

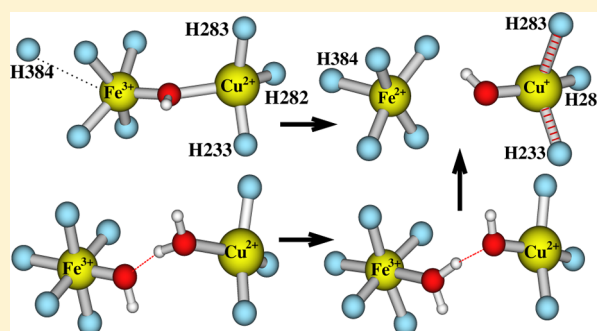
Broken Symmetry DFT Calculations/Analysis for Oxidized and Reduced Dinuclear Center in Cytochrome *c* Oxidase: Relating Structures, Protonation States, Energies, and Mössbauer Properties in *ba*₃ *Thermus thermophilus*

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S Supporting Information

ABSTRACT: The Fe_a³⁺...Cu_B²⁺ dinuclear center (DNC) structure of the as-isolated oxidized *ba*₃ cytochrome *c* oxidase (CcO) from *Thermus thermophilus* (*Tt*) is still not fully understood. When the proteins are initially crystallized in the oxidized state, they typically become radiolytically reduced through X-ray irradiation. Several X-ray crystal structures of reduced *ba*₃ CcO from *Tt* are available. However, depending on whether the crystals were prepared in a lipidic cubic phase environment or in detergent micelles, and whether the CcO's were chemically or radiolytically reduced, the X-ray diffraction analysis of the crystals showed different Fe_a^{3+/2+}...Cu_B^{2+/1+} DNC structures. On the other hand, Mössbauer spectroscopic experiments on reduced and oxidized *ba*₃ CcOs from *Tt* (Zimmermann et al., *Proc. Natl. Acad. Sci. USA* 1988, 85, 5779–5783) revealed multiple ⁵⁷Fe_a^{3+/2+} and ⁵⁷Fe_a³⁺ components. Moreover, one of the ⁵⁷Fe_a³⁺ components observed at 4.2 K transformed from a proposed “low-spin” state to a different high-spin species when the temperature was increased above 190 K, whereas the other high-spin ⁵⁷Fe_a³⁺ component remained unchanged. In the current Article, in order to understand the heterogeneities of the DNC in both Mössbauer spectra and X-ray crystal structures, the spin crossover of one of the ⁵⁷Fe_a³⁺ components, and how the coordination and spin states of the Fe_a^{3+/2+} and Cu_B^{2+/1+} sites relate to the heterogeneity of the DNC structures, we have applied density functional OLYP calculations to the DNC clusters established based on the different X-ray crystal structures of *ba*₃ CcO from *Tt*. As a result, specific oxidized and reduced DNC structures related to the observed Mössbauer spectra and to spectral changes with temperature have been proposed. Our calculations also show that, in certain intermediate states, the His233 and His283 ligand side chains may dissociate from the Cu_B²⁺ site, and they may become potential proton loading sites during the catalytic cycle.



1. INTRODUCTION

Located in the inner mitochondrial membrane in eukaryotes or alternatively in the periplasmic membrane in aerobic bacteria, cytochrome *c* oxidase (CcO) is the terminal enzyme in the respiratory chain that reduces O₂ to H₂O and pumps protons across the membrane.^{1–4} The catalytic site of CcO, which binds and reduces O₂, contains a heme A (Fe_a) and a Cu ion (Cu_B), forming the dinuclear (or binuclear) center (DNC or BNC). Cu_B is in the proximity (~5 Å) of Fe_a.^{5–15} In addition, CcO protein contains another two redox centers: a homodinuclear Cu dimer (Cu_A), which serves as the initial site of electron entry to CcO,^{16,17} and another heme, which is heme A (Fe_a) in the case of the *aa*₃ type of CcO or heme B (Fe_b) in the *ba*₃ type.

Zimmermann et al. reported Mössbauer spectra of the as-isolated oxidized and reduced *ba*₃ CcO from *Thermus thermophilus* (*Tt*).¹⁸ In the reduced state, two high-spin ferrous components (at 4.2 K) were observed for the Fe_a site with the majority component (86%) having an isomer shift $\delta = 0.95$ mm

s⁻¹ and quadrupole splitting $\Delta E_Q = 1.43$ mm s⁻¹ and a minority species (14%) with $\delta = 0.92$ mm s⁻¹ and $\Delta E_Q = 2.34$ mm s⁻¹.¹⁸ The oxidized state was even more complicated. When the temperature was between 4.2 and 190 K, the Mössbauer spectra revealed two components attributable to ⁵⁷Fe_a³⁺: a quadrupole doublet with splitting $\Delta E_Q = 0.71$ mm s⁻¹ and $\delta = 0.41$ mm s⁻¹, which was proposed to be a high-spin (HS) ferric heme and accounts for ~46% of ⁵⁷Fe_a³⁺, and a second doublet (54%) with $\Delta E_Q = 2.24$ mm s⁻¹ and $\delta = 0.29$ mm s⁻¹, which was proposed to be a low-spin (LS) ferric heme.¹⁸ When the temperature was >190 K, the “low-spin” ⁵⁷Fe_a³⁺ component was reported to undergo a transition to “high-spin” that was essentially complete at 245 K. However, the new “high-spin” compound yielded $\Delta E_Q \approx 1$ mm s⁻¹, and the two distinct “high-spin” species ($\Delta E_Q = 0.7$ and ~1 mm s⁻¹) coexisted at

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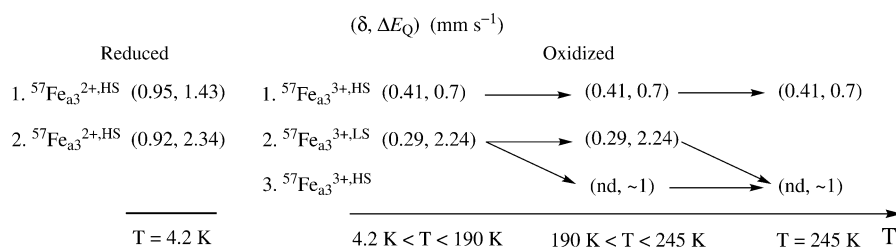


Figure 1. Data summarized from ref 18. The observed ⁵⁷Fe_{a3}²⁺ and ⁵⁷Fe_{a3}³⁺ Mössbauer isomer shifts (δ, mm s⁻¹) and quadrupole splittings (ΔE_Q, mm s⁻¹) for *ba*₃ CcO from *Tt* and the spectra variation with temperature (*T*).¹⁸ Note that only ΔE_Q ≈ 1 mm s⁻¹ was given in ref 18. for the third ⁵⁷Fe_{a3}³⁺ species (HS state), which was transformed from the second ⁵⁷Fe_{a3}³⁺ species (supposed to be in LS state) as the temperature increased to >190 K, and the transition was complete at 245 K. “nd” stands for “not determined from experiment”.

Table 1. Resolutions (Å) and the Main DNC Geometrical Parameters (Å) of the Reduced X-ray Crystal Structures of *ba*₃ CcO from *Tt* and the Experimental ⁵⁷Fe_{a3}²⁺ Mössbauer Isomer Shifts (δ, mm s⁻¹) and Quadrupole Splittings (ΔE_Q, mm s⁻¹) for the Reduced DNC in Different Cytochrome Oxidase Proteins

crystal structure	resolution	Fe–N (H384)	Fe–O/Fe–O1	O1–O2	Cu–O/Cu–O2	Fe...Cu	Cu–N (H233)	Cu–N (H282)	Cu–N (H283)	O...O (Y237)	ref
1EHK	2.4	3.31	2.26		2.31	4.40	2.10	2.22	2.13	2.52	10
1XME	2.3	2.48	2.44		2.07	4.39	1.96	1.96	1.97	2.67	11
3EH4	2.9	2.39	3.08		2.20	4.73	1.96	2.03	1.97	2.47	12
3EH3	3.1	2.39				5.06	1.92	2.05	2.06	2.91	12
3EH5	2.8	2.10				5.04	2.16	1.94	2.18	2.61	12
3S8F	1.8	2.09	2.25	1.54	2.29	4.87	1.94	1.99	1.91	2.56	15
3S8G	1.8	2.22	2.39	1.52	2.25	4.92	1.95	2.03	1.98	2.66	15
cytochrome oxidase						δ	ΔE _Q			ref	
<i>ba</i> ₃ from <i>T. thermophilus</i>			1 st :			0.95	1.43			18	
			2 nd :			0.92	2.34			18	
<i>c</i> ₁ <i>aa</i> ₃ from <i>T. thermophilus</i>						0.96 ± 0.03	2.06 ± 0.05			54	
<i>aa</i> ₃ from bovine heart						0.93 ± 0.06	1.85 ± 0.10			55	

245 K.¹⁸ For easier comparison, we assembled the ⁵⁷Fe_{a3}^{3+/2+} Mössbauer experimental data of *ba*₃ CcO from *Tt* in Figure 1.

Currently, detailed DNC structures and comparative energies in the as-isolated, fully oxidized, and fully reduced states of CcO are still not clear.^{1–3,6,7,10–15,19–22} Although crystals of the fully oxidized as-isolated CcOs were well prepared, when exposed to X-ray irradiation, the metal sites are easily reduced.^{12,13,15} Several reduced X-ray crystal structures of *ba*₃ CcO from *Tt* have been obtained.^{10–12,15} However, depending on whether the crystals were prepared in a lipidic cubic phase environment or in detergent micelles, and whether the CcOs were chemically or radiolytically reduced, X-ray diffraction analyses of the crystals obtained from different protocols show different DNC structures.^{12,15} The PDB entries of these *Tt* X-ray structures are 1EHK,¹⁰ 1XME,¹¹ 3EH3,¹² 3EH4,¹² 3EH5,¹² 3S8F,¹⁵ and 3S8G,¹⁵ in which both 3S8F (wild-type) and 3S8G (A120F mutant) were obtained in a lipidic cubic phase environment and others in detergent micelles. 1EHK, 1XME, 3EH4, 3S8F, and 3S8G are radiation-reduced crystal structures. 3EH3 and 3EH5 represent chemically reduced X-ray crystal structures. However, 3EH3 was obtained by chemically reducing the crystals of the oxidized, as-isolated protein, and 3EH5 was obtained by a path in which the oxidized as-isolated proteins were reduced with dithionite in an anaerobic environment prior to being crystallized and frozen in liquid nitrogen.¹²

The resolutions and the main geometric parameters in the DNCs from these X-ray structures are compared in Table 1. As an example, the DNC of 1EHK is shown in Figure 2. In the observed structures, the Fe_{a3} site has one axial histidine ligand (His384), and the Cu_B site has three histidine ligands: His233, His282, and His283. His233 covalently links with Tyr237, a

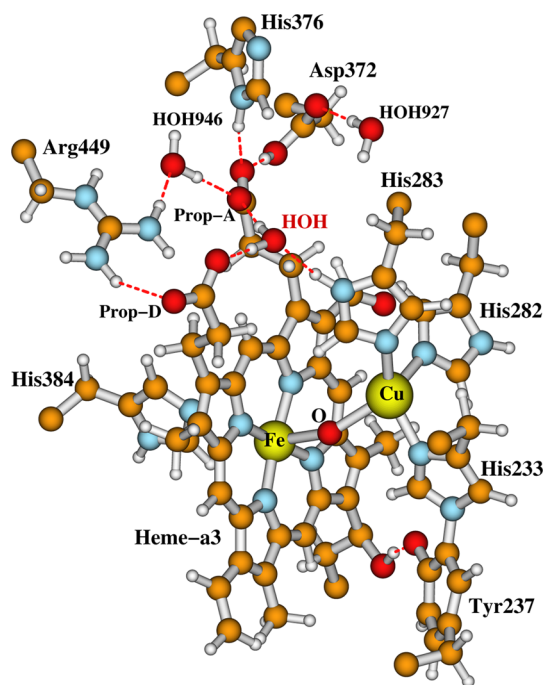


Figure 2. Fe–Cu DNC of the X-ray radiolytically reduced crystal structure 1EHK (2.4 Å resolution),¹⁰ which was obtained for the native *Tt* *ba*₃ CcO solubilized in detergent micelles. The protonation state of the Tyr237 side chain and the oxygen species between the Fe and Cu sites in the DNC are not known from the X-ray crystal structure alone. A water molecule (“HOH” in red) was added based on other X-ray structures.

linkage that is common to all CcOs but is otherwise unknown in metalloenzymes. These structures obviously vary in the $\text{Fe}_{\text{a}3}\cdots\text{Cu}_{\text{B}}$ distances, in what resides in the space between the $\text{Fe}_{\text{a}3}$ and Cu_{B} sites, and in the $\text{Fe}_{\text{a}3}$ –ligand distances. Because only the DNC of CcO is studied in the current Article, for simplicity, the $\text{Fe}_{\text{a}3}$ and Cu_{B} will be noted as Fe and Cu hereafter. An open $\text{Fe}^{2+}\cdots\text{Cu}^+$ (with no atoms between Fe^{2+} and Cu^+) DNC structure was obtained in 3EH3 and 3EH5.¹² A bridging oxygen species was observed between the Fe^{2+} and Cu^+ sites in 1EHK (Figure 2),¹⁰ where the His384 side chain was found to be far from the Fe atom with an $\text{Fe}\cdots\text{N}(\text{His384})$ distance of 3.31 Å. In 1XME¹¹ and 3EH4,¹² an oxygen species is also found in the space between Fe^{2+} and Cu^+ , but it appears bound only to the Cu^+ site, and the axial His384 side chain ligand is much closer to the Fe^{2+} site. Although the bound solvent was called “the water molecule residing between Fe and Cu” in later publications,¹² it is not evident whether this oxygen species in the DNC’s of 1EHK, 1XME, and 3EH4 is a water molecule (H_2O) or a hydroxide anion (OH^-).¹⁰

Further, a peroxide-type dioxygen species bridging the $\text{Fe}^{2+}\cdots\text{Cu}^+$ DNC was observed in both 3S8F and 3S8G.¹⁵ To evaluate whether this bridging dioxygen species is O_2^{2-} , O_2^- , HO_2^- , or H_2O_2 , in ref 23 we performed a series of density functional theory (DFT) calculations (using the OLYP^{24,25} functional) on the quantum cluster models based on 3S8G DNC and have proposed that the X-ray crystal structure 3S8G is likely to represent the superposition of the $\text{Fe}^{2+}\cdots(\text{HO}_2^-)\cdots\text{Cu}^+$ DNC’s in different states (Fe^{2+} in low-spin, intermediate-spin, or high-spin) with the majority species having the proton of the HO_2^- residing on the oxygen atom that is closer to the Fe^{2+} site in the $\text{Fe}^{2+}\cdots(\text{HO}-\text{O})^- \cdots \text{Cu}^+$ conformation.²³

Clearly, the X-ray crystal structures and the Mössbauer experiments show structural heterogeneity within the DNC of ba_3 CcO from *Tt*. Relating the detailed structures of the oxidized and reduced DNC’s is an essential step in analyzing the path(s) by which CcO reduces O_2 to H_2O and in finding what paths effectively couple O_2 reduction chemistry to proton pumping across the membrane. In the current Article, we continue our DFT calculations on DNC models starting from different X-ray crystal structures to address the following questions: (1) Is the bridging/Cu-binding oxygen species observed in the DNC’s of 1EHK, 1XME, and 3EH4 an H_2O molecule or an OH^- anion? (2) How do the axial His384 and nearby solvent ligands influence the $^{57}\text{Fe}^{2+}$ Mössbauer properties? (3) What are the DNC structures representing the two $^{57}\text{Fe}_{\text{a}3}^{2+}$ components observed in Mössbauer experiments, and what do the DFT calculations predict for Fe^{2+} spin states and their relative energies in the DNC? (4) Will the geometry optimized 3S8G- $\text{Fe}^{2+}\cdots(\text{HO}_2^-)\cdots\text{Cu}^+$ DNC structures obtained in ref 23 reproduce the Mössbauer properties of any of the two $^{57}\text{Fe}_{\text{a}3}^{2+}$ components? (5) What are the feasible DNC structures of the three $^{57}\text{Fe}_{\text{a}3}^{3+}$ components observed in the Mössbauer experiments? (6) How does spin-crossover link different states and structures in the transition of $\text{Fe}^{3+}(\text{state 2}) \rightarrow \text{Fe}^{3+}(\text{state 3})$ when the temperature is increased from 190 to 245 K (Figure 1)? (7) What do the comparative structures and energies tell us about potential reaction pathways and protonation states (especially for Tyr237) in this early part of the catalytic cycle?

2. MODELS AND CALCULATIONAL METHODS

Several water molecules are normally found above the DNC that have H-bonding interactions with the two propionate

carboxylate groups (prop-A and prop-D) and the side chains of Arg449 and Asp372. In many CcO X-ray crystal structures,^{6,11–15} a water molecule (“HOH” in red) was observed in a position to have hydrogen bonding interactions with both prop-A and prop-D and also with the His283 side chain (Figure 2). However, this water molecule was not present in the 1EHK.pdb file. We are not sure if this water molecule missed detection in 1EHK or if it is displaced (a water molecule HOH941 above this position was observed in 1EHK). To be consistent with the calculations for the DNC clusters of other X-ray crystal structures, we also placed a water molecule in this position (HOH) for the 1EHK DNC calculations.

