Protonation state of the Cu4S2 CuZ site in nitrous oxide reductase: redox dependence and insight into reactivity†

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Spectroscopic and computational methods have been used to determine the protonation state of the edge sulfur ligand in the Cu4S2 CuZ form of the active site of nitrous oxide reductase (N2OR) in its 3Cu1CuIV (1-hole) and 2Cu2CuII (2-hole) redox states. The EPR, absorption, and MCD spectra of 1-hole CuZ indicate that the unpaired spin in this site is evenly delocalized over Cu1, CuII, and CuIV. 1-hole CuZ is shown to have a μ2-thiolate edge ligand from the observation of S–H bending modes in the resonance Raman spectrum at 450 and 492 cm⁻¹ that have significant deuterium isotope shifts (−137 cm⁻¹) and are not perturbed up to pH 10. 2-hole CuZ is characterized with absorption and resonance Raman spectroscopies as having two Cu–S stretching vibrations that profile differently. DFT models of the 1-hole and 2-hole CuZ sites are correlated to these spectroscopic features to determine that 2-hole CuZ has a μ2-sulfide edge ligand at neutral pH. The slow two electron (+1 proton) reduction of N₂O by 1-hole CuZ is discussed and the possibility of a reaction between 2-hole CuZ and O₂ is considered.

1. Introduction

The main reductive part of the nitrogen cycle, known as bacterial denitrification, is performed by soil and marine bacteria as a means of anaerobic or microaerobic respiration. Denitrification involves the conversion of nitrate to dinitrogen via four successive reductive steps (NO3⁻ → NO2⁻ → NO → N₂O → N₂), each performed by a different metalloenzyme.1 The terminal product of denitrification can be either N₂O or N₂, depending on the regulatory control of the N₂O reduction process and whether the bacterium involved contains the gene cluster for nitrous oxide reduction (the nos cluster; nosZ encodes the nitrous oxide reductase enzyme).2,3 The N₂O reduction process and its regulation in vivo are of significant interest because N₂O is a potent greenhouse gas, with a global warming potential 300× that of CO2,4,5 and depletes the ozone layer.6 Anthropogenic sources of environmental N₂O, the majority of which is due to agricultural activity, is an increasing contribution to the global atmosphere.5 Soil studies have indicated that pH,7,8 temperature,9 acetylene,10 sulfide,11 and dioxygen12 all affect the production of N₂O, but the molecular basis of these effects is still not known. A molecular understanding of nitrous oxide reduction and how this process is regulated could enable mitigation of N₂O release from anthropogenic sources.5

Nitrous oxide reductase contains two copper sites: a binuclear site known as CuA that functions as an electron transfer site, and an unusual tetranuclear copper sulfide cluster active site, where N₂O binds and is reduced (Fig. 1). Two forms of this tetranuclear site have been structurally characterized. One, known as CuZ, has a μ4 sulfide ligand bridging all four coppers and a solvent derived ligand on an open edge (the CuI–CuIV edge) where N₂O is proposed to bind (Fig. 1A).13 This edge ligand has previously been assigned as a bridging hydroxide ligand, due to the presence of a vibration in the resonance Raman spectrum of CuZ that shifts in H2O solvent at high pH and the absence of significant spectroscopic differences between CuZ at high and low pH.13 The other form of the cluster, known as CuZ*, has an additional μ2 sulfur ligand bridging the CuI–CuIV edge (Fig. 1B).14 Whether the μ2 edge ligand in CuZ* is a thiolate (SH⁻) or a sulfide (S²⁻) and how its protonation depends on the redox state of the cluster are not known. The Cu₂S₂ CuZ form of the cluster is dominantly isolated when N₂OR is purified in the absence of...
oxygen or rapidly in the presence of oxygen, while the Cu$_4$S$_2$Cu$_2^+$ form is isolated when the purification is performed aerobically or anaerobically from mutants in the accessory genes; however, all purifications typically yield enzyme with a mixture of the two sites. Which structural form of the cluster is responsible for N$_2$O reduction in steady state assays is of some debate. As isolated, neither N$_2$OR containing a high percentage of Cu$_Z$ nor N$_2$OR containing a high percentage of Cu$_Z$ shows high enough specific activity in steady-state assays to be consistent with N$_2$OR activity in whole cells. N$_2$OR containing Cu$_Z$ can be activated by prolonged dialysis against base, while N$_2$OR that contains Cu$_Z^+$ can be reductively activated by preincubation with methyl viologen, which reduces Cu$_Z^+$ to the active fully reduced (4Cu$^0$) redox state. After activation, both Cu$_Z$ and Cu$_Z^+$ show specific activities consistent with whole cell N$_2$OR activity. However, it has recently been shown that the Cu$_Z$ site in its fully reduced redox state is the form of the cluster that is responsible for the rapid N$_2$O reduction in steady state assays with methyl viologen, based on its rapid single turnover reaction with N$_2$O. Alternatively, in single turnover studies Cu$_Z$ in its 1-hole redox state reduces N$_2$O but at a rate too slow to be catalytically relevant (10$^{-6}$ that of the fully reduced state of Cu$_Z$). Thus, the physiological role of the Cu$_Z$ site in nitrous oxide reduction and whether it participates in N$_2$O reduction in vivo is unknown.

The Cu$_Z$ site in nitrous oxide reductase has been extensively studied in N$_2$OR isolated from Pseudomonas stutzeri (PsN$_2$OR) and Paracoccus pantotrophus (PpN$_2$OR). In the latter enzyme, Cu$_Z$ has been shown to access two redox states, the resting 2Cu$^2$/2CuI (2-hole) redox state, and a 1 electron reduced 3Cu$^+$/2CuII (1-hole) redox state ($E^\circ = +60$ mV). Both redox states of Cu$_Z$ have previously been studied using EPR, absorption, MCD, and resonance Raman spectroscopies. However, these studies were performed before the elucidation of the presence of a second sulfur in the Cu$_Z$ cluster, and so yielded limited direct insight into the cluster and the protonation state of the edge sulfur. Additionally, the previous studies were performed in the presence of background spectroscopic features from $\sim$30% Cu$_Z^+$, which complicates the analysis. These limitations lead to the conclusion that Cu$_Z$ and Cu$_Z^+$ were very similar and perhaps differed only in the second sphere. These results are now extended and correlated to the structural insight that Cu$_Z$ contains an inorganic sulfur edge ligand. An understanding of the protonation state, electronic structure, and potential reactivity of the Cu$_Z$ site is necessary to gain insight into its reactivity and role in vivo.

