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Cyanobacterial cytochrome c_M: Probing its role as electron donor for Cu_A of cytochrome *c* oxidase

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ABSTRACT

It is well known that efficient functioning of photosynthetic (PET) and respiratory electron transport (RET) in cyanobacteria requires the presence of either cytochrome c_6 (Cytc_6) or plastocyanin (PC). By contrast, the interaction of an additional redox carrier, cytochrome c_M (Cytc_M), with either PET or RET is still under discussion. Here, we focus on the (putative) role of Cytc_M in cyanobacterial respiration. It is demonstrated that genes encoding the main terminal oxidase (cytochrome *c* oxidase, COX) and cytochrome c_M are found in all 44 totally or partially sequenced cyanobacteria (except one strain). In order to check whether Cytc_M can act as electron donor to COX, we investigated the intermolecular electron transfer kinetics between Cytc_M and the soluble Cu_A domain (i.e. the donor binding and electron entry site) of subunit II of COX. Both proteins from *Synechocystis* PCC6803 were expressed heterologously in *E. coli*. The forward and the reverse electron transfer reactions were studied yielding apparent bimolecular rate constants of $(2.4\pm0.1)\times10^5$ M^{-1} s^{-1} and $(9.6\pm0.4)\times10^3$ M^{-1} s^{-1} (5 mM phosphate buffer, pH 7, 50 mM KCl). A comparative analysis with Cytc_6 and PC demonstrates that Cytc_M functions as electron donor to Cu_A as efficiently as Cytc_6 but more efficient than PC. Furthermore, we demonstrate the association of Cytc_M with the cytoplasmic and thylakoid membrane fractions by immunobloting and discuss the potential role of Cytc_M as electron donor for COX under stress conditions.

1. Introduction

Cyanobacteria have uniquely accommodated both a photosynthetic oxygen-evolving electron transport chain (PET) and an oxygen-consuming respiratory electron transport chain (RET) within a single prokaryotic cell [1–3]. In PET, which is localized in intracytoplasmic membranes (ICM) or thylakoids, the type-1 copper protein plastocyanin (PC) and cytochrome c_6 (Cytc_6) act as alternative redox carriers between the membrane complexes b_6f and photosystem I [4]. In eukaryotic algae and many cyanobacteria synthesis of either PC or Cytc_6 is controlled by copper availability with PC being replaced by Cytc_6 under copper deficiency [4]. A recent genome analysis [3] has demonstrated that cyanobacteria have usually one gene (*petE*) encoding PC whereas the number of *petJ* genes (encoding Cytc_6) varies from 1 to 4.

Additionally, in cyanobacteria Cytc_6 and PC have been demonstrated to function as electron donor in RET, which is located in ICM and the cytoplasmic membrane (CM) [2,3,5–9]. RET in CM is essential to provide energy for various transport processes and its importance increases under stress conditions including nitrogen fixation [3,10,11]. The location of Cytc_6 and PC in the intrathylakoid lumen has been firmly established [3,4], whereas so far only Cytc_6 could be identified also as being periplasmically located [12]. The occurrence of PC in the periplasmic space of cyanobacteria has not yet been proved.

In 1994 a new type of cyanobacterial cytochrome *c* was detected in the genome of *Synechocystis* sp. PCC6803 [13]. Malakhov et al. identified an ORF corresponding to a gene designated *cytM* gene. Its amino acid sequence exhibited about 35% similarity to sequences of cytochromes c_6 and included the conserved heme-binding motif (–CXXC*H*–) with C representing the cysteines involved in heme to protein linkages and *H* representing the proximal heme ligand [13]. Due to its hydrophobic N-terminal domain, that might be either a transit peptide or a membrane anchor, the protein was labeled cytochrome c_M (Cytc_M) [13]. Both RET and PET in a mutant with a disrupted *cytM* gene were as efficient as they were in the wild-type

