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Insights into unknown foreign ligand in copper nitrite reductase

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ABSTRACT

Bifunctional copper nitrite reductase (CuNIR) catalyzes nitrite reduction to nitric oxide and dioxygen reduction to hydrogen peroxide. In contrast to the well-researched nitrite reduction mechanism, the oxygen reduction mechanism in CuNIR has been totally unknown, because mononuclear copper–oxygen complexes decompose so readily that their visualization has been challenging. Here, we provide spectroscopic evidence that a foreign ligand binds to the catalytic copper (T2Cu) site of CuNIR, and determine CuNIR structures displaying a diatomic molecule on T2Cu. This unknown ligand can be interpreted as dioxygen and may provide insights into the oxygen reduction mechanism of CuNIR.

1. Introduction

In the nitrogen cycle, nitrogen oxides are reduced to gaseous dinitrogen in a stepwise manner ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) [1]. This process, denitrification, is conducted by microorganisms and coupled with their anaerobic respiratory systems. Nitrite reduction to nitric oxide ($NO_2^- + e^- + 2H^+ \rightarrow NO + H_2O$) is the key step in denitrification. In some organisms, the reaction is catalyzed by copper nitrite reductase (CuNIR), which is composed of three monomeric units containing two copper sites: type 1 Cu (T1Cu) and type 2 Cu (T2Cu) [2–5]. The T1Cu site, coordinated by His95, His143, Cys135, and Met148 [the numbers of residues refer to the *Geobacillus thermodenitrificans* CuNIR (*Gt*NIR) sequence], receives an electron from electron-donor proteins. The received electron is subsequently transferred to the catalytic T2Cu site through juxtaposed Cys135 and His134, which are the ligands to T1Cu and T2Cu, respectively. The T2Cu atom is coordinated by His100, His134, and His294 whereby His294 comes from the adjacent monomer. In the resting state, the nitrite binding position on the T2Cu atom is occupied by water.

Typical CuNIRs can also catalyze the two-electron reduction of dioxygen to hydrogen peroxide, which degrades CuNIR itself [6–8]. The T2Cu site in CuNIR is similar to the catalytic site of Zn,Cu superoxide dismutase (Zn,Cu-SOD) [9,10] and shows SOD activity [10,11]. These facts indicate that the T2Cu site in CuNIR has a potential ability as a dioxygen binding site. However, while the mechanism of nitrite reduction by CuNIR has been extensively studied by determining crystal structures of CuNIR in complex with NO_2^- and NO [12,13], the detailed reactions involved in oxygen species has been unexplored because mononuclear copper-dioxygen complexes decompose so readily that their visualization is difficult.

In a previous study, we determined a crystal structure of *Gt*NIR, in which T2Cu was coordinated by a chloride ion contained in the purification buffer [14]. Therefore, our original motivation for this study had been to obtain a chloride-free structure of *Gt*NIR. However, we unexpectedly found a diatomic molecule on T2Cu in the chloride-free form. Because it can be interpreted as dioxygen and may provide insights into the oxygen reduction mechanism in CuNIR, here we want to report the detail.

Abbreviations: CuNIR, copper nitrite reductase; T1Cu, type 1 copper; T2Cu, type 2 copper; *Gt*NIR, *Geobacillus thermodenitrificans* copper nitrite reductase; *Ne*NIR, *Nitrosomonas europaea* copper nitrite reductase; *Af*NIR, *Alcaligenes faecalis* copper nitrite reductase; *Ax*NIR, *Achromobacter xylosoxidans* copper nitrite reductase; *Ac*NIR, *Achromobacter cycloclastes* copper nitrite reductase; MPD, 2-methyl-2,4-pentanediol; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(N-morpholino) ethanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; WT, wild type; Zn,Cu-SOD, Zn,Cu superoxide dismutase; LMCT, ligand-to-metal charge transfer.

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2. Materials and methods

2.1. Expression, purification, and crystallization of wild type (WT) and an H294M mutant of GtNIR

Preparation of WT *Gt*NIR was performed as described previously [14] with following modifications. HEPES buffer was used instead of Tris–HCl buffer in order to prevent chloride ions from binding to T2Cu. Additionally, $(NH_4)_2SO_4$ instead of NaCl was used to elute proteins from an anion exchange column. Crystallization was performed as described previously [14]. Crystals were soaked in the cryo-solution containing 0.1 M sodium acetate buffer pH 4.5, 5.5% (w/v) polyethylene glycol 4000, and 35% (v/v) 2-methyl-2,4-pentanediol (MPD) and flash-cooled by immersion in liquid nitrogen prior to data collection.

We made a *Gt*NIR mutant, in which T2Cu ligand His294 is replaced by methionine. The forward and reverse primers for the mutation were 5′-TCCGATCGTTACTATGCAGTTTAATCATGC-3′ and 5′-GCATGATTAAACTGCATAGTAACGATCGGA-3′, respectively. The sequence of the mutant plasmid (pET22b) was confirmed by DNA sequencing. The mutant was overexpressed, purified, and crystallized using the same method as that for the WT protein.

