Electronic Supporting Information

Protonation state of the Cu₄S₂CuZ site in nitrous oxide reductase: redox dependence and insight into reactivity

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S1 Experimental Methodology

S1.1 Materials

All reagents were purchased from Sigma-Aldrich and of the highest grade commercially available. Deuterated water (99.9%), deuterated sodium hydroxide (99+% ) and deuterated glycerol (99+% D) were obtained from Cambridge Isotopes. Nitrous oxide reductase (N₂OR) was isolated from Marinobacter hydrocarbonoclasticus 617 (formerly Pseudomonas nautica) grown under microaerobic conditions in the presence of nitrate, as described previously. MhN₂OR was isolated after two chromatographic steps that were performed aerobically, without added reductants, in Tris-HCl buffer at pH 7.6, as described by Dell’Acqua et. al. These purification conditions were shown to maximize the amount of Cu₄S₂CuZ content relative to Cu₄S CuZ* in the purified enzyme. Samples containing larger amounts of CuZ* 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 ºC for a long period. The total MhN₂OR concentration was determined by bicinchoninic acid (BCA) assay with bovine serum albumin as the protein standard; copper content was determined by the 2,2'-biquinoline assay, as previously described. MhN₂OR isolated by the first method had a copper content of 6.4 ± 0.2 Cu per monomer, while MhN₂OR isolated by the second method contained 6.2 ± 0.7 Cu per monomer, consistent with full occupancy of the copper sites in both the protein samples used. The total spin intensity quantified by EPR for the dithionite reduced MhN₂OR samples was one spin per monomer, consistent with the presence of CuZ or CuZ* in the 1-hole redox state and reduced CuA. The percentage of CuZ versus CuZ* in the samples used for this study was determined by EPR spin quantitation (Figure S1). The total spin of a dithionite reduced enzyme sample (where CuA and 2-hole CuZ are 1 electron reduced and only both 1-hole CuZ and 1-hole CuZ* contribute to the total spin) was compared to the total spin after 2 hours of reduction by 100 equivalents of reduced methyl viologen (which reduces 1-hole CuZ* to 4CuI but leaves 1-hole CuZ in the 3CuI/CuII state). Samples purified with high amounts of CuZ contained 60±10% CuZ, while samples purified to obtain more CuZ* contained 10±10% CuZ. Purified MhN₂OR in 100 mM Tris-HCl buffer at pH 7.6 was stored frozen at -80°C in small aliquots and thawed just prior to the preparation of spectroscopic samples.

S1.2 Spectroscopic studies

Spectroscopic samples of 1-hole and 2-hole CuZ were prepared in a glove box under N₂ atmosphere. Samples of 1-hole CuZ were prepared from MhN₂OR (60% CuZ...
and 40\% \text{Cu}_Z\text{*) that had been incubated with 100 equivalents of reduced methyl viologen. The methyl viologen was removed using a PD-10 Sephadex G-25 medium (GE HealthCare) desalting column with 100 mM phosphate at pH 7.6 as the elution buffer. The protein-containing column fractions were concentrated by centrifugation using Amicon Ultra concentrators with an Ultracell regenerated cellulose membrane (Millipore). For pH dependence studies, during the concentration step samples were buffer exchanged to 100 mM MES pD 6.0, 100 mM phosphate pD 7.6, or 100 mM CAPS pD 10. The total spin intensity observed by EPR was not changed by buffer exchanging to different pHs. To determine the effect of deuteration, samples were prepared in parallel at pH/pD 7.6 and pH/pD 10. Samples of 2-hole Cu\text{Z} were prepared by reducing \text{MhN}_2\text{OR} (60\% \pm 10\% \text{Cu}_Z, 40\% \pm 10\% \text{Cu}_Z\text{*) with 10 equivalents of sodium ascorbate, which reduces the Cu\text{A} site rapidly and the 2-hole Cu\text{Z} site very slowly, and spectra were collected within 1 hour so that minimal reduction of 2-hole Cu\text{Z} was observed. In parallel, \text{MhN}_2\text{OR samples containing 90\% \pm 10\% Cu}_Z\text{*) were reduced with 10 equivalents of sodium ascorbate to obtain the spectral features of 1-hole Cu\text{Z*}. Corrected absorption spectra of 2-hole Cu\text{Z} were obtained by subtracting the spectral contribution of the appropriate concentration of 1-hole Cu\text{Z*}. Ascorbate reduced samples of \text{MhN}_2\text{OR containing Cu}_Z were buffer exchanged by centrifugation to 100 mM MES pD 6.0, 100 mM phosphate pD 7.6, or 100 mM CAPS pD 10 for pH dependence experiments. Typical \text{MhN}_2\text{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. The concentration of the dimer \text{MhN}_2\text{OR was determined using the extinction coefficient of 7100 M}^{-1}\text{cm}^{-1} \text{at 640 nm for the dimer in the dithionite reduced spectrum in the purified protein and corrected according to the volume changes involved in spectroscopic sample preparation.}^4

