The two oxidized forms of the trinuclear Cu cluster in the multicopper oxidases and mechanism for the decay of the native intermediate

Jungjoo Yoon, Barry D. Liboiron, Ritimukta Sarangia, Keith O. Hodgson, Britt Hedman, and Edward I. Solomon

Multicopper oxidases (MCOs) catalyze the $4e^-$ reduction of $O_2$ to $H_2O$. The reaction of the fully reduced enzyme with $O_2$ generates the native intermediate (NI), which undergoes a slow decay to the resting enzyme in the absence of substrate. NI is a fully oxidized form, but its spectral features are very different from those of the resting form (also fully oxidized), because the type 2 and the coupled-binuclear type 3 Cu centers in the $O_2$-reducing trinuclear Cu cluster site are isolated in the resting enzyme, whereas these are all bridged by a $\mu_3$-oxy ligand in NI. Notably, the one azide-bound NI (NIAz) exhibits spectral features very similar to those of NI, in which the $\mu_3$-oxy ligand in NI has been replaced by a $\mu_3$-bridged azide. Comparison of the spectral features of NI and NIAz, combined with density functional theory (DFT) calculations, allows refinement of the NI structure. The decay of NI to the resting enzyme proceeds via successive proton-assisted steps, whereas the rate-limiting step involves structural rearrangement of the $\mu_3$-oxy-bridge from inside to outside the cluster. This phenomenon is consistent with the slow rate of NI decay that uncouples the resting enzyme from the catalytic cycle, leaving NI as the catalytically relevant fully oxidized form of the MCO active site. The all-bridged structure of NI would facilitate electron transfer to all three Cu centers of the trinuclear cluster for rapid proton-coupled reduction of NI to the fully reduced form for catalytic turnover.

Results and Discussion

Conversion of $R_{Az}$ to NI$_{Az}$. DFT calculations were used to obtain spectroscopically acceptable structures of $R_{Az}$ and NI$_{Az}$. First, a number of $R_{Az}$ structures with different starting azide positions were optimized where it can bridge the T2 Cu and T3 Cu centers. The T2 OH$^-$ ligand and T3 OH$^-$ bridge were kept as in the resting enzyme (20), because experiments show no change in the nature of these water-derived ligands upon azide binding. Two final structures were obtained: one with a $\mu_3$-1,1 bridge between T2 Cu and one of the T3 Cu atoms, in which the azide points away from the center of the trinuclear cluster [supporting information (SI) Fig. 9], and the other with an internal $\mu_3$-1,1,3-N$_3$ bridge, in which both Cu$_{1}$ and a T3 Cu (designated Cu$^{T3a}$) are $\mu_1$-1,1-bridged,

$(\text{NI}_{Az})$ can be trapped at 25 ms, which then decays to the one azide-bound resting Lc (R$_{Az}$) (B.D.L., J.Y., and E.I.S., unpublished manuscript). Alternatively, protonative uncoupling of the T3 OH$^-$ bridge in R$_{Az}$ can be achieved reversibly at low pH (13), in which the uncoupled species ($\approx$40% at pH 5.5) is in fact NI$_{Az}$, as evidenced by its spectral features. Thus, the two fully oxidized forms can interconvert via azide binding. Moreover, the reversibility of R$_{Az}$ and NI$_{Az}$ also suggests that the azide binding modes are similar in the two structures. Structural descriptions of R$_{Az}$ have been available from both spectroscopy and crystallography. Spectroscopy first determined that the azide must bridge the T2 and T3 Cu centers in R$_{Az}$ (14, 15). In this fact provided the first evidence for the presence of the trinuclear Cu clusters in biology, which was confirmed by the first crystal structure of an MCO, ascorbate oxidase (AO) (16). However, crystal structures of azide-bound AO (17), ceruloplasmin (Cp) (18), and CotA (19) later showed disagreement with spectroscopy (the three are also different from one another), indicating that the azide binding only occurs at the T3 site.

