Characterization of three bioenergetically active respiratory terminal oxidases in the cyanobacterium *Synechocystis* sp. strain PCC 6803

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Abstract

*Synechocystis* sp. PCC 6803 contains three respiratory terminal oxidases (RTOs): cytochrome *c* oxidase (Cox), quinol oxidase (Cyd), and alternate RTO (ARTO). Mutants lacking combinations of the RTOs were used to characterize these key enzymes of respiration. Pentachlorophenol and 2-heptyl-4-hydroxy-quinoline-N-oxide inhibited Cyd completely, but had little effect on electron transport to the other RTOs. KCN inhibited all three RTOs but the in vivo *K*<sub>i</sub> for Cox and Cyd was quite different (7 vs. 27 μM), as was their affinity for oxygen (*K*<sub>M</sub> 1.0 vs. 0.35 μM). ARTO has a very low respiratory activity. However, when uptake of 3-O-methylglucose, an active H<sup>+</sup> co-transport, was used to monitor energization of the cytoplasmic membrane, ARTO was similarly effective as the other RTOs. As removal of the gene for cytochrome *c*<sub>553</sub> had the same effects as removal of ARTO genes, we propose that the ARTO might be a second Cox. The possible functions, localization and regulation of the RTOs are discussed.

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Keywords: Respiration; Cytochrome *c* oxidase; Quinol oxidase; Bioenergetic electron transport; *petJ*; Glucose uptake

1. Introduction

Cyanobacteria are Gram-negative prokaryotes capable of oxygenic photosynthesis. However, all cyanobacteria are also able to respire in the dark with O<sub>2</sub> as the terminal electron acceptor. Respiratory and photosynthetic electron transport are intimately linked in cyanobacteria and there are two distinct bioenergetically active membranes, the thylakoids or intracytoplasmic membranes (ICM) and the cytoplasmic membrane (CM). All photosynthetic electron transport is localized in the ICM, while photosynthesis and respiration share components. In addition, there is ample evidence for the existence of respiratory chain(s) in the CM (for a review see [1]). Cyanobacterial respiratory terminal oxidases (RTOs) have no direct function in photosynthesis and therefore can be considered the key enzymes of respiration. All cyanobacteria that have been investigated so far contain several respiratory branches ending in different RTOs but their localization in the cell (ICM, CM or both) has not been definitely clarified for any strain. The best characterized cyanobacterium is *Synechocystis* sp. strain PCC 6803, for which the complete genomic sequence is available [2]. Three sets of genes for RTOs were found, the well characterized *aa3*-type cytochrome *c* oxidase (Cox, encoded by *coxBAC*) [3], a related set of genes also belonging to the heme-copper oxidase superfamily termed ARTO (alternate RTO, encoded by *ctaCII/ctaDIIEII*) [4,5], and two genes (*cydAB*) encoding a putative cytochrome *bd*-type quinol oxidase (Cyd) [5]. Howitt and Vermaas [5] have constructed mutant strains lacking all possible combinations of these RTOs and have shown that all three oxidases can be expressed in *Synechocystis*, but they could not detect respiratory activity (oxygen uptake in the dark) in a mutant strain in which the ARTO was the only remaining RTO. The purpose of the present work was to characterize the RTOs in *Synechocystis* in more detail and to determine whether the ARTO, for which homologs in several other cyanobacteria (*Anabaena* PCC 7120 (Kazusa DNA Research Institute (www.kazusa.or.jp/cyano/)), *Nostoc* ATCC 29133 (DOE Joint Genome Institute (www.jgi.doe.gov/)), and *Anabaena* ATCC 29413 (D. Pils, unpublished)) were found, is at the end of a bioenergetically active respiratory branch. Using the uptake of 3-O-methyl-D-glucose (3-OMG) as a...
sensor, we present evidence that all RTOs are bioenergetically active, specifically for the energization of the CM, and that cytochrome c553 is an essential part of the respiratory branch ending in ARTO.

2. Materials and methods

*Synechocystis* and its derivatives were grown photoautotrophically at 32°C in BG11TS medium [3] with about 0.25% CO2 in air and photoheterotrophically as described [4]. The *Synechocystis* mutant strains BC3 and BC3J were made by insertional inactivation as described [7], starting with strain B (Table 1) and using plasmids pGWUV9 (coxBAC::Km) and pDPUV2a (petJ::Cm) [4] to inactivate Cox and cytochrome c553, respectively. Homozygosity was confirmed by PCR as described earlier [4].

