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Immobility of phycobilins in the thylakoid lumen of a cryptophyte suggests that protein diffusion in the lumen is very restricted

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ABSTRACT

The thylakoid lumen is an important photosynthetic compartment which is the site of key steps in photosynthetic electron transport. The fluidity of the lumen could be a major constraint on photosynthetic electron transport rates. We used Fluorescence Recovery After Photobleaching in cells of the cryptophyte alga *Rhodomonas salina* to probe the diffusion of phycoerythrin in the lumen and chlorophyll complexes in the thylakoid membrane. In neither case was there any detectable diffusion over a timescale of several minutes. This indicates very restricted phycoerythrin mobility. This may be a general feature of protein diffusion in the thylakoid lumen.

1. Introduction

The chloroplast thylakoid lumen acts as a reservoir of protons for photosynthetic ATP synthesis. It also contains various proteins (including chaperones [1] and proteases [2]) and it is the site of photosynthetic electron transport between the cytochrome b_6f complex and Photosystem I, mediated by plastocyanin (PC).

PC is believed to transport electrons by lateral diffusion within the thylakoid lumen [3]. To date, there is very little direct information on the thylakoid lumen dynamics or fluidity (see e.g. [4] for review). For the opposite, stromal side of thylakoid membrane the high mobility of light-harvesting phycobiliproteins in cyanobacteria has been already demonstrated using the fluorescence recovery after photobleaching method (FRAP) [5–7]. Such an analysis of thylakoid lumen dynamics is still lacking as in most organisms there are no naturally fluorescent pigment–proteins in the lumen (see e.g. [4] for recent review). The introduction of a fluorescent marker protein such as Green Fluorescent Protein (GFP) into the thylakoid lumen might allow calculation of the effective viscosity from FRAP measurements [8] but an attempt to use the TAT system to import GFP into the lumen in a cyanobacterium resulted only in GFP accumulation in the periplasm [9]. In *Arabidopsis*, GFP was successfully imported into the lumen using the TAT system, however it could not be visualised in continuously illuminated chloroplasts, perhaps due to destabilisation at low pH [10].

Cryptophytes are taxonomically-distinct flagellate unicellular eukaryotic algae that contain highly-fluorescent phycobiliproteins in the thylakoid lumen (see e.g. [11]). These organisms have a specific composition of light-harvesting complexes that consist of membrane-integral chlorophyll *a,c*-binding proteins and lumenal proteins homologous to red algal phycobiliproteins [12]. The two-lobed chloroplast of *Rhodomonas salina* (sometimes called *Pyrenomonas salina)* contains thylakoid membrane pairs which extend over several microns roughly parallel to the long axis of the cell [13]. This layout of the thylakoid membranes is favourable for FRAP measurements, so we were able to use FRAP to study the phycoerythrin mobility in the thylakoid lumen of *Rhodomonas salina.* We could detect no diffusion of phycoerythrin in the lumenal space, suggesting a rather rigid structure of the lumen in *Rhodomonas.* We suggest that very restricted protein diffusion may be a general feature of the thylakoid lumen in photosynthetic organisms.

Abbreviation: Chl *a* (*c*), chlorophyll *a* (chlorophyll *c*); FRAP, fluorescence recovery after photobleaching; PBS, phycobilisome; PC, plastocyanin; PE, phycoerythrobilin.

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2. Materials and methods

2.1. Cell growth and sample preparation

The cryptophyte alga *Rhodomonas salina* (CCAP 978/27 strain) was obtained from the CCAP collection (Culture Collection of Protozoa, Argyll, Scotland, UK). Cells were grown in artificial seawater medium with f/2 nutrient addition [14]. Cells were cultivated in batch cultures in an illuminated orbital shaking incubator at 20 °C and 20 μmol quanta m^{-2} s^{-1}.

2.2. Fluorescence spectra

Room temperature fluorescence emission spectra of cell suspensions were measured using the LS50 Luminescence Spectrometer (Perkin Elmer, USA). The chlorophyll *a* emission was excited at 435 nm and the phycoerythrobilins at 550 nm. Excitation and emission slit-widths were set to 3 nm and 5 nm, respectively.

2.3. Absorbance measurements

Absorption spectra were recorded with the Unicam UV 550 spectrometer (Thermo Spectronic, UK) in an integrating sphere with 4 nm detection bandwidth. Cells were collected on membrane filters (pore diameter 0.6 μm), which were placed in the integrating sphere.

2.4. Preparation of cells for FRAP measurements

To immobilize cells for the FRAP measurement, cell suspensions were exposed for 5 min to medium buffered at pH 4.5 to disassemble the flagella [15]. The deflagellated cells were then mixed with low gelling temperature agarose (3% 2-Hydroxyethyl agarose, type VII) that was kept at 30 °C in a liquid form before mixing. The agarose with cells was spotted onto a microscope slide, covered with a glass coverslip and then used immediately for FRAP measurements at room temperature.

