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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,

preparation of Fe(II) Cytc_M was performed by addition of sodium dithionite either directly or from a freshly prepared anaerobic stock solution in a glove box (Meca-Plex, Neugebauer) with a positive pressure of nitrogen (25 mbar). All solutions were made anaerobic by flushing with nitrogen gas (oxygen <3 ppm) and discharged from the glove box in gas-tight syringes.

Stopped-flow measurements were made using PiStar-180 stopped-flow spectrophotometer from Applied Photophysics equipped with a 1 cm observation cell. Calculation of pseudo-first order rate constants (k_{obs}) from experimental time traces at 550 nm was performed with the SpectraKinetic work station (Version 4.38) interfaced to the instrument. Both the kinetics of oxidation of reduced Cytc_M by recombinant Cu_A domain (forward ET reaction) as well as the reduction of oxidized cytochrome c_M by reduced Cu_A domain (reverse ET reaction) was followed in the single mixing mode at 550 nm (peak of reduced Cytc_M). Various ionic strengths were adjusted by addition of KCl to 5 mM phosphate buffer, pH 7, at final ionic strengths of 0, 20, 50, 100, 200, 300 and 400 mM KCl.

Recombinant cytochrome c_6 and plastocyanin were tested under identical conditions. The experimental design has been described recently [6,7]. In all experiments the first data point was recorded 1.5 ms after mixing the two redox partners and 2000 data points were accumulated. Second order rate constants were calculated from the slope of the linear plot of pseudo-first-order rate constants *versus* protein concentration.

Additionally redox reactions were followed with a diode-array detector (Applied Photophysics PD.1) attached to the stopped flow machine and the XScan Diode Array Scanning software (Version 1.07). The Pro-K simulation program was used to analyze the normal data sets allowing the synthesis of artificial sets of time-dependent spectra as well as spectral analysis of enzyme intermediates.

2.6. Production of polyclonal antibody and Western blot analysis

Polyclonal antibodies against recombinant cytochrome c_M from *Synechocystis* were developed in rabbits at Invitrogen Corporation, Carlsbad (US) and purified under standard conditions by a chromatographic step with DEAE Affi Gel® Blue Gel (Bio-Rad). For Western blot analysis membranes or cytosolic proteins were separated by SDS-PAGE on 15% slab gels as described by Laemmli [25], and, finally, blotted onto nitrocellulose membrane (Hybond™-ECL™, Amersham Bioscience) using Mini protean III system (Bio-Rad). Goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) was used as secondary antibody and the immunoblots were developed after addition of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma).

2.7. Growth conditions and membrane separation

Axenic cultures of *Synechocystis* PCC6803, *Synechococcus elongatus* PCC6301 and *Nostoc* PCC7120 were cultured photoautotrophically in BG-11 medium [26], and including 0.5 M NaCl under salt stress conditions, for 2 weeks at 30 °C, under continuous white fluorescent light and bubbled with filtered air containing 1% ($^v/_v$) CO_2. Preparation of CM and ICM was performed according to the protocol published by Molitor [27].

2.8. Sequence analysis

Amino acid sequences used in this study were extracted from the NCBI-Blast (http://www.ncbi.nlm.nih.gov/BLAST/) protein data base (PHI and PSI blast). Multiple protein sequence alignments were performed by applying two different alignment programs, namely ClustalX, version 1.81, (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/) [28] with the following parameters: gap opening 10.0, gap extension 0.20 and the gonnet protein weight matrices and Kalign, version 2 (http://msa.cgb.ki.se/cgi-bin/msa.cgi) and gap opening 11.0, gap extension 0.85 and terminal gap 0.45 were set as parameters [29].

In order to analyze the N-terminal hydrophobic domain of cytochrome c_M and its putative role as either signal peptide or transmembrane helix several bioinformatic tools were used (selected organism group: gram negative prokaryotes): (i) SignalP, version 3.0 (http://www.cbs.dtu.dk/services/SignalP/) [30]; (ii) ProtCompB, version 3 (http://www.softberry.com/berry.phtml?topic=pcompb&group=programs&; subgroup=proloc); and (iii) PSORTB (http://www.psort.org/psortb/) [31].

The program Jpred (http://www.compbio.dundee.ac.uk/~www-jpred/) [32] was used to predict the secondary structure of cytochrome c_M.

3. Results and discussion

3.1. Genome and gene analysis

The present work has investigated the putative interplay of cytochrome c_M and RET in cyanobacteria. As a basis we have analyzed all 44 partially or totally sequenced cyanobacterial genomes (August 2008) for the occurrence of Cytc_6, PC and Cyt$_M$ and (aa_3-type) cytochrome *c* oxidase (COX) (for abbreviations and accession numbers see Supplemental material). Together with quinol (*bo*-type, QOX) oxidase, COX belongs to the superfamily of heme-copper oxidases and has been identified in cyanobacteria by various biochemical/physical techniques [33,34] as well as on a genetic basis [35,36]. Table 1 summarizes this analysis. QOX is included in order to underline that cyanobacteria have, besides COX, in addition other several terminal oxidases. COX appears to be the main terminal oxidase, as is reflected by its occurrence in all cyanobacteria. In diazotrophic *Nostocales* two COX paralogs are found. In addition many species contain the set of genes that encode QOX (Table 1). However, QOX lacks the binuclear Cu_A center found in subunit II of COX and thus cannot bind and oxidize soluble electron donors like Cytc_6 or PC [37]. So the present investigation focuses on COX.

