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Structural and functional characterisation of the cyanobacterial PetC3 Rieske protein family

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ABSTRACT

The cyanobacterium *Synechocystis* PCC 6803 possesses three Rieske isoforms: PetC1, PetC2 and PetC3. While PetC1 and PetC2 have been identified as alternative subunits of the cytochrome b_6f complex (b_6f), PetC3 was localized exclusively within the plasma membrane. The spatial separation of PetC3 from the photosynthetic and respiratory protein complexes raises doubt in its involvement in bioenergetic electron transfer.
Here we report a detailed structural and functional characterization of the cyanobacterial PetC3 protein family indicating that PetC3 is not a component of the b_6f and the photosynthetic electron transport as implied by gene annotation. Instead PetC3 has a distinct function in cell envelope homeostasis. Especially proteomic analysis shows that deletion of *petC3* in *Synechocystis* PCC 6803 primarily affects cell envelope proteins including many nutrient transport systems. Therefore, the observed downregulation in the photosynthetic electron transport – mainly caused by photosystem 2 inactivation – might constitute a stress adaptation. Comprehensive *in silico* sequence analyses revealed that PetC3 proteins are periplasmic lipoproteins tethered to the plasma membrane with a subclass consisting of soluble periplasmic proteins, i.e. their *N*-terminal domain is inconsistent with their integration into the b_6f. For the first time, the structure of PetC3 was determined by X-ray crystallography at an atomic resolution revealing significant high similarities to non-b_6f Rieske subunits in contrast to PetC1.
These results suggest that PetC3 affects processes in the periplasmic compartment that only indirectly influence photosynthetic electron transport. For this reason, we suggest to rename "Photosynthetic electron transport Chain 3" (PetC3) proteins as "periplasmic Rieske proteins" (Prp).

1. Introduction

Rieske proteins are electron transport modules involved in various cellular processes including photosynthesis and respiration [1,2], carotenoid biosynthesis [3], protein translocation [4], arsenite detoxification [5] and the biodegradation of toxic aromatic compounds [6,7]. They are characterized by their active site, a [2Fe-2S] cluster coordinated by the side chains of two histidine and two cysteine residues.

In cyanobacteria, multiple Rieske proteins have emerged as constituents of the cytochrome b_6f complex (b_6f) where they are involved in the photosynthetic and respiratory electron transport [8]. It has been shown that the major Rieske protein, PetC1, can be replaced by several isoforms as adaptation to different environmental conditions [9–11]. In the cyanobacterium *Synechocystis* PCC 6803 (here after *Synechocystis*), three different Rieske isoforms have been characterized [12]: Under normal growth conditions PetC1 is the major Rieske protein of b_6f, which is replaced by PetC2 under low oxygen [10] and high light stress [11].

A third Rieske protein from *Synechocystis* (sll1182) has also been assigned to the group of alternative b_6f subunits and named PetC3, although it exhibits a considerably lower sequence identity to PetC1 than PetC2 [12,13]. Further studies revealed, however, that PetC3 is exclusively localized in the plasma membrane (PM) whereas all other b_6f

Abbreviations: Δ*petC3*, *petC3* deletion strain (of *Synechocystis* PCC 6803); b_6f, cytochrome b_6f complex; C, inorganic carbon (in the form of CO_2 and HCO_3^-); E_m, midpoint redox potential; N, inorganic nitrogen; NDH-1, NADH dehydrogenase-like complex; NSAF, normalised spectral abundance factor; P, phosphate; PBP, penicillin-binding protein; PGN, peptidoglykan; PM, plasma membrane; PQ, plastoquinone/plastoquinol; Prp, periplasmic Rieske protein; PS1, photosystem 1; PS2, photosystem 2; RMSD, root-mean-square deviation; SP, signal peptidase; *Synechocystis*, *Synechocystis* PCC 6803; TAT, twin-arginine translocation pathway; TM, thylakoid membrane; WT, wild type.

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subunits are exclusively found in the thylakoid membrane [14,15]. Also, the midpoint redox potential (E_m) of PetC3 from *Synechocystis* (SyPetC3) - about 135 mV - is considerably lower than the E_m of PetC1 and PetC2 of about 300 mV. This prevents an involvement of PetC3 in the plastoquinone oxidation within the photosynthetic electron transport chain [12]. Generally, the E_m of Rieske proteins is strongly correlated with two residue positions within the conserved Box II motif [CxCHx(S,T,A,G)x(Y,F)] [16]: PetC1 is a high-potential Rieske protein with a conserved serine and tyrosine residue at positions which form hydrogen bonds with the [2Fe-2S] cluster and one of the cluster-coordinating cysteines, respectively. These conserved serine and tyrosine residues are replaced by alanine and phenylalanine in SyPetC3 and the absence of these hydrogen bonds may be the major reason for the lower E_m [12]. This is supported by studies showing that the Ser to Ala substitution in various Rieske proteins usually leads to a E_m drop of about 100 mV while the Tyr to Phe substitution leads to a 50 mV drop [7,16–18]. Physiological analyses on PetC3 are limited to *Synechocystis petC3* deletion strains (Δ*petC3*): In contrast to the Δ*petC1*/Δ*petC3* double deletion mutant, the Δ*petC1*/Δ*petC2* mutation appeared to be lethal. This indicated that PetC2, but not PetC3 can replace PetC1 in b_6f function and that PetC3 instead may belong to a functional distinct b_6f population in the PM [13]. As Δ*petC3* did not show the wild type-typical growth lag phase upon transfer of the cultures from low light to high light conditions [11], an involvement of PetC3 in high-light adaptation processes was proposed. Also, Δ*petC3* cells are characterized by a slightly larger cell size, a decreased linear and an increased cyclic electron flow [11,14]. Interestingly, another recent study showed that PetC3 was strongly upregulated in a *Synechocystis lepB1* deletion mutant which is unable to grow photoautotrophically [19]. In summary, a direct linkage of PetC3 to the photosynthetic electron transport remains elusive mainly because of its location in the PM [14,15].

