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# Enzymatic activity of the alternative complex III as a menaquinol:auracyanin oxidoreductase in the electron transfer chain of *Chloroflexus aurantiacus*

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ABSTRACT

**The surprising lack of the cytochrome $bc_1$ complex in the filamentous anoxygenic phototrophic bacterium *Chloroflexus aurantiacus* suggests that a functional replacement exists to link the cyclic electron transfer chain. Earlier work identified the alternative complex III (ACIII) as a substitute of cytochrome $bc_1$ complex. Herein, the enzymatic activity of ACIII is studied. The results strongly support the view that the ACIII functions as menaquinol:auracyanin oxidoreductase in the *C. aurantiacus* electron transfer chain. Among all the substrates tested, auracyanin is the most efficient electron acceptor of ACIII, suggesting that ACIII directly transfers the electron to auracyanin instead of cytochrome *c*-554. The lack of sensitivity to common inhibitors of the cytochrome $bc_1$ complex indicates a different catalytic mechanism for the ACIII complex.**


## 1. Introduction

The five phyla of bacteria that possess chlorophyll-based photosynthesis are purple bacteria, cyanobacteria, heliobacteria, green sulfur bacteria and green gliding bacteria (now commonly named filamentous anoxygenic phototrophs) [1]. Although each type of these bacteria has distinctive patterns of photosynthetic and in many cases respiratory electron transfer pathways, one component was until recently believed to be a constant constituent in all five different groups: the cytochrome $bc_1$ *or* $b_6f$ complex [2]. The general function of this complex is the electron shuttling between two mobile redox carriers, from the relatively lower potential quinol to the higher potential cytochrome *c* or plastocyanin. Such electron shuttling is coupled with the translocation of protons across the membrane (inner membrane in mitochondria or plasma membrane in bacteria), thus generating a proton-motive force in the form of an electrochemical proton and electrical potential, which can drive ATP synthesis or other energy requiring processes [3].

The only group of phototrophic bacteria for which there has not been either biochemical or genomic evidence for the existence of a related cytochrome $bc_1$ *or* $b_6f$ complex, are the filamentous anoxygenic phototrophs (FAPs) [4]. One well-studied representative of this group is the *Chloroflexus aurantiacus*. This thermophilic photosynthetic bacterium possesses an unusual electron transfer pathway due to the lack of both the cytochrome $bc_1$ complex and a soluble *c*-type cytochrome [5]. Instead, from the whole membrane of *C. aurantiacus*, a protein complex containing a *c*-type cytochrome but no cytochrome *b* or Rieske FeS factor was isolated [6]. It has been proposed that this protein may fulfill the role of complex III in the respiratory electron transfer chain or the cytochrome $bc_1$ complex in the photosynthetic electron transfer chain. A genome database search revealed that the presence of this protein complex in several bacteria from diverse taxa coincides with the absence of cytochrome $bc_1$ complex [7]. This evidence strongly suggests that this protein complex (now named alternative complex III [8,9] or ACIII) is a functional replacement for the cytochrome $bc_1$ complex in *C. aurantiacus*.

Based on these and other relevant findings, it has been proposed that the cyclic electron transport chain in *C. aurantiacus* includes the following steps. First, electrons are delivered from reduced photosynthetic reaction center to menaquinone, the sole quinone found in *C. aurantiacus* [10], to give menaquinol. Then, the menaquinol is oxidized by the ACIII complex, delivering electrons to a soluble copper protein auracyanin, which is attached to the periplasmic side of the inner cell membrane [11,12]. Auracyanin in turn transfers electrons to a 4-heme cytochrome *c*-554 [13] and re-reduces the oxidized reaction center, thereby completing the cyclic chain. Although functional studies of cytochrome $bc_1$ and $b_6f$ complexes in different other organisms have been reported [14,15], nevertheless,

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there has been no experimental evidence that ACIII substitutes the role of cytochrome $bc_1$ complex in *C. aurantiacus*, except for gene analysis of sequenced genomes of various species. In this work, we conduct the functional studies of ACIII to determine the role that ACIII plays in the photosynthetic electron transfer chain in *C. aurantiacus*. The enzyme kinetic analysis was shown to provide a reliable method to determine whether ACIII serves as a protein enzyme to catalyze the reduction of auracyanin (or cytochrome *c*) by menaquinone. Furthermore, characteristics of enzyme activity with respect to substrate concentration, pH, temperature and the presence/absence of inhibitors are described.

## 2. Materials and methods

### 2.1. Protein isolation and purification

*C. aurantiacus* strain J-10-fl was used and grown anaerobically under high-light conditions in modified medium D, under literature reported conditions with modifications [16]. ACIII protein was purified according to procedures reported previously [6,7,17].

