Materials and Methods

The C135A mutant of a thermostable CuNIR from *Geobacillus thermodenitrificans* NG80-2 was expressed and purified using previously published protocols (ref. S1). The crystal of the C135A mutant in complex with nitrite was prepared as described in ref S1. The obtained crystal was coated with paraffin oil to avoid drying. MicroLoop and MicroRT X-ray capillary (MiTeGen LLC, NY, USA) were used to mount the crystal on the goniometer of beamline BL38B1 at SPring-8 (Hyogo, Japan). Temperature of a cryostream controller was set to 320 K. After setting the crystal on the apparatus, the diffraction dataset was collected using an ADSC Quantum 315 CCD detector (Area Detector Systems Co., CA, USA). The beam size was 50 μm (height) × 88 μm (width). Oscillation angle and exposure time per image were set to 1 degree and 1.5 seconds, respectively. A total of 120 diffraction images were collected from the single crystal, where 5 min intervals were taken after collection of sets of 40 images to prevent the temperature of the crystal from increasing much higher than 320 K. The dataset was reduced, integrated and scaled with the HKL2000 package (ref. S2). Phase was determined by molecular replacement using the program MOLREP (ref. S3) from the CCP4 suite ver. 6.4.0 (ref. S4). A monomeric subunit of wild type CuNIR from *G. thermodenitrificans* [Protein Data Bank (PDB) code 3WKO] was used as the search model. The resulting model was refined with REFMAC5 (ref. S5). Manual model building was performed using COOT (ref. S6) through the refinement process. Water molecules were added to the model using the automated water-searching program built into COOT. Other water molecules, which were not found by COOT, were added manually according to the electron density maps. Anisotropic displacement parameters were introduced only to copper atoms after water molecules were built into the models.

When one water molecule (occupancy 1.0) or one nitrite molecule (occupancy 1.0) was modeled above the T2Cu atom, positive electron density or negative electron density was observed, respectively. When two water molecules (occupancy 1.0) were modeled at this site, the distance between them was too short (0.93 Å) for them to be present at the same time. When two water molecules (occupancy 0.5) were modeled at this site, positive electron density remained in the F_o-F_c map. Therefore, one nitrite molecule (occupancy 0.7) and one water molecule (occupancy 0.3) were assigned because this model showed the best agreement with the electron density. Occupancies of nitrite and water were determined by trying several different occupancies. We chose the best model which showed no significant positive and negative peaks around nitrite in the F_o-F_c map. The final model was checked for stereochemical quality using MolProbity (ref S7). Ramachandran favored, allowed, and outliers were 97.62, 2.38, and 0%, respectively. Data collection and refinement statistics are summarized in Table 1. The atomic coordinates and structure factor amplitude were deposited in the PDB. The PDB ID is 3X1N.

References

S1. Y. Fukuda *et al*. *J. Biochem*., 2014, **155**, 123.

S2. Z. Otwinowski and W. Minor, *Methods Enzymol*., 1997, **276**, 307.

S3. A. Vagin and A. Teplyakov, Acta Crystallogr., 2010, **D66**: 22.

S4. M. D. Winn *et al*., *Acta Crystallogr.*, 2011, **D67**, 235.

S5. G. N. Murshudov *et al*., *Acta Crystallogr*., 2011, **D67**, 355.

S6. P. Emsley *et al*., *Acta Crystallogr*., 2010, **D66**, 486.

S7. V. B. Chen *et al*., *Acta Crystallogr*., 2010, **D66**, 12.

