The Intramolecular Electron Transfer between the Type 1 Cu and the Type 2 Cu in a Mutant of *Hyphomicrobium* Nitrite Reductase

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The intramolecular electron transfer (ET) between the type 1 Cu and the type 2 Cu in the Cys114Ala mutant of *Hyphomicrobium denitrificans* nitrite reductase has been observed by pulse radiolysis. At pH 5.4–7.0, the ET rates of C114A having one type 1 Cu and one type 2 Cu per monomer are 10^4–10^5 times as large as those of the native enzyme containing two type 1 Cu sites and one type 2 Cu site.

Intramolecular ET reactions between redox centers are essential in many metabolic processes. The ET rates are generally regulated by biological processes coupled with the ET pathways. For example, the redox steps associated with the photosynthetic reaction centers are among the most rapid biological ET processes known (10^{12} s^{-1}). Meanwhile the intramolecular ET reactions from the type 1 Cu to the type 2 Cu in copper-containing nitrite reductases (NIRs), which are coupled with one-electron reduction of nitrite at the type 2 Cu, show the ET rate constants of 10^2–10^3 s^{-1}.[1,2]

We have recently found the unique NIR isolated from *Hyphomicrobium denitrificans* A3151 (HdNIR), which contains the two type 1 Cu sites and one type 2 Cu site per subunit (ca. 50 kDa).[3–5] On the other hand, the usual trimeric NIRs so far known, blue *Alcaligenes xylosoxidans* (Ax) and green *Achromobacter cycloclastes* (Ac) NIRs have one type 1 Cu and one type 2 Cu in one subunit (ca. 37 kDa).[1,2,6,7] In these two NIRs, the type 1 Cu ligated by four amino acid residues (2His, Cys, and Met) is bound to one of the two β-barrel domains inside a monomer and the type 2 Cu site bound to the interface between two adjacent monomers has three His ligands and a solvent ligand.[7–9] HdNIR is composed of the 15 kDa N-terminal domain having a type 1 Cu-binding motif (type 1 Cu_N) like plastocyanins and the 35 kDa C-terminal domain having one type 1 Cu (type 1 Cu_C) and one type 2 Cu like usual NIRs,[5] being proteolyzed to two protein fragments with subtilisin.[4]

Pulse radiolysis is a powerful technique for investigations of intramolecular ET processes in proteins having multiple redox centers. In AxNIR and AcNIR, the ET processes were observed as the recovery of the reduced 600-nm absorption band.[10,11] The apparent first-order rate constants (k_{ET}) of the intramolecular ET from the type 1 Cu to the type 2 Cu in AxNIR and AcNIR were estimated to be 1.9–2.0 × 10^3 s^{-1} at pH 6.0 and 20 °C in both the absence and presence of the substrate. Moreover, the pH-profiles of k_{ET} for AxNIR and AcNIR show bell-shaped curves having maxima around pH 6.0–6.5. On the other hand, the k_{ET} value (2.4 × 10^{-2} s^{-1}) of HdNIR at pH 6.0 is much smaller than those of AxNIR and AcNIR in the presence of the nitrite and no recovery of the reduced 600-nm band was observed in the absence of nitrite.[3] Moreover, the pH-profile of k_{ET} for HdNIR in the range of pH 4.5–7.5 shows a monotonous decrease with increasing pH.

In this work, we report the intramolecular ET process of the Cys114Ala mutant (C114A) of *Hyphomicrobium denitrificans* nitrite reductase to disclose the mechanism of the intramolecular ET in HdNIR having three Cu centers. The green C114A mutant, which the Cys114 ligand of the type 1 Cu in the N-terminal plastocyanin-like domain is replaced with Ala, has one type 1 Cu_C and one type 2 Cu in the 35 kDa C-terminal domain.[5]

The C114A was prepared by the previous method[5] and the ratio of the type 1 Cu to the type 2 Cu per subunit was estimated to be 1:1. The specific activity of C114A was determined to be $(2.03 \pm 0.15) \times 10^{10}$ units/(mol of protein)[12] by the steady-state method using dithionite/benzyl viologen as an electron donor, being slightly higher than that of the native enzyme $(1.23 \pm 0.20) \times 10^{10}$ units/(mol of protein). The visible electronic absorption spectrum of C114A shows two peaks at 455 (ε = 2600) and 600 nm (ε = 2800 M^{-1} cm^{-1}) and a shoulder near 700 nm. The EPR spectrum consists of a rhombic type 1 Cu_C signal (g_Z = 2.21 and A_Z = 5.5 mT) and an axial type 2 Cu signal ($g_{//}$ = 2.35 and $A_{//}$ = 13.5 mT), as shown in Figure 1. The four hyperfine peaks of the type 1 Cu in the range of 0.30–0.31 T show the existence of one type 1 Cu, which is assigned to the green type 1 Cu_C in the 35 kDa C-terminal domain.[5]

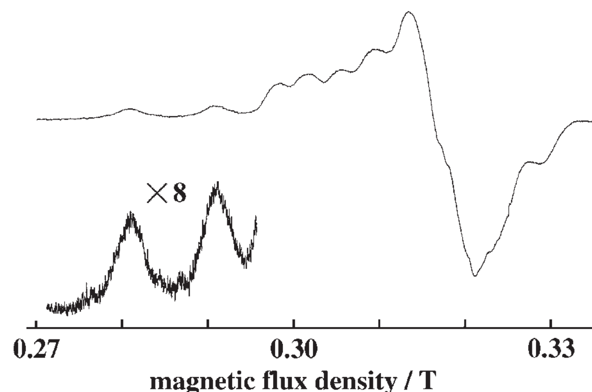

Figure 1. EPR spectrum of C114A at 77 K.

