Supplemental Information

Controllable phycobilin modification: an alternative photoacclimation response in cryptophyte algae

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Experimental Methods

Species and Pigment Information

*Hemiselmis pacifica* and *Proteomonas sulcata* were obtained from NCMA Bigelow National Laboratory. *H. pacifica* produces phycocyanin 577 (PC577) as its phycobiliprotein, which has a phycocyanobilin (PCB) bound to its α subunits, along with two PCBs and one 15,16-dihydrobiliverdin (DBV) on its β subunits.46 *P. sulcata* produces phycoerythrin 545 (PE545) as its phycobiliprotein, which contains three phycoerythroblins (PEB) on its β subunits and one DBV on its α subunits.47

Algae growth

All algae strains were grown under 12 hour light/12 hour dark cycle (light intensity ~5 umol m^-2 s^-1) at a constant temperature in ventilated culture flasks. *H. pacifica* was grown in Prov50 media (NCMA) inside an incubator at 15°C. *P. sulcata* was grown in L1 media (NCMA) at room temperature. Each species was recultured every two weeks by pipetting 10 mL of *H. pacifica* (5 mL of *P. sulcata*) into a fresh 75 mL of their respective media. Cellular concentration was monitored throughout growth by taking daily cell counts using a Neubauer Improved Hemocytometer (BulldogBio). Evan’s Blue dye was used to differentiate live and dead cells.

Restricted Light Growth Conditions

The restricted light conditions for *H. pacifica* and *P. sulcata* were produced by housing the culture flasks in covered cylinders constructed from JAS Green (738) and Jade (323) Lee Filters, respectively. Each cylinders had a top and bottom also made from the filter material to ensure that the cultures were completely isolated from white light. Restricted light cultures were grown in triplicate to confirm reproducibility. We matched the overall intensity of light for both white light and restricted light cultures to avoid alternative photoacclimation processes caused by restrictions in photosynthetically active light. In *H. pacifica*, we placed the white light cultures inside neutral density filter canisters in order to match the light intensity inside the green filter canisters. In *P. sulcata*, we placed the light restricted cultures closer to the light source until the light intensity was appropriately matched to the white light cultures.

Phycobiliprotein Harvesting and Purification

Following growth to saturation, algae were collected by centrifugation at 1000 x g and 10°C for 2 minutes. The supernatant was poured off and replaced with 5 mL of 100 mM phosphate buffer (pH 7.2 for PC577, pH 6.6 for PE545). The algae were then resuspended and frozen at -20°C for at least one day. To extract the proteins, the frozen resuspended cultures were allowed to thaw at room temperature in the dark for 1 hour. Following thawing, each culture was centrifuged at 5000 x g for 5 minutes. The supernatant containing the fluorescent proteins was collected into beakers, and ammonium sulfate was added for a final concentration of 0.23 g/mL. After the ammonium sulfate had dissolved, the beakers were covered tightly with Parafilm to prevent
evaporation and placed at 4°C overnight. The next day, the protein solutions were centrifuged at 6000 x g for 6 minutes and the supernatant syringe filtered (45 um) to remove any residual cell debris. Ammonium sulfate was added to raise the concentration another 0.1 g/mL, and the solution were placed at 4°C overnight. The next day another 0.2 g/mL of ammonium sulfate was added to the protein solutions and they were placed 4°C for at least 2 days. The precipitated phycobiliproteins were then collected using ultracentrifugation at 35000 rpm for 25 minutes (Beckman Coulter, Ti-60 rotor). The supernatants were carefully poured off and each pellet was resuspended in 1 mL of 100 mM phosphate buffer (7.2 pH, PC577 or 6.6 pH, PE545). Prior to spectroscopic analysis, the protein solutions were dialyzed against the corresponding pH 25 mM phosphate buffer overnight at 4°C.

