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Protonation state of the Cu$_4$S$_2$ Cu$_Z$ 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 Cu$_4$S$_2$ Cu$_Z$ form of the active site of nitrous oxide reductase (N$_2$OR) in its 3Cu$^{II}$Cu$^{II}$ (1-hole) and 2Cu$^{II}$Cu$^{III}$ (2-hole) redox states. The EPR, absorption, and MCD spectra of 1-hole Cu$_Z$ indicate that the unpaired spin in this site is evenly delocalized over Cu$_{II}$, Cu$_{III}$, and Cu$_{IV}$. 1-hole Cu$_Z$ is shown to have a µ$_2$-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 Cu$_Z$ 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 Cu$_Z$ sites are correlated to these spectroscopic features to determine that 2-hole Cu$_Z$ has a µ$_2$-sulfide edge ligand at neutral pH. The slow two electron (+1 proton) reduction of N$_2$O by 1-hole Cu$_Z$ is discussed and the possibility of a reaction between 2-hole Cu$_Z$ and O$_2$ is considered.

Nitrous oxide reductase contains two copper sites: a binuclear site known as Cu$_A$ that functions as an electron transfer site, and an unusual tetranuclear copper sulfide cluster active site, where N$_2$O binds and is reduced (Figure 1). Two forms of this tetranuclear site have been structurally characterized. One, known as Cu$_Z^*$, has a µ$_4$ sulfide ligand bridging all four copper and a solvent derived ligand on an open edge (the Cu$_{IV}$-Cu$_{IV}$ edge) where N$_2$O is proposed to bind (Figure 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 Cu$_Z^*$ that shifts in H$_2$O$_{18}$O solvent at high pH and the absence of significant spectroscopic

**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 (NO$_3^-$ $\rightarrow$ NO$_2^-$ $\rightarrow$ NO $\rightarrow$ N$_2$O $\rightarrow$ N$_2$), each performed by a different metalloenzyme. The terminal product of denitrification can be either N$_2$O or N$_2$, depending on the regulatory control of the N$_2$O 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 N$_2$O reduction process and its regulation in vivo are of significant interest because N$_2$O is a potent greenhouse gas, with a global warming potential 300x that of CO$_2$, and depletes the ozone layer. Anthropogenic sources of environmental N$_2$O, the majority of which is due to agricultural activity, is an increasing contribution to the global atmosphere.$^5$ Soil studies have indicated that pH,$^{5,8}$ temperature,$^9$ acetylene,$^{10}$ sulfide,$^{11}$ and dioxygen$^{12}$ all affect the production of N$_2$O, 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 N$_2$O release from anthropogenic sources.$^5$
differences between Cu$_2^*$ at high and low pH. The other form of the cluster, known as Cu$_2$, has an additional $\mu_2$ sulfur ligand bridging the Cu$_{11}$Cu$_{11}$ edge (Figure 1B). Whether the $\mu_2$ edge ligand in Cu$_2$ is a thiolate (SH) or a sulfide (S$^2-$) and how its protonation depends on the redox state of the cluster are not known. The Cu$_{11}$S$_2$ Cu$_2$ form of the cluster is dominantly isolated when N$_2$OR is purified in the absence of oxygen or rapidly in the presence of oxygen, while the Cu$_{11}$S 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 vivo is a matter of some debate. As isolated, neither N$_2$OR containing a high percentage of Cu$_2$ nor N$_2$OR containing a high percentage of Cu$_2^*$ 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$_2$ can be activated by prolonged dialysis against base, while N$_2$OR that contains Cu$_2^*$ can be reductively activated by preincubation with methyl viologen, which reduces Cu$_2^*$ to the active fully reduced (4Cu$^0$) redox state. After activation, both Cu$_2$ and Cu$_2^*$ show specific activities consistent with whole cell N$_2$OR activity. However, it has recently been shown that the Cu$_2^*$ 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$_2$ 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$_2^*$). Thus, the physiological role of the Cu$_2$ site in nitrous oxide reduction and whether it participates in N$_2$O reduction in vivo are unknown.

