*.

DISCUSSION

The genus *Chrysosphaera* was expanded and emended by Bourrelly (6) when he synonymized the genera *Epichrysis* and *Chrysobotrys* with *Chrysosphaera*. In many respects, the genus *Chrysosphaera* is atypical of the Chrysophyceae in that it possesses a distinct cell wall and a unique sporangia-like stage where autospores or zoospores are produced. Finally, a statospore is not known; this feature is diagnostic for the class and is known for most members.

Belcher (5) described *C. magna*, and demonstrated that this species was somewhat different from other members of the genus, particularly in that the sporangia produce up to 256 cells rather than 4 or 8 typical of the other species. This coccoid, freshwater unicell (10–15 μm in diameter) releases large numbers of biflagellated zoospores from sporangia (5). Cells contain a parietal chloroplast which can occupy over half of the cell volume.

Other freshwater and marine chrysophytes that have been examined possess Chl *a*, Chl c_1 and c_2 (3), β-carotene, and fucoxanthin as the major carotenoids (27). The present study demonstrates that *C. magna* possesses the typical major chrysosphycean pigments and antheraxanthin. In addition, traces (0.1% of the total) of phaeophytin were detected, though it is not possible to ascertain at this time whether this pigment is present *in vivo* or resulted from hydrocarbon extraction.

In the algae, a diversity of pigments function to harvest light for photosynthesis unlike terrestrial plants (16). The algal pigments include Chl *c*, peridinin, fucoxanthin, siphonaxanthin, and the phycobiliproteins (4, 7, 16, 20). We sought to determine whether antheraxanthin found in *C. magna* serves a light harvesting role in this alga. A short report by Haxo *et al.* (15) suggested that the xanthophyll carotenoid, diadinaxanthin, may function in light harvesting in the chloromonad alga, *Vacuolaria virescens*. They show significant contributions to fluorescence excitation spectra and photosynthetic action spectra at 494 nm which cannot be attributed to fucoxanthin because *V. virescens* lacks this carotenoid.

Other investigations (1, 9–11, 23, 24) have demonstrated the value of fluorescence excitation spectra in identifying pigments that transfer their absorbed energy to Chl *a* to support photosynthesis; fluorescence excitation data have been shown to confirm and mimic photosynthetic action spectra (1, 14, 18, 20). The use of corrected fluorescence excitation spectroscopy on whole cells of *C. magna* has revealed that the major excitation band at 487 nm is characteristic of these cells, and can be attributed to antheraxanthin. The pigment-protein organization of this carotenoid remains to be examined.

Since the *in vivo* absorption features of carotenoids, in general, is poorly understood or known, we have used three lines of evidence to make the assignment of antheraxanthin as a light-harvesting carotenoid in *C. magna*. First, the only two major xanthophylls identified in *C. magna* by HPLC and TLC of hydrocarbon extracts were fucoxanthin and antheraxanthin, and the fucoxanthin content of *C. magna* was reduced compared to other chromophyte algae. Second when fluorescence excitation features of *C. magna* were compared with the diatom *P. tricornutum* which lacks antheraxanthin (13), a major difference was resolved at 487 nm. Third, the 487 nm band in the fluorescence excitation spectrum for Chl *a* emission was a dominant component and when excited preferentially, the relative yield of Chl *a* emission was enhanced. Since the bulk of 680 nm fluorescence arises from PSII, it could be argued that antheraxanthin transfers energy predominantly to this photosystem (20).

The data presented support the assignment of antheraxanthin as a light harvesting carotenoid in the chromophyte alga, *C. magna*. This pigment extends light harvesting into the green portion of the spectrum and broadens the absorption cross-section for this alga (Figs. 2 and 4). This feature should not be taken as characteristic of the class or the genus; a preliminary survey of several species of Chrysophyceae has not revealed fluorescence excitation contributions in the 485 to 495 nm spectral region which can be attributed to antheraxanthin (RS Alberte, RA Andersen, unpublished data). The documented occurrence of antheraxanthin in *Poterioochromonas stipulata* (13) and one strain of the chrysophyte-like alga *Olisthodiscus luteus* (27) may deserve examination. However, since the reported levels of antheraxanthin in these algae are very small ($\leq$ 1%; ref. 27), it is not likely that antheraxanthin would make a significant contribution to photosynthetic light harvesting.

Corrected fluorescence excitation spectra of algae may provide diagnostic features of value in taxonomic distinctions and in assessment of species composition of natural populations (1, 11, 22). Though these spectroscopic techniques have yet to be fully exploited, they have been shown to provide valuable information on photosynthetic potential, growth rate, and physiological state of microalgae in culture (1, 2, 11).

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