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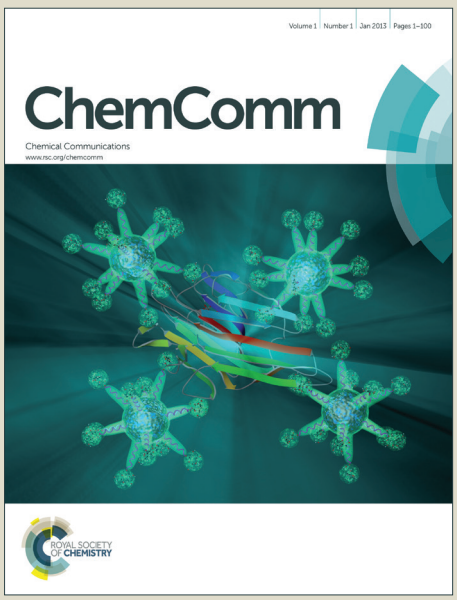

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High-temperature and high-resolution crystallography of thermostable copper nitrite reductase

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The 1.55 Å resolution thermostable copper nitrite reductase structure at 320 K displayed a near-bidentate binding mode of nitrite distinct from a monodentate mode in a cryogenic structure. To our knowledge, this is the first case that crystallography visualized the difference in substrate binding modes between cryogenic and high-temperature structures.

The consensus opinion on protein crystallography is that X-ray diffraction experiment has to be performed at low temperatures (<100 K) to reduce radiation damage to crystals;[1] more than 95% of protein crystal structures have therefore been determined using datasets collected below 100 K. However, conformational distributions of the side chains of amino acid residues involved in enzymatic activities are often different between cryogenic and room temperature structures.[2] Thus, it is unclear whether crystallographers can obtain information on the intrinsic dynamics of thermostable enzymes, which function at high temperatures, only from cryogenic structures. A popular experimental technique to reveal room- or high-temperature structures of biological macromolecules is nuclear magnetic resonance (NMR) analysis which can determine protein structures in solution.[3] It, however, cannot be applied to proteins that have high molecular weights or contain paramagnetic nuclei, as these make interpretation of NMR signals difficult.

Recent genomic analyses have revealed that more diverse microorganisms than expected have enzymes involved in denitrification and some of them have been predicted to have unique structures.[4] The investigation of these new enzymes is necessary to achieve a deeper understanding of the global nitrogen cycle. *Geobacillus* is a genus of gram-positive extremophiles and several of its species are thermophilic denitrifiers. They have genes encoding copper nitrite reductase (CuNIR), one of the well-studied enzymes related to denitrification.[5] CuNIR catalyses the one-electron reduction of nitrite (NO_2^-) to gaseous nitric oxide (NO), and has a homotrimeric structure consisting of 37 kDa monomers containing two distinct copper sites, called type 1 Cu (T1Cu) and type 2 Cu (T2Cu).[6] The T1Cu atom located near the molecular surface is tetrahedrally coordinated by one cysteine, one methionine, and two histidine residues and functions as an electron acceptor. The catalytic T2Cu site, positioned between two monomers, is ligated by three histidine residues, one of which comes from the adjacent monomer. In the resting state, one or two water molecules occupy the apical position of the T2Cu site, and these are replaced by nitrite in the course of nitrite reduction. CuNIR from thermophilic *Geobacillus* (*Geo*NIR) shows an overall structure similar to those of known CuNIRs, but has unique features such as a wide substrate channel, a rigid and compacted catalytic site, and an N-terminal arm structure involved in formation of a transient complex with an electron donor.[7] The crystal structure of *Geo*NIR in complex with nitrite shows an unusual η^1-O binding mode of nitrite to the T2Cu atom,[7a] whereas nitrite assumes an η^2-O,O binding manner in other CuNIRs.[6b,8] The rigid and compacted catalytic core in *Geo*NIR has been thought to prevent nitrite from intruding into the narrow space above the T2Cu site deeply enough to bind in the η^2-O,O mode. Based on structural information, we proposed a reaction mechanism of *Geo*NIR, in the course of which nitrite keeps monodentate.[7a] However, it has been unknown whether the rigidity of the structure and the η^1-O binding mode is really kept also at high temperatures suitable for the growth of *Geobacillus* (318–346 K),[9] because the crystal used for our previous experiment was prepared at room temperature (273 K) and the dataset was collected at 100 K. The catalytic site of *Geo*NIR is around the paramagnetic copper atom; therefore, the NMR technique cannot be applied to it. Instead, we introduced high-temperature X-ray crystallography to determine a high-temperature structure of *Geo*NIR in complex with nitrite.