Regarding (putative) electron donors for COX our genome analysis demonstrated the occurrence of usually one *petE* gene (encoding PC) in cyanobacteria (exception one *Synechococcus* sp. strain, *Thermosynechococcus elongatus* BP-1 and *Cyanothece* sp. PCC7427) (Table 1). With the exception of *Prochlorococcus marinus* str. MIT9515, all cyanobacteria contain 1–4 paralogs of cytochrome c_6 and one *cytM* gene (Table 1). Thus, cytochrome c_M, that exclusively occurs in cyanobacteria, seems to be relevant for cyanobacterial physiology.

Sequence alignment and analysis, secondary structure prediction (Fig. 1), and immunobloting (see below) suggest that these small redox proteins consist of a hydrophilic domain that anchors in membranes via an N-terminal α-helix. The soluble domain comprises three α-helices (cytochromes c_6: four α-helices) and two highly conserved regions, –CXXC*H*– (containing the proximal histidine and the cysteines involved in heme to protein linkage similar to other cytochromes *c*) at the end of helix I and the peculiar sequence –TPP*M*P– (containing the distal heme ligand methionine) between helices II and III. The succession of three prolines in the immediate neighborhood of the heme ligand suggest significant differences in the heme geometry and electron density at the heme iron compared to cytochromes c_6 of known structure [38] that have the sequence –XXMP– (Fig. 1). This is underlined by the lack of the 695 nm band in the absorption spectrum of oxidized Cytc_M (Fig. 2) that is usually viewed as diagnostic for methionine ligation at the sixth axial heme position [39] and also by its low $E^{\circ\prime}$ value of its Fe (III)/Fe(II) couple of 151 mV [15]. The calculated isoelectric points of the soluble Cytc_M domains (excluding the transmembrane helix) were calculated to vary to from 4.4 to 9.0, with no noticeable correlation between IP and cyanobacterial morphology (e.g. unicellular *versus* filamentous) or physiology (e.g. non N_2-fixing *versus* diazotrophic organisms) (Table 1).

Table 1
Putative electron donors for cytochrome *c* oxidases (COXs) in cyanobacteria

COX	QOX	Cyanobacteria	Classification	Geno mesize	N_2-fixation	Heterocysts	PC	CYT c_6	CYT c_M
1	0	*Acaryochloris marina* MBIC11017	Unclassified	6.5 Mb	N o	No	1	2	1 (8.8)
1[a]	0	*Gloeobacter violaceus* PCC7421	*Chroococcales*	4.6 Mb	No	No	2	2	1 (5.8)
1	0	*Microcystis aeruginosa* NIES-843	*Chroococcales*	5.8 Mb	No	No	1	1	1 (6.0)
1	1	*Prochlorococcus marinus* str. MIT9515	*Pleurocapsales*	1.7 Mb	No	No	1	0	0
1	0 90%	11 x *Prochlorococcus marinus*[b]	*Pleurocapsales*	1.6–2.7 Mb	No	No	1	0 35%	1
1	1 10%						1	1 45%	1 (4.4–5.9)
1							1	2 20%	1
1	0	*Synechococcus elongatus* PCC6301	*Chroococcales*	2.7 Mb	No	No	1	3	1 (8.0)
1	0	*Synechococcus elongatus* PCC7942	*Chroococcales*	2.7 Mb	No	No	1	3	1 (8.0)
1							1	1 15%	1
1	0 30%	14 x *Synechococcus sp.*[c]	*Chroococcales*	2.2–3.0 Mb	No	No	0	2 50%	1 (4.9–9.0)
1	1 70%						1	3 5%	1
1							1	4 30%	
1	1	*Synechocystis sp.* PCC6803	*Chroococcales*	3.6 Mb	No	No	1	1	1 (7.0)
1	0	*Thermosynechococcus elongatus* BP-1	*Chroococcales*	2.6 Mb	No	No	0	1	1 (5.4)
1	1	*Croscosphaera watsonii* WH8501*	*Chroococcales*	6.2 Mb	Yes	No	1	3	1 (6.9)
1	1	*Cyanothece sp.* ATCC51142	*Chroococcales*	4.9 Mb	Yes	No	1	2	1 (6.9)
1	1	*Cyanothece sp.* CCY0110*	*Chroococcales*	5.9 Mb	Yes	No	1	2	1 (8.7)
1	1	*Cyanothece sp.* PCC7427*	*Chroococcales*	6.4 Mb	Yes	No	2	2	1 (6.7)
1	0	*Cyanothece sp.* PCC8801*	*Chroococcales*	4.6 Mb	Yes	No	1	2	1 (7.8)
1	1	*Lyngbya sp.* PCC8106*	*Oscillatoriales*	7.0 Mb	Yes	No	1	2	1 (6.8)
1	1	*Trichodesmium erythraeum* IMS101	*Oscillatoriales*	7.7 Mb	Yes	No	1	2	1 (5.1)
2	2[a]	*Anabaena variabilis* ATCC29413	*Nostocales*	6.3 Mb	Yes	Yes	1	3	1 (7.0)
2	1	*Nodularia spumigena* CCY9414*	*Nostocales*	5.3 Mb	Yes	Yes	1	4	1 (6.2)
2	1[a]	*Nostoc punctiforme* PCC73102*	*Nostocales*	9.0 Mb	Yes	Yes	1	3	1 (6.0)
2	1	*Nostoc* (Anabaena) sp. PCC7120	*Nostocales*	6.4 Mb	Yes	Yes	1	2	1 (7.0)

