Cytochrome c_{551} Is a Mediator of Electron Transfer between Copper-Containing Nitrite Reductase and Azurin in a Denitrifying Bacterium, *Achromobacter xylosoxidans*

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Cu-containing nitrite reductase from a denitrifying bacterium, *Achromobacter xylosoxidans* GIFU1051 (the organism formerly known as *Alcaligenes xylosoxidans*), can accept an electron from three possible electron donors (cytochrome c_{551} and two azurins). We have kinetically demonstrated that cytochrome c_{551} functions as a mediator in the electron flow processes between the enzyme and two azurins.

Dissimilatory nitrite reductase (NIR) is a key enzyme in biological denitrification, catalyzing the first step that leads to gaseous products (NO, N_2O, and N_2).[1,2] There are two main categories of NIR: the heme-containing and Cu-containing enzymes. Generally, Cu-containing NIRs (CuNIRs) from *Achromobacter cycloclastes* (green *Ac*NIR), *Alcaligenes faecalis* (green *Af*NIR), and *Achromobacter xylosoxidans* (blue *Ax*NIR, the organism is formerly known as *Alcaligenes xylosoxidans*)[3] fold to a common trimeric structure, in which a monomer (ca. 37 kDa) contains one type 1 Cu (T1Cu) and one type 2 Cu (T2Cu).[2,4–6] The enzyme receives an electron from the specific electron-donor proteins at the T1Cu site and catalyzes one-electron reduction of NO_2^- to NO at the T2Cu site.

Recently, it has been reported that three electron-transfer (ET) proteins (azurin-I (*Ax*AzI), azurin-II (*Ax*AzII),[7,8] and cytochrome c_{551} (*Ax*Cyt c_{551})[9]) from *A. xylosoxidans* act as electron donors for *Ax*NIR. *Ax*AzI and *Ax*AzII are typical blue copper proteins, exhibiting an intense absorption band at ca. 600 nm in the visible spectra of their oxidized forms ($\varepsilon =$ ca. 5000 $M^{-1}\,cm^{-1}$). Based on the steady-state kinetics for the nitrite reduction of *Ax*NIR with *Ax*Az's, it has been reported that bimolecular ET rate constants between *Ax*NIR and *Ax*Az's are $(3.5–4.0) \times 10^3\,M^{-1}\,s^{-1}$.[8] The *Ax*NIR–AzII docking simulation model reveals that the distance between both redox Cu centers is extremely long (over 20 Å), indicating that the efficiency of the ET reaction between them is considerably low.[10] On the other hand, the ET reaction between *Ax*Cyt c_{551} and *Ax*NIR has been previously examined by cyclic voltammetry.[9] At pH 6.0, oxidized *Ax*Cyt c_{551} has two maximum peaks at 408 (Soret band, $\varepsilon = 118$) and 521 nm ($\varepsilon = 8.5$ $mM^{-1}\,cm^{-1}$), and the reduced protein gives the intense Soret, β, and α bands at 417 ($\varepsilon = 142$), 522 ($\varepsilon = 17$), and 551 nm ($\varepsilon = 22\,mM^{-1}\,cm^{-1}$), respectively. These findings indicate that both forms have a six-coordinate low-spin heme.[9] *Ax*Cyt c_{551} accepts an electron from an electrode and then donates it to *Ax*NIR in the presence of NO_2^-. Under these conditions, the shape of the voltammogram becomes sigmoidal with an increase of the catalytic current due to the regeneration of oxidized *Ax*Cyt c_{551}.[9] Very recently, stopped-flow kinetics experiments have demonstrated that an electron transfers rapidly from *Ax*Cyt c_{551} to *Ax*NIR.[11] The second-order ET rate constant (k) between *Ax*Cyt c_{551} and *Ax*NIR was estimated to be $(4.8 \pm 0.2) \times 10^6\,M^{-1}\,s^{-1}$ at 25 °C, being ca. 10^3 times as large as *Ax*Az's. Therefore, an electron donor protein for *Ax*NIR has been the focus of considerable debate.

