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The pH-Dependent Changes of Intramolecular Electron Transfer on Copper-Containing Nitrite Reductase[1]

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Electron transfer over 12.6 Å from the type 1 copper (T1Cu) to the type 2 copper (T2Cu) was investigated in the copper-containing nitrite reductases from two denitrifying bacteria (*Alcaligenes xylosoxidans* GIFU 1051 and *Achromobacter cycloclastes* IAN 1013), following pulse radiolytical reduction of T1Cu. In the presence of nitrite, the rate constant for the intramolecular electron transfer of the enzyme from *A. xylosoxidans* decreased 1/2 fold to 9×10^2 s^{-1} (20°C, pH 7.0) as compared to that for the same process in the absence of nitrite. However, the rate constant increased with decreasing pH to become the same (2×10^3 s^{-1}) as that in the absence of nitrite at pH 6.0. A similar result was obtained for the enzyme from *A. cycloclastes*. The pH profiles of the two enzymes in the presence of nitrite are almost the same as that of the enzyme activity of nitrite reduction. This suggests that the intramolecular electron transfer process is closely linked to the following process of catalytic reduction of nitrite. The difference in redox potential (ΔE) of T2Cu minus T1Cu was calculated from equilibrium data for the electron transfer. The pH-dependence of ΔE was in accord with the equation: $\Delta E=\Delta E^0+0.058$ log $(K_r[H^+]+[H^+]^2)/(K_0+[H^+])$, where K_r and K_0 are the proton dissociation constants for the oxidized and reduced states of T2Cu, respectively. These results raise the possibility that amino acid residues linked by the redox of T2Cu play important roles in the enzyme reaction, being located near T2Cu.

Key words: intramolecular electron transfer, nitrite reductase, pulse radiolysis, type 1 copper, type 2 copper.

Denitrification is the dissimilatory reduction of nitrate or nitrite leading to the production of usually dinitrogen by prokaryotic organisms (*1*). It comprises a cascade of anaerobic respiration processes which are catalyzed in either the cytoplasmic membrane or the periplasm by the corresponding oxidoreductases. Some of these steps must be coupled with ATP synthesis. Dissimilatory nitrite reductase, which is located in the periplasm, catalyzes the reduction of nitrite to nitric oxide.

$$NO_2^- + e^- + 2H^+ \longrightarrow NO + H_2O$$

Two types of nitrite reductase (NIR) containing Cu or hemes *c* and d_1 are known so far (*1, 2*). The recent description of high-resolution crystal structures for the two types of the Cu-containing enzyme (*3-8*), and the cytochrome cd_1 enzyme (*9-11*) provided a framework for understanding the reduction of nitrite to nitric oxide.

The structures of Cu-NIR isolated from *Achromobacter cycloclastes* IAM 1013 (AciNIR) (*3, 4*), *Alcaligenes faecalis* S-6 (*5, 6*), and *Alcaligenes xylosoxidans* (AxgNIR) (*7, 8*) have been determined. These enzymes exhibit a high degree of amino acid sequence homology and their three-dimensional structures are very similar. Each enzyme is a trimer, in which the monomer (36 kDa) contains two different types of Cu, type 1 Cu (T1Cu) and type 2 Cu (T2Cu). T1Cu is an electron acceptor site in the enzyme from the respiratory chain *via* molecules of cytochrome c_{551} or pseudoazurin, and mediates the electron transfer (ET) to the catalytic site of T2Cu. In the T1Cu site, four amino acid residues (His-95, Cys-136, His-145, and Met-150) form a flattened tetrahedron in AciNIR (*3-6*) and a distorted tetrahedron in AxgNIR (*7, 8*), respectively. This difference in geometry in T1Cu is reflected in the color, *i.e.* blue (AxgNIR) and green (AciNIR). The T2Cu site binds, with a distorted tetrahedral geometry, a solvent molecule and 3 His residues of each of two monomers in the trimer. The distance between T1Cu and T2Cu is about 12.6 Å and these Cu atoms are bound by adjacent amino acid residues in the sequence, as shown in Fig. 1.

