Supporting Information

Residual electron density at T2Cu site of SR1 and SFX structure

In the SR1 data, ellipsoidal residual electron density was observed around WatC with full occupancy (Fig. S4a). This indicates the presence of a diatomic molecule, and it may be an oxygen species because *Gt*NiR can reduce dioxygen and spectroscopic data implied that a Cu-O_2 complex is generated at the T2Cu site by X-ray irradiation (ref. 30). However, here we refined the model without a dioxygen model, because more research is needed to support this hypothesis. In other SR data, the electron density of WatD disappeared and there was no residual electron density around WatC (Fig. 1a). The residual electron density at the position of WatC was observed also in the SFX data (Fig. S4b). However, the electron density was weaker and the shape was more spherical than that in the SR1 data, thus ruling out the presence of the diatomic molecule in the SFX structure. Our recent study showed that an unknown diatomic molecule binds to T2Cu during synchrotron data collection (ref. 30). The present microfocus SRX study showed that the binding of a diatomic molecule and the dissociation of WatD occurred on T2Cu during X-ray irradiation (Fig 1a, S4). The binding of the diatomic molecule may expel WatD from the catalytic site. Considering the fact that SR2–SR7 data did not show the residual electron density around WatC, it is reasonable to guess that the diatomic ligand was gradually decomposed by further X-ray irradiation.

Data collection and refinement for 3X1E

A SRX diffraction dataset for PDB code ID 3X1E was collected with a single position method at 100 K on beamline BL44XU at SPring-8, using an MX-300HE detector (Rayonics LLC, IL, USA). The beam size was 100 μm (H) × 100 μm (W). X-ray beams were attenuated by a 1.3 mm Al attenuator. Oscillation angle and exposure time per image were 0.5 degree and 1.0 second, respectively. A total of 720 diffraction images were collected from the one position of a single crystal. Estimated total X-ray dose of the exposed region was ~ 0.43 MGy. The

dataset was reduced, integrated and scaled with the HKL-2000 package. Phase was determined by molecular replacement using the program Molrep from the CCP4 suite. A monomeric subunit of *Gt*NIR (PDB code 4ZK8), which included T1Cu and T2Cu atoms, was used as the search model. The resulting models were refined with Refmac5. Manual model building was carried out using Coot through the refinement process. Water molecules were added to the model using the automated water-searching program built into Coot. Anisotropic displacement parameters were introduced after water molecules were built into the models because resolutions of data were high enough to do so. The final models were checked for stereochemical quality using MolProbity. Data-collection and refinement statistics are summarized in Table SI.

The T2Cu site structure of 3X1E

The distance between T2Cu and the ligand plane in the 3X1E structure was 0.687 Å, which is longer than that in the SR3 structure and shorter than that in the SR2 structure. The electron density above the T2Cu atom in the 3X1E structure was ellipsoidal, although two water molecules were assigned to it without positive or negative residual electron density peaks (Fig. S10). These observations are reasonable because the 3X1E structure is thought to represent the state between the SR1 and SR2 data only based on X-ray dose, although there is no linear relationship between 3X1E and other cryo SR data because of the difference in data collection methods. The X-ray dose for the 3X1E data was higher than those for the 3WNI and 3WNJ data, which were reported previously and showed an obvious diatomic ligand on T2Cu (ref. 30). An anomalous peak showed that a copper ion with 0.3 occupancies bound to His255 (Fig. S6), as mentioned in the main text. The conformation of His255 in the 3X1E structure was the rotated form as in other SRX structures (Fig. S11). In summary, the 3X1E data, which was previously determined independently of the present study, is compatible with our present data.