Because X-ray irradiation easily reduces the T1Cu site and sequential intramolecular electron transfer (ET) to the T2Cu site causes reduction of nitrite bound to the T2Cu atom,[10] we used, as the earlier study, the C135A mutant of *Geo*NIR, in which one of the T1Cu ligand, Cys135, is replaced with alanine and intramolecular ET is inhibited. A crystal of the C135A mutant complexed with nitrite (C135A-NO_2) was prepared as described previously,[7a] and data collection was carried out at 320 K. In the pioneering and exclusive work on high-temperature protein X-ray crystallography performed by Petsko's group, in which crystal structures were determined for ribonuclease A at nine different temperatures, ranging from 98 to 320 K, overall expansion of the unit cell with increasing temperature was observed.[11] The dataset for C135A-NO_2 collected at 320 K showed that the cell parameters were about 1.5 Å longer than those at 100 K (Table 1).

Table 1. Data collection and refinement statistics

PDB code	3X1N
Wavelength (Å)	1.00000
Space group	*R*3
Unit cell dimensions	
a = *b*, *c* (Å)	116.3, 85.65 [114.9, 84.04][a]
Bragg spacings (Å)	50.0-1.55 (1.58-1.55)[b]
R_{sym} (%)[c]	6.6 (37.7)
R_{pim} (%)[d]	3.8 (24.1)
$CC_{1/2}$, *CC**	(0.846), (0.957)
Completeness (%)	96.6 (98.9)
Unique reflections	60,565 (3,082)
$\langle I/\sigma(I)\rangle$	22.1 (2.5)
Redundancy	3.9 (3.7)
Resolution range (Å)	34.8-1.55
R_{work} (%)[e]/ R_{free} (%)[e]	11.5/13.1
RMSD bond length (Å)	0.025
RMSD bond angle (°)	2.37

[a] Values of the cryogenic structure (PDB code 3WKP[7a])
[b] Values in parentheses are for the highest-resolution shell.
[c] R_{sym} is calculated as $\Sigma_{hkl}\Sigma_i|I_i(hkl)-\langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i\langle I(hkl)\rangle$, where $I_i(hkl)$ is the intensity of an individual measurement of the reflection with Miller indices *hkl* and <I(hkl)> is the average intensity from multiple observations.
[d] R_{pim} is calculated as $\Sigma_{hkl}(1/n-1)^{1/2}\Sigma_i|I_i(hkl)-\langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i\langle I(hkl)\rangle$.
[e] R_{work} is calculated as $\Sigma_{hkl}||F_{obs}|-|F_{calc}||/\Sigma_{hkl}|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure-factor amplitudes, respectively.
[e] The free *R* factor, R_{free}, is computed in the same manner as R_{work} but using only a small set (5%) of randomly chosen intensities that were not used in the refinement of the model.

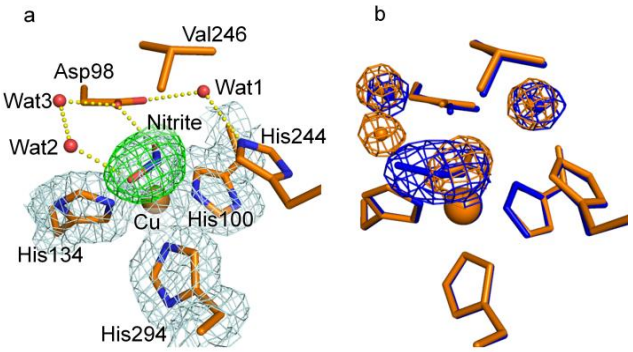

Fig. 1 (a) The T2Cu site in the C135A-NO_2^{high} structure. $2F_o$-F_c map contoured at 1.0 σ is shown by cyan meshes. The green mesh around nitrite represents a F_o-F_c map contoured at 6.0 σ, which was calculated for the model without nitrite. The copper ion is shown as a brown sphere. Carbon, oxygen, and nitrogen atoms are shown in orange, red, and blue, respectively. Hydrogen bonds are drawn as yellow dashed lines. (b) Comparison of electron density maps for nitrite and water molecules between high-temperature (orange) and cryogenic (blue) data. Residues and nitrite are represented by sticks. Copper ions and water molecules are shown as spheres. Meshes represent $2F_o$-F_c maps contoured at 1.0 σ. Superimposed maps were generated with PHENIX. [12]