Supplementary Figures and Tables

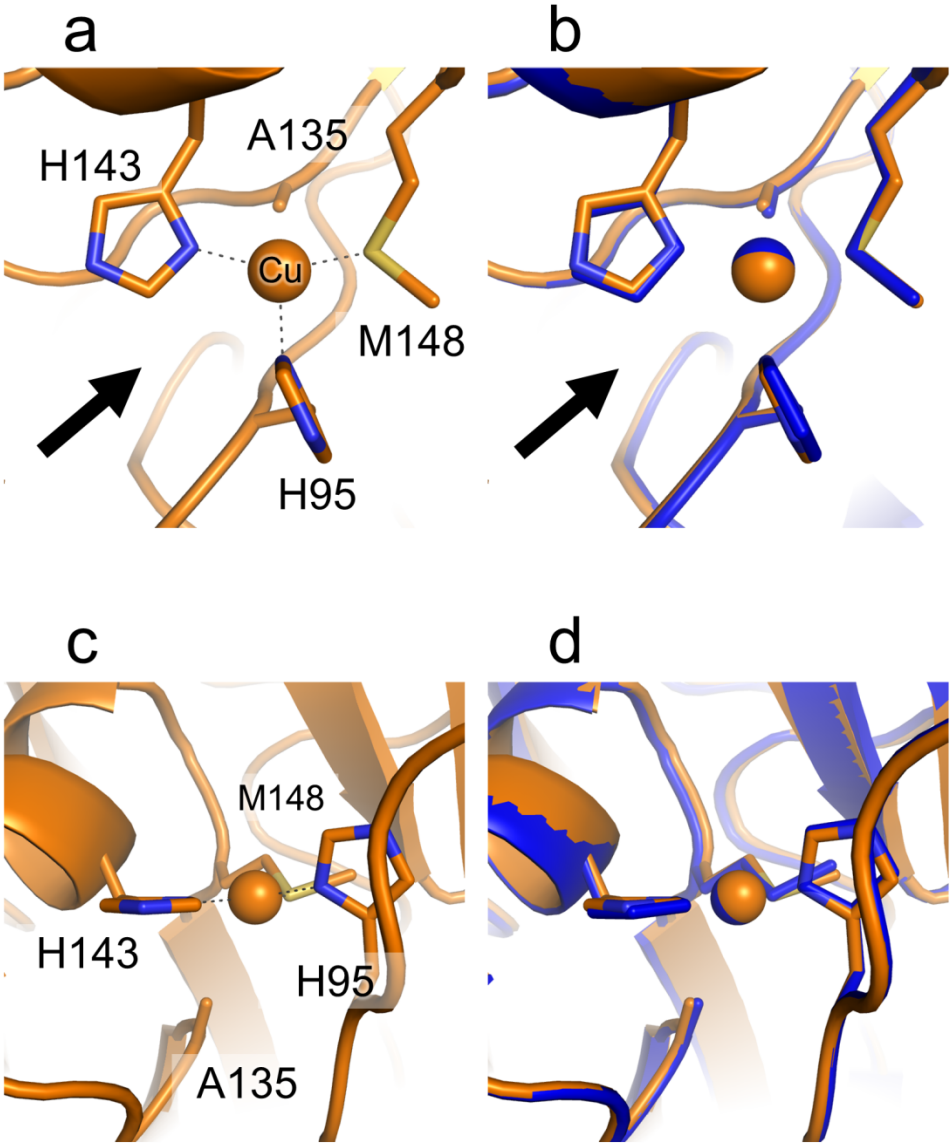

Figure S1. The planar T-shape T1Cu site in the C135A mutant structure. (a) The T1Cu site in the C135A-NO_2^{high} structure and (b) superimposition of it on the C135A-NO_2^{low} structure (blue). (c) and (d) are viewed from the directions shown by black arrows in (a) and (b), respectively. Coordination bonds are shown in (a) and (c) as dashed black lines. The distances between the T1Cu atom and ligands are shown in Table S1.