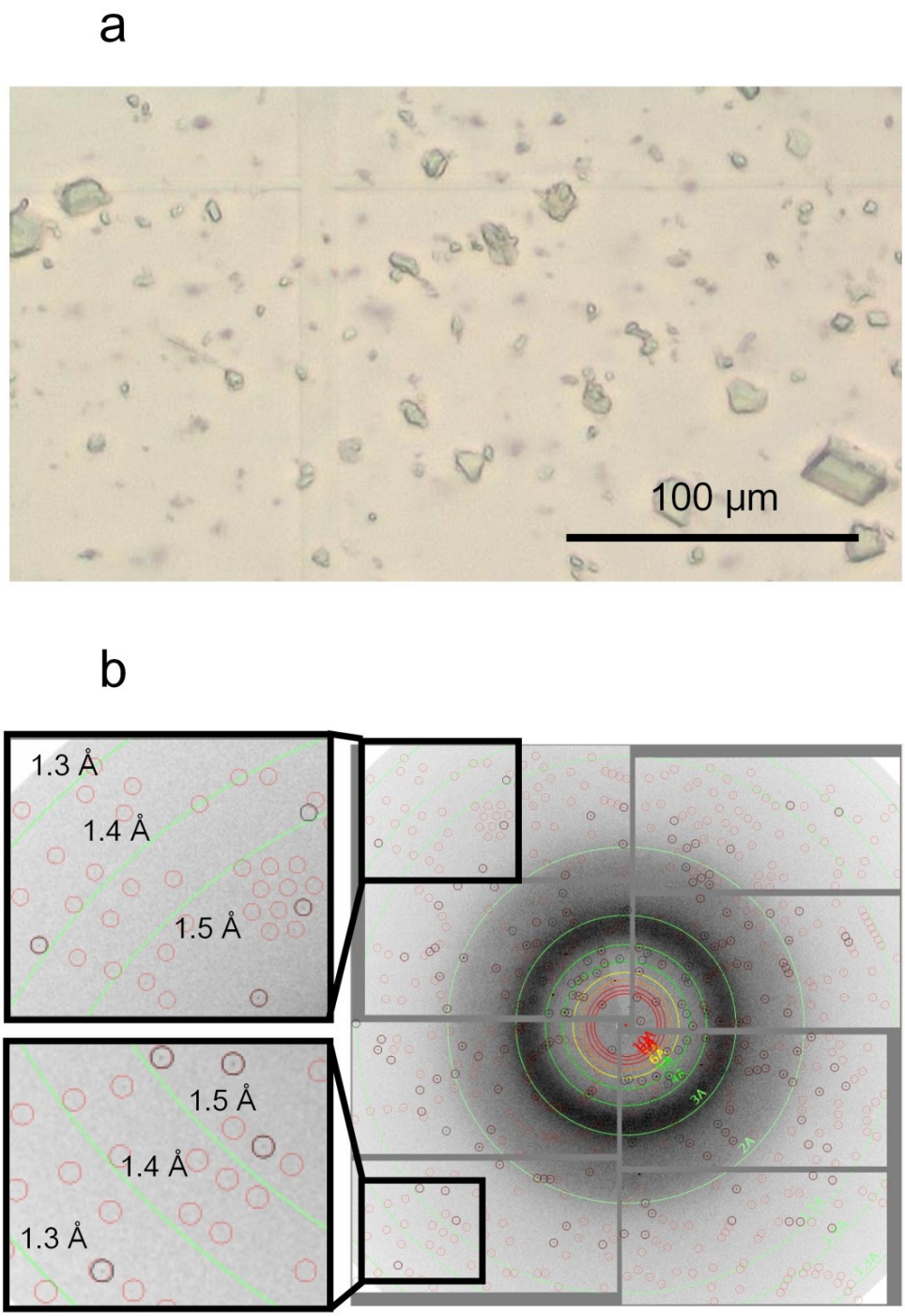

Fig. S1. Serial femtosecond experiment of *Gt*NiR. (a) Microcrystals of *Gt*NiR in the oxidized form. The sizes of the microcrystals were distributed between 2 and 30 µm. (b) Diffraction pattern of a *Gt*NiR microcrystal. The spots circled in black were used for indexing, and the red circles show the expected positions of the diffraction spots.

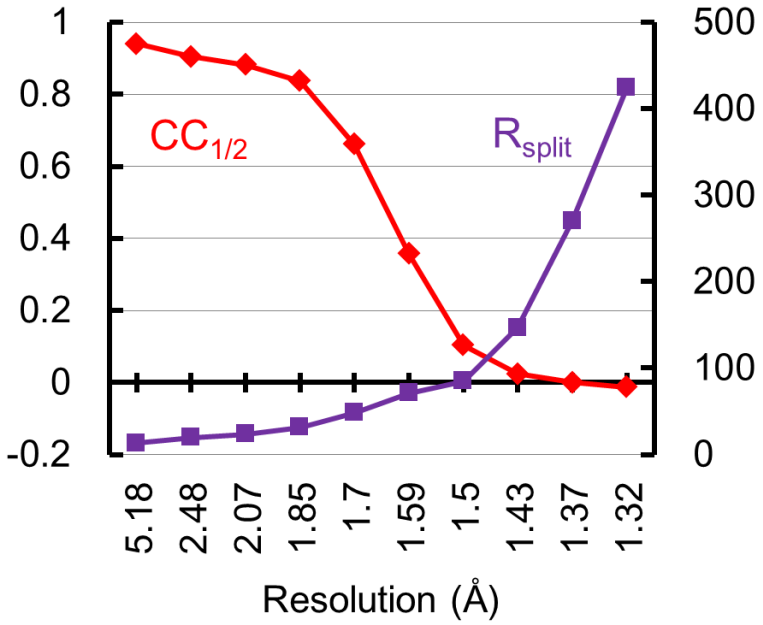

Fig. S2. $CC_{1/2}$ and R_{split} of the *Gt*NiR SFX data.