Absorption spectra were acquired in a Teflon-sealed 3 mm small volume quartz cell at room temperature using an Agilent 8453 UV-visible spectrophotometer with deuterium and tungsten sources. MCD samples were prepared by mixing protein samples in deuterated buffer 1:1 with deuterated glycerol. MCD spectra were collected on CD spectropolarimeters (Jasco J810 with an S20 PMT detector for the 300-900 nm region and a Jasco J730 with an InSb detector for the 600-2000 nm region) with sample compartments modified to insert magnetocryostats (Oxford Instruments SM4-7T). Low temperature absorption spectra were additionally obtained from the samples used for MCD using a double-beam Cary 500 spectrophotometer modified to accommodate a liquid helium cryostat (Janis Research Super Vari-Temp). Low temperature absorption spectra were corrected by subtracting the background spectrum from a cell containing a 50:50 mixture of buffer and deuterated glycerol and an additional scattering correction to account for the differences in glassing between the protein and background samples. EPR and resonance Raman samples were frozen in 4 mm diameter quartz sample tubes. EPR spectra were collected using a Bruker EMX spectrometer with an ER 041 XG microwave bridge, and an ER4102ST sample cavity for X-band and an ER 051 QR microwave bridge, an ER 5106QT resonator, and an Oxford continuous-flow CF935 cryostat for Q-band. X-band samples were run at 77 K in a liquid nitrogen finger dewar. Q-band samples were run at 77 K using a cooling He gas flow. EPR spectra were baseline corrected using WinEPR (Bruker) and simulated using Simfonia (Bruker). Resonance Raman spectra were collected using a series of lines from a Kr^+ ion laser (Coherent 190CK), a Ti-sapphire laser (M-squared SolsTice, pumped by a 12 W Lighthouse
Photonics Sprout diode pumped solid state laser), and a Dye laser (Rhodamine 6G, Coherent 699) with incident power of 20-30 mW arranged in a 130° backscattering configuration. The scattered light was dispersed through a triple monochromator (Spex 1877 CP, with 1200, 1800, and 2400 grooves mm\(^{-1}\) gratings) and detected with a back-illuminated CCD camera (Andor iDus model). Resonance Raman samples were immersed in a liquid nitrogen finger dewar at 77 K. The spectrum of black carbon in an identical quartz EPR tube was subtracted to remove the spectral contribution from quartz scattering. The intensity of the ice peak at ~229 cm\(^{-1}\) was used to normalize the intensities of vibrations to obtain resonance Raman excitation profiles.