This result indicates that data collection was conducted at high temperature. The statistics shown in Table 1 confirm that data collection was completed before the crystal suffered severe radiation damage. The high-temperature structure of the nitrite-bound C135A-NO_2 mutant (C135A-NO_2^{high}) was determined at 1.55 Å resolution and refined to R_{work} and R_{free} factors of 11.5 and 13.1%, respectively. These small values showed the accuracy of the structure. Figure S1 shows that the T1Cu site displayed the same planar T-shape coordination geometry as that in the C135A-NO_2 structure at 100 K [C135A-NO_2^{low}, Protein Data Bank (PDB) code 3WKP[7a]]. On the other hand, the electron density map around the T2Cu site of C135A-NO_2^{high}, which indicated the presence of a small molecule (Figure 1a), was different from that of C135A-NO_2^{low} (Figure 1b). We assigned a mixture of 0.7-occupancy nitrite and 0.3-occupancy water to the electron density above the T2Cu atom of C135A-NO_2^{high} (Figures 1a and S2). Comparison between high-temperature and cryogenic structures clearly revealed that at 320 K, nitrite no longer bound to the T2Cu atom in the η^1-O binding mode, but assumed the coordination manner similar to η^2-O,O (Figure 2a). All three atoms of nitrite were located near enough to interact with the T2Cu atom in the C135A-NO_2^{high} structure. The distances of Cu–$O_{proximal}$, Cu–O_{distal}, and Cu–N were 2.13, 2.52, and 2.21 Å, respectively (The detailed coordination structure of both the T1Cu and T2Cu sites are shown in Table S1). While in the C135A-NO_2^{low} structure, the $O_{proximal}$ atom of nitrite was far from the $O^{\delta1}$ atom of

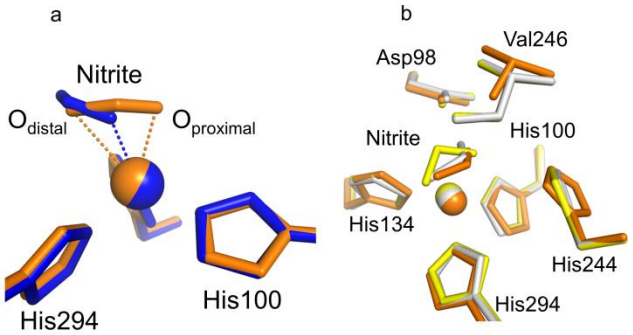

Fig. 2 (a) Comparison of binding modes of nitrite between C135A-NO_2^{high} (orange) and C135A-NO_2^{low} (blue). Coordination bonds between the T2Cu atom and oxygen atoms are shown by dashed lines. (b) Superimposition of the C135A-NO_2^{high} structure (orange) on typical CuNIR structures in complex with nitrite. The yellow and white structures are CuNIR from *Alcaligenes faecalis* (PDB code 1SJM[8a]) and *Achromobacter cycloclastes* (PDB code 2BWD[8b]), respectively.

Asp98 (3.38 Å), the distance was shorter in the C135A-NO_2^{high} structure (2.47 Å), as was observed in other CuNIRs.[8] Figure 2b shows that the nitrite binding mode in the C135A-NO_2^{high} structure was similar to those in other CuNIRs, especially CuNIR from *Achromobacter cycloclastes*.[8b] Isoleucine above the T2Cu site, which is conserved in typical CuNIRs, plays a key role in controlling the binding modes of the substrate and other small molecules.[13] Probably because this isoleucine is replaced with smaller valine (Val246) in *Geo*NIR (Figure 2b), nitrite was not fixed in the complete η^2-O,O mode. Actually, in the crystal structure of the mutant CuNIR from *Alcaligenes faecalis*, in which isoleucine was replaced with valine, displays a more asymmetric η^2-O,O manner of nitrite than wild type.[13a] Additionally, structural flexibility at high temperatures can cause such asymmetric binding. The fact that occupancy of nitrite was 0.7 and the low-occupancy water molecule was observed indicates that rapid exchange of external ligands occurred at the T2Cu site.