In the vicinity of the T2Cu site, there is a hydrogen bond network extending from the solvent ligand of T2Cu which involves the Asp-98 and His-255 residues (*4, 6*). The solvent ligand is hydrogen bonded to the carboxylate group

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Abbreviations: AciNIR, NIR from *Achromobacter cycloclastes* IAM 1013; AxgNIR, NIR from *Alcaligenes xylosoxidans* GIFU 1051; ET, electron transfer; NMA, *N*-methylnicotinamide; NIR, nitrite reductase; T1Cu, type 1 Cu; T2Cu, type 2 Cu; T3Cu, type 3 Cu.

of Asp-98, which in turn forms a H_2O-bridged hydrogen bond with the imidazole nitrogen atom of His-255. It has been suggested from the results of crystallographic studies on NO_2^--bound AciNIR at pH 5.4 (*4*) that His-255 and Asp-98, and the water bound between them are the most probable sources of the two protons required for NO_2^- reduction. Recently, the structures of the oxidized, reduced, and nitrite-soaked reduced forms of the NIR from *A. faecalis* S-6 were determined by X-ray diffraction (*6*). Based on comparison of the structures, Murphy *et al.* proposed that the protonated species of Asp-98 forming the hydrogen-bond to the nitrite oxygen facilitates proton transfer to nitrite ligated to T2Cu.

A powerful approach for investigating the ET process within proteins is pulse radiolysis, through which an electron can be introduced rapidly and selectively into one redox center of an enzyme (*12-22*). Our previous studies showed that the *N*-methylnicotinamide (NMA) radical caused rapid and specific reduction of T1Cu of AciNIR (*12, 13*). After the reduction, intramolecular ET was successfully observed with a first-order rate constant (k_{ET}) of $1.4 \times 10^3\ s^{-1}$ at pH 7.0. In the presence of nitrite, however, k_{ET} was decreased by a factor of approximately 1/2 fold at pH 7.0, as compared to that for the same process in the absence of nitrite (*13*). Then, the question arose of why k_{ET} decreased on the binding of NO_2^- to T2Cu. In the present study, the effect of pH on the rate of intramolecular ET was examined with two different types (blue and green) of AxgNIR and AciNIR, respectively. Based on the kinetic and equilibrium data, the reaction mechanism is discussed here.

MATERIALS AND METHODS

Two NIRs were isolated and purified from the denitrifying bacteria, *A. xylosoxidans* GIFU 1051 and *A. cycloclastes* IAM 1013, according to a slight modification of the previous method (*13*). The enzymes, AxgNIR and AciNIR, with absorbance ratios of $A_{280}/A_{593} = 13$ and $A_{280}/A_{460} = 16$, were obtained as pure enzymes, respectively. The concentrations of AxgNIR and AciNIR were determined using molar extinction coefficients of 3.8 $mM^{-1} \cdot cm^{-1}$ at 593 nm and 2.4 $mM^{-1} \cdot cm^{-1}$ at 460 nm, respectively. The total copper contents of the enzymes were determined by atomic absorption spectroscopy with a Nippon Jarrell-Ash AA mark II. The T2Cu/T1Cu ratios in AxgNIR and AciNIR were 0.7 and 0.4, respectively. Since T2Cu is bound at the interface of two subunits, it would be partially removed under drastic conditions such as those for the enzyme preparation (*23*).