This study uses EPR, absorption, MCD and resonance Raman spectroscopies coupled with DFT calculations to determine the protonation state of the edge sulfur ligand in the 1-hole and 2-hole redox states of Cu$_Z$ in Marinobacter hydrocarbonoclasticus N$_2$OR (Mh$_n$N$_2$OR) and to define the electronic structures of these states. This leads to insight into the nature of the reactivity of the 1-hole and 2-hole states of Cu$_Z$ and the origin of the spectroscopic similarity between 1-hole Cu$_Z$ and 1-hole Cu$_Z^+$, despite significant differences in edge ligation in the two sites.

2. Methodology

2.1 Summary of experimental methodology

Full experimental methodology and computational details can be found in the ESI, while a summary is presented here. Nitrous oxide reductase (N$_2$OR) was isolated from Marinobacter hydrocarbonoclasticus 617 (formerly Pseudomonas nautica) grown under microaerobic conditions in the presence of nitrate after two aerobic chromatographic steps without added reductant, as described previously. These purification conditions were shown to maximize the amount of Cu$_4$S$_2$ Cu$_2^+$ content relative to Cu$_4$S$_2$Cu$_2^+$ in the purified enzyme. Samples containing larger amounts of Cu$_Z^+$ were purified in parallel with three chromatographic purification steps from a batch of cells grown under anaerobic conditions in the presence of nitrate, and that had been stored at $-80^\circ$C for a long period. Both Mh$_n$N$_2$OR samples showed copper quantitation results consistent with full occupancy of the Cu$_A$ and Cu$_Z$/Cu$_Z^+$ sites (6.4 $\pm$ 0.2 and 6.2 $\pm$ 0.7 respectively). The percentage of Cu$_Z$ versus Cu$_Z^+$ in the samples used for this study was determined by EPR spin quantitation (Fig. S1†). Samples purified with high amounts of Cu$_Z$ contained 60 $\pm$ 10% Cu$_Z^+$, while samples purified to obtain more Cu$_Z^+$ contained 10 $\pm$ 10% Cu$_Z$. Spectroscopic samples of 1-hole and 2-hole Cu$_Z$ were prepared in a glove box under N$_2$ atmosphere. Samples of 1-hole Cu$_Z$ were prepared from Mh$_n$N$_2$OR (60% Cu$_Z$ and 40% Cu$_Z^+$) that had been incubated with 100 equivalents of reduced methyl viologen, with subsequent removal of the methyl viologen using a desalting column. Samples of 2-hole Cu$_Z$ were prepared by reducing Mh$_n$N$_2$OR (60 $\pm$ 10% Cu$_Z$, 40 $\pm$ 10% Cu$_Z^+$) with 10 equivalents of sodium ascorbate, which reduces the Cu$_Z$ site rapidly and the 2-hole Cu$_Z$ site very slowly, and spectra were collected within 1 hour so that minimal reduction of 2-hole Cu$_Z$ was observed. In parallel, Mh$_n$N$_2$OR samples containing 90 $\pm$ 10% Cu$_Z^+$ were reduced with 10 equivalents of sodium ascorbate to obtain the spectral features of 1-hole Cu$_Z^+$. For pH and deuteration studies, samples of 1-hole and 2-hole Cu$_Z^+$ were buffer exchanged by centrifugation into different pH or pH buffers. Typical Mh$_n$N$_2$OR concentrations used for spectroscopic samples were 0.1–0.3 mM for absorption, MCD and EPR, and up to 0.5 mM for resonance Raman.

![Fig. 1](image-url) The two forms of the tetranuclear copper sulfide active site of nitrous oxide reductase. (A) Cu$_Z^+$ in PsN$_2$OR isolated aerobically (PDB ID 1FWX). (B) Cu$_Z$ in PsN$_2$OR isolated anaerobically (PDB ID 3SBP).
2.2 Computational modeling

A computational model of CuZ was built from the atomic coordinates of the crystal structure of *Pseudomonas stutzeri* N3BP, the only known structure of the Cu$_4$S$_2$ cluster (PDB ID 3SBP, resolution 1.7 Å). The model included the Cu$_4$S$_2$ core and 7 ligating His residues, where the z carbon and distal nitrogen were constrained at their crystallographic positions. A computational model for CuZ$_2$ with a hydroxide bridging ligand and identical z carbon and distal nitrogen constraints was constructed from the crystal structure of *Paracoccus denitrificans* N3OR (PDB ID 1FWX). Calculations were performed using Gaussian 09 (version d01). Geometry optimizations were performed using the B3LYP functional, the TZVP basis set on all core atoms (Cu$_4$S) and the ligating His nitrogens, and the SV basis set on all remaining atoms, and solvation was modeled with a PCM of 4.0. A larger basis set and different functionals were also explored, as described in the text. The optimized structures were then used for frequency, TD DFT, and single point calculations. To determine the relative energy of deprotonation ($\Delta \Delta E$) of the edge SH$^-$ in the 2-hole versus 1-hole redox state, larger models were optimized that included two second sphere carboxylates, Asp127 and Asp240, which hydrogen bond to the His ligands of Cu$_4$Z and Cu$_4$Z$_2$. The energy of an internal proton transfer from the edge SH$^-$ to Asp127 was calculated for the 1-hole and 2-hole redox states and compared to obtain the $\Delta \Delta E$.

3 Results and analysis

3.1 Spectroscopy of 1-hole CuZ

Previous spectroscopic studies of CuZ$_2$, undertaken before identification of the presence of a second sulfur, were performed on samples of PpN$_2$OR and *Ps*N$_2$OR that contained mixtures of the CuZ$_2$ and CuZ$_2^*$ sites (in a 7 : 3 ratio for PpN$_2$OR) without a way to resolve the spectral features of the CuZ$_2$ site from the mixture. Recently, it has been found that the two-sulfur CuZ$_2$ site cannot be reduced by methyl viologen, which reduces both the CuZ$_2$ site and the CuZ$_2^*$ form of the cluster. This provides an opportunity to cleanly resolve the spectral features of 1-hole CuZ$_2$ by studying methyl viologen reduced samples after removal of the reductant. This approach allows correlation of the electronic structure of 1-hole CuZ$_2$, obtained from spectroscopy, with the recently determined Cu$_4$S$_2$ structure of the cluster, to determine the nature of the edge sulfur ligand in its 1-hole and resting 2-hole redox states.

