Kinetics of interprotein electron transfer between cytochrome c_6 and the soluble Cu_A domain of cyanobacterial cytochrome *c* oxidase

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Received 30 June 2004; revised 26 August 2004; accepted 26 August 2004

Available online 11 September 2004

Edited by Stuart Ferguson

Abstract Cytochrome c_6 is a soluble metalloprotein located in the periplasmic space and the thylakoid lumen of many cyanobacteria and is known to carry electrons from cytochrome b_6f to photosystem I. The Cu_A domain of cytochrome *c* oxidase, the terminal enzyme which catalyzes the four-electron reduction of molecular oxygen in the respiratory chains of mitochondria and many bacteria, also has a periplasmic location. In order to test whether cytochrome c_6 could also function as a donor for cytochrome c oxidase, we investigated the kinetics of the electron transfer between recombinant cytochrome c_6 (produced in high yield in *Escherichia coli* by coexpressing the maturation proteins encoded by the *ccm*A-H gene cluster) and the recombinant soluble Cu_A domain (i.e., the donor binding and electron entry site) of subunit II of cytochrome *c* oxidase from *Synechocystis* PCC 6803. The forward and the reverse electron transfer reactions were studied by the stopped-flow technique and yielded apparent bimolecular rate constants of $(3.3 \pm 0.3) \times 10^5$ M^{-1} s^{-1} and $(3.9 \pm 0.1) \times 10^6$ M^{-1} s^{-1}, respectively, in 5 mM potassium phosphate buffer, pH 7, containing 20 mM potassium chloride and 25 °C. This corresponds to an equilibrium constant K_{eq} of 0.085 in the physiological direction ($\Delta_r G'^0 = 6.1$ kJ/mol). The reduction of the Cu_A fragment by cytochrome c_6 is almost independent on ionic strength, which is in contrast to the reaction of the Cu_A domain with horse heart cytochrome c, which decreases with increasing ionic strength. The findings are discussed with respect to the potential role of cytochrome c_6 as mobile electron carrier in both cyanobacterial electron transport pathways.

Keywords: Cytochrome c_6; Electron transfer; Photosynthesis; Respiration; Cyanobacterium; *Synechocystis* PCC 6803; Cytochrome oxidase; Cu_A domain; Stopped-flow kinetics

1. Introduction

The electron carrier cytochrome c_6 (Cyt c_6), formerly known as algal cytochrome c_{553}, is a member of the ubiquitous family of cytochrome *c* proteins. The high potential monoheme protein of class I is mainly characterized by its highly conserved N-terminal heme-coordination sequence C–X–X–C–H and a low-spin heme-iron with methionine and histidine as axial ligands [1,2]. It serves as intrathylakoidal one-electron shuttle between cytochrome b_6f and photosystem I (PSI) of cyanobacteria and various algae [3]. Database analysis has also revealed a gene for Cyt c_6 in higher plants but significant differences to cyanobacterial or algal Cyts c_6 suggest a modified function [4,5]. In higher plants, the analogous monocopper protein plastocyanin (PC) replaces Cyt c_6, whereas many cyanobacteria and algae alternate between PC and Cyt c_6 in response to iron and copper availability [6,7].

In cyanobacteria, both oxygenic photosynthesis and aerobic respiration are uniquely accommodated within a single prokaryotic cell [8]. In the cyanobacterial thylakoid membrane (ICM), both photosynthetic and respiratory electron transfer (ET) are taking place. They share the plastoquinone pool and the cytochrome b_6f complex. It has long been argued [9,10] and recently corroborated [11] that the normal functioning of the cyanobacterial photosynthetic and respiratory chains obligatorily depends on either Cyt c_6 or PC (see Fig. 1) [9–11]. Both metalloproteins possess similar physicochemical and surface structural properties, which make them capable of replacing each other [12,13]. The location and functional activity of Cyt c_6 and PC in the intrathylakoid space has been firmly established, still under discussion is the nature of the periplasmic mobile carrier serving as electron donor to cytochrome *c* oxidase (C*c*O) in the cytoplasmic membrane (CM), which contains only respiratory chain components and no photosynthetic reaction centers [10,14]. The occurrence of PC in the periplasmic space of cyanobacteria has not yet been proved, but a periplasmic cytochrome c_6 was identified as a potential donor to CM-bound C*c*O [15,16].