The Cu$_2$ 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$_2$ has been shown to access two redox states, the resting 2Cu$^2$/2Cu$^{II}$ (2-hole) redox state, and a 1 electron reduced 3Cu$^0$/Cu$^{II}$ (1-hole) redox state (E$^\circ$ = +60 mV). Both redox states of Cu$_2$ 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$_2$ 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 ~30% Cu$_2^*$, which complicates the analysis. These limitations lead to the conclusion that Cu$_2$ and Cu$_2^*$ were very similar and perhaps differed only in the second sphere. These results are now extended and correlated to the structural insight that Cu$_2$ contains an additional inorganic sulfur edge ligand. An understanding of the protonation state, electronic structure, and potential reactivity of the Cu$_2$ 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$_2$ in Marinobacter hydrocarbonoclasticus N$_2$OR (MhN$_2$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$_2$ and the origin of the spectroscopic similarity between 1-hole Cu$_2$ and 1-hole Cu$_2^*$, 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 Electronic Supporting Information, 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$_{11}$S$_2$ Cu$_2$ content relative to Cu$_{11}$S Cu$_2^*$ in the purified enzyme. Samples containing larger amounts of Cu$_2^*$ 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 °C for a long period. Both MhN$_2$OR showed copper quantitation results consistent with fully occupancy of the Cu$_A$ and Cu$_B$/Cu$_C^*$ sites (6.4±0.2 and 6.2±0.7 respectively). The percentage of Cu$_A$ versus Cu$_B$ in the samples used for this study was determined by EPR spin quantitation (Figure S1). Samples purified with high amounts of Cu$_2$ contained 60±10% Cu$_2$, while samples purified to obtain more Cu$_2^*$ contained 10±10% Cu$_2$. Spectroscopic samples of 1-hole and 2-hole Cu$_2$ were prepared in a glove box under N$_2$ atmosphere. Samples of 1-hole Cu$_2$ were prepared from MhN$_2$OR (60% Cu$_2$ and 40% Cu$_2^*$) 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$_2$ were prepared by reducing MhN$_2$OR (60±10% Cu$_2$, 40±10% Cu$_2^*$) with 10 equivalents of sodium ascorbate, which reduces the Cu$_A$ site rapidly and the 2-hole Cu$_2$ site very slowly, and spectra were collected within 1 hour so that minimal reduction of 2-hole Cu$_2$ was observed. In parallel, MhN$_2$OR samples containing 90±10% Cu$_2^*$ were reduced with 10 equivalents of sodium ascorbate to obtain the spectral features of 1-hole Cu$_2^*$. For pH and deuterium studies, samples of 1-hole and 2-hole Cu$_2$ were buffer exchanged by centrifugation into different pH or pD buffers. Typical MhN$_2$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.

2.2 Computational Modeling
A computational model of Cu_2 was built from the atomic coordinates of the crystal structure of *Pseudomonas stutzeri* N_2OR, the only known structure of the Cu_2S_2 cluster (PDB ID 3SBP, resolution 1.7 Å). The model included the Cu_2S_2 core and 7 ligating His residues, where the α carbon and distal nitrogen were constrained at their crystallographic positions. A computational model for Cu_2* with a hydroxide bridging ligand and identical α carbon and distal nitrogen constraints was constructed from the crystal structure of *Paracoccus denitrificans* N_2OR (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_2S) 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 (∆∆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_1 and Cu_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 ∆∆E.

### 3 Results and Analysis

#### 3.1 Spectroscopy of 1-hole Cu_2

Previous spectroscopic studies of Cu_2, undertaken before identification of the presence of a second sulfur, were performed on samples of *PpN_2OR* and *PsN_2OR* that contained mixtures of the Cu_2 and Cu_2* sites in a 7:3 ratio for *PpN_2OR* without a way to resolve the spectral features of the Cu_2 site from the mixture. Recently, it has been found that the two-sulfur Cu_2 site cannot be reduced by methyl viologen, which reduces both the Cu_2 site and the Cu_2* form of the cluster. This provides an opportunity to cleanly resolve the spectral features of 1-hole Cu_2 by studying methyl viologen reduced samples after removal of the reductant. This approach allows correlation of the electronic structure of 1-hole Cu_2, obtained from spectroscopy, with the recently determined Cu_2S_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 Cu_2 are given in Figure 2. The EPR spectrum is axial with g || > g _⊥ > 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 d_2zy orbital 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_ _⊥_ region (Figure 2A inset and Figure S2). Simulation of the X-band, X-band 2nd derivative, and Q-band EPR spectra yields the g and A values for 1-hole Cu_2 listed in Table 1. The g values for 1-hole Cu_2 are very similar to those previously obtained for 1-hole Cu_2* (Table 1) and to those obtained for Cu_2 in *PpN_2OR*. This is interesting, considering that an edge SH (thiolate) or S^(2-) (sulfide) in Cu_2 would be expected to be a more covalent ligand than the hydroxide in Cu_2*. This would lead to a lower g value for Cu_2*. However, in 1-hole Cu_2* there is a high energy d-d transition that is not present in the 1-hole Cu_2 spectrum (vide infra). This transition has previously been assigned as a d_2zy → d_2zy excitation localized on Cu_1.

![Figure 2](image_url) **Figure 2:** EPR spectra of 1-hole Cu_2 (black) with simulations (red). A) X-band at 77 K, 9.6349 GHz. inset: 2^{nd} derivative of the X-band. B) Q-band at 77 K, 34.082 GHz.