ORFs and genes of cyanobacterial heme-copper oxidases (including quinol oxidase, QOX) and their (putative) electron carriers in 44 completely or partially (*) sequenced strains (including genome size). Nitrogen-fixing cyanobacteria are highlighted in grey, heterocyst-forming species are highlighted in dark-grey. Protparam was used to calculate isoelectric points of cytochrome c_M from proteins (excluding putative transmembrane helix).

[a] Genome analysis shows the presence of additional but incomplete operons for COX or QOX (absence of subunit III).

[b] The *Prochlorococcus marinus* genus includes following strains: Pro9601, Pro9211, Pro9215, Pro9301, Pro9303, Pro9312, Pro9313, ProNATL1A, ProNATL2A, Pro1375, Pro1986.

[c] The following strains are included in the *Synechococcus* group: Syn107*, Syn9311, Syn9605, Syn9902, SynJA23, SynJA33, Syn307, Syn7002, Syn9916*, Syn9917*, Syn5701*, Syn7803, Syn7805*, Syn8102.

3.2. Recombinant Cytc_M and Cu_A domain

In order to probe the ability of cytochrome c_M to donate electrons for COX we have cloned and expressed Cytc_M from *Synechocystis* without its N-terminal hydrophobic region. The yield of redox-active protein was about 4–5 times higher than the first expression of a Cytc_M described by Cho et al. [15], since in the present work the host was co-transformed with the genes required for cytochrome *c* maturation [21,22]. This suggests that the same chaperones are active in post-translational modification of different *c*-type cytochromes despite distinctions in heme geometry and overall structure.

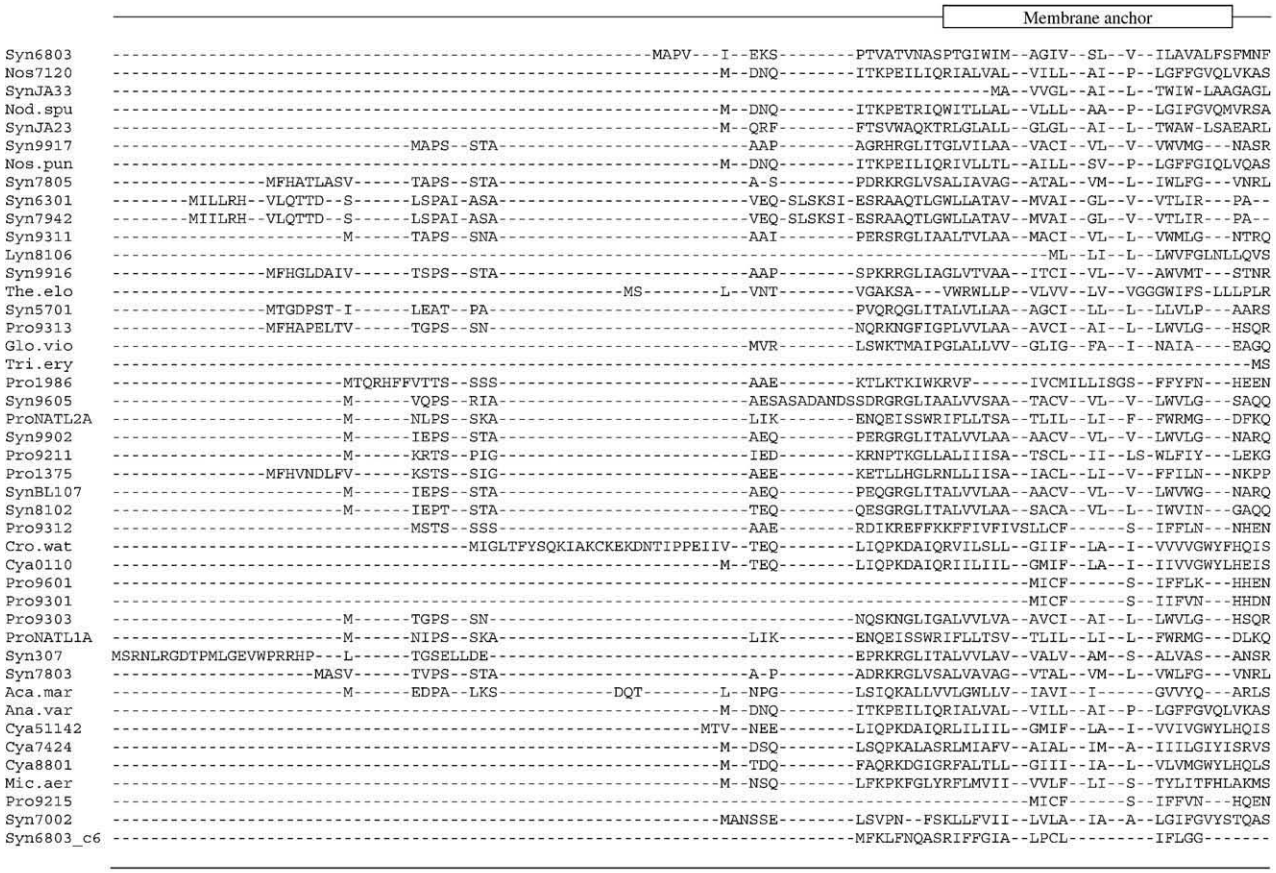
The mass obtained by spectrometric analysis (8990 Da) (see Fig. 2A) is consistent with the theoretical mass of 8975 Da calculated from the amino acid sequence (8359 Da) including the heme (616 Da) without the N-terminal methionine.