Respiratory activity (defined as KCN-inhibitable oxygen uptake in the dark) of photoautotrophically grown cultures was measured with a Clark-type electrode at the growth temperature as described [4]. The KM of the RTOs for O2 in vivo was determined from two independent cultures of mutant strains with only one remaining RTO at low oxygen concentrations using a Lineweaver-Burk plot.

For the uptake of U-14C-labeled D-glucose (glc) and 3-OMG in the dark cells were grown photoautotrophically, harvested at an OD730 of about 1, and resuspended in BG11TS or an OD730 growth temperature as described [4]. The KM for the uptake in the dark) of photoautotrophically grown cultures was 50 μg ml−1 of kanamycin (Km), 50 μg ml−1 of spectinomycin (Sp), 10 μg ml−1 of chloramphenicol (Cm) and 10 μg ml−1 of erythromycin (Em).

### Table 1

**Synechocystis strains used in this work**

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviated name</th>
<th>Relevant genotype</th>
<th>Remaining oxidase(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCC 6803</td>
<td>WT</td>
<td>wild-type</td>
<td>Cox, ARTO, Cyd</td>
<td>[6]</td>
</tr>
<tr>
<td>GWC</td>
<td>C3</td>
<td>coxBAC::Km</td>
<td>ARTO, Cyd</td>
<td>[4]</td>
</tr>
<tr>
<td>ctaDIEI+</td>
<td>A</td>
<td>ctaDIEI::Sm'</td>
<td>Cox, Cyd</td>
<td>[5]</td>
</tr>
<tr>
<td>cydAB</td>
<td>B</td>
<td>cydAB::Em'</td>
<td>Cox, ARTO</td>
<td>[5]</td>
</tr>
<tr>
<td>ctaDIEI+</td>
<td>cydAB+</td>
<td>ctaDIEI::Sm'</td>
<td>Cox</td>
<td>[5]</td>
</tr>
<tr>
<td>ctaDIEI+</td>
<td>ctaDIEI+</td>
<td>ctaDIEI::Sm', coxA::Km'</td>
<td>Cyd</td>
<td>[5]</td>
</tr>
<tr>
<td>ctaDIEI+</td>
<td>ctaDIEI+</td>
<td>ctaDIEI::Sm', cydAB::Em', coxA::Km'</td>
<td>ARTO</td>
<td>[5]</td>
</tr>
<tr>
<td>ctaDIEI+</td>
<td>ctaDIEI+</td>
<td>ctaDIEI::Sm', cydAB::Em', coxA::Km'</td>
<td>none</td>
<td>[5]</td>
</tr>
<tr>
<td>PDBC3</td>
<td>BC2</td>
<td>cydAB::Em', coxBAC::Km'</td>
<td>ARTO</td>
<td>this work</td>
</tr>
<tr>
<td>PDBC3</td>
<td>BC3</td>
<td>cydAB::Em', coxBAC::Km'</td>
<td>ARTO</td>
<td>this work</td>
</tr>
<tr>
<td>GS1</td>
<td>GS1</td>
<td>gtr::Km</td>
<td>(like WT)</td>
<td></td>
</tr>
</tbody>
</table>

a The coxBAC genes have also been called ctaCIDIEI.

b C3 means that only the genes for subunits I and III (coxAC) are interrupted and C3 that the entire Cox locus (coxBAC) is missing.

c Antibiotic concentrations were: 50 μg ml−1 kanamycin (Km), 50 μg ml−1 spectinomycin (Sp), 10 μg ml−1 chloramphenicol (Cm) and 10 μg ml−1 erythromycin (Em).

d The list of strains used in this work is presented in Table 1. All strains were confirmed to be homozygous at all mutated loci. All except the glucose transporter deficient strain (GS1) were capable of photoheterotrophic growth. For almost all strains total respiratory activity was only slightly dependent on the RTOs present (Table 2), as has been noted earlier [5]. The only exceptions were strains BC2, BC3, and ABC2. Strain ABC2 lacks all RTOs known in *Synechocystis* and is non-respiring. When measured in very dense suspensions, the respiratory activity of ARTO in strains BC2 or BC3 was clearly non-zero and inhibitable by KCN, but could not be induced or activated by growth under high salinity conditions (BG11TS+0.5 M NaCl), growth at 20°C or 40°C (in BG11TS with about 0.1% CO2 in air), or semi-anaerobic incubation (sparging with pure N2 or a mixture of 99% N2 and 1% O2) in the light (40 μmol quanta m−2 s−1) or in the dark in the presence of glc for 12 h. In WT cells, respiratory activity was measured in the growth phase (40 μmol quanta m−2 s−1).