S1.3 Computational Details

A computational model of Cu\(_{2}\) was built from the atomic coordinates of the crystal structure of Pseudomonas stutzeri N\(_{2}\)OR, the only known structure of the Cu\(_{4}\)S\(_{2}\) cluster (PDB ID 3SBP, resolution 1.7 Å).\(^5\) The model included the Cu\(_{4}\)S\(_{2}\) 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\(_{2}\)OR (PdN\(_{2}\)OR, PDB ID 1FWX).\(^6\) Two larger structural models were also optimized: (1) including a second sphere Lys-Glu salt bridge near the Cu\(_{1}\)-Cu\(_{IV}\) edge in both sites (Lys397 for PdN\(_{2}\)OR and Lys454 for PsN\(_{2}\)OR)\(^7\) for the 1-hole redox state of Cu\(_{2}\) with an SH– edge ligand and for 1-hole Cu\(_{2}\)\(^*\) with an OH– edge ligand and (2) including two second sphere carboxylate residues, Asp127 and Asp240 (which hydrogen bond to the His ligands of Cu\(_{I}\) and Cu\(_{II}\)), in optimizations of 1-hole and 2-hole Cu\(_{2}\) with SH– or S\(^2\)– edge ligation. Including the second sphere residues did not significantly perturb the core Cu-S bond lengths (Table S3), geometries or the spin distribution of the cluster, so the smaller computational model lacking second sphere residues was used for the analysis of the spectroscopic properties of Cu\(_{2}\). Calculations were performed using Gaussian 09 (version d01).\(^8\) Molecular structures and frequencies were visualized using Avogadro, an open source molecular builder and visualization tool (Version 1.1.1).\(^9\) VMD 1.9.1 was used to visualize molecular orbitals,\(^10\) and QMForge was used to obtain Mulliken spin populations of different orbitals and to analyze TD-DFT calculations.\(^11\) Geometry optimizations were performed using the B3LYP functional, the TZVP basis set on all core atoms (Cu\(_{4}\)S\(_{2}\)) and the ligating His nitrogens, and the SV basis set on all remaining atoms. The optimizations of the large model (2), including two second sphere Asp residues, were additionally performed using a larger basis set with TZVP on the Cu\(_{4}\)S\(_{2}\) core and all His ring heavy atoms. The resulting structures, spin distributions, and relative energies of the singlet and triplet ground states did not differ significantly from the smaller basis set optimizations (see Tables S4-S6), thus the structures optimized with TZVP only on the Cu\(_{4}\)S\(_{2}\) core and ligating nitrogens were used for frequency, TD DFT, and single point calculations. Optimizations, single points, frequencies, and TD-DFT calculations were performed with PCM values of 4.0 and 10.0 and no significant change in the spin distribution, frequencies, or TD-DFT was observed. Spin distributions, frequencies, and TD-DFT results reported are from calculations with a PCM of 4.0. TD-DFT calculations were additionally performed with the functional B98, which has previously shown to predict the experimental spectrum of a Cu\(_{3}\)S\(_{2}\) model complex.\(^12\) As described in the Analysis,
models with an edge S$^{2-}$ or SH$^-$ were optimized for both 1-hole and 2-hole redox states and the 2-hole models were optimized in both triplet and broken symmetry singlet spin states (the singlet states were always lower in energy). This was further tested with the functionals M06L, M06, and TPSSh, which all predict that singlet ground states are lower in energy by at least 5 kcal/mol for the 2-hole redox state; however, these functionals predict a restricted singlet ground state wavefunction for the 2-hole Cu$_Z$ site, while B3LYP predicts a more chemically reasonable spin polarized wavefunction.

To determine the relative energy of deprotonation ($\Delta\Delta E$) of the edge SH$^-$ in the 2-hole versus 1-hole redox state, the larger structural models including two second sphere Asp residues were used. The directly deprotonated versions of the 2Asp model (with loss of H$^+$ to solvent) were considered and the energies of an internal proton transfer from the edge SH$^-$ to Asp127 were also calculated for the 1-hole and 2-hole redox states. The PCM dependence of the $\Delta\Delta E$ of deprotonation was also evaluated by obtaining the singlet point energies with different PCM values for structures optimized with a PCM of 4.0. The $\Delta G$ for deprotonation of 1-hole Cu$_Z$ was also approximated from frequency calculations using structures with identical fixed atom constraints and the same number and magnitude for the imaginary frequencies associated with the fixed atom constraints. To minimize the error introduced into the vibrational energy correction by the fixed atom constraints, the masses of the fixed atoms were artificially increased until the calculated $\Delta G$ correction showed no further significant dependence on the fixed atom masses (~200 Da). The resulting $\Delta G$ was very similar to the calculated $\Delta E$, indicating that the energy differences are electronic in origin.

S2 Supporting Figures, Tables, and Schemes

Figure S1: Methyl viologen reduction of three dithionite reduced samples of N$_2$OR to quantify the % Cu$_Z$ from the % spin remaining after reduction. Black and red: N$_2$OR prepared with two aerobic chromatographic steps (“Form I”), containing ~60% Cu$_Z$ and ~40% Cu$_Z^*$. Green: N$_2$OR prepared with three aerobic chromatographic steps (“Form II”), containing ~90% Cu$_Z^*$ and ~10% Cu$_Z$. 
Figure S2: Second derivative (black) of the X band EPR spectrum of 1-hole CuZ at 77 K, 9.6349 GHz, with simulation in red.

