Effect of Arginine at Type 2 Cu Site on Spectroscopic Features and Enzymatic Activity of Copper-containing Nitrite Reductase

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Spectroscopic features and enzymatic activity of H135R nitrite reductase mutant, in which a His ligand at the type 2 copper site is replaced with Arg, have been investigated to get information on unique nitrite reductases having one Arg at the type 2 copper site. Although the substitution of Arg for His slightly perturbed the structure of copper sites, the enzymatic activity was considerably lower than that of wild-type enzyme.

Copper-containing nitrite reductase (CuNIR) plays a key role in biological denitrification, which is an environmentally significant process that the dissimilatory reduction of nitrate or nitrite into dinitrogen by prokaryotic organisms.[1] CuNIR is classified into three subgroups based on the spectroscopic properties of type 1 Cu, namely blue CuNIR, green CuNIR, and other CuNIR.[2] The identical trimeric CuNIRs in blue and green subgroups generally have two Cu centers, one type 1 Cu and one type 2 Cu per 37-kDa monomer. The type 1 Cu accepts one electron from an external electron-donor protein, while the type 2 Cu, which accepts an electron from the reduced type 1 Cu site, is the reduction center of substrate (NO_2^-) into NO. The type 2 Cu site is connected via a His–Cys bridge to the type 1 Cu site. The Cu ion at the type 2 Cu site is ligated by three His imidazolyls and a water molecule (Figure 1). Recently, bioinformatic study[3] has been reported new gene sequences[4] of CuNIRs from *Burkholderia mallei* (YP_105511) and *B. pseudomallei* (YP_111458), in which an adjacent amino acid residue to the Cys ligand of the type 1 Cu is not a His, but an Arg at the type 2 Cu site. As any CuNIRs having the Arg residue instead of the His at the type 2 Cu site had been unknown except the CuNIRs from *B. mallei* or *B. pseudomallei*, the His binding to the type 2 Cu has been considered to be essential for the enzymatic function. Unfortunately, the CuNIRs from *B. mallei* or *B. pseudomallei* have not been isolated yet. One of the reasons might be that *B. mallei* is regarded as a potential biological weapon; a Centers for Disease Controland Prevention category B agent. Therefore, in order to reveal the effect of the Arg residue at the type 2 Cu site of CuNIR for the enzymatic function, we investigated the spectroscopic features and enzymatic activity of H135R CuNIR mutant, in which the His residue at the type 2 Cu site is replaced with Arg.

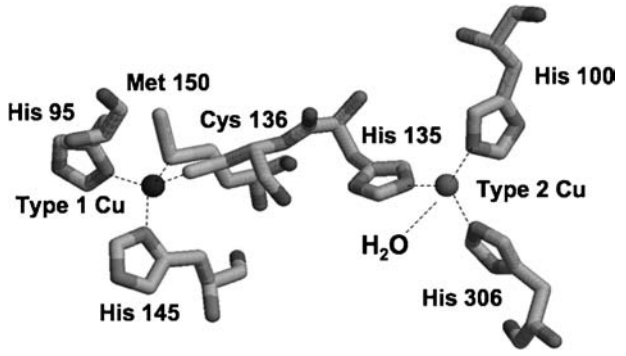

Figure 1. Structural representation of the type 1 Cu and type 2 Cu sites in CuNIR. The residue numbers refer to the *Achromobacter cycloclastes* NIR sequence and all of the residues are conserved in blue and green CuNIRs.

The H135R mutant of green CuNIR from *Achromobacter cycloclastes* IAM1013 (AcNIR) was prepared by site-directed mutagenesis.[5] The electronic and CD spectra of H135R with those of wild-type AcNIR in 20 mM Tris-HCl buffer (pH 7.0) are represented in Figures S1A and S1B, respectively.[8] The visible absorption spectrum of H135R displays three bands at 400 ($\varepsilon = 1.77 \times 10^3\,M^{-1}\,cm^{-1}$), 459 ($\varepsilon = 2.08 \times 10^3\,M^{-1}\,cm^{-1}$), and 586 nm ($\varepsilon = 1.72 \times 10^3\,M^{-1}\,cm^{-1}$) and a broad band at 689 nm ($\varepsilon = 1.42 \times 10^3\,M^{-1}\,cm^{-1}$), which are characteristic of the type 1 Cu. In wild-type AcNIR having the flattened tetrahedral type 1 Cu center,[9,10] two intense bands (460 and 589 nm), a shoulder near 400 nm and a broad band at 690 nm are assigned to N(His) $\rightarrow$ Cu, S(Cys) $\rightarrow$ Cu, and S(Met) $\rightarrow$ Cu charge-transfer transitions and d–d transition of type 1 Cu, respectively.[11] Although the molar absorption coefficient (ε) at 459 of H135R is smaller than that of wild-type AcNIR, the spectral features of H135R are almost the same as that of wild-type AcNIR. In the visible region, the CD spectrum of H135R exhibits two positive peaks at 391 ($\Delta\varepsilon = +5.22\,M^{-1}\,cm^{-1}$) and 1.99 ($\Delta\varepsilon = +1.99\,M^{-1}\,cm^{-1}$) nm and two negative peaks at 467 ($\Delta\varepsilon = -7.54\,M^{-1}\,cm^{-1}$) and 677 ($\Delta\varepsilon = -8.51\,M^{-1}\,cm^{-1}$). The pattern of CD spectrum for H135R is also almost identical with that of wild-type AcNIR. Accordingly, the type 1 Cu in H135R is considered to have extremely similar coordination geometry to that in wild-type AcNIR. The spectroscopic results suggest that the mutation of His135, which is next to the Cys136 ligand of the type 1 Cu, little influences the coordination structures of the type 1 Cu site in AcNIR.