The $\text{O}\cdots\text{O}$ distance between the carboxylate groups of Prop-A and Asp372 side chain in 1EHK is only 2.49 Å. Therefore, the Asp372 side chain is likely in the neutral protonated state and has a H-bonding interaction with the anionic carboxylate group of prop-A.¹⁷ Prop-A also H-bonds to $\text{HN}_{\delta 1}$ of His376 side chain with an $\text{O}\cdots\text{N}_{\delta 1}$ distance of 2.90 Å. $\text{N}_{\epsilon 2}$ (near the top of Figure 2) of the His376 side chain is within a strong H-bonding distance (2.68 Å) with one of the oxygen atoms of the carboxylate group of Glu126B side chain (not shown in Figure 2). Therefore, the His376 side chain is likely in the neutral state, and its $\text{N}_{\epsilon 2}$ site H-bonds to the neutral protonated Glu126B side chain.

The initial geometries of our DNC models are taken from the different X-ray crystal structures. The size of the model clusters taken from 1EHK, 3EH3, 3EH4, and 3EH5 is the same as shown in Figure 2 (but with different species between the Fe and Cu sites). No water molecules were reported in 3EH3.pdb, we therefore manually added the three water molecules above the DNC in that structure according to their relative positions in 3EH5.pdb. The ending $\text{C}_{\alpha}/\text{C}_{\gamma}/\text{C}_{\text{geranyl-side chain}}$ atoms shown in Figure 2 were each replaced by a link hydrogen (H_{link}) atom along the original C–C direction with the C– H_{link} distance of 1.09 Å. The positions of the H_{link} atoms that replace the $\text{C}_{\alpha}/\text{C}_{\gamma}$ atoms were fixed during all geometry optimization calculations.

Additional crystallographic water molecules near the DNC were observed in 3S8G and 3S8F compared to the other structures. The size of the model cluster taken from 3S8G, which was used in both ref 23 and in the current study is a little larger than that in Figure 2 by including the Gly232 residue and four more water molecules that are in the positions of HOH604, HOH608, HOH607, and HOH610 in 3S8G (see Figures 1 and 2 of ref 23).

On the basis of the calculations for relative spin-state energetics of Fe^{2+} and Fe^{3+} heme models performed by Vancoillie et al.,²⁶ none of the tested density functionals (B3LYP, B3LYP*, OLYP, BP86, TPSS, TPSSh, M06, or M06-L) consistently provide better accuracy than CASPT2 (multi-configurational perturbation theory) for all their model complexes against available high-level coupled cluster singles and doubles (CCSD) results. However, the pure functional OLYP yields similar results to the hybrid functionals B3LYP* and B3LYP. And for their large heme models, the results of OLYP, B3LYP, and B3LYP* are reasonably close to the best estimate of the spin-splittings with errors typically ≤ 6 kcal mol^{-1} .²⁶ Radoń and Pierloot also investigated the performance of the CASSCF/CASPT2 approach and several DFT functionals (PBE0, B3LYP, BP86, and OLYP) in calculating the bonding of CO, NO, and O_2 molecules to two model heme systems.²⁷ They found that the experimentally available binding energies are best reproduced by the CASPT2 method and with the OLYP functional. The CASSCF spin populations most

closely correspond to the results obtained with the pure OLYP or BP86 rather than with the hybrid functionals.²⁷ Therefore, we used the OLYP functional in studying the 3S8G-DNC²³ geometric and energetic properties and will continue using this functional in the current study.

The DNC model geometries were optimized using the Amsterdam Density Functional Package (ADF2012.01)^{28–30} with integration grid accuracy parameter 4.0 within the conductor-like screening (COSMO) solvation model.^{31–34} Because both the cluster and the surrounding protein environment are quite polar and contain many water molecules, to be consistent with refs 17 and 23, a large dielectric constant of a simple ketone ($\epsilon = 18.5$) was applied to the environment in all COSMO calculations. The van der Waals radii of 1.5, 1.4, 1.7, 1.52, 1.55, and 1.2 Å were used for atoms Fe, Cu, C, O, N, and H, respectively.^{17,23} During geometry optimizations, the all-electron triple- ζ plus polarization (TZP) Slater-type basis set was applied for the major atoms at the DNC, including Fe^{3+/2+}, Cu^{2+/1+}, the oxygen species (H₂O or OH⁻) between Fe^{3+/2+} and Cu^{2+/1+}, and all the N atoms binding with Fe^{3+/2+}/Cu^{2+/1+}, and the double- ζ plus polarization (DZP) basis set with frozen cores of C(1s), N(1s), and O(1s) was applied to the rest of the atoms.

The Fe²⁺ and Fe³⁺ sites may exist in one of the three spin states: low-spin (LS) with $S_{\text{Fe}^{2+}} = 0$ and $S_{\text{Fe}^{3+}} = 1/2$, intermediate-spin (IS) with $S_{\text{Fe}^{2+}} = 1$ and $S_{\text{Fe}^{3+}} = 3/2$, or high-spin (HS) with $S_{\text{Fe}^{2+}} = 2$ and $S_{\text{Fe}^{3+}} = 5/2$. The Cu⁺ has spin $S_{\text{Cu}^+} = 0$, and Cu²⁺ has $S_{\text{Cu}^{2+}} = 1/2$. Further, the Fe³⁺ site may couple with the Cu²⁺ site ferromagnetically (F) with a total spin $S_{\text{total}} = S_{\text{Fe}^{3+}} + S_{\text{Cu}^{2+}}$ or antiferromagnetically (AF) with $S_{\text{total}} = S_{\text{Fe}^{3+}} - S_{\text{Cu}^{2+}}$. The AF-coupling spin state cannot be obtained directly from the normal DFT calculations. As in previous work,^{23,35–42} we represent the AF spin-coupled state in DFT by a “broken-symmetry” (BS) state,^{43–45} where a spin-unrestricted determinant is constructed in which the Fe³⁺ site has spin-up electrons as majority spin and the Cu²⁺ site has majority spin-down electrons.

After geometry optimizations, to obtain more accurate energies (which will be reported in the Results section) and the Mössbauer isomer shift and quadrupole splitting properties, we applied a single-point energy calculation at each optimized geometry using an all-electron/all-TZP basis set to all atoms within the COSMO solvation model. Our own program is then used to calculate the electron density $\rho(0)$ at the Fe nuclei.^{40,46,47} The Mössbauer isomer shift δ is calculated based on $\rho(0)$

$$\delta = \alpha[\rho(0) - A] + C \quad (1)$$

A is a constant and taken as 11877. In our previous study,⁴⁰ $\alpha = -0.328$, $C = 0.622 \text{ mm s}^{-1}$ for ⁵⁷Fe²⁺ and $\alpha = -0.307$, $C = 0.385 \text{ mm s}^{-1}$ for ⁵⁷Fe³⁺ were obtained from linear regression between the measured isomer shifts and OLYP-calculated electron densities at Fe nuclei of 17 Fe^{2+,2.5+} complexes (31 total Fe sites) and 19 Fe^{2.5+,3+,3.5+,4+} complexes (30 total Fe sites), respectively.

The electric field gradient (EFG) at the Fe nucleus obtained from the all-electron/all-TZP single-point energy calculation is used to calculate the Mössbauer quadrupole splitting (ΔE_{Q}) property. Normally, the EFG tensors V are diagonalized and the eigenvalues are reordered such that $|V_{zz}| \geq |V_{yy}| \geq |V_{xx}|$. The asymmetry parameter η is defined as

$$\eta = (V_{xx} - V_{yy})/V_{zz} \quad (2)$$

Then, the ΔE_{Q} for ⁵⁷Fe of the nuclear excited state ($I = 3/2$) can be calculated as

$$\Delta E_{\text{Q}} = (1/2)eQV_{zz}(1 + \eta^2/3)^{1/2} \quad (3)$$

where e is the electrical charge of a positive electron, and Q is the nuclear quadrupole moment of Fe. Recently, the ADF software package determines the ΔE_{Q} value using $eQ = 0.16$ electron-barn.⁴⁸ We will report the ADF-calculated ΔE_{Q} values in the current study.

A few comments outlining how different state energies are compared should prove helpful. Using the ADF code, state energies are defined with respect to a sum of reference fragment energies (reference atoms in the present case). Then, these electronic energies E can be directly compared when the states have the same atom compositions (types and numbers of atoms). Such states are isomers (tautomers). When there are proton transfers between tautomers, ΔE can be defined by adding the ΔZPE difference between states (see below). Further, when considering net deprotonation/protonation, the H⁺ reference energy in the gas phase and in aqueous solution must be included, as discussed next. Systems differing in the total number of water molecules cannot be directly compared in energies because an H₂O (in bulk aqueous solvent) reference energy is also needed. This H₂O gas phase and H₂O in solvent energy reference was presented in our earlier works (refs 17 and 42) but is not a focus in this paper.

To determine the protonation state of the oxygen species residing between the Fe and Cu sites and of the Tyr237 side chain, we performed $\text{p}K_{\text{a}}$ calculations for certain states using^{23,42}

$$1.37\text{p}K_{\text{a}} = E(\text{A}^-) - E(\text{AH}) + E(\text{H}^+) + \Delta G_{\text{sol}}(\text{H}^+, 1 \text{ atm}) - T\Delta S_{\text{gas}}(\text{H}^+) + \Delta \text{ZPE} + (5/2)RT \quad (4)$$

where $E(\text{A}^-)$ and $E(\text{AH})$ are the calculated energies of the deprotonated and protonated states, $\Delta G_{\text{sol}}(\text{H}^+, 1 \text{ atm})$ is the solvation free energy of a proton at 1 atm pressure. We use the “best available” experimental value of $-264.0 \text{ kcal mol}^{-1}$ for this term based on analysis of cluster-ion solvation data.^{49–51} Unlike in ref 23, where $E(\text{H}^+)$ was calculated as the energy of a proton (in gas-phase with OLYP potential) with respect to a spin-restricted hydrogen atom, here, we take the empirically corrected $E(\text{H}^+) = 12.71 \text{ eV} = 293.1 \text{ kcal mol}^{-1}$ based on experimental standard hydrogen electrode energy and the proton solvation free energy (see appendix in ref 42). The translational entropy contribution to the gas-phase free energy of a proton is taken as $-T\Delta S_{\text{gas}}(\text{H}^+) = -7.8 \text{ kcal mol}^{-1}$ at 298 K and 1 atm pressure.⁵² $(5/2)RT = 1.5 \text{ kcal mol}^{-1}$ includes the proton translational energy $(3/2)RT$ and $PV = RT$.⁵² The term ΔZPE is the zero point energy difference for the deprotonated state (A^-) minus the protonated state (AH), and it was estimated as $\Delta \text{ZPE} = -7.6$ and $-7.9 \text{ kcal mol}^{-1}$ for the OH⁻/H₂O and the Tyr237⁻/Tyr237 systems, respectively, by only optimizing the geometries (and then performing frequency calculations) of an OH⁻ and an H₂O molecule and the linked His233–Tyr237 side chains in both neutral and anionic His233–Tyr237 forms within the COSMO solvation model (with $\epsilon = 18.5$ and all-electron/all-TZP basis set). Similarly, we also obtained $\Delta \text{ZPE} = -8.1 \text{ kcal mol}^{-1}$ for the His376 side chain to estimate its $\text{p}K_{\text{a}}$ in section 3.4.

Table 2. Calculated Properties for the Reduced Fe²⁺...Cu⁺(Y237/Y237⁻) DNC Models Generated Starting from the DNCs of 3EH5, 3EH3, and 1EHK X-ray Crystal Structures^a

starting structure	models ^b	geometry						<i>E</i>	p <i>K</i> _a (Y237)	<i>Q</i>	net spin		⁵⁷ Fe ²⁺ Mössbauer	
		Fe–N (H384)	Fe...Cu	Cu–N (H233)	Cu–N (H282)	Cu–N (H283)	O...O (Y237)				Fe ²⁺	Cu ⁺	δ	ΔE_Q
3EH5	Fe ^{2+,LS} ...Cu ⁺ (Y237)	1.96	5.74	1.99	2.08	2.03	3.34	-41.8	7.0	0	0.00	0.00	0.64	2.43
	Fe ^{2+,IS} ...Cu ⁺ (Y237)	2.38	5.48	2.00	2.07	2.04	3.43	-50.3	9.0	0	2.31	0.00	0.73	2.02
	Fe ^{2+,HS} ...Cu ⁺ (Y237)	2.22	5.62	2.00	2.08	2.04	3.33	-49.6	7.8	0	3.90	0.00	0.92	2.21
	Fe ^{2+,LS} ...Cu ⁺ (Y237 ⁻)	1.95	5.82	1.99	2.08	2.04	2.82	-47.1		-1	0.00	0.00	0.64	2.41
	Fe ^{2+,IS} ...Cu ⁺ (Y237 ⁻)	2.34	5.55	1.99	2.08	2.04	2.80	-52.9		-1	2.32	0.00	0.73	2.08
	Fe ^{2+,HS} ...Cu ⁺ (Y237 ⁻)	2.21	5.73	1.99	2.09	2.04	2.80	-53.8		-1	3.91	0.00	0.91	2.16
3EH3 ^c	Fe ^{2+,HS} ...Cu ⁺ (Y237 ⁻)	2.39	5.06	2.03	2.28	1.99	2.75	-38.6		-1	3.85	0.00	0.92	2.44
1EHK ^d	Fe ^{2+,HS} ...Cu ⁺ (Y237 ⁻) ⁽¹⁾	3.00	5.09	2.03	2.08	2.03	2.67	-31.5		-1	3.86	0.00	0.90	2.90
	Fe ^{2+,HS} ...Cu ⁺ (Y237 ⁻) ⁽²⁾	2.71	5.36	2.02	2.10	2.03	2.68	-37.8		-1	3.87	0.00	0.94	2.67
	Fe ^{2+,HS} ...Cu ⁺ (Y237 ⁻) ⁽³⁾	2.45	5.59	2.02	2.11	2.04	2.70	-42.4		-1	3.88	0.00	0.96	2.43
	Fe ^{2+,HS} ...Cu ⁺ (Y237 ⁻) ⁽⁴⁾	2.33	5.74	2.02	2.10	2.04	2.70	-44.4		-1	3.89	0.00	0.96	2.34

^aThe properties include geometries (Å), electronic energies (*E*, offset by -25100 kcal mol⁻¹) obtained from the all-electron/all-TZP single-point energy calculations on the optimized geometries, p*K*_a's, the net charge (*Q*) of the clusters, Mulliken net spin polarizations for the Fe and Cu sites, and the ⁵⁷Fe Mössbauer isomer shifts (δ , mm s⁻¹) and quadrupole splittings (ΔE_Q , mm s⁻¹). ^bLS, low-spin; IS, intermediate-spin; HS, high-spin. ^cThe Cartesian coordinates of N(His384), Fe, and Cu were fixed during geometry optimization. ^dHere, we deleted the bridging oxygen atom from the 1EHK structure, and geometry optimized the DNC cluster to see how the Fe...N(His384) distance and the Mössbauer properties will change. The Fe^{2+,HS}...Cu⁺(Y237⁻)⁽¹⁾, Fe^{2+,HS}...Cu⁺(Y237⁻)⁽²⁾, and Fe^{2+,HS}...Cu⁺(Y237⁻)⁽³⁾ are three points along the geometry optimization pathway. Fe^{2+,HS}...Cu⁺(Y237⁻)⁽⁴⁾ is the lowest energy structure obtained.

3. RESULTS AND DISCUSSION OF FE²⁺/CU⁺ REDUCED STATE DNC MODEL CALCULATIONS

3.1. Fe²⁺...Cu⁺ DNC Calculations—Models Taken from 3EH5, 3EH3, and 1EHK. Feasible Structure of the Second Observed ⁵⁷Fe_{a3}²⁺ Component? No substantial electron density was observed directly between the Fe and Cu sites in the DNC's of the chemically reduced X-ray crystal structures of 3EH5 and 3EH3.¹² To see if such a DNC will yield the Mössbauer properties observed for the reduced *Tt ba*₃ CcO, we first built up our Fe²⁺...Cu⁺ DNC model (similar to Figure 2, but without the oxygen species between the Fe and Cu sites) based on the 3EH5 Cartesian coordinates. Both the Fe²⁺...Cu⁺(Y237) (180 atoms with neutral Tyr237 side chain) and Fe²⁺...Cu⁺(Y237⁻) (179 atoms with deprotonated anionic Tyr237⁻ side chain) clusters were geometry optimized with Fe²⁺ in LS, IS, and HS states. The calculated properties of these DNC models, including the main geometric parameters (Å), energies (*E*, off set by -25100 kcal mol⁻¹), Mulliken net spin polarizations on Fe²⁺ and Cu⁺, and the ⁵⁷Fe²⁺ Mössbauer isomer shifts (δ) and quadrupole splittings (ΔE_Q) are given in Table 2 (in the 3EH5 section).

For both the Fe²⁺...Cu⁺(Y237) and Fe²⁺...Cu⁺(Y237⁻) cases, the Fe^{2+,HS} and Fe^{2+,IS} states yield similar electronic energies, which are much lower than the corresponding Fe^{2+,LS} state energy. Further, the calculated Mössbauer properties of $\delta = 0.92$ mm s⁻¹ and $\Delta E_Q = 2.21$ mm s⁻¹ for the Fe^{2+,HS}...Cu⁺(Y237) state and $\delta = 0.91$ mm s⁻¹ and $\Delta E_Q = 2.16$ mm s⁻¹ for the Fe^{2+,HS}...Cu⁺(Y237⁻) state agree very well with the observed Mössbauer properties ($\delta = 0.92$ mm s⁻¹, $\Delta E_Q = 2.34$

mm s⁻¹) of the second ⁵⁷Fe_{a3}²⁺ component (Figure 1). Conversely, the calculated Mössbauer isomer shifts (0.73 mm s⁻¹) for both the ⁵⁷Fe^{2+,IS} sites are too small compared with the observed $\delta = 0.92/0.95$ mm s⁻¹ values. Therefore, our Mössbauer property calculations support that the ⁵⁷Fe_{a3}²⁺ species observed in the Mössbauer experiments are in high-spin states. The OLYP potential may underestimate the energy gap between the Fe^{2+,IS} and Fe^{2+,HS} states. However, although not included in the present calculations, both vibrational entropy and vibrational zero-point-energy terms most likely favor the Fe^{2+,HS} state over the Fe^{2+,IS} state. The Fe²⁺ site of the reduced DNC of *ba*₃ CcO in *Tt* then most likely exists only in the high-spin state at the *Thermus* optimum growth temperature of 50–80 °C.⁵³ We will therefore only report the results of the Fe^{2+,HS} state for the rest of the reduced-DNC calculations in the current study.