### 2.2. Enzymatic activity assays

The enzyme activity of ACIII was monitored using reduced vitamin K2 as electron donor and either oxidized cytochrome *c* or oxidized auracyanin as electron acceptors.

#### 2.2.1. Spectrophotometric assay of ACIII complex using vitamin K2 and cytochrome c as substrates

The enzyme activity of ACIII as menaquinol:cytochrome *c* oxidoreductase was measured at 25 °C by UV–vis spectroscopy on a Perkin Elmer Lambda 950 spectrophotometer. The reaction was followed by the increase in absorption at 550 nm, due to the simultaneous menaquinol-4 (reduced vitamin K2) oxidation with horse heart cytochrome *c* reduction. The differential extinction coefficient for cytochrome *c* is 18.5 $mM^{-1}$ $cm^{-1}$ at 550 nm [18]. The preparation of menaquinol-4 was performed according to Ragan [19] with modifications. The ACIII activity assays were performed in an anaerobic 1-ml cuvette at final concentrations of 80 μM menaquinol-4, 50 μM cytochrome *c*, and *ca*. 3.0 nM ACIII. All the assays were carried out in 50 mM phosphate buffer (pH 7.3) in the presence of 0.2 mM EDTA, 1 mM $NaN_3$, 250 mM sucrose, 0.1% BSA and 0.02% *n*-dodecyl-β-D-maltoside. The reaction was started by the addition of menaquinol-4 to the mixture of the cytochrome *c* and the ACIII in buffer and then followed for 5 min at 550 nm. The activity was calculated from the initial quasilinear reduction rate of cytochrome *c*. Non-enzymatic cytochrome *c* reduction was measured and subtracted when calculating enzyme activity.

#### 2.2.2. Spectrophotometric assay of ACIII complex using vitamin K2 and auracyanin A as substrates

The measurement of ACIII activity using auracyanin A as substrate utilized a similar protocol except that the reaction was followed by monitoring a decrease in absorption at 594 nm due to auracyanin A reduction. Auracyanin A was purified from *C. aurantiacus*, and has an extinction coefficient at 594 nm of 3.0 $mM^{-1}$ $cm^{-1}$ [20].

### 2.3. Enzyme kinetics of ACIII complex as a function of substrate concentration

The Michaelis–Menten curves were obtained by measuring the initial reaction rates as a function of graded concentrations of three substrates (menaquinone-4, cytochrome *c* and auracyanin). The initial rate data were fitted to the Michealis–Menten equation using a non-linear fitting program. The enzyme activity was expressed in terms of the turnover number, $s^{-1}$ (mol of cytochrome *c* or auracyanin A reduced/mol of ACIII/s).

### 2.4. The effects of pH, temperature and enzyme inhibitor

The pH and temperature effects on the activity of ACIII as well as the inhibitor effects were also measured using menaquinol-4 and cytochrome *c* instead of auracyanin as substrates, taking advantage of the higher extinction coefficient of cytochrome *c* [15]. Antimycin A and stigmatellin, which are commonly used inhibitors for cytochrome $bc_1$ complexes, were chosen for the inhibitor effect test.

## 3. Results and discussion

Current evidence suggests that ACIII is the functional substitute of the cytochrome $bc_1$ complex, completing the cyclic electron transfer from the reaction center and utilizing the quinone pool. This work seeks to test this hypothesis by direct enzyme assay analysis of ACIII and its putative substrates, vitamin K2 and auracyanin. To serve as the functional replacement for cytochrome $bc_1$, ACIII has to catalyze the enzymatic reaction as a menaquinol:cytochrome *c*/auracyanin oxidoreductase. Therefore, menaquinol-4 (reduced vitamin K2) and auracyanin purified from *C. aurantiacus* were chosen as substrates. Horse heart cytochrome *c* was also used as an alternative, although nonphysiological substrate. The reduction of cytochrome *c* and auracyanin was monitored by continuously recording the α band absorbance at 550 nm and the auracyanin absorbance peak at 594 nm, respectively. The rate of the reduction reactions showed a significant increase when air-oxidized ACIII was added, indicating enzymatic activity. Fig. 1A shows the reduction of cytochrome *c* by menaquinol-4, in the presence or absence of protein enzyme ACIII, and Fig. 1B shows the same reduction reaction of auracyanin A, which is the proposed direct electron acceptor of ACIII in the photosynthetic electron transfer chain [20]. When reduced, the absorption for cytochrome *c* increases at 550 nm, while the absorption for auracyanin at 594 nm decreases, leading to positive and negative slopes of the curves. For the latter, the absorption difference between oxidized and reduced states is much smaller compared with that of cytochrome *c* due to a much lower extinction coefficient of auracyanin A. It was also found that the reduced ACIII had no cytochrome *c* oxidase activity, suggesting that it can only pass the electron from an electron donor to an electron acceptor while three components are present. The reduction rate showed saturation as substrate concentration increased (Fig. 2). All three curves exhibited typical characteristics of a Michealis–Menten reaction. The enzyme activity kinetics at room temperature and pH 7.3 are summarized in Table 1. By comparing the turnover numbers as well as the $k_{cat}/K_m$ values of the enzyme using either auracyanin or cytochrome *c* as substrate, it is demonstrated that auracyanin is more efficient than cytochrome *c* as a direct electron acceptor for the ACIII complex by showing a higher turnover number and $k_{cat}/K_m$. The isoelectric point of auracyanin was found to be around 4 [20], suggesting an alkaline binding site on the ACIII complex. This may also explain why mitochondrial cytochrome *c* (alkaline electron acceptor) is a less efficient acceptor than auracyanin (acidic electron acceptor).