In this study, the unique spectral features of NI and NI$_{Az}$ were compared and combined with DFT calculations to demonstrate the presence of an all-bridged Cu(II) trimeric structure in NI$_{Az}$, as well as in R$_{Az}$. Furthermore, these results provide the basis for evaluating the structural relationship between the two fully oxidized enzymatic forms, NI and resting, the mechanism of the slow decay of NI to the resting site, and the relevance of these two fully oxidized forms of the enzyme to the catalytic cycle of the MCOs.


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Abbreviations: AO, ascorbate oxidase; CT, charge transfer; DFT, density functional theory; EXAFS, extended x-ray absorption fine structure; FT, Fourier transform; Lc, laccase; MCD, magnetic circular dichroism; MCO, multicopper oxidase; NI, native intermediate; NI$_{Az}$, one azide-bound NI; R$_{Az}$, one azide-bound resting Lc; SOC, spin-orbit coupling; Tr, type n.

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whereas the other two Cu pairs are μ-1,3-bridged (Fig. 1, R_Az). Of these, the latter is found to be energetically favored (by ~23 kcal/mol)\(^\text{4}\), because the open coordinate positions of the three Cu centers toward the center of the trimuclear cluster facilitate a favorable internal azide binding, whereas the external azide binding in the former is sterically hindered by the His ligands.

Three unpaired electrons in R_Az reside in the d\(_{x^2-y^2}\) orbitals of the three Cu atoms, where CuT\(^2\) d\(_{x^2-y^2}\) is oriented perpendicular to the Cu3 plane, whereas CuT3a and CuT3b d\(_{x^2-y^2}\) are nearly in the Cu3 plane (Fig. 2A). The azide in R_Az is not in the Cu3 plane but slants out-of-plane at the N\(^3\) end where it coordinates to CuT3b (Fig. 1). Consequently, whereas CuT2 and CuT3a d\(_{x^2-y^2}\) interact with the N\(^3\) \(\pi^*\) orbital (the N\(^3\) \(\pi^*\) HOMO with \(p\) orbitals in the Cu3 plane), CuT3b d\(_{x^2-y^2}\) interacts with the N\(^1\) \(\pi^*\) orbital (the N\(^1\) \(\pi^*\) HOMO with \(p\) orbitals orthogonal to the Cu3 plane). CuT3a and CuT3b d\(_{x^2-y^2}\) orbitals also form \(\sigma\)-bonds with the \(O\)-orbitals of the T3 OH\(^-\) bridge as in the resting enzyme. The relative energies of the three Cu d\(_{x^2-y^2}\)-based MOs are CuT3a < CuT3b (< 690 cm\(^{-1}\)) < CuT2 (+6,690 cm\(^{-1}\)),\(^\text{5}\) where the high MO energy of CuT2 derives from the strong ligand field of the OH\(^-\) donor ligand. The CuT3b-based MO is slightly higher in energy than the CuT3a-based MO as the CuT3b geometry is more distorted; one of the His ligands on CuT3 is found to be ligated at δN in all reported crystal structures of the MCOs. The calculated isotropic exchange constants (\(J\)) indicate a doublet ground state for R_Az (Table 1), where the superexchange contribution is mainly from the CuT3a-CuT3b AF coupling, effectively leaving CuT2 isolated as in the resting enzyme. This phenomenon is consistent with the 77 K T2 EPR signal in R_Az being unperturbed from that of the resting enzyme [but the absorption and MCD spectra do show an Ni\(_1\) → CuT2 charge transfer (CT) transition indicating coordination to CuT2] (13).

The structure of NIAz was then obtained by protonating the T3 OH\(^-\) bridge in R_Az. During the geometry optimization, the protonated T3 OH\(^-\) bridge (i.e., H\(_2\)O) dissociated from the trimuclear Cu cluster and therefore, the final structure was obtained without the dissociated H\(_2\)O (Fig. 1, NIAz). The azide is now in the Cu3 plane in NIAz, in contrast to the stunted configuration in R_Az (Fig. 1). The loss of the T3 OH\(^-\) bridge and change in the azide binding mode result in a significant change in the ground state description of NIAz. Whereas the CuT2 geometry is relatively unperturbed, the CuT3a-CuT3b geometries are changed from square pyramid to distorted tetrahedral. The three unpaired electrons still occupy the three Cu d\(_{x^2-y^2}\) orbitals, although the T3 Cu d\(_{x^2-y^2}\) orbitals are differently oriented to accommodate the changes in the T3 Cu geometries (Fig. 2B). The relative MO energies of the Cu d\(_{x^2-y^2}\)-based MOs are CuT3a < CuT3b < CuT2 (+1,020 cm\(^{-1}\)) < CuT2 (+15,900 cm\(^{-1}\)) in NIAz. As in R_Az, the high energy of the CuT2-based MO derives from the strong ligand field of the OH\(^-\) ligand. However, the loss of the OH\(^-\) ligation stabilizes the CuT3a-CuT3b-based MOs (which are antibonding MOs) and results in a larger energy splitting from the CuT2-based MO.