Fig. 2B shows the absorbance spectrum of reduced cytochrome c_M in comparison with the recombinant Cu_A domain in its oxidized form, which was produced as described by Paumann et al. [24]. Ferrous recombinant Cytc_M has its absorbance maxima at 550 nm (α peak) and 521 nm (β peak) in 20 mM phosphate buffer, pH 7.0, consistent with the spectral features reported in the literature [15]. The oxidized (purple) copper A domain of SUII of COX from *Synechocystis* features two strong absorbance maxima at 482 nm and 535 nm as well as two additional maxima at 359 nm and 785 nm. In its reduced form the copper protein is colorless [15]. Calculated/and measured IP values of recombinant Cytc_M and Cu_A were 7.0/5.6 [17] and 4.3/5.5 [24], respectively. In order to allow a comparative functional analysis of electron transfer between Cytc_M and the Cu_A domain, in addition Cytc_6 and PC from *Synechocystis* PCC6803 were recombinantly produced and investigated under identical conditions. The IP of both recombinant Cytc_6 and PC are very close to that of Cytc_M (5.6) [17].

3.3. Kinetics of electron transfer between cytochrome c_M and the Cu_A domain of COX

Molina-Heredia et al. have investigated the ability of recombinant *Synechocystis* Cytc_6, PC and soluble Cytc_M to reduce photosystem I by a comparative laser flash-induced kinetic analysis [17]. The obtained bimolecular rate constant for the overall reaction was up to 100 times lower with cytochrome c_M than with Cytc_6 or PC suggesting that Cytc_M does not act as electron shuttle between the two membrane complexes cytochrome b_6f and PSI in cyanobacterial ICM [17]. Here, we intended to compare these three electron carriers in their ability to donate electrons to the donor-binding and electron-entry domain (i.e. Cu_A) of SUII of COX in a comparative stopped-flow study.

Fig. 1. Amino acid sequence alignment of cyanobacterial cytochromes c_M, for abbreviations and accession numbers see Supplemental material. For comparison the sequence of cytochrome c_6 from *Synechocystis* PCC6803 is shown. The secondary structure for cytochrome c_M from *Synechocystis* was predicted by the program Jpred (http://www.compbio.dundee.ac.uk/~www-jpred/) [32]. Helices are indicated by rectangles and marked by Roman numerals. Arrows indicate the first amino acid of the soluble recombinant form of cytochrome c_M and cytochrome c_6, respectively from *Synechocystis* PCC6803.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I
II
III
-NCXXCH-
-TPPMP-
IV

