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Role of pigments and subunits in the cytochrome b_6f complex of *Synechocystis* PCC6803

D Schneider[1], S-O Wenk[1], S Berry[1], U Boronowsky[1], C Jäger[1], FL de Weerd[2], JP Dekker[2], M Rögner[1]

[1]*Plant Biochemistry, Ruhr-University Bochum, D-44780 Bochum, Germany. matthias.roegner@ruhr-uni-bochum.de*

[2]*Dept. Physics & Astronomy, Biophysics, Vrije Universiteit Amsterdam, 1081 HV Amsterdam, The Netherlands. dekker@nat.vu.nl*

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Introduction

The cytochrome b_6f complex of cyanobacteria consists of the four main subunits cytochrome *f* (PetA), cytochrome b_6 (PetB), the Rieske protein (PetC), subunit IV (PetD) and additional small subunits named PetG, PetM and PetN. In contrast to higher plants and algae, no gene encoding the subunits PetL and PetO can be found in the genome of the completely sequenced cyanobacterium *Synechocystis* PCC 6803. The function of the additional small subunits in cyanobacteria is not yet clear: While PetM seems to have a regulatory role (Schneider et al., 2001), deletion of *petG* and *petN* did not yield completely segregated mutants (Schneider, unpubl.). In contrast to all other subunits which are encoded by single genes, the genome of *Synechocystis* shows a family of three *petC* genes (*sll1316 = petC1, slr1185 = petC2, sll1182 = petC3*), the reason for which is unknown. Also, the role of one chlorophyll and one carotenoid per monomeric b_6f complex in both pro- and eukaryotic cyt b_6f preparations (Bald et al., 1992; Pierre et al., 1997, Zhang et al., 1999) is still unresolved.

Results and discussion

Hemes, chlorophyll and carotenoids

Reduction of the highly purified cyt b_6f complex (Wenk et al., 1998) with dithionite caused a 1-nm red shift in the absorbance spectrum of the chlorophyll molecule (Fig. 1A). As such a shift was not observed with ascorbate, which reduces cyt *f* but not cyt b_6, a charge interaction of the chlorophyll molecule with one or both hemes of cyt b_6 is strongly suggested. This is supported by identical kinetics of the chlorophyll absorbance shift and the cyt b_6 redox change (Fig. 1B), yielding a linear relationship between these two events as shown in Fig. 1C.

The role of the bound carotenoid was investigated in more detail with a *Synechocystis* mutant strain containing an interrupted *crtO* gene. This gene codes for the β-carotene ketolase, CrtO, which is required for the synthesis of echinenone, the carotenoid selectively bound by the cyt b_6f complex of *Synechocystis.* Pigment analysis of the cyt b_6f complex isolated from this mutant showed the replacement of echinenone by three other carotenoids: β-carotene, zeaxanthine and a mono-hydroxy β-carotene (possibly cryptoxanthine). All three were 9-*cis* isomers, showing a characteristic 4-5 nm blue-shift, increased absorption at 340 nm and decreased absorption at 280 nm similar to β-carotene.

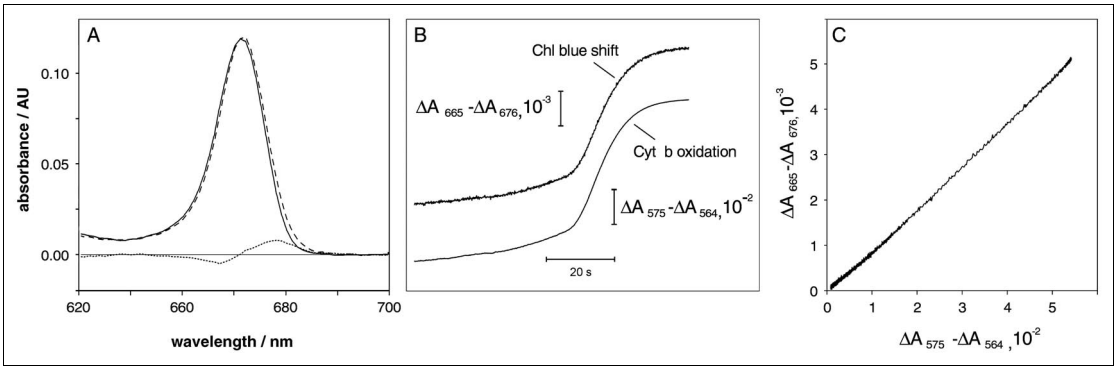

Fig. 1. A: 4 K absorbance spectrum of chlorophyll associated with the cyt b_6f complex. Solid line: Sample oxidized by 100 µM ferricyanide, followed by reduction of cyt *f* with 2 mM ascorbate. Dashed line: Cyt b_6 reduced by dithionite. Dotted line: Difference spectrum of solid and dashed line. B: Reoxidation kinetics (by air) of cyt b_6 combined with the kinetics of chlorophyll absorbance shift after reduction by 0.5 mM dithionite. C: Kinetics of the cyt b_6 redox change plotted against the chlorophyll absorbance shift.

