Respiratory cytochrome *c* oxidase can be efficiently reduced by the photosynthetic redox proteins cytochrome c_6 and plastocyanin in cyanobacteria

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Abstract Plastocyanin and cytochrome c_6 are two small soluble electron carriers located in the intrathylacoidal space of cyanobacteria. Although their role as electron shuttle between the cytochrome b_6f and photosystem I complexes in the photosynthetic pathway is well established, their participation in the respiratory electron transport chain as donors to the terminal oxidase is still under debate. Here, we present the first time-resolved analysis showing that both cytochrome c_6 and plastocyanin can be efficiently oxidized by the aa_3 type cytochrome *c* oxidase in *Nostoc* sp. PCC 7119. The apparent electron transfer rate constants are ca. 250 and 300 s^{-1} for cytochrome c_6 and plastocyanin, respectively. These constants are 10 times higher than those obtained for the oxidation of horse cytochrome *c* by the oxidase, in spite of being a reaction thermodynamically more favourable.

Keywords: Cyanobacteria; Cytochrome c_6; Cytochrome *c* oxidase; Photosynthesis; Plastocyanin; Respiration

1. Introduction

Photosynthesis and respiration are physically separated in eukaryotic cells but share a common plastoquinone pool and cytochrome (Cyt) b_6f complex in cyanobacteria [1]. In the photosynthetic electron transport chain, it is well established that plastocyanin (Pc) and Cyt c_6 act as alternative redox carriers between the membrane complexes b_6f and photosystem I, with their expression levels being regulated by copper availability: Cyt c_6 and Pc are produced in the absence and presence of copper, respectively [2]. In the respiratory electron transport chain, however, the nature of the electron donor to Cyt *c* oxidase (C*c*O) is still under debate as cyanobacteria cannot produce respiratory Cyt *c*. Thus Cyt c_6 and Pc have been proposed as candidates to serve such a respiratory function as well [3–6].

In the last years, a lot of work has been devoted to analyze photosystem I reduction by Pc and Cyt c_6 (reviewed in [7,8]), but the information concerning the possible involvement of both electron carriers in the respiratory electron transfer pathway is rather scarce. To the best of our knowledge, no functional study using the whole cyanobacterial C*c*O has been reported yet, even though a kinetic analysis has recently been published showing that the recombinant, soluble Cu_A domain of the C*c*O subunit II from *Synechocystis* is reduced by Cyt c_6 and Pc with bimolecular rate constants of ca. 5×10^5 and $5\times10^4\ M^{-1}s^{-1}$, respectively [9–11]. Here, we report the first time-resolved functional study using whole C*c*O to demonstrate that both Cyt c_6 and Pc can be involved in respiration as well.

2. Materials and methods

Purification of Cyt c_6 and Pc from the cyanobacterium *Nostoc* sp. PCC 7119 was carried out as previously described [12]. Partial purification of the aa_3 type C*c*O from *Nostoc* heterocysts was performed as described in [13], with the following modifications: Triton X-100 was used in place of Genapol x-080; the two FPLC steps were replaced by two standard Mono-Q chromatography stages, and a final step of precipitation with 20% ammonium sulfate was added. Protein concentrations were determined using the following absorption coefficients: $\Delta\varepsilon_{red-ox} = 19.7\ mM^{-1}cm^{-1}$ [14] and $16.2\ mM^{-1}cm^{-1}$ [12] for Cyt *c* and Cyt c_6, at 550 and 552 nm, respectively; $\Delta\varepsilon_{604-630} = 12.0\ mM^{-1}cm^{-1}$ for the difference spectra (reduced *minus* oxidized) of C*c*O (heme *a*) [13,15], and $\varepsilon_{597} = 4.5\ mM^{-1}cm^{-1}$ for oxidized Pc [12].