The strongest AF-coupling is now present in the CuT2-CuT3b pair in NIAz (\(J = -106 \text{ cm}^{-1}\), Table 1), which changed from weak ferromagnetic in R_Az (\(J = +8 \text{ cm}^{-1}\)) due to the change in the binding mode of the azide. In R_Az, the CuT2 and CuT3b-based magnetic orbitals have Ni\(_1\) \(\pi^*\) \(\text{Nb}\) and Ni\(_2\) \(\pi^*\) \(\text{Nb}\) characters, respectively, that are orthogonal at the bridging azide, favoring a ferromagnetic interaction (Fig. 2A). In NIAz, however, both magnetic orbitals have Ni\(_1\) \(\pi^*\) \(\text{Nb}\) characters that lead to favorable AF coupling (Fig. 2B). The CuT3a-CuT3b pair is also AF coupled in NIAz (\(J = -49 \text{ cm}^{-1}\)), although the superexchange interaction now involves the \(\mu-1,3\)-Ni bridge via the Ni\(_3\) \(\pi^*\) \(\text{Nb}\) orbital.

Note that extended x-ray absorption fine structure (EXAFS) indicates the presence of a Cu–Cu distance of 3.46 Å (see below), which is ~0.8 Å less than the shortest Cu–Cu distance in the optimized NIAz. This discrepancy likely originated from constraints on the NIAz geometry imposed to reflect the features of the crystal structure, which may not have allowed for changes in the protein environment upon azide binding. Thus, the NIAz structure was re-optimized with the shortest CuT2-CuT3a distance fixed at 3.46 Å (NIE_XAFS). The resulting energy difference between NIAz and NIE_XAFS is relatively small (8.0 kcal/mol) and the main structural features remain unchanged. The \(J\) values are similar to those of NIAz for the CuT2-CuT3b and CuT3a-CuT3b pairs (Table 1), although the smaller CuT2-Ni\(_2\)-CuT3a angle (122° vs. 153° in NIAz) (SI Table 2) results in a ferromagnetic CuT2-CuT3a interaction. Nevertheless, the combination of all three \(J\) values yields a doublet ground state, with doublet-quartet energy very similar to that of NIAz (Table

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\(^4\)These energies were obtained using B3LYP functional and double-\(\zeta\) basis sets (6-31G\(^*\) for Cu and coordinated N/O atoms and 3-21G\(^*\) for the rest). All the other energies not referred to this note were obtained using B3LYP functional and triple-\(\zeta\) TZV basis sets.