Phycobiliprotein Subunit Separation and Isolation

Phycobiliprotein subunit separation and isolation were preformed using the methods of Laos et al, and are briefly summarized here. Dialyzed solutions of PC577 and PE545 were concentrated to an absorbance of ~2 OD and final volume of 0.5 mL using centrifuge concentrators (30kDa cut off, Amicon). Next, 17 uL of 1 M HCl was added to each solution, causing a change in solution color indicating protein disassembly. The solutions were immediately separated using HPLC (Agilent) on a C4 Prep Column (Waters). The α and β subunits were collected manually at the absorbance peak centers. Following separation, the subunit populations were concentrated using centrifuge concentrators (3KDa for α subunits, 10kDa cut-off for the β subunits). Immediately prior to spectroscopic analysis, the β subunit populations were titrated back to pH 7 by first adding NaKPO₄ to reach a final concentration of 25 mM, then slow addition of 1 M NaOH until the pH reached 7.2 (PC577) or 6.6 (PE545). An additional step was performed for PC577 prior to titration: the buffer was replaced with 80% HCl/20% acetonitrile to help with β subunit stability in the titrated solution. The concentration of the β subunit solution was kept between 5-10 µM to prevent aggregation. Subunit separation was performed 10 times for both PC577 and PE545 to ensure the reproducibility of protein refolding and changes to the spectroscopic measurements.

Protein Crystallization

Protein crystals were grown using sitting drop vapor diffusion with highly concentrated PC577 mixed 1:1 with precipitant solution, utilizing a precipitant of 25% 3350 PEG, 50 mM HEPES, 100 mM MgCl₂. PC577 was concentrated until the absorbance intensity measured at 570 nm was at least 15 a.u. PC577 crystals grew as flat plates within 3 days.

Crystal Structure Determination

Crystals of PC577 were cryoprotected in crystallization solution supplemented with 15% (v/v) ethylene glycol by rapid equilibration immediately prior to flash-cooling in a nitrogen gas stream at 100 K. Data were collected on flash-cooled crystals using a Rigaku 007HF generator (Rigaku Corporation) and a Dectris Pilatus R 300K detector (Dectris Ltd) and processed with the
HKL suite. Data processing statistics are summarized in Table S2. The crystals had cell dimensions of $a=41$ Å $b=96$ Å $c=125$ Å in space group $P2_12_12_1$ with an $\alpha_2/\beta_2$ in the asymmetric unit. The structure of PC577 was determined using the program PHASER\textsuperscript{49} using PDB entry 4LM6\textsuperscript{34,50} as a model. Iterative model rebuilding with COOT\textsuperscript{51} and refinement with phenix.refine\textsuperscript{52} yielded a native structure comprising four polypeptide chains with a total of eight chromophore molecules covalently attached. Chromophores were added to unassigned difference density after the first cycles of refinement, unambiguously establishing both their identities and also their protein attachment points. The structure of photoacclimated PC577 was determined by rigid-body refinement using the native PC577 structure as a starting point.

**Spectroscopic Measurements**

Absorbance spectroscopy was performed using a UV-Vis spectrometer with an integrating sphere attachment (Cary 6000i). The fluorescence of each sample was measured using a standard fluorometer with excitation and emission correction (Horiba Fluorolog). Whole cell algae and assembled phycobiliproteins were measured in a quartz cuvette with a pathlength of 1 cm (Starna). Isolated subunits were measured using a 50 uL microcuvette with a pathlength of 1 cm (Starna). CD measurements were performed at room temperature using a 1 mm pathlength cuvette (Chirascan CD spectrometer, Applied Photophysics). ESI-MS measurements were performed on 10 uL injections of protein subunits following separation on the HPLC (Agilent 6220 Accurate-Mass Time-of-Flight LC/MS). During ESI-MS measurements, the $\alpha$ and $\beta$ subunits separate due to low pH conditions of the measurement.