For comparison, the experimental Mössbauer δ and ΔE_Q values for the reduced ⁵⁷Fe_{a3}²⁺ sites in *c*₁*aa*₃ from *Tt*⁵⁴ and *aa*₃ from a bovine heart⁵⁵ are also given in Table 1. The observed δ values for all Fe_{a3}²⁺ sites are similar (0.92–0.96 mm s⁻¹) and are >0.9 mm s⁻¹, indicating they all are in the high-spin state. The ΔE_Q value for the ⁵⁷Fe_{a3}²⁺ in *c*₁*aa*₃ *Tt* (2.05 mm s⁻¹) is close to the second ΔE_Q (2.34 mm s⁻¹) observed in *ba*₃ *Tt*. These two may have very similar DNC structural features.

From the Mössbauer property calculations, one cannot tell under the conditions of the Mössbauer experiments whether the Tyr237 side chain is in a neutral or deprotonated anionic state. Taking $E[\text{Fe}^{2+,HS}\dots\text{Cu}^+(\text{Y237}^-)]$ and $E[\text{Fe}^{2+,HS}\dots\text{Cu}^+(\text{Y237})]$ from Table 2 for the *E*(A⁻) and *E*(AH) terms in eq 4, we then obtained the p*K*_a(Y237) = 7.8. Because this

calculated pK_a value is close to 7, considering the uncertainty of DFT calculations, the limited size of the quantum cluster model, and the fact that the pK_a calculations are for the equilibrium state at room temperature, it is not certain whether the Tyr237 side chains of the $Fe^{2+}\cdots Cu^+$ -type DNC's are in a neutral or anionic state.

The experimental H-bonding O \cdots O distance between the Tyr237 side chain and the geranyl –OH of the a_3 -heme is only 2.61 Å in the X-ray crystal structure of 3EH5 (2.8 Å resolution). The DFT OLYP calculations predict 2.80 Å and a longer 3.33 Å for this O \cdots O distance in the $Fe^{2+,HS}\cdots Cu^+(Y237^-)$ and $Fe^{2+,HS}\cdots Cu^+(Y237)$ states, respectively. Therefore, the geometry optimization calculations support that the Tyr237 side chain in the X-ray crystal structure of 3EH5 is in anionic deprotonated state.

So far, our calculations show that the DNC of the second (minority) $Fe_{a_3}^{2+}$ component observed in the Mössbauer experiment may have the $Fe^{2+,HS}\cdots Cu^+(Y237^-/Y237)$ structure. Now the question is, what is the DNC structure of the first (majority) $Fe_{a_3}^{2+}$ component observed in the Mössbauer experiment?

We noticed that the DNC of 3EH3 is similar to that of 3EH5 but with a longer Fe–N(His384) distance (2.39 Å in 3EH3 relative to 2.10 Å in 3EH5 and 2.21 Å in the OLYP-optimized $Fe^{2+,HS}\cdots Cu^+(Y237^-)$ structure). Next, we would examine how a longer Fe–N(His384) and a shorter $Fe^{2+,HS}\cdots Cu^+$ distance (5.06 Å in 3EH3 relative to 5.73 Å in the OLYP-optimized $Fe^{2+,HS}\cdots Cu^+(Y237^-)$) will affect the $^{57}Fe^{2+}$ Mössbauer spectra, and if such a $Fe^{2+,HS}\cdots Cu^+$ DNC in 3EH3 would represent the first $Fe_{a_3}^{2+}$ component observed in the Mössbauer experiment.

A new $Fe^{2+}\cdots Cu^+(Y237^-)$ model was then constructed based on the DNC coordinates in 3EH3. Because we wanted to keep the Fe–N(His384) (2.39 Å in 3EH3) and $Fe^{2+,HS}\cdots Cu^+$ (5.06 Å in 3EH3) distances, the atom positions of N(His384), Fe, and Cu were fixed during geometry optimization. Then, the Mössbauer property calculation on this partially optimized $3EH3-Fe^{2+,HS}\cdots Cu^+(Y237^-)$ model yielded $\delta = 0.92$ mm s $^{-1}$ and $\Delta E_Q = 2.44$ mm s $^{-1}$ (also given in Table 2, 3EH3 section), which is similar to the Mössbauer parameters of $\delta = 0.91$ mm s $^{-1}$ and $\Delta E_Q = 2.16$ mm s $^{-1}$ obtained for the $3EH5-Fe^{2+,HS}\cdots Cu^+(Y237^-)$ model. Because the experimentally observed component 1 of $^{57}Fe_{a_3}^{2+}$ has a smaller $\Delta E_Q = 1.43$ mm s $^{-1}$,¹⁸ whereas the model $3EH3-Fe^{2+,HS}\cdots Cu^+(Y237^-)$ yields an even larger $\Delta E_Q = 2.44$ mm s $^{-1}$, this $3EH3-Fe^{2+,HS}\cdots Cu^+(Y237^-)$ model does not represent the DNC structure of the first $Fe_{a_3}^{2+}$ component observed in the Mössbauer experiment.

As mentioned earlier in 1EHK, the axial His384 side chain was farther away from the Fe atom with the Fe \cdots N(His384) distance of 3.31 Å. An oxygen species (probably originating from the oxidized state) was found between the Fe and Cu sites. To see if this long Fe \cdots N(His384) distance will remain and how it affects the $^{57}Fe^{2+}$ Mössbauer properties when the bridging oxygen species is omitted, we took the DNC model (Figure 2) from 1EHK, deleted the bridging oxygen atom, and optimized the geometry in the $Fe^{2+,HS}\cdots Cu^+(Y237^-)$ state. The geometric and Mössbauer properties of the three structures $Fe^{2+,HS}\cdots Cu^+(Y237^-)^{(1-3)}$ along the optimization trajectory and the final optimized structure $Fe^{2+,HS}\cdots Cu^+(Y237^-)^{(4)}$ are also shown in Table 2 (in the 1EHK section). Without the bridging oxygen species, the $Fe^{2+,HS}$ and the Cu^+ sites move away from each other during the geometry optimization. The Fe \cdots N(His384) distance is gradually shortened, and because the His384 side chain cannot move freely (H_{link} is fixed during

geometry optimization), Fe^{2+} moves toward the N(His384) atom. The final structure $Fe^{2+,HS}\cdots Cu^+(Y237^-)^{(4)}$ is 9.4 kcal mol $^{-1}$ higher in energy than that of the $3EH5-Fe^{2+,HS}\cdots Cu^+(Y237^-)$ optimized geometry.

The calculated Mössbauer properties on $Fe^{2+,HS}\cdots Cu^+(Y237^-)^{(1-4)}$ show that when the Fe \cdots N(His384) distance is very long (3.0 Å), the calculated Mössbauer isomer shift is relatively smaller ($\delta = 0.90$ mm s $^{-1}$), and the quadrupole splitting value is very large ($\Delta E_Q = 2.90$ mm s $^{-1}$). As the Fe \cdots N(His384) distance gets shorter, the calculated δ gets larger and ΔE_Q becomes smaller. Therefore, the $Fe^{2+,HS}\cdots Cu^+(Y237^-)$ model with a long Fe \cdots N(His384) distance does not represent the DNC structure of the experimentally observed component one, which has a large $\delta = 0.95$ mm s $^{-1}$ and a small $\Delta E_Q = 1.43$ mm s $^{-1}$.¹⁸

Next, we study whether the bridging oxygen species in 1EHK is H_2O or OH^- and if the $Fe^{2+}-H_2O-Cu^+/Fe^{2+}-OH^- -Cu^+$ structures will yield the Mössbauer properties of the first $^{57}Fe_{a_3}^{2+}$ component observed in the Mössbauer experiment.

3.2. Bridging H_2O ($Fe^{2+}-H_2O-Cu^+$) or OH^- ($Fe^{2+}-OH^- -Cu^+$) in the DNC of 1EHK? From the X-ray crystal structure, the Tyr237 side chain in 1EHK is also likely in the deprotonated anionic state because the H-bonding O \cdots O distance between the Tyr237 side chain and the geranyl –OH of the a_3 -heme is only 2.52 Å. Therefore, we optimized the geometry of the $1EHK-Fe^{2+,HS}-H_2O-Cu^+(Y237^-)$ DNC cluster (Figure 2, starting with bridging H_2O) with the deprotonated Tyr237 side chain. The calculated geometrical parameters of the DNC, the energy (E), and the $^{57}Fe^{2+}$ Mössbauer properties are given in Table 3.

In 1EHK, the Fe \cdots Cu distance is very short (4.40 Å); the distance of Fe–O (2.26 Å) is similar to the distance of Cu–O (2.31 Å), and the bridging O is close to the line connecting the Fe and Cu sites with $\angle Fe-O-Cu = 148^\circ$. During DFT geometry optimization, the proposed bridging H_2O molecule gradually moves away from both the Fe^{2+} and Cu^+ sites, and finally detaches from them. The Fe^{2+} and Cu^+ sites also move apart from each other. The final Fe \cdots O, Cu \cdots O, and Fe \cdots Cu distances are 2.41, 3.57, and 5.26 Å, respectively. Therefore, the H_2O molecule does not bind with either the $Fe^{2+,HS}$ or Cu^+ site. The bridging oxygen species in 1EHK is therefore probably not a water molecule.

The central portion of the optimized $Fe^{2+,HS}-H_2O-Cu^+(Y237^-)$ geometry is shown in Figure S1 in the Supporting Information. Although the H_2O molecule is 2.41 Å away from the $Fe^{2+,HS}$ site, it prevents the $Fe^{2+,HS}$ from moving toward the His384 side chain. Without this H_2O molecule (see section 3.1 and $Fe^{2+,HS}\cdots Cu^+(Y237^-)^{(4)}$ in Table 2), $Fe^{2+,HS}$ would bind to His384, and the calculated Fe \cdots N(His384) distance would be shortened to 2.33 Å. This H_2O molecule only moderately affects the Mössbauer properties of $^{57}Fe^{2+,HS}$. From $3EH5-Fe^{2+,HS}\cdots Cu^+(Y237^-)$ (see Table 2) to the current $1EHK-Fe^{2+,HS}-H_2O-Cu^+(Y237^-)$, the calculated isomer shift decreases from 0.91 to 0.90 mm s $^{-1}$, and the quadrupole splitting increases from 2.16 to 2.60 mm s $^{-1}$. Overall, the $\delta = 0.90$ mm s $^{-1}$ and $\Delta E_Q = 2.60$ mm s $^{-1}$ values are still in reasonable agreement with the experimental results (the second $^{57}Fe_{a_3}^{2+}$ component) of $\delta = 0.92$ mm s $^{-1}$ and $\Delta E_Q = 2.34$ mm s $^{-1}$. It is not clear if there is a water molecule trapped in the DNC of the enzyme under the conditions of the Mössbauer experiments.

To test if an OH^- would stay in the bridging position of the oxygen species found in the DNC of 1EHK, we also geometry optimized the $1EHK-Fe^{2+,HS}-OH^- -Cu^+(Y237^-)$ model. The

Table 3. Calculated Properties of the Reduced $\text{Fe}^{2+,\text{HS}}\text{-H}_2\text{O-Cu}^+(\text{Y237}^-)$ Model with Bridging H_2O and $\text{Fe}^{2+,\text{HS}}\text{-OH}^- \text{-Cu}^+(\text{Y237}^-/\text{Y237}^-)$ Models with Bridging OH^- Generated from the DNC of the 1EHK X-ray Crystal Structure^a

state	geometry										net spin		⁵⁷ Fe ²⁺ Mössbauer			
	Fe-N (H384)	Fe-O	Cu-O	∠Fe-O-Cu	Fe...Cu	Cu-N (H233)	Cu-N (H282)	Cu-N (H283)	O...O (Y237)	E	pK _a (Y237)	pK _a (H ₂ O)	Q	δ	ΔE _Q	
$\text{Fe}^{2+,\text{HS}}\text{-H}_2\text{O-Cu}^+(\text{Y237}^-)$	3.15	2.41	3.57	122	5.26	2.01	2.10	2.04	2.72	-357.7	17.3	-1	3.86	0.00	0.90	2.60
$\text{Fe}^{2+,\text{HS}}\text{-OH}^- \text{-Cu}^+(\text{Y237}^-)$	4.07	1.95	2.31	135	3.95	2.11	2.15	2.16	2.73	-349.2		-2	3.90	0.05	0.80	1.82
$\text{Fe}^{2+,\text{HS}}\text{-OH}^- \text{-Cu}^+(\text{Y237}^-)$	4.17	1.94	2.39	138	4.05	2.10	2.14	2.14	3.15	-352.3	13.1	-1	3.88	0.03	0.80	1.89
exp. (1EHK) ^b	3.31	2.26	2.31	148	4.40	2.10	2.21	2.13	2.52							

^aThe properties include geometries (Å, °), electronic energies (E, offset by -25100 kcal mol⁻¹) obtained from the all-electron/all-TZP single-point energy calculations on the optimized geometries, pK_a's, the net charge (Q) of the clusters, Mulliken net spin polarizations for the Fe and Cu sites, and the ⁵⁷Fe Mössbauer isomer shifts (δ, mm s⁻¹) and quadrupole splittings (ΔE_Q, mm s⁻¹). ^bA bridging oxygen species was found in between the Fe and Cu sites in the DNC of the radiation-reduced X-ray crystal structure of 1EHK (2.4 Å resolution).¹⁰ Here we study if this bridging oxygen species is a H₂O molecule or a hydroxide anion.

OH⁻ clearly stays in the bridging position. The optimized structure is given in Figure S2 in the Supporting Information. The calculated Fe-O (1.95 Å) and Cu-O (2.31 Å) distances in this model (see Table 3) are much closer to the corresponding distances (2.26 and 2.31 Å) in 1EHK than in the calculated $\text{Fe}^{2+,\text{HS}}\text{-H}_2\text{O-Cu}^+(\text{Y237}^-)$ model. Therefore, the bridging oxygen species in 1EHK is better represented by a hydroxide anion than by a water molecule.

The optimized $\text{Fe}^{2+,\text{HS}}\text{-OH}^- \text{-Cu}^+(\text{Y237}^-)$ structure has a shorter Fe...Cu distance and a longer Fe...N(His384) distance compared to the X-ray crystal structure (1EHK). The radiolytically reduced protein is likely not in an equilibrium state. The structure 1EHK may represent a mixture of the $\text{Fe}^{2+,\text{HS}}\text{-OH}^- \text{-Cu}^+(\text{Y237}^-)$ and the open $\text{Fe}^{2+,\text{HS}}\text{...Cu}^+(\text{Y237}^-)$ states (Table 2). A minority contribution from $\text{Fe}^{2+,\text{HS}}\text{-H}_2\text{O-Cu}^+(\text{Y237}^-)$ to 1EHK is also possible.

We further optimized the geometry of the $\text{Fe}^{2+,\text{HS}}\text{-OH}^- \text{-Cu}^+(\text{Y237}^-)$ state with neutral Tyr237; its calculated properties are provided in Table 3. Because the calculated pK_a for the Tyr237 side chain is 13.1, the Tyr237 energetically favors the neutral protonated form in the equilibrium state at room temperature. Further, comparing tautomers, the energy of $\text{Fe}^{2+,\text{HS}}\text{-H}_2\text{O-Cu}^+(\text{Y237}^-)$ is 5.4 kcal mol⁻¹ lower than that of the $\text{Fe}^{2+,\text{HS}}\text{-OH}^- \text{-Cu}^+(\text{Y237}^-)$ state, indicating that $\text{Fe}^{2+,\text{HS}}\text{-H}_2\text{O-Cu}^+(\text{Y237}^-)$ is energetically more stable than the $\text{Fe}^{2+,\text{HS}}\text{-OH}^- \text{-Cu}^+(\text{Y237}^-)$ state, and therefore also more stable than the $\text{Fe}^{2+,\text{HS}}\text{-OH}^- \text{-Cu}^+(\text{Y237}^-)$ state. This conclusion is also supported by the calculated pK_a value of 17.3 for the H₂O molecule in $\text{Fe}^{2+,\text{HS}}\text{-H}_2\text{O-Cu}^+(\text{Y237}^-)$. Therefore, the radiolytically reduced DNC of $\text{Fe}^{2+,\text{HS}}\text{-OH}^- \text{-Cu}^+(\text{Y237}^-)$ in 1EHK is not in an equilibrium state. The bridging OH⁻ is likely trapped, originating from 2e⁻ reduction of the oxidized $\text{Fe}^{3+}\text{-OH}^- \text{-Cu}^{2+}(\text{Y237}^-)$ state at low temperature with an effective kinetic barrier to proton transfer from the K-path.