In this work, the involvement of *Ax*Cyt c_{551} in the electron flow processes from *Ax*Az's to *Ax*NIR has been studied. The rapid reductions of *Ax*Cyt c_{551} with *Ax*Az's were investigated by monitoring the increasing curve of the Soret band of *Ax*Cyt c_{551}. Unusual kinetics has been observed in the case of the *Ax*AzI–Cyt c_{551} redox pair because the reaction exhibited two phases, fast (second-order ET rate constant, $k_{fast} = (8.4 \pm 0.8) \times 10^5\,M^{-1}\,s^{-1}$) and slow ($k_{slow} = (4.9 \pm 0.3) \times 10^4\,M^{-1}\,s^{-1}$) phases. On the other hand, the reduction of *Ax*Cyt c_{551} with *Ax*AzII followed monophasic kinetics with $k = (1.2 \pm 0.2) \times 10^6\,M^{-1}\,s^{-1}$ (Figure 1). Their kinetic behavior seems to be due to the conformational difference around the His35 residues in both Az's.[12–16] In the other denitrifying bacterium *Pseudomonas aeruginosa*, the ET reaction between Az (*Pa*Az) and Cyt c_{551} (*Pa*Cyt c_{551}) also exhibits biphasic behavior like that between *Ax*AzI and *Ax*Cyt c_{551}. Through pH titrations in NMR and kinetics studies, it has been demonstrated that protonation of His35 affected by solvent exposed environments in the *Pa*Az molecule, is an important factor in the ET kinetic behavior.[13,14]

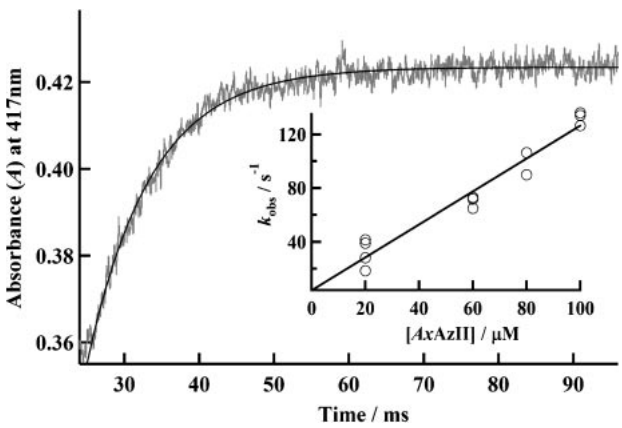

Figure 1. Rapid increasing absorbance of *Ax*Cyt c_{551} by a stopped-flow spectrophotometer. 100 μM reduced *Ax*AzII and 2 μM *Ax*Cyt c_{551} were mixed in 10 mM potassium phosphate buffer (pH 6.0) at 25 °C. The solid line is a single-exponential fitting curve. Inset shows plots of k_{obs} values versus *Ax*AzII concentrations.

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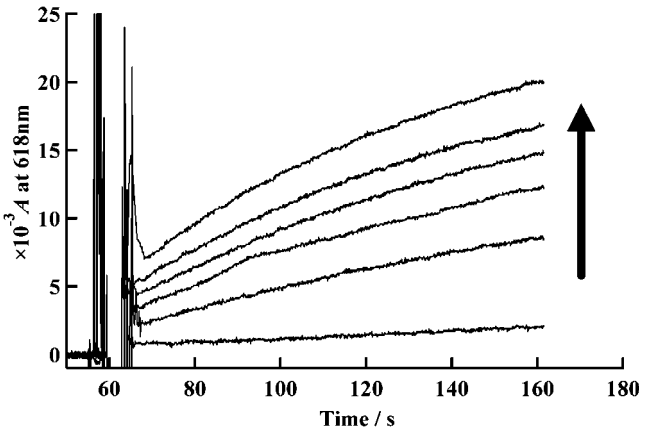


Figure 2. Electron-transfer reactions from *Ax*AzII to *Ax*NIR mediated by *Ax*Cyt c_{551} in the presence of $NaNO_2$. The reaction was monitored by oxidation of *Ax*AzII in a 5 mm cell containing 10 mM potassium phosphate buffer (pH 7.0). *Ax*NIR (50 nM) was added to a mixture of *Ax*Cyt c_{551}, *Ax*AzII (40 μM), and $NaNO_2$ (1 mM), which was incubated for 60 s at 25 °C. Upward arrow means the acceleration of turnover by adding *Ax*Cyt c_{551} (0, 200, 400, 600, 800, and 1000 nM).

