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Quantitation of Cytochromes b_{559}, b_6, and *f*, and the Core Component of Photosystem I P_{700} in Cyanobacterial Cells

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[Abstract] Cytochrome (Cyt) b_{559}, an important and essential core component of photosystem II in the photosynthetic electron transport system, is a heme-bridged heterodimer protein composed of an alpha subunit (*PsbE*) and a beta subunit (*PsbF*), and its reduced form has an absorption maximum in the α-band at 559 nm. The amounts of Cyt b_{559} can be determined by spectrophotometrical measurement of reduced minus oxidized difference spectra that are normalized with absorbance of isosbestic point at 580 nm. The authors use differential extinction coefficients of Cyt b_{559} [$\Delta\varepsilon_{(559\text{-}580\ nm)}$ = 15.5 $mM^{-1}\cdot cm^{-1}$], which have been reported by Garewal and Wasserman (1974). In addition to the Cyt b_{559}, this procedure can be used for quantitation of Cyt b_6 and Cyt *f,* the subunits of the Cyt b_6f complex, and P_{700}, one of the core components of photosystem I. This protocol, which is adapted from Fujita and Murakami (1987), is used in a cyanobacterium, *Synechococcus elongatus* PCC 7942, and also in other cyanobacterial strains including *Synechocystis* sp. PCC 6803.

Materials and Reagents

1. 50 ml polypropylene centrifuge tubes with conical bottom (Corning, Falcon®, catalog number: 352070)
2. 20 ml standard glass test tubes (Thermo Fisher Scientific, Fisher Scientific, catalog number: S63289)
3. 10 ml Oak Ridge high-speed PPCO centrifuge tubes (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 3119-0010)
4. 1.5 ml polypropylene microcentrifuge tubes (Eppendorf, catalog number: 0030125150)
5. 0.20 µm cellulose acetate membrane filter, sterile (Toyo Roshi Kaisha, catalog number: 25CS020AS)
6. Micro spatula
7. *Synechococcus elongatus* PCC 7942 (http://cyanobacteria.web.pasteur.fr/)
8. BG11 as a culture medium (Rippka *et al.*, 1979)
9. 0.1 mm diameter Zirconia/Silica beads (BioSpec, catalog number: 11079101z)
10. Sodium ascorbate (Sigma-Aldrich, catalog number: A7631)
11. Sodium hydrosulfite (Sigma-Aldrich, catalog number: 157953)
12. Methanol (Wako Pure Chemical Industries, catalog number: 139-13995)

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13. HEPES (Dojindo Molecular Technologies, catalog number: GB10)
14. Sodium hydroxide (NaOH) (Wako Pure Chemical Industries, catalog number: 198-13765)
15. Sodium chloride (NaCl) (Wako Pure Chemical Industries, catalog number: 191-01665)
16. Tricine (Dojindo Molecular Technologies, catalog number: GB19)
17. Hydrochloric acid (HCl) (Wako Pure Chemical Industries, catalog number: 080-01066)
18. Potassium ferricyanide (III) (Sigma-Aldrich, catalog number: 702587)
19. Hydroquinone (Sigma-Aldrich, catalog number: H9003)
20. HN buffer (see Recipes)
21. 50 mM tricine buffer (pH 7.5) (see Recipes)
22. 100 mM potassium ferricyanide solution (see Recipes)

Equipment

1. Vortex mixer (Scientific Industries, model: Vortex-Genie 2)
2. Tabletop centrifuge (KUBOTA, model: 5220) equipped with ST-720M swing rotor (16 x 50 ml)
3. High speed refrigerated centrifuge (KUBOTA, model: 7700) equipped with RA-150 rotor (12 x 12 ml)
4. High speed refrigerated micro centrifuge (Tomy, model: MX-301) equipped with 1.5 ml/2.0 ml rotor
5. Ultrasonic cell disruptor (Branson, model: Sonifier SFX250) equipped with tapered microtip
6. Spectrophotometer (Beckman Coulter, model: DU 640)
7. 300 µl micro cuvette, 10 mm optical pathlength (Hellma, catalog number: 105-QS)
8. PTFE coated nickel stainless steel micro spatula (Kokugo, catalog number: 101-378-51)

Procedure

1. Culture the cyanobacterial cells at 30 °C in 200 ml of BG11 medium, with illumination (10 W m^{-2}) and aeration, to the exponential phase of growth (optical density at 730 nm *ca.* 0.3 to 0.4).
2. Harvest cells in 4 x 50 ml tubes by centrifuging at 3,000 *x g* for 5 min at room temperature (Tabletop centrifuge). Discard supernatant by decantation and collect precipitated cells in one of these 50 ml tubes by resuspending them in 50 ml of ice-chilled HN buffer. Repeat the cell-washing step, *i.e.*, centrifugation-resuspension step, twice, with the cells finally resuspended in 3 ml of the same buffer. Transfer the cell suspension into a glass test tube. Work under a dim light on ice for all following steps.
3. Agitate the cell suspension with 2 g of Zirconia/Silica beads by a vortex mixer to disrupt the cells. Agitation for 30 sec at the maximal speed is repeated 4 times with ice-cooling intervals of 60 sec. Transfer the whole cell extracts into a PPCO centrifuge tube.
4. Centrifuge the cell extracts at 3,000 *x g* for 5 min at 4 °C for removal of cell debris (high speed refrigerated centrifuge). Transfer supernatant into a PPCO centrifuge tube.