The $\text{Fe}^{2+,\text{HS}}\text{-OH}^- \text{-Cu}^+(\text{Y237}^-)$ and $\text{Fe}^{2+,\text{HS}}\text{-OH}^- \text{-Cu}^+(\text{Y237}^-)$ clusters yield essentially the same Mössbauer isomer shift (0.80 mm s⁻¹) and quadrupole splitting (1.82/1.89 mm s⁻¹) properties. Compared with the open $\text{Fe}^{2+,\text{HS}}\text{...Cu}^+(\text{Y237}^-/\text{Y237}^-)$ structures in Table 2, the bridging OH⁻ in $\text{Fe}^{2+,\text{HS}}\text{-OH}^- \text{-Cu}^+(\text{Y237}^-/\text{Y237}^-)$ lowers both the ⁵⁷Fe^{2+,\text{HS}}} Mössbauer isomer shift and quadrupole splitting values. Because the calculated δ = 0.80 mm s⁻¹ is well below the observed values of 0.92/0.95 mm s⁻¹, the radiolytically reduced $\text{Fe}^{2+,\text{HS}}\text{-OH}^- \text{-Cu}^+(\text{Y237}^-)$ DNC in 1EHK does not represent either Fe₃²⁺ component observed in the Mössbauer experiments.

3.3. Terminal H₂O or OH⁻ in the DNC of 3EH4? As mentioned earlier, in the radiolytically reduced X-ray crystal structures 3EH4 and 1XME (Table 1), the oxygen species found in the DNC is much closer to the Cu⁺ site. We now start from the DNC of 3EH4 to study whether a water molecule or an OH⁻ can be terminally bound to the Cu⁺ site. We geometry optimized the $\text{Fe}^{2+,\text{HS}}\text{...H}_2\text{O-Cu}^+(\text{Y237}^-/\text{Y237}^-)$ and $\text{Fe}^{2+,\text{HS}}\text{...OH}^- \text{-Cu}^+(\text{Y237}^-/\text{Y237}^-)$ DNC clusters in both anionic Tyr237⁻ and neutral Tyr237 states and also calculated the ⁵⁷Fe^{2+,\text{HS}}} Mössbauer properties. The calculated results are given in Table 4.

As shown in Figure 3, during geometry optimizations, the Cu⁺-terminally bound H₂O molecule in both $\text{Fe}^{2+,\text{HS}}\text{...H}_2\text{O-Cu}^+(\text{Y237}^-/\text{Y237}^-)$ models dissociated from the Cu⁺ site. It stayed in between Fe²⁺ and Cu⁺ but did not bind to either site. Therefore, the oxygen species observed in the DNC of 3EH4 cannot be a water molecule.

Table 4. Calculated Properties of the Reduced $\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O}-\text{Cu}^+(\text{Y237}^-/\text{Y237})$ and $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237}^-/\text{Y237})$ DNC Models Generated from the DNC of the X-ray Crystal Structure of 3EH4^a

state	geometry										net spin		⁵⁷ Fe ²⁺ Mössbauer			
	Fe-N (H384)	Fe...O	Cu-O	∠Fe-O-Cu	Fe...Cu	Cu-N (H233)	Cu-N (H282)	Cu-N (H283)	O...O (Y237)	E	pK _a (Y237)	pK _a (H ₂ O)	Q	ΔE _Q	δ	ΔE _Q
$\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O}-\text{Cu}^+(\text{Y237}^-)$	2.17	4.05	3.30	100	5.65	1.99	2.11	2.01	2.81	-366.5	27.4	-1	3.88	0.00	0.91	2.31
$\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O}-\text{Cu}^+(\text{Y237})$	2.18	3.93	3.25	104	5.68	2.00	2.09	2.04	3.29	-366.8	25.0	0	3.88	0.00	0.91	2.20
$\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237})$	2.22	4.01	1.95	113	5.10	2.18	2.07	2.29	2.89	-344.2		-2	3.83	0.28	0.93	1.01
$\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237}^-)$	2.22	3.82	1.93	116	4.98	2.31	2.02	2.42	3.29	-347.7		-1	3.84	0.23	0.92	1.20
exp. (3EH4) ^b	2.39	3.08	2.20	126	4.73	1.96	2.03	1.97	2.47							

^aThe properties include geometries (Å, °), electronic energies (E, offset by -25100 kcal mol⁻¹) obtained from the all-electron/all-TZP single-point energy calculations on the optimized geometries, pK_a's, the net charge (Q) of the clusters, Mulliken net spin polarizations for the Fe and Cu sites, and the ⁵⁷Fe Mössbauer isomer shifts (δ, mm s⁻¹) and quadrupole splittings (ΔE_Q, mm s⁻¹). ^bAn oxygen species between the Fe²⁺ and Cu⁺ sites but much closer to the Cu⁺ site was observed in the DNC of the X-ray crystal structure of 3EH4 (2.9 Å resolution).¹² Here we study if this oxygen species is a terminally bound (to Cu⁺) H₂O molecule or a hydroxide anion.

The optimized $\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O}-\text{Cu}^+(\text{Y237}^-/\text{Y237})$ clusters yield very similar ⁵⁷Fe^{2+,\text{HS}}} Mössbauer properties (δ = 0.91 mm s⁻¹, ΔE_Q = 2.31/2.20 mm s⁻¹), which are very close to the calculated ⁵⁷Fe^{2+,\text{HS}}} Mössbauer properties (δ = 0.91/0.92 mm s⁻¹, ΔE_Q = 2.16/2.21 mm s⁻¹) for the open $\text{Fe}^{2+,\text{HS}}\cdots\text{Cu}^+(\text{Y237}^-/\text{Y237})$ structures calculated for the 3EH5 DNC (see Table 2) and are also consistent with the second ⁵⁷Fe_{a3}²⁺ component (δ = 0.92 mm s⁻¹, ΔE_Q = 2.34 mm s⁻¹) observed in the Mössbauer experiments.¹⁸

Although this dangling H₂O molecule between the Fe²⁺ and Cu⁺ sites has little effect on the ⁵⁷Fe^{2+,\text{HS}}} Mössbauer properties, it does affect the pK_a of the Tyr237 side chain. The Fe²⁺...Cu⁺ distance is very similar with or without the weak H₂O bridge. Here, with a water molecule in the DNC, the pK_a of the Tyr237 side chain is estimated as 11.1, which is a 3.3-unit increase from the corresponding calculated pK_a (7.8) for the 3EH5 open DNC $\text{Fe}^{2+,\text{HS}}\cdots\text{Cu}^+(\text{Y237}^-/\text{Y237})$ models in Section 3.1 (Table 2). The sensitivity of the pK_a of this unique Tyr237 residue in response to this structural and component change in the DNC is suggestive of a potential role in proton transfer and proton pumping during the catalytic cycle. For example, proton transfer from Tyr237 toward the reaction center or above it could be promoted in the “open” form.

By contrast, during geometry optimizations, the terminally bound OH⁻ in both $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237}^-/\text{Y237})$ clusters remained tightly bound to the Cu⁺ site (see Figure 4). In these two optimized geometries, the Fe...O and Fe...Cu distances are longer and the Fe-N(His384) and Cu-O distances are shorter than the corresponding values in the radiolytically reduced X-ray crystal structure 3EH4. It is possible that the DNC of 3EH4 represents a mixture of the structures $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237}^-)$, $\text{Fe}^{2+,\text{HS}}\cdots\text{Cu}^+(\text{Y237}^-)$, and/or $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237}^-)$ (bridging OH⁻).

The $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237}^-/\text{Y237})$ states also yield very similar Mössbauer properties (δ = 0.93/0.92 mm s⁻¹, ΔE_Q = 1.01/1.20 mm s⁻¹), which are so far the closest calculated results to the majority first ⁵⁷Fe_{a3}²⁺ component property (δ = 0.95 mm s⁻¹, ΔE_Q = 1.43 mm s⁻¹) observed in Mössbauer experiments.¹⁸

The calculated pK_a (27.4/25.0) of the H₂O molecule in the DNC and the pK_a (11.1/13.4) of the Tyr237 side chain indicate that, when there is an oxygen species in between the Fe^{2+,\text{HS}}} and Cu⁺ sites, the $\text{Fe}^{2+,\text{HS}}\cdots\text{Cu}^+$ DNC strongly favors a H₂O molecule at thermodynamic equilibrium rather than a hydroxide anion, and the Tyr237 side chain energetically prefers the protonated charge-neutral state. In 3EH4, the O...O distance between the Tyr237 side chain and the geranyl -OH of the a₃-heme is very short (2.47 Å). It is likely that the Tyr237 side chain in 3EH4 is in the deprotonated anionic state. Therefore, the $\text{Fe}^{2+}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237}^-)$ DNC structure of 3EH4 probably originates from the oxidized state. That is, if the oxidized as-isolated DNC has a structure like $\text{Fe}^{3+}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237}^-)$ when it is radiolytically reduced by the X-ray beam at low temperature with limited proton mobility, the Cu-bound OH⁻ is then trapped in the reduced state, and the Tyr237 side chain remains in the anionic state.

However, comparing the optimized geometries of the $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237}^-)$ and $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237})$ states (in Table 4), we notice that, in $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237})$, both the Cu-O and Cu-N(H282) distances are shorter and both the Cu-N(H233) and Cu-N(H283) distances are longer than the corresponding Cu-ligand distances in $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237}^-)$. In other words, upon proto-

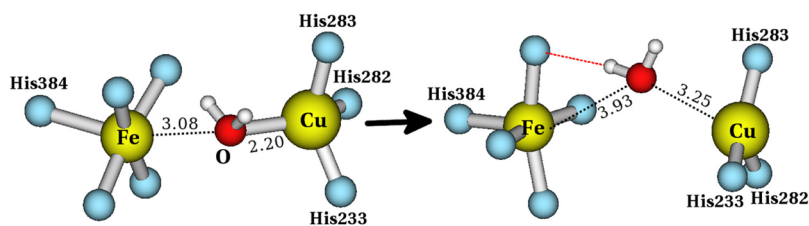


Figure 3. Left: The central portion of the initial $\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O}-\text{Cu}^+(\text{Y237}/\text{Y237})$ structure, which was taken from the DNC of the 3EH4 X-ray crystal structure. Right: During geometry optimizations, the H_2O molecule dissociated from the Cu^+ site. Distances (Å) on the right are taken from the optimized $\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O}-\text{Cu}^+(\text{Y237})$ structure.

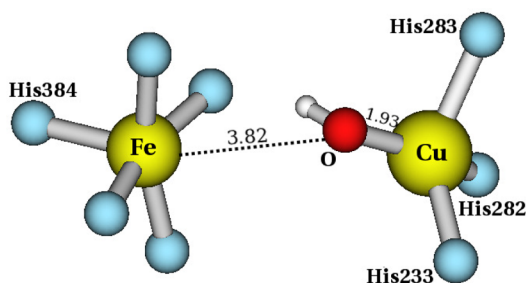


Figure 4. Central portion of the optimized $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+(\text{Y237})$ structure with OH^- binding with Cu^+ . Labeled distances are in Å.

nation of the Tyr237 side chain, the OH^- ligand will bind even stronger with Cu^+ . Therefore, even when Tyr237 is protonated in the $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+$ state, because of the proton transfer barrier from neutral Tyr237 to the OH^--Cu^+ , the $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+(\text{Y237})$ state may still be trapped at low temperature in the Mössbauer experimental conditions.

3.4. Changing of the Cu^+ Coordination State and Feasible DNC Structure of the first $^{57}\text{Fe}_{\text{a}_3}{}^{2+}$ Component Observed in the Mössbauer Experiments. The overall charge of the $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+(\text{Y237})$ quantum cluster is -1 , whereas the total charge of the $\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O}-\text{Cu}^+(\text{Y237})$ state is 0. It is also possible that the $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+(\text{Y237})$ state is stabilized by an extra proton in the proton loading site (PLS), which is close to the DNC. It is still not known where the PLS exactly locates in the ba_3 CcO from *Tt*. For simplicity, Fee et al. protonated the His376 side chain (the positive imidazolium form) to represent having a proton at the PLS.^{17,42} According to Gennis and co-workers' mutagenesis and kinetics studies of proton pumping in ba_3 CcO from *Tt*, His376 is probably close to the proton exit path but not absolutely required.⁵⁶ However, to compare how the energy changes when an extra proton exists in the $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+(\text{Y237})$ cluster, starting from the optimized $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+(\text{Y237})$ structure, we also protonated the His376 side chain and performed geometry optimization calculations. This His376-protonated cluster is named $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+(\text{Y237})(\text{H376H}^+)$. Interestingly, we observed that during the geometry optimization the His283 side chain gradually tilted up and dissociated from the Cu^+ site, and the Cu^+ changed from 4-coordinate (4c) state to 3-coordinate (3c) configuration. In the meantime, the His233 side chain also slowly moved away from the Cu^+ and both the OH^- ligand and the His282 side chain were closer to the Cu^+ . Finally, the His233 side chain also dissociated from Cu^+ , and the optimized structure ended with a 2-coordinate (2c) Cu^+ site with a near-linear $\text{OH}^--\text{Cu}^+_{2c}-\text{N}(\text{His282})$ configuration (see Figure 5). The calculated properties of this structure are given in the first row of Table 5 (footnote b). Its relative energy ($-364 \text{ kcal mol}^{-1}$) is very close to that ($-366.8 \text{ kcal mol}^{-1}$) of

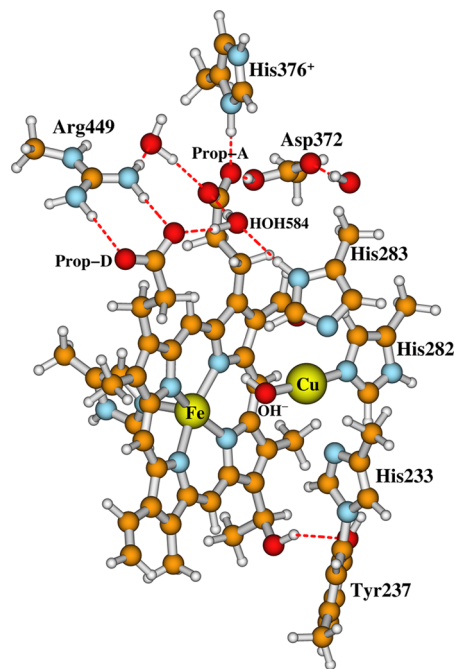


Figure 5. Optimized structure of the $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+(\text{Y237})(\text{H376H}^+)$ cluster with a neutral Tyr237 and a cationic protonated His376⁺ side chain. During the geometry optimization process, the Cu^+ site changes from 4-coordinate to 2-coordinate (2c) configuration with both His283 and His233 side chains gradually dissociating from the Cu^+ .

the $\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O}-\text{Cu}^+(\text{Y237})$ state (in Table 4). These two tautomers are therefore close in energy but differ strongly in Cu^+ coordination geometry and proton location.

We then deleted the added proton on His376⁺ and optimized the geometry to obtain the $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+_{2c}(\text{Y237})$ structure with the 2-coordinated Cu^+ . The calculated properties of the optimized $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+_{2c}(\text{Y237})$ cluster are given in the second row of Table 5 (footnote c). During the geometry optimization, the $\text{Fe}\cdots\text{Cu}$, $\text{Cu}\cdots\text{N}(\text{His233})$, and $\text{Cu}\cdots\text{N}(\text{His283})$ distances are lengthened further. However, overall, the geometries of the optimized $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+_{2c}(\text{Y237})(\text{H376H}^+)$ and $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+_{2c}(\text{Y237})$ structures are very similar. In both structures, the angle of $\text{OH}^--\text{Cu}-\text{N}(\text{His282})$ is 176° , which is close to a linear structure. The relative energy of the $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+_{2c}(\text{Y237})$ state is $\sim 17 \text{ kcal mol}^{-1}$ lower than that of its corresponding 4-coordinate Cu^+ state $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--\text{Cu}^+(\text{Y237})$, as given in Table 4. Therefore, with OH^- binding to Cu^+ , the DNC favors the 2-coordinate Cu^+ structure. The calculated Mössbauer properties of both the $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^--$