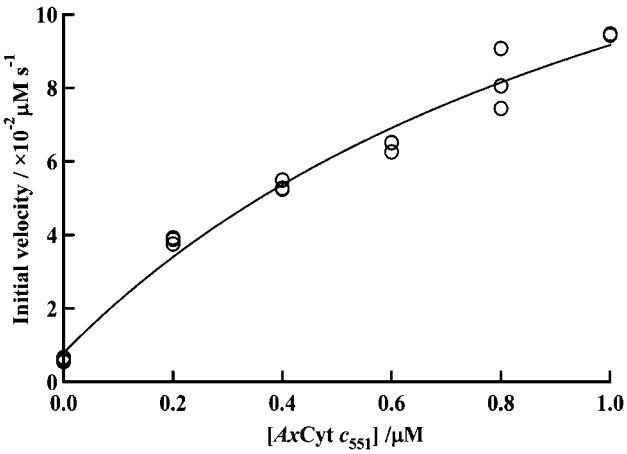
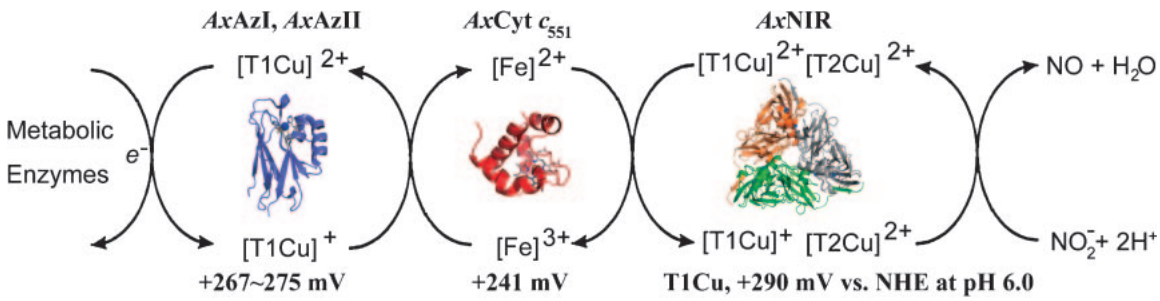


Figure 3. Plots of intitial oxidation velocities of *Ax*AzII versus *Ax*Cyt c_{551} concentrations. The line is a fitting curve using eq 2 (see Experimental).



Scheme 1. A possible electron transport chain in *A. xylosoxidans* GIFU1051.

Comparison of the second-order ET rate constants between *Ax*Az (I and II), *Ax*Cyt c_{551}, and *Ax*NIR strongly suggests that *Ax*Cyt c_{551} acts as a mediator in the electron flow between *Ax*NIR and *Ax*Az's. To obtain direct evidence, the *Ax*Cyt c_{551} concentration dependence on the nitrite reduction assay of *Ax*NIR using reduced *Ax*AzII was investigated. The assay mixtures contained sodium nitrite (1 mM), reduced *Ax*AzII (40 μM), *Ax*NIR (50 nM), and various concentrations of *Ax*Cyt c_{551} in 10 mM potassium phosphate buffer (pH 7.0). The reactions were started by addition of the enzyme, and the initial velocities (v) were monitored by the increasing absorbance at 618 nm. Oxidation of *Ax*AzII was slightly observed in the absence of *Ax*Cyt c_{551} ($v = 0.0061\ \mu M\ s^{-1}$), but was accelerated dramatically by addition of *Ax*Cyt c_{551} (Figure 2).

The values of the initial oxidation velocities of *Ax*AzII were plotted against *Ax*Cyt c_{551} concentrations (Figure 3). When 1 μM *Ax*Cyt c_{551} was added to the mixture, the initial velocity was about 15 times as fast as that in the absence of *Ax*Cyt c_{551}. This result clearly indicates that *Ax*Cyt c_{551} functions as a mediator in the electron flow from *Ax*AzII to *Ax*NIR. Moreover, curve fitting analysis shown in Figure 3 means that *Ax*Cyt c_{551} mediates the ET process from *Ax*AzII to *Ax*NIR, but the backward ET process from *Ax*Cyt c_{551} to *Ax*AzII occurs simultaneously.

Recent biochemical studies have demonstrated that electrons derived from some metabolic processes are transferred to the respiratory chains via various ET proteins.[17] In the denitrifying bacterium *A. xylosoxidans*, at least three ET proteins, *Ax*AzI, *Ax*AzII, and *Ax*Cyt c_{551}, function as one-electron donors for *Ax*NIR, as shown in Scheme 1. The electron flow process mediated by *Ax*Cyt c_{551} is far more effective than that of the *Ax*Az–NIR pair only. Although the redox potentials of *Ax*Az and *Ax*Cyt c_{551} show the unfavorable uphill ET reaction from copper to heme, the formation of an ET complex between them might bring their redox potentials close to each other.[18–20] The high-resolution crystal structure of the ET complex between *Ax*NIR and *Ax*Cyt c_{551} will be presented for supporting these kinetics results.[11]

Experimental

Materials. All chemicals reagents used in this study are commercial products of the highest available purity and were used as received.

Purification of *Ax*AzI, *Ax*AzII, *Ax*Cyt c_{551}, and *Ax*NIR. Isolation and purification of *Ax*NIR,[21] *Ax*AzI,[22] *Ax*AzII,[22] and

$$\mathrm{Az_{red}} + \mathrm{Cyt_{ox}} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{Az : Cyt} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \mathrm{Az_{ox}} + \mathrm{Cyt_{red}}$$

$$\mathrm{Cyt_{red}} + \mathrm{NIR} \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \mathrm{Cyt : NIR} \xrightarrow{k_4} \mathrm{Cyt_{ox}} + \mathrm{NIR}$$

Scheme 2.