In contrast to the abundance of knowledge regarding the direct interactions of Cyt c_6 and PC with the cytochrome b_6f complex and PSI in cyanobacteria [13,17,18], nothing is known about the mode of direct interaction between Cyt c_6 and cyanobacterial aa_3-type cytochrome oxidase. Therefore, the present stopped-flow kinetic analysis was performed. Since this

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Abbreviations: Cyt c_6, cytochrome c_6; hh Cyt *c*, horse heart cytochrome *c*; C*c*O, cytochrome *c* oxidase; SUII, subunit II; PC, plastocyanin; PSI, photosystem I; ET, electron transfer; PCC, Pasteur Culture Collection; CM, cytoplasmic (plasma) membrane; ICM, intracytoplasmic (thylakoid) membrane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylene diamine tetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride

0014-5793/$22.00
doi:10.1016/j.febslet.2004.08.070

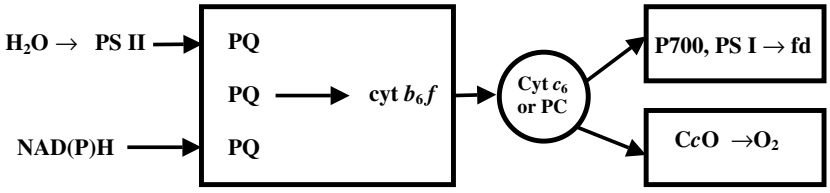

Fig. 1. The dual-function of the photosynthetic and respiratory ET assembly in cyanobacterial ICM. As demonstrated recently [11], either Cyt c_6 or PC is absolutely indispensable for integral electron transport in both photosynthesis and respiration. C*c*O, cytochrome *c* oxidase; PSI, photosystem I; PSII, photosystem II; fd, ferredoxin; PQ, plastoquinone.

technique requires much protein and conventional heterologous expression of Cyt c_6 in *Escherichia coli* resulted in relatively poor yields [19,20], the *ccmA-H* gene cluster from *E. coli* was co-expressed with the *petJ* gene, which allowed expression of Cyt c_6 in the periplasm of *E. coli* at high levels [21–23]. Here, we report (i) the expression of *Synechocystis* Pasteur Culture Collection (PCC) 6803 cytochrome c_6 in the *E. coli* periplasm, carried out with coexpression of the *E. coli* cytochrome *c* maturation genes, its purification and characterization, (ii) the kinetics of electron transfer between Cyt c_6 and the soluble Cu_A domain of *Synechocystis* C*c*O subunit II (SUII), and (iii) the effect of ionic strength on the interprotein ET rate. The findings are discussed with respect to the physiological role of respiration in cyanobacterial metabolism.

2. Materials and methods

2.1. Materials

Horse heart cytochrome *c* (hh Cyt *c*), standard chemicals and biochemicals were obtained from Sigma Chemical Co. at the highest grade available. Platinum Pfx DNA polymerase was from Invitrogen and DNA ligase from MBI Fermentas; restriction enzymes (*Nco*I, *Bam*HI) and alkaline phosphatase were purchased from New England Biolabs, pET-27b(+) expression vector was from Novagen; GFX PCR DNA and Gel Band Purification Kit, Phenyl Sepharose 6 Fast Flow (high sub), Superdex 75 HR 10/30 FPLC column and Chelating Sepharose Fast Flow were purchased from Amersham Pharmacia Biotech.

2.2. Cloning and heterologous overexpression

The DNA and deduced amino acid sequence of Cyt c_6 were provided by Cyanobase, the genome database for *Synechocystis* sp. PCC 6803 (http://www.kazusa.or.jp/cyano/Synechocystis). Cloning, heterologous overexpression and purification of the soluble domain of SUII of *Synechocystis* C*c*O have been described by Paumann et al. [24].

