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Pulse Radiolysis of Hexameric Nitrite Reductase Containing Two Type 1 Cu Sites in a Monomer

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Pulse radiolysis of hexameric nitrite reductase containing the two type 1 Cu sites in a monomer has been carried out. Reduction of the enzyme with *N*-methylnicotinamide radical follows biphasic kinetics, and the fast and slow phases were assigned to reductions of type 1 Cu_N and type 1 Cu_C, respectively.

Denitrification, an important step in the terrestrial nitrogen cycle, is the dissimilatory reduction of nitrate or nitrite to produce N_2 via NO and N_2O by prokaryotic organisms.[1–3] Copper-containing nitrite reductase (NIR) catalyzes one-electron reduction of NO_2^- to NO. Well-known NIRs from *Achromobacter cycloclastes* (AcNIR),[4,5] *Alcaligenes faecalis*,[6] *Alcaligenes xylosoxidans* (AxNIR),[7,8] and *Rhodobacter sphaeroides*[9] form a homotrimer, in which each monomer composed of two consecutive Greek-key β barrel folding domains[10] contains one type 1 Cu having four ligands (2His, Cys, and Met) and one type 2 Cu having three His ligands. The distance between the two Cu sites connected through the sequence segment (–Cys–His–) is ca. 12.5 Å. The enzyme receives one electron at the type 1 Cu from an electron donor protein and catalyzes one-electron reduction of NO_2^- to NO at the type 2 Cu, which intramolecularly accepts an electron from the reduced type 1 Cu.

We have recently determined the X-ray crystal structure of hexameric NIR (HdNIR) from a methylotrophic denitrifying bacterium, *Hyphomicrobium denitrificans* (Figure 1).[11] The 50-kDa monomer of HdNIR is composed of ca. 14-kDa N-terminal domain having one blue type 1 Cu (type 1 Cu_N) and ca. 35-kDa C-terminal domain possessing one green type 1 Cu (type 1 Cu_C) and one type 2 Cu (Scheme 1).[12] The N-terminal and C-terminal domains are similar to a cupredoxin containing type 1 Cu and a monomer of well-known NIRs, respectively. Hexameric HdNIR is a dimer of trimers and altogether contains twelve type 1 Cu and six type 2 Cu atoms. In order to investigate a binding site of HdNIR to cytochrome c_{550}, which is a physiological electron-donor protein, the reductions of HdNIR and the two mutants (Scheme 1) with cytochrome

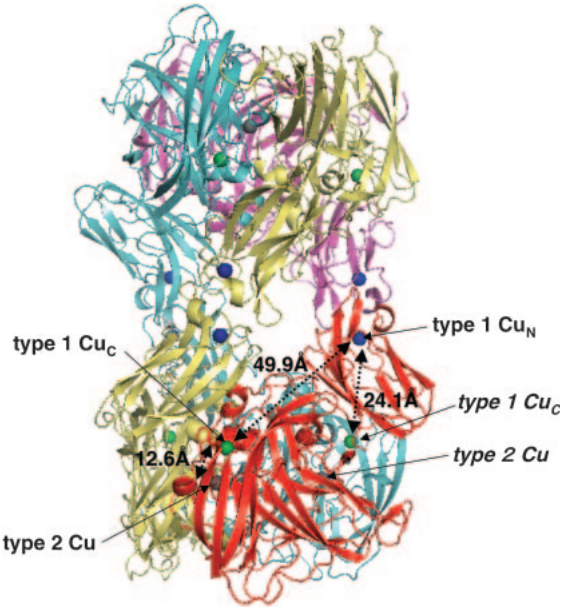
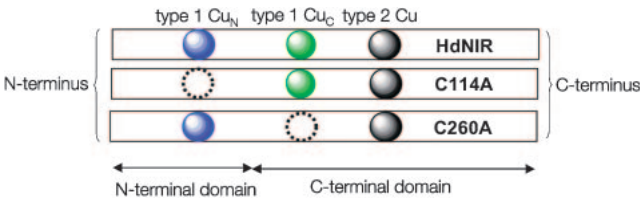


Figure 1. Arrangement of the type 1 Cu_N, type 1 Cu_C, and type 2 Cu in a monomer (red) of hexameric HdNIR (PDB 2DV6). Italics indicate the Cu centers in the adjacent monomer.