EPR. The X-band and Q-band EPR spectra of a methyl viologen reduced sample of 1-hole CuZ$_2$ are given in Fig. 2. The EPR spectrum is axial with $g_{||} > g_{\perp} > 2.0$ and a pattern of five evenly space hyperfine lines in the $A_{||}$ region. The axial nature of the spectrum indicates that, while the spin density is delocalized over multiple copper nuclei, it resides in dominantly dx$_{2-y}$z orbitals on each Cu site that contributes to the ground state. The $A_{||}$ hyperfine features can be further resolved in the second derivative of the X-band EPR spectrum, as can hyperfine features in the $A_{\perp}$ region (Fig. 2A inset and S2†). Simulation of the X-band, X-band 2nd derivative, and Q-band EPR spectra yields the $g$ and $A$ values for 1-hole CuZ$_2$ given in Table 1. The $g$ values for 1-hole CuZ$_2$ are very similar to those previously obtained for 1-hole CuZ$_2$ (Table 1) and to those obtained for CuZ$_2$ in PpN$_2$OR.$^{15,29}$ This is interesting, considering that an edge SH$^-$ (thiolate) or S$_2^-$ (sulfide) in CuZ$_2$ would be expected to be a more covalent ligand than the hydroxide in CuZ$_2$ and this would lower the $g$ values. However, in 1-hole CuZ$_2$ there is a high energy d-d transition that is not present in the 1-hole CuZ spectrum (wide infra). This transition has previously been assigned as a d$_{xy} \rightarrow$ d$_{x^2-y^2}$ excitation localized on Cu$_{I}$. The $g_{||}$ value is inversely proportional to the d$_{xy}$ to d$_{x^2-y^2}$ energy splitting, so the presence of a high energy d$_{xy}$ to d$_{x^2-y^2}$ transition in CuZ$_2$ but not in CuZ$_2$ would lead to a lower $g_{||}$ value for CuZ$_2$ than would be expected from covalency alone, which could result in similar $g_{||}$ values between 1-hole CuZ$_2$ and the more covalent 1-hole CuZ$_2$ site. The ligand field origin of the lower energy d$_{xy}$ to d$_{x^2-y^2}$ transition in 1-hole CuZ$_2$ is considered below.

The $A_{||}$ and $A_{\perp}$ values for 1-hole CuZ$_2$ are similar in magnitude to those for CuZ$_2$, but fitting the hyperfine pattern requires three equivalent contributions rather than the $\sim 5 : 2$ ratio of hyperfine values observed for CuZ$_2$ (Table 1). This indicates that in the ground state of 1-hole CuZ$_2$ the spin is distributed over three copper centers in dominantly dx$_{2-y}$z orbitals. The three coppers involved are likely Cu$_{II}$, Cu$_{III}$, and Cu$_{IV}$, since these copper centers are in the same plane as the two sulfur ligands and bonding with the strong donor $\mu_4$ sulfide and $\mu_2$ sulfur ligands should define a common x, y plane for these coppers, with the z axis of the local $g$ tensor of each copper oriented perpendicular to the Cu$_4$S$_2$ plane. This is consistent with the

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axial nature of the $g$ values and with the DFT calculations reported below.

**Absorption and MCD.** The low temperature absorption and MCD spectra of a methyl viologen reduced sample of 1-hole Cu$_Z$ are presented in Fig. 3A. The absorption maximum of 1-hole Cu$_Z$ occurs at 14 600 cm$^{-1}$ ($\varepsilon = 3000$ M$^{-1}$ cm$^{-1}$), 1000 cm$^{-1}$ lower than the absorption maximum of 1-hole Cu$_2$ (Fig. 3B). There are no additional low energy intense absorption features due to the edge sulfur. The low temperature absorption and MCD spectra can be simultaneously fit to yield a total of 11 transitions, which can be assigned by considering their energies and Cu/$D_0$ ratios, following ref. 15 (Table S1†). Comparison of the transition assignments and energies of the 1-hole Cu$_Z$ and 1-hole Cu$_2$ sites reveals some key differences. While the absorption maximum of Cu$_Z$ occurs at lower energy than that of Cu$_{2z}$ from MCD the three $\mu_4S$ to Cu CT transitions, assigned in Cu$_2$,$^{35}$ occur at very similar energies in the two sites (bands 5, 6, and 7, numbering given in Fig. 3). The shift in the absorption maximum therefore arises from a different intensity pattern for these transitions, where in Cu$_Z$ the lowest energy transition at 14 600 cm$^{-1}$ is the most intense (band 5) and the transition at 15 600 cm$^{-1}$ is weaker (band 6), but in Cu$_Z$ this is reversed. The $\mu_4S$ to Cu CTs in Cu$_Z$ have previously been assigned as transitions from the three different $3p$ orbitals of the $\mu_4S$ to the $\beta$ LUMO of the cluster. From our current study of resting 1-hole Cu$_Z$ and Cu$_Z$ with different edge ligands,$^{15,33}$ the $\beta$ LUMO is delocalized in the plane that contains Cu$_{II}$, Cu$_{IV}$, Cu$_{IV}$, and the $\mu_4S$, with different amounts of spin distributed over Cu$_{II}$, Cu$_{III}$, and Cu$_{IV}$, depending on the edge ligation. Two of the $\mu_4S$–$p$ orbitals are in the plane, oriented between Cu$_{II}$ and Cu$_{IV}$ ($S_{p_y}$) and between Cu$_{III}$ and Cu$_{IV}$ ($S_{p_z}$), while the third is perpendicular to the plane ($S_{p_x}$).