2.3. Periplasmic coexpression of Cyt c_6 and cytochrome c maturation proteins and purification of Cyt c_6

For the periplasmic expression of the amino acid residues 36–120 of the *petJ* gene, primers were designed excluding the first 35 amino acids which represent the signal peptide [20]. The synthetic oligonucleotide primers were purchased from genXpress (Maria Wörth, Austria). Primer 1 (5′-CAT GCC ATG GCG GAC CTA GCC CAC GGT AAA GCT ATC-3′) introduced an *Nco*I restriction site, an ATG start codon and removed a naturally occurring *Nco*I restriction site. With primer 2 (5′-CGC GGA TCC CTA CCA GCC CTT TTC AGC TTG ATC-3′), a *Bam*HI restriction site and a TAG termination codon were introduced. A cell suspension of *Synechocystis* PCC 6803 grown in BG11 medium [25] for three weeks at 30 °C in a shaker under illumination was used as a template for PCR. PCR was carried out under the following conditions: 95 °C for 2 min, hot start; 30 cycles of 94 °C for 40 s, 55 °C for 30 s, 68 °C for 30 s; followed by a final step of 68 °C for 10 min. A single PCR product of the expected size (276 bp) was obtained and purified using the GFX PCR DNA and Gel Band Purification Kit. The PCR product was digested with the restriction enzymes *Nco*I and *Bam*HI and cloned into the *Nco*I and *Bam*HI digested, alkaline phosphatase-treated expression vector pET-27b(+) [26], which produces a signal sequence fusion to facilitate protein translocation into the periplasm. The insert was sequenced by the dideoxy chain termination method [27]. Competent *E. coli* BL21 Star was transformed with the pET-27b(+) by electroporation (Gene Pulser, Bio-Rad). Positive clones carrying the recombinant plasmid were selected by PCR and transformed with the pEC86 plasmid which encodes the *ccm*A-H gene cluster of *E. coli*. In *E. coli*, cytochrome *c* maturation is achieved by the Ccm (cytochrome *c* maturation) proteins which are organized in a membrane-bound protein complex which selects for apocytochrome *c* and reduces and covalently links it to heme in a stereospecific way [28,29]. Selected clones were grown overnight in standard Luria Bertani (LB) medium [30] containing 50 μg/ml kanamycin and 25 μg/ml chloramphenicol on an orbital shaker at 200 rpm and 37 °C. LB medium supplemented with the same antibiotics was inoculated with the overnight culture in a 1:50 ratio and grown at 37 °C and 200 rpm to an $OD_{600} = 1.6$. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM and carried out at room temperature on an orbital shaker at 200 rpm. 0.1% glycerol was added at the time of induction. 18 h after the induction, cells were harvested by centrifugation ($5000\times g$, 10 min, room temperature) and stored at −80 °C.

The cell pellet (obtained from 1 l *E. coli* culture) was thawed and resuspended on ice in 100 ml of 200 mM sodium borate buffer, pH 8.0, 160 mM NaCl, 1 mM ethylene diamine tetraacetic acid, 5 μM leupeptin, 5 μM pepstatin A and 1 mM phenylmethylsulfonyl fluoride. The suspension was kept on ice for 2 h. The spheroplasts were sedimented by 20 min of centrifugation at $4500\times g$. Cyt c_6 was purified from the periplasmic fraction by column chromatography in two steps.

To the periplasmic fraction solid ammonium sulfate was added to a final concentration of 0.7 M. The fraction was loaded on a Phenyl Sepharose 6 Fast Flow (high sub) column (1.6×15 cm), which was equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 0.8 M ammonium sulfate. After washing with equilibration buffer Cyt c_6 was eluted with 50 mM potassium phosphate buffer, pH 7.0. The Cyt c_6 fraction was concentrated with Centriprep concentrators YM-3 and loaded on a Superdex 75 HR 10/30 FPLC column, which was equilibrated with 67 mM potassium phosphate buffer, pH 7.0, containing 150 mM KCl. Cyt c_6 fractions were pooled, concentrated and stored at −80 °C. All steps with the exception of the Superdex 75 column were carried out at 4 °C.

2.4. Mass spectrometry

Mass spectrometry was performed on a Waters Micromass Q-TOF Ultima Global with offline infusion using PicoTips™ (New Objectives, MA). For tuning and calibration purposes Glu-Fibrinopeptide B (Sigma, Vienna) was used. Spectra were acquired under the following conditions: Capillary voltage: 1.5 kV, cone voltage: 35 V, cone gas: 60 l/h, sample solution was delivered by a syringe pump at a flow rate of approx. 300 nl/min. Prior to analysis, samples were diluted in the same volume of acetonitrile containing 1% formic acid. The protein concentration in this solution was about 40 pmol/μl. An *m/z* range from 600 to 2000 was analyzed. Spectra were deconvoluted by the MaxEnt1 function of MassLynx 4.0 software.