In Figure 2, the 77-K EPR spectrum of H135R is depicted. The EPR parameters of H135R were estimated to be $g_\parallel = 2.18$, $A_\parallel = 7.8$ mT, and $g_\perp = 2.06$, which are assigned to the type 1 Cu, and $g_\parallel = 2.30$, $A_\parallel = 13.0$ mT, and $g_\perp = 2.06$, which are assigned to the type 2 Cu. The spin density ratio of the type 1 Cu and the type 2 Cu in H135R is 1:0.6. The EPR spectrum of H135R is very similar to that of wild-type ANIR ($g_\parallel = 2.19$, $A_\parallel = 7.3$ mT for the type 1 Cu, and $g_\parallel = 2.33$, $A_\parallel = 13.0$ mT and $g_\perp = 2.06$ for the type 2 Cu).[12] The EPR data show the existence of a type 2 Cu in H135R. From the atomic absorption spectra of the holo and type 2 Cu-depleted H135R, the molar ratio of the type 1 Cu and the type 2 Cu in H135R was calculated to be 0.70.

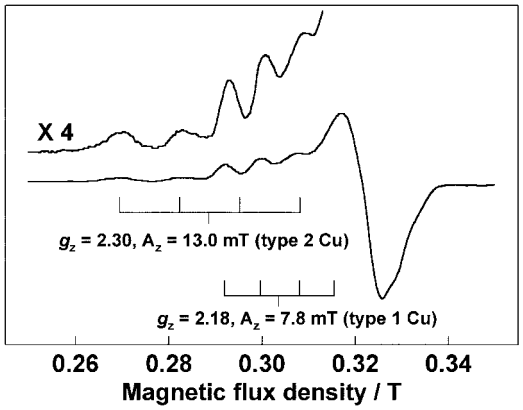

Figure 2. EPR spectrum of H135R mutant in 20 mM Tris-HCl buffer (pH 7.0) at 77 K.

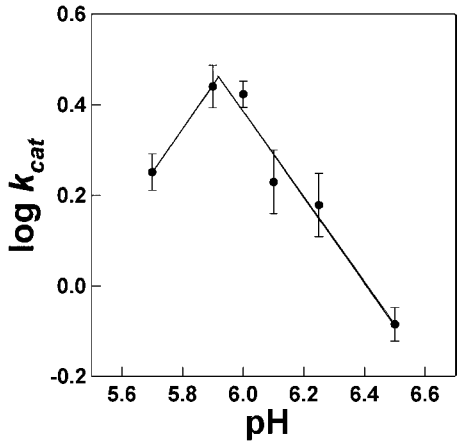

Figure 3. pH Dependence of the enzymatic activity of H135R mutant.

The nitrite reduction activity of H135R was determined by two steady-state methods using a reduced benzyl viologen at 25.0 °C.[13] The mutant H135R exhibited 10^3-fold smaller k_{cat} value (2.8 s^{-1} at pH 5.9) than that of the wild-type NIR (2.3 × 10^3 s^{-1} at pH 6.0).[12] The K_m value of H135R (0.45 mM at pH 6.0) was determined to be quite similar to that of wild-type NIR (0.5 mM).[14] The pH dependence of activity for H135R exhibits a maximum of approximately 5.9 (Figure 3), which is closely resembled the optimum pH of wild-type NIR (pH 6.0).[7] The pH profile implies the presence of two dissociation residues. The pulse radiolysis study of wild-type CuNIR also revealed the existence of proton dissociation groups having pK_a values of 5.0 and 7.0.[15] On the basis of kinetic studies of D98A and H255A CuNIRs,[7] it have been known that Asp and His around the type 2 Cu site work as general acid–base catalysts, which provide the two protons required for nitrite reduction. Accordingly, the functional hydrogen-bonding network of Asp–water–His around the type 2 Cu site would be conserved in H135R.

In summary, the electronic absorption, CD, and EPR spectra and optimum pH value of H135R are nearly identical to those determined for the wild-type enzyme, suggesting that the substitution of Arg for His135 only slightly perturbs the structure of type 1 Cu and type 2 Cu sites. However, the enzymatic activity of H135R was considerably lower than that of wild-type NIR. The results suggest that the proteins coded by the new gene sequences from *B. mallei* and *B. pseudomallei* would be CuNIRs having very low NIR activities. As the coordinational structure of the type 2 Cu site and the intramolecular electron transfer from the type 1 Cu to the type 2 Cu in H135R might be key to solving the reason why H135R mutant has quite low activity, X-ray crystal structure analysis and pulse radiolysis study of H135R are in progress.

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