Scheme S1† reflects the orientation and simplified composition of these orbitals determined for 1-hole Cu$_Z$ from DFT calculations. The CT intensities reflect the overlap of these three $3p$ orbitals with the $\beta$ LUMO. Since bands 5 and 6 show the highest intensity in the 1-hole forms of Cu$_{II}$ and Cu$_{IV}$, these must reflect charge transfer from the in-plane $S_{p_x}$ (band 6, dominant in Cu$_Z$ due to higher overlap with Cu$_{IV}$) and $S_{p_y}$ (band 5) orbitals. Bands 6 and 5 form a pseudo-$A$ feature in the MCD spectrum (*i.e.* derivative-shaped) and thus must arise from two transitions with orthogonal transition moments that spin–orbit couple in a third, mutually perpendicular direction (*i.e.* $L_z$). Since band 6 arises from a transition to Cu$_{II}$ (from its dominant intensity in Cu$_2$), band 5 must reflect a transition to Cu$_{IV}$, since the Cu$_{I}$–S and Cu$_{IV}$–S bonds are close to perpendicular (96° from crystallography) while the Cu$_{II}$–S and Cu$_{IV}$–S bonds are close to parallel (160°). The change in relative intensities of the $\mu_4S$ to Cu CT transitions in Cu$_Z$ relative to Cu$_Z$ where band 6 decreases in intensity while band 5 increases in intensity, thus indicates that there is less spin on Cu$_{II}$ and more spin on Cu$_{IV}$ in 1-hole Cu$_Z$ relative to 1-hole Cu$_Z$. This is consistent with the EPR hyperfine values, which suggest that the spin in Cu$_Z$ is delocalized $1:1:1$ over Cu$_{II}$, Cu$_{III}$, and Cu$_{IV}$, while from ref. 35 in Cu$_Z$ the spin is delocalized $\sim 5:2$ over Cu$_{II}$ and Cu$_{IV}$. Additionally, in Cu$_2$, a band at 18 000 cm$^{-1}$ (band 8) was assigned as a high energy d–d transition due to its high Cu/$D_0$ ratio; this was assigned as a localized $d_{xy} \rightarrow d_{z^2}$ transition on Cu$_{II}$, where most of the 1-hole is localized.$^{35}$ No equivalent high energy d–d transition is observed in the MCD spectrum of Cu$_Z$. The lower energy of the d–d transitions in Cu$_Z$ relative to Cu$_Z$ is likely due to the decreased spin on Cu$_{II}$, the only four coordinate site, relative to Cu$_{II}$ and Cu$_{IV}$, which are both 3 coordinate and have a weaker ligand field.

**Resonance Raman.** The resonance Raman spectrum of 1-hole Cu$_Z$ and the enhancement profiles of the vibrations are presented in Fig. 4A and B, respectively. Seven vibrations are enhanced in the most intense S to Cu CT transition (band 5), including three intense vibrations at 203, 378, and 492 cm$^{-1}$. The vibration at 378 cm$^{-1}$ occurs at the same energy as a Cu–S

![Fig. 3 Low temperature absorption and MCD spectra of (A) 1-hole Cu$_Z$, 10 K absorption, 5 K and 7 T MCD. (B) 1-hole Cu$_Z$, 5 K absorption, 5 K and 7 T MCD (adapted from ref. 15).](image1)

![Fig. 4 (A) Resonance Raman spectrum of 1-hole Cu$_Z$ at 77 K, excitation energy 697 nm. (B) Excitation profile of the 203, 378, and 492 cm$^{-1}$ vibrations. (C) H/D isotope shift of the vibrations of 1-hole Cu$_Z$ performed in pH or pD 7.8, 100 mM phosphate, excitation energy 676 nm. (D) Comparison of SH bending vibrations at pH/pD 7.8 (green) and pH/pD 10 (blue).](image2)
stretch of the Cu$_Z$ site (Fig. S3†) and the previously reported $^{34}$S isotope sensitivities of both vibrations are similar (−5.8 and −4.7 cm$^{-1}$, respectively), indicating that the 378 cm$^{-1}$ vibration in 1-hole Cu$_Z$ can be assigned as a Cu–S vibration of the μ$_4$ sulfide. In contrast, the 203 cm$^{-1}$ vibration is significantly lower in energy than the vibrations of Cu$_Z^*$ and thus can be assigned as a Cu–S vibration of the μ$_2$ sulfur ligand that is only present in Cu$_Z$. Further, there are two high energy vibrations in Cu$_Z$ at 450 and 492 cm$^{-1}$ that show significant deuterium isotope sensitivity, shifting down in energy by −137 cm$^{-1}$ (for the 492 cm$^{-1}$ vibration) in deuterated buffer (Fig. 4C). This shift requires their assignment as S–H bending modes. Thus, we can definitively identify the edge ligand in 1-hole Cu$_Z$ as a μ$_2$SH$.^*$ The S–H bending modes at 492 and 450 cm$^{-1}$ are present at both pH 7.8 and pH 10 (Fig. 4D), indicating that the pK$_a$ of the edge thiolate is ~11 or higher. This is further supported by the lack of pH dependence observed in the MCD and EPR spectra of 1-hole Cu$_Z$ between pH 6 and pH 10 (Fig. S4†). Since the second pK$_a$ of free hydrogen sulfide in water is 12, a pK$_a$ range of 11–12 can be estimated for the edge thiolate ligand in 1-hole Cu$_Z$.

3.2 Spectroscopy of 2-hole Cu$_Z$

Absorption. The 2-hole redox state has been previously shown to be the resting redox state of Cu$_Z$. 2-hole Cu$_Z$ is diamagnetic from MCD.$^{19}$ The absorption features of 2-hole Cu$_Z$ in as-isolated N$_5$OR are present with additional spectral contributions from oxidized Cu$_A$ and some amount of 1-hole Cu$_Z$. To remove these contributions, the absorption spectrum of 2-hole Cu$_Z$ (Fig. 5) was obtained after reduction with sodium ascorbate, which reduces the Cu$_A$ site faster than it reduces 2-hole Cu$_Z$, and subtraction of the spectral contribution of 1-hole Cu$_Z$, obtained from a separately purified N$_5$OR sample containing 90 ± 10% Cu$_Z$. An intense absorption maximum for 2-hole Cu$_Z$ is observed at 18 300 cm$^{-1}$ ($\epsilon \approx 10 000$ M$^{-1}$ cm$^{-1}$) with a weaker low energy shoulder, consistent with absorption spectra previously reported for ascorbate reduced samples containing high amounts of 2-hole Cu$_Z$.$^{19,32}$ The low temperature absorption spectrum of 2-hole Cu$_Z$ (Fig. S5†) resolves this absorption maximum into three distinct absorption bands. Simulation of the room temperature absorption spectrum with transition energies derived from the low temperature spectrum distinguishes five transitions, all with absorption intensities higher than 1000 M$^{-1}$ cm$^{-1}$, sufficiently intense to be S to Cu CT transitions from the μ$_4$S$^2$– or the μ$_2$S ligand (Fig. 5). The two most intense transitions (bands 2 and 3) are either from different ligands (μ$_2$S and μ$_4$S$^2$–) or from the same ligand to two different acceptor orbitals (the α and β holes of the broken symmetry singlet ground state). Based on the correlation of resonance Raman excitation profiles of the vibrations of 2-hole Cu$_Z$ to DFT calculations (vide infra), the assignment of the two transitions as CT transitions from the μ$_4$S$^2$– to two different holes is preferred.