2.5. Reduction of Cyt c_6 and the soluble Cu_A domain

The metalloproteins were reduced by the addition of a 10-fold molar excess of sodium ascorbate, which was subsequently removed by gel filtration (PD-10 columns from Amersham-Pharmacia). The proteins were always reduced freshly before measurements and kept on ice in

the dark. Under these conditions, the metalloproteins were insensitive to autoxidation.

2.6. Spectral and kinetic investigations

Steady-state spectrophotometric measurements were made on a diode-array spectrophotometer Specord S10 (Zeiss) and a Hitachi Model U-3000 spectrophotometer, respectively. The concentration of fully reduced and desalted Cyt c_6 was determined using the extinction coefficients of 23 300 M^{-1} cm^{-1} at 553 nm (this work) or 6990 M^{-1} cm^{-1} at 280 nm (calculated from the known amino acid composition according to Pace et al. [31]). The concentration of the oxidized desalted protein was determined spectrophotometrically after the addition of solid sodium ascorbate to the cuvette, applying the extinction coefficient of 23 300 M^{-1} cm^{-1} at 553 nm. The concentration of the soluble Cu_A center was determined by using the extinction coefficient of 3080 M^{-1} cm^{-1} at 535 nm [24].

Stopped-flow measurements were made using the model SX-18MV stopped-flow spectrophotometer from Applied Photophysics equipped with a 1 cm observation cell thermostated at 25 °C. Calculation of pseudo-first-order rate constants (k_{obs}) from experimental traces at 553 nm was performed with the SpectraKinetic workstation v4.38 interfaced to the instrument. The kinetics of both the oxidation of reduced Cyt c_6 by the recombinant Cu_A domain (forward ET reaction) and the reduction of oxidized Cyt c_6 by the reduced Cu_A domain (reverse ET reaction) was followed in the single mixing mode at 553 nm (peak of reduced Cyt c_6). Various ionic strengths were adjusted by addition of KCl to 5 mM potassium phosphate buffer (pH 7) to final ionic strengths of 10, 20, 45, 80, 120, 160, 200, 300 and 400 mM. The first data point was recorded 1.5 ms after mixing 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. The reactions were also investigated using the diode-array detector (Applied Photophysics PD.1) attached to the stopped-flow machine and the XScan Diode Array Scanning v1.07 software.

3. Results and discussion

3.1. Expression of Cyt c_6 in E. coli

The *petJ* gene coding for mature Cyt c_6 (amino acid residues 36–120) was fused to the pelB leader sequence of the pET-27b(+) plasmid in order to achieve the export of apocytochrome c_6 into the periplasmic space concomitant with the cleavage of the signal sequence. Coexpression of the *ccm*A-H gene cluster resulted in a high protein yield of mature Cyt c_6. Approximately 8 mg Cyt c_6 per 1 l cell culture could be separated successfully from other periplasmic proteins in two chromatographic steps. To the best of our knowledge, this is the highest protein yield of recombinant *Synechocystis* Cyt c_6 that has been reported so far. The purified Cyt c_6 was obtained in its reduced state and showed to be insensitive to autoxidation. By contrast, only about 300 μg/l Cyt c_6 in its oxidized state could be purified when Cyt c_6 was expressed in the periplasm without the Ccm proteins (not shown). These findings clearly demonstrate the necessity of the coexpression of the Ccm proteins for proper cytochrome c_6 maturation in high yield.

3.2. Spectral characterization of cytochrome c_6 and the Cu_A domain

The mobility of purified recombinant Cyt c_6 during sodium dodecyl sulfate–polyacrylamide gel electrophoresis (inset to Fig. 2) is similar to that of native type Cyt c_6 purified from *Synechocystis* (apparent molecular mass of about 6.5 kDa) [20]. The mass obtained by mass spectrometric analysis (9434.5 Da) is consistent with the theoretical mass of 9436.9 Da [i.e., the mature apoprotein with an N-terminal methionine plus