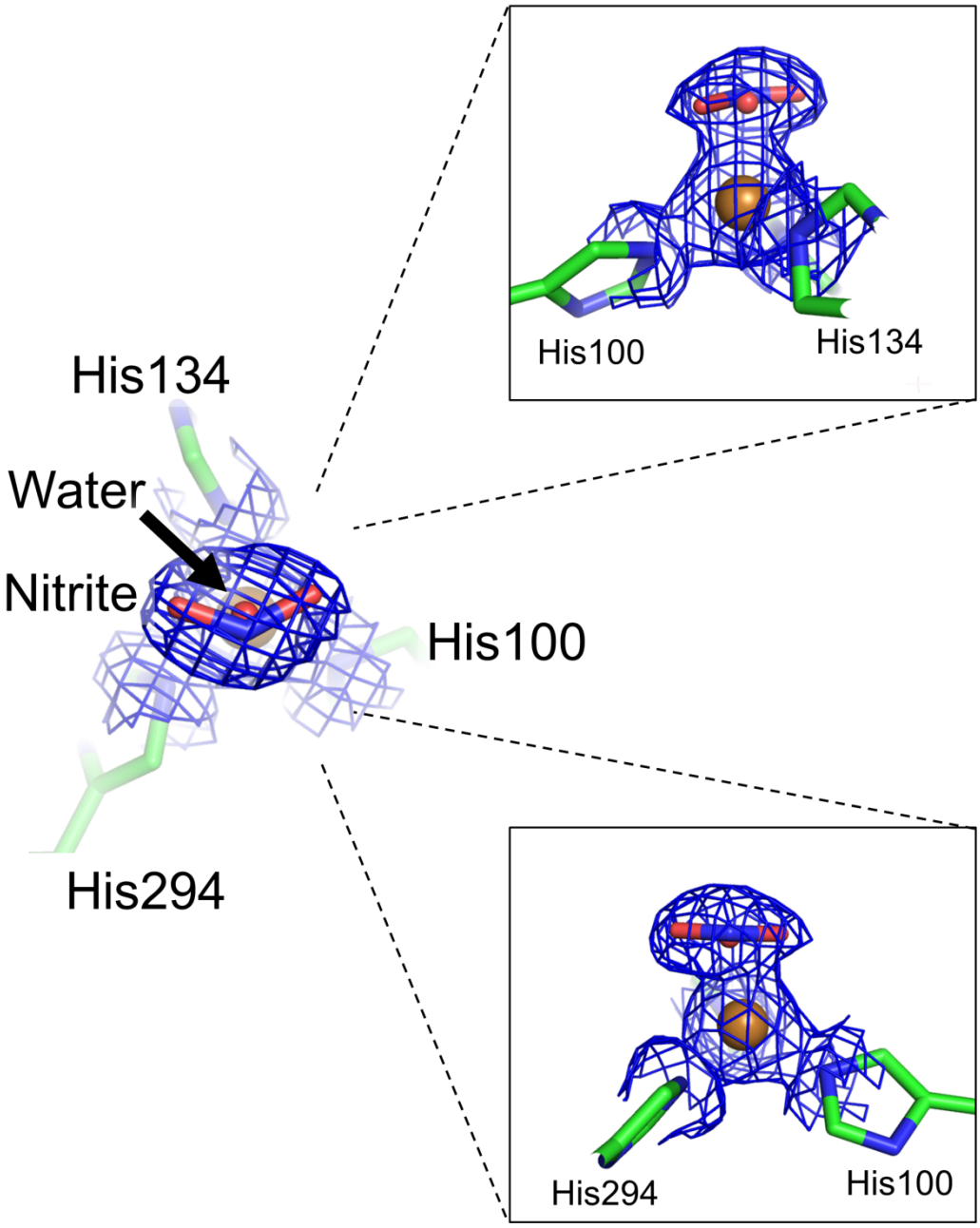

Figure S2. The detailed structure of the T2Cu site in the C135A-NO_2^{high} structure. Blue meshes show the $2F_o$-F_c map contoured at 1.0 σ. Carbon, oxygen, and nitrogen atoms are colored green, red, and blue, respectively. The T2Cu atom and the ligand water molecule are shown as brown and small red spheres, respectively. The distances between the T2Cu atom and ligands are summarized in Table S1.