Scheme 1. Schematic structures of monomers of native and two mutant HdNIRs. Dotted circles show the depletion of the type 1 Cu.

c_{550} have been observed by stopped-flow method.[11,13] The reduction time course of HdNIR was fitted by biexponential function, suggesting that the fast and slow reduction processes of HdNIR correspond to reductions of the type 1 Cu_C and type 1 Cu_N, respectively. The reduction of the type 1 Cu_C close to the type 2 Cu smoothly leads to the subsequent electron transfer to the type 2 Cu where nitrite is reduced.

Pulse radiolyses of well-known trimeric NIRs have been carried out to observe intramolecular electron-transfer reactions from the type 1 Cu to the type 2 Cu.[14–16] A hydrated electron generated by pulse radiolysis in an aqueous solution is unable to reduce the type 1 Cu. However, by using *N*-methylnicotinamide (NMA) as a mediator, the generated NMA radical can effectively reduce it. Although C260A containing type 1 Cu_N and type 2 Cu exhibited no intramolecular electron transfer, the rate constant ($1.0 \times 10^3\ s^{-1}$) of the intramolecular electron transfer from the type 1 Cu_C to the type 2 Cu in C114A was similar to those from the type 1 Cu to the type 2 Cu in AcNIR and AxNIR ($(1.9–2.0) \times 10^3\ s^{-1}$) in the presence of nitrite at pH 6.0.[16] On the other hand, the apparent first-order rate constant for the intramolecular electron transfer from the type 1 Cu to the type 2 Cu in native HdNIR was calculated to be $2.4 \times 10^{-2}\ s^{-1}$ at pH 6.0,[17] being very much smaller than those of AcNIR,[14] AxNIR,[14] and C114A.[16] Accordingly we

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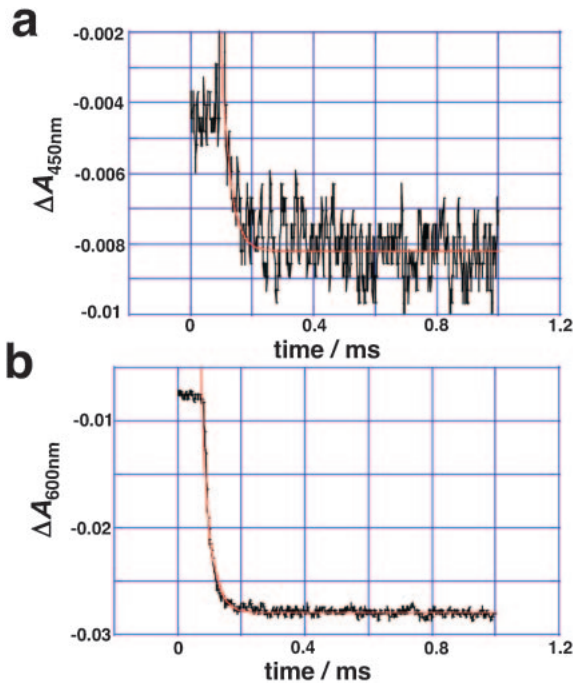

Figure 2. Absorption changes after pulse radiolysis of HdNIR measured at 600 (a) and 450 nm (b) in 10 mM (1 M = 1 mol dm^{-3}) potassium phosphate buffer (pH 7.0) at 20.0 °C. Concentrations: HdNIR, 130 μM; *N*-methylnicotinamide, 1 mM; *tert*-butyl alcohol, 0.1 M. The red lines are fitting curves using exponential functions.

have studied the reactions of an NMA radical with two type 1 Cu centers to elucidate the very slow intramolecular electron-transfer process.