\(^5\)The \(\nu\) lowest unoccupied molecular orbital (\(\nu\text{UOMO})\) energies of the three broken-symmetry states, (CuT2-CuT3-CuT3b = |\(\text{lowf}\), \(\text{lowp}\), \(\text{lowf}\rangle\), plane), are compared. The \(\nu\text{UOMO}\) of each broken-symmetry state is localized magnetic-orbital centered at the Cu center with \(\mu\)-spin, which is useful in describing the ligand field at each Cu center.
the smaller tetragonal Cu(II) complexes. This is consistent with the electron MCD features of NIAz. The spectrum of NIAz was obtained (no feature at 77 K), in which a pattern from spin-orbit coupling (SOC), which provide structural information on the associated chromophore. First, the liquid He EPR spectrum of NIAz was obtained (no feature at 77 K), in which a signal with a low g_{eff} of 1.86 and a seven-line hyperfine splitting pattern \( |J| \approx 93 \times 10^{-4} \text{ cm}^{-1} \) centered at \( g_{eff} \) of 2.33 are observed (Fig. 3). In addition, variable-temperature variable-field (VT VH) MCD data indicate a doublet ground state and the presence of a low-lying excited doublet state at \( \approx 30 \text{ cm}^{-1} \) (B.D.L, J.Y., and E.L.S., unpublished manuscript). These properties cannot be derived from an isolated mono- or binuclear Cu site (21) but from an all-bridged Cu(II) trimer with three comparable energy splittings (22, 23). The \( |J| \) value of \( 93 \times 10^{-4} \text{ cm}^{-1} \) is \( \approx 60\% \) of that of typical tetragonal Cu(II) complexes. This is consistent with the electron density distribution in the ground state of an exchange coupled isosceles Cu(II) trimer (24); in NIAz, CuT2 and CuT3 would contribute \( \approx 67\% \) to the \( S_{tot} = 1/2 \) ground state coefficients (plus the smaller \( \approx 33\% \) contribution from CuT3 that broadens these hyperfine lines).

Alternatively, the low \( g_{eff} \) can be explained by a SOC effect called the antisymmetric exchange (25). We have demonstrated that this effect is responsible for field-induced mixing of the ground and low-lying excited doublets (split by \( \approx 65 \text{ cm}^{-1} \)) of a D3-symmetric Tris-hydroxy-bridged Cu(II) trimer, resulting in a dramatic shift in \( g_{eff} \) from 2.32 down to \( \approx 1.2 \) (23). In NIAz, we estimate that there is \( \approx 3\% \) mixing of the ground with the low-lying excited doublet state by using the experimental low-lying excited state energy of \( \approx 30 \text{ cm}^{-1} \) from variable-temperature variable-field (VT VH) MCD and \( g_{eff} = 2.33 \) and \( g_{eff} = 1.86 \) (Fig. 3). This spin-orbit mixing leads to narrowing of the Zeeman splitting in both the ground and excited doublets and shifts the EPR transition to higher field, yielding the low \( g_{eff} \) of 1.86 (Fig. 3B). The antisymmetric exchange originates from a combination of efficient ground-to-ground and ground-to-excited state superexchange interactions. These are made possible by the \( \mu_3-1,1,3-N_3 \) bridge, where the \( \sigma \)-bonds between the three Cu \( d_{x^2-y^2} \) ground state orbitals and \( N_3 \pi_{d^b} \) leads to ground-to-ground superexchange, whereas the \( \pi \)-bond between the CuT3 \( d_{x^2-y^2} \) excited state orbital and \( N_3 \pi_{d^b} \) leads to ground-to-excited superexchange in the CuT2/CuT3 and CuT3/CuT5 pairs (Fig. 3C).

The \( \mu_3-1,1,3-N_3 \) -bridged NIAz structure is further corroborated by the unique excited state features in the MCD spectrum (Fig. 4). NIAz, \( \pi^* \rightarrow \text{Cu(II)} \) CT transitions are observed in the 22,000–30,000 cm\(^{-1}\) energy region (bands 7–11).\(^4\) Notably, at least two overlapping MCD pseudo-\( \alpha \) terms (a pair of field- and temperature-dependent MCD C terms with equal magnitude but opposite signs) are associated with these transitions (bands 7/9 and 9/11), in which each pseudo-\( \alpha \) term derives from two perpendicular transitions that are spin-orbit coupled in the third orthogonal direction (26). In NIAz, all \( N_3 \rightarrow \text{Cu(II)} \) CT transitions are \( \pi \)-polarized along the Cu-N bonds, because the azide is in the Cu3 plane (i.e., in x,y plane). Consequently, SOC must be present in the z direction normal to the Cu3 plane, which in fact must derive from a meta-stable SOC centered at the CuT3 center, in which the CuT3 \( d_{x^2-y^2} \) character in...
the CuTβ2-based acceptor MO in the N3− πσ* → CuTβ2 CT transition spin-orbit rotate into the CuTβ2 dσ characters in either the CuTβ2 or CuTβ2-based acceptor MOs (SI Fig. 10); note that ligand-centered (i.e., azide-centered) SOC would be limited, because the N3− πσ* cannot rotate into N3− πσ* in the z direction, and N3− mixing from the N3− MO would be too small to account for the high MCD intensities. Detailed analysis (given in SI Text) indicates that two pseudo-4 terms involving the CuTβ2-based SOC are possible overall, in which the transition pair N3− πσ* → CuTβ2 and CuTβ2 is predicted to have negative lower energy and positive higher energy C term components, whereas the transition pair N3− πσ* → CuTβ2 and CuTβ2 is predicted to have positive lower energy and negative higher energy C term components. These are consistent with the experimental MCD features of the N3− πσ* → CuTβ2 transition (Fig. 4 Lower, bands 7, 9, and 11).