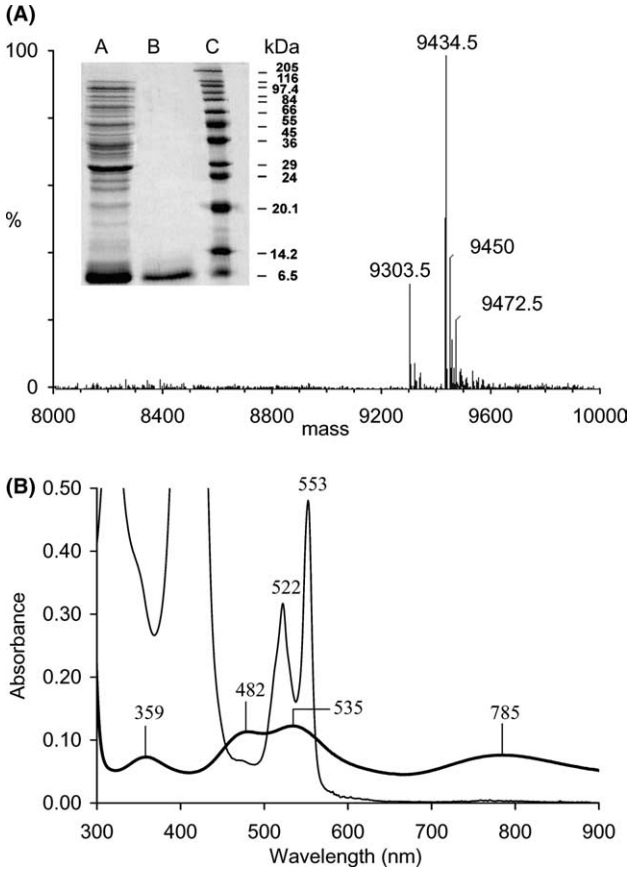

Fig. 2. (A) Mass spectrum of cytochrome c_6 from *Synechocystis* PCC 6803. The inset shows the polypeptide pattern (Coomassie-stained) of cytochrome c_6 preparations after different purification steps. Lane A, Cyt c_6 fraction after Phenyl Sepharose 6 Fast Flow column (15 μg protein); lane B, Cyt c_6 fraction (2 μg protein) after Superdex 75 column; C, molecular mass standard. (B) Absorbance spectra of 20 μM Cyt c_6 (thin line) in 50 mM potassium phosphate buffer, pH 7, and 40 μM soluble Cu_A domain of C*c*O SUII (bold line) in 20 mM potassium phosphate buffer, pH 7.

heme] (Fig. 2A). The second most dominant mass spectrometric peak of 9303.5 Da could originate from the mature protein which has lost its methionine at the N-terminus. The theoretical isoelectric point of Cyt c_6 is 5.47 (using ExPASy Molecular Biology Server at http://us.expasy.org/tools/pi_tool.html).

In its reduced state at neutral pH, recombinant Cyt c_6 exhibits absorbance maxima at 553 nm (α peak), 522 nm (β peak), 416 nm (Soret or γ peak) and 318 nm (δ peak) (Fig. 2B). Upon oxidation with ferricyanide the Soret peak shifts to 410 nm combined with hypochromicity, whereas the α and β peak convert into one broad peak with a maximum at 525 nm concomitant with hypochromicity. The δ peak disappears, whereas a peak at 360 nm is observed instead. These spectral features are identical to those reported for native cytochrome c_6 purified from *Synechocystis* [12]. The extinction coefficient at pH 7.0 was determined to be 23 300 M^{-1} cm^{-1} at 553 nm for reduced Cyt c_6. The absorbance ratio $A_{416}/A_{280\,nm}$ of recombinant Cyt c_6 of 7.3 underlines both its purity and high heme occupancy at the active site.

In its oxidized form the Cu_A domain has a characteristic purple color exhibiting two strong absorbance maxima at 482

nm ($\varepsilon = 2820\ M^{-1}\ cm^{-1}$) and 535 nm ($\varepsilon = 3080\ M^{-1}\ cm^{-1}$), as well as two additional maxima at 359 and 785 nm (Fig. 2B). In its reduced form the copper protein is colorless [24].

3.3. Kinetics of electron transfer between cytochrome c_6 and the Cu_A domain

Since ascorbate was used for the reduction of both Cyt c_6 (forward ET reaction) and the Cu_A center (reverse ET reaction), we have also determined the second-order rate constants for the ascorbate reduction of oxidized Cyt c_6 and the Cu_A domain. These reactions are relatively slow when compared to the interprotein ET (see below). The rate constants were calculated to be $(3.1 \pm 1) \times 10^2$ and $(3.9 \pm 0.4) \times 10^2\ M^{-1}\ s^{-1}$, respectively. Ascorbate was routinely removed from the reduced proteins by size exclusion chromatography but any remaining traces could not have re-reduced Cyt c_6 or the Cu_A domain in any of the experiments reported in the present paper.

