An Alternative Electron Donor for *Hyphomicrobium* Copper-containing Nitrite Reductase

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Cu-containing nitrite reductase from *Hyphomicrobium denitrificans* catalyzes the reduction of nitrite to nitric oxide by an electron from a specific electron donor protein, cytochrome c_{550}. However, we have recently found that another periplasmic soluble cytochrome *c*, cytochrome c_L, also donates an electron to this enzyme. The detailed electron-transfer mechanism was investigated kinetically.

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] There are two main categories of NIR: the heme-containing and Cu-containing enzymes. Generally, Cu-containing NIRs (CuNIRs) from *Achromobacter cycloclastes* (green AcNIR), *Alcaligenes faecalis* (green AfNIR), and *Alcaligenes xylosoxidans* (blue AxNIR) fold a trimeric structure, in which a monomer (ca. 37 kDa) contains one type 1 Cu and one type 2 Cu.[2] The enzyme receives one electron at the type 1 Cu from a specific electron donor protein and catalyzes one electron reduction of NO_2^- to NO at the type 2 Cu. The type 2 Cu site is connected via a His–Cys bridge to the type 1 Cu site. Compared with these well-characterized CuNIRs, the enzyme from a methylotrophic denitrifying bacterium, *Hyphomicrobium denitrificans* A3151 (greenish-blue HdNIR) has a larger molecular mass, 50-kDa per monomer, and exhibits different spectroscopic and functional features.[3,4] Recently, crystal structure of a novel hexameric HdNIR has been presented (Figure 1).[5] The HdNIR molecular structure reveals a trigonal-prism-shaped homohexamer (a tightly associted dimer of trimers), in which the monomer consisting of 447 amino acids and three Cu atoms is organized. The monomer is hydrolyzed into two protein fragments, N-terminal region protein containing the type 1 Cu_N and C-terminal region protein containing the type 1 Cu_C and type 2 Cu, with subtilisin.[6]

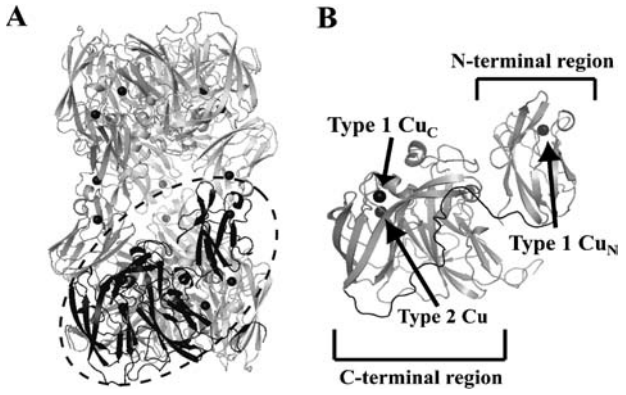

Figure 1. Ribbon diagram of the hexameric structure (A) and a monomer (B) of HdNIR. The broken line represents a monomer region in the hexamer.

The reduction of HdNIR with a physiological electron donor protein, cytochrome c_{550} (Cyt c_{550}) has been investigated by monitoring the decay of the Soret band of Cyt c_{550}.[5] Interestingly, unusual kinetics was observed because the reaction exhibited two phases, fast ($k = (1.4 \pm 0.28) \times 10^5\ M^{-1}\ s^{-1}$) and slow ($k = (9.4 \pm 1.9) \times 10^3\ M^{-1}\ s^{-1}$) phases. On the other hand, reductions of HdNIR mutants, C114A and C260A,[7] in which Cys114 and Cys260 ligands are replaced with Ala, followed monophasic kinetics with second-order rate constants as follows: C114A, $k = (8.4 \pm 1.0) \times 10^5\ M^{-1}\ s^{-1}$ and C260A, $k = (3.5 \pm 0.21) \times 10^3\ M^{-1}\ s^{-1}$. Therefore, it has been suggested that Cyt c_{550} is an excellent electron donor for the type 1 Cu_C.

Recently, we reported that another cytochrome *c* (cytochrome c_L, Cyt c_L) from *H. denitrificans* A3151 is a physiological electron acceptor for methanol dehydrogenase (MDH).[8] Cyt c_L is also localized in the periplasmic space with HdNIR and Cyt c_{550}. To investigate the electron transfer from Cyt c_L to HdNIR, the reduction of HdNIR was monitored spectrophotometrically in the presence of one or both MDH and Cyt c_L (Figure 2). At first the oxidized HdNIR was mixed with MDH. The visible spectrum was not so changed. However, further addition of Cyt c_L to the mixture results in rapid bleaching of the blue band of HdNIR. The reduction kinetics of HdNIR with Cyt c_L was further analyzed by stopped-flow spectrophotometry.[9] The decay curve at 415 nm exhibits two phases, and the second-order rate constants of the fast and slow phases were calculated as follows: $k_{fast} = (6.42 \pm 0.24) \times 10^4\ M^{-1}\ s^{-1}$ and $k_{slow} =$

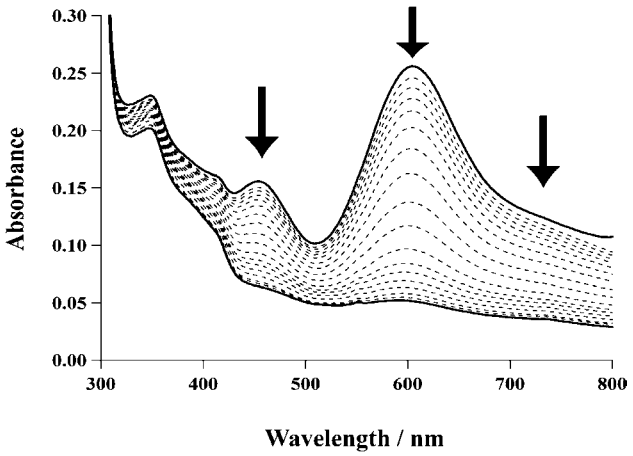

Figure 2. Intermolecular electron-transfer reaction from MDH to HdNIR in the presence of Cyt c_L. Thick solid line: the visible absorption spectrum of the mixture of 44 μM HdNIR and 19 μM MDH in 20 mM Mes–NaOH buffer (pH 6.0) containing 400 mM methanol. Broken line: the spectra recorded with the 2 min interval, after 0.07 μM Cyt c_L was added to the mixture.