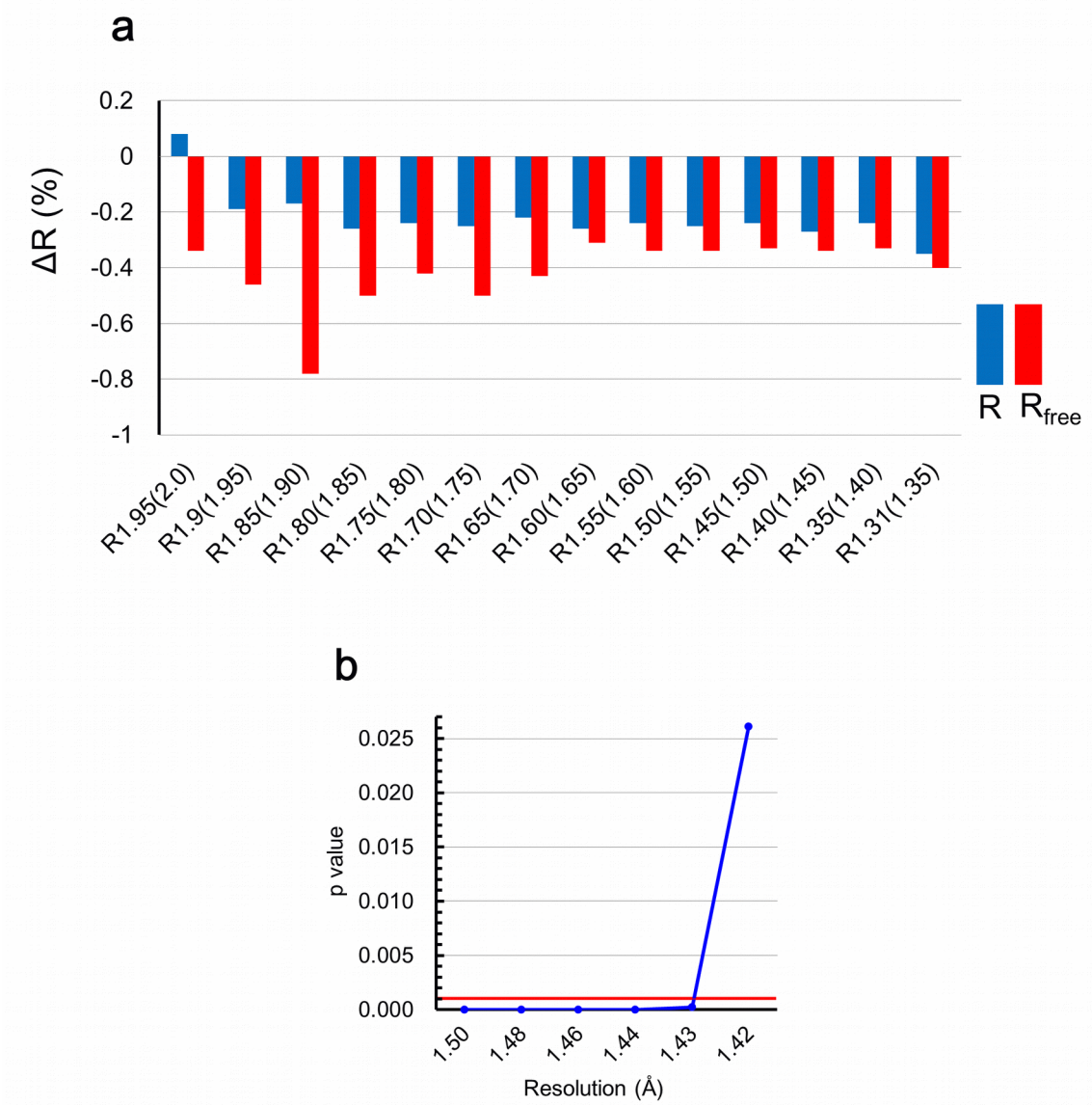

Fig. S3. "Nominal resolution" of SFX structure. (a) Results of paired refinement. R*x* (*y*) means that *R* values calculated at *x* Å resolution with the model refined at *y* Å resolution were compared with those using the model refined at *x* Å resolution. (b) *P* values plotted against resolution. *P* values were calculated using the number of observations and $CC_{1/2}$ at each resolution shell. The red line shows the significance level.