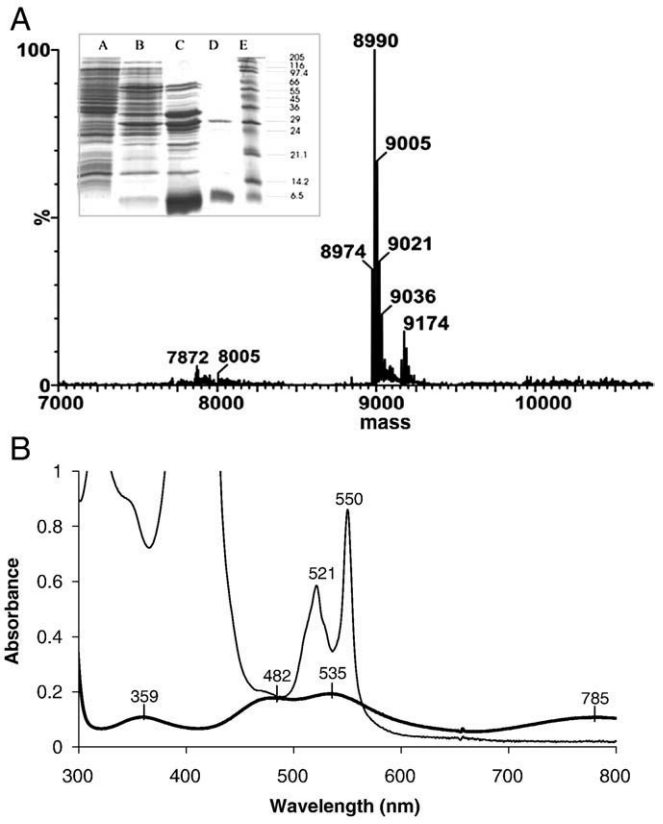

Fig. 2. (A) Mass spectrum of recombinant soluble cytochrome c_M from *Synechocystis* PCC6803. The inset depicts SDS-PAGE after Coomassie staining showing the *E. coli* cell lysate of the soluble fraction (lane A), pooled protein after DEAE chromatography (lane B), pooled protein after CM chromatography (lane C), and protein after Superdex75™ chromatography (lane D). For details see Materials and methods. (B) Absorbance spectra of reduced Cytc_M (40 μM, thin line) in 20 mM phosphate buffer, pH 7, and oxidized soluble Cu_A domain (30 μM, bold line) of COX SUII in 20 mM phosphate buffer, pH 7.

To probe the interprotein ET between recombinant Cytc_M and the Cu_A center the conventional stopped-flow technique was applied and reactions were followed either at 550 nm or by using a diode array detector attached to the stopped-flow machine. A typical time trace of the forward electron transfer reaction is displayed in Fig. 3A, which was recorded when reduced Cytc_M was mixed with 2 μM oxidized Cu_A domain. At all concentrations the observed phases could be fitted to a single exponential time course. Fig. 3B shows the resulting plot of the observed pseudo-first order rate constants (k_{obs}) *versus* cytochrome c_M concentration. The apparent rate constant (k_f) was calculated from the slope of the linear plot: $(2.4\pm0.1)\times10^5$ M^{-1} s^{-1} in 5 mM phosphate buffer, pH 7.0 containing 50 mM KCl (Fig. 3B). The ionic strength dependence of the reaction showed a (moderate) bell-shaped profile with a maximum at an ionic strength of 50 mM (Fig. 3C). Taking into account that both redox partners are slightly acidic proteins with similar IP values (5.6 and 5.5, respectively) and that the kinetic data did not suggest a kinetically detectable (transient) complex (see above), this profile might reflect a relatively unstable (not electrostatically optimised) complex between Cytc_M and the Cu_A domain that requires certain rearrangements involving hydrophobic interactions.

Reduction of oxidized Cytc_M by reduced Cu_A center was followed by an increase of absorbance at 550 nm. Reverse ET was much slower and biphasic, with a rapid phase responsible for about 70% of the absorbance increase at 550 nm. Fitting the first rapid and the following linear phase by using a single-exponential and slope equation, and plotting the first-order rate constants, k_{obs}, of the exponential phase *versus* cytochrome c_M concentration, gave linear plots (not shown) that allowed calculation of the apparent second-order rate constant for the reverse ET (k_r) reaction to be $(9.6\pm0.4)\times10^3$ M^{-1} s^{-1} (5 mM phosphate buffer, pH 7, 50 mM KCl).

Upon using k_f and k_r, an apparent equilibrium constant for the potential physiological direction (i.e. reduction of COX by electron donor) was calculated to be 21, which compares with a value of 75 calculated from thermodynamic data (i.e. published $E^{\circ\prime}$ values of Cytc_M and Cu_A) (Table 2). The observed discrepancy in K_{eq} values could result in uncertainties of $E^{\circ\prime}$ values and/or errors in estimation of k_r, the latter being evident from the fact that $k_r \ll k_f$. In any case the calculated negative free reaction enthalpies ($\Delta_rG^{\circ\prime}$) (Table 2) clearly indicate the higher stability of reduced Cu_A domain over oxidized Cytc_M which fits with $E^{\circ\prime}${Cytc_M [Fe(III)/Fe(II)]}$<E^{\circ\prime}${Cu_A [$(Cu_{A,ox})/(Cu_{A,red})$]}.