| Band | Energy (cm$^{-1}$) | $C_0/D_0$ | Assignment | Energy (cm$^{-1}$) | $C_0/D_0$ | Assignment |
|------|-------------------|-----------|------------|-------------------|-----------|------------|
| 1    | 8900              | -0.137    | d-d        | 8000              | -0.016    | CuI dz$^2$ |
| 2    | 11400             | ---       | IT         | 10000             | ----      | IT         |
| 3    | 12000             | -1.565    | d-d        | 11100             | -0.218    | CuI dxz    |
| 4    | 13100             | -0.054    | d-d        | 12900             | -0.194    | CuI dyz    |
| 5    | 14600             | -0.273    | S p$^x$   | 14300             | -0.327    | S p$^x$   |
| 6    | 15800             | 0.565     | S p$^y$   | 15700             | 0.196     | S p$^y$   |
| 7    | 16900             | 0.144     | S p$^z$   | 16500             | 0.091     | S p$^z$   |
| 8    | 18000             |           |            | 18000             | 0.170     | CuI dxy    |
| 9    | 18500             |           | His $\pi_1$ | 19800             | -0.029    | His $\pi_1$ |
| 10   | 21400             |           | His $\pi_1$ | 21000             | -0.045    | His $\pi_1$ |
| 11   | 22200             | -0.256    | His $\pi_1$ | 22300             | 0.193     | His $\pi_1$ |
| 12   | 25900             | -0.027    | His $\pi_1$ | 24000             | -0.047    | His $\pi_1$ |
| 13   | 28800             | -0.005    | His $\pi_2$ | 28100             | -0.002    | His $\pi_2$ |
| 14   | 31600             | 0.005     | His $\pi_2$ |                    |           |            |

Table S1: Transition energies, $C_0/D_0$ ratios, and assignments from simultaneous fitting of the low temperature absorption and MCD spectra of 1-hole CuZ and 1-hole CuZ* (values and assignments for CuZ* reproduced from Ref. 7).
Scheme S1: Simplified acceptor and donor MOs for sulfide charge transfer transitions in resting Cu$_Z^*$, derived from DFT calculations of the Cu$_Z^*$ cluster with a hydroxide bridged edge (B3LYP, tzvp on Cu$_4$SON$_7$, sv on remainder, PCM=4.0).
Figure S3: Resonance Raman spectrum of resting Cu$_Z^*$ at 77 K and 605 nm excitation; excitation profile of resting Cu$_Z^*$. Reproduced from Ref. 7.

Figure S4: Lack of pH dependence of the MCD, resonance Raman, and EPR spectra of 1-hole Cu$_Z$ at pH 6 (red), pH 7.6 (green) and pH 10 (blue). Note that the presence of weak S-H bends in the resonance Raman spectra at pH 6 and pH 7.6 is due to incomplete deuteration of the samples.

Figure S5: Absorption spectrum of 2-hole Cu$_Z$ at 10 K, after ascorbate reduction and before (blue) or after (black) subtraction of the spectral contribution of 1-hole Cu$_Z^*$.

Figure S6: Resonance Raman spectra of ascorbate reduced N$_2$OR containing 2-hole Cu$_Z$ and 1-hole Cu$_Z^*$ at pH 6.0 (red), pH 7.6 (green), and pH 10 (blue), with energies of the vibrations of the 2-hole Cu$_Z$ site labeled.
| Bond Lengths (Å) | 1-hole SH | 1-hole S¹ | 2-hole SH | 2-hole S¹ |
|-----------------|-----------|-----------|-----------|-----------|
| Cu_Ir-S₁       | 2.437     | 2.641     | 2.385     | 2.383     |
| Cu_Ir-S₂       | 2.264     | 2.262     | 2.270     | 2.288     |
| Cu_Ir-S₃       | 2.229     | 2.226     | 2.232     | 2.229     |
| Cu_Ir-S₄       | 2.255     | 2.244     | 2.217     | 2.202     |
| Cu_Ir-S₅       | 2.351     | 2.350     | 2.340     | 2.229     |
| Cu_Ir-S₆       | 2.478     | 2.508     | 2.448     | 2.278     |
| S₁-S₂           | 3.379     | 3.402     | 3.164     | 3.010     |
| Cu_Ir-N₁₄      | 2.098     | 2.057     | 2.030     | 2.066     |
| Cu_Ir-N₁₈      | 2.198     | 2.270     | 2.100     | 2.215     |
| Cu_Ir-N₂₂      | 2.116     | 2.085     | 2.193     | 2.043     |
| Cu_Ir-N₂₆      | 2.060     | 2.051     | 2.045     | 2.058     |
| Cu_Ir-N₃₀      | 2.026     | 2.028     | 1.998     | 2.009     |
| Cu_Ir-N₃₄      | 2.076     | 2.088     | 2.051     | 2.066     |
| Cu_Ir-N₄₈      | 2.037     | 2.034     | 2.086     | 2.001     |