### 3. Results

A list of strains used in this work is presented in Table 1. All strains were confirmed to be homozygous at all mutated loci. All except the glucose transporter deficient strain (GS1) were capable of photoheterotrophic growth. For almost all strains total respiratory activity was only slightly dependent on the RTOs present (Table 2), as has been noted earlier [5]. The only exceptions were strains BC2, BC3, and ABC2. Strain ABC2 lacks all RTOs known in *Synechocystis* and is non-respiring. When measured in very dense suspensions, the respiratory activity of ARTO in strains BC2 or BC3 was clearly non-zero and inhibitable by KCN, but could not be induced or activated by growth under high salinity conditions (BG11TS+0.5 M NaCl), growth at 20°C or 40°C (in BG11TS with about 0.1% CO2 in air), or semi-anaerobic incubation (sparging with pure N2 or a mixture of 99% N2 and 1% O2) in the light (40 μmol quanta m−2 s−1) or in the dark in the presence of glc for 12 h. In WT cells, respiratory activity...
of photoautotrophically grown cells in the absence of exogenous glucose varied considerably from culture to culture and often was not of zero order with respect to O$_2$ uptake. Therefore glucose was always added to obtain reproducible activities. However, the addition of exogenous glucose always led to a significant (about two-fold) increase of respiratory activity. A similar behavior was observed in most of the mutant strains with the notable exception of strains AC2, on whose (well reproducible) activity glucose had no influence, and strains BC2 and BC3, whose small activity could not be enhanced by exogenous glucose. Interestingly, the protonophoric uncoupler FCCP (10 μM) did not enhance the respiratory rate of strains WT, AB, AC2, BC2, and BC3, both in the absence and in the presence of exogenous glucose (data not shown).

Indirect evidence had led us earlier to propose that the target for the inhibitors 2-heptyl-4-hydroxy-quinoline-N-oxide (HQNO) and pentachlorophenol (PCP) is the putative cytochrome bd-type quinol oxidase Cyd [4]. Indeed, strain AC2, in which Cyd is the only RTO present, was inhibited 100% by both inhibitors (using the same concentrations as previously [4]), while they had little effect on strain AB (Table 2).

NaCl is known to enhance the respiratory activity of cyanobacteria (‘salt respiration’ [8]). This is probably due to additional energy requirements for the extrusion of Na$^+$ ions via one or several of the Na$^+/H^+$ antiporters that exist in Synechocystis [9] or via a direct Na$^+$ pump as identified in Escherichia coli [10]. We have used strains AB, AC2, BC2, and BC3 to determine which respiratory branches are responsible for salt respiration. Table 2 shows that the presence of Cox is necessary and sufficient to obtain the salt respiration rate of the wild-type.

<table>
<thead>
<tr>
<th>Strain(s)</th>
<th>+Glucose$^a$</th>
<th>+HQNO$^b$</th>
<th>+PCP$^b$</th>
<th>+NaCl$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.083 (0.034)</td>
<td>42.4 (5.1)</td>
<td>50.0 (6.5)</td>
<td>22.0 (7.2)</td>
</tr>
<tr>
<td>C3</td>
<td>0.055 (0.015)</td>
<td>72.5 (2.5)</td>
<td>83.0 (4.0)</td>
<td>n.d.</td>
</tr>
<tr>
<td>A</td>
<td>0.090 (0.028)</td>
<td>20.2 (4.8)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>B</td>
<td>0.094 (0.024)</td>
<td>20.0 (6.2)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>AB</td>
<td>0.100 (0.030)</td>
<td>25.4 (3.3)</td>
<td>13.1 (7.8)</td>
<td>26.1 (6.2)</td>
</tr>
<tr>
<td>AC2</td>
<td>0.120 (0.035)$^d$</td>
<td>100 (0.0)</td>
<td>100 (0.0)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>BC2, BC3</td>
<td>~0.001$^e$</td>
<td>n.d.</td>
<td>n.d.</td>
<td>no increase</td>
</tr>
<tr>
<td>ABC2</td>
<td>0.0</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

The values in parentheses are the variations between different experiments. The concentrations used were: 10 mM glucose, 50 μM HQNO, 1 mM PCP, 1 mM KCN, and 108 mM NaCl. n.d., not determined.

$^a$Respiration in the presence of glucose is given as μmol O$_2$ h$^{-1}$ (ml at OD$_{730}$ = 1)$^{-1}$. The addition of KCN led to 100% inhibition in all cases.

$^b$The numbers denote percent inhibition of the glucose-supported respiration.

$^c$The numbers denote percent increase of the glucose-supported respiration.

$^d$In this strain respiration was not increased by the addition of glucose.

