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Chlorophyll *d* in Rhodophyceae: Presence and function

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Introduction

Photosynthetic pigments are important to capture solar energy, thus sometimes they will be critical for survival or competition with other organisms in one specific habitat. Distribution of pigments is also an index of taxonomy of photosynthetic organisms. Chlorophyll (Chl) *a* is a common pigment in all oxygenic photosynthetic organisms, except for *Prochlorococcus marinus* that possesses divinyl Chl *a*. Chl *b* is present in green algae and land plants, and the photosynthetic prokaryotes, *Prochloron* and *Prochlorothrix*. Chl *d* was for the first time reported to be an antenna pigment in marine red alga, *Erythrophyllum delesseriodes* in 1943 by Manning and Strain, however it was discarded from an antenna pigment because Chl *d* was reported to be a degradation product during an isolation process of Chl's (Holt and Morley 1959), and a content of Chl *d* was not constant and usually very low (Holt 1961). Chl *d* has been found in cell extracts in several species of red seaweed (Sagromsky 1958, 1960, 1964, Estigneev and Cherkashina 1969). Recently, Chl *d* was identified in the photosynthetic prokaryote, *Acaryochloris marina* (Miyashita *et al.* 1996), where Chl *d* is a major antenna pigment and also a constituent of the primary electron donor in photosystem (PS) I (Hu *et al*. 1998). This finding leads us to hypothesize that Chl *d* is a real component in red algae, and it may function as an antenna pigment. Thus we have investigated the presence of uncommon photosynthetic pigments in red algae and found that Chl *d* is present in the extract from marine red algae. However, it was suggested that Chl *d* is not a real component of red algae but comes from photosynthetic epiphyte(s) that attaches to the surface of thalli of the red algae.

Materials and Methods

Red seaweed *Ahnfeltiopsis flabelliformis* (Harvey) Masuda was collected in the rocky intertidal seashore, Awaji Island, Hyogo prefecture, Japan. Thalli were washed several times with filtrated seawater, and used for measurements.

Absorption and fluorescence spectra were measured with a Hitachi 557 dual wavelength spectrophotometer and a Hitachi F-4500 spectrofluorometer, respectively. For the low temperature fluorescence spectra, a custom-made Dewar bottle and its holder were introduced.

Pigments were extracted with methanol and analyzed by HPLC using reverse phase column (Nova-Pack C18 column, Waters, USA) equipped with a photodiode array detector (MD-915, JASCO, Japan). Chl *d* was purified by the method

reported previously (Omata and Murata 1983) after a minor modification. The nuclear magnetic resonance (NMR) spectrum was measured with a Varian Unity 500 NMR spectrometer (500 MHz) as described previously (Miyashita *et al*. 1997). The fast atom bombardment mass spectroscopy (FABMS) was measured with a JEOL JMS-SX102 mass spectrometer (Miyashita *et al*. 1997).

Micro-spectrophotometery was performed with use of an optical system developed with a high sensitivity spectro-multichannel-photodetector (MCPD-7000, Otsuka Electronics, Japan) under an epifluorescence microscope (E600, Nikon, Japan). An area for measurement of the absorption and fluorescence spectra was limited to 10 μm in a diameter with the optical fiber.

Ultrastructure of the organism was observed with a transmission electron microscope (JEM-1010D, JEOL, Japan). Thalli were fixed with 2% glutaraldehyde and postfixed in 2% osmium tetroxide. The samples were dehydrated through a graded acetone series, followed by three changes in 100% acetone, and embedded in Spurr's epoxy resin. The samples were cut with a ultramicrotome (ULTRACUT D, Reichert-Jung, Australia). Ultrathin sections were stained with uranyl acetate and lead citrate.