Kinetic and thermodynamic data obtained from the reactions between the Cu_A domain and Cytc_6 and PC at pH 7.0 and 20 mM KCl are summarized in Table 2. In addition, apparent rate constants with hhCyt*c* are included. The k_f values of the reaction between Cytc_M with Cu_A [i.e. $(2.0\pm0.1)\times10^5$ M^{-1} s^{-1}] is 2.7 times lower than that with cytochrome c_6 ($(5.4\pm0.3)\times10^5$ M^{-1} s^{-1}), but 4 times higher than with PC [$(5.1\pm0.2)\times10^4$ M^{-1} s^{-1}], and 14 times higher than with hhCyt*c* ($(1.37\pm0.02)\times10^4$ M^{-1} s^{-1}), respectively (Table 2). The calculated K_{eq} and $\Delta_rG^{\circ\prime}$ values of the forward reaction strongly reflect the different reduction potentials of the donors: +350 mV (Cytc_6 and PC) [17] *versus* 151 mV (Cytc_M) [15]. Generally, the calculated rate constants are one to two orders of magnitude lower

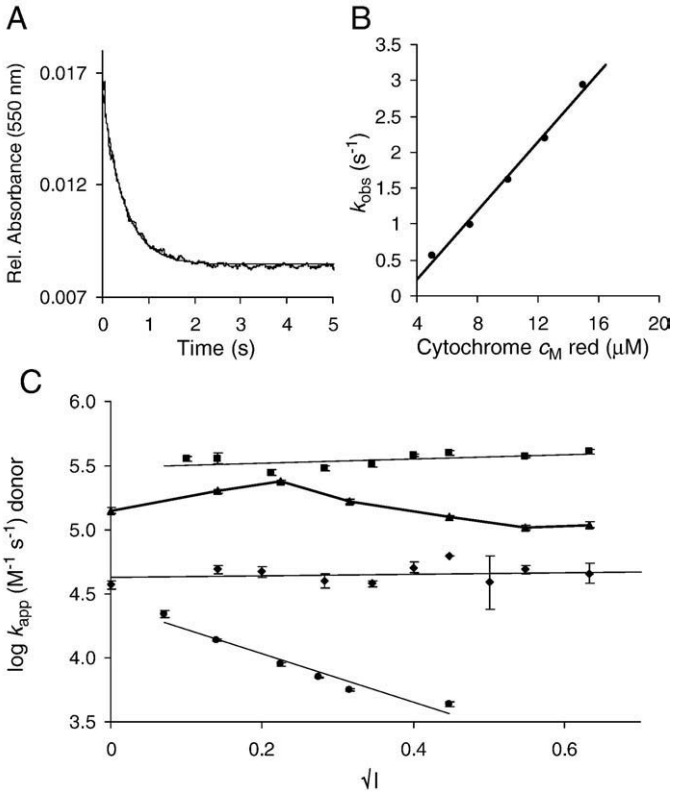

Fig. 3. Kinetics of intermolecular ET between Cytc_M and the Cu_A domain. (A) Typical time trace and fit of the forward ET reaction between 25 μM reduced Cyt c_M and 2 μM oxidized Cu_A domain. Oxidation of Cytc_M was followed at 550 nm. (B) Plot of k_{obs} values against concentration of cytochrome c_M for the forward ET reaction. Reaction conditions: 2 μM Cu_A domain, 5–30 μM Cytc_M in 5 mM phosphate buffer, pH 7.0, and 50 mM potassium chloride. (C) Dependence of the apparent bimolecular rate constant (k_f) of the reaction between *Synechocystis* Cu_A domain and plastocyanin (PC), cytochrome c_6 (Cytc_6), cytochrome c_M (Cytc_M) and horse heart cytochrome *c* (hhCyt*c*) on the square root of ionic strength ($\sqrt{I}$). Conditions: 2 μM oxidized Cu_A domain, 5–30 μM cytochromes *c* and 10–100 μM plastocyanin, respectively, 5 mM phosphate buffer, pH 7, adjusted by addition of KCl to a final ionic strength of 0–400 mM. Mean values±SD (n=4) are presented.

Table 2
Kinetic and thermodynamic parameters of the reaction between the Cu_A domain of subunit II of cytochrome *c* oxidase from *Synechocystis* PCC6803 and its putative soluble electron donors cytochrome c_6 ($Cytc_6$), plastocyanin (PC) and cytochrome c_M ($Cytc_M$)

Soluble redox carrier	Ionic strength KCl (mM)	$E^{\circ\prime}$ (mV)	$\Delta_r G^{\circ\prime}$ (kJ/mol)	Bimolecular rate constant (M^{-1} s^{-1})			
				k_f ($\times 10^4$)	k_r ($\times 10^4$)	K_{eq}*	K_{eq}**
hh Cytc	20	265 [43]	0.96	1.37±0.02	n. d.	–	1.5
$Cytc_6$	20	324 [17]	6.08	54±3	390±10	0.14	0.08
PC	20	360 [17]	9.55	5.1±0.2	85±4	0.06	0.02
$Cytc_M$	20	150 [15]	−10.71	20±1	0.96±0.04	21	75