<table>
<thead>
<tr>
<th></th>
<th>1-hole Cu_2</th>
<th>1-hole Cu_2*</th>
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<tr>
<td>g <em>∥</em></td>
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<tr>
<td>A <em>⊥</em> (cm^(-1))</td>
<td>20×10^4</td>
<td>25×10^4</td>
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The _A_ _∥_ and _A_ _⊥_ values for 1-hole Cu_2 are similar in magnitude to those for Cu_2*, but fitting the hyperfine pattern requires three equivalent contributions rather than the ~5:2 ratio of hyperfine values observed for Cu_2*. This indicates that in the ground state of 1-hole Cu_2 the spin is distributed over three copper centers in dominantly d_2zy orbitals. The three copper involved are likely Cu_1, Cu_1*, and Cu_2*, since these copper centers are in the same plane as the Cu_2S_2 ligands and bonding with the strong donor µ_4 sulfide and µ_2 sulfur ligands should define a common x,y plane for these copers, with the z axis of the local g tensor of each copper oriented perpendicular to the Cu_2S_2 plane. This is
consistent with the 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$_2$, are presented in Figure 3A. The absorption maximum of 1-hole Cu$_2$ occurs at 14,600 cm$^{-1}$ ($\varepsilon \approx 3,000$ M$^{-1}$ cm$^{-1}$), 1,000 cm$^{-1}$ lower than the absorption maximum of 1-hole Cu$_2^*$. (Figure 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 C$_p$/D$_p$ ratios, following Ref. 15 (Table S1). Comparison of the transition assignments and energies of the 1-hole Cu$_2$ and 1-hole Cu$_2^*$ sites reveals some key differences. While the absorption maximum of Cu$_2$ occurs at lower energy than that of Cu$_2^*$, from MCD the three $\mu_4S$ to Cu CT transitions, assigned in Cu$_2^*$, occur at very similar energies in the two sites (bands 5, 6, and 7, numbering given in Figure 3). The shift in the absorption maximum therefore arises from a different intensity pattern for these transitions, where in Cu$_2$, 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$_2^*$ this is reversed. The $\mu_4S^2$ to Cu CTs in Cu$_2$ have previously been assigned as transitions from the three different 3p orbitals of the $\mu_4S^2$ to the $\beta$LUMO of the cluster. From our current study of resting 1-hole Cu$_2^*$ and Cu$_2$ with different edge ligands,15, 15 the $\beta$LUMO is delocalized in the plane that contains Cu$_6$, Cu$_{11}$, Cu$_{16}$, and the $\mu_4S^2$, with different amounts of spin distributed over Cu$_6$, Cu$_{11}$, and Cu$_{16}$ depending on the edge ligation. Two of the $\mu_4S^2$ p orbitals are in the plane, oriented between Cu$_6$ and Cu$_{11}$ (S $p_x$) and between Cu$_{11}$ and Cu$_{16}$ (S $p_y$), while the third is perpendicular to the plane (S $p_z$). Scheme S1 reflects the orientation and simplified composition of these orbitals determined for 1-hole Cu$_2^*$ from DFT calculations. The CT intensities reflect the overlap of these three S p orbitals with the $\beta$LUMO. Since bands 5 and 6 show the highest intensity in the 1-hole forms of Cu$_2$ and Cu$_2^*$, these must reflect charge transfer from the in-plane S $p_x$ (band 6, dominant in Cu$_2^*$ due to higher overlap with Cu$_6$) 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. $Z_x$). Since band 6 arises from a transition to Cu$_6$ (from its dominant intensity in Cu$_2^*$), band 5 must reflect a transition to Cu$_{11}$, since the Cu$_6$-S and Cu$_{11}$-S bonds are close to perpendicular (96° from crystallography) while the Cu$_6$-S and Cu$_{11}$-S bonds are close to parallel (160°).13 The change in relative intensities of the $\mu_4S^2$ to Cu CT transitions in Cu$_2$ relative to Cu$_2^*$, while band 6 decreases in intensity while band 5 increases in intensity, thus indicates that there is less spin on Cu$_6$ and more spin on Cu$_{11}$ in 1-hole Cu$_2$ relative to 1-hole Cu$_2^*$. This is consistent with the EPR hyperfine values, which suggest that the spin in Cu$_2$ is delocalized 1:1:1 over Cu$_6$, Cu$_{11}$, and Cu$_{16}$, while from Ref. 15 in Cu$_2^*$ the spin is delocalized 5:2 over Cu$_6$ and Cu$_{11}$. 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 C$_p$/D$_p$ ratio; this was assigned as a localized $d_{xy} \rightarrow d_{x^2-y^2}$ transition on Cu$_6$, where most of the 1-hole is localized.15 No equivalent high energy d-d transition is observed in the MCD spectrum of Cu$_2$. The lower energy of the d-d transitions in Cu$_2$ relative to Cu$_2^*$ is likely due to the decreased spin on Cu$_6$, the only four coordinate site, relative to Cu$_6$ and Cu$_{11}$, which are both 3 coordinate and have a weaker ligand field.