Resonance Raman. The resonance Raman spectrum of 2-hole Cu$_Z$ was obtained upon excitation into the intense absorption maximum at 18 300 cm$^{-1}$ (Fig. 6A). Two vibrations are enhanced at 350 and 405 cm$^{-1}$. The $^{34}$S isotope shifts of these vibrations have been previously reported to be −5.6 and −5.8 cm$^{-1}$, respectively, indicating that they are Cu–S stretches.$^{24}$ In contrast to 1-hole Cu$_Z$, no higher energy S–H bending vibration is observed (up to 800 cm$^{-1}$). The excitation profile of the Cu–S stretching vibrations shows that they are enhanced differently in the most intense absorption bands 2 and 3 (Fig. 6B). The lower energy vibration at 350 cm$^{-1}$ is enhanced in both transitions, while the higher energy vibration at 405 cm$^{-1}$ is dominantly enhanced in the lower energy transition (band 2) and only weakly enhanced in band 3. This difference in profiling behavior is consistent with the Cu–S vibrations obtained computationally for a Cu$_4$S$_2$ cluster with a μ$_4$S$^2$– and μ$_2$S$^2$– and with the predicted enhancements of key vibrations in transitions from the μ$_4$S$^2$– to the α and β holes (see 3.3).

The resonance Raman spectrum 2-hole Cu$_Z$ shows no significant shift in the energies of the 350 and 405 cm$^{-1}$ vibrations between pH 6 and pH 10 (Fig. S6†). This suggests that the edge ligand has a pK$_a$ either lower than 5.5 or higher than 10.5. A pK$_a$ higher than 10.5 in the 2-hole redox state is not consistent with observed pK$_a$ of 11–12 for 1-hole Cu$_Z$, as the increased charge of the 2-hole state will lead to a lower pK$_a$ relative to the 1-hole redox state. The possibility of a pK$_a$ less than 5.5 for 2-hole Cu$_Z$ is evaluated computationally below.

3.3 Calculations

1-hole Cu$_Z$. A computational model of 1-hole Cu$_Z$ was constructed based on the crystal structure of Pseudomonas stutzeri...
N2OR (PDB ID 3SBP, resolution 1.7 Å). On the basis of the resonance Raman data, the edge sulfur was modeled as an SH ligand bridging the CuI–CuIV edge (Fig. 7A). This will be compared to an experimentally validated model of the Cu2 site, which has a hydroxide ligand bridging the CuI–CuIV edge (Fig. 7B). The optimized structure of the 1-hole SH cluster agrees well with the bond lengths and angles observed in the crystal structure (2.35 Å and 2.48 Å for the CuI–μ3SH and CuIV–μ2SH bonds computationally, relative to 2.61 Å and 2.49 Å crystallographically with a resolution of 1.7 Å, Table S2†). Since the crystal was grown from the “purple” resting form of PnN2OR, containing the resting 2-hole redox state of the Cu2 site, the Cu2 site in the crystal may have some photo-reduction due to exposure to X-ray radiation.37 The calculated structures and spin distributions are not significantly perturbed when a triple zeta basis set is used on all His ring atoms (Tables S4 and S5†). Including the second sphere residues Lys397 and Glu435 in the computational model also does not affect the structure or spin distribution, consistent with the small effect on the spectral features of 1-hole Cu2 observed experimentally upon deprotonation of Lys397. Thus, the structures including only first sphere ligands were used to model the Cu2 and Cu2’ sites in this study.

The 1-hole model with an SH edge ligand reproduces the key spectral features observed for the 1-hole Cu2 site. The Mulliken atomic spin distribution of the cluster with an SH edge ligand is delocalized over CuI, CuII, and CuIV in a 2 : 1 : 1 ratio. In going from Cu2 to Cu2’, the calculated spin on CuI changes from 26% to 17% (Table 2), which is consistent with the decrease in intensity of band 6 observed in the absorption and MCD data for 1-hole Cu2 and leads to a more equal distribution of spin over CuI, CuII, and CuIV, consistent with the EPR hyperfine values. The LUMO of the Cu2 model contains dxy–g character on CuII, CuIV, and CuIV, which are aligned, consistent with the ground state predicted from the EPR g values (Fig. S7†). It also contains significant antibonding μS2– and μ2SH character, explaining why Cu–S stretching vibrations of both the μ4S2– and μ2SH are enhanced in the charge transfer transitions to this acceptor orbital. Additionally, the computational model predicts the Cu2 site to be more covalent than the Cu2’ site, with 10% less Cu character in the ground state wavefunction, reflecting delocalization of the spin from CuI onto the edge SH ligand. The low g∥ value for 1-hole Cu2 is also predicted by the computational model (Table S7†). However, in contrast to experiment, the calculated g∥ values for the Cu2 and Cu2’ models differ, with a higher calculated g∥ value for Cu2’ than that observed experimentally. This suggests that the calculated model of Cu2’ does not accurately predict the ligand field on CuI that leads to the higher energy dxy → dyz–g transition observed experimentally.

The Cu–S stretching vibrations and S–H bending vibrations for the 1-hole SH model of Cu2 are given in Table S8 and Fig. S8.† The model predicts two S–H bending modes at 426 and 461 cm⁻¹ with H/D isotope shifts of −125 cm⁻¹ and −123 cm⁻¹, respectively, similar to the vibrations observed experimentally at 450 and 492 cm⁻¹ (with a shift of −137 cm⁻¹ for the 492 cm⁻¹ vibration; the 450 cm⁻¹ vibration cannot be observed after deuteration due to overlap with the ice scattering peak). Equivalent O–H bends are predicted for the OH bridged Cu2 model at higher energies, but these are not experimentally observed. The Cu2 model also predicts the presence of a low energy Cu–S stretching vibration of the μ2SH (178 cm⁻¹, observed at 203 cm⁻¹ experimentally) and both models show similar energies for the Cu–μ2S stretching vibrations. The absolute energies of the Cu–S stretching vibrations for both the μ4 sulfide and μ2 thiolate are underestimated, as has been found for computational models of the Cu2 site.55–57 The TD DFT calculated absorption spectrum for the Cu2 model is also very similar to the calculated absorption spectrum for the Cu2 model both with B3LYP and with the functional B98, which has been shown to predict the experimental absorption spectrum of a Cu4S2 model complex reasonably well.18 Interestingly, neither the experimental absorption spectrum nor the TD-DFT calculation predicts an intense low energy charge transfer transition from the μ2SH ligand (Fig. S9†). While some weak transitions predicted computationally at lower energy than the μ2S2– to CuCT transitions have μ2SH to CuCT character, they are predicted to lack intensity and are thus difficult to distinguish from the Cu d to d transitions that are also observed in this energy region.