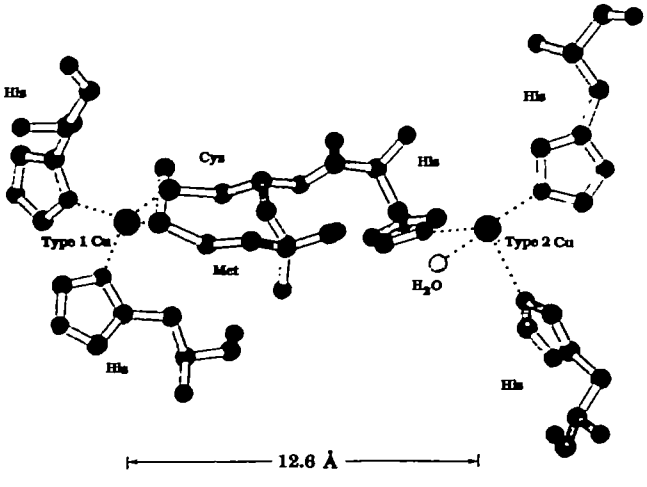


Fig. 1. **Structures of the T1Cu and T2Cu sites of Cu-containing NIR.**

Pulse radiolysis was carried out with the pulse width of 8 ns and the energy of 27 MeV, respectively, as described previously (*12, 13, 16-19*). The concentration of the NMA radical generated on pulse radiolysis was determined as the absorbance change at 420 nm using a millimolar extinction coefficient of 3.2 $mM^{-1} \cdot cm^{-1}$ (*22*). The concentration of the NMA radical was approximately 20 μM. This concentration could be adjusted by varying the electron beam dose. The reactions were carried out at 20°C.

Samples for pulse radiolysis were prepared as follows. Solutions of NIR containing 2 mM NMA and 0.1 M *tert*-butylalcohol (for scavenging OH radicals) in 10 mM phosphate buffer (pH 5-8) were deoxygenated in sealed cells by repeated evacuation and flushing with argon. The quartz cells had an optical path length of 1 cm. For each pulse, a new sample was used, even though pulse radiolysis did not cause any damage to a sample, as judged from its visible absorption spectrum. Each data point was the average for two to four pulses.

Optical absorption spectra were obtained with a Hitachi U-3400 spectrometer.

RESULTS AND DISCUSSION

A pulse radiolysis experiment involves the almost instantaneous generation of the NMA radical, which in turn can reduce T1Cu of NIR (*12, 13*). This is apparent from the decrease in the absorbance due to T1Cu (blue copper). After the reduction, the slow recovery of the absorbance was observed on a millisecond timescale. In the enzyme with T2Cu selectively removed, on the other hand, such a recovery process was not observed, although the reduction of T1Cu was not affected (*12*). Based on the results, the reaction after a pulse can be interpreted by the following sequence events, as shown in Scheme 1. In this process, rapid equilibrium of ET between the two types of Cu is attained. Thus, the observed k_{ET} consists of the sum of k_f (forward) and k_b (backward). In the presence of nitrite, there is essentially quantitative transfer to T2Cu, due to a non-equilibrium process due to subsequent reduction of nitrite to nitric oxide.

In the present study, the effect of pH on k_{ET} was examined with two different types of NIR (AxgNIR and AciNIR). The NMA radical reacted very rapidly with AxgNIR, resulting in the reduction of T1Cu. A decrease in the absorbance at 590 nm reflected the reduction of T1Cu (Fig. 2). Whereas the second-order rate constants ($3.4 \times 10^8\ M^{-1} \cdot s^{-1}$) for the reduction T1Cu by the NMA radical was not affected by pH (data not shown), the rate of the recovery process in the presence of nitrite decreased considerably with increasing pH, as compared to that for the same process in the absence of nitrite. The k_{ET} values are plotted against pH in Fig. 3A. The k_{ET} values in the presence of nitrite decreased with increasing pH in the pH range of 6.0 to 8.0. In the absence of nitrite, the k_{ET} values decreased gradually with increasing pH. In the pH range of

5.0 to 6.0, the pH-profile was not affected by the presence of nitrite.

Similar experiments were carried out with a green type of NIR, AciNIR. The k_{ET} values and pH profiles in Fig. 4 are almost the same as those of a blue type NIR, AxgNIR. This reflects the similar protein structures of the two types of NIR.

It is important to note that the pH-k_{ET} curves of AxgNIR and AciNIR in the presence of nitrite are almost the same as that of the pH-dependence NIR activity (*24, 25*). The activity increased with decreasing pH in the range from 8.0 to 5.2, exhibiting an optimum at pH 5.2 and a plateau between pH 6.1 and 5.8 (*24*). This suggests that the intramolecular ET process in the presence of nitrite is closely linked to the following catalytic reduction of NIR. In addition, such pH-rate constant curves were only seen in the presence of a substrate for NIR. In the absence of nitrite or in the presence of NO_3^- (data not shown), the k_{ET} values were not affected so much by pH.