Because NIAz is reversibly formed from RAz, a similar μ3-1,1,3-N3−-bridged structure is also likely for RAz as obtained by DFT calculations (Fig. 1). The fact that the T2 EPR signal in the resting enzyme is unperturbed in RAz and that the N3− πσ* → CuTβ2 CT transition is observed at ∼20,000 cm−1 in the MCD spectrum (13, 15) are consistent with the T2 site being isolated from the AF-coupled T3 site in RAz. In addition, the similar energies of the N3− πσ* → CuTβ2 CT transition in NI Az (Fig. 4, band 6) and RAz reflect the similar T2 site geometries in the two forms. Alternatively, the N3− πσ* → CuTβ2 CT transitions at ∼29,400 cm−1 in the absorption and CD spectra of RAz (14) are ∼3,000–6,000 cm−1 higher in energy than those of NIAz, consistent with having the additional coordination of the OH− bridging ligand to the T3 Cu atoms in RAz (27).

Our spectroscopically derived structures of RAz and NIAz (i.e., the uncoupled RAz form at low pH) are different from the crystallographic structures of azide-bound AO (17), ceruloplasmin (Cp) (18), and CoAT (19), which also differ from one another. However, the spectral features of azide-bound AO (different from those of RAz and NIAz in LC) are also inconsistent with its crystal structure, particularly in EPR and MCD spectra, in which only T2 features are observed with no additional features from the uncoupled T3 Cu atoms present in its crystal structure (28). These inconsistencies suggest that the protein environment has a large effect on azide binding. A Glu residue near the T3 site appears to play a significant role in stabilizing the structure found in the AO and CoAT crystals, because it is well positioned for hydrogen bonding with the azide(s), directly or via water molecules. This Glu (E510 in AO and E509 in CoAT) is in the putative solvent channel (19, 29), and the azide may well have been trapped by this residue in the crystals (prepared by soaking the resting crystals in azide solution). Notably, DFT calculations indicate that the NIAz structure is lower in energy than the T3 μ3-1,1 end-on bridged structure of CoAT (SI Fig. 11) by ∼1.7 kcal/mol,4 suggesting that the NIAz structure is thermodynamically accessible. Thus, the discrepancy between crystallography and spectroscopy appears to reflect the high kinetic barrier in the rigid crystal for rearrangement of the azide conformation, which is lowered in more flexible protein in solution.

Alternatively, F−-bound forms of different MCOs exhibit very similar spectral features, where F− binds strongly (Kd ∼ 105 M−1) at the μ3-position of the trinuclear site (13, 20, 28, 30). With its small size and high affinity, F− would bind to the trinuclear site unobstructed by the protein environment (N3− has an ∼200-fold lower Kd). Importantly, the μ3-oxo-bridged NI (8), which is structurally similar to the F−-bound forms of the MCOs, is also observed in a number of MCOs with very similar spectral features (3–7). Thus, the formation of NI is less affected by the protein environment, and thus NI is a key step in the catalytic cycles of all MCOs.

Correlation of NI to NI. We have previously shown that NI exhibits unique spectral features similar to those of NI Az evaluated above. In NI, the low g eff is observed at ∼1.65, which is due to ∼6% spin-orbit mixing (31) of the ground and low-lying excited doublet states split by ∼150 cm−1 (3). MCD pseudo-4 terms associated with O2− → CuTβ2 CT transitions are also observed at 316(−) and 364(+) nm. The striking resemblance of the spectral features of NI and NI Az originates from their all-bridged structures.