Thus, a computational model of the tetranuclear copper cluster with an SH edge ligand bridging CuI and CuIV provides a good structural model of 1-hole Cu2 that reproduces its key spectral features. This spectroscopically calibrated model was then extended to the 2-hole redox state of the Cu2 site, for which less experimental data are accessible.

2-hole Cu2. Two possible computational models were developed for 2-hole Cu2, one with an edge thiolate ligand (Cu4S(SH)) and one with an edge sulfide (Cu4S2). These were optimized in both the triplet (S = 1) and broken symmetry

![Fig. 7](https://example.com/fig7.png)

**Fig. 7** Computational models of (A) 1-hole Cu2 and (B) 1-hole Cu2’ (B3LYP, TZVP on Cu, S, and ligating N atoms, and SV on all remaining atoms, PCM of 4.0).

<table>
<thead>
<tr>
<th>Edge ligand</th>
<th>CuI</th>
<th>CuII</th>
<th>CuIII</th>
<th>CuIV</th>
<th>μ4S2–</th>
<th>μ2L–</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH– bridge</td>
<td>0.17</td>
<td>0.11</td>
<td>0.06</td>
<td>0.10</td>
<td>0.34</td>
<td>0.16</td>
</tr>
<tr>
<td>OH– bridge</td>
<td>0.26</td>
<td>0.09</td>
<td>0.04</td>
<td>0.13</td>
<td>0.31</td>
<td>0.10</td>
</tr>
</tbody>
</table>
singlet ($S = 0$) ground spin states. For both models the singlet is lower in electronic energy, by $-8.0$ kcal mol$^{-1}$ for the sulfide and $-3.4$ kcal mol$^{-1}$ for the thiolate (spin corrected energies using B3LYP). The singlet state was verified to be the ground state using a variety of functionals, including M06L, M06, and TPSSh. Thus, both structures would be consistent with the experimentally determined singlet ground state of 2-hole Cu$_2$. The optimized structure of the 2-hole Cu$_4$S(SH) model is similar to that of the 1-hole SH$^-$ model of 1-hole Cu$_2$, with slightly shorter Cu$_{4}$/Cu$_{5}$–SH$^-$ and Cu$_{6}$/Cu$_{7}$–µ$_3$S bonds (Table S9†). The α LUMO is dominantly localized on Cu$_{4}$ and has equal µ$_3$S$^2$– and µ$_3$SH$^-$ antibonding character (Table 3) while the β LUMO is delocalized equally over Cu$_{4}$ and Cu$_{5}$ and has more µ$_3$S$^2$– antibonding character. Upon deprotonation of the edge SH$^-$, the 2-hole Cu$_4$S$_2$ model has significantly shorter bonds between the edge sulfide and Cu$_{4}$/Cu$_{5}$ and similar µ$_3$S$^2$––Cu bond lengths to the 2-hole SH$^-$ model (Table S9†). In this model, the α LUMO is localized on Cu$_{4}$ while the β LUMO is localized on Cu$_{5}$ (Fig. 8 and Table 3). Both holes have significant µ$_3$S$^2$– character, indicating that the edge sulfide copper bonds are highly covalent.

The energy of deprotonation calculated from the 2-hole models was compared to the calculated energy of deprotonation of the 1-hole SH$^-$ model, where the experimentally estimated pK$_a$ of the edge thiolate is 11–12 (vide supra). Examination of the energy required to deprotonate the 1- and 2-hole SH$^-$ models shows that deprotonation of the 1-hole is not energetically favored ($\Delta E = 26$ kcal mol$^{-1}$, relative to an energy of $-268$ kcal mol$^{-1}$ for a solvated proton)$^{39}$ while deprotonation of the 2-hole is favorable ($\Delta E = -9$ kcal mol$^{-1}$). However, the two models have different charges (+2/+1 and +3/+2 for protonated and deprotonated 1-hole and 2-hole models, respectively) and this will significantly affect the relative energies of deprotonation. To minimize the charge effect, the computational models were expanded to include two second sphere Asp residues near the Cu$_2$ site, such that the 1-hole Cu$_2$ model with an edge thiolate is neutral and the 2-hole Cu$_2$ Cu$_4$S(SH) model has a +1 charge. The proton transfer was performed internally to one of the Asp residues, so the total charge of the model does not change upon deprotonation (Fig. S10†). The $\Delta \Delta E$ for deprotonation of the 2-hole edge thiolate relative to the 1-hole species is calculated to be $-25$ kcal mol$^{-1}$ (with a dielectric of 4.0). This value is dependent on the dielectric (Fig. S11†) and, at high dielectric values, converges to a $\Delta \Delta E$ of $-12$ kcal mol$^{-1}$. To estimate the difference in pK$_a$ between 1-hole and 2-hole Cu$_2$, the $\Delta \Delta G$ was estimated from the $\Delta \Delta E$ using frequency calculations for structures with identical fixed atom constraints and thus the same number and magnitude of imaginary frequencies (these $\Delta \Delta E$ corrections vary by only 0.3 kcal mol$^{-1}$ between the protonated and deprotonated 1-hole structures). This gives a $\Delta \Delta G$ of $-12$ kcal mol$^{-1}$ for deprotonation of 2-hole versus 1-hole Cu$_2$, which corresponds to a ΔpK$_a$ of $\sim 9$. Given the experimental pK$_a$ value of 11–12 for the edge thiolate in 1-hole Cu$_2$, these calculations predict a pK$_a$ for a thiolate in 2-hole Cu$_2$ of 3 or less, consistent with the absence of a pH effect in resonance Raman of 2-hole Cu$_2$ at pH 6. This strongly suggests that 2-hole Cu$_2$ is a two sulfide cluster at neutral pH.