The specificity of ACIII complex for various electron donors and acceptors were also studied (Table 2). Electron donors other than menaquinol-4 were observed to be less efficient. For example, the shorter chain menadione gives high non-enzymatic reduction of cytochrome *c*. For the electron acceptors, the ACIII catalytic efficiency for auracyanin A from *C. aurantiacus* is the highest, followed by horse heart cytochrome *c*. Cytochrome *c*-554 ($\varepsilon$ = 28 $mM^{-1}$ $cm^{-1}$ at 554 nm [13]) from *C. aurantiacus* has slightly lower catalytic effi-

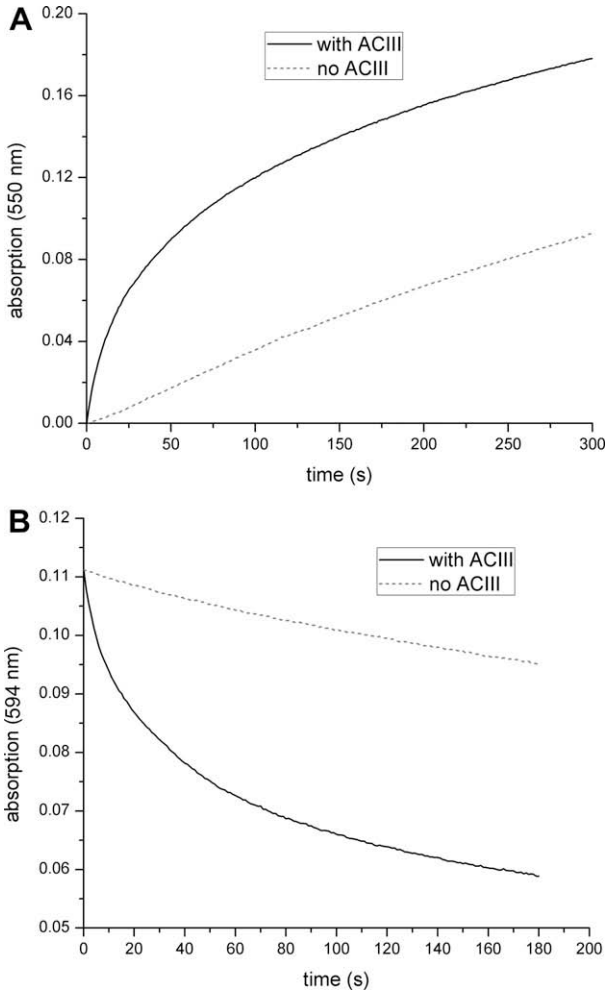


**Fig. 1.** (A) Quinol:cytochrome *c* oxidoreductase activity of the ACIII complex purified from *C. aurantiacus* with solid line representing the absorption change at 550 nm when adding the ACIII complex as an enzyme and the dashed line representing the absorption change without the ACIII complex. (B) Quinol:auracyanin oxidoreductase activity of the ACIII complex with solid line representing the absorption change at 594 nm when adding the ACIII complex as an enzyme and the dashed line representing the absorption change without the ACIII complex.

ciency than horse heart cytochrome *c*. Interestingly, although the auracyanin purified from *Roseiflexus castenholzii* is supposed to possess a very similar structure and protein property to the auracyanin from *C. aurantiacus*, it shows a zero efficiency to react with ACIII. DCPIP was also tested because of its similar redox potentials to auracyanin A. However, it gave extremely high rate of non-enzymatic reduction so was not suitable as a substrate.