Table 5. Calculated Properties for the DNC Models with a 3- or 2- Coordinate (3c/2c) Cu⁺ Site in which His233/His283 Dissociated from the Cu²⁺^a

state	geometry										net spin		⁵⁷ Fe ²⁺ Mössbauer	
	Fe-N (H384)	Fe...O	Cu-O	∠Fe-O-Cu	Fe...Cu	Cu-N (H233)	Cu-N (H282)	Cu-N (H283)	∠O-Cu-N (H282)	Q	Fe ²⁺	Cu ⁺	δ	ΔE _Q
Fe ²⁺ ,HS...OH ⁻ -Cu ⁺ _{2c} (Y237) (H376H ⁺) ^b	2.20	3.95	1.85	101	4.66	3.02	1.90	4.03	176	0	3.89	0.00	0.91	2.17
Fe ²⁺ ,HS...OH ⁻ -Cu ⁺ _{2c} (Y237) ^c	2.19	4.19	1.86	97	4.78	3.09	1.90	4.25	176	-1	3.89	0.00	0.91	2.28
Fe ²⁺ ,HS...OH ⁻ -Cu ⁺ _{3c} (Y237) (H376H ⁺) ^{(1),d}	2.21	3.86	1.92	113	4.94	2.32	2.00	2.60	160	0	3.85	0.20	0.92	1.32
Fe ²⁺ ,HS...OH ⁻ -Cu ⁺ _{3c} (Y237) (H376H ⁺) ^{(2),d}	2.20	3.86	1.91	111	4.89	2.36	1.97	2.83	163	0	3.86	0.15	0.92	1.51
Fe ²⁺ ,HS...OH ⁻ -Cu ⁺ _{3c} (Y237) (H376H ⁺) ^{(3),d}	2.21	3.82	1.89	110	4.80	2.44	1.95	3.09	166	0	3.87	0.11	0.92	1.75
Fe ²⁺ ,HS...OH ⁻ -Cu ⁺ _{2c} (Y237) (H376H ⁺) ^{(4),d}	2.21	3.78	1.89	108	4.71	2.64	1.92	3.30	172	0	3.88	0.04	0.91	2.02
Fe ²⁺ ,HS...OH ⁻ -Cu ⁺ _{3c} (Y237) ^{(1),e}	2.22	3.85	1.91	114	4.94	2.32	2.00	2.60	159	-1	3.85	0.20	0.92	1.31
Fe ²⁺ ,HS...OH ⁻ -Cu ⁺ _{3c} (Y237) ^{(2),e}	2.21	3.85	1.90	112	4.89	2.36	1.97	2.83	162	-1	3.86	0.15	0.92	1.52
Fe ²⁺ ,HS...OH ⁻ -Cu ⁺ _{3c} (Y237) ^{(3),e}	2.21	3.83	1.89	109	4.80	2.44	1.95	3.09	166	-1	3.87	0.11	0.92	1.75
Fe ²⁺ ,HS...OH ⁻ -Cu ⁺ _{2c} (Y237) ^{(4),e}	2.21	3.77	1.87	108	4.71	2.64	1.92	3.30	171	-1	3.88	0.04	0.92	2.06
Fe ²⁺ ,HS...H ₂ O-Cu ⁺ _{3c} (Y237) ^f	2.19	4.19	2.35	106	5.35	1.99	1.94	4.45	132	0	3.89	0.00	0.91	2.31
Fe ²⁺ ,HS...H ₂ O...Cu ⁺ _{2c} (Y237) ^f	2.20	4.76	3.13	95	5.92	1.92	1.91	5.40	-378.7	0	3.88	0.00	0.91	2.24

^aThe properties include geometries (\AA , °), electronic energies (E , offset by -25100 kcal mol⁻¹) obtained from the all-electron/all-TZP single-point energy calculations on the optimized geometries, pK_a 's, the net charge (Q) of the clusters, Mulliken net spin polarizations for the Fe and Cu sites, and the ⁵⁷Fe Mössbauer isomer shifts (δ , mm s⁻¹) and quadrupole splittings (ΔE_Q , mm s⁻¹). ^bBoth His233 and His283 side chains dissociated from the Cu⁺ site during the geometry optimization. ^cAfter deleting the H⁺ of His376⁺ in Fe²⁺,HS...OH⁻-Cu⁺_{2c}(Y237)(H376H⁺), and geometry optimizing the structure, the Cu⁺ site remained in the 2c configuration. ^dFour structures (1–4) with a 3c or 2c Cu⁺ site, which were taken from the geometry optimization trajectory of the Fe²⁺,HS...OH⁻-Cu⁺_{2c}(Y237)(H376H⁺) state. ^eSee footnote d above. These are the four corresponding structures of the Fe²⁺,HS...OH⁻-Cu⁺_{2c}(Y237) state. ^fWhen protonating the OH⁻ ligand in Fe²⁺,HS...OH⁻-Cu⁺_{2c}(Y237) and optimizing the geometry, the Cu⁺ coordination state changes from Fe²⁺,HS...H₂O-Cu⁺_{3c}(Y237) (left in Figure 7) to Fe²⁺,HS...H₂O-Cu⁺_{2c}(Y237) (middle in Figure 7) and to Fe²⁺,HS...H₂O...Cu⁺_{2c}(Y237) (right in Figure 7).

$\text{Cu}^+_{2c}(\text{Y237})(\text{H376H}^+)$ and $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{2c}(\text{Y237})$ states ($\delta = 0.91 \text{ mm s}^{-1}$, $\Delta E_Q = 2.17/2.28 \text{ mm s}^{-1}$) are now similar to those of the open $\text{Fe}^{2+, \text{HS}}\cdots\text{Cu}^+(\text{Y237}^-/\text{Y237})$ structures given in Table 2 and the structure with a dangling H_2O molecule (in Table 4) between the $\text{Fe}^{2+, \text{HS}}$ and Cu^+ sites. In other words, the $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{2c}(\text{Y237})(\text{H376H}^+)$ and $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{2c}(\text{Y237})$ states with 2-coordinate Cu^+ do not represent the DNC of the first (majority) $^{57}\text{Fe}_{a3}^{2+}$ component observed in the Mössbauer experiments¹⁸ but are entirely compatible with the observed second (minority) $^{57}\text{Fe}_{a3}^{2+}$ Mössbauer experimental parameters ($\delta = 0.92 \text{ mm s}^{-1}$, $\Delta E_Q = 2.34 \text{ mm s}^{-1}$).

Reviewing the X-ray crystal structure of 3EH4, we found that the side chain of Trp229 is very close (with the closest atom to atom distance being 3.4 Å) and parallel to the side chain of His283 (see Figure 6). During the $\text{Cu}^+_{4c} \rightarrow \text{Cu}^+_{2c}$ transition in

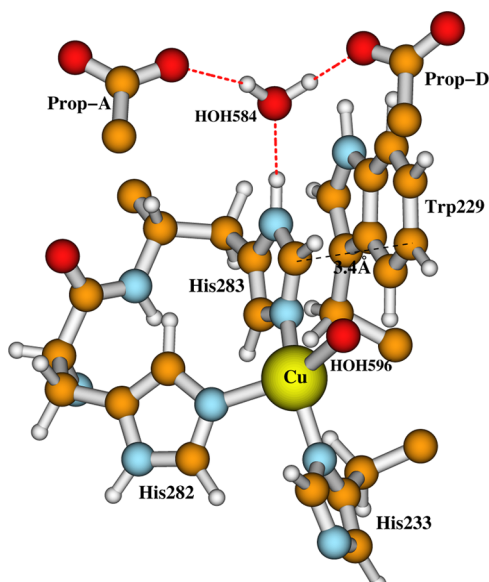


Figure 6. Taken from the X-ray crystal structure of 3EH4, which shows that the side chain of Trp229 is very close (with the closest atom to atom distance being 3.4 Å) and parallel to the side chain of His283. The steric interaction between the two side chains prevents His283 from freely moving away from the Cu^+ site.

the geometry optimization of the $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237}) - (\text{H376H}^+)$ state, the side chain of His283 tilted up and away from the Cu^+ and moved toward the side chain of Trp229 (not included in the current model calculation). Therefore, we propose that because of the steric interactions and the potential energy barriers encountered upon moving the His233 and His283 side chains away from Cu^+ , the $\text{OH}^- - \text{Cu}^+$ site is frequently trapped in the Cu^+_{4c} or Cu^+_{3c} configurations, particularly under the low temperature in Mössbauer experiments.

To test this idea, we picked four structures with a “maximum gradient” of $<0.005 \text{ au}/\text{Å}$ along the geometry optimization trajectory of $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237})(\text{H376H}^+)$ to represent the middle structures of the $\text{Cu}^+_{4c} \rightarrow \text{Cu}^+_{2c}$ transition. The calculated properties of the four $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{3c/2c}(\text{Y237}) - (\text{H376H}^+)^{(1-4)}$ structures from all-electron/all-TZP single-point energy calculations are also given in Table 5 (rows 3–6 with footnote d). We noticed that the structural and coordination state of the Cu^+ site indeed influences the electric field at the Fe^{2+} site and therefore affects the $^{57}\text{Fe}^{2+}$ Mössbauer,

especially the quadruple splitting property. The Cu^+_{4c} structure of $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237}^-)$ in Table 4 yields a large calculated isomer shift (0.93 mm s^{-1}) and a small quadrupole splitting value (1.01 mm s^{-1}). Next, in the Cu^+_{4c} structure of $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+(\text{Y237})$ in Table 4, the $\text{Cu}-\text{N}(\text{His233})$ and $\text{Cu}-\text{N}(\text{His283})$ distances are increased and the $\text{Cu}-\text{O}$, $\text{Cu}-\text{N}(282)$, and $\text{Fe}\cdots\text{Cu}$ distances are decreased; meanwhile, the calculated $^{57}\text{Fe}^{2+}$ Mössbauer isomer shift (0.92 mm s^{-1}) is decreased, and its quadrupole splitting value (1.20 mm s^{-1}) is increased. Further, the trend continues in the four $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{3c/2c}(\text{Y237})(\text{H376H}^+)^{(1-4)}$ structures in Table 5, that is, with a decrease in the Cu^+ coordination number and the $\text{Fe}^{2+}\cdots\text{Cu}^+$ distance, the calculated $^{57}\text{Fe}^{2+}$ Mössbauer isomer shift decreases and its quadrupole splitting value increases. In particular, the ΔE_Q values (1.32 and 1.51 mm s^{-1}) of $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{3c}(\text{Y237})(\text{H376H}^+)^{(1)}$ and $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{3c}(\text{Y237})(\text{H376H}^+)^{(2)}$ structures are very close to the observed $\Delta E_Q = 1.43 \text{ mm s}^{-1}$ of the first $^{57}\text{Fe}_{a3}^{2+}$ Mössbauer component.¹⁸ It is therefore probable that the DNC structure of the first (majority) $^{57}\text{Fe}_{a3}^{2+}$ component observed in the Mössbauer experiment is trapped at a structure similar to the ones in $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{3c}(\text{Y237})(\text{H376H}^+)^{(1)/(2)}$ at low temperature.

It is not evident whether the His376 side chain is typically protonated or not. To see how the protonation state of His376 affects the Mössbauer properties of the $^{57}\text{Fe}^{2+}$ site, we deleted the added proton on His376⁺ in $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{3c/2c}(\text{Y237}) - (\text{H376H}^+)^{(1-4)}$ and optimized the geometries with fixed atom positions of Fe, Cu, N(His233), N(His283), and N(His283). The calculated properties of these four partially optimized DNC structures, $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{3c/2c}(\text{Y237})^{(1-4)}$ are also given in Table 5 (rows 7–10 with footnote e). The calculated net spin populations on Fe^{2+} and Cu^+ , and the Mössbauer isomer shifts and quadrupole splittings of the four $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{3c/2c}(\text{Y237})^{(1-4)}$ structures, are essentially the same as the corresponding ones of $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{3c/2c}(\text{Y237}) - (\text{H376H}^+)^{(1-4)}$. Therefore, the protonation state of the His376 side chain does not affect the Mössbauer properties of the $^{57}\text{Fe}^{2+}$ site.

The calculated $\text{p}K_a$ s of His376- H^+ are larger than 10, which indicates that the system favors this charge neutral state. Without including the carboxylate group of the Glu126B side chain (which is within a strong H-bonding distance of N_{e2} of the His376 side chain) in the DNC quantum cluster model, it is not clear whether the His376 side chain would be protonated in a realistic protein environment.

If we use the energies of $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{2c}(\text{Y237})$ (second row of Table 5) and $\text{Fe}^{2+, \text{HS}}\cdots\text{H}_2\text{O} - \text{Cu}^+(\text{Y237})$ (second row of Table 4) to calculate the $\text{p}K_a$ of the H_2O molecule, we obtain a value of 12.7. These structures differ very significantly in Cu^+ coordination geometry. We then protonated the OH^- ligand in the optimized structure $\text{Fe}^{2+, \text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{2c}(\text{Y237})$ (see the left structure in Figure 7) and performed geometry optimization to see if the linear $\text{H}_2\text{O} - \text{Cu}^+_{2c} - \text{N}(\text{His282})$ configuration would persist. However, during the geometry optimization, the $\text{H}_2\text{O} - \text{Cu}^+$ distance was gradually lengthened and the $\text{Cu}^+ - \text{N}(\text{His233})$ distance shortened, and the Cu^+ site became 3-coordinate with ligands His233, His282, and H_2O (see the middle structure in Figure 7).

The relative energy of a middle $\text{Fe}^{2+, \text{HS}}\cdots\text{H}_2\text{O} - \text{Cu}^+_{3c}(\text{Y237})$ state ($-368.9 \text{ kcal mol}^{-1}$) (footnote f in Table 5) is similar to that ($-366.8 \text{ kcal mol}^{-1}$) of the $\text{Fe}^{2+, \text{HS}}\cdots\text{H}_2\text{O} - \text{Cu}^+(\text{Y237})$ state (with three His ligands at the Cu^+ site and a weak $\text{Cu}^+\cdots\text{H}_2\text{O}$

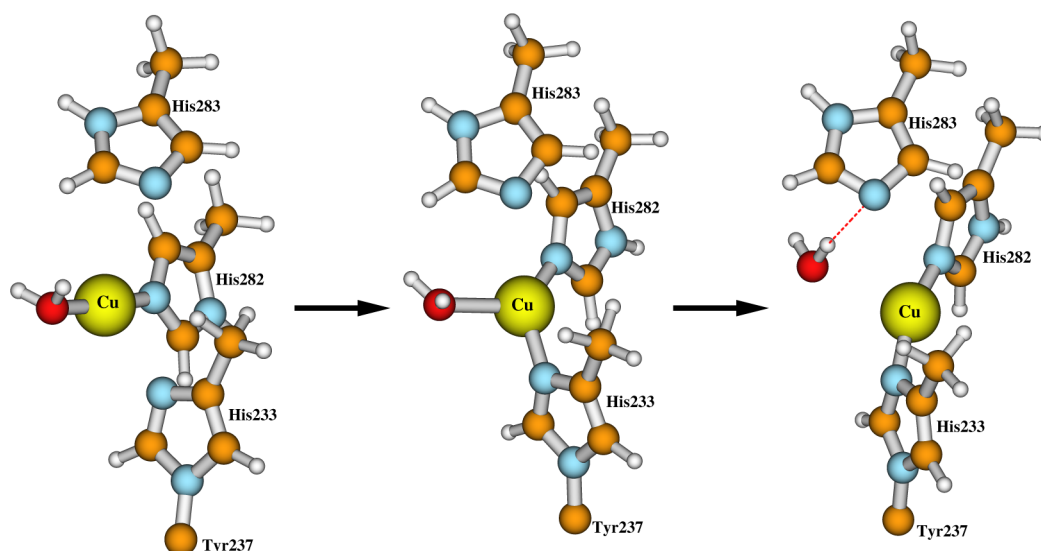


Figure 7. Changing of the Cu^+ coordination state during the geometry optimization calculation after manually protonating the OH^- ligand in $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- - \text{Cu}^+_{2c}(\text{Y237})$ (second row of Table 5). Left: The initial linear $\text{H}_2\text{O} - \text{Cu}^+_{2c} - \text{N}(\text{His282})$ structure when adding a proton to the OH^- ligand. Other parts of the model cluster are similar to those in Figure 5 but with a neutral His376 side chain. Middle: During the geometry optimization, the $\text{O} - \text{Cu}^+$ distance lengthens, the His233 side chain rebinds with Cu^+ , and a 3-coordinate Cu^+ configuration is formed (see $\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O} - \text{Cu}^+_{3c}(\text{Y237})$ with footnote f in Table 5). Right: The H_2O ligand eventually dissociates from the Cu^+ site, and a 2-coordinate $\text{N}(\text{His233}) - \text{Cu}^+_{2c} - \text{N}(\text{His282})$ configuration is obtained (see $\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O}\cdots\text{Cu}^+_{2c}(\text{Y237})$ in Table 5).

interaction), as given in Table 4. As the geometry optimization continued, the H_2O molecule and the Cu^+ center continued moving away from each other, and finally the H_2O dissociated from the Cu^+ site and H-bonded with the His283 side chain. The 2-coordinate $\text{His233} - \text{Cu}^+_{2c} - \text{His282}$ center is shown on the right side of Figure 7, and the calculated properties of this model cluster are given in the last row of Table 5. This structure has a very long $\text{Fe}\cdots\text{Cu}$ distance of 5.92 Å and is ~ 10 kcal mol $^{-1}$ lower in energy than the middle $\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O} - \text{Cu}^+_{3c}(\text{Y237})$ state. We do not know if such an $\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O}\cdots\text{Cu}^+_{2c}(\text{Y237})$ state is feasible during the catalytic cycle given other protein geometric constraints. For example, both $\text{Fe}\cdots\text{Cu}$ (5.92 Å) and $\text{Cu} - \text{N}(\text{His283})$ (5.40 Å) distances are very long. However, our calculations indeed show the mobility of the His233 and His283 side chains, which can be potential proton loading sites during the catalytic cycle and proton pumping.