**Resonance Raman** The resonance Raman spectrum of 1-hole Cu$_2$ and the enhancement profiles of the vibrations are presented in Figures 4A and 4B, respectively. Seven vibrations

![Figure 3: Low temperature absorption and MCD spectra of A) 1-hole Cu$_2$, 10 K absorption, 5 K and 7 T MCD. B) 1-hole Cu$_2^*$, 5 K absorption, 5 K and 7 T MCD (adapted from Ref. 35).](image)

![Figure 4: A) Resonance Raman spectrum of 1-hole Cu$_2$ 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$_2$, 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).](image)
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 stretch of the Cu\(_2\) site (Figure S3) and the previously reported 34\(^{34}\)S isotope sensitivities of both vibrations are similar (-5.8 and -4.7 cm\(^{-1}\), respectively),\(^{28}\) indicating that the 378 cm\(^{-1}\) vibration in 1-hole Cu\(_2\) can be assigned as a Cu-S vibration of the \(\mu_4\) sulfur ligand that is only present in Cu\(_2\). In contrast, the 203 cm\(^{-1}\) vibration is significantly lower in energy than the vibrations of Cu\(_2\)*, and thus can be assigned as a Cu-S vibration of the \(\mu_2\) sulfur ligand that is also present in Cu\(_2\). Further, there are two high energy vibrations in Cu\(_2\) 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 (Figure 4C). This shift requires their assignment as S-H bending modes. Thus, we can definitively identify the edge ligand in 1-hole Cu\(_2\) as a \(\mu_2\)SH. The S-H bending modes at 492 and 450 cm\(^{-1}\) are present at both pH 7.8 and pH 10 (Figure 4D), indicating that the pKa of the edge thiolate is ~11 or higher. This is further supported by the second pKa of free hydrogen sulfide in water is 12, a pKa range definitively identify the edge ligand in 1-hole Cu\(_2\). The vibrations at 492 and 450 cm\(^{-1}\) (Figure S3) and the previously reported 34\(^{34}\)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.\(^{28}\) In contrast to 1-hole Cu\(_2\), 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 (Figure 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\(_2\)S\(_2\) cluster with a \(\mu_4\)S\(_2\) and \(\mu_2\)S\(_2\) and with the predicted enhancements of key vibrations in transitions from the \(\mu_4\)S\(_2\) to the \(\alpha\) and \(\beta\) holes (see 3.3).

The resonance Raman spectrum 2-hole Cu\(_2\) shows no significant shift in the energies of the 350 and 405 cm\(^{-1}\) vibrations between pH 6 and pH 10 (Figure S6). This suggests that the edge ligand has a pKa either lower than 5.5 or higher than 10.5. A pKa higher than 10.5 in the 2-hole redox state is not consistent with observed pKa of 11-12 for 1-hole Cu\(_2\), as the increased charge of the 2-hole state will lead to a lower pKa
be compared to an experimentally validated model of the Cu$_{2}$$^*$ site, which has a hydroxide ligand bridging the Cu$_{1}$-Cu$_{IV}$ edge (Figure 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 Cu$_{1}$-$\mu$SH and Cu$_{IV}$-$\mu$SH bonds, respectively), with a resolution of 1.7 Å (Table S2). Since the crystal was grown from the “purple” resting form of PsN$_2$OR, containing the resting 2-hole redox state of the Cu$_Z$ site, the Cu$_Z$ site in the crystal may have some photo-reduction due to exposure to X-ray radiation. The calculated structures and spin distributions are not significantly perturbed when a tetrahedral basis set was 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 Cu$_Z$* observed experimentally upon deprotonation of Lys397. Thus, the structures including only first sphere ligands were used to model the Cu$_Z$ and Cu$_Z$* sites in this study.

The 1-hole model with an SH$^*$ edge ligand reproduces the key spectral features observed for the 1-hole Cu$_Z$ site. The Mulliken atomic spin distribution of the cluster with an SH$^*$ edge ligand is delocalized over Cu$_{II}$, Cu$_{III}$, and Cu$_{IV}$ in a 2:1:1 ratio. In going from Cu$_Z$* to Cu$_Z$ the calculated spin on Cu$_{II}$ 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 Cu$_Z$ and leads to a more equal distribution of spin over Cu$_{II}$, Cu$_{III}$, and Cu$_{IV}$, consistent with the EPR hyperfine values. The LUMO of the Cu$_Z$ model contains $d_{x^2-y^2}$ character on Cu$_{II}$, Cu$_{III}$, and Cu$_{IV}$, which are aligned, consistent with the ground state predicted from the EPR $g$ factor values (Figure S7). It also contains significant antibonding $\mu_4S^2^*$ and $\mu_2SH^*$ character, explaining why Cu-S stretching vibrations of both the $\mu_4S^2^*$ and $\mu_2SH^*$ are enhanced in the charge transfer transitions to this acceptor orbital. Additionally, the computational model predicts the Cu$_Z$ site to be more covalent than the Cu$_Z$* site, with 10% less Cu character in the ground state wavefunction, reflecting delocalization of the spin from Cu$_{II}$ onto the edge SH$^*$ ligand. The low $g_{||}$ value for 1-hole Cu$_Z$ is also predicted by the computational model (Table S7).