**Table 1**
Kinetic parameters of the menaquinol:cytochrome *c*/auracyanin oxidoreductase. The assay is described in Section 2. The data were analyzed with the Michaelis–Menten equation using a non-linear fitting program.

| Substrate | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $\mu M^{-1}$) |
|---|---|---|---|
| Menaquinol-4 | 14.0 | 45.8 | 3.2 |
| Cytochrome *c* (horse heart) | 4.3 | 51.7 | 12.0 |
| Auracyanin A (*C. aurantiacus*) | 2.6 | 70.3 | 27.0 |

The pH value and temperature were found to have an effect on enzyme activity, which are shown in Fig. 3A and B, respectively. In Fig. 3A, the optimal pH for ACIII as menaquinol:cytochrome *c* oxidoreductase is *ca.* 7, which is slightly lower than the optimum growth pH of *C. aurantiacus* (pH 8.0), but comparable with the pH optimum observed for *R. sphaeroides* cytochrome $bc_1$ complex [21]. The activities have been corrected for the non-catalyzed reaction, which became prominent at higher pH values. The temperature effect on the catalytic activity of ACIII is shown in Fig. 3B. At *ca.* 60 °C, which is coincidentally the temperature of growth maximum of the bacterium, the enzyme gave the highest activity.

In addition to studying the pH and temperature effects, common inhibitors for the cytochrome $bc_1$ complex were also tested for ACIII. Antimycin A and stigmatellin, being two specific inhibitors for almost all cytochrome $bc_1$ complexes binding at the $Q_i$ and $Q_o$ sites, respectively, have been used to examine the involvement of $bc_1$ or $b_6f$ complex in the electron pathways of the microorganism [22]. However, antimycin A and stigmatellin (data not shown) did not lead to decreased reaction rate for ACIII-catalyzed reductions. Considering the ACIII is fundamentally different from cytochrome $bc_1$ or $b_6f$ complexes in structure, its insensitivity to common inhibitors could suggest a different catalytic mechanism of the ACIII (Fig. 3C). Such lack of sensitivity of the ACIII complex for inhibitors might be due to the absence of the cytochrome *b* subunit present in cytochrome $bc_1$ complexes. As a result, ACIII showed very low degree of sensitivity to inhibitors specific to the cytochrome *b* subunit. It has also been reported that the whole membrane of *C. aurantiacus* has antimycin A and myxothiazol inhibition patterns similar to the green plant cytochrome $b_6f$ complex [23,24].

In summary, we have performed functional studies of the *C. aurantiacus* integral membrane protein Alternative Complex III and have established that it is a menaquinol:auracyanin/cytochrome *c* oxidoreductase. To this end, enzymatic activity assays were performed using a variety of electron donors and acceptors. The presence of ACIII significantly accelerated the reduction reaction for both cytochrome *c* and auracyanin, demonstrating the

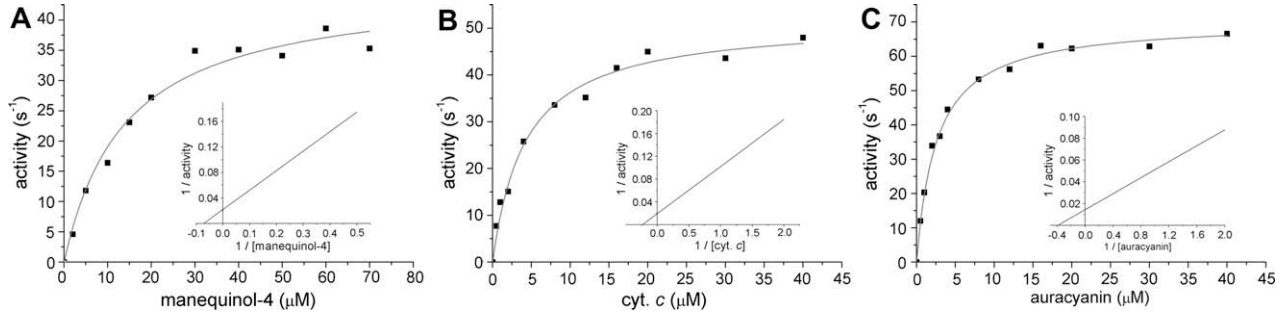


**Fig. 2.** Dependence of the initial rate on the concentration of different substrates. Each point shows the initial rate value *vs.* the concentration of the substrate. Solid curve is the Michaelis–Menten fit curve and the Lineweaver–Burk plot (using the parameters from Table 1) is shown in the inset. (A) menaquinol-4 (reduced $VK_2$). (B) cytochrome *c*. (C) auracyanin A.