To further compare NI and NI Az, EXAFS and corresponding Fourier transform (FT) data have been obtained (Fig. 5). The FT EXAFS data from NI are consistent with those reported in ref. 3, indicating at least one strong bridging interaction within the trinuclear site with a Cu–Cu distance of ∼3.37 Å (SI Table 3). The beat pattern at k = 8 Å−1 is present in NI, whereas NI Az has higher intensity between k = 7–11 Å−1 (Fig. 5 Inset). A comparison of the nonphase-shift-corrected FT shows that the outer shell amplitude is reduced, and the double peak between R = 2–3.5 Å is split more in NI Az with the intensity at ∼2.8 Å moving to ∼3.0 Å. The NI Az first shell EXAFS data were fit with 3 Cu-N/O interactions at 1.96 Å (SI Table 3). The second and third shell single and multiple scattering contributions from His imidazole rings were insufficient in fitting the intensity at ∼3.0 Å and inclusion of a Cu–Cu contribution at ∼3.46 Å was necessary to obtain a good fit to the data. The best fit to the data indicates a coordination number of 4.5 (σ = 0.0063) for the Cu–Cu interaction at 3.46 Å, indicating that of the four Cu centers in LC, two are present at a distance of 3.46 Å. This distance is longer than that in NI by ∼0.1 Å, indicating that the NI structure has been perturbed upon replacing the μ3-oxo with the μ3-azide ligand. In addition, a higher coordination number of the first shell Cu-N/O interaction (3.5 in NI vs. 3 in NI Az) suggests the prototactically dissociated T3 OH− bridge in NI Az is intact in the structure of NI Az.

DFT calculations were performed to further refine the structure of NI to evaluate whether the additional T3 OH− bridge (derived from the prototactic reduction of the OH− bond) is in fact present in NI. NI structures with both the μ3-oxo and T3 OH− ligands (with either H2O or OH− as the water-derived T2 ligand, referred to as NI1,0,0 and NI0,0,1, respectively) were first obtained. A proton was then added to either the T3 OH− or the μ3-oxo center, which are the two most likely sites for protonation in NI1,0,0/NI0,0,1. As a result, the NI1,0,0 and NI0,0,1 structures with protonated T3 OH− (i.e., with uncoupled H2O bound to CuTβ2) is found to be less stable by 17 and 25 kcal/mol,5 respectively, compared with those with protonated μ3-oxo (i.e., μ3 OH), because of the higher electron density at the

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3The EXAFS of RAz was also obtained and is presented in SI Figs. 13 and 14. In SI Fig. 15, the nonphase-shift-corrected FT data of NI Az and RAz are compared. In RAz, the peak at ∼3.46 Å present in NI Az is absent, indicating the absence of a Cu–Cu contribution.

4The protonation state of the T2 water-derived ligand in NI is still elusive.
The addition of a second proton to either the Cu pairs results in the spin-frustrated doublet ground states in NIOH (Table 4). Thus, comparable exchange coupling between all three singly protonated NIOH and NIH2O results in similar structures in which the T3 OH in magnitude than those of the singly bridged CuT2–CuT3 pairs, which is due to the short CuT3–CuT3 distance (associated with two single-atom bridges) that renders smaller CuT3–O–CuT3 angles (∼100°) compared with the CuT2–O–CuT3 angles (∼130°, SI Table 4). Thus, comparable exchange coupling between all three Cu pairs result in the spin-frustrated doublet ground states in NIOH and NIH2O. (See SI Fig. 12 for contour plots of the magnetic orbitals and SI Table 5 for MO compositions.)

Mechanistic Implications. The decay of NI to the resting enzyme is a slow process, which is accelerated at low pH (t1/2 = 12–13 s at pH 7.4 vs. t1/2 = 0.33–1.0 s at pH 4.0–4.2; refs. 4 and 32). DFT calculations indicate that the first proton binds at the μ3-oxo center in NIOH, NIH2O as described above (Fig. 7 Upper, second structure). The addition of a second proton to either the μ3OH or T3 OH− in the singly protonated NIOH and NIH2O results in similar structures in which the T3 OH− bridge is protonated (the proton added to μ3OH migrates to the T3 OH− bridge), uncoupling CuT3 from the other two Cu atoms (Fig. 7 Upper, third structure). The uncoupled T3 OH−, now H2O bound to CuT3, would be readily exchanged with the ambient water, consistent with the 18O isotope ratio MS (IRMPS) data that showed only one of the O atoms from O2 remains in the resting enzyme (10, 11).

