Pulse Radiolysis Studies on Nitrite Reductase from *Achromobacter cycloclastes* IAM 1013: Evidence for Intramolecular Electron Transfer from Type 1 Cu to Type 2 Cu

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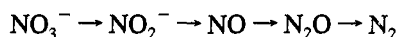
Denitrification is the dissimilatory reduction on nitrate or nitrite to produce usually dinitrogen by prokaryotic organisms.[1]



$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

In a cascade of anaerobic respiration processes, nitrite reductase catalyzes the reduction of nitrite ion to nitrogen oxide. Dissimilatory nitrite reductases containing Cu or hemes c and d_1 have so far been known.[1,2] Copper-containing nitrite reductase from *Achromobacter cycloclastes* IAM 1013 shows the electron paramagnetic resonance (EPR) signal attributable to two different kinds of Cu and the unique absorption spectrum having intense bands near 460 and 600 nm.[3,4] Recently the crystal structure has been determined to 2.3 Å resolution by isomorphous replacement.[5] The enzyme is a trimer, where the monomer (M_r = 36 000) contains two kinds of Cu. The type 1 Cu site, having four ligands (2His, Cys, and Met), lies about 4 Å from the Connolly surface of the protein, and its geometry is described as a flattened tetrahedron. The type 2 Cu site is bound with nearly perfect tetrahedral geometry by total 3 His residues from each of two monomers in the trimer. The two Cu sites (T1Cu and T2Cu) are about 12.5 Å apart and are bound by adjacent residues in the sequence (T2Cu-His(135)Cys(136)-T1Cu).

In this work, pulse radiolysis studies on *Achromobacter* nitrite reductase have been performed in order to elucidate the roles of two types of Cu. Pulse radiolysis is useful for investigations of electron transfers in proteins having multiple electron-accepting sites such as cytochrome oxidase,[6] xanthine oxidase,[7] and ascorbate oxidase.[8] In these enzymes, it can be anticipated that the hydrated electrons or organic radicals reduce the primary redox site and subsequently an electron flows to the other electron-accepting site by intramolecular migration until equilibrium is reached. Here we succeeded in observing intramolecular electron transfer from type 1 Cu to type 2 Cu in *Achromobacter* nitrite reductase.

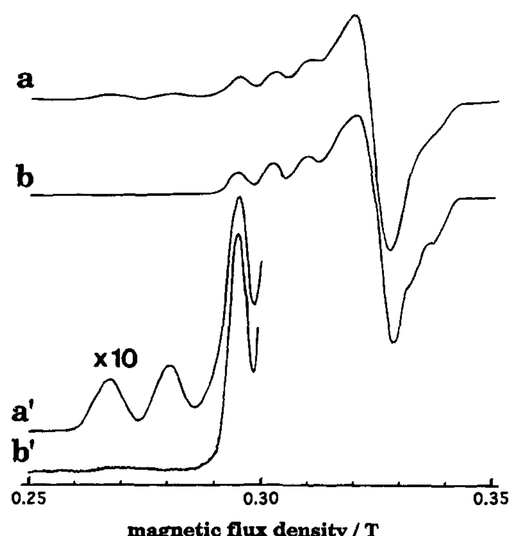


Figure 1. EPR spectra of native (a and a′) and T2D (b and b′) nitrite reductases isolated from *A. cycloclastes* at 77 K; buffer, 0.1 M Tris-HCl (pH 7.0).

Native nitrite reductase containing two kinds of Cu (type 1 Cu:type 2 Cu 1:0.5)[9] was isolated from *A. cycloclastes* IAM 1013 by the previous method.[3] Figure 1 shows the EPR spectra of native and type 2 Cu depleted (T2D) nitrite reductases at 77 K. T2D nitrite reductase was prepared by the selective removal of type 2 Cu from the native enzyme.[10,11] The EPR signal of T2D nitrite reductase reveals that there is no residual type 2 Cu, and the signal of type 1 Cu has a rhombic character.[12] The visible absorption[13] and CD spectra of the T2D enzyme are quite similar to those of the native enzyme (data now shown),[14] which indicates that type 2 Cu does not contribute in this region. This enzyme activity obtained electrochemically is about 5-fold lower than that of the native enzyme.[11]