Here we present proteomic data on PetC3 based on highly standardized continuous growth conditions. *In silico* sequence analyses from various cyanobacterial species provide new insights into processing, localization and phylogenetic distribution of PetC3 proteins. Also, the three-dimensional structure of the soluble domain of SyPetC3 was determined to a resolution of 1.7 Å by X-ray crystallography. The combined data analysis suggests a completely new role for PetC3.

2. Material and Methods

2.1. Cultivation of Synechocystis PCC 6803

For proteomic analysis, the glucose-tolerant *Synechocystis* PCC 6803 wild type (*Synechocystis* WT) and its Δ*petC3* strain [13] were cultivated in a 5 L flat-bed photobioreactor system (KSD Innovations GmbH, Hattingen, Germany) according to [20]. Cells were grown in Y-BG11 at 30 °C under 100 μmol m^{-2} s^{-1} white light (LED panels) and aeration with air enriched by 3% CO_2 in the absence of glucose and other reduced carbon sources.

Cultures for the phosphorus depletion experiment were grown in BG11 medium to an optical density (680 nm) of 1.0, followed by three-times washing with phosphate-free BG11 (K_2HPO_4 replaced by equimolar amounts of KCl) and adjustment to an optical density (680 nm) of 0.3.

2.2. Measurements of oxygen evolution, $P700^+$ reduction kinetics and 77 K fluorescence spectra

In vivo photosystem 2 (PS2) activity was measured via oxygen production - using a fiber-optic oxygen sensor (PreSens, Regensburg, Germany) - in the presence of the artificial electron acceptors 2.6-dichloro-p-benzoquinone (DCBQ) and ferrocyanide according to [21].

$P700^+$ reduction kinetics was recorded with a Dual-Pam-100 system (Walz GmbH, Effeltrich, Germany) according to [22].

77 K fluorescence spectra were recorded from whole cells with 3 μg chlorophyll *a* per ml culture using an Aminco-Bowman Series 2 spectrofluorometer (SLM Spectronic Instruments). All spectra were normalised to the fluorescence maximum of photosystem 1 (PS1) (around 725 nm).

2.3. Determination of the phosphorus content

Phosphorus content was determined by inductively coupled plasma optical emission spectrometry (ICP-OES; Thermo Scientific iCAP 6500). Dried cells with known cell number - determined by Z2 Coulter counter (Beckman Coulter, Fullerton, CA) - were lysed in 65% HNO_3 at 80 °C (30 min), 100 °C (30 min) and 120 °C (60 min).

2.4. Proteomic analysis

The photobioreactor culture was cooled down immediately by adding ice and harvested by centrifugation (all steps at 4 °C). After suspension of the cell pellets in 20 mM HEPES buffer pH 7.4 (10 mM EDTA, 5 mM NaCl and 20% (*w*/*v*) sucrose) cells were broken by a French press treatment (2 × 2000 psi) in presence of DNase and protease inhibitors (PMSF, TLCK, E64 and Pefabloc) according to manufacturer instructions. Cell debris were removed from the lysate by centrifugation (1000 g, 10 min). Membrane and soluble fractions were separated by ultracentrifugation (200,000 g, 45 min), with protein aliquots of each fraction being frozen in liquid nitrogen and stored at −80 °C.

For tryptic digest, 5 μg proteins were focused by SDS-PAGE into one single band at the interface of stacking and separating gel [23]. The resulting bands were cut off and digested with trypsin according to [24]; peptide extraction, mass spectrometric measurement and analysis according to [23].

2.5. Immunoblot analysis

Plasma membrane fraction of *Synechocystis* WT has been prepared by sucrose density gradient centrifugation as previously described [14]. Subsequently, the proteins were separated in an SDS-PAGE. Electroblotting and immunodetecting with the anti-PetC3 antibody have been done according to Ref [12].

2.6. Cloning, expression and purification of recombinant SyPetC3

PetC3 was expressed from a pASK-IBA37 (IBA GmbH) vector coding for a shortened version of SyPetC3 with the *N*-terminal 23 amino acids being replaced by 16 artificial residues (MASRGSHHHHHHIEGR) including a 6-times histidine tag and a factor Xa protease recognition site. The vector was transformed in *E. coli* Bl21 cells (Rosetta, Novagen). For overexpression, cells were grown in LB medium (37 °C, 200 rpm) to an optical density (600 nm) around 0.6 and then induced by adding 200 μg anhydrotetracycline per liter culture. After 4–6 h expression, the cells were harvested by centrifugation and disrupted by lysozyme treatment and sonication. Subsequent purification via Ni-NTA resin (Qiagen) and removal of the artificial residues with the Factor Xa Cleavage Capture Kit (Merck Millipore) was done according to the manufacturer's instructions. The protein was further purified by size exclusion chromatography with a Superdex 75 10/300 GL column (GE Healthcare) in 10 mM Tris-HCl buffer (pH 7.3) with 150 mM NaCl. After desalting the main peak was applied onto a Resource™ S cation exchange chromatography column equilibrated in 10 mM Tris-HCl pH 7.3 and eluted by an increasing NaCl gradient. The main peak was collected and the buffer was replaced by 10 mM Tris-HCl pH 7.3. SyPetC3 aliquots were frozen in liquid nitrogen and stored at −80 °C.

2.7. EPR Measurements

Continuous wave (cw) X-Band measurements of recombinant SyPetC3 were carried out using an X-band Bruker ESP 300E instrument