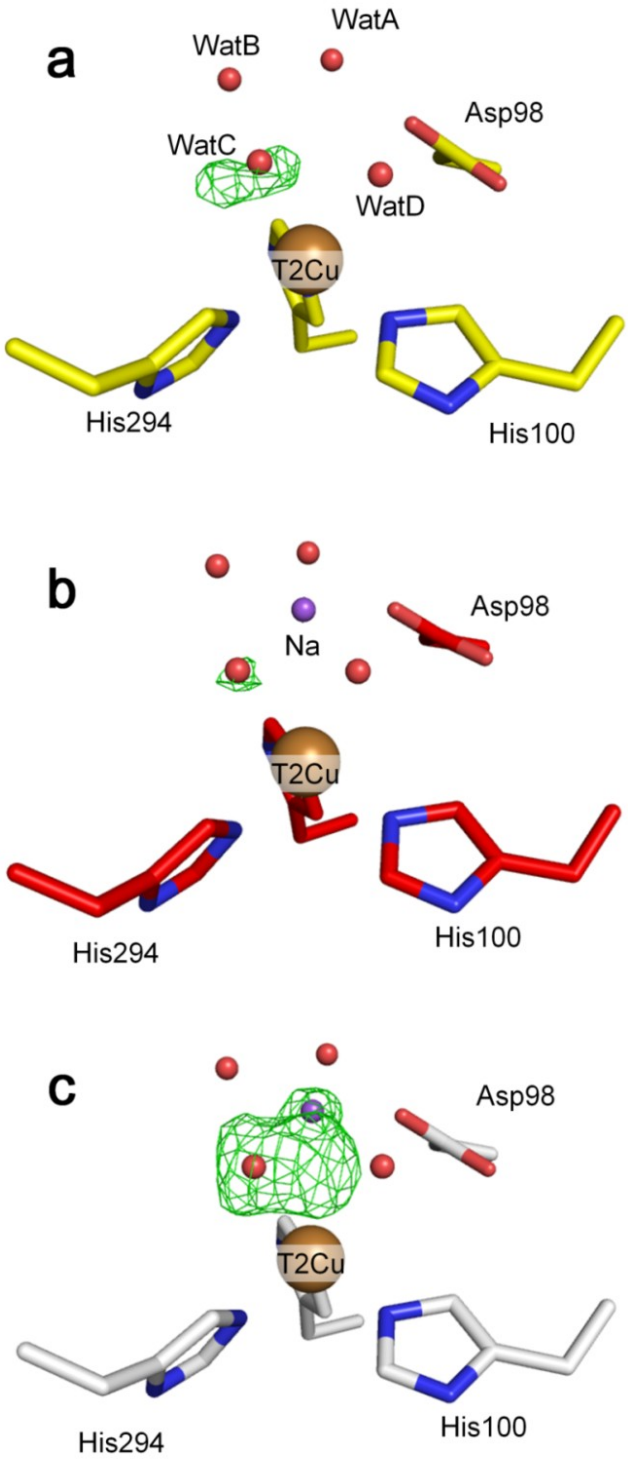

Fig. S4. Comparison of residual positive electron density around WatC for the (a) SR1, (b) SFX, and (c) RT SRX structures. F_o–F_c maps contoured at 4.5 σ are shown as green meshes. Water molecules and Na^+ are shown as red and purple spheres, respectively.