We note that the calculated Mössbauer properties of both the $\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O} - \text{Cu}^+_{3c}(\text{Y237})$ and $\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O}\cdots\text{Cu}^+_{2c}(\text{Y237})$ states ($\delta = 0.91$ mm s $^{-1}$, $\Delta E_{\text{O}} = 2.31/2.24$ mm s $^{-1}$) are also similar to those of the open $\text{Fe}^{2+,\text{HS}}\cdots\text{Cu}^+(\text{Y237}^-/\text{Y237})$ structures given in Table 2 and are consistent with the observed Mössbauer properties for the second $^{57}\text{Fe}_{\text{a}_3^{2+}}$ component. Therefore, up until now, one cannot draw an exclusive conclusion about the DNC structure of the second observed $^{57}\text{Fe}_{\text{a}_3^{2+}}$ component in the Mössbauer experiments.¹⁸

3.5. $\text{Fe}^{2+} - (\text{HO}_2)^- - \text{Cu}^+$ DNC Models from 3S8G. In ref 23, on the basis of a series of structural calculations and comparisons, we proposed that the dioxygen species found in the DNC of 3S8G is HO_2^- with the majority species having the proton of the HO_2^- residing on the oxygen atom that is closer to the Fe^{2+} site in the $\text{Fe}^{2+} - (\text{HO} - \text{O})^- - \text{Cu}^+$ conformation. Details of the optimized 3S8G- $\text{Fe}^{2+,\text{LS/IS/HS}} - (\text{HO}_2)^- - \text{Cu}^+(\text{Y237}^-/\text{Y237})$ DNC structures are given in Tables 4–8 of ref 23. We have now performed Mössbauer property calculations on those structures. It turns out that the calculated Mössbauer properties of the 3S8G- $\text{Fe}^{2+,\text{LS/IS/HS}} - (\text{HO}_2)^- - \text{Cu}^+(\text{Y237}^-/\text{Y237})$ DNC models, which are given in Tables

S1 and S2 in the Supporting Information, are not consistent with either of the observed $^{57}\text{Fe}_{\text{a}_3^{2+}}$ Mössbauer properties. This also supports the conclusion that the HO_2^- species found in the 3S8G X-ray crystal structure arises from the recombination of two radiation produced $\text{HO}\cdot$ radicals (product of water radiolysis) formed very near or even between the two metal sites,¹⁵ or comes from the photoreaction of the $\text{H}_2\text{O}/\text{OH}^-$ between the as-isolated oxidized Fe^{3+} and Cu^{2+} sites in the X-ray beam.²³ Next, we will examine if $\text{Fe}^{3+} - \text{OH}^- \cdots \text{H}_2\text{O} - \text{Cu}^{2+}$ - and $\text{Fe}^{3+} - \text{H}_2\text{O} \cdots \text{OH}^- - \text{Cu}^{2+}$ -type model clusters represent the DNC structures observed in the Mössbauer experiments for the oxidized ba_3 CcO from *Tt*.

4. RESULTS AND DISCUSSION ON THE $\text{Fe}^{3+}/\text{Cu}^{2+}$ OXIDIZED STATE DNC MODEL CALCULATIONS

4.1. The $\text{Fe}^{3+} - \text{OH}^- \cdots \text{H}_2\text{O} - \text{Cu}^{2+}$ and $\text{Fe}^{3+} - \text{H}_2\text{O} \cdots \text{OH}^- - \text{Cu}^{2+}$ DNC Models from 3S8G. The initial model structures in this subsection are constructed by replacing the dioxygen atoms observed in the DNC of 3S8G (Figures 1 and 2 of ref 23) with the $\text{OH}^- \cdots \text{H}_2\text{O}/\text{H}_2\text{O} \cdots \text{OH}^-$ species. The central structures of the $\text{Fe}^{3+} - \text{OH}^- \cdots \text{H}_2\text{O} - \text{Cu}^{2+}$ and $\text{Fe}^{3+} - \text{H}_2\text{O} \cdots \text{OH}^- - \text{Cu}^{2+}$ model clusters are shown in Figures 8 and 9, respectively.

We have optimized the geometries and calculated the Mössbauer properties of the $\text{Fe}^{3+} - \text{OH}^- \cdots \text{H}_2\text{O} - \text{Cu}^{2+}$ and $\text{Fe}^{3+} - \text{H}_2\text{O} \cdots \text{OH}^- - \text{Cu}^{2+}$ model clusters with the Tyr237 side chain in either the deprotonated (Y237^-) or neutral protonated (Y237) state and with Fe^{3+} in either the LS, IS, or HS state, which is F- or AF-coupled to the Cu^{2+} site. The calculated properties are given in Tables 6 and 7, respectively, for the $\text{Fe}^{3+} - \text{OH}^- \cdots \text{H}_2\text{O} - \text{Cu}^{2+}$ and $\text{Fe}^{3+} - \text{H}_2\text{O} \cdots \text{OH}^- - \text{Cu}^{2+}$ model clusters.

In Table 6, we failed to obtain the optimized $\text{Fe}^{3+,\text{IS}} - \text{OH}^- \cdots \text{H}_2\text{O} - \text{Cu}^{2+}(\text{Y237}^-)(\text{AF})$ and $\text{Fe}^{3+,\text{HS}} - \text{OH}^- \cdots \text{H}_2\text{O} - \text{Cu}^{2+}(\text{Y237}^-)(\text{AF})$ structures, but instead, the geometry optimizations of these two states led to the lower-energy $\text{Fe}^{3+,\text{LS}} - \text{OH}^- \cdots \text{H}_2\text{O} - \text{Cu}^{2+}(\text{Y237}^-)(\text{F})$ and $\text{Fe}^{3+,\text{IS}} - \text{OH}^- \cdots \text{H}_2\text{O} - \text{Cu}^{2+}(\text{Y237}^-)(\text{F})$ states, respectively. The properties given for $\text{Fe}^{3+,\text{IS}} - \text{OH}^- \cdots \text{H}_2\text{O} - \text{Cu}^{2+}(\text{Y237}^-)(\text{AF})$ and $\text{Fe}^{3+,\text{HS}} - \text{OH}^- \cdots \text{H}_2\text{O} - \text{Cu}^{2+}(\text{Y237}^-)$

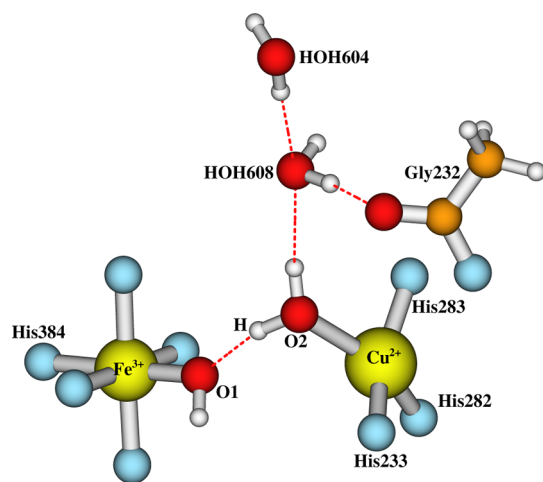


Figure 8. Detailed central portion of the $\text{Fe}^{3+}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}$ cluster generated by modifying the oxidized 3S8G DNC model. The rest of the model cluster is shown in Figures 1 and 2 of ref 23.

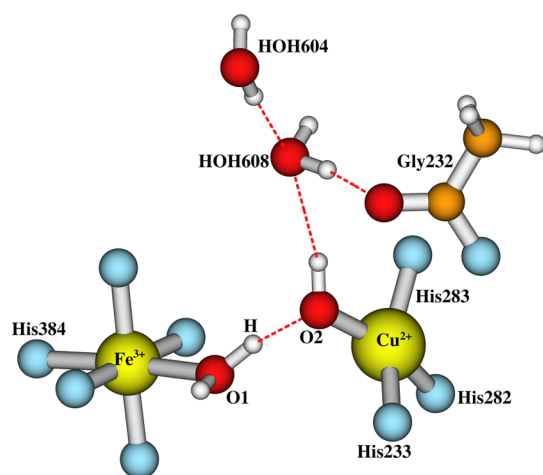


Figure 9. Detailed central portion of the $\text{Fe}^{3+}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}$ cluster generated by modifying the oxidized 3S8G DNC model. The rest of the model cluster is shown in Figures 1 and 2 of ref 23.

(AF) states were obtained from the broken-symmetry single-point energy calculations on the optimized $\text{Fe}^{3+,\text{LS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237}^-)(\text{F})$ and $\text{Fe}^{3+,\text{HS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237}^-)(\text{F})$ geometries, respectively. We did not obtain the optimized $\text{Fe}^{3+,\text{LS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237})(\text{F}/\text{AF})$ structures (neutral Tyr237) either because the proton on the Cu^{2+} -bound H_2O ligand, which originally H-bonded to the $\text{Fe}^{3+,\text{LS}}$ -bound OH^{\ominus} ligand, spontaneously transferred to the OH^{\ominus} during the geometry optimizations, and the $\text{Fe}^{3+,\text{LS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}(\text{Y237})(\text{F}/\text{AF})$ states were obtained.

The calculated pK_a values (3.4–5.5) in Table 6 show that the Tyr237 side chain favors the deprotonated anionic state (Y237⁻) when the DNC has the $\text{Fe}^{3+}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}$ -type structure no matter whether Fe^{3+} is in LS or HS state and F- or AF-coupled with Cu^{2+} .

The $\text{Fe}^{3+,\text{LS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237}^-)(\text{F})$ and $\text{Fe}^{3+,\text{LS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237}^-)(\text{AF})$ states have very similar energies (with only 0.5 kcal mol^{-1} difference), and they are $2\text{--}3\text{ kcal mol}^{-1}$ and $>5\text{ kcal mol}^{-1}$ lower in energy than the $\text{Fe}^{3+,\text{LS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237})(\text{F}/\text{AF})$ and $\text{Fe}^{3+,\text{HS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237})(\text{F}/\text{AF})$ states, respectively. Therefore, if the oxidized DNC has the $\text{Fe}^{3+}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}$ -type structure at 4.2 K, it is expected to

be in the $\text{Fe}^{3+,\text{LS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ state. The calculated $\Delta E_Q = 2.53/2.16\text{ mm s}^{-1}$ for the $\text{Fe}^{3+,\text{LS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states are in good agreement with the experimentally observed value $\Delta E_Q = 2.24\text{ mm s}^{-1}$ for the “low-spin” $^{57}\text{Fe}_{\text{a}3}^{3+}$ species at $4.2\text{ K} < T < 190\text{ K}$.¹⁸ The calculated $\delta = 0.19/0.21\text{ mm s}^{-1}$ of these two states are $\sim 0.1\text{ mm s}^{-1}$ smaller than, but are still reasonably consistent with, the corresponding observed value of $\delta = 0.29\text{ mm s}^{-1}$ (see Figure 1).

For the $\text{Fe}^{3+}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}$ DNC model calculations in Table 7, during geometry optimizations starting from the $\text{Fe}^{3+,\text{LS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ clusters (see Figure 9), the proton on the $\text{Fe}^{3+,\text{LS}}$ -bound H_2O ligand, which originally H-bonded to the Cu^{2+} -bound OH^{\ominus} ligand, transferred to the OH^{\ominus} . As a result, the $\text{Fe}^{3+,\text{LS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states (in Table 6 and Figure 8) were obtained. Therefore, Table 7 shows only the $\text{Fe}^{3+,\text{LS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}$ and $\text{Fe}^{3+,\text{HS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}$ state calculations; these forms are stable under geometry optimization.

The calculated pK_a s of the Tyr237 side chain for the $\text{Fe}^{3+,\text{LS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}$ and $\text{Fe}^{3+,\text{HS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}$ clusters are 6.6–7.2 (Table 7). Considering the uncertainty of DFT calculations and the fact that the pK_a calculations are for the equilibrium state at room temperature, it is not certain whether the Tyr237 side chains of the $\text{Fe}^{3+,\text{LS/HS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}$ -type DNCs are in the neutral or anionic state under the Mössbauer experimental conditions. The very short O \cdots O distance (2.66 Å) between the Tyr237 side chain and the geranyl side chain of a₃-heme in 3S8G implies that the Tyr237 is in the deprotonated anion form in this radiolytically reduced X-ray crystal structure.²³ However, in ref 23, our calculations have shown that Tyr237 energetically favors the neutral protonated form in the reduced $\text{Fe}^{2+,\text{LS/IS/HS}}\text{-(HO}_2\text{)}^{\ominus}\text{-Cu}^{2+}$ DNC. Therefore, Tyr237 is likely in the anionic state in the oxidized protein before the X-ray irradiation and is trapped at very low temperature after the irradiation.

The $\text{Fe}^{3+,\text{LS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states are 4.3–4.7 kcal mol⁻¹ lower in energy than the $\text{Fe}^{3+,\text{HS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states (Table 7). In addition, the energies of the $\text{Fe}^{3+,\text{LS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states (Table 7) are very similar to the energies of the $\text{Fe}^{3+,\text{LS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states (Table 6). The calculated quadrupole splittings for the $\text{Fe}^{3+,\text{LS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states ($2.42/2.42\text{ mm s}^{-1}$) are also similar to those for the $\text{Fe}^{3+,\text{LS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states ($2.53/2.16\text{ mm s}^{-1}$). Further, the average (avg) of the four calculated isomer shifts and quadrupole splittings ($\delta^{\text{avg}} = 0.30\text{ mm s}^{-1}$, $\Delta E_Q^{\text{avg}} = 2.38\text{ mm s}^{-1}$) of the $\text{Fe}^{3+,\text{LS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ and $\text{Fe}^{3+,\text{LS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states agrees very well with the experimentally observed spectra ($\delta = 0.29\text{ mm s}^{-1}$, $\Delta E_Q = 2.24\text{ mm s}^{-1}$), which was suggested as a “low-spin” $^{57}\text{Fe}_{\text{a}3}^{3+}$ species at $4.2\text{ K} < T < 190\text{ K}$.¹⁸ We therefore propose that this “low-spin” $^{57}\text{Fe}_{\text{a}3}^{3+}$ species observed in the Mössbauer experiment at $4.2\text{ K} < T < 190\text{ K}$ is a mixture of the $\text{Fe}^{3+,\text{LS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ and $\text{Fe}^{3+,\text{LS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states.

It was reported that this “low-spin” $^{57}\text{Fe}_{\text{a}3}^{3+}$ component underwent a transition to a “high-spin” species with $\Delta E_Q \approx 1\text{ mm s}^{-1}$ when the temperature was increased above 190 K, and the transition was complete at 245 K.¹⁸ The isomer shift of this new “high-spin” $^{57}\text{Fe}_{\text{a}3}^{3+}$ species was not given in ref 18. We notice that the calculated ΔE_Q values of the $\text{Fe}^{3+,\text{LS}}\text{-OH}^{\ominus}\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ (in Table 6) and $\text{Fe}^{3+,\text{HS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ (in Table 6) and $\text{Fe}^{3+,\text{HS}}\text{-H}_2\text{O}\cdots\text{OH}^{\ominus}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states are in good agreement with the experimentally observed value $\Delta E_Q = 2.24\text{ mm s}^{-1}$ for the “low-spin” $^{57}\text{Fe}_{\text{a}3}^{3+}$ species at $4.2\text{ K} < T < 190\text{ K}$.¹⁸ The calculated $\delta = 0.19/0.21\text{ mm s}^{-1}$ of these two states are $\sim 0.1\text{ mm s}^{-1}$ smaller than, but are still reasonably consistent with, the corresponding observed value of $\delta = 0.29\text{ mm s}^{-1}$ (see Figure 1).

Table 6. Calculated Properties of the Oxidized $\text{Fe}^{3+}\text{-OH}^-\cdots\text{H}_2\text{O-Cu}^{2+}(\text{Y237}^-/\text{Y237})$ Clusters Generated from the DNC of the X-ray Crystal Structure 3S8G^a

state ^b	geometry										net spin		⁵⁷ Fe ³⁺ Mössbauer			
	Fe-N (H384)	Fe-O1	Cu-O2	O1...O2	Fe...Cu	Cu-N (H233)	Cu-N (H282)	Cu-N (H283)	O...O (Y237)	E ^c	pK _a (Y237)	Q	Fe ³⁺	Cu ²⁺	δ	ΔE _Q
Fe ^{3+,LS} -OH ⁻ ...H ₂ O-Cu ²⁺ (Y237 ⁻)(F)	2.09	1.84	2.36	2.66	5.47	2.05	2.09	2.04	3.04	-58.8		0	1.04	0.33	0.19	2.53
Fe ^{3+,LS} -OH ⁻ ...H ₂ O-Cu ²⁺ (Y237 ⁻)(AF)	2.09	1.85	2.32	2.63	5.40	2.05	2.09	2.04	3.02	-58.3		0	0.95	-0.35	0.21	2.16
Fe ^{3+,HS} -OH ⁻ ...H ₂ O-Cu ²⁺ (Y237 ⁻)(F)	2.43	2.00	2.20	2.51	5.31	2.07	2.09	2.06	3.08	-56.1		0	2.71	0.38	0.39	1.57
Fe ^{3+,HS} -OH ⁻ ...H ₂ O-Cu ²⁺ (Y237 ⁻)(AF) ^d	2.42	1.95	2.23	2.59	5.24	2.08	2.07	2.05	3.01	-51.1		0	4.10	0.38	0.36	0.21
Fe ^{3+,HS} -OH ⁻ ...H ₂ O-Cu ²⁺ (Y237 ⁻)(AF) ^e										-53.0		0	4.10	-0.37	0.36	0.21
Fe ^{3+,LS} -OH ⁻ ...H ₂ O-Cu ²⁺ (Y237)(F)	2.08	1.83	2.28	2.68	5.45	2.06	2.10	2.03	3.29	-49.7	5.0	1	1.28	0.39	0.14	2.07
Fe ^{3+,LS} -OH ⁻ ...H ₂ O-Cu ²⁺ (Y237)(AF)	2.09	1.85	2.13	2.58	5.41	2.07	2.08	2.02	3.39	-48.1	3.4	1	0.85	-0.46	0.19	2.25
Fe ^{3+,HS} -OH ⁻ ...H ₂ O-Cu ²⁺ (Y237)(F)	2.38	1.99	2.05	2.48	5.26	2.07	2.05	2.02	3.42	-43.8	5.5	1	4.12	0.51	0.39	0.32
Fe ^{3+,HS} -OH ⁻ ...H ₂ O-Cu ²⁺ (Y237)(AF)	2.42	1.97	2.06	2.49	5.25	2.07	2.06	2.02	3.50	-45.5	5.4	1	4.10	-0.50	0.36	0.39

^aThe properties include geometries (Å), electronic energies (E, offset by -28200 kcal mol⁻¹ in this table and in Table 7) obtained from the all-electron/all-TZP single-point energy calculations on the optimized geometries, pK_a's, the net charge (Q) of the clusters, Mulliken net spin polarizations for the Fe and Cu sites, and the ⁵⁷Fe Mössbauer isomer shifts (δ, mm s⁻¹) and quadrupole splittings (ΔE_Q, mm s⁻¹). ^bF stands for ferromagnetically coupled and AF for antiferromagnetically coupled. ^cThe energies calculated for the AF-coupled states are broken-symmetry state energies. ^dA broken-symmetry single-point energy calculation on the Fe^{3+,HS}-OH⁻...H₂O-Cu²⁺(Y237⁻)(F) optimized geometry. ^eA broken-symmetry single-point energy calculation on the Fe^{3+,LS}-OH⁻...H₂O-Cu²⁺(Y237⁻)(F) optimized geometry.