The absorption changes after pulse radiolysis of HdNIR in the absence of nitrite were monitored at 600 and 450 nm (Figure 2). HdNIR has two visible absorption bands at 454 ($\varepsilon = 2900\,M^{-1}\,cm^{-1}$) and 605 nm ($\varepsilon = 6300$).[12] The absorption curve measured at 600 nm was fitted by a double-exponential function, that is, the reduction behavior of HdNIR follows biphasic kinetics. The initial fast phase was associated with an amplitude of ca. 80% of the total absorbance change. On the other hand, the absorption curve observed at 450 nm was fitted by a single-exponential function. Figure 3 shows the enzyme-concentration dependence of rate constants obtained at 450 and 600 nm. Both rate constants of the fast and slow phases observed at 600 nm linearly increase with the concentrations of the enzyme. Therefore, the second-order rate constants of the fast and slow phases were calculated to be $(1.3 \pm 0.3) \times 10^9$ and $(3.1 \pm 0.9) \times 10^8\,M^{-1}\,s^{-1}$, respectively (Table 1). The rate constant obtained at 450 nm is also proportional to the enzyme concentration and the second-order rate constant is in accord with that of the slow phase. These findings suggest that the slow phase reaction observed at 600 nm is cognate with the reaction monitored at 450 nm.

The kinetic difference absorption spectrum, which was obtained by subtracting the absorption spectrum of oxidized HdNIR from the time-resolved absorption spectrum at 200 μs after pulse radiolysis of the enzyme, is shown in Figure 4 with the visible absorption spectra[12] of the type 1 Cu_N in C260A and the type 1 Cu_C in C114A. Although an intense absorption

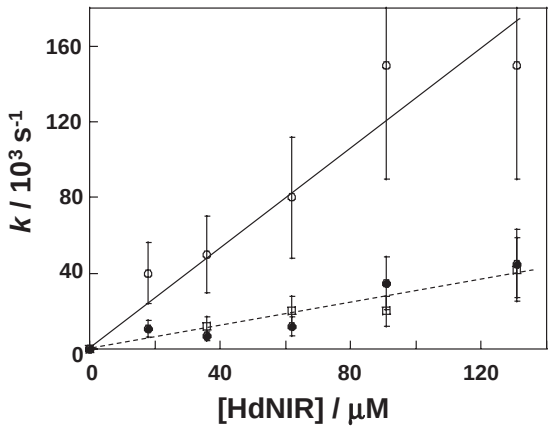

Figure 3. Enzyme-concentration dependence of rate constants determined at 600 nm (fast phase, open circle; slow phase, closed circle) and 450 nm (open square) in 10 mM potassium phosphate buffer (pH 7.0) at 20.0 °C. Concentrations: *N*-methylnicotinamide, 1 mM; *tert*-butyl alcohol, 0.1 M.

Table 1. Second-Order Rate Constants (k_2) of Reduction of the Type 1 Cu with the NMA Radical at pH 7.0

Enzyme	k_2/M^{-1} s^{-1}	Ref.
HdNIR at 600 nm	$(1.3 \pm 0.3) \times 10^9$	this work
	$(3.1 \pm 0.9) \times 10^8$	
HdNIR at 450 nm	$(3.1 \pm 0.9) \times 10^8$	this work
C260A (type 1 Cu_N)	2.4×10^9	unpublished
C114A (type 1 Cu_C)	8.3×10^8	16