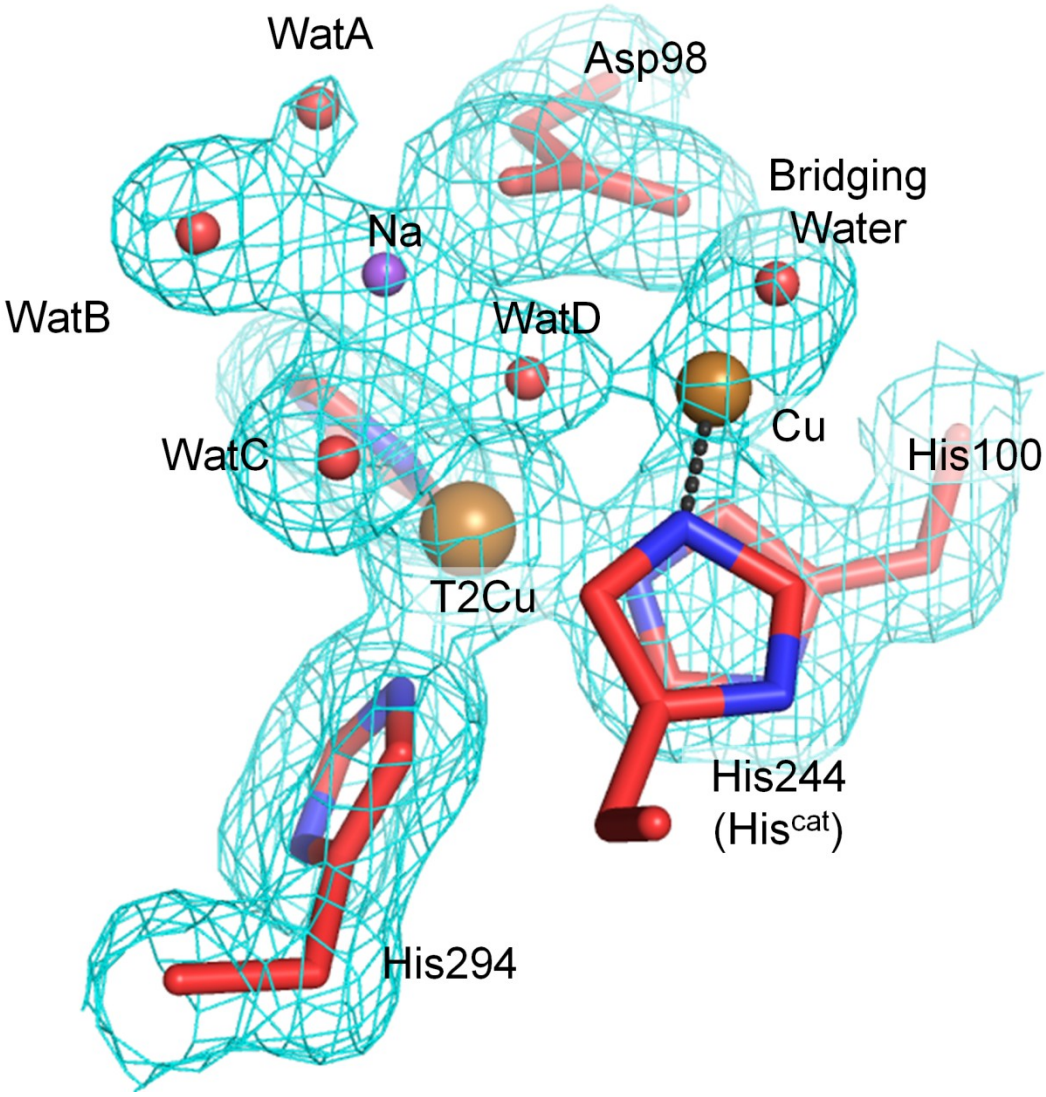

Fig. S5. The T2Cu site in the SFX structure. The $2F_o–F_c$ maps contoured at 1.0 σ are shown as cyan meshes. The coordination bond between His^{cat} and a Cu atom is shown by dashed line.

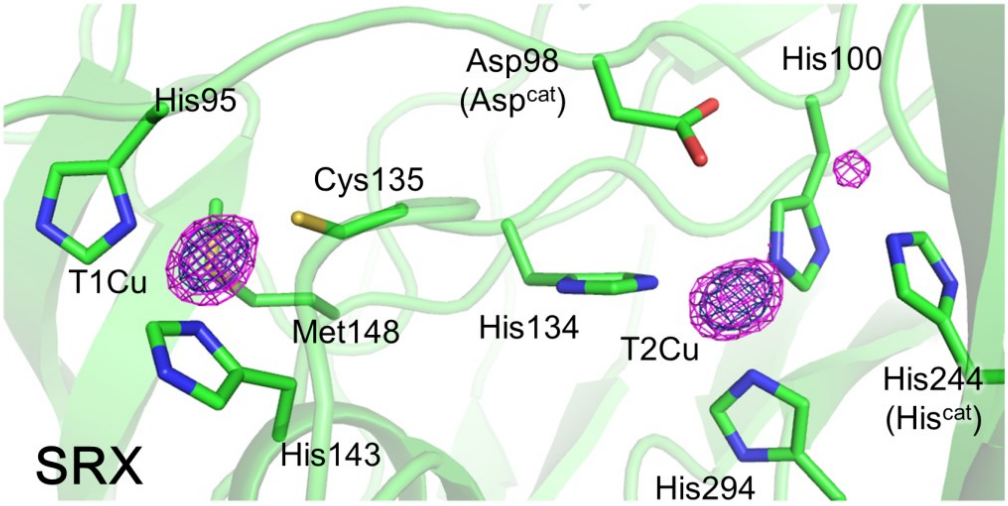

Fig. S6. Copper binding sites in the previously determined cryogenic SRX structure (PDB code ID 3X1E). The anomalous Fourier maps are contoured at 4.0 (magenta) and 12 σ (dark blue).