Abbreviations: Cytc_M, cytochrome c_M; Cytc_6, cytochrome c_6; PC, plastocyanin; hhCytc, horse heart cytochrome *c*; *cytM*, gene encoding cytochrome c_M; *petJ*, gene encoding cytochrome c_6; *petE*, gene encoding plastocyanin; COX, cytochrome *c* oxidase; SUII, subunit II; QOX, quinol oxidase; RET, respiratory electron transport chain; PET, photosynthetic electron transport chain; ET, electron transfer; PSI, photosystem I; PSII, photosystem II; ORF, open reading frame; IP, isoelectric point; EDTA, ethylene diamine tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride

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Synechocystis sp. PCC6803 strain. No discernible phenotype was observed when the cells were grown under normal conditions [13].

To elucidate the role of Cytc_6, PC and Cytc_M in the transfer of PS II-generated electrons to terminal oxidase(s) in RET, deletion constructs for genes for these proteins were introduced into a PS I-less *Synechocystis* sp. PCC6803 strain. Loss of Cytc_6 or PC decreased the rate of electron flow out of PS II [14]. Neither a double mutant lacking both Cytc_6 and PC nor a mutant lacking Cytc_M could be obtained [14]. This was the first indication that Cytc_M is actually expressed and might play a role in RET. Due to sequence similarities with cytochrome *c* associated with SUII of cytochrome *c* oxidase (COX) of *Thermus thermophilus* and *Bacillus* sp. (i.e. *caa*$_3$-type COX) it has been suggested that Cytc_M might be a component of COX in cyanobacteria serving as redox shuttle between Cytc_6 or PC and COX [14]. However, a systematic analysis of all available Cytc_M sequences in the present study clearly indicates that these *caa*$_3$-type COXs miss the Cytc_M-typical environment of the distal heme ligand methionin (*M*) represented by the peculiar sequence –TPP*M*P– with three prolines in its immediate neighborhood.

Finally, Cytc_M could be both detected by Western Blot analysis in crude cell extracts of *Synechocystis* sp. PCC6803 as well as was produced in recombinant form in *E. coli* as soluble protein (8.3 kDa) without the hydrophobic region at the N-terminal end [15]. Recombinant *Synechocystis* Cytc_M exhibited a quite low standard reduction potential of +151 mV and in its reduced form had the Soret band at 416 nm and the α and β bands at 550 and 521 nm, respectively [15].

Nowadays, it seems to be well established that the efficient functioning of both PET and RET in *Synechocystis* sp. PCC6803 strictly requires the presence of either Cytc_6 or PC under normal conditions [8]. By contrast, the physiological role of Cytc_M is still under discussion. Although the data of Metzger et al. [16] indicate that Cytc_M can operate in PET between the cytochrome b_6f complex and PS I, it seems unlikely that this is its primary role, at least under optimal growth conditions, where both Cytc_6 and PC function more efficiently, as has been demonstrated in a comparative functional laser flash-induced kinetic analysis of PSI reduction [17]. Northern blotting analysis revealed that under stress conditions like low temperature or high-intensity light, *petJ* and *petE* transcription is suppressed, whereas expression of *cytM* (that is scarcely expressed under normal growth conditions) was enhanced [18,19]. Additionally, it is a well known phenomenon that under stress conditions cyanobacteria usually shut down PET, whereas RET is significantly enhanced [10,20]. This suggests that under stress conditions Cytc_6 and PC are replaced for cytochrome c_M that might function as alternative electron carrier between cytochrome b_6f and cytochrome *c* oxidase.

In the present work we have analyzed all 44 partially or totally sequenced cyanobacterial genomes for the occurrence of *cytM* and its putative electron acceptor cytochrome *c* oxidase (COX). In order to probe the kinetics of electron transfer between Cytc_M and the Cu_A site of SUII in COX both proteins have been produced in recombinant form and their redox reactivity was tested by stopped-flow spectroscopy. In a comparative study recombinant cytochrome c_6 and plastocyanin were investigated under identical conditions. Western blot analysis using a polyclonal antibody raised against recombinant cytochrome c_M demonstrates the association of Cytc_M with both CM and ICM. The findings are discussed with respect to the physiological role of cytochrome c_M in cyanobacterial ET.