As shown in Fig. 2, the absorbance of T1Cu did not return the initial value. The ratios of the recovery of the absorbance to the initial decrease at 590 nm were plotted against pH (Fig. 3B). In the presence of nitrite, the values were almost identical to the content of T2Cu (70%) in the enzyme and were not affected by pH in the range from 5 to 7.5. Therefore, quantitative ET to T2Cu essentially took place in this pH range. In the absence of nitrite, on the other hand, the recovery ratios were less than the content of T2Cu, indicating that rapid equilibrium of the ET between T1Cu and T2Cu was attained. Thus, the equilibrium constants for the ET were calculated from the recovery of the absorbance in the absence of nitrite. Since a linear relationship between the T2Cu/T1Cu ratio and the reoxidation of T1Cu was observed in our previous study (*12*), the equilibrium constants calculated were not affected by the amount of T2Cu in the sample. The redox potentials of T1Cu in AxgNIR and AciNIR at pH 7.0 were obtained by

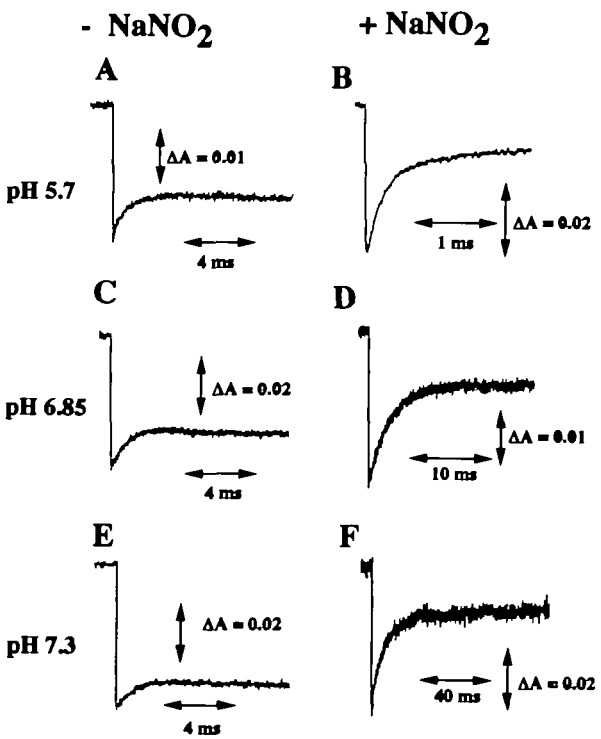


Fig. 2. **Absorbance changes at 590 nm after pulse radiolysis of AxgNIR at pH 5.7 (A), pH 5.7 in the presence of 500 μM $NaNO_2$ (B), pH 6.85 (C), pH 6.85 in the presence of 500 μM $NaNO_2^-$ (D), pH 7.3 (E), and pH 7.3 in the presence of 500 μM $NaNO_2^-$ (F). All samples contained 102 μM AxgNIR.**

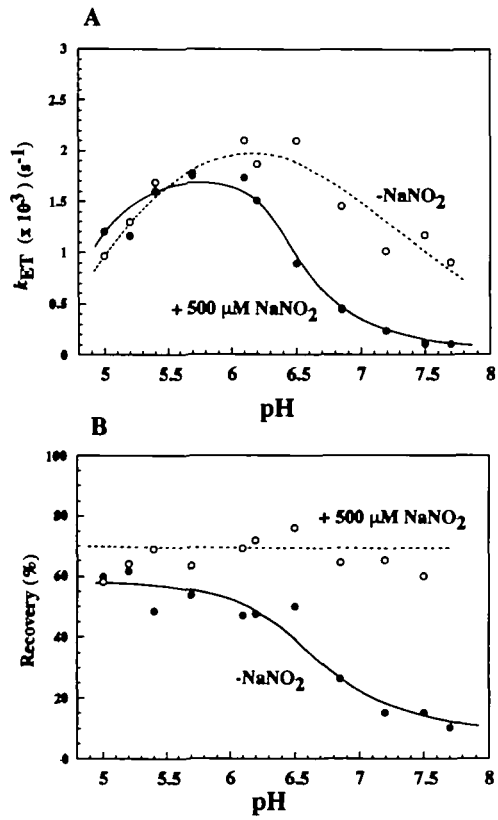
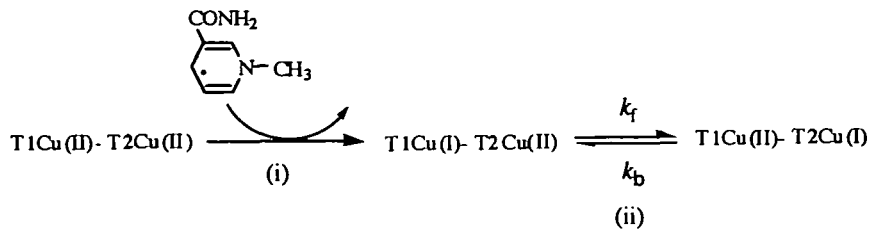