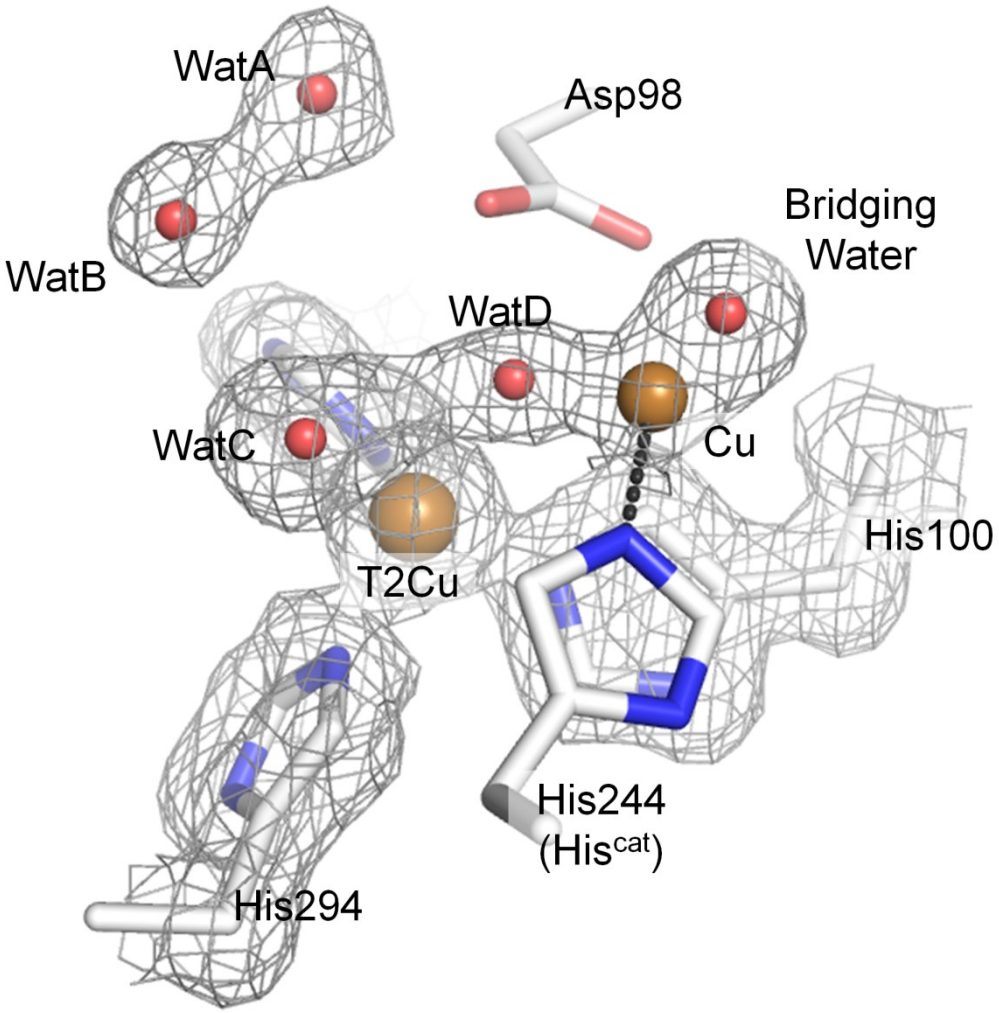

Fig. S7. Hydration structure of the T2Cu site in the RT SRX structure. The $2F_o–F_c$ maps contoured at 1.0 σ are shown as gray meshes. The coordination bond between His^{cat} and a putative Cu atom is shown by dashed line. We regarded the Cu atom and bridging water were alternative structures. The occupancies of the Cu atom and bridging water were 20% and 80%, respectively.

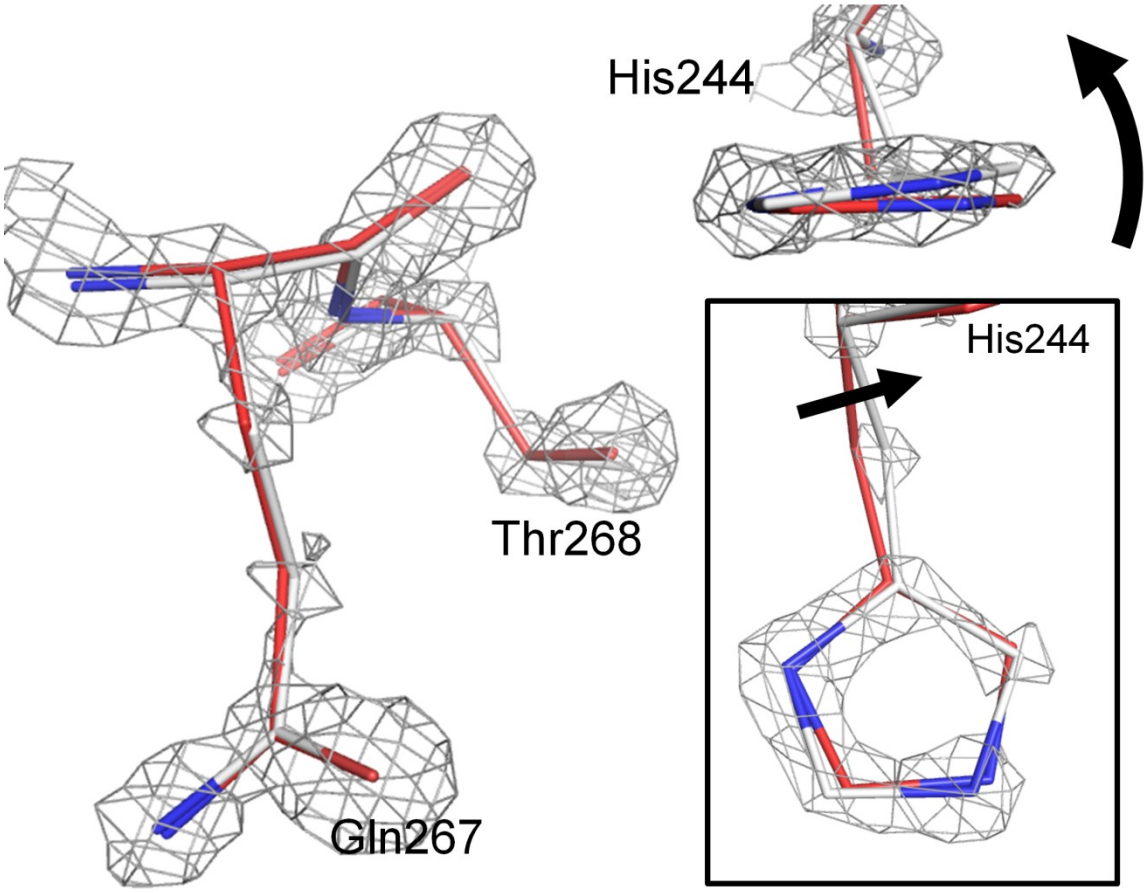

Fig. S8. Electron density maps (contoured at 4.5 σ) around His^{cat} in the RT SRX structure (white). The His^{cat} model in the SFX structure (red) cannot be fit to the RT SRX data.