Table S2: Comparison of the important bond lengths and angles for the Cu₇ site obtained from X-ray crystallography of Ps₅N₂OR (PDB ID 3SBP, resolution 1.7 Å) and the optimized structure of the 1-hole Cu₇ site with an SH¹ edge ligand (B3LYP, TZVP on Cu, S, and ligating N atoms, and SV on all remaining atoms, PCM of 4.0). For comparison, the optimized structure of the OH bridged model of resting Cu₇* is also included.

| Bond Lengths (Å) | 1-hole SH | 1-hole S² | 2-hole SH | 2-hole S² |
|-----------------|-----------|-----------|-----------|-----------|
| Cu_Ir-S₁       | 2.437     | 2.641     | 2.385     | 2.383     |
| Cu_Ir-S₂       | 2.264     | 2.262     | 2.270     | 2.288     |
| Cu_Ir-S₃       | 2.229     | 2.226     | 2.232     | 2.229     |
| Cu_Ir-S₄       | 2.255     | 2.244     | 2.217     | 2.202     |
| Cu_Ir-S₅       | 2.351     | 2.350     | 2.340     | 2.229     |
| Cu_Ir-S₆       | 2.478     | 2.508     | 2.448     | 2.278     |
| S₁-S₂           | 3.379     | 3.402     | 3.164     | 3.010     |
| Cu_Ir-N₁₄      | 2.098     | 2.057     | 2.030     | 2.066     |
| Cu_Ir-N₁₈      | 2.198     | 2.270     | 2.100     | 2.215     |
| Cu_Ir-N₂₂      | 2.116     | 2.085     | 2.193     | 2.043     |
| Cu_Ir-N₂₆      | 2.060     | 2.051     | 2.045     | 2.058     |
| Cu_Ir-N₃₀      | 2.026     | 2.028     | 1.998     | 2.009     |
| Cu_Ir-N₃₄      | 2.076     | 2.088     | 2.051     | 2.066     |
| Cu_Ir-N₄₈      | 2.037     | 2.034     | 2.086     | 2.001     |

Table S3: Comparisons of key bond lengths for small computational models (white columns) of 1-hole and 2-hole Cu₇ and large computational models (grey columns) including two second sphere Asp residues (optimized with B3LYP; tzvp Cu₄S₂N₇/sv; PCM=4.0).
| Bond Lengths (Å) | 1-hole SH 2Asp | 1-hole S² 2Asp | 2-hole SH⁻ 2Asp | 2-hole S² 2Asp |
|------------------|----------------|----------------|-----------------|----------------|
| **CuI-S₁**       | 2.464          | 2.565          | 2.422           | 2.406          |
| **CuII-S₁**      | 2.262          | 2.258          | 2.269           | 2.285          |
| **CuIII-S₁**     | 2.226          | 2.229          | 2.229           | 2.225          |
| **CuIV-S₁**      | 2.244          | 2.267          | 2.215           | 2.194          |
| **CuI-S₂**       | 2.350          | 2.216          | 2.338           | 2.215          |
| **CuIV-S₂**      | 2.508          | 2.319          | 2.470           | 2.293          |
| S₁-S₂            | 3.502          | 3.443          | 3.206           | 3.074          |
| **CuI-N₂₄**      | 2.057          | 2.021          | 1.997           | 2.018          |
| **CuII-N₂₄**     | 2.085          | 2.156          | 2.022           | 2.071          |
| **CuIII-N₂₄**    | 2.051          | 2.055          | 2.033           | 2.046          |
| **CuIV-N₂₄**     | 2.028          | 2.048          | 2.003           | 2.013          |
| **CuI-N₃₀**      | 2.270          | 3.028          | 2.149           | 2.394          |
| **CuII-N₃₀**     | 2.270          | 2.956          | 2.171           | 2.484          |
| **CuIII-N₃₀**    | 2.028          | 2.048          | 2.033           | 2.046          |
| **CuIV-N₃₀**     | 2.034          | 2.086          | 2.061           | 2.073          |

Table S4: Basis set dependence of the calculated structures of 1-hole and 2-hole Cu₄ for large computational models (white: tzvp Cu₄S₂N₇/sv; grey: tzvp Cu₄S₂His₇/sv, B3LYP, PCM=4.0).