2. Materials and methods

2.1. Materials

Standard chemicals and biochemicals were obtained from Sigma Chemicals Co. at the highest grade available. DEAE Sepharose Fast Flow, CM Sepharose Fast Flow and Superdex 75 HR were purchased from GE Healthcare Europe GmbH. For dialysis Membra-Cel® from Serva (MWCO 3500) was used. The stirred ultrafiltration cells (Models 8010 and 8200) were from Amicon, and the ultrafiltration membranes, regenerated cellulose (PLBC), NMWC 3000 from Millipore Corporation.

2.2. Cloning and heterologous overexpression

Competent *E. coli* MC1061 cells were co-transformed by electroporation (Gene Pulser, Bio Rad) with the pESCM [17] (a kind gift from Fernando P. Molina-Heredia, Universidad de Sevilla y CSIC, Spain) and pEC86 [21,22] which encodes the *ccm*A-H gene cluster of *E. coli*. Positive clones were grown overnight in standard Luria Bertani (LB) medium [23] containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol on an orbital shaker at 180 rpm and 37 °C. LB medium supplemented with the same antibiotics was inoculated with the overnight culture in a 1:100 ratio and grown at 37 °C and 180 rpm to an OD_{600}=0.7. Protein expression was carried out at 30 °C and 24 h after inoculation glycerol (0.2% $^{v}/_{v}$) was added. Cells were harvested by centrifugation (6000 g, 6 min, room temperature) 48 h after induction and stored at −80 °C.

The frozen cell pellet (obtainted from 1 L *E. coli* culture) was defrosted and resuspended in 50 mL lysis buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 20% ($^{w}/_{w}$) sucrose, 500 μg/mL lysozyme and 1 mM PMSF). Subsequently the suspension was centrifuged 20 min at 9000 rpm and 4 °C to remove the cell debris. After dialysis Cytc_M was purified from the supernatant by column chromatography in three steps.

2.3. Protein purification

The orange-pinkish colored supernatant containing cytochrome c_M was dialysed against 10 mM Tris–HCl, pH 8.0, for 16 h to remove sucrose. The protein solution was loaded on a DEAE Sepharose Fast Flow column (2.5×12 cm) equilibrated with 10 mM Tris–HCl, pH 8.0. After washing with equilibration buffer cytochrome c_M was eluted with 10 mM Tris–HCl buffer, pH 8.0, containing 50 mM NaCl. Cytc_M-containing fractions were pooled and concentrated by ultrafiltration to a final volume of 20 mL and loaded on a Carboxymethyl-Sepharose Fast Flow to remove lysozyme. The column was equilibrated and washed after protein application with 50 mM phosphate buffer, pH 8.0. Cytochrome c_M did not bind to the column and was found in the flow-through fractions. The orange protein solution was concentrated to a final volume of 1 mL. Portions of 100 μL were loaded on a Superdex75™ HR 10/30 FPLC column equilibrated with 67 mM phosphate buffer, pH 7.0, containing 150 mM KCl. Cytc_M fractions were pooled, concentrated as described above and stored at −80 °C.

Recombinant production and purification of Cytc_6 and PC as well as of Cu_A domain of SUII of COX from *Synechocystis* sp. PCC6803 were described previously [6,7,24].

2.4. Mass spectrometry

ESI Q-TOF MS was carried out on a Q-TOF Ultima Global (Waters Micromass, UK). The sample was diluted to a concentration of approximately 1 pmol/μL in 50% acetonitrile containing 0.1% formic acid. This sample solution was subjected to offline ESI Q-TOF mass spectrometer to acquire a spectrum.

2.5. Spectral and kinetic investigations

Steady-state spectrophotometric measurements were made on a diode-array spectrophotometer (Specord S10, Zeiss). The concentration of the protein was determined spectrophotometrically using the absorption coefficients described by Cho et al. [15]. Since recombinant ferrous cytochrome c_M was very susceptable to reoxidation,