Redox-coupled proton transfer mechanism in nitrite reductase revealed by SPring-8 and SACLA

Since the invention of the Haber–Bosch process, the amount of fixed nitrogen in soil and water has been increasing, and this trend has a significant influence on the global environment. Fixed nitrogen is oxidized to nitrite (NO_2^-) by nitrification and then converted to gaseous dinitrogen (N_2) by microbial denitrification, which closes the nitrogen cycle. The reduction of NO_2^- to toxic nitric oxide ($NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$) is referred to as the key step in denitrification and is catalyzed by copper nitrite reductase (CuNiR).

CuNiR is a homotrimeric protein comprised of two distinct Cu sites per monomer (Fig. 1). Type 1 Cu (T1Cu) with a Cys–Met–His_2 ligand set is an electron acceptor, whereas type 2 Cu (T2Cu) with a His_3 ligand set is a catalytic center. Spaced ~12.5 Å apart, the two Cu sites are linked by an electron pathway, a Cys–His bridge. Two conserved residues, Asp98 and His255 (*Alcaligenes faecalis* numbering), are located at the T2Cu site and are bridged by a water molecule called bridging water. They are essential to the CuNiR activity because they assist proton-coupled electron transfer (PCET) to the substrate NO_2^-. However, the catalytic mechanism of CuNiR is controversial.

Analyses of the nitrite reduction mechanism in CuNiR using conventional synchrotron radiation crystallography (SRX) have encountered difficulties because X-ray photoreduction changes the native structures of metal centers and the enzyme–substrate complex. In recent years, serial femtosecond crystallography (SFX) using ultrabright femtosecond pulses from X-ray free electron lasers (XFELs) has opened a new age of structural biology. Using the high photon density in the focused XFEL beam, which achieves single-pulse diffraction within femtosecond exposure time, SFX enables protein structure determination from micrometer- to submicrometer-size crystals at ambient temperature. The femtosecond pulse duration allows the "diffraction-before-destruction" approach by circumventing radiation damage (photoreduction) of the sample because the diffraction process can be terminated in a timescale shorter than that of the damage process.

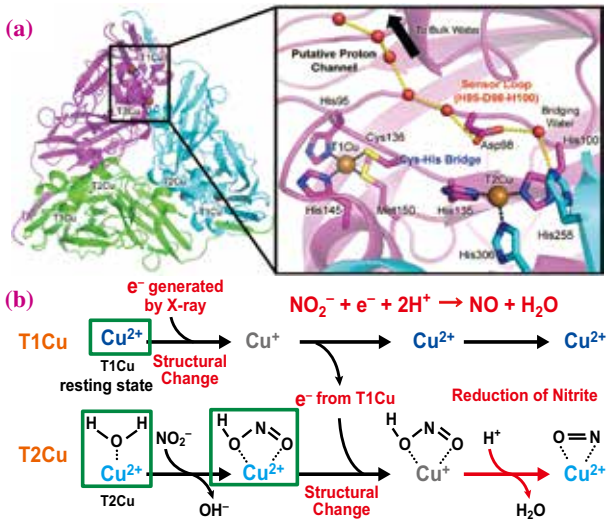

Fig. 1. **(a)** Overall structure (left) and catalytic site (right) of CuNiR (PDB ID: 1AS7 [3]) drawn as ribbon models. The yellow dashed lines and black dashed lines represent hydrogen bonds and coordination bonds, respectively. **(b)** Catalytic scheme of CuNiR. The structures surrounded by green rectangles are in oxidized states, which are susceptible to photoreduction by X-rays.

In this study, we utilized photoreduction in SRX at SPring-8 **BL26B1** and **BL26B2** to initiate a catalytic reaction of CuNiR from *Alcaligenes faecalis* (*Af*NiR) and to trap enzymatically produced intermediary structures [1]. Furthermore, to visualize intact CuNiR structures, we applied SFX at SACLA **BL3**, which enables damage-free structural determination and evaluation of the native conformational population at room temperature. By comparing SRX and SFX data, we obtained new insights into PCET and nitrite reduction in CuNiR.

The $N^{\delta 1}$ atom of catalytically important His255 can form a hydrogen bond (H-bond) with the carbonyl O atom of Glu279 and/or the hydroxyl O atom of Thr280, and this Glu–Thr pair is conserved in CuNiRs. Compared with the imidazole ring of His255 in the intact SFX resting-state structure refined at 2.03 Å resolution, the imidazole ring in the photoreduced SRX resting-state structure at 1.20 Å resolution is rotated by about 20° (Fig. 2(a)). Hence, by the reduction of the copper center, the H-bond partner of His255 was changed from Glu279 to Thr280. Moreover, we recently showed that the imidazole ring of His244 in CuNiR from *Geobacillus thermodenitrificans* (*Gt* NiR), which corresponds to His255 in *Af*NiR, rotates as a result of photoreduction [2]. These results indicate that the rotation of His is a universal phenomenon during CuNiR catalysis.