A characteristic difference in the carotenoid content was also suggested by the absorbance spectrum of the isolated mutant cyt b_6f complex (Fig. 2): Reduction of cyt b_6 caused a red shift by about 1.5 nm of the bands at 496 and 462 nm, which did not occur upon reduction of cyt *f*. Such small band shifts could not be observed in the WT cyt b_6f complex containing echinenone due to the structureless absorbance spectrum of echinenone.

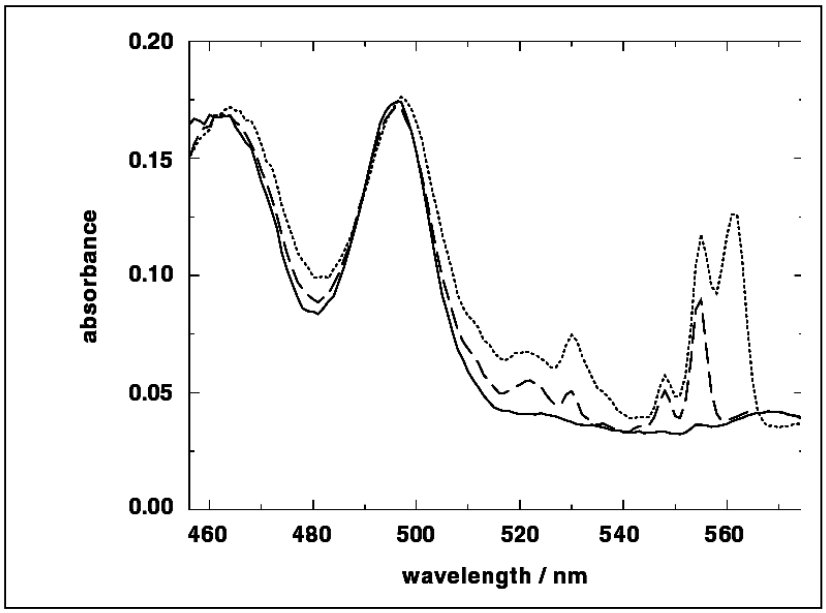

Fig. 2. Absorbance spectra of the cyt b_6f complex from the *CrtO*-minus mutant at 4 K recorded in the presence of 100 µM ferricyanide (solid line), 20 mM ascorbate (dashed line) or after addition of a few grains of dithionite (dotted line).

To characterize the specific binding sites of the two pigments, the isolated cyt b_6f complex was dissociated into its individual subunits by a mild detergent treatment, followed by chromatographic separation of the native proteins as outlined in (Boronowsky et al., 2001).

Characterization of the native subunits showed that both pigments are exclusively bound to the cyt b_6 subunit. This location fulfils all requirements which have been imposed by previous results:

1) The extremely short fluorescence lifetime of the chlorophyll (Peterman et al., 1998) suggested a binding of Chl in a specific pocket of the cyt b_6f complex, where a heme or an

amino acid is able to quench the excited state of the chlorophyll in order to protect the protein from oxidative damage.

2) The red-shift of the chlorophyll peak simultaneously with the reduction of the *b*-type hemes suggests a short distance between these two components.

3) Also, the red shift of the carotene peaks with the reduction of the *b*-type hemes suggests a short distance between them.

4) The proximity of both pigments is required for the suggested function of the carotenoid to prevent the generation of singlet oxygen by photoexcited chlorophyll *a* (Zhang et al., 1999).

Modelling of the b_6-structure based on the known bc_1-complex structure reveals that the most probable Chl binding site is located just in between the two heme groups. This enables speculations on a possible role of Chl as light sensor, which could have impact on the Q-cycle of the b_6f complex.