Results

1. Presence of Chl *d* in red algae

Red algal Chl *d* was detected by an absorption spectrum of whole thalli; the peak was located at approximately 680 nm but in the wavelength region longer than 700 nm, a tail was prolonged to 720 nm, suggesting the presence of a component in addition to Chl *a*. Presence of Chl *d* was much easily detected by fluorescence spectra of thalli at –196°C. By excitation at 440 nm, a typical PS I Chl *a* fluorescence was observed at 715 nm. This was a little shorter than that of higher plants, but it was usual properties of red algae. By excitation at 460 nm, two new peaks were detected at 738 and 748 nm. These most probably came from Chl *d*. At room temperature, one new band was detected at 728 nm. When pigments were extracted with methanol and an absorption spectrum was measured, a clear shoulder was detected at 697 nm. This was consistent with the peak of Chl *d* isolated from *A. marina* (Mimuro et al. 1999). Chl *d* was also confirmed by fluorescence spectrum. When acetone extracts were excited at 435 nm, Chl *a* emission was detected at 670 nm, and when excited at 455 nm, the main peak appeared at 712 nm in addition to 670 nm. The peak locations were sensitive to solvents for both absorption and fluorescence spectra as in the case of other Chl's. The peak location of Chl *d* was red-shifted in cells, thus it is suggested that Chl *d* was not in the free form, but was protein-bound.

2. Identification of Chl *d*

Chl *d* in the extracts from red algae was identified by several methods. First, it was analyzed by HPLC; its retention time and absorption spectrum measured with the multichannel photodiode array detector were identical to those of Chl *d* purified from *A. marina*. The molecular structure of Chl *d* in the algal extracts was analyzed by the NMR spectroscopy. Chl *d* in the algal extracts was purified by dioxane-precipitation and two kinds of column chromatography, and its molecular structure was shown to be identical to that of Chl *d* of *A. marina* (Miyashita et al. 1997). Based on the absorption, fluorescence, NMR spectroscopy and HPLC analyses, the presence of Chl *d* in the extracts from red algae was proved. This is a re-discovery of Chl *d* in red algae originally reported more than 50 years ago (Manning and Strain 1943).

3. Localization of Chl *d*

A content of Chl *d* was not constant: we collected many samples from the seashore and estimated Chl *d* content by absorption spectra on acetone extracts. It varied sample to sample. A Chl *d*/*a* ratio varied from 0.03 to 0.3, more than 10 times difference. Sometimes, no Chl *d* was detected in *A. flabelliformis*, even if samples were collected at the same habitat. Thus, we estimated the Chl *d* content of thalli by separating into two parts, tip and bottom. In many cases, the Chl *d* content was higher in the bottom than in the tip. This seemed to be crucial for the function; if Chl *d* is a component of antenna, it is to be synthesized in a certain amount under every condition. However, it was not necessarily the case. This evoked a new approach to the presence of Chl *d*.

4. Confirmation of localization

We noticed that there were several small patches in a dark color on the surface of thalli of *A. flabelliformis* (Fig. 1), and a frequency of its appearance was not constant. By a light microscope, a patch seemed a cluster of organisms of green in color and attached to the surface. The image of the fluorescent microscope of patches was uniformly distributed red in color, on the contrary, thalli showed orange fluorescence coming from phycoerythrin and fluorescence was not distributed uniformly, which indicate the presence of chloroplasts in cells. Based on these results, the organism in patches did not seem a part of thalli, thus was assigned to an epiphyte. For further analyses, isolation of an epiphyte was necessary, however it was very hard to isolate, thus we performed the optical fiber spectroscopy under the light microscope.

At first, we measured the fluorescence spectrum of the epiphyte in longitudinal section of thalli (Fig. 2a). The diameter of the monitoring beam was less than 10 μm; it was shorter than the thickness of thalli and size of the patches, thus it was easy to discriminate the observation area, thalli and an epiphyte. The excitation wavelengths were limited to bright lines of a mercury lamp and optical filters, that is, 400 - 440 nm and 450 - 490 nm, thus Chl *a* was mainly excited under our measuring conditions. Under this condition, two fluorescence peaks were detected at 686 and 717 nm (Fig. 2a). Since the Chl *a* fluorescence was located at 686 nm, the latter was spectrally distinct, most probably coming from Chl *d*. Measurements were repeated on several different patches, and all spectral data indicated the presence of Chl *d* (Fig. 2a). As a reference, micro-spectrophotometry was applied to the thalli without an epiphyte. The fluorescence with the maximum at 717 nm was hardly detected. Sometimes it was observed, but its intensity was very low, indicating that Chl *d* was not present in the thalli.