Using mutated *Af*NiR, we further proved that the rotated state of His255 is a transient conformation important for the CuNiR activity. The activities of the T280V and T280S mutants were, respectively, 20% and 29% of the WT activity. Because the T280V

mutant lacks the hydroxyl O atom that can form an H-bond with His255, the rotation of His255 is inhibited in this mutant. Although the T280S mutant maintains a hydroxyl group in the side chain, it rotates more flexibly than that of Thr, which means that His255 is not always able to form an H-bond with Ser280. Therefore, the T280S mutant showed lower activity than that of WT but higher activity than that of T280V.

We propose that His255 is a redox-coupled switch for proton transfer (Fig. 2(b)). Because the hydroxyl O atom of Thr280 is less negatively charged than the carbonyl O atom of Gln279, the $N^{\delta1}$ atom of His255 forms a longer and weaker H-bond with Thr280. As a result, the H atom is more attracted to the $N^{\delta1}$ atom, and a proton on the $N^{\varepsilon2}$ atom moves to the bridging water. It is considered that a proton is supplied to NO_2^- via the bridging water (or even Asp98). It is an interesting fact that the proton transfer from His255 via the bridging water has also been supported by recent computational studies.

The vertical binding mode is known to occur in many biomimetic model complexes of Cu(II)–NO_2^- and supported by computational chemistry. However, the SRX CuNiR structures reported previously have shown the near face-on modes. In this study, an SFX NO_2^- complex structure was solved at 1.60 Å resolution by the single-wavelength anomalous diffraction (SAD) method using Cu as a phasing element. This is the first example of phase determination with natural heavy atom binding sites using the data obtained with SFX. Then, two SRX NO_2^- complex structures at a cryogenic temperature (Cryo) and room temperature (RT) were determined at 1.30 and 1.54 Å resolutions, respectively.

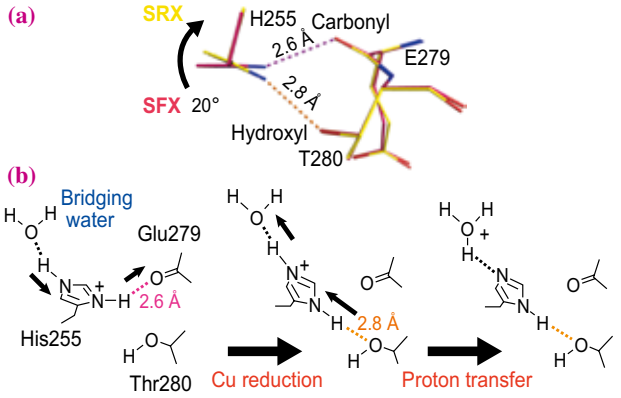

Fig. 2. **(a)** Switching of H-bond partners. The SFX and SRX resting-state structures are shown in pink and yellow, respectively. Dashed lines represent H-bonds. **(b)** Proposed mechanism of efficient proton transfer driven by the rotation of His255. Dashed lines represent H-bonds. Thin black arrows illustrate the directions in which H atoms are attracted.

The NO_2^- binding mode in the SFX structure was vertical, whereas that in the Cryo SRX or RT SRX structure was near face-on (Fig. 3). It is most probable that the conformational change of NO_2^- from vertical to near face-on is induced by photoreduction. Furthermore, the N atom of NO_2^- becomes closer to His255 when NO_2^- changes its conformation from vertical to near face-on, meaning that owing to steric hindrance (<3.5 Å), near face-on NO_2^- might inhibit the reverse rotation of His255 and hence reverse proton transfer.

Our study has demonstrated that combining SRX and SFX technology is extremely effective as a powerful tool for studying the catalytic process of redox enzymes.

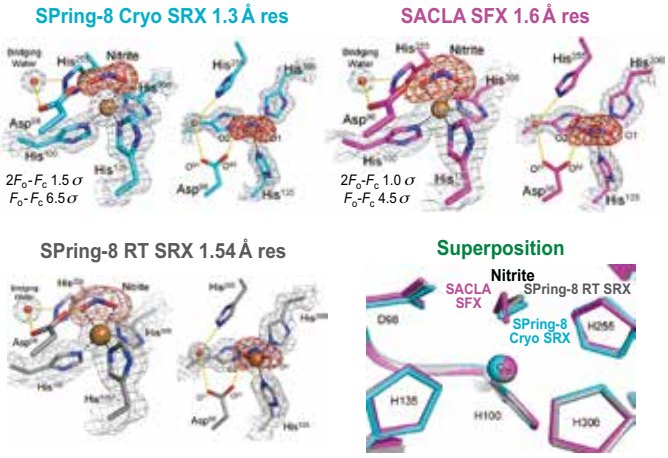

Fig. 3. NO_2^- complex structures of CuNiR determined by SFX (magenta), Cryo SRX (cyan), and RT SRX (gray). Superposition of the structures indicates that the binding modes of NO_2^- on T2Cu are different among the structures.

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