Rieske proteins

In order to investigate the role of multiple Rieske genes in *Synechocystis*, all three encoded proteins were heterologously overexpressed in *E. coli* BL21(DE3). In addition, for *in vivo* studies, *Synechocystis* mutant strains lacking one or two Rieske genes were created and characterized.

After overexpression of the three full length Rieske proteins, two of them (PetC2 and PetC3) were found in a native form in the cytoplasmic membrane of *E. coli* with incorporated iron-sulfur cluster. As the third protein (PetC1) could not be obtained in an active form, the overproduced protein was purified from inclusion bodies and the Fe-S cluster was reconstituted enzymatically *in vitro* (Schneider et al., 2000). EPR-measurements showed the typical g-values for all three Rieske proteins (Fig. 3) and enabled the determination of the redox potentials by titration: In contrast to the main Rieske protein PetC1 (+320 mV) and to PetC2 (about +300 mV) with rather high E_m values, PetC3 showed an unusual low midpoint potential of only +135 mV. In consequence, plastoquinone would not be able to donate electrons to PetC3 and only menaquinone is a potential electron donor due to its low redox potential.

Further experiments were done to elucidate the physiological role of the three Rieske proteins in the cytochrome b_6f complex. Expression studies showed that all three proteins are expressed (Schneider et al., 2001). Single gene deletion experiments revealed a nonessential function of any of the individual Rieske proteins. However, deletion of the main gene *petC1* affected the cells considerably more than deletion of *petC2* which had no phenotype: The Δ*petC1* strain showed effects on the activity of the cyt b_6f complex, PS2 and the cyt *bd* oxidase in the thylakoid membrane. The observation that the double gene deletion mutants Δ*petC1/C3 and* Δ*petC2/C3*, but not Δ*petC1/C2* completely segregated also confirm that PetC2 can partly replace the function of PetC1.

The existence of free MQ in thylakoid or cytoplasmic membrane and of a special MQ-oxidizing cyt b_6f complex remains further to be investigated. In combination with the different Rieske proteins they may represent mechanisms of physiological adaptations to environmental (stress) conditions as has already been shown for three copies of the *psbA* gene in *Synechocystis,* coding for the D1 protein in PS2.

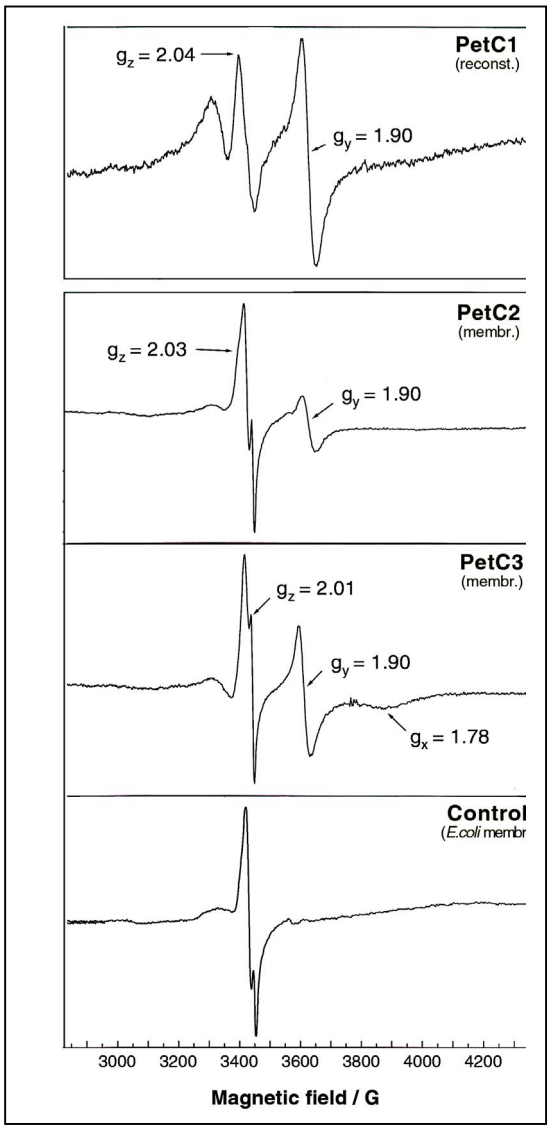

Fig. 3. EPR spectra of *Synechocystis* Rieske proteins after purification and reconstitution (PetC1) or of *E. coli* membranes with incorporated PetC2 and PetC3 and of control membranes recorded at 15 K.

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