We previously predicted that the imidazole ring of His244 above the T2Cu site should rotate so that the space above the T2Cu site becomes wider and nitrite can bind to the T2Cu atom in the η^2-O,O manner.[7a] Such a conformational change was not detected in the C135A-NO_2^{high} structure. However, distances from the centre of the catalytic site (T2Cu) to residues located on the substrate pocket were longer in the C135A-NO_2^{high} structure than those in the C135A-NO_2^{low} structure (Figures S3 and Table S2), which means that the substrate pocket became wider at 320 K. Because 320 K is slightly lower than the optimum temperature for *Geobacillus* (333 K),[9] temperatures higher than 320 K, at which the thermal energy enables residues around the catalytic site to move more flexibly, may be needed for the complete η^2-O,O binding mode. However, based on

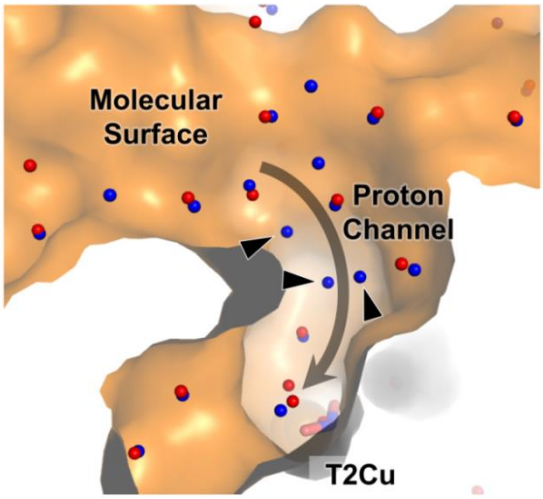

Fig. 3 (a) Water molecules located in the putative proton channel. Water molecules observed in the C135A-NO_2^{high} and C135A-NO_2^{low} structures are shown in red and blue spheres, respectively. Water molecules observed specifically in the cryogenic structure are shown by black triangles.

the C135A-NO_2^{high} structure, it can be concluded that the reaction mechanism of *Geo*NIR at high temperature is the same as those of typical CuNIRs.

There still remain some issues to be resolved, such as at which temperature nitrite changes its binding mode and whether room temperature is too low for nitrite to assume the bidentate mode. Cryogenic structures of CuNIRs from mesophilic organisms showed that the product of nitrite reduction, NO, binds to the T2Cu atom in the side-on manner,[6b,8a,b,13c] whereas spectroscopic and computational studies demonstrated that NO is likely end-on bound in solution.[14] The flexibility of isoleucine above the T2Cu site in solution is thought to be the key factor for the end-on binding mode of NO.[14c] To reveal the detailed reaction mechanism of CuNIR, room temperature structures in complex with NO should be determined because product binding modes may differ at different temperatures, too. These are the next subjects of our study.

Water plays important roles in structural stabilization and chemical reactions of proteins.[15] The number of observed water molecules in crystal structures decreases at high temperatures.[11] Thus, water found even in high-temperature structures must be important. Several studies have suggested that water molecules lined up from the molecular surface to the catalytic site of CuNIR form a channel for supply of protons, which is consumed by nitrite reduction.[7a,8b,16] In the C135A-NO_2^{high} structure, water molecules in this proton channel were clearly observed at the molecular surface and the catalytic core, showing that *Geo*NIR partially retains the hydrated structure in the proton channel at high temperatures. On the other hand electron densities of water molecules at the middle of the proton channel were ambiguous, meaning that water molecules

move rapidly there (Figure 3). This combination of fixed and flexible water molecules may be involved in efficient proton transfer at high temperatures. Wat2 in Figure 1a was found only in the high-temperature *Geo*NIR structure (Figure 1b). The hydrogen bond between the O_{distal} atom of nitrite and Wat2 may stabilize the η^2-O,O binding mode of nitrite and facilitate nitrite reduction. Indeed, this hydrogen bond between nitrite and water is also found in high resolution structures of other CuNIRs.[8a-c]

In conclusion, using high-temperature and high-resolution X-ray crystallography, we demonstrated that a cryogenic structure of a thermostable enzyme does not always reflect a structure under the physiological high-temperature environment. To the best of our knowledge, this is the first time that X-ray crystallographic technique has shown that binding modes of substrate molecules differ at different temperatures. Proteins from thermophilic organisms have been used as model proteins to investigate structure-function relationships because purification and crystallization of them are easier than their non-thermostable homologs. However, we think that high-temperature X-ray crystallography should be applied to such thermostable targets. It will open up a new horizon for structural biochemistry.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Information on materials, experimental details, additional figures and tables. The atomic coordinate and structure factor (PDB codes 3X1N) have been deposited into the Protein Data Bank.. See DOI: 10.1039/c000000x/

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