For comparison data obtained with horse heart cytochrome *c* (hhCytc) are included. Bimolecular rate constants of reduction of oxidized Cu_A (k_f) and oxidation of reduced Cu_A (k_r) were determined by using the stopped-flow technique (5 mM phosphate buffer, pH 7.0 and 25 °C) as described in Materials and methods (n.d., not determined). Equilibrium constants (K_{eq}) were calculated from kinetic* ($K_{eq} = k_f/k_r$) and thermodynamic** data [$\Delta_r G^{\circ\prime} = -RT\ln K_{eq} = -nF\Delta E^{\circ\prime}$ and $\ln K_{eq} = -(F/RT)\Delta E^{\circ\prime}$, with F=96485 $JV^{-1}mol^{-1}$, R=8.31 JK^{-1} mol^{-1} and T=298 K]. The standard reduction potential of $E^{\circ\prime}$ of Cu_A of *Synechocystis* is 261 mV [24].

than that obtained by laser flash-induced kinetic analysis of the interaction of cytochrome c_6 and plastocyanin with preparations of intact COX from *Nostoc* PCC7119 [9]. These differences could arise from the different organisms and systems used in these studies. In any case, the bimolecular rate constant k_f of the reaction between $Cytc_M$ or $Cytc_6$ with Cu_A is similar.

Regarding the dependence of k_f on the ionic strength the following was found. In Fig. 3C the logarithms of apparent k_f values are plotted against the square root of ionic strength ($\sqrt{I}$). The apparent bimolecular rates obtained with hhCytc decrease at higher salt concentrations, which indicates that the electron transfer is affected by electrostatic interactions and this is in accordance with the isoelectric points of the reaction partners, i.e. cationic hhCytc and anionic Cu_A domain at pH 7.0. Neither the bimolecular rate constants obtained with PC nor with $Cytc_6$ show any major dependence on ionic strength, suggesting that ET is likely based on hydrophobic interactions. As already mentioned, the ionic strength dependence of $Cytc_M$ exhibits a bell-shaped profile with k_f slightly increasing to a maximum value followed by a modest decrease (Fig. 3C). Since the IP values of all three electron donors are around 5.6 and there is no pronounced dependence of reaction on ionic strength (except with hhCytc), the three cyanobacterial donor proteins could follow a similar mechanism of interaction with Cu_A.

Regarding the interaction of these donors with PSI it has been demonstrated that for both $Cytc_6$ and PC the rate constants increase with increasing salt concentrations suggesting an repulsive interaction of PSI with both donors [17], whereas in case of $Cytc_M$ rate constants with PSI were independent of ionic strength. This was explained by a completely different pattern of surface electrostatic potentials of $Cytc_6$ and PC (same distribution and orientation) and $Cytc_M$. Besides the fact that $Cytc_M$ was slowest in reaction with PSI this was taken as further evidence against a role of $Cytc_M$ as third electron donor between cytochrome b_6f and PSI [17].

3.4. Localization of cytochrome c_M

Cytochrome c_M has been postulated to be terminally processed, presumably after translocation into the lumen [13]. Depending on the actual processing site the remaining part of the N-terminus of the mature protein can be more hydrophobic than presumed and might suggest the possibility that $Cytc_M$ is an integral membrane protein. Cho et al. [15] have analyzed crude cell extracts (mixture of cytosol and membranes) of *Synechocystis* by immunoblotting using a polyclonal antibody raised against recombinant soluble $Cytc_M$ (see Fig. 6 in [15]) and the resulting band of the total protein extract in Western blot exhibited a higher MW than that of recombinant soluble (His-tagged) $Cytc_M$, which could indicate the presence of a hydrophobic tail in the native protein.

In order to get more detailed information about the actual localization of cytochrome c_M in cyanobacteria, we have analyzed the available sequences with various bioinformatic tools that allow predictions about the occurrence of signal peptide or membrane anchor. All available $Cytc_M$ sequences were analyzed and compared with cytochromes c_6, which are known to be soluble, not membrane-associate proteins. Upon using SignalP 3.0 [30] with almost all $Cytc_6$ sequences the N-terminus was attributed as signal peptide, whereas from the 43 available $Cytc_M$ sequences only 8 (with low probability) were attributed as signal peptide. Less defined was the output of the programs ProtCompB and PSORTB. With both tools the majority of the sequences were predicted to contain an N-terminal transmembrane segment, but with a relatively high percentage of sequences (25–40%) the probability was low or even the presence of a signal peptide was predicted.