The final step in NI decay requires the reorganization of the internal O atom to the external side of the T2 site in the resting enzyme (Fig. 7 Upper, fourth structure), as indicated by 17O EPR data (12). The energy profile of this step is presented in Fig. 7 Lower, along with the optimized structures of doubly protonated NH2O (red) and NI (blue) on the far left side, then of those with the central O atom rotated 45° and 90° relative to its original positions (the line that connects CuT2 and eN of the T2 His ligand above the plane was used as the rotating axis), then of the final decayed products (as the resting enzyme has OH− on CuT2, the H2O ligand in NH2O would deprotonate). Notably, the proton on the CuT3 H2O ligand transfers to the rotating OH− with the reformation of the T3 OH− bridge at the initial 45° rotation. Then, the proton on the now rotating H2O in the doubly protonated NH2O transfers to the external CuT2 OH− ligand upon 90° rotation. In the final decayed products, the original water-derived ligand bound externally to CuT2 is replaced by the rotating OH− (from O2) in NH2O and by the rotating H2O in NH2O. The rotation energy barrier is found to be lower for the doubly protonated NI (Fig. 7 Lower, blue plot) than in NH2O due to the energy stabilization from the strong CuT2(II)–OH− bond. The lower limits for the energy barrier (ΔE‡) for the structural rearrangement of the doubly protonated NH2O and NH2O are 8.5 and 18.2 kcal/mol, respectively, which are consistent with the experimental enthalpy of activation of 8.8–13.9 kcal/mol (32), indicating that this step is likely the rate limiting step in the slow decay of NI.

Fig. 6. Optimized structures of NI with H2O and OH− on the CuT2 center (NH2O and NI, respectively). Geometric parameters are listed in SI Table 4.

Fig. 7. Proposed decay mechanism of NI to the resting enzyme. The O atoms in red indicate that these are from O2 (Upper) and the energy profiles of the rotation of the internal O atom to the external side of the T2 site in doubly protonated NH2O (in red) and NI (in blue) (Lower). A water molecule near the T3 site found in all MCO crystal structures is also included in these calculations.

Fig. 8. Possible mechanisms for the reduction of NI to the fully reduced enzyme.

1The calculated ground doublet-quartet splittings are 219 and 64 cm−1 for NI and NI with protonated T3 OH−, respectively.
The all-bridged structure of NI is consistent with the rapid 4e− reduction of NI to the fully reduced enzyme, because the µ3-oxo bridge would allow electron delocalization over the three Cu centers for facile electron transfer (ET) through the cluster. In Fig. 8, a reaction coordinate for the four-electron reduction of NH₄OH or NaN₃ is presented, where the three Cu centers in the trinuclear cluster are sequentially reduced by reducing substrate via the T1 site [the fourth electron reduces the T1 site (data not shown)]. CuT₃ is expected to reduce first as the hole on CuT₃ is lower in energy than those of CuT₃ and CuT₂; this is based on our recent study in which a negatively charged Asp residue near the CuT₂ and CuT₃ centers is found to significantly lower the reduction potentials of these Cu centers below that of CuT₃ (note that the electronic structure of NI is not significantly affected by this Asp as the Cu sites are all oxidized in NI) (J.Y. and E.L.S., unpublished manuscript). The reduction of CuT₃ would likely be coupled to protonation of the µ3-oxo center as well as the OH− ligand, which further dissociate from the reduced CuT₃ already ligated by three His ligands. Next, CuT₂ would likely reduce, where in NH₄OH, protonation of the T2 OH− ligand would be coupled to reduction of CuT₂ to raise its reduction potential relative to that of CuT₃. Importantly, the OH− bridge between CuT₁ and CuT₃ should play a critical role in the rapid ET from CuT₂ to CuT₁; however, CuT₂ before CuT₃ would result in protonation of the OH− bridge, leading to the loss of the CuT₂-CuT₃ electronic coupling for rapid ET to CuT₂. Note that this model predicts formation of a mixed-valence Cu pair. In fact, NI decays in the presence of excess reductant to a species with a new reduction potential relative to that of CuT₃, which is not significantly affected by this Asp as the Cu sites are all oxidized in NI (J.Y. and E.L.S., unpublished manuscript). Further reduction of the remaining CuT₃ would be fast via the Cys−His pathway between T1 and T3 centers and would be accompanied by protonation of the OH− bridge and dissociation of the two water molecules from the trinuclear site.