Table 7. Calculated Properties of the Oxidized $\text{Fe}^{3+}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237}^-/\text{Y237})$ Clusters Generated from the DNC of the X-ray Crystal Structure 3S8G^a

state ^b	geometry										E^c	pK_a (Y237)	Q	net spin		⁵⁷ Fe ³⁺ Mössbauer	
	Fe–N (H384)	Fe–O1	Cu–O2	O1...O2	Fe...Cu	Cu–N (H233)	Cu–N (H282)	Cu–N (H283)	O...O (Y237)	Fe ³⁺				Cu ²⁺	δ	ΔE_Q	
$\text{Fe}^{3+,LS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237}^-)(\text{F})$	2.27	2.29	1.94	2.58	5.48	2.09	2.06	2.06	2.93	–58.7	0	2.74	0.50	0.40	2.42		
$\text{Fe}^{3+,LS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237}^-)(\text{AF})$	2.26	2.28	1.94	2.57	5.48	2.08	2.06	2.06	2.91	–58.4	0	2.74	–0.50	0.39	2.42		
$\text{Fe}^{3+,HS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237}^-)(\text{F})$	2.20	2.31	1.93	2.60	5.53	2.09	2.05	2.05	3.02	–54.1	0	4.06	0.51	0.52	1.49		
$\text{Fe}^{3+,HS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237}^-)(\text{AF})$	2.19	2.29	1.93	2.58	5.53	2.09	2.05	2.05	2.99	–54.0	0	4.07	–0.52	0.50	1.40		
$\text{Fe}^{3+,LS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237})(\text{F})$	2.27	2.29	1.93	2.59	5.49	2.10	2.07	2.05	3.39	–51.9	6.6	1	2.75	0.52	0.39	2.49	
$\text{Fe}^{3+,LS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237})(\text{AF})$	2.27	2.27	1.93	2.57	5.47	2.11	2.06	2.05	3.44	–52.3	6.9	1	2.74	–0.52	0.40	2.47	
$\text{Fe}^{3+,HS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237})(\text{F})$	2.21	2.24	1.93	2.57	5.51	2.11	2.06	2.05	3.46	–48.8	7.0	1	4.11	0.52	0.47	0.88	
$\text{Fe}^{3+,HS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237})(\text{AF})^d$										–48.9	7.2	1	4.11	–0.52	0.47	0.88	

^aThe properties include geometries (Å), electronic energies (E , offset by $-28200 \text{ kcal mol}^{-1}$ in this table and in Table 6) obtained from the all-electron/all-TZP single-point energy calculations on the optimized geometries, pK_a 's, the net charge (Q) of the clusters, Mulliken net spin polarizations for the Fe and Cu sites, and the ⁵⁷Fe Mössbauer isomer shifts (δ , mm s^{-1}) and quadrupole splittings (ΔE_Q , mm s^{-1}). ^bF stands for ferromagnetically coupled and AF for antiferromagnetically coupled. ^cThe energies calculated for the AF-coupled states are broken-symmetry state energies. ^dA broken-symmetry single-point energy calculation on the optimized $\text{Fe}^{3+,HS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237})(\text{F})$ geometry.

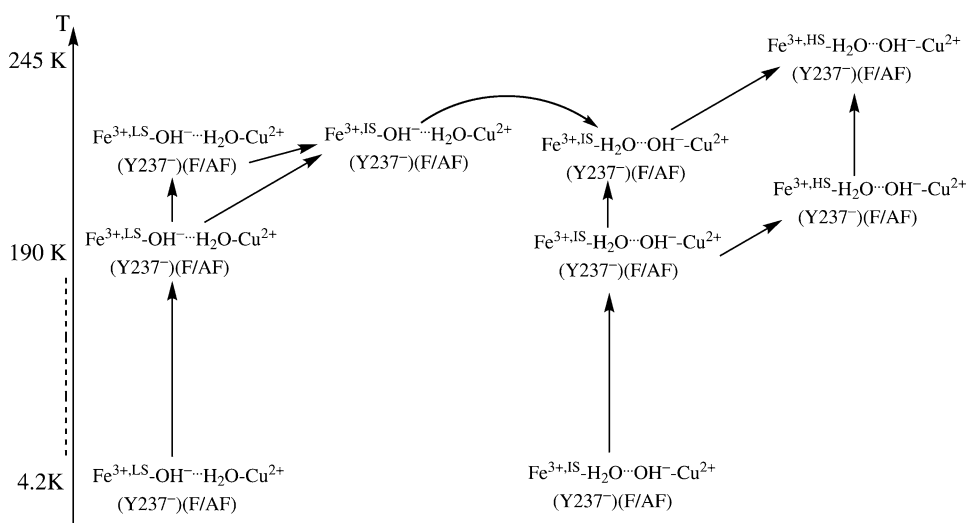


Figure 10. We propose that the so-called “low-spin” ⁵⁷Fe_{a3}³⁺ species observed in the Mössbauer experiments at $4.2 \text{ K} < T < 190 \text{ K}$ is a mixture of the $\text{Fe}^{3+,LS}\text{-OH}\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ and $\text{Fe}^{3+,IS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states, which transform to the $\text{Fe}^{3+,HS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states with increasing temperature.

$\text{Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ (in Table 7) states are very similar ($1.4\text{--}1.6 \text{ mm s}^{-1}$) and are close to the observed $\Delta E_Q \approx 1 \text{ mm s}^{-1}$. It is likely that when the temperature is increased the $\text{Fe}^{3+,LS}\text{-OH}\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ and $\text{Fe}^{3+,IS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states start to transform to the $\text{Fe}^{3+,IS}\text{-OH}\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ and $\text{Fe}^{3+,HS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states, respectively. Qualitatively, one expects the vibrational entropies to increase ($\Delta S_{\text{vib}} > 0$) when $\text{Fe}^{3+,LS} \rightarrow \text{Fe}^{3+,IS}$ and $\text{Fe}^{3+,IS} \rightarrow \text{Fe}^{3+,HS}$. The vibrational entropy differences ($\Delta G_{\text{vib}} = -T\Delta S_{\text{vib}}$) are not in our current models. They require careful frequency calculations on fairly large models.

When the Fe_{a3}^{3+} site is in the intermediate-spin state, the $\text{Fe}^{3+,IS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ DNC clusters (in Table 7 and Figure 9) are $2\text{--}3 \text{ kcal mol}^{-1}$ lower in energy than the $\text{Fe}^{3+,LS}\text{-OH}\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ (in Table 6 and Figure 8) clusters. We have mentioned above that, during geometry optimizations of the $\text{Fe}^{3+,IS}\text{-OH}\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ structures (neutral Tyr237), the proton on the Cu^{2+} -bound H_2O ligand transferred to the Fe^{3+} -bound OH^- ligand, and the $\text{Fe}^{3+,IS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ states are obtained. Therefore, the proton transfer barrier for the transition of $\text{Fe}^{3+,LS}\text{-OH}\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF}) \rightarrow \text{Fe}^{3+,IS}\text{-H}_2\text{O}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$ is small. With a further increase in temperature, the $\text{Fe}^{3+,IS}\text{-OH}\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)(\text{F}/\text{AF})$

(F/AF) states (anionic tyrosine) also likely undergo proton transfer and change to the $\text{Fe}^{3+,\text{IS}}\text{-H}_2\text{O}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (F/AF) states, which then transform to the $\text{Fe}^{3+,\text{HS}}\text{-H}_2\text{O}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (F/AF) states. Therefore, all these transformations will end in $\text{Fe}^{3+,\text{HS}}\text{-H}_2\text{O}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (F/AF) states at 245 K. This proposed transformation process is shown schematically in Figure 10.

To estimate the proton transfer barrier in the transformation of $\text{Fe}^{3+,\text{IS}}\text{-OH}^-\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)$ (F) \rightarrow $\text{Fe}^{3+,\text{IS}}\text{-H}_2\text{O}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (F) (energy difference $\Delta E = -2.6$ kcal mol $^{-1}$), starting from the optimized geometry of $\text{Fe}^{3+,\text{IS}}\text{-OH}^-\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)$ (F), we gradually moved the proton on O2 (see Figure 8) toward the oxygen atom O1 along the H \cdots O1 direction, then optimized the cluster with the position of this proton (labeled as H in Figure 8) fixed. The electronic energies of these optimized clusters with different fixed-H positions are very similar to the originally optimized $\text{Fe}^{3+,\text{IS}}\text{-OH}^-\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)$ (F) cluster. The highest energy structure we obtained is only 2.0 kcal mol $^{-1}$ higher than the energy of the lowest-energy $\text{Fe}^{3+,\text{IS}}\text{-OH}^-\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)$ (F) structure. The central portion of this highest-energy structure is shown in Figure 11 in which the proton H is at equal distance (1.20 Å)

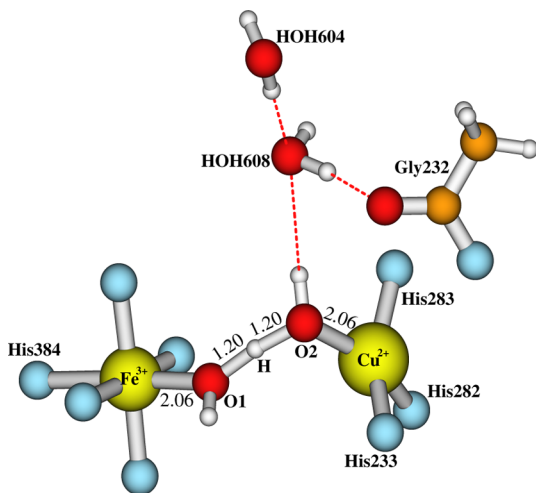


Figure 11. To estimate the energy barrier of the proton (H) transfer of $\text{Fe}^{3+,\text{IS}}\text{-OH}^-\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)$ (F) \rightarrow $\text{Fe}^{3+,\text{IS}}\text{-H}_2\text{O}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (F) from the position shown in Figure 8 to the position in Figure 9, we gradually moved the proton along the H \cdots O1 direction toward the O1 atom and then optimized the cluster with the fixed position of this proton. This figure shows the detailed central portion of the highest-energy DNC structure obtained among these H-fixed optimized clusters.

with O1 and O2. The distances of Fe–O1 and Cu–O2 are also the same (2.06 Å). Because of the size of the system, we did not perform transition state search and frequency calculations. According to the features of this structure, it is reasonable to assume that it is close to the transition state of the proton transfer. Then, the barrier for this proton transfer should also be ~ 2 kcal mol $^{-1}$, which is smaller than the energy differences (3.3/5.0 kcal mol $^{-1}$) between the $\text{Fe}^{3+,\text{IS}}\text{-OH}^-\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)$ (F/AF) and $\text{Fe}^{3+,\text{HS}}\text{-OH}^-\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)$ (F/AF) states (Table 6). Therefore, the $\text{Fe}^{3+,\text{IS}}\text{-OH}^-\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)$ (F/AF) states will transform more easily to the $\text{Fe}^{3+,\text{IS}}\text{-H}_2\text{O}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (F/AF) states than to the $\text{Fe}^{3+,\text{HS}}\text{-OH}^-\cdots\text{H}_2\text{O}\text{-Cu}^{2+}(\text{Y237}^-)$ (F/AF) states with increasing temperature. Such a proton transfer from the water molecule

ligating the Cu^{2+} ion to the hydroxyl ligand of Fe^{3+} was also obtained in DFT calculations on the oxidized states from two *aa*₃ CcO DNCs (bacterial, *Paracoccus denitrificans*; and mammalian, bovine) by Sharma et al.⁵⁷

In addition, a high-spin $^{57}\text{Fe}_{\text{a}3}^{3+}$ species with ($\delta = 0.41$ mm s $^{-1}$, $\Delta E_{\text{Q}} = 0.7$ mm s $^{-1}$, see Figure 1) was also observed in the Mössbauer experiments on *Tt*, which did not change through the temperature range from 4.2 to 245 K.¹⁸ Next, we present our studies on DNC models generated from the X-ray crystal structures 3EH4 and 1EHK to see if this high-spin $^{57}\text{Fe}_{\text{a}3}^{3+}$ species could be represented by the DNC structure containing a Cu^{2+} -terminally bound OH^- or bridging OH^- between Fe^{3+} and Cu^{2+} .

4.2. $\text{Fe}^{3+}\cdots\text{OH}^-\text{-Cu}^{2+}$ DNC Models from 3EH4. Our calculations in sections 3.3 and 3.4 imply that the $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^-\text{-Cu}^{2+}$ DNC in 3EH4 is probably trapped from the earlier oxidized $\text{Fe}^{3+,\text{HS}}\cdots\text{OH}^-\text{-Cu}^{2+}$ (or $\text{Fe}^{3+,\text{HS}}\text{-H}_2\text{O}\cdots\text{OH}^-\text{-Cu}^{2+}$) structure after X-ray irradiation. We now geometry optimize the $\text{Fe}^{3+,\text{HS}}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-/\text{Y237})$ (F/AF) clusters. However, the calculations for the $\text{Fe}^{3+,\text{HS}}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-/\text{Y237})$ (AF) states lead instead to $\text{Fe}^{3+,\text{IS}}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-/\text{Y237})$ (F) states. Therefore, we present the calculated properties of both $\text{Fe}^{3+,\text{IS/HS}}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-/\text{Y237})$ (F) states in Table 8. In these models (and all the $\text{Fe}^{3+}\cdots\text{Cu}^{2+}$ DNC models in the current paper), the three histidine ligands of Cu^{2+} bind strongly with typical $\text{Cu}^{2+}\text{-N}(\text{His})$ distances of 2.02–2.15 Å.

The calculated pK_{a} values for the Tyr237 side chain show that the $\text{Fe}^{3+,\text{IS/HS}}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (F) states with deprotonated anionic Tyr237 $^-$ are energetically more stable than the corresponding $\text{Fe}^{3+,\text{IS/HS}}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237})$ (F) states. Further, the $\text{Fe}^{3+,\text{IS}}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (F) state is 2.3 kcal mol $^{-1}$ lower in energy than the $\text{Fe}^{3+,\text{HS}}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (F) state. Therefore, if the $\text{Fe}^{3+}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ -type DNC exists, it will be in the $\text{Fe}^{3+,\text{IS}}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ state at low temperature and will likely transform to the $\text{Fe}^{3+,\text{HS}}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ state as the temperature increases, a feature similar to the $\text{Fe}^{3+,\text{IS}}\text{-H}_2\text{O}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-) \rightarrow \text{Fe}^{3+,\text{HS}}\text{-H}_2\text{O}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ transition proposed in the previous section. The calculated Mössbauer properties ($\delta = 0.41/0.57$ mm s $^{-1}$, $\Delta E_{\text{Q}} = 2.38/1.93$ mm s $^{-1}$) of the $\text{Fe}^{3+,\text{IS/HS}}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (F) states are also fairly similar to the corresponding properties ($\delta = 0.40/0.52$ mm s $^{-1}$, $\Delta E_{\text{Q}} = 2.42/1.49$ mm s $^{-1}$) of the $\text{Fe}^{3+,\text{IS/HS}}\text{-H}_2\text{O}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (F) states. Therefore, the $\text{Fe}^{3+,\text{HS}}\cdots\text{OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ DNC model does not represent the high-spin $^{57}\text{Fe}_{\text{a}3}^{3+}$ species observed with $\delta = 0.41$ mm s $^{-1}$ and $\Delta E_{\text{Q}} = 0.7$ mm s $^{-1}$ through 4.2–245 K in the Mössbauer experiments.¹⁸

4.3. $\text{Fe}^{3+}\text{-OH}^-\text{-Cu}^{2+}$ DNC Models from 1EHK. The 1EHK- $\text{Fe}^{3+}\text{-OH}^-\text{-Cu}^{2+}$ DNC model shown in Figure 2 was geometry optimized with Fe^{3+} in LS, IS, and HS states F- or AF-coupled with the Cu^{2+} site and with Tyr237 side chain in neutral or an anionic state. The main calculated properties of these 1EHK- $\text{Fe}^{3+}\text{-OH}^-\text{-Cu}^{2+}$ cluster models are given in Table 9. Note that we did not obtain the optimized structures for the $\text{Fe}^{3+,\text{LS}}\text{-OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (F) and $\text{Fe}^{3+,\text{IS}}\text{-OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (F) states. Calculations for these two states led to the $\text{Fe}^{3+,\text{IS}}\text{-OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (AF) and $\text{Fe}^{3+,\text{HS}}\text{-OH}^-\text{-Cu}^{2+}(\text{Y237}^-)$ (AF) states, respectively.

The calculated $\text{pK}_{\text{a}}(\text{Y237})$ values (−0.4 and 0.6) for the $\text{Fe}^{3+,\text{HS}}\text{-OH}^-\text{-Cu}^{2+}(\text{Y237})$ (F/AF) models clearly show that the $\text{Fe}^{3+,\text{HS}}\text{-OH}^-\text{-Cu}^{2+}$ -type DNC energetically favors the anionic form of the Tyr237 side chain.