Fig. 3. **(A) pH-dependence of the intramolecular electron transfer rate constants of AxgNIR determined as the recovery of the absorbance at 590 nm in the presence (●) and absence of 500 μM $NaNO_2$ (○). (B) pH-dependence of the ratios of the recovery of the absorbance to the initial decrease at 590 nm in the presence (●) and absence of 500 μM $NaNO_2$ (○). The buffer used was 10 mM phosphate buffer.**



Scheme 1

cyclic voltammetry (*23*). From the results of the present experiment, the difference in the redox potentials (ΔE) of T2Cu minus T1Cu can be calculated from the equilibrium data. The E^0 of the T1Cu and T2Cu centers at pH 6 and 7 are presented in Table I. In Fig. 5, the differences in the redox potentials (ΔE) of T2Cu minus T1Cu are plotted. It is noteworthy that the ΔE values are relatively small in the absence of nitrite. In addition, since the ΔE are negative above pH 7, the reduction of T2Cu by T1Cu is not energetically favored. But, it is considered that the binding of nitrite to T2Cu is the trigger positively shifting the redox potential of T2Cu to induce the catalytic electron transfer. A similar result was obtained for *Rhodobacter sphaeroides* NIR (*26*), the redox potential of T1Cu (247 mV) being positively shifted compared with that of T2Cu (200 mV) in the absence of nitrite. Spectroscopic evidence (Q-band electron nuclear double resonance) that the redox potential of T2Cu shifted positively on the binding of nitrite to T2Cu has been presented (*27*).

Figure 5 shows that the ΔE values nicely fit Eq. 1 (*28*),

$$\Delta E = \Delta E^0 + 0.058 \log (K_r[H^+] + [H^+]^2)/(K_o + [H^+]) \quad (1)$$

where K_r and K_o are the redox-coupled proton dissociation constants for the reduced and oxidized forms of T2Cu, respectively. Here, pK_r (AxgNIR) = 7.2, pK_r (AciNIR) = 7.0, and pK_o = 5.0 (AxgNIR and AciNIR). Judging from the results, a proton dissociation group linked by the redox of T2Cu exists in the vicinity of T2Cu, its pK_a value changing from 5.0 to 7.0 with the reduction of T2Cu.

The reaction scheme after 1-electron reduction of NIR in the presence of nitrite can be schematically presented as in Scheme 2. Following the initial reduction of the NO_2^- bound form of NIR at T1Cu, the intramolecular ET from T1Cu to T2Cu takes place to yield a Cu(I) nitrite complex of T2 Cu [step (i)]. Then, the ET from copper to the nitrite ligand, rapid protonation, and a loss of water give rise to an intermediate, NO, transiently bound to T2Cu [step (ii)]. Finally, NO is dissociated from T2Cu to give the final product [step (iii)]. Among these reaction sequences, only process (i) could be followed spectrophotometrically as the oxidation of T1Cu. The other processes could not be observed directly. The speculated species, Cu(I)-NO_2^- (*29*) and Cu(II)-NO (*30*), may be formed as intermediates, since these complexes have been used as biomimetic models.

