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Intersite Structural Rearrangement of the Blue Copper Site Induced by Substrate Binding: Spectroscopic Studies of a Copper-Containing Nitrite Reductase from *Alcaligenes xylosoxidans* NCIMB 11015

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A copper-containing nitrite reductase from *Alcaligenes xylosoxidans* NCIMB 11015 has its own unique blue or type 1 copper protein resonance Raman spectrum in the usual Cu–S_{Cys} stretching region, ν(Cu–S_{Cys}), with a pair of strong peaks at 412 and 420 cm^{-1} and a weak peak at 364 cm^{-1}. The predominantly ν(Cu–S_{Cys}) Raman bands at 412, 420, and 364 cm^{-1} of the type 1 copper site all shifted to higher frequencies upon binding of nitrite to the type 2 copper site, and the resonance Raman difference spectra progressively intensified with the increments of nitrite ion concentration. Positive support for substrate binding to the type 2 copper is provided by the ν(Cu–S_{Cys}) bands in the resonance Raman spectrum of a type 2 copper-depleted enzyme, which is insensitive to the presence of NO_2^-. The shift to higher frequency of the Raman bands of the type 1 copper center with the addition of nitrite ions suggests a stronger Cu–S_{Cys} interaction in the substrate-bound *A. xylosoxidans* nitrite reductase.

Nitrite reductase (NiR) of denitrifying bacteria is an enzyme that catalyzes the reduction of NO_2^- to NO, as a part of the anaerobic respiration of nitrate to dinitrogen. NiRs are divided into two types, heme-cd_1 and copper-containing enzymes.[1] It has been observed that the electronic absorption spectrum of the copper-containing NiRs exhibits an intense absorption band in the visible region due to a $(Cys)S^- \rightarrow Cu^{2+}$ charge-transfer (CT) transition. Electron paramagnetic resonance (EPR) spectra of the reductases indicate that the enzyme has two different types of copper atoms, type 1 (blue) copper (T1Cu) and type 2 (nonblue) copper (T2Cu).[2] It has been found that the T1Cu site accepts an electron from a soluble electron-carrier protein, such as azurin, pseudoazurin, or *c*-type cytochrome. The T1Cu site also functions as an electron donor to the T2Cu site, where nitrite ion binds and is reduced to nitrogen oxide.

Hasnain and colleagues reported the three-dimensional X-ray structure of *Alcaligenes xylosoxidans* NiR (*Ax*NiR) containing one T1Cu and one T2Cu per one subunit,[3] and the enzyme exists as a trimer composed of three identical 36 kDa subunits,[3,4] as seen in structures of the reductases from *Achromobacter cycloclastes*[5] and *Alcaligenes faecalis* S-6.[6] The coordination environment of the T1Cu site is consistent with all other blue copper electron-transfer proteins. The T1Cu site of *Ax*NiR is coordinated by two histidines, one methionine, and one cysteine in a distorted tetrahedral arrangement. The two copper sites of *Ax*NiR are directly connected through adjacent residues Cys130 and His129, which are ligated to the T1Cu and T2Cu centers, respectively. This linkage provides a 12.5-Å pathway for efficient electron transfer from the T1Cu to T2Cu site. A very similar electron-transfer pathway of the direct Cys−His linkage, which provides large electronic coupling, can be seen in the structures of ascorbate oxidase and laccase.[7]

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X-ray crystallographic studies on the NO_2^--soaked NiR crystals have revealed that T2Cu is the substrate binding site, with an asymmetric coordination of NO_2^- to the Cu.[3,5,6] Murphy and co-workers suggested a nearly side-on copper−nitrosyl coordination at the T2Cu site for an intermediate formed during the catalytic cycle of copper-containing NiR, based on the X-ray crystallographic analysis of the NO-bound enzyme from *A. faecalis* S-6.[8] Direct electrochemistry and related electrochemical studies of the *A. xylosoxidans* enzyme using a modified gold electrode have been reported, and the redox potential of the T1Cu site of the *A. xylosoxidans* enzyme was evaluated to be 260 mV vs NHE.[9]

Resonance Raman (RR) spectroscopy has proven to be a valuable technique for probing the active site structure in blue copper proteins.[10] Hence, the RR spectroscopic investigation of a blue copper-containing *A. xylosoxidans* NiR has been performed, and the structural rearrangements at the electron-transfer site of T1Cu caused by substrate binding (NO_2^-) and the depletion of T2Cu are presented in this report.