The Cu-S stretching vibrations and SH$^*$ bending vibrations for the 1-hole SH$^*$ model of Cu$_Z$ are given in Table S8 and Figure 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 -135 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 Cu$_{2}$$^*$ model at higher energies, but these are not experimentally observed. The Cu$_Z$ model also predicts the presence of a low energy Cu-S stretching vibration of the $\mu_2SH^*$ (178 cm$^{-1}$, observed at 203 cm$^{-1}$ experimentally) and both models show similar energies for the Cu$_{IV}$-$\mu$S stretching vibrations. The absolute energies of the Cu-S stretching vibrations for both the $\mu_4S^2^*$ and $\mu_2S$ are underestimated, as has been found for computational models of the Cu$_Z$* site. The TD DFT calculated absorption spectrum for the Cu$_Z$ model is also very similar to the calculated absorption spectrum for the Cu$_Z$* model both with B3LYP and with the functional B98, which has been shown to predict the experimental absorption spectrum of a Cu$_Z$S$_2^*$ model complex reasonably well.

Interestingly, neither the experimental absorption spectrum nor the TD-DFT calculation predicts an intense low energy charge transfer transition from the $\mu_2SH^*$ ligand (Figure S9). While some weak transitions predicted computationally at lower energy than the $\mu_4S^2^*$ to Cu CT transitions have $\mu_4S^2^*$ to Cu CT 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 Cu$_{II}$ and Cu$_{IV}$ provides a good structural model of 1-hole Cu$_Z$ 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 \((\text{Cu}_2\text{S(SH)})\) and one with an edge sulfide \((\text{Cu}_2\text{S}_2)\). These were optimized in both the triplet \((S=1)\) and broken symmetry singlet \((S=0)\) ground spin states. For both models the singlet is lower in electronic energy, by \(-8.0 \text{ kcal/mol}\) for the sulfide and \(-3.4 \text{ kcal/mol}\) 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 Cu₂.¹⁹

The optimized structure of the 2-hole Cu₂S(SH) model is similar to that of the 1-hole SH⁻ model of 1-hole Cu₂, with slightly shorter Cu/S(CuIV-SH) and Cu/S(CuIV-μ₂S) bonds (Table S9). The α LUMO is dominantly localized on Cu and has equal μ₂S²⁻ and μ₂SH⁻ antibonding character (Table 3) while the β LUMO is delocalized equally over Cu₁ and Cu₁V and has more μ₂S²⁻ antibonding character. Upon deprotonation of the edge SH⁻, the 2-hole Cu₂S₂ model has significantly shorter bonds between the edge sulfide and Cu/S(CuIV) and similar μ₂S²⁻-Cu bond lengths to the 2-hole SH⁻ model (Table S9). In this model, the α LUMO is localized on Cu while the β LUMO is localized on Cu₁V (Figure 8 and Table 3). Both holes have significant μ₂S²⁻ 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 pKa of the edge thiolate is 11-12 (vide supra). Examination of the energy required to deprotonate the 1-hole and 2-hole SH⁻ models shows that deprotonation of the 1-hole is not energetically favored \((ΔE = 26 \text{ kcal/mol}, \text{ relative to an energy of } -268 \text{ kcal/mol for a solvated proton})\)⁹ while deprotonation of the 2-hole is favorable \((ΔE = -9 \text{ kcal/mol})\). 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 Cu₂ site, such that the 1-hole Cu₂ model with an edge thiolate is neutral and the 2-hole Cu₂ Cu₂S(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 (Figure S10). The ΔE for deprotonation of the 2-hole edge thiolate relative to the 1-hole species is calculated to be -25 kcal/mol (with a dielectric of 4.0). This value is dependent on the dielectric (Figure S11) and, at high dielectric values, converges to a ΔE of -12 kcal/mol. To estimate the difference in pKa between 1-hole and 2-hole Cu₂, the ΔAG was estimated from the ΔE using frequency calculations for structures with identical fixed atom constraints and thus the same number and magnitude of imaginary frequencies (these ΔG corrections vary by only 0.3 kcal/mol between the protonated and deprotonated 1-hole structures). This gives a ΔAG of -12 kcal/mol for deprotonation of 2-hole versus 1-hole Cu₂, which corresponds to a ΔpKa of -9. Given the experimental pKa value of 11-12 for the edge thiolate in 1-hole Cu₂, these calculations predict a pKa for a thiolate in 2-hole Cu₂ of 3 or less, consistent with the absence of a pH effect in resonance Raman of 2-hole Cu₂ at pH 6. This strongly suggests that 2-hole Cu₂ is a two sulfide cluster at neutral pH.