The present data show that binding of a substrate to T2Cu above pH 7.0 considerably decreases k_{ET} of the two NIRs. Interestingly, this is in contrast with in the case of ascorbate oxidase, where the presence of the substrate, oxygen, enhances the intramolecular k_{ET} from T1Cu to T3Cu (*21*). The effect of the substrate in NIR cannot be explained by a change in the redox potential. Process (i) in Scheme II is closely linked to the following catalytic reduction of nitrite. It is likely that the rate-determining step in Scheme 2 is process (ii), not process (i), because the k_{cat} of 4×10^2 s^{-1} at pH 5.2 (*24*) is much slower than k_{ET} [process (i)]. Here we propose that the structural change around the T2Cu site occurring during the catalytic reduction of NIR is responsible for the internal ET. The equilibrium resulting in the absence of nitrite (Fig. 5) shows that the reduction of T2Cu induces a change in the pK_a value of the amino acid residue from 5.0 to 7.0. We could not determine which residue is responsible for the change in pK_a, but the most likely one in the oxidized form is the carboxylate group of Asp-98, which is hydrogen bonded to water-bound T2Cu. In the reduced enzyme, on the other

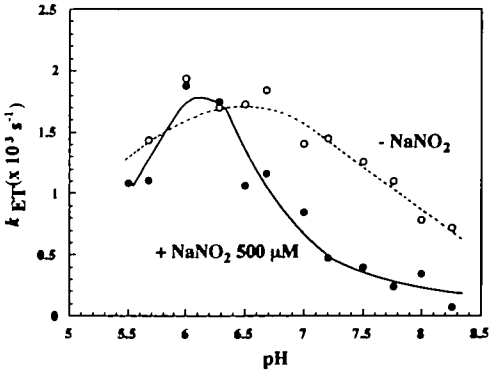


Fig. 4. **pH-dependence of the intramolecular electron transfer rate constants of AciNIR determined as the recovery of the absorbance at 460 nm in the presence (●) and absence of 500 μM $NaNO_2$ (○).** The buffer used was 10 mM phosphate buffer.

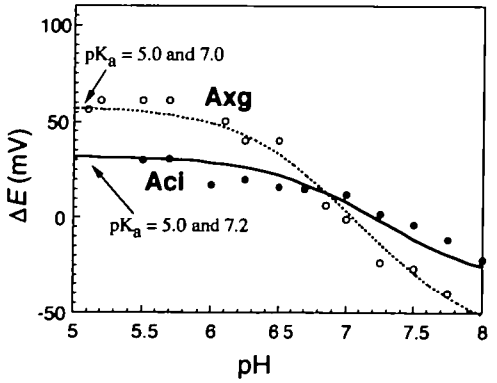
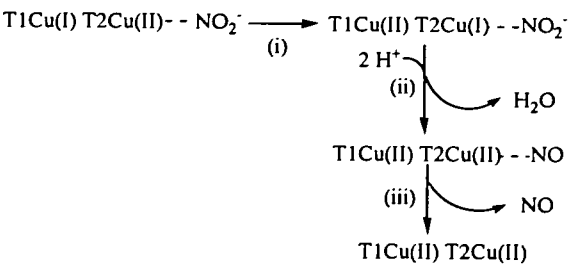


Fig. 5. **pH-dependence of the difference in redox potentials (ΔE) (Cu^{2+}/Cu^+) of T2Cu minus T1Cu in AxgNIR and AciNIR.** The lines represent the best fit curves calculated.

TABLE I. **Redox potentials of AxgNIR and AciNIR at 20°C.**

NIR	pH	E^0 (V) (*vs.* NHE) T1Cu	T2Cu[a]
AxgNIR	6.0	+0.29	+0.34
AxgNIR	7.0	+0.28	+0.28
AciNIR	6.0	+0.27	+0.30
AciNIR	7.0	+0.24	+0.25

[a]The E^0 values of T2Cu reported previously (*23*) should be corrected to the above values.



Scheme 2

hand, the pK_a value of 7.0 may be due to the imidazole group of His-255, rather than the carboxylate group of Asp. The reduction of T2Cu may result in specific pH-dependent relocation of His-255 or a water molecule bound to His-255, with accompanying loss of water bound to T2Cu. Thus, the amino acid residues linked by the redox of T2Cu play an important role in the catalytic process. It would be interesting to determine the effect of mutation of Asp-98 and His-255 on the intramolecular electron transfer process in NIR.

Finally, the results presented here show that the kinetic and equilibrium data for the blue and green types of NIRs are the same, although the structures of the two T1Cu sites are different. Therefore, the proposed mechanism may be common to the NIR reactions of various types Cu NIR from other denitrifying bacteria.

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