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5. Centrifuge the supernatant at 30,000 *x g* for 20 min at 4 °C (high speed refrigerated centrifuge). Discard supernatant by pipetting and resuspend precipitate in 5 ml of pre-chilled HN buffer. Repeat this centrifugation-resuspension step twice, with the precipitate finally resuspended in a small volume (< 0.5 ml) of the same prechilled buffer.
6. Transfer the resuspended membranes sample for cytochromes and P_{700} measurement into a micro centrifuge tube. Divide the sample into five aliquots to be stored at -80 °C until use.
7. Dilute the sample with 50 mM Tricine buffer (pH 7.5) to a chlorophyll *a* concentration of 50 to 70 µM (see Note 1).
8. Sonicate the diluted sample twice for 1 sec at 20 W with 10 sec interval on ice to disperse the membranes (Ultrasonic cell disruptor). Transfer 200 µl of the sonicated sample into a micro cuvette.
9. Add 2 µl of 100 mM potassium ferricyanide solution (final concentration 1 mM) to the sample in the micro cuvette and mix it well with a micro spatula. Incubate the sample for several minutes at room temperature to completely oxidize cytochromes and P_{700}.
10. Set the micro cuvette in a spectrophotometer and record the absorbance spectra at the range of wavelengths from 500-750 nm at room temperature. The complete oxidation can be confirmed by observation of no further change in the spectrum (see Note 2).
11. Add a few powder particles of hydroquinone to the sample in the micro cuvette and mix it well with a micro spatula to reduce Cyt *f*. Record the absorbance spectra at the same range of wavelengths in step 10 at room temperature. Check that Δλ maximum in difference spectra of this step minus step 10 is exhibited at 556.5 nm. Repeat step 11 several times until Cyt *f* is completely reduced, *i.e.*, until the spectrum shows no further change (the final amount of hydroquinone, *ca.* 0.02 to 0.09 mg) (see Note 2).
12. To reduce Cyt b_{559} and P_{700} in the sample, add a few powder particles of sodium ascorbate to the sample in the micro cuvette and mix it well with a micro spatula. Record the absorbance spectra at room temperature. Check that Δλ maximum in Cyt b_{559} difference spectra of this step minus step 11 is exhibited at 559 nm. Also check that Δλ maximum in P_{700} difference spectra of this step minus step10 is exhibited at 700 nm. Repeat step 12 several times until Cyt b_{559} and P_{700} are completely reduced, *i.e.*, until the spectrum shows no further change (the final amount of sodium ascorbate, *ca.* 0.04 to 0.16 mg) (see Note 2).
13. Add a few powder particles of sodium hydrosulfite to the sample in the micro cuvette and mix it well with a micro spatula to reduce Cyt b_6. Record the absorbance spectra at room temperature. Check that Δλ maximum in Cyt b_6 difference spectra of this step minus step 12 is exhibited at 563 nm. Repeat step 13 several times until Cyt b_6 is completely reduced, *i.e.*, until the spectrum shows no further change (the final amount of sodium hydrosulfite, *ca.* 0.03 to 0.14 mg) (see Note 2).
14. Quantitate Cyt b_{559}, Cyt b_6, Cyt *f* and P_{700} by using following formulas, [1], [2], [3] and [4], respectively (see Note 3). The quantitative results should be presented by mean values ± standard deviations from at least three experiments.

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Cytochrome b_{559} [μM] = 64.5 x {(A_{559} - A_{580}) reduced (step12) - (A_{559} - A_{580}) oxidized (step 11)} [1]

Cytochrome b_6 [μM] = 71.4 x {(A_{563} - A_{580}) reduced (step13) - (A_{563} - A_{580}) oxidized (step 12)} [2]

Cytochrome *f* [μM] = 46.5 x {($A_{556.5}$ - $A_{544.5}$) reduced (step11) - ($A_{556.5}$ - $A_{544.5}$) oxidized (step 10)} [3]

P_{700} [μM] = 15.6 x {(A_{700} - A_{730}) reduced (step12) - (A_{700} - A_{730}) oxidized (step 10)} [4]

Representative data

Figures 1 and 2 show representative examples of difference spectra with the use of thylakoid membranes of *Synechocystis* sp. PCC 6803.