2.5. FRAP measurements and data analysis

FRAP measurements were carried out with a laser-scanning confocal microscope (Nikon PCM2000) equipped with a 100 mW Ar laser (a 488 nm line was used) and with a 60× oil immersion lens (numerical aperture 1.4). A 20 μm confocal pinhole gave a resolution of about 1.3 μm in the *Z*-direction (full width at half-maximum of the point-spread function). A dichroic mirror transmitting above 565 nm separated excitation and fluorescence light. Chlorophyll fluorescence was selected with a Schott RG665 red glass filter (transmitting wavelengths longer than about 665 nm) and phycoerythrin fluorescence was selected with an interference band-pass filter (590–618 nm). Fluorescence signals were detected simultaneously in two channels.

The pre-bleach images were recorded with laser power decreased to 3%. The bleach line across the cell was induced with full laser power (for about 4 s) by switching the confocal microscope to X-scanning. Post-bleach images were recorded typically every 30 s for 6 min with (laser power of 3%). The series of FRAP images were analyzed by public domain Java image processing program ImageJ 1.32b (see e.g. [16]). A one-dimensional bleaching profile was obtained by integration across the cell in the *X*-direction [5]. The baseline fluorescence from the unbleached cell was subtracted.

3. Results

3.1. Specific detection of chlorophyll a and phycoerythrobilin fluorescence

Fig. 1 shows fluorescence emission and absorption spectra for a suspension of *Rhodomonas salina* cells. As expected, the absorption spectrum (Fig. 1B) shows major peaks for chlorophyll *a* (435 nm and 676 nm), phycoerythrin (~550 nm) and Chl c_2 (~470 nm) and alloxanthin (~496 nm). When fluorescence emission is excited at 435 nm, the main fluorescence emission observed is from Chl *a* at 685 nm (Fig. 1A). Excitation at 550 nm results in two main fluorescence peaks, from phycoerythrobilin (PE) at 591 nm and from Chl *a* at 685 nm (Fig. 1B). Chl *a* is probably excited mainly by the efficient energy transfer from PE [17,18]. Excitation at wavelength used for confocal microscopy (488 nm) results in both PE and Chl *a* fluorescence emission (Fig. 1A). The presence of the PE peak indicates that there is significant excitation of PE with 488 nm excitation. Chlorophyll c_2 emission is not observed (Fig. 1A) because of the efficient energy transfer from chlorophyll c_2 to reaction centers.

For confocal microscopy and FRAP we combined excitation at 488 nm with simultaneous fluorescence detection in a two-channel detector at 590–618 nm and >665 nm. This allows us to visual-

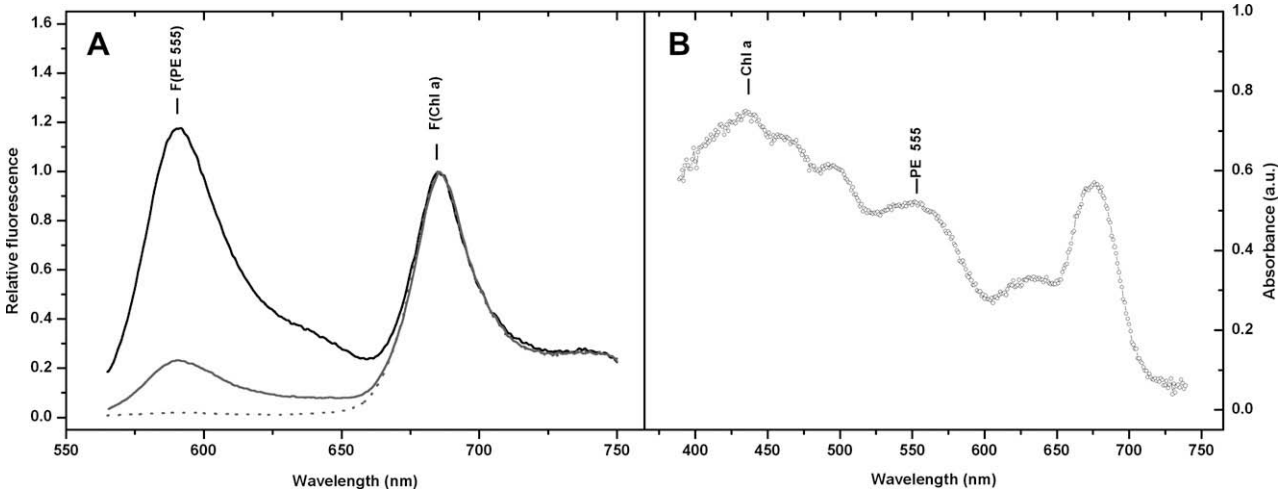

Fig. 1. Fluorescence emission spectra (Panel A) and absorption spectra (Panel B) of *Rhodomonas salina* cells. Fluorescence (Panel A) was excited at the absorbance maximum of phycoerythrobilin (550 nm – black line), at the absorbance maximum of chlorophyll *a* (435 nm – dotted line) and at the excitation wavelength used for FRAP measurements (488 nm – gray line). Fluorescence spectra were normalized to the 685 nm chlorophyll *a* fluorescence peak. In the absorption spectrum (Panel B) the main absorption maxima of chlorophyll *a* (~435 nm) and phycoerythrobilin (~555 nm) are indicated.