Pulse radiolysis[15] was carried out in 10 mM phosphate buffer (pH 7.0) containing native or T2D nitrite reductase in the presence of 0.1 M *tert*-butyl alcohol for scavenging OH radical. The samples were deaerated by repeated evacuation and Ar gas flushing. Since the hydrated electron hardly reduced the Cu chromophore in the enzyme, *N*-methylnicotinamide (NMA) was used as a mediator. The typical pulse radiolysis traces of native and T2D nitrite reductases are displayed in Figure 2. Both of the absorbances at 460 nm decreased quickly after the pulses.[16] After the initial phase of the bleaching in Figure 2a, the slow recovery of the absorbance is observed, but it does not return to the initial level. Contribution to the recovery of absorption

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(9) The amount of type 1 Cu is spectroscopically obtained by using the molar absorptivity (ϵ = 2400 M^{-1} cm^{-1}) at 460 nm. The concentration of type 2 Cu is calculated by subtracting the amount of type 1 Cu from that of total Cu, which is determined by atomic absorption spectroscopy.
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(12) Spin Hamiltonian parameters: g_z = 2.19, g_y = 2.06, g_x = 2.02, A_z = 7.3 mT, A_x = 4.2 mT.
(13) The visible absorption spectrum exhibits three absorption bands at 460 (ϵ = 2400), 583 (ϵ = 1900), and 690 nm (ϵ = 1600 M^{-1} cm^{-1}).
(14) Suzuki, S.; Kohzuma, T.; Masuko, M.; Shidara, S.; Iwasaki, H., to be submitted.
(15) Pulse radiolysis experiments were performed with an electron linear accelerator at the Institute of Scientific and Industrial Research, Osaka University. The pulse width and energy were 8 ns and 27 MeV, respectively. The spectral data after the first or the second pulse were collected, since many pulses inflict damage on the protein.

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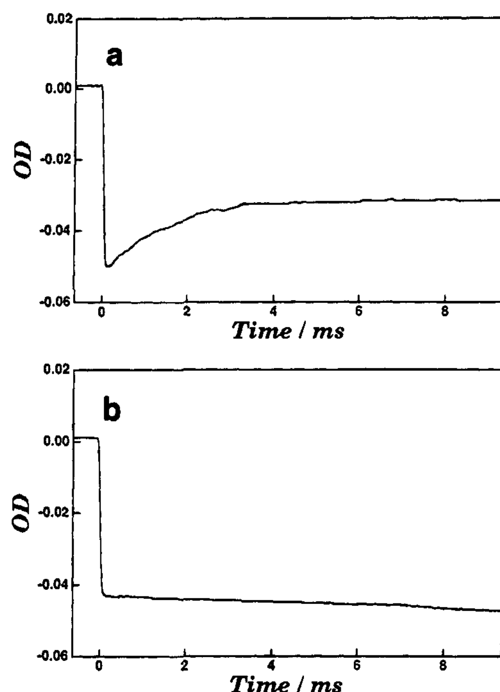

Figure 2. Pulse radiolysis traces for the reduction behaviors of native (a) and T2D (b) nitrite reductases in 10 mM potassium phosphate buffer (pH 7.0) at 460 nm. Concentrations: native enzyme, 216 μM; T2D enzyme, 212 μM; *N*-methylnicotinamide, 1 mM; *tert*-butyl alcohol, 0.1 M.