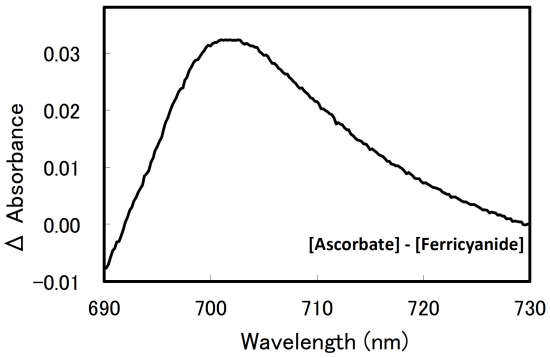

Figure 1. A difference spectrum of P_{700} in *Synechocystis* sp. PCC 6803. The sample included thylakoid membranes equivalent to 60 μM chlorophyll *a*.

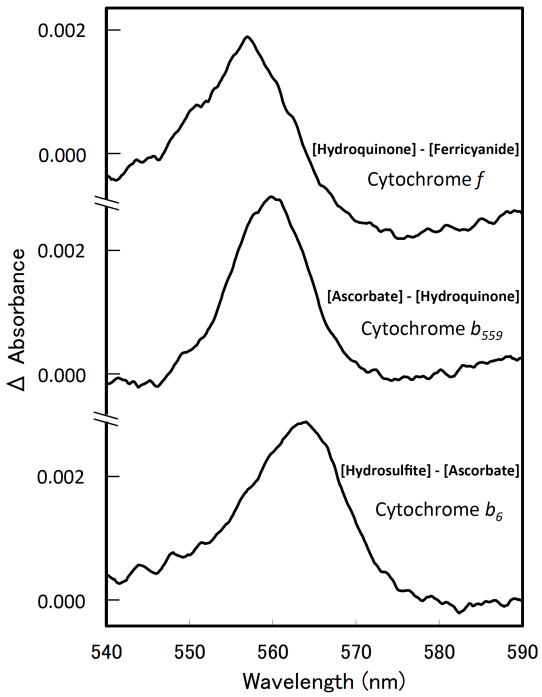

Figure 2. Difference spectra of the cytochromes in *Synechocystis* sp. PCC 6803. Each sample included thylakoid membranes equivalent to 60 μM chlorophyll *a*.

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Notes

1. Chlorophyll *a* concentration is determined according to the method of Porra *et al.* (1989). After extracting chlorophyll *a* in the membranes sample with 100% methanol, measure the absorbance at 650.0, 665.2 and 730 nm, and calculate the chlorophyll *a* concentration by using following formula [5].
   Chlorophyll *a* [μM] = 18.22 x ($A_{665.2}$ - A_{730}) – 9.55 x ($A_{652.0}$ - A_{730}) [5]
2. Be careful to avoid addition of oxidizing or reducing reagents in excess, since it will cause aggregation and/or bleaching of the sample.
3. Differential extinction coefficients used were $\Delta\varepsilon_{(700 - 730\ nm)}$ = 64 $mM^{-1}\cdot cm^{-1}$ (P_{700}), $\Delta\varepsilon_{(559 - 580\ nm)}$ = 15.5 $mM^{-1}\cdot cm^{-1}$(Cyt b_{559}), $\Delta\varepsilon_{(563 - 580\ nm)}$ = 14.0 $mM^{-1}\cdot cm^{-1}$ (Cyt b_6), and $\Delta\varepsilon_{(556.5 - 544.5\ nm)}$ = 21.5 $mM^{-1}\cdot cm^{-1}$ (Cyt *f*), which has been reported by Hiyama and Ke (1972), Garewal and Wasserman (1974), Stuart and Wasserman (1973), and Böhme *et al.* (1980), respectively.

Recipes

1. HN buffer (5 mM HEPES-NaOH [pH 7.5], 10 mM NaCl)
   Dissolve 1.19 g of HEPES and 0.58 g of NaCl to ~800 ml of ddH_2O
   Adjust pH to 7.5 with NaOH
   Add ddH_2O to final volume of 1,000 ml
   Sterilized by filtration (0.20 μm cellulose acetate membrane filter)
   Store at room temperature
2. 50 mM tricine buffer (pH 7.5)
   Dissolve 8.96 g of tricine to ~800 ml of ddH_2O
   Adjust pH to 7.5 with HCl
   Add ddH_2O to final volume of 1,000 ml
   Sterilized by filtration (0.20 μm cellulose acetate membrane filter)
   Store at room temperature
3. 100 mM potassium ferricyanide solution
   Dissolve 0.329 g of potassium ferricyanide (III) to ddH_2O in a final volume of 10 ml
   This solution should be prepared just before use and store in dark

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