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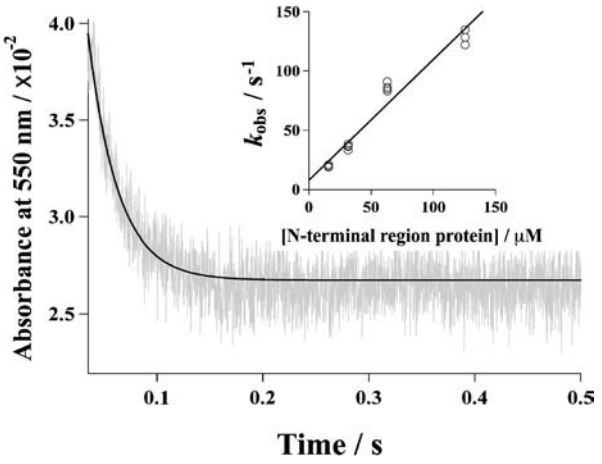

Figure 3. Rapid decay curve observed by a stopped-flow spectrophotometer. 31.5 μM N-terminal region protein and 3.5 μM Cyt c_L were contained in 10 mM Mes–NaOH buffer (pH 5.5). *Insert* shows the plot of k_{obs} vs. N-terminal region protein concentration.

$(1.87 \pm 0.05) \times 10^4\,M^{-1}\,s^{-1}$. The reduction of C260A also followed biphasic kinetics with second-order rate constants as follows: $k_{fast} = (6.27 \pm 0.22) \times 10^4\,M^{-1}\,s^{-1}$ and $k_{slow} = (1.16 \pm 0.10) \times 10^4\,M^{-1}\,s^{-1}$. On the other hand, the k_{obs} of C114A was too small to determine the precise rate constant, $<1.0 \times 10^3\,M^{-1}\,s^{-1}$. Furthermore, the detailed kinetics of intermolecular electron transfer between Cyt c_L and the N-terminal region protein consisting of 145 amino acids[10] is shown in Figure 3.

The k_{obs} values were obtained under the conditions that the reaction mixture contains 3.5 μM Cyt c_L and 18–125 μM N-terminal region protein at pH 5.5. The rapid oxidation of Cyt c_L is monophasic and obeys pseudo-first-order kinetics. From the slope of the plot of k_{obs} vs. the N-terminal region protein concentration, the second-order rate constant was determined to be $(1.02 \pm 0.06) \times 10^6\,M^{-1}\,s^{-1}$ at 25 °C (Figure 3, inset). The midpoint potential ($E_{1/2}$) of Cyt c_L is +210 mV at pH 7.0,[8] and the cyclic voltamogram of the N-terminal region protein shows a $E_{1/2}$ of +310 mV vs. NHE at pH 6.0. These findings clearly supports that the rapid electron-transfer event occurs at the intermolecule between Cyt c_L and the type 1 Cu_N in the N-terminal region of HdNIR.

In the present kinetics study, it has been demonstrated that Cyt c_L is an electron donor protein for the type 1 Cu_N of HdNIR. Both Cyt c_L and Cyt c_{550} are soluble proteins localized in the periplasmic space. Therefore, the physiological electron donation to HdNIR ocurrs not only to the type 1 Cu_C but also to the type 1 Cu_N. The type 1 Cu_N is known to be essential for dimerization of the trimers in the HdNIR molecule.[5] Although the other functions of the type 1 Cu_N remains unknown, these findings will be helpful for understanding the roles of the N-terminal region in hexameric HdNIR.

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9 The reduction of HdNIR, C260A, or C114A with Cyt c_L was recorded at 415 nm by monitoring the decay of the Soret band of Cyt c_L. Their kinetics were studied under the pseudo-first-order condition with 30 μM HdNIR or its mutants and 2.5 μM Cyt c_L in 20 mM potassium phosphate buffer (pH 6.5). While the reduction of the N-terminal region protein with Cyt c_L was recorded at 550 nm by monitoring the decay of the α-band of Cyt c_L. These kinetic traces were acquired at 25 °C by using a RA-2000 stopped-flow spectrophotometer (Otsuka Electronics). Pseudo-first-order rate constants were calculated by nonlinear regression with IgorPro version 4.02 (WaveMetrics).

10 For preparation of the N-teminal region protein, we performed a PCR using oligonucleotide primers, i.e., 5′-acggatccgatgctccggccatg-3′ and 5′-ccaagcttatttcatctcggcgcgattgcccgg-3′ with pQHdNIR2 as template.[4] The amplified DNA fragment was digested with restriction enzymes (*Bam*HI and *Hind*III) and inserted into pMal–c2x vector (New England Biolab.). The N-terminal region protein was expressed in *E. coli* JM109 as maltose-binding domain fused recombinant protein. The protein was purified, as decribed previously.[11] The spectroscopic characters of the N-terminal region protein are quite similar to those of the type 1 Cu_N in HdNIR.

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