The kinetic experiments were based on the analysis of typical laser flash-induced, flavin-mediated redox reactions. In an anaerobic, 1 cm path-length cuvette, 5-deazariboflavin (dRf) is photoexcited by the laser flash and reduced by EDTA, with the resulting radicals (dRfH$^\bullet$) reducing in turn any oxidized protein [16,17]. In our case, the oxidized form of Cyt c_6, Pc or Cyt *c* was first reduced, as they were well in excess over C*c*O in the reaction cell, and the reduction of the terminal oxidase was further monitored by following the absorbance changes of Cyt c_6, Pc and Cyt *c* at 552, 597 and 550 nm, respectively. The standard reaction mixture contained, in a final volume of 1 ml, 10 mM sodium phosphate, pH 7.0, 2.0 mM EDTA, 100 μM dRf, 30 μM oxidized donor protein and 2 μM C*c*O. To study the dependence of the rate constant on donor protein concentration, the effective concentrations of reduced donor proteins (<1.5 μM) were modified by placing neutral filters that turn the intensity of the laser beam down, whereas the ionic strength was adjusted by adding small volumes of a concentrated solution of NaCl. All experiments were performed under pseudo-first-order conditions. Each kinetic trace was the average of 6–8 measurements, with 20 s time spacing between flashes. Kinetic analyses were performed according to the reaction mechanisms previously proposed [18].

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Abbreviations: C*c*O, cytochrome *c* oxidase; Cyt, cytochrome; dRf, 5-deazariboflavin; k_{bim}, bimolecular rate constant; k'_{et}, apparent electron transfer rate constant; k_{obs}, observed pseudo first-order rate constant; Pc, plastocyanin

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3. Results and discussion

The lack of knowledge on cyanobacterial C*c*O's is due to serious obstacles concerning the purification of these complexes, mainly the extremely low concentration of the complex in the cell and its instability throughout the purification process [1,13]. However, the heterocysts of N_2-fixing cyanobacteria – which exhibit a greater respiration activity to keep the dioxygen concentration as low as possible and avoid nitrogenase inactivation [13] – are the most suitable material to purify cyanobacterial oxidases. Here, partially purified aa_3 type C*c*O from *Nostoc* heterocysts was obtained from 1 kg (wet weight) of cells by following the procedure previously described in [13]. The main spectral characteristics of the resulting preparation were as previously described [13]: the γ-peak of the oxidized enzyme is at 420 nm, whereas the difference spectrum (reduced *minus* oxidized) shows absorption peaks at 440, 517 and 604 nm (Fig. 1), with chlorophyll being the most relevant pigment in the visible region of the spectrum [13]. Such spectral features unequivocally indicate that C*c*O is the only redox active species, ensuring the absence of any optical interference due to redox changes in the spectral region under analysis (Fig. 1).

Fig. 2A (upper trace) shows the fast reduction kinetics of *Nostoc* Cyt c_6 by dRfH·. In the absence of C*c*O, Cyt c_6 remains reduced for more than a second. When C*c*O is added to the reaction cell containing Cyt c_6 (Fig. 2A, lower trace), the laser-induced absorbance changes fit well with the first, fast reduction of Cyt c_6 followed by the oxidation of the heme protein at longer time-scale. In fact, the slower decrease in absorbance at 552 nm does not reach the level of the preflash baseline, thus indicating that Cyt c_6 reoxidation is not complete [16]. The relatively small extent of Cyt c_6 oxidation can be explained by assuming that the similar redox potential values of the two proteins make the electron transfer reaction reversible and close to equilibrium [19], according to the following scheme:

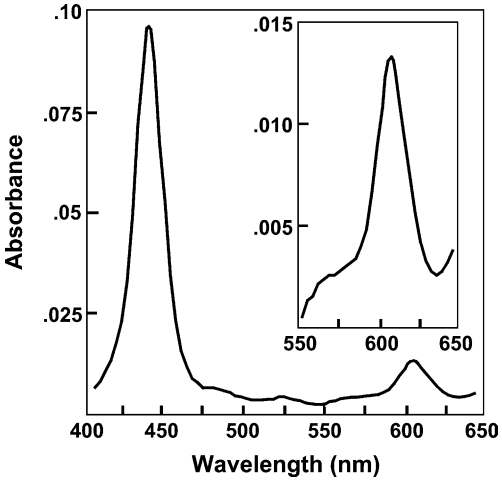

Fig. 1. Difference spectrum of *Nostoc* cytochrome *c* oxidase. The spectrum was obtained by subtracting the native, oxidized cytochrome *c* oxidase spectrum from that recorded after reduction by sodium dithionite. This spectrum corresponds mainly to cytochrome aa_3, with minor contributions arising from the Soret band of chlorophyll. The contributions of the latter obscure the usual 'negative' bands seen in difference spectra of cytochromes.