The electronic absorption spectrum of *Ax*NiR shows the intense absorption band due to a T1Cu (Cys)S^- → Cu^{2+} CT transition at 597 nm.[9,10] The T2Cu site does not contribute to the visible spectrum. The RR spectra of blue copper proteins (T1Cu) are enhanced upon excitation in the (Cys)S^- → Cu^{2+} CT band, leading to characteristic spectra, generally, with one or two Raman bands in the 250−280-cm^{-1} region and multiple Raman bands in the 330−490-cm^{-1} region. The bands at the lower and higher frequency regions are assigned to the Cu−N_{His} and Cu−S_{Cys} stretching vibrations, respectively.[10a,b] Trace a in Figure 1A shows the RR spectrum of BlueNiR obtained by excitation at 647.1 nm. *Ax*NiR has its own unique T1Cu spectrum in the ν(Cu−S_{Cys}) region, with one strong peak at 412 cm^{-1}, a prominent shoulder at ∼420 cm^{-1}, and an additional small feature at 364 cm^{-1}. Dooley et al. reported a similar RR spectroscopic pattern for the NiR enzyme from *A. cycloclastes* upon excitation at 590 nm at 290 K.[11] To investigate the spectral contribution of T2Cu, the RR spectra of type 2 copper-depleted (T2D) *Ax*NiR were measured. Trace b in Figure 1A shows that the T2D enzyme gave an almost identical spectrum of the holo-enzyme (trace a in Figure 1A). The

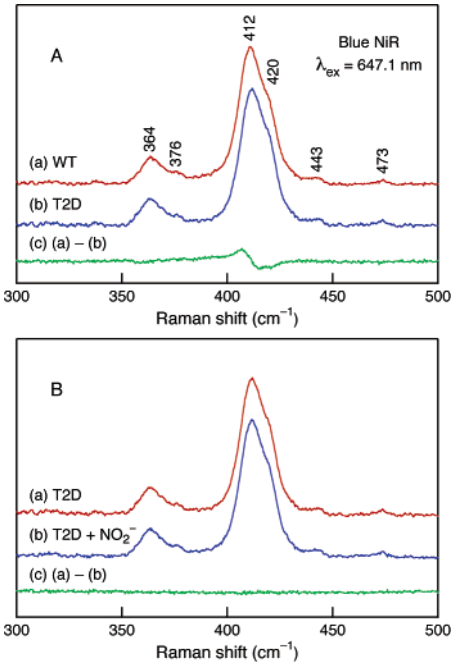

Figure 1. (A) RR spectra of (a) holo-*Ax*NiR (WT) and (b) its T2Cu-depleted protein (T2D) in 20 mM phosphate buffers (pH 7.0) and (c) their difference spectrum. (B) RR spectra of T2D *Ax*NiR in the absence (a) and presence (b) of nitrite and their difference spectrum (c).

difference spectrum (trace c in Figure 1A) between T2D and holo-enzyme revealed that the Raman bands associated with stretching of the Cu−S_{Cys} bond, particularly those at 412 and 420 cm^{-1}, are slightly shifted to higher frequencies upon removal of the T2Cu ion. In the case of laccase, a significant decrease of the Raman intensity from ν(Cu−S_{Cys}) was induced by depletion of the copper atom from the T2Cu site. The changes observed in the Raman spectrum of T2D laccase have been attributed to the T1Cu and T2Cu intersite structural rearrangement.[12] We, therefore, also attribute the observed shift to higher frequency of the RR bands in T2D *Ax*NiR to the subtle rearrangement of the T1Cu−Cys130−His129−T2Cu intersite structure.

RR spectra of *Ax*NiR in the presence of the various nitrite concentrations were also measured, and the difference spectra at different nitrite concentrations were calculated (Figure 2). The RR spectra of blue NiR recorded in the presence and absence of nitrite do not show significant changes (Figure 2a,b), but their difference spectra (Figure 2i) clearly show that the substrate binding alters the structure of the T1Cu site in NiR. The Raman bands of ν(Cu−S_{Cys}) at 412, 420, and 364 cm^{-1} all slightly shift to higher energies in the presence of nitrite, and the intensity of the difference spectra depends on the concentration of nitrite (Figure 2c−i). The spectral changes that we observe upon treatment of the enzyme with aliquots of increasing NO_2^- concentration implies that nitrite does not enter a coordination sphere of the T1Cu center in NiR but rather binds to its T2Cu ion, as had been previously established by X-ray crystallography.[3,5,6] The increased frequencies of the Raman bands at 412, 420,