In order to investigate the kinetics of interprotein ET, the conventional stopped-flow technique was applied and reactions were followed at 553 nm as well as by using the diode-array detector attached to the stopped-flow machine. Fig. 3A shows a typical time trace, which was obtained when reduced Cyt c_6 was mixed with 2 μM oxidized Cu_A center (forward ET reaction). At all concentrations the observed phases could be fitted to a single-exponential time course. The clear isosbestic points at 337, 433 and 561 nm, which are obtained by following the forward reaction with the diode-array detector (not shown), strongly suggest a direct ET reaction between Cyt c_6 and the copper protein. Furthermore, the decrease of absorbance at 553 nm (oxidation of Cyt c_6) fully corresponded to the decrease at 785 nm (formation of reduced Cu_A center) when the reaction was followed in a diode-array spectrophotometer. The results of the forward ET reaction are shown in Fig. 3C in which the observed fitted rate constants are plotted as a function of Cyt c_6 concentration. From the slope of the linear plot the apparent bimolecular rate constant was determined to be $(3.3 \pm 0.3) \times 10^5\ M^{-1}\ s^{-1}$ in 5 mM potassium phosphate buffer, pH 7, containing 20 mM potassium chloride, which is approximately 16 times faster than the reaction between reduced hh Cyt c and the Cu_A domain [24].

Reduction of oxidized Cyt c_6 by the reduced Cu_A domain was followed by an increase of absorbance at 553 nm. Reverse ET was faster and biphasic, with a rapid first phase responsible for about 85% of the absorbance increase at 553 nm. A typical time trace is shown in Fig. 3B. Fitting the first rapid phase by using a single-exponential equation from which pseudo-first-order rate constants, k_{obs}, were obtained, showed that the k_{obs} values linearly increased with the concentration of Cyt c_6 (Fig. 3D). The slope yielded the apparent second-order rate constant for the reverse ET reaction, namely $(3.9 \pm 0.1) \times 10^6\ M^{-1}\ s^{-1}$ in 5 mM potassium phosphate buffer, pH 7, containing 20 mM potassium chloride.

From forward and reverse bimolecular rate constants, an apparent equilibrium constant for the physiological direction was calculated to be 0.085 corresponding to a reaction Gibbs energy ($\Delta_r G'^0$) of 6.1 kJ/mol (25 °C), which is similar to values published for other c-type cytochromes and soluble Cu_A domains [32,33]. Thermodynamically this indicates a higher stability of reduced Cyt c_6 over oxidized Cu_A domain, but it has to be kept in mind that the Cu_A domain is only the electron entry site of CcO. In an intact aa_3 oxidase, the following in-