*Ax*Cyt c_{551}[9] from *Achromobacter xylosoxidans* GIFU 1051 were performed according to previously described procedures.

Stopped-Flow Kinetics of ET Reaction between *Ax*Az and *Ax*Cyt c_{551}. The rapid reductions of *Ax*Cyt c_{551} with two *Ax*Az's were recorded at 417 nm by monitoring the increasing curve of the Soret band of *Ax*Cyt c_{551}. The kinetic traces were acquired at 25.0 °C with an RA-2000 stopped-flow spectrophotometer (Otsuka Electronics, Osaka, Japan) using the single-wavelength mode of the machine under Ar atmosphere. The kinetics were analyzed under the pseudo-first-order conditions with 2 μM *Ax*Cyt c_{551} and 20–100 μM *Ax*Az's in 10 mM potassium phosphate buffer (pH 6.0). Pseudo-first-order rate constants were calculated by nonlinear regression with IgorPro version 5.0 (WaveMetrics, Lake Oswego, OR, USA).

Steady State Kinetics. Figure 3 shows plots of initial oxidation velocities of *Ax*AzII versus *Ax*Cyt c_{551} concentrations. Since *Ax*Cyt c_{551} behaves like an enzyme, the initial velocity ($v = \mathrm{d[Az_{ox}]}/\mathrm{d}t$) is expected to be linearly dependent on the *Ax*Cyt c_{551} concentration, but actually exhibits a hyperbolic dependence. The following simple kinetic model was used for interpreting the behavior (Scheme 2).

In this scheme, the backward ET process from reduced *Ax*Cyt *c* ($\mathrm{Cyt_{red}}$) to oxidized *Ax*Az ($\mathrm{Az_{ox}}$) is involved. According to steady-state analysis, $\mathrm{d[Az_{ox}]}/\mathrm{d}t$ is given as eq 1, which means that oxidation of *Ax*AzII is restrained by the backward ET (k_{-2}).

$$\begin{aligned} v = {} & k_1k_2k_3k_4[\mathrm{E}][\mathrm{Az_{red}}][\mathrm{C_0}]/\{k_3k_4(k_{-1}+k_2)[\mathrm{E}] \\ & + k_1k_2(k_{-3}+k_4)[\mathrm{Az_{red}}] + k_1k_3(k_2+k_4)[\mathrm{E}][\mathrm{Az_{red}}] \\ & + k_{-1}k_{-2}(k_{-3}+k_4)[\mathrm{Az_{ox}}] \\ & + k_1k_{-2}(k_{-3}+k_4)[\mathrm{Az_{ox}}][\mathrm{Az_{red}}]\} \end{aligned} \quad (1)$$

In eq 1, [E] is concentration of NIR, $[\mathrm{C_0}]$ is total concentration of *Ax*Cyt c_{551}. Two second-order rate constants (ca. $10^6\,\mathrm{M^{-1}\,s^{-1}}$) of the ET processes from *Ax*Cyt c_{551} to *Ax*NIR and from *Ax*Az to *Ax*Cyt c_{551} are similar to each other. Since reduced *Ax*AzII is in excess compared with *Ax*Cyt c_{551} and NIR, the rate limiting step is the ET reaction from *Ax*Cyt c_{551} to *Ax*NIR. Oxidized *Ax*AzII exists in the same concentration with total *Ax*Cyt c_{551} concentration just after mixing, that is, $[\mathrm{Az_{ox}}] = [\mathrm{Az_{ox}}]_{\mathrm{init}} + \Delta[\mathrm{Az_{ox}}]$, where $[\mathrm{Az_{ox}}]_{\mathrm{init}}$ is initial concentration of *Ax*Az and equal to $[\mathrm{C_0}]$. Therefore, eq 1 can be simply expressed as

$$v = V[\mathrm{C_0}]/(K + [\mathrm{C_0}]) + d \quad (2)$$

where *d*, *V*, and *K* are the initial oxidation velocity of *Ax*AzII in the absence of *Ax*Cyt c_{551}, $k_2k_3k_4[\mathrm{E}]/k_{-2}(k_{-3}+k_4)$, and $\{k_2(k_{-3}+k_4)+k_3(k_2+k_4)\}/k_{-2}(k_{-3}+k_4)$, respectively. The plot of *v* shows good curve fitting to eq 2. The oxidation of *Ax*AzII is clearly accelerated by adding Cyt c_{551}, although it is restrained by the backward ET.

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