$^e$Respiratory activity of this strain was measured in suspensions of at least OD$_{730}$ = 5 and was just above the sensitivity of the method. An inhibition by KCN was detectable.

Strains AB and AC2 allowed us to determine the affinity of the Cox and Cyd oxidases, respectively, for O$_2$ by measuring the respiratory rate at low O$_2$ concentrations. The apparent in vivo K_M values for O$_2$ uptake are about 1 μM for Cox and 0.35 μM for Cyd. The respiratory rate of strains BC2 or BC3 was too low to yield reliable values. All RTOs were inhibited completely by 1 mM KCN. The individual sensitivities to KCN of Cox and Cyd were determined in strains AB and AC2 (K_I 7 μM and 27 μM, respectively, Fig. 1). WT showed essentially the same behavior as strain AC2.

Very little is known about the bioenergetic processes in the CM of cyanobacteria. We have used the uptake of 3-OMG in the dark as an in vivo sensor for the ability of the different respiratory branches to sustain a proton gradient across the CM. Glucose, its derivatives 3-OMG and 6-DG [11], and fructose [12] are transported into the cells in the light through the Gtr transporter [13] that catalyzes an H$^+$/sugar symport [11]. Since the glc transporter is only present in the CM and 3-OMG is not metabolized by the cells [11], uptake of 3-OMG specifically measures the status of the proton gradient across the CM. Fig. 2A,D shows that the uptake of 3-OMG in the dark was mediated through the Gtr transporter (see strain GS1 and the inhibition by the competitive substrate 6-DG) and was dependent on respiratory activity (see inhibition by KCN and the lack of uptake in the non-respiring mutant ABC2). Fig. 2B–D shows that all three RTOs were able to energize the CM, since in strains AB, AC2, BC2, and BC3 uptake of 3-OMG was observed that was inhibitable by KCN and the protonophore FCCP. Similar results were obtained with glc (Fig. 2E,F), showing that the observed effects are essentially independent of the transported substance.

To determine whether cytochrome c$_{553}$ is an essential part of the electron transport branch ending in ARTO as proposed earlier [4], we constructed a mutant strain (BC3) lacking the entire coxBAC locus and the cyd genes. BC3 differs from BC2 in lacking Cox subunit II (coxB), which is the docking site for cytochrome c. In view of the high sequence similarity between the coxB and ctaCH genes, we wanted to ascertain that the ctaCH gene product was the only such protein available in the cell. 3-OMG uptake by strain BC3 was identical to strain BC2, but strain BC3J that additionally lacks the petJ gene encoding cytochrome c$_{553}$ displayed an uptake as low as strain ABC2 (Fig. 2D), indicating that cytochrome c$_{553}$ is an essential part of the electron transport branch ending in ARTO.

4. Discussion

The purpose of this work was to find a possible function for each of the electron transport branches ending in the three RTOs of Synechocystis sp. strain PCC 6803 (see Fig.
3 for a proposed scheme). Our data clearly demonstrate that all three RTOs are at the end of bioenergetically active branches.

Respiratory activity through Cox constitutes the bioenergetically most proficient branch. This is demonstrated by the fact that Cox minus mutants do not grow chemoheterotrophically [4], that the branch ending in Cox is the only one that displays salt respiration (Table 2), and that the capacity of strain AB for active $H^+/3$-OMG uptake is almost the same as for WT (Fig. 2A,B). However, all three respiratory branches can energize the CM to allow uptake of 3-OMG or glc (Fig. 2B–F). This is especially remarkable for strains BC2 and BC3 (Fig. 2D) that show extremely low respiratory activity (Table 2). Their uptake of 3-OMG is quite similar to that of strain AC2 (Fig. 2C) that has almost the same respiratory activity as
WT cells (Table 2). The removal of all three RTOs (strain ABC2) reduces 3-OMG uptake to the level observed in the presence of KCN (Fig. 2A,D). Therefore, the ARTO is bioenergetically active. Although oxidation of horse heart cytochrome \(c\) by isolated membranes of strain C3 was below the detection limit [4], the data presented here and earlier [4] make it likely that cytochrome \(c_{553}\) (encoded by \(petJ\)) is involved in electron transport to ARTO. Specifically, independent of the genetic background, the phenotypes of strains lacking either \(ctaII\) genes or \(petJ\) are similar: inhibition of respiratory activity by HQNO in strains A (Table 2) and PDJ [4] is lower than in the WT, whereas inhibition by HQNO or PCP is 100% in strains AC2 (Table 2) and PDCJ [4]. Furthermore, both strain BC3J and strain ABC2 show 3-OMG uptake as low as WT inhibited with KCN (Fig. 2A,D). We propose therefore that the ARTO might be a genuine Cox with cytochrome \(c_{553}\) as its donor. This is unusual, because the sequence of the \(cta\) gene product, the putative subunit II, lacks the presumed CuA binding site [5] that has been inferred to be the primary acceptor of electrons from cytochrome \(c\) [14]. However, electron transfer into Cox circumventing the CuA site has also been discussed [15]. Serrano et al. [16] have found that the majority of the cytochrome \(c_{553}\) in the cyanobacterium \(Anabaena\) sp. ATCC 29413 is located in the periplasm. Therefore the ARTO might be localized in the CM (Fig. 3), and its small bioenergetic turnover is used for energization of the CM. In the following way, the activity of the ARTO can be estimated to be sufficient to energize the observed transport of 3-OMG, assuming the typical stoichiometry of 1:1 for H\(^+\)/sugar symport.