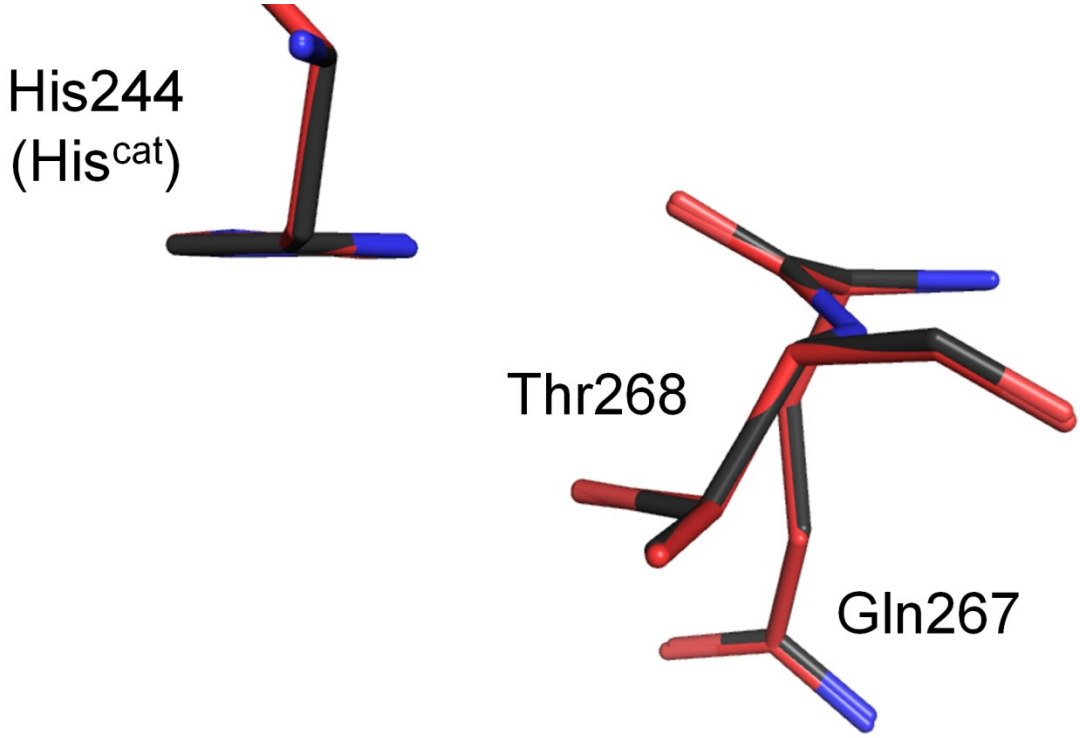

Fig. S9. Comparison of His^{cat} of the SFX structure refined against data at 1.43 Å (red) and 1.65 Å (black) resolution.

Fig. S10. The T2Cu site in the 3X1E structure. The $2F_o–F_c$ maps contoured at 1.5 σ are shown as gray meshes.