2.2. Oxygen reduction assay

The *o*-dianisidine (3,3′-dimethoxybenzidine dihydrochloride) dependent assay was performed as described previously [8,15]. A saturated *o*-dianisidine solution was prepared by stirring excess *o*-dianisidine in 80 mM MES-HEPES buffer, pH 7.0 overnight. Insoluble *o*-dianisidine powders were eliminated by filtering with a 0.22 μm filter. The reaction was initiated by adding WT or the H294M mutant of *Gt*NIR. Final concentration of the enzymes was 1.6 μM. Oxidation of *o*-dianisidine was monitored by the increase in absorbance at 460 nm (11,300 M^{-1} cm^{-1}) with a UV-2550 UV–vis spectrophotometer (Shimadzu, Kyoto, Japan). The measurement temperature was kept at 25 °C by a TCC-240A temperature controller (Shimadzu). The assay was performed three times for WT and the H294M mutant, respectively.

2.3. Microspectroscopy

The UV–vis spectra of a *Gt*NIR crystal (~0.3 × 0.4 × 0.1 mm) were measured using a microspectrophotometer connected with an X-ray diffractometer installed at beamline BL38B1 at SPring-8 (Hyogo, Japan) [16]. All UV–vis microspectrum data were measured in a 100 K cryostream and were collected four times to increase signal to noise ratios. The spectra were recorded every 45 s X-ray irradiation. The X-ray dose for 45 s irradiation, which was calculated with RADDOSE [17], was 0.041 MGy. Differences of UV–vis absorption due to different crystal thicknesses were avoided by fixing the direction of the crystal and the measurement position for each measurement. The UV–vis spectra only of the cryo-solution were measured with the same cryoloop as that used for the *Gt*NIR crystal.

2.4. Crystal structure determination

The dataset for one of the two WT crystals (WT-OXY1) was collected at 100 K on beamline BL38B1 at SPring-8 using an ADSC Quantum 315 CCD detector (Area Detector Systems Co., CA, USA). This is the same crystal as the crystal that was used in the microspectroscopic analysis described above. The higher-resolution dataset for the other *Gt*NIR crystal (WT-OXY2) grown under the same condition as WT-OXY1 was collected at 100 K on beamline BL1A at Photon Factory (Ibaraki, Japan) using a Pilatus 2M-F detector (DECTRIS Ltd., Baden, Switzerland). Total X-ray doses for WT-OXY1 and WT-OXY2 were ~0.16 and ~0.18 MGy, respectively. The HKL2000 package [18] was used to reduce, integrate and scale the collected data. The phases were determined by molecular replacement using the program MOLREP [19] from the CCP4 suite [20]. A monomeric subunit of *Gt*NIR (PDB code 4ZK8) was used as the search model. The resulting models were refined by REFMAC5 [21]. Manual model building was performed using COOT [22]. The final models were checked for stereochemical quality using MolProbity [23]. Data collection and refinement statistics are summarized in Table 1.

The diffraction dataset for a H294M crystal was collected at 100 K on beamline BL38B1 at SPring-8 using the ADSC Quantum 315 CCD detector. The dataset was reduced, integrated and scaled with the HKL2000 package. Phasing was performed by molecular replacement using MolRep. The monomeric subunit of WT *Gt*NIR (PDB code 4ZK8) was used as the search model. The resulting model was refined with Refmac5. Manual model building was carried out using COOT through the refinement process. The final model was checked for stereochemical quality using MolProbity. Data collection and refinement statistics are summarized in Table 1.

3. Results

3.1. Oxygen reduction assay

o-dianisidine and dioxygen can be an electron donor and a terminal electron acceptor in the reaction system, respectively [8,15]. Addition of WT *Gt*NIR or the H294M mutant to the saturated *o*-dianisidine solution caused a jump of absorbance at 460 nm (Fig. 1A), because oxidized *Gt*NIR has an absorption peak at around 450 nm (Fig. 1B). Addition of WT *Gt*NIR showed a linear increase in absorbance (Fig. 1A). Conversely, the H294M mutant did not show the oxygen reduction ability (Fig. 1A). The dioxygen reduction rate of WT *Gt*NIR was $2.4 \times 10^{-4} \pm 1.3 \times 10^{-5}$ s^{-1}, which is about 17 times slower than 4.2×10^{-3} s^{-1} of CuNIR from mesophilic *Alcaligenes faecalis* (*Af*NIR) [8].

3.2. Microspectroscopic analysis

The UV–vis spectrum of the *Gt*NIR crystal before X-ray irradiation (red line in Fig. 1C) shows absorption bands with peaks at around 450 and 600 nm, which correspond to peaks observed in the solution UV–vis spectrum of aerobically oxidized *Gt*NIR (Fig. 1B). These bands are derived from ligand-to-metal charge transfer (LMCT) transitions from the sulfur atom of Cys135 to oxidized (Cu^{2+}) T1Cu [24]. Moreover, weak absorption originating from a *d*–*d* transition of Cu^{2+} was observed between 650 and 750 nm (Fig. 1C).

Synchrotron X-ray beams is known to cause reduction of the metal sites in metalloproteins [25,26]. As the dose of X-ray increased, the intensities of absorption at around 450 and 600 nm became weaker (Fig. 1C and D). The band of the *d*–*d* transition decreased, too. Conversely, the absorption intensity between 300 and 400 nm increased during exposure of the crystal to X-ray (Fig. 1C and D). These changes occurred sequentially. In other words, the decreases of absorption, derived from oxidized copper sites (at 450, 600, and ~700 nm), were first recorded and then the increase of absorption between 300 and 400 nm became conspicuous (Fig. 1C and D). The UV–vis spectrum between 300 and 400 nm only of the crystallization buffer was not significantly changed by X-ray irradiation (Fig. S1).

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