From Fig. 2D, the uptake of 3-OMG is about 0.7% in 25 min, therefore about 1.7% h\(^{-1}\) and 0.017 \(\mu\)mol 3-OMG h\(^{-1}\) (ml at OD\(_{730}\) = 1)\(^{-1}\). From Table 2, the activity of the ARTO is about 0.001 \(\mu\)mol O\(_2\) h\(^{-1}\) (ml at OD\(_{730}\) = 1)\(^{-1}\). Since four electrons are necessary to reduce one O\(_2\) molecule to water, and assuming that six H\(^+\) ions are translocated across the membrane per electron, a total of 0.024 \(\mu\)mol H\(^+\) h\(^{-1}\) (ml at OD\(_{730}\) = 1)\(^{-1}\) is translocated by the respiratory chain of the CM. Energization of the CM by the other two respiratory branches, whose RTOs are probably localized in the ICM (see below), might function via ATP synthesis at the ICM and ATP hydrolysis at the CM.

The Cyd protein is a quinol oxidase located in the ICM (Berry et al., Biochemistry, submitted). Consistently, Cyd is the major target of the quinol oxidase inhibitors HQNO and PCP (Table 2). The respiratory activity of strain AC2 was as high as that of the WT (Table 2), but the uptake of 3-OMG was similar to that of the almost non-respiring strains BC2 and BC3. This may indicate that the branch ending in Cyd is not very effective in energizing the CM, possibly due to its location in the ICM and/or the lower amount of H\(^+\) ions translocated per electron characteristic for \(bd\)-type quinol oxidases [17].

Currently no method is available to determine the relative activities of the respiratory branches in the WT under any condition. The total respiratory activity of the WT
cannot be estimated by adding the respiratory activities of the mutants or from the percentages of inhibition by HQNO or PCP (Table 2). Even specific inhibition of an RTO or deletion of its genes do not necessarily yield the same result. This is exemplified by inhibition of Cyd by HQNO in Cox minus strain C3, after which a respiratory activity of about 28% remains (Table 2), which can only be attributed to the activity of the ARTO. In contrast, strains BC2 and BC3 containing only the ARTO genes have a respiratory activity of only 2% of strain C3. Thus, the expression of the RTOs might be influenced by the presence of the other RTO genes by a regulatory mechanism yet unknown. In any case removing or inhibiting an RTO leads to a major redistribution of electrons among the remaining respiratory branches. This may also be the explanation for the similar KCN inhibition curves for WT and AC2 in Fig. 1. Of several interpretations possible we favor that electrons originally passing through the Cox branch are gradually transferred to the Cyd branch upon the gradual inhibition of the Cox by rising concentrations of KCN. A similar redistribution of electrons between a Cox and a quinol oxidase – regulated by the redox state of the quinone pool – has been observed for Rhodobacter capsulatus [18]. This is only possible if both branches reside in the same membrane. In Synechocystis, Cox is largely or exclusively located in the ICM, when in Fig. 3 both the Cox and Cyd branches are placed in the ICM.

The uncoupler FCCP did not enhance respiratory activity in any of the strains used, although FCCP de-energized the CM for 3-OMG uptake (Fig. 2). This implies that the coupling mechanism is not rate limiting for respiration in Synechocystis. Our data do not allow to state with confidence which reaction(s) is/are the rate limiting step(s) for the different respiratory branches. Since the addition of exogenous glc (and NaCl) enhanced respiratory activity of strain AB containing only the Cox branch, but not of strain AC2 containing only the Cyd branch (Table 2), in the latter case the Cyd itself might be the rate limiting enzyme.

Acknowledgements

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References