The calculated spectral features for the Cu₂S₂ 2-hole Cu₂ 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 (Figure S12). The predicted vibrations of the 2-hole Cu₂S₂ model are given in Table S10 and Figure 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-μ₂S²⁻ bonds in the 2-hole Cu₂S₂ cluster. In particular, the μ₂S²⁻-CuI stretch now occurs at a similar energy to and mixes with vibrations of the μ₂S²⁻, leading to symmetric and antisymmetric combinations of the μ₂S²⁻-CuI and μ₂S²⁻-CuV stretches (predicted at 312 and 309 cm⁻¹, respectively, see SI). The symmetric combination is allowed in resonance Raman and will be

### Table 3: Mulliken spin densities on Cu and S atoms in the α and β LUMOs of 2whole Cu₂

<table>
<thead>
<tr>
<th>Mulliken Spin Density</th>
<th>μ₂L</th>
<th>Cu₁</th>
<th>Cu₁V</th>
<th>Cu₁III</th>
<th>Cu₁V</th>
<th>μ₂S²⁻</th>
<th>μ₂S⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-hole SH⁻ S=0</td>
<td>α LUMO</td>
<td>0.16</td>
<td>0.37</td>
<td>0.06</td>
<td>0.06</td>
<td>0.04</td>
<td>0.20</td>
</tr>
<tr>
<td>2-hole S²⁻ S=0</td>
<td>α LUMO</td>
<td>0.33</td>
<td>0.22</td>
<td>0.06</td>
<td>0.04</td>
<td>0.07</td>
<td>0.23</td>
</tr>
<tr>
<td>2-hole S²⁻ S=0</td>
<td>β LUMO</td>
<td>0.37</td>
<td>0.05</td>
<td>0.06</td>
<td>0.05</td>
<td>0.14</td>
<td>0.27</td>
</tr>
</tbody>
</table>

LUMO is delocalized equally over Cu₁ and Cu₁V and has more μ₂S²⁻ antibonding character. Upon deprotonation of the edge SH⁻, the 2-hole Cu₂S₂ model has significantly shorter bonds between the edge sulfide and Cu/S(CuIV) and similar μ₂S²⁻-Cu bond lengths to the 2-hole SH⁻ model (Table S9). This gives a ΔAG of -12 kcal/mol for deprotonation of 2-hole versus 1-hole Cu₂, which corresponds to a ΔpKa of -9. Given the experimental pKa value of 11-12 for the edge thiolate in 1-hole Cu₂, these calculations predict a pKa for the thiolate in 2-hole Cu₂ of 3 or less, consistent with the absence of a pH effect in resonance Raman of 2-hole Cu₂ at pH 6. This strongly suggests that 2-hole Cu₂ is a two sulfide cluster at neutral pH.
enhanced in all transitions due to the high amount of $\mu_4$S$^2^-$ character in both the $\alpha$ and $\beta$ holes. This is a good candidate for the 350 cm$^{-1}$ vibration observed experimentally that profiles in both intense absorption bands (see Figure 6B). The highest energy core vibration of the 2-hole $\mu_4$S$^2^-$ cluster is a symmetric Cu$_{11}$-$\mu_4$S$^2^-$Cu$_{11}$ stretching mode at 344 cm$^{-1}$ which will be selectively enhanced in a transition to the $\beta$ LUMO localized on Cu$_{11}$ (Figure 8). A symmetric Cu$_{11}$-$\mu_4$S$^2^-$Cu$_{11}$ stretching mode 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 Cu$_{11}$ (378 and 405 cm$^{-1}$ respectively, $\Delta$v of +27 cm$^{-1}$). Thus, the 2-hole Cu$_{11}$S$_2$ model qualitatively predicts a high energy Cu-S vibration that will be selectively enhanced only in a transition to the $\beta$ 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 Figure 6B. This establishes that a $\mu_4$S$^2^-$ bridge is energetically favored and consistent with the spectral features of 2-hole Cu$_{11}$.

4. Discussion

A combination of spectroscopic methods and DFT calculations has been used to define the protonation state of the $\mu_4$S sulfur ligand on the Cu$_{11}$-Cu$_{11}$ edge in 1-hole and 2-hole Cu$_{11}$. This leads to insight into the spectroscopic similarities between 1-hole Cu$_{11}$ and 1-hole Cu$_{11}^*$, the redox reactivity of 1-hole Cu$_{11}$ in the slow 2 electron reduction of N$_2$O, and the interconversion between Cu$_{11}$ and Cu$_{11}^*$, the reactive form of the cluster for N$_2$O reduction in vitro.