Pulse radiolysis was carried out in 10 mM phosphate buffer (pH 5.4, 6.0, and 7.0) containing C114A (ca. 150 µM), *tert*-butyl alcohol (0.1 M, a hydroxyl radical scavenger), and *N*-methylnicotinamide (2 mM, a mediator) at 20 °C.[10,11,13] As a substrate, 1 mM sodium nitrite was employed. The typical pulse radiolysis traces of C114A at 600 nm are illustrated in Figure 2. The 600-nm band quickly disappears following pulse electron beam radiation to the enzyme solution. The spectral behavior indicates that the type 1 Cu site is reduced with a half-life period of ca. 10 µs.

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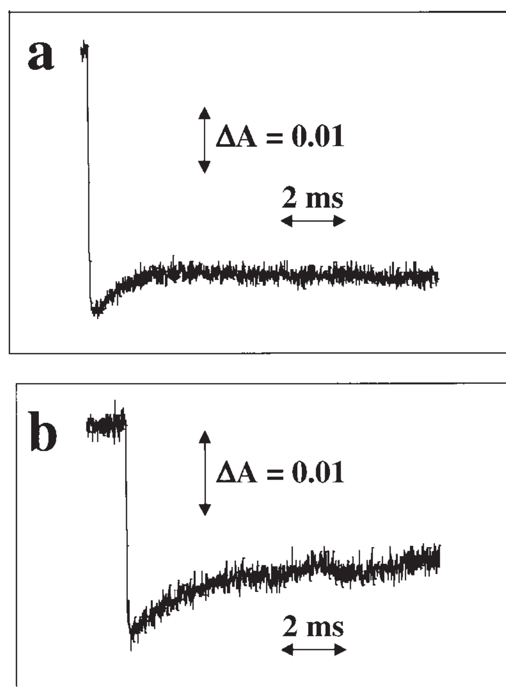

Figure 2. Time-resolved absorption changes of C114A in the absence (a) and presence (b) of nitrite at pH 7.0.

The second-order rate constants of the type 1 Cu reduction with *N*-methylnicotinamide radical were determined to be 1.1×10^9 (pH 5.4), 9.6×10^8 (pH 6.0), and $8.3 \times 10^8\ M^{-1}s^{-1}$ (pH 7.0) regardless of the existence of nitrite. After the reduction of the type 1 Cu site, a slow recovery of the absorbance was observed on the millisecond time scale, suggesting that the intramolecular ET reaction from the type 1 Cu to the type 2 Cu occurs. The rate constants of the ET reactions are tabulated in Table 1. At pH 5.4 and 6.0 no intramolecular ET reactions were observed in the absence of nitrite, in contrast to pH 7.0. These results are explained by the difference of redox potentials between two Cu centers, that is, at pH 7.0 the more negative redox potential of the type 1 Cu_C (+321 mV)[5] than that of the type 2 Cu would result in the ET, but at pH 5.4 and 6.0 the former would become to be more positive than the latter. In Figure 2b, the reduced 600-nm absorbance even in the presence of excess nitrite doesn't return to the initial value, whereas the almost complete recovery occurs in AxNIR and AcNIR under the same conditions.[11] A similar incomplete recovery was also observed in the pulse radiolysis of the Asp98Ala AxNIR mutant.[13] Thus the reduced type 1 Cu in the mutants nonstoichiometrically transfers an electron to the nitrite-binding type 2 Cu. At the present stage, we have no clear explanation for the incomplete recovery of the 600-nm band in the presence of the substrate.

Table 1. Intramolecular ET rate constants (k_{ET}) of C114A in the absence (−) or presence (+) of nitrite at 20 °C

pH	k_{ET}/s^{-1} − nitrite	k_{ET}/s^{-1} + nitrite (1 mM)
5.4	—	6.1×10^2
6.0	—	1.0×10^3
7.0	1.9×10^3	5.9×10^2

In Table 1, the k_{ET} values of C114A are similar to those of usual AxNIR and AcNIR,[11] but are quite larger than those of the native enzyme by a factor of 10^4–10^5 in the same pH range.[3] Moreover, the pH-dependence of k_{ET} for C114A is similar to those of AxNIR and AcNIR. These findings suggest that *N*-methylnicotinamide radical gives an electron to the type 1 Cu coupled with the type 2 Cu in the enzymes having two copper centers, but HdNIR having three copper centers might accept an electron at the type 1 Cu (type 1 Cu_N) in the N-terminal plastocyanin-like domain. In the native enzyme, the extremely small k_{ET} value ($2.4 \times 10^{-2}\ s^{-1}$) might result from the very slow ET between the type 1 Cu_N and Cu_C sites or an effect of type 1 Cu_N on the ET from the type 1 Cu_C to the type 2 Cu. The more detailed analysis of the intramolecular ET of HdNIR is in progress.

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