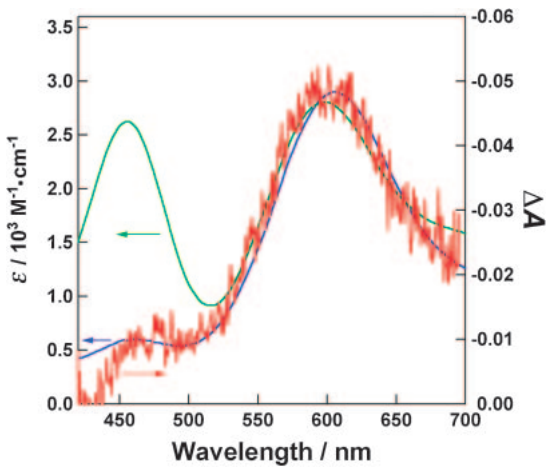

Figure 4. Kinetic difference absorption spectrum at 200 μs after pulse radiolysis of HdNIR at 20.0 °C (red line) and visible absorption spectra of the type 1 Cu_C in C114A (green line) and the type 1 Cu_N in C260A (blue line). Concentrations: HdNIR, 68 μM; *N*-methylnicotinamide, 1 mM; *tert*-butyl alcohol, 0.1 M; 10 mM potassium phosphate buffer (pH 5.8).

band of the NMA radical at 420 nm disturbs the absorption spectrum in the range of 420–450 nm, a similarity between the kinetic difference spectrum and the absorption spectrum of the type 1 Cu_N indicates that the fast process of reduction

of HdNIR is probably assigned to reduction of the type 1 Cu_N.[18] Consequently the slow process is probably assigned to reduction of the type 1 Cu_C, which shows an intense 450-nm absorption band.[12] The NMA radical gives an electron to the type 1 Cu_N and type 1 Cu_C at the fast and slow phases, respectively. Likewise the type 1 Cu_N in C260A is more quickly reduced than the type 1 Cu_C in C114A, as shown in Table 1. The type 1 Cu_N in HdNIR is more exposed to bulk solvent than the type 1 Cu_C, and hence the type 1 Cu_N is quickly reduced with the small molecule of the NMA radical compared to the type 1 Cu_C. The reduction of the type 1 Cu_C leads to the catalytic reaction of nitrite at the type 2 Cu. However, the reduction of the type 1 Cu_N is unfavorable for catalytic reaction, because the reduced type 1 Cu_N gives an electron to the type 2 Cu through the type 1 Cu_C in the adjacent monomer. The type 1 Cu_C is located 24.1 Å apart from the type 1 Cu_N (Figure 1). Therefore, the long-distance electron transfer between these type 1 Cu sites might explain the fact that the apparent intramolecular electron transfer rate constant of HdNIR is much smaller than that of C114A.

We have recently demonstrated that cytochrome c_L, which occurs in the periplasmic space with cytochrome c_{550} and HdNIR, is also an electron-donor protein for the type 1 Cu_N of HdNIR.[19] The type 1 Cu_N is known to be essential for dimerization of the trimers in HdNIR.[11] In addition, the reduction of the type 1 Cu_N might bring about some physiologically significant functions such as electron storage and encouragement of catalytic reaction. Studies are in progress to ascertain the multifunction of the type 1 Cu_N.

Experimental

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

Purification of HdNIR. *Hyphomicrobium denitrificans* A3151 cells were grown at 30 °C for 5 days under static conditions in an aqueous medium containing 1% (vol/vol) methanol, 1% (wt/vol) potassium nitrate, and several mineral salts. The isolation and purification of HdNIR was carried out according to a previous method.[12] The cells suspended in 0.01 M phosphate buffer (pH 7.0) were sonicated for 30 min. After centrifugation of the sonicated sample for 1 h, cell-free extract was treated with ammonium sulfate (40% wt/vol). The precipitate containing crude HdNIR was collected by centrifugation. The precipitate was dissolved in distilled water and the resulting solution was applied to a Sephadex G-200 column equilibrated with 0.01 M phosphate buffer (pH 7.0). The blue fraction was eluted with the same buffer and purified with DEAE-Sephadex and Sephadex G-150. The sample showed a single band (49 kDa) by SDS–polyacrylamide gel electrophoresis.