Thus, in order to clarify these uncertainties we have cultured *Synechocystis* PCC6803 under normal and salt-stress conditions and probed the occurrence of $Cytc_M$ in the cytosolic protein fraction as well as in separated chlorophyll-free CM and ICM fractions by immunoblotting using a polyclonal antibody raised against

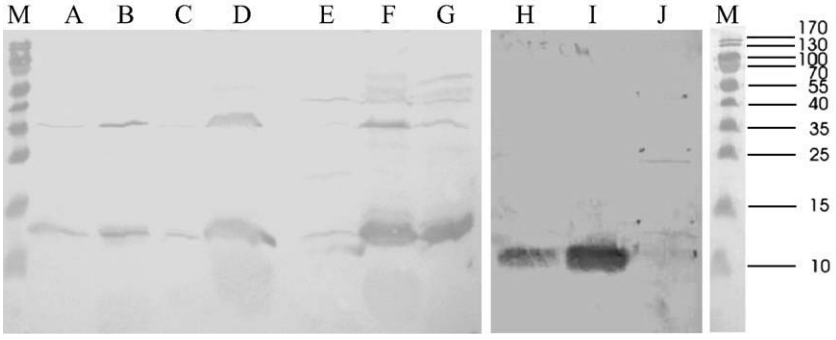

Fig. 4. (A) Analyses of various cyanobacterial membrane preparations by immunobloting using a polyclonal antibody raised against soluble cytochrome c_M shows cross-reactions at around 13 kDa. Separated cytoplasmic (CM) and thylakoid (ICM) membranes from 3 different cyanobacterial species grown under standard condition and salt stress, respectively, have been tested. Lane A: ICM, *Nostoc* PCC7120; lane B: ICM from *Synechococcus elongatus* PCC6301; lane C: CM from *Synechococcus elongatus* PCC6301; lanes D and E: ICM (D) and CM (E) from *Synechocystis* PCC6803 grown under normal conditions; lanes F and G: ICM (F) and CM (G) from *Synechocystis* PCC6803 grown under salt stress (0.5 M NaCl); lanes H and I: recombinant soluble cytochrome c_M from *Synechocystis* [0.75 μg protein (H) and 7.5 μg protein (I)]; lane J: cytochrome c_6 from *Synechocystis*; lanes M: marker proteins.

recombinant Cytc_M from *Synechocystis* (Fig. 4). The antisera recognized recombinant soluble Cytc_M in a concentration dependent manner (lanes H and I) around 9 kDa, whereas cytochrome c_6 even at high concentrations showed no cross-reaction with the antiserum (lane J). Most interestingly, although the bands are much less intensive than those obtained with the recombinant protein, membrane fractions but not cytosolic extracts gave a specific and consistent pattern of immunological cross-reactions at around 13 kDa, suggesting that Cytc_M is associated with both CM and ICM via an N-terminal membrane anchor. Generally, the cross-reactivity with ICM preparations was much more pronounced than with CM. Heterologous cross-reaction with membrane fractions of *Nostoc* PCC7120 and *Synechococcus elongatus* PCC6301 (lanes A–C in Fig. 4) suggest that this could be a structural feature common to all cyanobacteria. Comparison of lanes D and E with F and G in Fig. 4 indicates that salt stress induces expression of cytochrome c_M. Thus, enhancement of expression of Cytc_M in stress conditions is a general phenomenon irrespective of the applied stress, being either low temperature or high-intensity light [18,19] or salt-stress (present work). It has to be mentioned that in the ICM fractions (see Fig. 4) another band is visible at around 36 kDa. Formation of dimeric cytochrome c_M can be excluded since size exclusion chromatography unequivocally showed the presence of a monomer. Whether this is an association of Cytc_M with another membrane protein due to incomplete solubilization and dissociation is not clear at the moment. Clearly more work is needed to elucidate the exact (co-) localization of Cytc_M in cyanobacterial membranes.

3.5. Conclusion

Genes encoding cytochrome *c* oxidase and cytochrome c_M are found in all so far sequenced cyanobacteria (except one strain) (Table 1). The comparative stopped-flow study demonstrates that Cytc_M can function as electron donor to Cu_A at rates similar to Cytc_6 but faster than PC or hhCyt*c*. COX is well known to be localized in both ICM and CM [3] and the present analysis indicates that also Cytc_M is associated with both distinct bioenergetically competent cyanobacterial membrane systems. These findings – together with the low efficiency of Cytc_M in PSI reduction [17] – suggest that Cytc_M might function as electron donor for COX. However, from a thermodynamic point of view, it is difficult to see how Cytc_M, with a midpoint redox potential of +151 mV [15], could be reduced by cytochrome b_6f, with a redox potential of +320 mV [16].

Under stress conditions it has been demonstrated that oxygen consumption is significantly enhanced [10,20] and COX is the primary terminal oxidase under these conditions [3,40]. Especially the pivotal role of the CM and its respiratory chain for the adaptation of cyanobacterial growth to stress conditions has been pointed out in numerous physiological [3,10,41] and ultrastructural [42] studies. Additionally, under stress conditions cyanobacteria usually shut down PET and also suppress the expression of both Cytc_6 and PC [18,19], whereas expression of *cytM* (that is scarcely expressed under normal growth conditions) is enhanced [18,19]. This gives further evidence that cytochrome c_M participates in COX reduction. Its function could be to act as electron sink in particular conditions like light, temperature or salt stress, thereby diverting the electron flow (via plastoquinol?) towards COX.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2008.12.003.

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