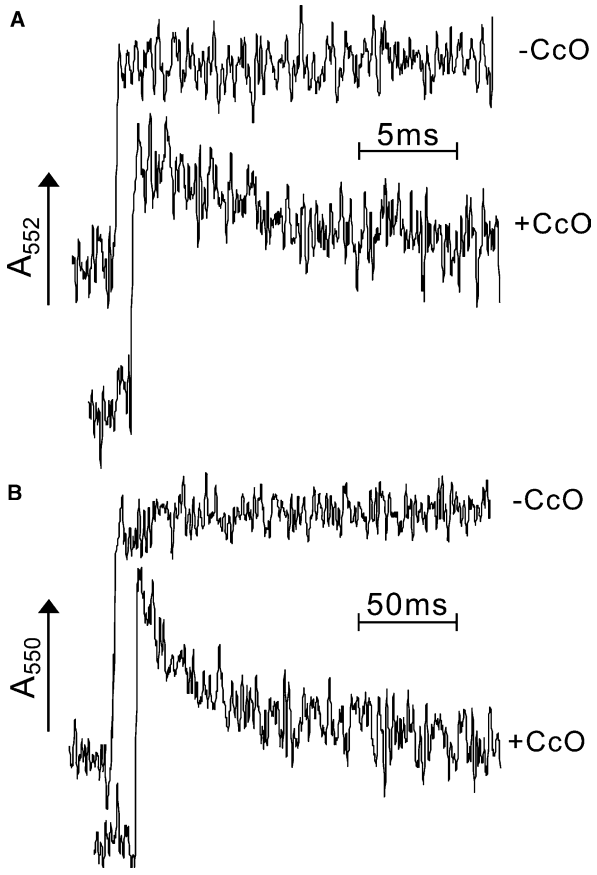

Fig. 2. Kinetic traces showing fast reduction of *Nostoc* cytochrome c_6 (A) or horse mitochondrial cytochrome *c* (B) by dRfH·, and subsequent oxidation of the heme proteins by *Nostoc* cytochrome *c* oxidase. Pre-flash oxidized cytochromes c_6 and *c* concentration was 30 μM, whereas the concentration of reduced cytochrome generated by the laser-flash was 0.2 and 0.3 μM, respectively. The concentration of cytochrome *c* oxidase was 2 μM. Other conditions were as described in Section 2.

$$\mathrm{Cyt}c_{6\mathrm{red}} + \mathrm{C}c\mathrm{O}_{\mathrm{ox}} \overset{K_A}{\rightleftarrows} [\mathrm{Cyt}c_{6\mathrm{red}} \cdots \mathrm{C}c\mathrm{O}_{\mathrm{ox}}] \underset{k_{\mathrm{reverse}}}{\overset{k_{\mathrm{forward}}}{\rightleftarrows}} \mathrm{Cyt}c_{6\mathrm{ox}} + \mathrm{C}c\mathrm{O}_{\mathrm{red}}$$

where K_A stands for the complex association constant. Moreover, due to the low extent of photoreduction of the Cyt (see above), the redox equilibrium favours the electron to be on the heme protein.

The k_{obs} values for Cyt c_6 oxidation by C*c*O at low ionic strength are independent of donor protein concentration within the range analyzed (Fig. 3, upper), indicating that the system has a very high association constant ($K_A \geqslant 10^7$ M^{-1}) and allowing the estimation of an apparent first-order intracomplex electron transfer rate constant (k'_{et}). The k'_{et} value of 250 s^{-1} ($t_{1/2}$ = 2.8 ms) calculated from these experiments (Table 1) reveals the high efficiency of the *Nostoc* system as compared with the much slower kinetics (in a time scale of seconds) determined for the interaction of the isolated subunit II of *Synechocystis* 6803 with both Cyt c_6 and Pc [10,11]. The value here reported is of the same order of magnitude as that described for the oxidation of bovine Cyt *c* by bovine C*c*O (k'_{et} = 630 s^{-1} at low ionic strength) [16]. The low signal-to-noise ratio, ascribed to the low extent of oxidation of Cyt c_6 (see above), prevented a reliable estimation of the forward (Cyt $c_6 \rightarrow$ C*c*O) and reverse (C*c*O $\rightarrow$ Cyt c_6) electron transfer