4.1 Protonation states of 1-hole and 2-hole Cu$_{11}$

The protonation state of the edge ligand in 1-hole Cu$_{11}$ has been directly determined by resonance Raman spectroscopy. Two high energy vibrations are enhanced in the most intense $\mu_4$S$^2^-$ to Cu CT transition of 1-hole Cu$_{11}$ 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 $\mu_4$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 $\mu_4$S\textsuperscript{8} bridging the Cu$_{11}$-Cu$_{11}$ edge. The EPR spectrum of 1-hole Cu$_{11}$ indicates a ground state in which the spin is delocalized over 3 Cu$_{11}$-$\mu_4$S$_2^-$-Cu$_{11}$ orbitals in the absorption and MCD spectra show three $\mu_4$S$^2^-$ to Cu charge transfer transitions that have very similar energies to those observed for 1-hole Cu$_{11}$* (which has a hydroxide bridged Cu$_{11}$-Cu$_{11}$ edge) but a different intensity pattern, consistent with a change in spin distribution in the cluster from dominantly on Cu$_{11}$ in 1-hole Cu$_{11}$* to more evenly delocalized over Cu$_{11}$, Cu$_{11}$*, and Cu$_{11}$V. This ground state spin distribution is consistent with that predicted from DFT calculations for the $\mu_4$S\textsuperscript{8}. Based on the absence of a pH effect in 1-hole Cu$_{11}$ up to a pH of 10, the pKa of the edge thiolate in 1-hole Cu$_{11}$ is estimated to be 11-12.

The 2-hole state of Cu$_{11}$ 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 $\mu_4$S\textsuperscript{8} ligand in 2-hole Cu$_{11}$ relative to 1-hole Cu$_{11}$ were used to determine that 2-hole Cu$_{11}$ likely has a sulfide edge ligand. Deprotonation of a $\mu_4$S\textsuperscript{8} ligand in the 2-hole redox state is at least 12 kcal/mol more favorable than in the 1-hole redox state, after accounting for charge and dielectric effects. This yields a calculated pKa for a $\mu_4$S\textsuperscript{8} ligand in 2-hole Cu$_{11}$ of 3 or less, which strongly suggests that 2-hole Cu$_{11}$ has an edge sulfide ligand at physiological pH. The calculated spectroscopic properties of a model of 2-hole Cu$_{11}$ with a $\mu_4$S$^2^-$ ligand are also consistent with those observed experimentally.

4.2 Similarities between 1-hole Cu$_{11}$ and 1-hole Cu$_{11}^*$

It has previously been observed that the spectral features of 1-hole Cu$_{11}$ are rather similar to those of 1-hole Cu$_{11}^*$, despite the change in the nature of the edge ligand from a thiolate to a hydroxide.\textsuperscript{19, 28} The spectroscopic similarities between 1-hole Cu$_{11}$ and 1-hole Cu$_{11}^*$ reflect similar bonding interactions between the $\mu_4$S$^2^-$ and the in plane copper (Cu$_{11}$, Cu$_{11}$*, and Cu$_{11}$V) 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 $\mu_4$S$^2^-$ to Cu charge transfer, and a similar intense core Cu-$\mu_4$S$^2^-$ 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 Cu$_{11}$, where the more covalent $\mu_4$S\textsuperscript{8} leads to delocalization of the spin on Cu$_{11}$ (dominant in Cu$_{11}$*) onto the edge SH. Despite the higher covalency of the Cu$_{11}$ site, the g values in the EPR spectra are similar for Cu$_{11}$ and Cu$_{11}^*$, as the localization of spin on the four coordinate Cu$_{11}$ in 1-hole Cu$_{11}^*$ leads to higher energy d-d transitions, opposing the decreased covalency, leading to the net low g values also observed experimentally for Cu$_{11}$. The difference in edge ligation in the two sites is observed primarily in the resonance Raman enhanced vibrations, where a low energy Cu$_{11}$-$\mu_4$S\textsuperscript{8} stretch at 203 cm$^{-1}$ and higher energy S-H bending modes at 450 and 492 cm$^{-1}$ are additionally enhanced in the dominant $\mu_4$S$^2^-$ to Cu CT transition in Cu$_{11}$ but not Cu$_{11}^*$, due to the more covalent interaction between the copper and the edge SH. Thus, the spectral similarities between 1-hole Cu$_{11}$ and 1-hole Cu$_{11}^*$ reflect similar bonding with the $\mu_4$S$^2^-$ ligand and the distribution of spin over Cu$_{11}$, Cu$_{11}$*, and Cu$_{11}$V. The differences in the vibrational spectra of the two sites reflect the $\mu_4$S\textsuperscript{8} versus $\mu_4$OH$^-$ edge ligation.