Table 8. Calculated Properties of the Oxidized Fe³⁺...OH⁻-Cu²⁺(Y237⁻/Y237) Models Generated from the DNC of the X-ray Crystal Structure 3EH4^a

state ^b	geometry							<i>E</i>	p <i>K</i> _a (Y237)	<i>Q</i>	net spin		⁵⁷ Fe ³⁺ Mössbauer	
	Fe–N	Fe...O	Cu–O	Fe...Cu	Cu–N (H233)	Cu–N (H282)	Cu–N (H283)				Fe ³⁺	Cu ²⁺	δ	Δ <i>E</i> _Q
Fe ^{3+,LS} ...OH ⁻ - Cu ²⁺ (Y237 ⁻)(F)	2.22	4.08	1.91	5.13	2.08	2.07	2.06	-165.4		0	2.62	0.52	0.41	2.38
Fe ^{3+,HS} ...OH ⁻ - Cu ²⁺ (Y237 ⁻)(F)	2.10	4.29	1.91	5.32	2.08	2.07	2.06	-163.1		0	4.02	0.53	0.57	1.93
Fe ^{3+,LS} ...OH ⁻ - Cu ²⁺ (Y237)(F)	2.19	4.11	1.91	5.18	2.11	2.07	2.05	-158.8	6.1	1	2.72	0.53	0.39	2.47
Fe ^{3+,HS} ...OH ⁻ - Cu ²⁺ (Y237)(F)	2.08	4.25	1.91	5.33	2.11	2.07	2.05	-155.1	5.0	1	4.11	0.53	0.49	0.34

^aThe properties include geometries (Å), electronic energies (*E*, offset by -25100 kcal mol⁻¹) obtained from the all-electron/all-TZP single-point energy calculations on the optimized geometries, p*K*_a's, the net charge (*Q*) of the clusters, Mulliken net spin polarizations for the Fe and Cu sites, and the ⁵⁷Fe Mössbauer isomer shifts (δ, mm s⁻¹) and quadrupole splittings (Δ*E*_Q, mm s⁻¹). ^bF stands for ferromagnetically coupled.

Table 9. Calculated Properties of the Oxidized Fe³⁺-OH⁻-Cu²⁺(Y237⁻/Y237) Clusters Generated from the DNC of the 1EHK X-ray Crystal Structure^a

state ^b	geometry								<i>E</i> ^c	p <i>K</i> _a (Y237)	<i>Q</i>	net spin		⁵⁷ Fe ³⁺ Mössbauer	
	Fe–N (H384)	Fe–O	Cu–O	Fe...Cu	Cu–N (H233)	Cu–N (H282)	Cu–N (H283)	O...O (Y237)				Fe ³⁺	Cu ²⁺	δ	Δ <i>E</i> _Q
Fe ^{3+,LS} -OH ⁻ - Cu ²⁺ (Y237 ⁻)(AF)	3.72	1.81	2.72	4.31	2.02	2.08	2.04	2.80	-157.3		0	0.99	-0.29	0.24	3.03
Fe ^{3+,LS} -OH ⁻ - Cu ²⁺ (Y237 ⁻)(AF)	3.78	1.98	2.15	3.93	2.06	2.11	2.15	2.77	-163.0		0	2.65	-0.32	0.37	2.02
Fe ^{3+,HS} -OH ⁻ - Cu ²⁺ (Y237 ⁻)(F)	4.05	1.91	2.31	3.97	2.05	2.09	2.09	2.83	-166.1		0	4.09	0.34	0.39	0.54
Fe ^{3+,HS} -OH ⁻ - Cu ²⁺ (Y237 ⁻)(AF)	4.08	1.91	2.30	3.97	2.05	2.10	2.09	2.82	-167.5		0	4.08	-0.29	0.38	0.51
Fe ^{3+,HS} -OH ⁻ - Cu ²⁺ (Y237)(F)	4.13	1.94	2.19	3.90	2.05	2.06	2.04	3.08	-150.6	-0.4	1	4.09	0.49	0.41	0.42
Fe ^{3+,HS} -OH ⁻ - Cu ²⁺ (Y237)(AF)	4.11	1.93	2.21	3.91	2.05	2.07	2.05	3.05	-153.4	0.6	1	4.04	-0.43	0.39	0.40

^aThe properties include geometries (Å), electronic energies (*E*, offset by -25100 kcal mol⁻¹) obtained from the all-electron/all-TZP single-point energy calculations on the optimized geometries, p*K*_a's, the net charge (*Q*) of the clusters, Mulliken net spin polarizations for the Fe and Cu sites, and the ⁵⁷Fe Mössbauer isomer shifts (δ, mm s⁻¹) and quadrupole splittings (Δ*E*_Q, mm s⁻¹). ^bF stands for ferromagnetically-coupled and AF for antiferromagnetically-coupled. ^cThe energies calculated for the AF-coupled states are broken-symmetry state energies.

Both Fe^{3+,HS}-OH⁻-Cu²⁺(Y237⁻)(F) and Fe^{3+,HS}-OH⁻-Cu²⁺(Y237⁻)(AF) states yield very similar geometric and Mössbauer properties. The Fe^{3+,HS}-OH⁻-Cu²⁺(Y237⁻)(AF) state yields the lowest energy, which is 10.2, 4.5, and 1.4 kcal mol⁻¹ lower in energy than the Fe^{3+,LS}-OH⁻-Cu²⁺(Y237⁻)(AF), Fe^{3+,LS}-OH⁻-Cu²⁺(Y237⁻)(AF), and Fe^{3+,HS}-OH⁻-Cu²⁺(Y237⁻)(F) states, respectively. Therefore, if the oxidized DNC exists in the Fe³⁺-OH⁻-Cu²⁺ form with a bridging hydroxo, it would stay in the Fe^{3+,HS}-OH⁻-Cu²⁺(Y237⁻)(AF) state. Further, we noticed that the calculated Mössbauer properties (δ = 0.38 mm s⁻¹, Δ*E*_Q = 0.51 mm s⁻¹) of this Fe^{3+,HS}-OH⁻-Cu²⁺(Y237⁻)(AF) state are in very good agreement with the observed Mössbauer spectra (δ = 0.41 mm s⁻¹, Δ*E*_Q = 0.7 mm s⁻¹).¹⁸ Therefore, it is very likely that the Fe^{3+,HS}-OH⁻-Cu²⁺(Y237⁻)(AF) DNC structure represents the observed (δ = 0.41 mm s⁻¹, Δ*E*_Q = 0.7 mm s⁻¹) ⁵⁷Fe₃₊₃ high-spin species, which exists through the 4.2–245 K range in the the Mössbauer experiments.¹⁸

5. CONCLUSIONS

The X-ray crystal structures (PDB entries: 1EHK,¹⁰ 1XME,¹¹ 3EH3,¹² 3EH4,¹² 3EHS,¹² 3S8F,¹⁵ and 3S8G¹⁵) of the reduced *ba*₃ cytochrome *c* oxidase (CcO) from *Thermus thermophilus* (*Tt*) showed different Fe₃₊₂₊...Cu_B⁺ dinuclear center (DNC)

structures. Mössbauer experiments on the *ba*₃ CcO from *Tt* also revealed DNC structural heterogeneities in both oxidized and reduced states. Both radiolytic reduction and chemical reduction^{10–12,15} with dithionite⁵⁸ will typically generate high-energy reduced state intermediates compared to the physiological donor cytochrome *c*, which has a midpoint redox potential of approximately +0.20 to +0.25 V relative to SHE. By contrast, the dithionite midpoint potential is quite negative and depends also on the dithionite initial (and final) concentrations. Use of the dithionite chemical reduction data from Zimmermann et al.,¹⁸ and from Liu et al.¹² in combination with Mayhew's⁵⁸ analysis, yields estimated dithionite midpoint potentials of about -0.50 and -0.45 V, respectively. So there is an additional reductive driving force of about 0.7 to 0.8 V using dithionite relative to cytochrome *c*. Radiolytic reduction of the X-ray structures in the synchrotron X-ray beam is also highly reducing^{10–12,15} although less predictable. The resulting unstable or metastable intermediates can be trapped at low temperatures (the Cryogenic X-ray structures are at ~100 K). The X-ray structures in Table 1 (particularly 3EH3 and 3EHS) are consistent with the view that chemical reduction prior to freezing in liquid N₂ and X-ray irradiation yield more geometrically and energetically relaxed structures.

Using DFT OLYP potential, we have performed geometric, energetic, and Mössbauer property calculations on the DNCs of the X-ray crystal structures listed above. Our calculations support that the $^{57}\text{Fe}_{\text{a}3}{}^{2+}$ sites observed in the Mössbauer experiments are in high-spin states. The oxygen species between the Fe^{2+} and Cu^+ sites found in 1EHK,¹⁰ 1XME,¹¹ and 3EH4,¹² is better represented by a hydroxide anion (OH^-). The first (majority) $^{57}\text{Fe}_{\text{a}3}{}^{2+}$ component observed in the Mössbauer experiment is best represented by the DNC in the $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- \text{-Cu}^+$ state, where OH^- terminally binds with Cu^+ and the Cu^+ is in a 4- or 3-coordinate configuration. With OH^- as a ligand, the Cu^+ site energetically favors a linear $\text{OH}^- \text{-Cu}^+ \text{-N}(\text{His}282)$ 2-coordinate ($2c$) configuration with the His233 and His283 side chains dissociating from the Cu^+ site. However, steric interactions, especially from the side chain of Trp229 (not included in the current model calculations), which is beside the His283 side chain, the potential energy barriers for geometry rearrangement, and the low temperature under the experimental conditions may prevent the formation of the $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- \text{-Cu}^+_{2c}$ state. Further, a reduced DNC with OH^- between the $\text{Fe}^{2+,\text{HS}}$ and the Cu^+ sites are not in the equilibrium state. The system strongly favors a water molecule (if there is solvent oxygen species) rather than a hydroxide anion in the DNC. Therefore, the reduced $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- \text{-Cu}^+ / \text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- \text{-Cu}^+$ DNCs found in the Mössbauer experiments/X-ray crystal structures should arise from the earlier oxidized state with structures like $\text{Fe}^{3+,\text{HS}}\cdots\text{OH}^- \text{-Cu}^{2+} / \text{Fe}^{3+,\text{HS}}\cdots\text{OH}^- \text{-Cu}^{2+}$ and be trapped at very low temperature.

Our calculations indeed have shown that the DNC structure of $\text{Fe}^{3+,\text{HS}}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y}237^-)(\text{AF})$, in which a hydroxo bridges the AF-coupled high-spin Fe^{3+} and Cu^{2+} sites, yields very similar Mössbauer properties ($\delta = 0.38 \text{ mm s}^{-1}$, $\Delta E_{\text{Q}} = 0.51 \text{ mm s}^{-1}$) to the observed $^{57}\text{Fe}_{\text{a}3}{}^{3+}$ high-spin species ($\delta = 0.41 \text{ mm s}^{-1}$, $\Delta E_{\text{Q}} = 0.7 \text{ mm s}^{-1}$) in the temperature range of 4.2–245 K. We have also found that the DNC structure of the observed “low-spin” $^{57}\text{Fe}_{\text{a}3}{}^{3+}$ species at 4.2–190 K with ($\delta = 0.29 \text{ mm s}^{-1}$, $\Delta E_{\text{Q}} = 2.24 \text{ mm s}^{-1}$)¹⁸ is likely a mixture of both the $\text{Fe}^{3+,\text{LS}}\cdots\text{H}_2\text{O} \text{-Cu}^{2+}(\text{Y}237^-)(\text{F}/\text{AF})$ and $\text{Fe}^{3+,\text{LS}}\cdots\text{H}_2\text{O} \text{-Cu}^{2+}(\text{Y}237^-)(\text{F}/\text{AF})$ states, and they gradually transition to the $\text{Fe}^{3+,\text{HS}}\cdots\text{H}_2\text{O} \text{-Cu}^{2+}(\text{Y}237^-)(\text{F}/\text{AF})$ states with increasing temperature (see Figure 10), which explains the observed transition of “low-spin” $^{57}\text{Fe}_{\text{a}3}{}^{3+}$ to high-spin $^{57}\text{Fe}_{\text{a}3}{}^{3+}$ in the Mössbauer experiment when the temperature was increased from 190 to 245 K.¹⁸

Previous resonance Raman experiments on ba_3 CcO from *Tt* (at ambient temperature) also discovered two high-spin $\text{Fe}_{\text{a}3}{}^{3+}$ species with one in an in-plane (6-coordinated) and another in an out-of-plane (5-coordinated) configuration.⁵⁹ The high-spin $\text{Fe}_{\text{a}3}{}^{3+}$ DNCs we have proposed here, $\text{Fe}^{3+,\text{HS}}\cdots\text{H}_2\text{O} \text{-Cu}^{2+}(\text{Y}237^-)(\text{F}/\text{AF})$ and $\text{Fe}^{3+,\text{HS}}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y}237^-)(\text{AF})$, are indeed in 6- and 5- coordinated configurations, respectively. The average dihedral angle between Fe^{3+} and three of the four ligating interior N atoms in the $\text{Fe}^{3+,\text{HS}}\cdots\text{H}_2\text{O} \text{-Cu}^{2+}(\text{Y}237^-)(\text{F}/\text{AF})$ structures is 5° , whereas the corresponding value in $\text{Fe}^{3+,\text{HS}}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y}237^-)(\text{AF})$ is -20° . The Fe^{3+} ion in the $\text{Fe}^{3+,\text{HS}}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y}237^-)(\text{AF})$ structure is certainly in the out-of-plane position toward the bridging OH^- .

Very recently, Sharma et al. presented their computational study of a metastable but activated ferric/cupric state (O_H in their notation) in the catalytic cycle of aa_3 CcO.⁵⁷ The experimental evidence suggests that the DNC in the catalytically relevant O_H state may have high-spin $\text{Fe}_{\text{a}3}{}^{3+}$ with a strongly H-bonded distal hydroxide ligand and $\text{Cu}_\text{B}{}^{2+}$ with a

raised redox potential.⁵⁷ On the basis of their DFT calculations, Sharma et al. have proposed that the high energy and high redox potential of Cu_B in the O_H state may stem from a 3-coordinated near-planar geometry of $\text{Cu}_\text{B}{}^{2+}$ (first alternative).⁵⁷ As a second alternative, they have also proposed that the DNC may be compact in state O_H with a very short Fe–Cu distance and a bridging μ -hydroxo between the two metals.⁵⁷ Note that this proposed second alternative O_H structure has a very similar DNC structure to our calculated $\text{Fe}^{3+,\text{HS}}\cdots\text{OH}^- \text{-Cu}^{2+}(\text{Y}237^-)(\text{AF})$ state.

To date, several DNC structures in our calculations yield very similar $^{57}\text{Fe}^{2+,\text{HS}}$ Mössbauer properties, which are very close to the properties of the second $^{57}\text{Fe}_{\text{a}3}{}^{2+}$ component observed in the Mössbauer experiments (Figure 1).¹⁸ These structures include: (1) the open $\text{Fe}^{2+,\text{HS}}\cdots\text{Cu}^+$ DNC structure as observed in the X-ray crystal structures 3EH3 and 3EH5,¹² where no oxygen species is between the $\text{Fe}^{2+,\text{HS}}$ and Cu^+ sites, and the Cu^+ binds with all three His233, His282, and His283 side chains; (2) similar structures to the open $\text{Fe}^{2+,\text{HS}}\cdots\text{Cu}^+$ structure, but with one (or more) water molecule(s) staying between but not binding with the $\text{Fe}^{2+,\text{HS}}$ and Cu^+ sites; (3) the $\text{Fe}^{2+,\text{HS}}\cdots\text{OH}^- \text{-Cu}^+_{2c}$ -His282 DNC structure, where the His233 and His283 side chains dissociate from Cu^+ and the 2-coordinate Cu^+ site linearly binds with OH^- and the His282 side chain; (4) the $\text{Fe}^{2+,\text{HS}}\cdots\text{H}_2\text{O} \text{-Cu}^+_{3c}$ -type structure, in which the 3-coordinate Cu^+ site binds with an H_2O and the His233 and His282 side chains; and (5) the $\text{Fe}^{2+,\text{HS}}\cdots\text{His}233\text{-Cu}^+_{2c}$ -His282 structure with the H_2O ligand in structure (4) dissociating from the Cu^+ site and the Cu^+ binding with only the His233 and His282 side chains.

It is not known if the low coordination Cu^+_{2c} state exists in the ba_3 CcO from *Tt* during its catalytic cycle. However, the variation of the Cu^+ coordination state and the mobility of the His233 and His283 side chains shown in our calculations do suggest that the His233 and His283 side chains can be potential proton loading sites during the catalytic cycle and in the proton pumping process. Considering the high optimal growth temperature of *Thermus thermophilus*, such variation of the Cu^+ coordination state is possible. If His283 side chain dissociates and is then protonated to form a His283⁺ cation, the nearby Trp229 side chain could potentially stabilize this state through a cation- π interaction,⁶⁰ but the relative His283-Trp229 geometry must then be nearly optimal. Further calculations with larger DNC models, including the Trp229 side chain, are planned in the near future.

■ ASSOCIATED CONTENT

📄 Supporting Information

Some additional figures, the detailed Mössbauer property calculations described in section 3.5 ($\text{Fe}^{2+} \text{-}(\text{HO}_2)^- \text{-Cu}^+$ DNC Models from 3S8G), and the Cartesian coordinates for some representative OLYP-optimized clusters discussed in Tables 2–9. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorgchem.5b00700.

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Notes

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