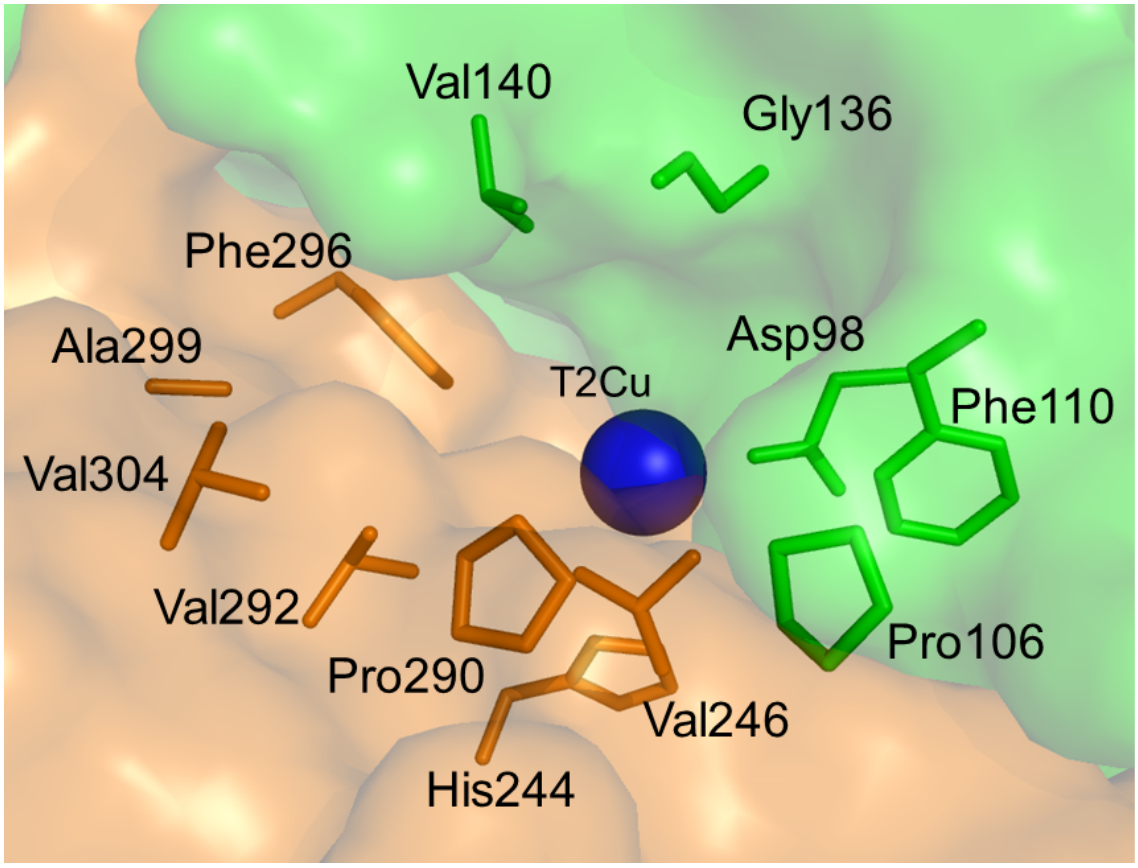

Figure S3. Residues located on the substrate pocket. Different monomers are colored green and orange, respectively. The T2Cu atom is shown as a blue sphere. The distances from the T2Cu atom to the atoms of the shown residues are summarized in Table S2.

Table S1. Copper site geometries

Parameter	C135A-NO_2^{high}	C135A-NO_2^{low} (3WKP)
I. Type 1 Cu-Ligand Distances (Å)		
T1Cu-H95$N^{\delta 1}$	2.08	2.14
T1Cu-H143$N^{\delta 1}$	2.01	1.96
T1Cu-M148S^{δ}	2.13	2.07
II. Type 2 Cu-Ligand Distances (Å)		
T2Cu-H100$N^{\varepsilon 2}$	2.07	1.96
T2Cu-H134$N^{\varepsilon 2}$	2.00	1.95
T2Cu-H294$N^{\varepsilon 2}$	1.98	2.01
T2Cu-Wat	2.02	n/a
III. Type 2 Cu-Nitrite Distances (Å)		
T2Cu-$O_{proximal}$	2.13	1.97
T2Cu-N	2.21	2.85
T2Cu-O_{distal}	2.52	3.41

Table S2. The distances from the T2Cu atom (Å)

	(a) 320K	(b) 100K	(a) - (b)
Asp98 $O^{\delta1}$	4.70	4.72	-0.02
Asp98 $O^{\delta2}$	3.99	3.66	0.33
Pro106 C^{β}	9.8	9.65	0.15
Phe110 C^{β}	9.44	9.28	0.16
Phe110 $C^{\delta2}$	7.85	7.66	0.19
Phe110 $C^{\varepsilon2}$	7.52	7.39	0.13
Gly136 C^{α}	7.60	7.46	0.14
Val140 $C^{\gamma1}$	6.27	6.26	0.01
Val140 $C^{\gamma2}$	7.68	7.59	0.09
His244 C^{δ}	4.01	3.94	0.07
His244 $N^{\varepsilon2}$	3.99	3.96	0.03
Val246 $C^{\gamma1}$	6.06	6.03	0.03
Val246 $C^{\gamma2}$	5.49	5.24	0.25
Pro290 C^{β}	8.94	8.56	0.38
Pro290 C^{δ}	9.51	9.25	0.26
Pro290 C^{γ}	9.29	9.02	0.27
Val292 $C^{\gamma1}$	7.21	6.97	0.24
Val292 $C^{\gamma2}$	5.78	5.66	0.12
Ala299 C^{β}	9.43	9.25	0.18
Phe296 $C^{\varepsilon1}$	6.75	6.65	0.10
Phe296 $C^{\varepsilon2}$	4.80	4.80	0
Phe296 C^{ζ}	5.44	5.26	0.18
Val304 $C^{\gamma1}$	11.00	10.73	0.27