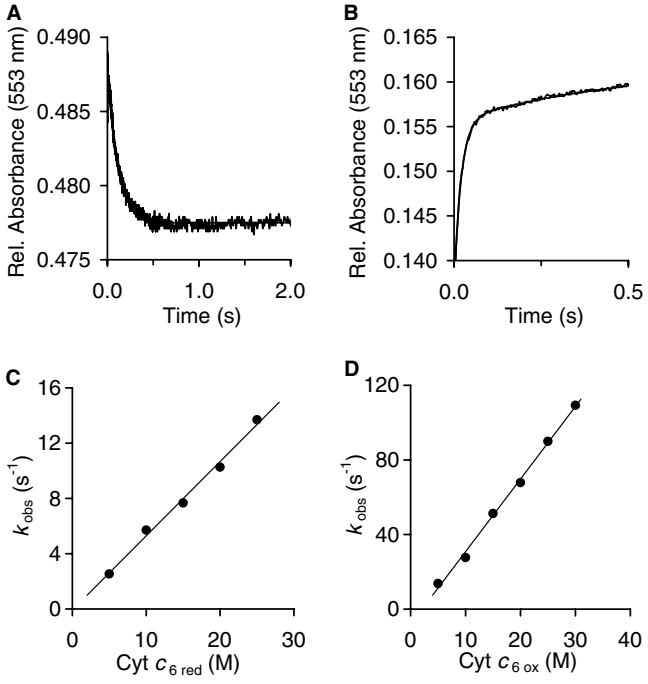

Fig. 3. Kinetics of intermolecular ET between Cyt c_6 and the Cu_A domain. (A, B) Typical time traces of the absorbance change of the forward (A) and reverse (B) ET reaction, respectively, using 15 μM Cyt c_6 and 2 μM Cu_A domain. The oxidation and reduction of Cyt c_6 was followed at 553 nm. (C, D) Plots of k_{obs} values against concentration of Cyt c_6 for the forward (C) and reverse (D) ET reaction. Reaction conditions: 2 μM Cu_A domain, 5–25 μM reduced Cyt c_6 (forward reaction) and 5–30 μM oxidized Cyt c_6 (reverse reaction) in 5 mM potassium phosphate buffer, pH 7.0, and 20 mM potassium chloride.

tramolecular ET reactions from the Cu_A center to the low spin heme *a* and the a_3-Cu_B center that eventually account for O_2 reduction are exergonic thus driving the thermodynamically unfavorable reaction in the physiological direction. Therefore, the findings clearly suggest that cytochrome c_6 acts as an electron donor to both PSI and cytochrome *c* oxidase.

With $\Delta_r G'^0 = 6.1$ kJ/mol and the known standard reduction potential of cytochrome c_6 from *Synechocystis* (324 mV) [12] the standard reduction potential E'^0 of the Cu_A domain can be calculated by using $-\Delta_r G'^0/nF = \Delta E'^0$ with $n = 1$ and $F = 96\,860$ J V^{-1} mol^{-1}. The obtained value of 261 mV for E'^0 ($Cu_{A_{ox}}/Cu_{A_{red}}$) is similar to the values published for other soluble Cu_A domains [34,35].

In order to investigate the electrostatic nature of the interaction, a detailed analysis of the effect of ionic strength on the interprotein electron transfer rate was performed. The apparent bimolecular rate constant of the reaction between hh Cyt *c* and the Cu_A domain was determined to be 2.1×10^4 M^{-1} s^{-1} at pH 7 and low ionic strength [24] and the rates decrease at higher salt concentrations. In Fig. 4, the logarithms of k_{app} are plotted against the square root of ionic strength ($I^{0.5}$). The decrease of the rates in this Bronstedt plot indicates the existence of attractive electrostatic interactions between the reaction partners that are overwhelmed at high ionic strength. Using the Bronsted law $\log k_{app} = \log k_0 + 2Bz_A z_B \sqrt{I}$, the number of charges on the protein interfaces that control the interaction between the two proteins can be estimated: k_{app} is the bimolecular rate constant at ionic strength *I*, k_0 is the bimolecular rate constant at $I = 0$, and *B* is a temperature dependent term, whose value at 25 °C is about 0.6. The slope of the plot (the $z_A z_B$ product with z_A and z_B representing the electron transfer sensitive charges on the protein surfaces) is −1.9, which indicates that about one effective charge of opposite sign on each protein interface interacts in the reaction between *Synechocystis* Cu_A domain and hh Cyt *c*. This is evident by looking at the isoelectric points of the reaction partners, with hh Cyt *c* being a cationic protein with a strong positive surface potential and *Synechocystis* Cu_A domain being anionic at pH 7 [24]. By contrast, the apparent bimolecular rate constants of the reaction between cytochrome c_6 and the Cu_A domain do not show any major dependence on ionic strength. The slope of the plot is +0.2. At each ionic strength, the observed rate constants (k_{obs}) for Cu_A reduction increase with increasing donor protein concentration indicating that there is no formation of any kinetically detectable (or long-lived) intermediate complex between the two proteins. No electrostatically governed pre-orientation seems to occur with the *Synechocystis* protein couple. The structure of the cyanobacterial Cu_A fragment is unknown, but sequence analysis, secondary and tertiary structure prediction [24] showed several peculiarities of the cyanobacterial C*c*O SUII, which could be responsible for the observed interaction mode. Several conserved acidic amino acids of *Paracoccocus* or bovine heart C*c*O, which have been shown to be important in binding the donor cytochrome *c* [33,36], are not present in *Synechocystis* C*c*O. The putative electron entry in cyanobacterial C*c*O SUII (Tyr179 corresponding to Trp149 in *P. denitrificans* C*c*O SUII) is part of a conserved sequence rich in aromatic amino acids, and whether the *Synechocystis*-specific insertion (Pro120-Gly166) and the extended C-terminus contribute to the interaction with the donor is unknown at the moment [24].