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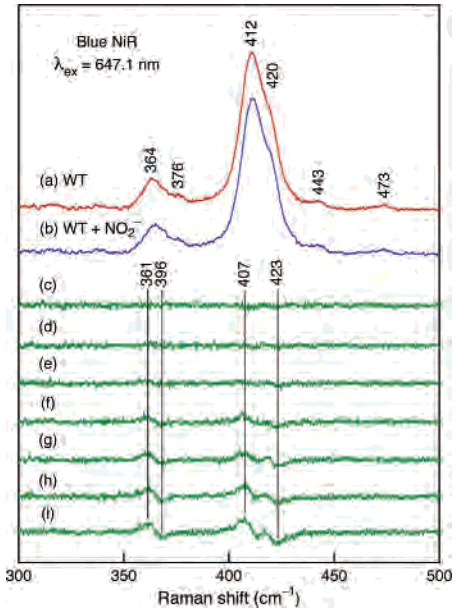

Figure 2. RR spectra of (a) holo-*Ax*NiR (0.5 mM) and (b) its complex with nitrite (10 mM). Traces c−i are difference RR spectra between those in the absence and presence of nitrite: (c) none; (d) 0.5 mM; (e) 1.25 mM; (f) 2.5 mM; (g) 4.0 mM; (h) 5.0 mM; (i) 10 mM.

and 364 cm^{-1} in a nitrite-bound enzyme suggest that there could be a stronger Cu−S_{Cys} bonding than that in the absence of substrate, or a weakened H bonding on the coordinated S ligand of cysteine, as a result of the T1Cu−T2Cu intersite structural rearrangement. That substrate binding occurs at the T2Cu site has been supported by the RR spectra of T2D *Ax*NiR measured in the presence and absence of nitrite. The results are presented in Figure 1B, where it can be seen that the spectrum of T2D *Ax*NiR does not show any changes in the ν(Cu−S_{Cys}) bands by the addition of nitrite, as is evident from the nitrite-treated T2D minus untreated T2D difference spectrum (trace c in Figure 1B). This observation is consistent with the ENDOR study of *A. xylosoxidans* T2D NiR.[13] Strange et al. pointed out that the His129−Cys130 and His89−Asp92−His94 loops are utilized for electron transfer and for communication of the status of the type 2 copper site, respectively.[3b] Very recently, Huber and co-workers reported that the EPR spectroscopic changes on the T2Cu site of NiR from *A. faecalis* were triggered by the mutation of a T1Cu ligand His145 residue to alanine.[14] It is obviously suggested that T2Cu is essential for substrate binding, and the information about substrate binding at T2Cu can be transferred to the T1Cu site.

The extended X-ray absorption fine structure,[15] EPR,[16] and X-ray crystallographic studies of *Ax*NiR have demonstrated the binding of nitrite to the T2Cu center. Strange et al. reported that the substrate replaces a putative water ligand and is accommodated by a lengthening of one of the T2Cu−His bonds by approximately 0.08 Å.[15] The changes in the electronic and geometrical properties of the T2Cu site induced by the structural changes at the T1Cu site were observed and reported by EPR spectroscopy.[16b,c] The fact that the T1Cu marker Raman bands occur at higher frequencies in the presence of nitrite suggests that the binding of nitrite to the T2Cu site causes subtle conformational changes in the bridging amino acid residues cysteine and histidine, which are covalently bonded to each other and strongly coordinated to T1Cu and T2Cu, respectively, in copper-containing NiRs. Such structural changes may alter the electronic coupling to the T2Cu site in the NiR. Crystal structures of the blue copper proteins show an unusually short Cu−S_{Cys} bond distance in their mononuclear T1Cu active sites, which is improved by a highly covalent bonding character according to the detailed spectroscopic analyses and calculation of the electronic structure.[17] Solomon and co-workers[18] pointed out that the electronic structure of the T1Cu site in the multicopper proteins is optimized for efficient long-range electron transfer to the trinuclear copper site by providing a large electronic coupling into the pathway through the Cu−S_{Cys} bond. A recent tunneling pathway analysis for the electron-transfer reaction has proposed that the electron-transfer efficiency through the covalent bond is larger than that through the noncovalent bond.[19] Our present RR data on *Ax*NiR indicate that it is likely that the Cu−S_{Cys} bond becomes slightly more covalent when the substrate binds to the T2Cu site, and the increased covalency of the Cu−S_{Cys} bonding may then provide a larger electronic coupling between the T1Cu and T2Cu sites in NiR. The structural rearrangement around the T1Cu site may be caused by substrate binding at the T2Cu site for optimization of the intramolecular electron transfer.

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