In summary, the unique spectral features of the one-azide-bound forms of Lc (Rₐz and NIAz) originate from the µ3-oxo-bridged trinuclear Cu cluster site. Deeper insight into the exchange-coupled trinuclear Cu cluster site in NIAz further led to refinement of the NI structure in which the T3 OH− bridge is found necessary to achieve the spin-frustrated doublet ground state observed experimentally. The decay of NI is initiated by successive protonation of the electron-rich µ3-oxo ligand, then the T3 OH− bridge, to uncouple the three Cu centers in the trinuclear site. The rate-limiting step is associated with the rotation of the protonated µ3-oxo ligand in NI that must overcome steric hindrance of the cluster. The slow decay of NI is due to the barrier for this rearrangement, implicating NI as the catalytically relevant fully oxidized form of the MCoOs. Indeed, the all-bridged structure of NI would facilitate ET to all three Cu centers of the trinuclear cluster for rapid proton-coupled reduction of NI to the fully reduced enzyme for catalytic turnover.

Materials and Methods

Preparation of Samples. Japanese Rhus vernicifera Lc and its T2-depleted derivative were purified and assayed according to published procedures (35, 36). Rapid freeze-quenched (RFQ) NI samples were obtained as reported in ref. 3. NIAz was obtained either by mixing the reduced Lc in the presence of 10 equivalent of NaN₃ with equal volume of O₂-saturated buffer or by mixing the reduced Lc with O₂-saturated buffer allowed to age for 100 ms where most of the NI is still not decayed (τdecay = 6.6–7.6 × 10⁻³ s⁻¹ (ref. 3)), followed by mixing with buffer containing 10 equivalents of NaN₃, then RFQ trapping at 25 ms. RFQ samples were loaded directly into EPR tubes or dissolved in glycerol (vol/vol = 50/50) at −30°C into MCD or XAS sample cells (3).

Computational Methods. DFT calculations were performed on Gaussian 03 (37), implementing the broken symmetry method (38, 39). All geometry optimizations were performed with B3LYP functional (40) and double-ζ basis sets (6–31G* for Cu and coordinated N/O atoms and 3–21G* for the rest). Rₐz and NIAz were optimized at the [Cu³Cu⁴T₃Cu⁵] = αββ symmetry state, whereas all of the other structures were optimized at their high-spin states (ααα). The starting geometry of the trinuclear Cu site was adapted from the crystal structure of Trametes versicolor Lc (1GYC. Res. 1.9 Å) (29), in which the His ligands were replaced by imidazolyl ligands. To fully reflect the features of the crystal structure, (i) the positions of the H atoms that replaced the side chains to the protein backbone and those bound to His N not bound to Cu (all involved in hydrogen bonds) were fixed; (ii) the angle of the O atom on the T2 water-derived ligand relative to the plane of the two T2 His rings was fixed to prevent it from artificial bonding to the nearby T3 His ligands; and (iii) the three N−Cu₃−C−Cu₃−N dihedral angles, where N is the coordinated atom of the eclipsed His ligand, were fixed to keep the eclipsed conformation as found in the crystal structures of all MCoOs. The optimized geometries of Rₐz, NIEK₃₃, NIOH− and NH₂O were used for single point calculations at the B3LYP/TZV level (41). The reported energies and MO compositions (obtained by using PyMOlize; ref. 42) are based on B3LYP/TZV, unless otherwise noted. Further details are available in SI Text.

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