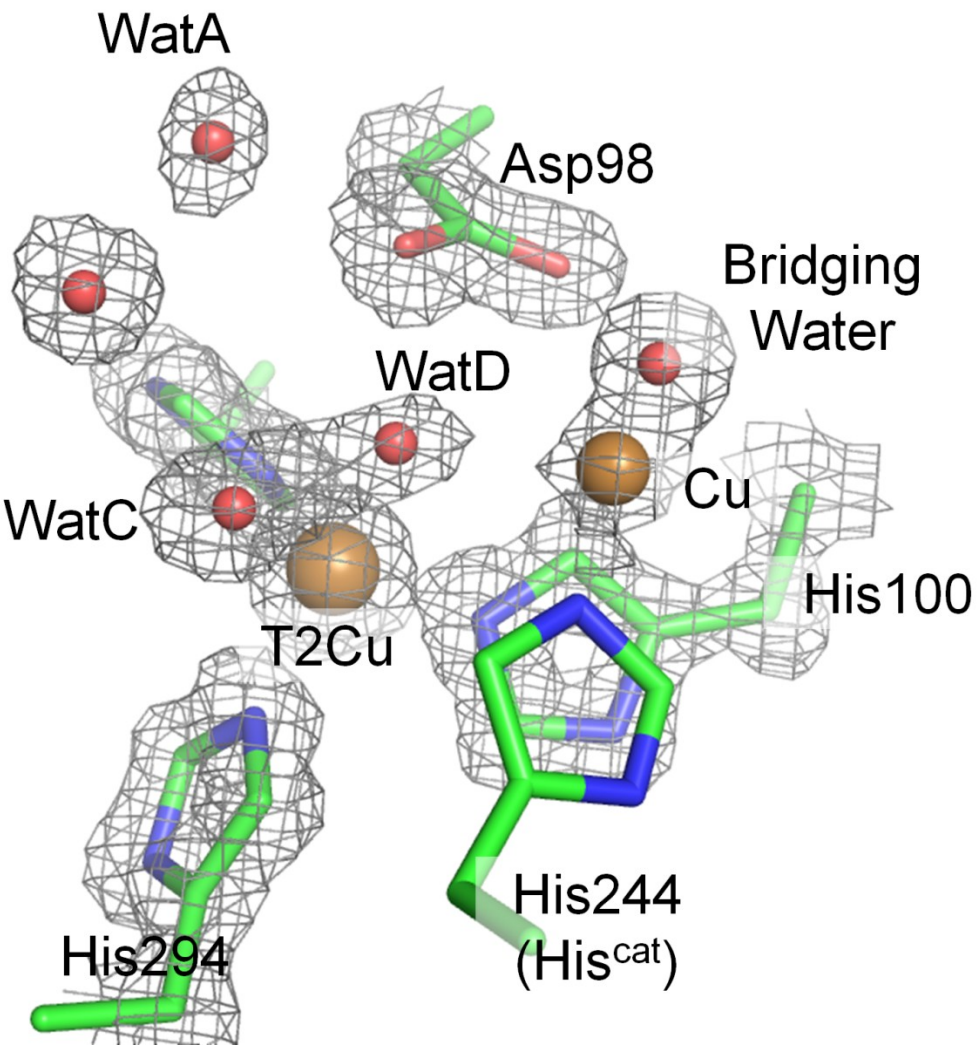

Fig. S11. Comparison of the His^{cat} conformation between the 3X1E structure (green) and the SFX structure (red).

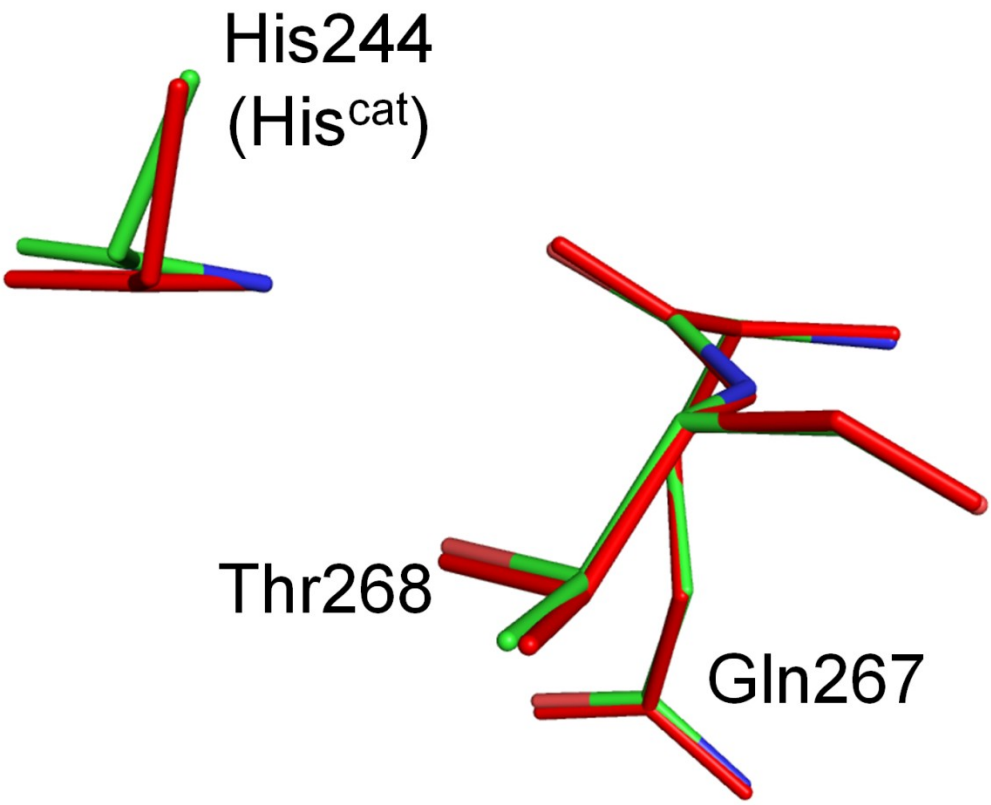

Table SI | Data collection with a single position method and refinement statistics

Data collection at BL38B1 of SPring-8 (Wavelength 0.9000 Å)	
Space group	*R*3
Unit cell *a* = *b*, *c* (Å)	114.9, 84.2
Resolution range (Å)	50.0-1.25 (1.29-1.25)
R_{sym} (%)	11.4 (37.0)
Completeness (%)	99.9 (99.9)
Unique reflections	114,829 (11,494)

<I/σ (I)>	16.5 (3.6)
Redundancy	7.5 (5.7)
Refinement	
Resolution (Å)	50.0-1.25
R_{work} (%)/ R_{free} (%)	14.1/17.0
PDB code	3X1E