To confirm the presence of Chl *d* in the epiphyte, an absorption spectrum was measured under the microscope (Fig. 2b). The absorption maximum of Chl *a* was located at 680 nm, and in the longer wavelength region than the maximum, there was a tail up to 710 nm. However, it was not clear whether Chl *d* was present in the epiphyte. When absorption spectrum was measured on the thalli without an epiphyte, there was almost no tail in the longer wavelength region than the maximum. The difference in the absorption spectrum suggests the presence of Chl *d* in the epiphyte.

As the second step of analyses, a part of thalli including the epiphyte were isolated manually under a stereoscopic microscope and subjected to measurements. Another part of thalli not including the epiphyte was also isolated and used as a reference; a purity was higher than 80% however separation was not complete. Fluorescence spectrum is much sensitive to detect Chl *d*, thus was applied to methanol-extracts from two parts. We found that the pigment content was clearly different; the major pigment of the epiphyte was, of course, Chl *a*, however there

was a clear fluorescence from Chl *d*. On the other hand, methanol-extract of thalli did not show the presence of Chl *d*. The same results were obtained by the absorption spectrum, even though difference was small.

5. Morphology of the epiphyte

In order to survey the epiphytic organism of *A. flabelliformis*, the transmission electron microscopy was applied. Electron micrographs showed that the epiphytic organism was cluster of large prokaryotic cells packed with many thylakoid membranes. The size of this unicellular organism was 20 - 45 μm in a long axis and 10 -15 μm in a short axis.

6. Function of Chl *d*

In the case of *A. marina*, Chl *d* is the major pigment and used for both antenna and primary electron donor in PS I (Hu *et al*. 1998). On the contrary, Chl *d* is not the major pigment in this case, thus the function of Chl *d* is determined by a content and energy levels in cells (or in the complex). By the time-resolved spectroscopy on the epiphyte, a long-lived fluorescence component was not detected in the wavelength region of Chl *d*, thus Chl *d* is not the primary electron donor of PS II. A long-lived component was observed at approximately 685 nm coming from Chl *a*, as in the case of other organisms (Arnold 1991).

The steady-state fluorescence spectra of the epiphyte showed the Chl *d* fluorescence when Chl *a* was excited. Since Chl *d* was a minor component, direct absorption by Chl *d* was limited to a small fraction. Therefore energy transfer from Chl *a* to Chl *d* was suggested. The energy level of Chl *d* was lower than that of Chl *a*, thus Chl *d* might be an energy sink. However as shown on *A. marina*, up-hill energy transfer from Chl *d* to Chl *a* is also expected (Mimuro *et al.* 2000). In this sense, Chl *d* might be an energy reservoir in the antenna system.

Discussion

1. An actual organism that contains Chl *d*

Presence of Chl *d* in red algae was reported many times for the last 50 years (Manning and Strain 1943, Sagromsky 1958, 1960, 1964, Holt 1961, Estigneev and Cherkashina 1969), however those reports are sometimes inconsistent or contradictory. Chl *d* has been reported to an artificial product in the course of Chl isolation (Holt and Morley 1959). These lead to the doubt of the presence of Chl *d* in red algae. In the course of our analyses, we thought that Chl *d* was a component of red algae, however it was not the case. Light and electron microscopy and spectral analyses indicate that a prokaryotic epiphyte of the red alga contains a significant amount of Chl *d*. Even if we cannot completely exclude that red alga *A. flabelliformis* itself contains Chl *d*, however, it is more probable that cells attaching outside of red algae contain Chl *d*. The epiphyte seems cyanobacteria-like on the basis of image of fluorescence microscope; there was no clear localization of red fluorescent spot. Electron micrograph also suggests that the organism is prokaryote, even though it has not yet been identified. This is a striking result. If this is the case, distribution of Chl *d* might be limited to prokaryotes at the moment.