is 35% of the total absorption change for the initial reduction. Moreover, the difference spectra (400–800 nm) obtained at 100 μs and 2 ms after the pulse are identical to the visible absorption spectrum of the native enzyme. From these findings, it can be confirmed that type 1 Cu is initially reduced by NMA radical and subsequently reoxidized. The second-order rate constant of the reduction of type 1 Cu with NMA radical is estimated to be $3.4 \times 10^8\ M^{-1}\ s^{-1}$ in the native enzyme.[17] On the other hand, no reoxidation process in T2D nitrite reductase is observed (Figure 2b), though the reduction process of type 1 Cu is not affected.[18] In addition, a linear relationship between the amount of type 2 Cu and the percentage of the reoxidation of type 1 Cu is obtained (Figure 3). Therefore, it is supported that the slower process in the native enzyme is due to the intramolecular electron transfer from type 1 Cu to type 2 Cu. The observed first-order rate constant of the electron transfer is calculated to be $1.4 \times 10^3\ s^{-1}$ at pH 7.0.[19] This value is independent of the enzyme concentration. The recent pulse radiolysis study on the oxidized form of ascorbate oxidase from zucchini squash disclosed that there are two intramolecular electron-transfer steps (fast process, 166 s^{-1}, and slow second stage, 2.8 s^{-1} at 25 °C) between the type 1 Cu site and the trinuclear site composed of types 2 and 3 Cu;[8] two sites are about 12 Å apart and are bound by adjacent residues in the sequence[20] like nitrite reductase. The observed rate constant of nitrite reductase is larger than that of the fast electron-transfer process in ascorbate oxidase by a factor of about 10.[8,21,22] It is noted that these half-life periods have the orders of 10^{-4}–10^{-3} s.

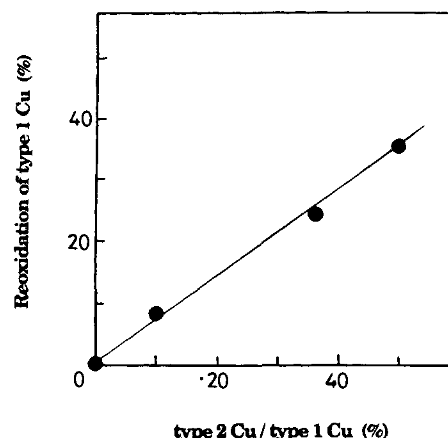

Figure 3. Relationship between type 2 Cu/type 1 Cu ratio and reoxidation of type 1 Cu.

The incomplete reoxidation of type 1 Cu can be explained by a rapid equilibrium for the electron transfer between the two types of Cu. In the case of the intact form, which contains type 1 Cu:type 2 Cu 1:1, the reoxidation of reduced type 1 Cu is estimated by extrapolation to be 70% in Figure 3. This might reflect the difference between the redox potentials of two Cu sites. The midpoint potential ($E_{1/2}$) of type 1 Cu in *Achromobacter* nitrite reductase was obtained to be +240 mV (*vs* NHE) by cyclic voltammetry.[11] Consequently, the $E_{1/2}$ value of type 2 Cu is estimated to be ca. +260 mV (*vs* NHE).

The present investigation indicates that type 1 Cu in native *Achromobacter* nitrite reductase mediates an electron from a blue copper protein, pseudoazurin (a natural electron donor),[4,23] to type 2 Cu, which is probably the binding and reduction center of nitrite ion.[11] Pulse radiolysis experiments on nitrite reductases from the other denitrifying bacteria are currently in progress.

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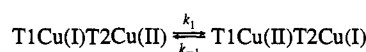
(16) The reaction of NMA radical with native nitrite reductase obeys pseudo-first-order kinetics when 8.6 μM NMA radical ($\epsilon = 3.2\ mM^{-1}\ cm^{-1}$ at 420 nm: Hill, R.; Anderson, R. F. *J. Biol. Chem.* **1991**, *266*, 5608–5615) is generated in a solution containing 216 μM enzyme. The absorbance of the native enzyme at 460 nm was decreased with a half-life of 7 μs.

(17) The second-order rate constant of the reduction of blue copper (type 1 Cu) in *Achromobacter* pseudoazurin with NMA radical is calculated to be $9.6 \times 10^8\ M^{-1}\ s^{-1}$ under the same conditions.

(18) The second-order rate constant is estimated to be $3.5 \times 10^8\ M^{-1}\ s^{-1}$ in T2D nitrite reductase.

(19) The electron-transfer reaction is interpreted in terms of a rapid equilibrium:



$$\mathrm{T1Cu(I)T2Cu(II)} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{T1Cu(II)T2Cu(I)}$$

Therefore, the observed first-order rate constant consists of the sum of k_1 (forward) and k_{-1} (backward). The k_1 and k_{-1} are estimated to be 980 and 420 s^{-1}, respectively, on the basis of 70% reoxidation of the reduced type 1 Cu in the enzyme containing type 1 Cu: type 2 Cu 1:1 (Figure 3).

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