4.3 Insights into reactivity of 1-hole and 2-hole Cu$_{11}$

1-hole Cu$_{11}$ has been shown to perform a slow 2 electron reduction of N$_2$O under single turnover conditions, with oxidation of both 1-hole Cu$_{11}$ and reduced Cu$_{11}$ to generate resting 2-hole Cu$_{11}$ and 1 electron oxidized Cu$_{11}$*.$^26$ A structure of Pr$_5$N$_2$O$_2$ obtained from crystals pressurized with N$_2$O shows a
linear N₂O molecule binding above the Cu₁₇-Cu₁₎ edge of the Cu₂ cluster (Figure 9). The O of N₂O is thought to be oriented towards a solvent-filled cavity between Cu₂ and Cu₃α, where there is a hydrogen bonding interaction with a localized solvent molecule, while the N end of the molecule is 2.8 Å from Cu₁₇ and 3.5 Å from the μ₂SH ligand. The spectroscopically and computationally defined protonation states for 1-hole and 2-hole Cu₂ indicate that the 1-hole Cu₂ site will donate both an electron and a proton upon oxidation, due to the significantly decreased pKa of the μ₂SH in the 2-hole redox state. The participation of a proton in the reduction of N₂O by 1-hole Cu₂ avoids the thermodynamically unfavorable 1-electron reduction of N₂O to N₂, which is endergonic by 25.4 kcal/mol, while the proton-coupled reduction of N₂O to form N₂ and a hydroxyl radical is exergonic by 7.4 kcal/mol. However, a substantial barrier exists for this process due to the fact that N₂O is not activated through direct interaction with Cu₁₇ (the rate of N₂O reduction by 1-hole Cu₂ is 2x10⁻⁴ s⁻¹). Thus the N₂O may alternatively be oriented with the O atom pointed towards Cu₂, where it can directly accept a proton and an electron from the μ₂SH to break the N-O bond and generate resting 2-hole Cu₂, with transfer of the second electron from Cu₃α. Since no intermediate is observed in the reduction of N₂O by 1-hole Cu₂, 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 Cu₂ cluster.

The 2-hole resting state of Cu₂ has been defined as having a highly covalent sulfide ligand bridging the Cu₁₇-Cu₁₅ edge. This resting species is potentially the starting point for the chemical conversion of Cu₂ to Cu₂*, the reactive form of the cluster for N₂O reduction. In vitro, the presence of O₂ is thought to promote the conversion of Cu₂ to Cu₂*, as isolation of N₂O in the presence of O₂ results in samples with a high proportion of resting 1-hole Cu₂*, while the resting 2-hole state of Cu₂ is obtained when the purification is performed in the absence of oxygen. DFT calculations on the μ₂S²⁻ model of 2-hole Cu₂ suggest that there are frontier molecular orbitals (FMOs) available to interact with O₂. The α and β HOMOs of 2-hole Cu₂ are occupied μ₂S²⁻ orbitals with dominant S pₓ character (50% and 66% μ₂S²⁻ respectively, Figure 8). This μ₂S²⁻ pₓ orbital is oriented perpendicular to the Cu₃S₂ plane, towards the solvent-filled cavity where N₂O, and by analogy O₂, would access the Cu₂ cluster. Based on these FMOs, reaction of the Cu₂ site with O₂ would proceed via oxidation of the edge sulfide, rather than by a Cu-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 Cu₂ and Cu₂* in vivo, since resting Cu₂* 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 Cu₂ and Cu₂*, which is required to maintain N₂OR in the reactive Cu₂* 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 Cu₂. From resonance Raman spectroscopy, 1-hole Cu₂ has a μ₂ thiolate ligand with a pKa 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 μ₂SH ligand reproduce the key spectral features of 1-hole Cu₂. The computational modeling of the 2-hole Cu₂ site indicates that the edge ligand is a μ₂S²⁻ with a pKa of 3 or less, which is consistent with the absorption and resonance Raman features of 2-hole Cu₂. The nature of this edge ligand has been used to obtain insight into the slow reduction of N₂O by 1-hole Cu₂ and suggest how 2-hole Cu₂ might react with O₂, a possible route for the conversion of Cu₂ to Cu₂* in vitro.

6. Acknowledgements

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Electronic Supplementary Information (ESI) available: Full experimental and computational methodology, EPR quantification of the Cu₂*/Cu₂
ratio, 2nd derivative of the X band EPR of 1-hole Cu2, table of absorption band energies and assignments for 1-hole Cu2 and 1-hole Cu4+, resonance Raman spectrum and profile of 1-hole Cu4+, pH dependence of the spectral features of 1-hole and 2-hole Cu2, low temperature absorption spectrum of 2-hole Cu2, and computational structures, vibrations, and TDDFT absorption spectra for models of 1-hole and 2-hole Cu2 (1-hole SH and OH and 2-hole SH and S2 models). See DOI: 10.1039/b000000x/


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