2. Solution of ambiguous description on the presence of Chl *d*

After the discovery of Chl *d* in the red seaweed *E. delesseriodes* in 1943 by Manning and Strain, Chl *d* was found in extracts of whole cells of the several species of red algae. However, distribution of Chl *d* was limited to marine macroalgae (Strain 1958, Lev 1999). In most cases, a relative content of Chl *d* to Chl *a* was extremely low.

however only in one case, the maximum amount of Chl *d* was reached about 30% of Chl *a* (Strain 1958). A content of Chl *d* was variable even in the same species; we observed this phenomena on *A. flabelliformis*, even though collected in the same habitat and at the same time. These ambiguous or inconsistent descriptions will be solved when Chl *d* is not a real component of red algae, but the epiphyte of the red algae contains Chl *d*. At the moment, we are not sure whether the epiphyte is only one species or plural species that contain Chl *d*, however it will be solved soon.

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References

Arnold WA (1991) *Photosynthe Res* **27**, 73-82.
Estigneev VB and Cherkashina NA (1969) *Biokhimiya* **35**, 48-52.
Holt AS and Morley HV (1959) *Can J Chem* **37**, 507-514.
Holt AS (1961) *Can J Botany* **39**, 327-331.
Hu Q, Miyashita H, Iwasaki I, Kurano N, Miyachi S, Iwaki M and Itoh S (1998) *Proc Natl Acad Sci USA* **95**, 13319-13323.
Ley RE (1999) *Phycology*, (3rd ed.), Cambridge University Press, Cambridge, UK.
Manning MM and Strain HH (1943) *J Biol Chem* **151**, 1-19.
Mimuro M, Akimoto S, Yamazaki I, Miyashita M and Miyachi S (1999) *Biochim Biophys Acta **1412***, 37-46.
Mimuro M, Hirayama K, Uezono K, Miyashita H and Miyachi S (2000) *Biochim Biophys Acta* **1456**, 27-34.
Miyashita H, Ikemoto H, Kurano N, Adachi K, Chihara M and Miyachi M (1996) *Nature* **383**, 402.
Miyashita H, Adachi K, Kurano N, Ikemoto H, Chihara M and Miyachi S (1997) *Plant Cell Physiol* **38**, 274-281.
Omata T and Murata N (1983) *Plant Cell Physiol* **24**, 1093-1100.
Sagromsky H (1958) *Ber Det Botan Ges* **71**, 435-438 .
Sagromsky H (1960) *Ber Det Botan Ges* **73**, 358-362.
Sagromsky H (1964) *Ber Det Botan Ges* **77**, 323-326.
Strain HH (1958) *Chloroplast pigments and chromatographic analysis*. 32nd Annual Priestley Lecture. Penn State Univ. Press. USA.

Fig. 1. Micrograph of thin section of *Ahnfeltiopsis flabelliformis* thalli. An epiphyte was indicated by an arrow. A horizontal full length is 650 μm.

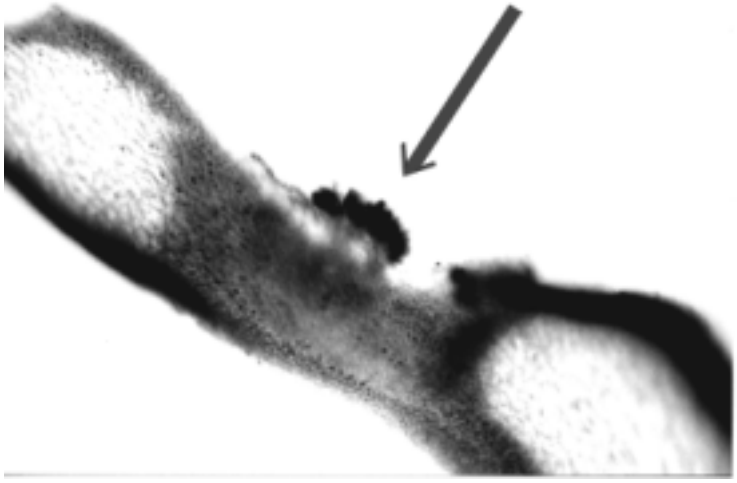

Fig. 2. Fluorescence (a) and absorption (b) spectra of the epiphyte attached on the red alga *Ahnfeltiopsis flabelliformis* measured with microspectrophotometry at room temperature.