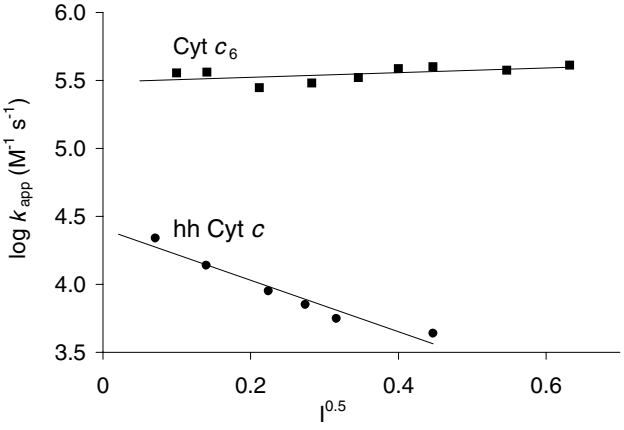

Fig. 4. Dependence of the apparent bimolecular rate constant (k_{app}) of the reaction of *Synechocystis* Cu_A domain with (■) Cyt c_6 and with (●) hh Cyt *c* on ionic strength (*I*). Conditions for the reaction with Cyt c_6: 2 μM oxidized Cu_A domain, 5–25 μM reduced cytochrome c_6, 5 mM potassium phosphate buffer, pH 7.0, adjusted by the addition of KCl to the final ionic strengths of 10–400 mM. Conditions for the reaction with hh Cyt *c*: 2 μM oxidized Cu_A domain, 5–30 μM reduced hh cytochrome *c*, 5 mM potassium phosphate buffer, pH 7.0, adjusted by the addition of KCl to final ionic strengths of 5–200 mM.

3.4. Physiological relevance

Our work clearly showed that the proteinaceous maturation factors, the Ccm proteins, of *E. coli* which mediate the transfer and assembly of *E. coli* cytochrome *c*, can also mediate the biosynthesis of a cyanobacterial cytochrome c_6.

Both cytochrome c_6 and the Cu_A domain of C*c*O are located on the P-side of the CM and the ICM of *Synechocystis* [10]. The present study strongly suggests that Cyt c_6 is at least one of the endogenous electron donors of C*c*O. In contrast to the periplasm, in the thylakoid lumen Cyt c_6 can deliver electrons to two completely different acceptors, namely PSI and cytochrome *c* oxidase (Fig. 1). At low ionic strength and pH 7, the bimolecular rate constant determined for the reduction of the Cu_A domain by Cyt c_6 is smaller than PSI reduction by Cyt c_6 (8.6×10^6 M^{-1} s^{-1}) and the difference in rates increases with increasing ionic strength [17]. In the case of PSI reduction by Cyt c_6 this was interpreted by a repulsive interaction between the two proteins and finally underlined by site-directed mutagenesis [17]. The mode of interaction between Cyt c_6 and the Cu_A fragment is different and most probably based on hydrophobic interactions similar to the *Thermus thermophilus* ba_3 oxidase, but different to C*c*O of *Paracoccus denitrificans*, in which the primary electrostatic encounter closely matches the mitochondrial situation [33,36].

Though cyanobacteria are phototrophic organisms par excellence, respiration is needed for the generation of maintenance energy in the dark and whenever photosynthesis does not work such as in certain stress conditions. Irrespective of the stress type, both activity and concentration of respiratory components increase significantly in CM and/or ICM in a species-dependent manner, while photosynthesis slows down [37]. In these periods, respiratory electron transport temporarily substitutes for the lack of ATP supply from photophosphorylation. Cyt c_6 as the common mobile carrier facilitates rapid switching between photosynthesis and respiration.

Acknowledgements: We thank Linda Thoeny-Meyer for the generous gift of the pEC86 plasmid and Daniel Kolarich for performing mass spectrometry measurements with the Q-TOF Global, a donation of the Austrian Council for Research and Technology Development. This work was supported by the Austrian Science Foundation (FWF Project P13069-CHE).

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