**Transient Absorption**

The transient absorption spectroscopy measurements were performed using a commercial pump-probe setup (Ultrafast system Helios) and are briefly described here. The initial laser pulses, centered at 800 nm and $\sim$45 fs in length, were generated using a 1 kHz regeneratively amplified Ti:sapphire pulsed laser system (Coherent Libra, Santa Clara, CA). These pulses are split using a high-efficiency 50:50 beamsplitter; one branch was directed to a commercial optical parametric amplifier (OPerA Solo, Vilnius) which was used to generate a 60 fs pump pulse centered at 500 nm. The other branch is directed into the Helios system, where it is physically delayed using a delay stage which controls the time delay between the pump and probe pulse. Following the delay stage, the pulse is directed through a sapphire crystal to generate a white light spectrum ranging from 400-800 nm. The probe pulse passes through a final beam splitter, with the transmitted pulse passing through the sample and finally onto a detector, and the reflected pulse directed to an additional detector for balance detection measurements. The pump pulse passes through a chopper which blocks every other pump pulse, and is then overlapped with the probe pulse on the sample cuvette. The detector measures the probe spectra for each on/off pump-probe pair, outputting a difference signal between the two ($\Delta A$). Each time point during the scan was integrated for 2 seconds. Each scan was performed 10 times and averaged to improve the signal to noise ratio. The pump and probe were set at the magic angle to prevent polarization effects. The assembled protein
solutions were measured in a 1 mm flow cell cuvette using a flow rate of 1 mL/min with inline filtering to remove precipitates.
Figure S1. Growth curves of *H. pacifica* (left) and *P. sulcata* (right) grown under full and restricted light conditions. *H. pacifica* grown under full white light and white light with a neutral density filter are shown to demonstrate the slower growth rates, but overall reached the same maximum cellular concentration after 2 weeks.

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**Table S1.** Mass of each protein subunit obtained using ESI-MS

| Phycobiliprotein           | $\alpha_1$ | $\alpha_2$ | $\beta$ |
|---------------------------|------------|------------|---------|
| PE545                     | 7.7        | 8.8        | 20.3    |
| Photoacclimated PE545     | 7.7        | 8.8        | 20.3    |
| PC577                     | 7.2        | -          | 20      |
| Photoacclimated PC577     | 7.2        | -          | 20      |

**Table S2.** X-ray data collection and structure refinement statistics

| Data collection               | PC577 Native | PC577 Photoacclimated |
|-------------------------------|--------------|-----------------------|
| Space group                   | P2$_1$2$_1$2$_1$ | P2$_1$2$_1$2$_1$ |
| Cell dimensions               |              |                       |
| $a,b,c$ (Å)                   | 41.4, 95.9, 125.0 | 41.1, 95.7, 124.9 |
| $\alpha, \beta, \gamma$ (°)  | 90., 90., 90. | 90., 90., 90. |
| Resolution (Å)                | 30 - 1.80 (1.83 - 1.80) | 35 - 2.35 (2.39 - 2.35) |
| $R_{\text{meas}}$             | 0.092 (0.809) | 0.180 (0.926) |
| $R_{\text{pim}}$              | 0.044 (0.455) | 0.081 (0.492) |
| $I/\sigma(I)$                 | 11.9 (1.6)    | 8.1 (1.4)              |
| Completeness (%)              | 98.9 (94.3)   | 97.5 (95.0)            |
| Redundancy                    | 4.0 (2.8)     | 4.3 (3.0)              |

**Refinement**

| Resolution                  | 30 – 1.80 (1.84 – 1.80) | 30 – 2.35 (2.39 – 2.35) |
| No. reflections             | 46551                   | 20735                  |
| $R_{\text{work}}/R_{\text{free}}$ | 0.172/0.220 (0.297/0.357) | 0.182/0.255 (0.224/0.332) |
| No. atoms                   |                         |                       |
| Protein                     | 3482                    | 3464                   |
| Ligand/ion                  | 344                     | 344                    |
| Water                       | 705                     | 342                    |
| B factors (Å$^2$)            |                         |                       |
| Protein                     | 21.2                    | 28.0                   |
| Ligand/ion                  | 19.5                    | 25.6                   |
| Water                       | 29.7                    | 28.5                   |
| R.M.S. Deviations           |                         |                       |
| Bond Lengths (Å)            | 0.005                   | 0.006                  |
| Bond Angles (°)             | 0.765                   | 0.850                  |
| Ramachandran Plot           |                         |                       |
| Favored (%)                 | 98.3                    | 96.8                   |
| Outlier (%)                 | 0.0                     | 0.2                    |

| PDB ID                      | 7S96                    | 7S97                   |

*Values in parentheses are for highest-resolution shell.*
Figure S4. Absorbance spectra of purified $\alpha$ subunits at pH 2 from a) PE545 and b) PC577. PE545 has two $\alpha$ subunits with slightly different mass values and were thus purified and measured independently.

Figure S5. CD spectra of PE545 $\beta$ subunits from *P. sulcata* grown in full light and restricted light, showing no change in tertiary structure after TA measurements.