**Table 2**
Enzyme activity of ACIII with different electron donors and electron acceptors. The assay is described in Section 2. The mixture for assay contained 80 μM electron donor (using 50 μM horse heart cytochrome *c* as electron acceptor) or 50 μM electron acceptor (using 80 μM menaquinol-4 as electron donor) as listed in the table to reach quasi-saturating conditions.

| Electron donor | Activity ($s^{-1}$) | Electron acceptor | Activity ($s^{-1}$) |
|---|---|---|---|
| Menaquinol (VK2) | 40.2 ± 0.7 | Auracyanin A, *C. aurantiacus* | 63.7 ± 0.5 |
| Phylloquinol (VK1) | 9.0 ± 0.2 | Auracyanin, *R. castenholzii* | 0 |
| Menadione | / | Cytochrome *c*, horse heart | 45.6 ± 0.8 |
| | | Cytochrome *c*-554, *C. aurantiacus* | 41 ± 1 |
| | | DCPIP | / |

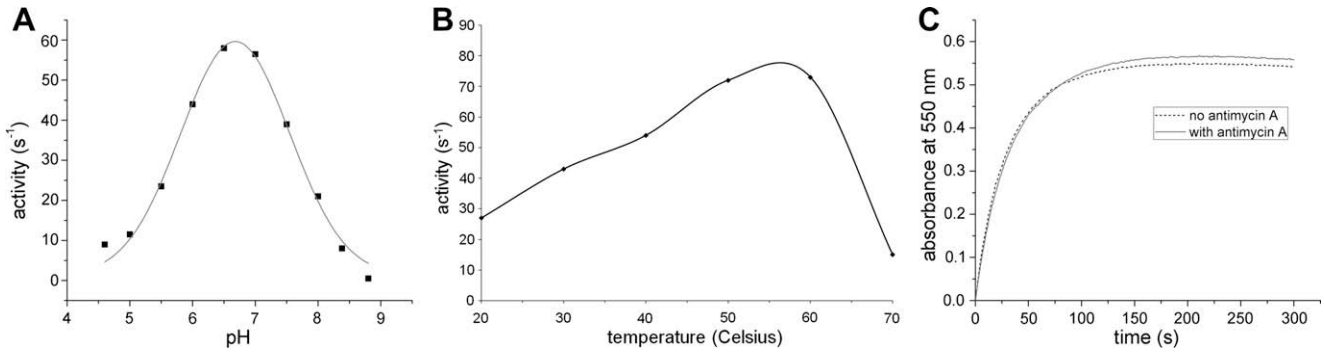


**Fig. 3.** (A) The pH profiles of quinol:cytochrome *c* oxidoreductase activity of the ACIII complex at room temperature. (B) The temperature profiles of quinol:cytochrome *c* oxidoreductase activity of the ACIII complex. (C) Effect of antimycin A on quinol:cytochrome *c* oxidoreductase activity of the ACIII complex with solid line representing adding inhibitor antimycin A and dashed line representing without inhibitor.

ability for ACIII to be the functional replacement of the cytochrome $bc_1$ complex. It was found that auracyanin was a better substrate for ACIII compared with cytochrome *c*, showing a higher turnover number and $k_{cat}/K_m$. The activity of isolated ACIII is comparable to that of the isolated cytochrome $bc_1$ complex in *Rhodospirillum rubrum* [25] or in *Ectothiorhodospiraceae* family [26]. However, the rates may not be directly comparable because the ACIII complex may use an entirely different mechanism from that of cytochrome $bc_1$ complex or cytochrome $b_6f$ complex, due to the completely different protein subunit structure and the insensitivity to common inhibitors of cytochrome $bc_1$ complexes (antimycin A and stigmatellin).

These functional studies clearly suggest that the ACIII complex is the functional replacement of cytochrome $bc_1$ to serve the function of electron transfer and proton pumping. However, the mechanisms of proton shuttling by ACIII are not yet understood. ACIII may translocate protons through the Lundegardh mechanism [27], oxidizing menaquinol and depositing the protons into the periplasm, without active pumping of protons. This is the case for bacterial reaction centers and most succinate dehydrogenase complexes, showing an $H^+/e^-$ ratio of 1. Alternatively, an active proton pumping may take place, giving an $H^+/e^-$ ratio of 2, as found for cytochrome $bc_1$ [28]. To further investigate the proton pumping mechanism of ACIII, more studies using protein reconstitution into liposomes are underway.

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