The calculated spectral features for the Cu$_4$S$_2$ 2-hole Cu$_2$ model can be compared with those determined experimentally. The TD DFT predicted absorption spectrum (using both B3LYP and B98) is qualitatively similar to the experimental absorption spectrum, showing two intense absorption maxima with a higher energy shoulder (Fig. S12†). The predicted vibrations of the 2-hole Cu$_4$S$_2$ model are given in Table S10 and Fig. S13.† All of the calculated vibrations are shifted up in energy in comparison to those of the 1-hole SH$^-$ model, with the most significant energy differences observed for the sulfur edge vibrations, due to the short and highly covalent Cu–µ$_3$S$^2$– bonds in the 2-hole Cu$_4$S$_2$ cluster. In particular, the µ$_3$S$^2$––Cu$_{4}$ stretch now occurs at a similar energy to and mixes with vibrations of the µ$_3$S$^2$–, leading to symmetric and antisymmetric combinations of the µ$_3$S$^2$––Cu$_{4}$ and µ$_3$S$^2$––Cu$_{4}$ stretches (predicted at 312 and 309 cm$^{-1}$, respectively, see ESIF). The symmetric combination is allowed in resonance Raman and will be enhanced in all transitions due to the high amount of µ$_3$S$^2$– character in both the α and β holes. This is a good candidate for the 350 cm$^{-1}$ vibration observed experimentally that profiles in both intense absorption bands (see Fig. 6B). The highest energy core vibration of the 2-hole µ$_2$S$^2$– cluster is a symmetric Cu$_{4}$–µ$_3$S$^2$––Cu$_{4}$ stretch predicted at 344 cm$^{-1}$ which will be
selectively enhanced in a transition to the β LUMO localized on CuIV (Fig. 8). A symmetric CuI=μ_S2−stretch is also computationally predicted in the 1-hole SH− model at 320 cm−1 and the calculated shift in energy of this mode between the 1-hole and 2-hole models (+24 cm−1) is similar to the energy increase of the highest energy Cu−S stretches observed experimentally in 1-hole and 2-hole CuZ (378 and 405 cm−1 respectively, Δν of +27 cm−1). Thus, the 2-hole Cu_S2 model qualitatively predicts a high energy Cu−S vibration that will be selectively enhanced only in a transition to the β hole and a lower energy Cu−S vibration that will be enhanced in both intense transitions. This is consistent with the enhancement profiles of the two vibrations observed experimentally in Fig. 6B. This establishes that a μ_S2− bridge is energetically favored and consistent with the spectral features of 2-hole CuZ.

4. Discussion

A combination of spectroscopic methods and DFT calculations has been used to define the protonation state of the μ_S sulfur ligand on the Cu_I−Cu_V edge in 1-hole and 2-hole CuZ. This leads to insights into the spectroscopic similarities between 1-hole CuZ and 1-hole CuIVZ, the redox reactivity of 1-hole CuZ in the slow 2 electron reduction of N2O and the interconversion between CuZ and CuIVZ, the reactive form of the cluster for N2O reduction in vitro.

4.1 Protonation states of 1-hole and 2-hole CuZ

The protonation state of the edge ligand in 1-hole CuZ has been directly determined by resonance Raman spectroscopy. Two high energy vibrations are enhanced in the most intense μ_S2−to Cu CT transition of 1-hole CuZ at 450 and 492 cm−1, and have large isotope shifts upon solvent deuteration (−137 cm−1 for the 492 cm−1 mode). This is consistent with S−H bending modes, indicating that the μ_S ligand is a thiolate. The energy and solvent isotope shift of these S−H bending modes are as predicted by DFT calculations for a model with a μ_SH− bridging the Cu_I−Cu_V edge. The EPR spectrum of 1-hole CuZ indicates a ground state in which the spin is delocalized over 3 coppers in dominantly dx−dy orbitals. The absorption and MCD spectra show three μ_S2− to Cu charge transfer transitions that have very similar energies to those observed for 1-hole Cu_Z (which has a hydroxide bridged Cu_I−Cu_V edge) but a different intensity pattern, consistent with a change in spin distribution in the cluster from dominantly Cu_I in 1-hole CuZ to more evenly delocalized over Cu_I, Cu_H, and Cu_V. This ground state spin distribution is consistent with that predicted from DFT calculations for the μ_SH−. Based on the absence of a pH effect in 1-hole CuZ up to a pH of 10, the pK_a of the edge thiolate in 1-hole CuZ is estimated to be 11–12.

The 2-hole state of CuZ was also spectroscopically defined, but no direct spectroscopic evidence for the protonation state of the edge ligand was obtained. DFT calculations of the deprotonation of a μ_SH− ligand in 2-hole CuZ relative to 1-hole CuZ were used to determine that 2-hole CuZ likely has a sulfide edge ligand. Deprotonation of a μ_SH− ligand in the 2-hole redox state is at least 12 kcal mol−1 more favorable than in the 1-hole redox state, after accounting for charge and dielectric effects. This yields a calculated pK_a for a μ_SH− ligand in 2-hole CuZ of 3 or less, which strongly suggests that 2-hole CuZ has an edge sulfide ligand at physiological pH. The calculated spectroscopic properties of a model of 2-hole CuZ with a μ SH− ligand are also consistent with those observed experimentally.