Pulse Radiolysis of HdNIR. The sample of HdNIR for pulse radiolysis was prepared as follows: Ar gas was passed over the surface of 10 mM phosphate buffer (pH 6–7) containing HdNIR (20–140 μM), NMA (1 mM), and *tert*-butyl alcohol (0.1 M) for scavenging of OH radical. No effect of *tert*-butyl alcohol on the optical absorption spectrum of HdNIR was observed. Pulse radiolysis experiments were carried out with an electron linear accelerator at the Institute of Scientific and Industrial Research, Osaka University. A fresh solution was used for each pulse experiment. Pulse width and energy were 8 ns and 27 MeV, respectively. Light source was a 200-W Xe lamp and the light was passed through a quartz cell having an optical path length of 10 mm. Absorption change was measured using a spectrophotometric system composed a Nikon monochrometer, an R-982 photomultiplier, and a Unisoku data analyzing system. For time-resolved transient absorption spectral measurement, the monitored light was focused into a quartz optical fiber, which transported the electron pulse-induced transmittance changes to a gated spectrometer (Unisoku, TSP-601-02). The resolution time of spectrophotometer is 10 ns.

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References

1 W. G. Zumft, *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 533.
2 S. Suzuki, K. Kataoka, K. Yamaguchi, *Acc. Chem. Res.* **2000**, *33*, 728.
3 P. Tavares, A. S. Pereira, J. J. G. Moura, I. Moura, *J. Inorg. Biochem.* **2006**, *100*, 2087.
4 E. T. Adman, J. W. Godden, S. Turley, *J. Biol. Chem.* **1995**, *270*, 27458.
5 S. V. Antonyuk, R. W. Strange, G. Sawers, R. R. Eady, S. S. Hasnain, *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 12041.
6 M. E. P. Murphy, S. Turley, E. T. Adman, *J. Biol. Chem.* **1977**, *45*, 28455.
7 F. E. Dodd, S. S. Hasnain, Z. H. L. Abraham, R. R. Eady, B. E. Smith, *Acta Crystallogr., Sect. D* **1997**, *53*, 406.
8 M. A. Hough, M. J. Ellis, S. V. Antonyuk, R. W. Strange, G. Sawers, R. R. Eady, S. S. Hasnain, *J. Mol. Biol.* **2005**, *350*, 300.
9 F. Jacobson, H. Guo, K. Olesen, M. Okvist, R. Neutze, L. Sjolin, *Acta Crystallogr., Sect. D* **2005**, *61*, 1190.
10 E. T. Adman, M. E. P. Murphy, in *Handbook of Metalloproteins*, ed. by A. Messerschmidt, R. Huber, T. Poulos, K. Wieghardt, Wiley, Chichester, UK, **2001**, Vol. 2, pp. 1381–1390.
11 M. Nojiri, Y. Xie, T. Inoue, T. Yamamoto, H. Matsumura, K. Kataoka, Deligeer, K. Yamaguchi, Y. Kai, S. Suzuki, *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 4315.
12 K. Yamaguchi, K. Kataoka, M. Kobayashi, K. Itoh, A. Fukui, S. Suzuki, *Biochemistry* **2004**, *43*, 14180.
13 In a solution, HdNIR and C260A are hexamers, but C114A is a trimer.[11]
14 S. Suzuki, Deligeer, K. Yamaguchi, K. Kataoka, K. Kobayashi, S. Tagawa, T. Kohzuma, S. Shidara, H. Iwasaki, *J. Biol. Inorg. Chem.* **1997**, *2*, 265.
15 K. Kobayashi, S. Tagawa, Deligeer, S. Suzuki, *J. Biochem.* **1999**, *126*, 408.
16 S. Suzuki, T. Maetani, K. Yamaguchi, K. Kobayashi, S. Tagawa, *Chem. Lett.* **2005**, *34*, 36.
17 Deligeer, R. Fukunaga, K. Kataoka, K. Yamaguchi, K. Kobayashi, S. Tagawa, S. Suzuki, *J. Inorg. Biochem.* **2002**, *91*, 132.
18 The kinetic difference spectrum taken at 100 ms after pulse radiolysis of AcNIR was also in accord with the visible absorption spectrum of the type 1 Cu, indicating the spectrum is attributable to the reduction of the type 1 Cu.[14]
19 M. Nojiri, A. Agatahama, R. Kobayashi, K. Yamaguchi, S. Suzuki, *Chem. Lett.* **2007**, *36*, 788.