4.2 Similarities between 1-hole CuZ and 1-hole CuIVZ

It has previously been observed that the spectral features of 1-hole CuZ are rather similar to those of 1-hole CuIVZ, despite the change in the nature of the edge ligand from a thiolate to a hydroxide. The spectroscopic similarities between 1-hole CuZ and 1-hole CuIVZ reflect similar bonding interactions between the μ_S2− and the in plane coppers (Cu_I, Cu_V, and Cu_H) which are not significantly perturbed by the nature of edge ligand. This results in similar transition energies in the absorption and MCD spectra, as the dominant transitions are due to μ_S2− to Cu charge transfer, and a similar intense core Cu−μ_S2− stretching mode in the resonance Raman spectrum, observed at 378 cm−1 in both sites. Small quantitative differences in the EPR hyperfine values and transition absorption and MCD intensities between the two sites arise from a perturbation of the spin density distribution of the cluster in 1-hole CuZ, where the more covalent μ_SH− leads to delocalization of the spin on Cu_I (dominant in CuIVZ) onto the edge SH−. Despite the higher covalency of the CuIV site, the g values in the EPR spectra are similar for CuZ and CuIVZ as the localization of spin on the four coordinate Cu_I in 1-hole CuZ leads to higher energy d−d transitions, opposing the decreased covalency, leading to the net low g values also observed experimentally for CuZ. The difference in edge ligation in the two sites is observed primarily in the resonance Raman enhanced vibrations, where a low energy Cu−μ_SH− stretch at 203 cm−1 and higher energy S−H bending modes at 450 and 492 cm−1 are additionally enhanced in the dominant μ_SH− to Cu CT transition in CuIVZ but not CuZ due to the more covalent interaction between the coppers and the edge SH−. Thus, the spectral similarities between 1-hole CuZ and 1-hole CuIVZ reflect similar bonding with the μ_SH2− ligand and the distribution of spin over Cu_I, Cu_H, and Cu_V. The differences in the vibrational spectra of the two sites reflect the μ_SH− versus μ_OH− edge ligation.

4.3 Insights into reactivity of 1-hole and 2-hole CuZ

1-hole CuZ has been shown to perform a slow 2 electron reduction of N2O under single turnover conditions, with oxidation of both 1-hole CuZ and reduced CuZ to generate resting 2-hole CuZ and 1 electron oxidized CuZ. A structure of PbN2O5 obtained from crystals pressurized with PbO shows a linear N2O molecule binding above the Cu_I−Cu_V edge of the CuZ cluster (Fig. 9). The O of N2O is thought to be oriented towards a solvent filled cavity between CuZ and CuIV where there is a hydrogen bonding interaction with a localized solvent molecule, while the N end of the molecule is 2.8 Å from CuIV and 3.5 Å from the μ_SH− ligand. The spectroscopically and computationally defined protonation states for 1-hole and...
2-hole Cu$_2$ indicate that the 1-hole Cu$_2$ site will donate both an electron and a proton upon oxidation, due to the significantly decreased pK$_a$ of the $\mu$SH$^-$ in the 2-hole redox state. The participation of a proton in the reduction of N$_2$O by 1-hole Cu$_2$ avoids the thermodynamically unfavorable 1-electron reduction of N$_2$O to N$_2$O$^-$, which is endergonic by 25.4 kcal mol$^{-1}$, while the proton-coupled reduction of N$_2$O to form N$_2$ and a hydroxyl radical is exergonic by 7.4 kcal mol$^{-1}$.

However, a substantial barrier exists for this process due to the fact that N$_2$O is not activated through direct interaction with Cu$_2$ (the rate of N$_2$O reduction by 1-hole Cu$_2$ is $2 \times 10^{-4}$ s$^{-1}$). Thus the N$_2$O may alternatively be oriented with the O atom pointed towards Cu$_2$, where it can directly accept a proton and an electron from the $\mu$SH$^-$ to break the N-O bond and generate resting 2-hole Cu$_2$, with transfer of the second electron from CuA. Since no intermediate is observed in the reduction of N$_2$O by 1-hole Cu$_2$, the hydroxide product that would be formed after N-O bond cleavage would likely be rapidly protonated and released into the nearby solvent-filled cavity, rather than coordinating to the Cu$_2$ cluster.

The 2-hole resting state of Cu$_2$ has been defined as having a highly covalent sulfide ligand bridging the Cu$_1$–Cu$_{iv}$ edge. This resting species is potentially the starting point for the chemical conversion of Cu$_2$ to Cu$_2^*$, the reactive form of the cluster for N$_2$O reduction. In vitro, the presence of O$_2$ is thought to promote the conversion of Cu$_2$ to Cu$_2^*$, as isolation of N$_2$O in the presence of O$_2$ in samples with a high proportion of resting 1-hole Cu$_2$, while the resting 2-hole state of Cu$_2$ is obtained when the purification is performed in the absence of oxygen. DFT calculations on the $\mu_2S^{2-}$ model of 2-hole Cu$_2$ suggest that there are frontier molecular orbitals (FMOs) available to interact with O$_2$. The $\alpha$ and $\beta$ HOMOs of 2-hole Cu$_2$ are occupied $\mu_2S^{2-}$ orbitals with dominant $s_p$ character (50% and 66% $\mu_2S^{2-}$ respectively, Fig. 8). This $\mu_2S^{2-}$ $p_z$ orbital is oriented perpendicular to the Cu$_2$S$_2$ plane, towards the solvent-filled cavity where N$_2$O, and by analogy O$_2$, would access the Cu$_2$ cluster. Based on these FMOs, reaction of the Cu$_2$ site with O$_2$ would proceed via oxidation of the edge sulfide, rather than by a Cu$_2$-based oxidation. Since this is a four electron process, there will in principle also be electrons available from the sulfide for the reduction of the copper site, dependent on the nature of the oxidized sulfur product. However, it is unlikely that this is the mechanism involved in interconversion of Cu$_2$ and Cu$_2^*$ in vivo, since resting Cu$_2$ has been isolated under exclusion of oxygen conditions from anaerobically grown cells in bacterial strains with accessory genes knocked-out. Thus, the in vivo mechanism for interconversion of Cu$_2$ and Cu$_2^*$, which is required to maintain N$_2$O in the reactive Cu$_2$ form, and the role of accessory proteins in this process, remain to be identified.

5. Conclusions

We have used a combination of spectroscopies and DFT calculations to determine the protonation states of the edge sulfur in the 1-hole and 2-hole redox states of Cu$_2$. From resonance Raman spectroscopy, 1-hole Cu$_2$ has a $\mu_2$ thiolate ligand with a pK$_a$ of 11–12, due to the presence of S–H bending modes that are not perturbed up to pH 10. DFT calculations of a 1-hole cluster with a $\mu_2$SH$^-$ ligand reproduce the key spectral features of 1-hole Cu$_2$. The computational modeling of the 2-hole Cu$_2$ site indicates that the edge ligand is a $\mu_2S^{2-}$ with a pK$_a$ of 3 or less, which is consistent with the absorption and resonance Raman features of 2-hole Cu$_2$. The nature of this edge ligand has been used to obtain insight into the slow reduction of N$_2$O by 1-hole Cu$_2$ and suggest how 2-hole Cu$_2$ might react with O$_2$, a possible route for the conversion of Cu$_2$ to Cu$_2^*$ in vitro.

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Notes and references