| Model          | Mulliken Atomic Spin Density |
|----------------|-----------------------------|
| 1-hole SH⁻ 2Asp| CuI 0.17, CuII 0.11, CuIII 0.05, CuIV 0.10, μ₄S² 0.34, μ₂S 0.16 |
| 1-hole S² 2Asp | CuI 0.15, CuII 0.04, CuIII 0.01, CuIV 0.09, μ₄S² 0.27, μ₂S 0.40 |

Table S5: Basis set dependence of the Mulliken atomic spin density distribution of 1-hole Cu₄ with 2 Asp residues (white: tzvp Cu₄S₂N₇/sv; grey: tzvp Cu₄S₂His₇/sv, B3LYP, PCM=4.0).

| Mulliken Atomic Spin Density | CuI 0.37, CuII 0.06, CuIII 0.06, CuIV 0.04, μ₄S² 0.20, μ₂S 0.16 |
|-----------------------------|-----------------------------|
| 2-hole SH⁻ S=0              | α LUMO 0.03, β LUMO 0.17, α LUMO 0.36, β LUMO 0.03 |
| 2-hole S² S=0               | α LUMO 0.22, β LUMO 0.05, α LUMO 0.18, β LUMO 0.07 |

Table S6: Basis set dependence of the Mulliken atomic spin density in the α and β LUMOs of 2-hole Cu₄S₂ and 2-hole Cu₄S(SH) (white: tzvp Cu₄S₂N₇/sv, grey: tzvp Cu₄S₂His₇/sv, B3LYP and PCM=4.0).
Figure S7: Ground state wavefunction of the 1-hole SH model of \( \text{Cu}_Z \).

|                | \( g_x \) | \( g_y \) | \( g_z \) |
|----------------|----------|----------|----------|
| **SH bridge**  | 2.044    | 2.057    | 2.158    |
| **OH bridge**  | 2.055    | 2.076    | 2.243    |

Table S7: Computationally predicted g values for SH\(^{-}\) bridged model of 1-hole \( \text{Cu}_Z \) (B3LYP, tzvp on \( \text{Cu}_4\text{S}_2\text{N}_7 \), sv on remainder, PCM=4.0) and OH\(^{-}\) bridged model of 1-hole \( \text{Cu}_Z^* \) with second sphere Lys and Glu (B3LYP, tzvp on \( \text{Cu}_4\text{SN}_7\text{O} \), sv on remainder, PCM=4.0), calculated using Orca.\(^{13}\)

| 1-hole SH\(^{-}\) bridge | 1-hole OH bridge |
|---------------------------|------------------|
| **Energy (cm\(^{-1}\))**  | **Vibration**    | **Energy (cm\(^{-1}\))**  | **Vibration**    |
| (H/D shift)               |                  | (H/D shift)               |                  |
| 461 (-125)                | S-H bend \text{||} to Cu\(_{\text{I}}\)-Cu\(_{\text{IV}}\) | 682 (-175)       | O-H bend \text{||} to Cu\(_{\text{I}}\)-Cu\(_{\text{IV}}\) |
| 426 (-123)                | S-H bend \text{\perp} to Cu\(_{\text{I}}\)-Cu\(_{\text{IV}}\) | 510 (-128)       | O-H bend \text{\perp} to Cu\(_{\text{I}}\)-Cu\(_{\text{IV}}\) |
| 396 (-8)                  |                   |                       |                  |
| 320 (+1)                  | Cu\(_{\text{II}}\)-\(\mu_2\)S-Cu\(_{\text{IV}}\) sym | 341 (+1)           | Cu\(_{\text{II}}\)-S-Cu\(_{\text{III}}\)-Cu\(_{\text{IV}}\) |
| 310 (+6)                  | Cu\(_{\text{III}}\)-\(\mu_4\)S | 312 (-3)           | Cu\(_{\text{III}}\)-S-Cu\(_{\text{IV}}\) sym |
| 299 (-2)                  | Cu\(_{\text{II}}\)-\(\mu_4\)S-Cu\(_{\text{IV}}\) sym | 285 (0)            | Cu\(_{\text{III}}\)-S-Cu\(_{\text{IV}}\) antisym |
| 268 (-3)                  |                   |                       | Cu\(_{\text{IV}}\)-OH |
| 242 (-2)                  | Cu\(_{\text{II}}\)-\(\mu_2\)S |                   |                  |
| 178 (0)                   | Cu\(_{\text{IV}}\)-\(\mu_2\)S |                   |                  |
| 162 (0)                   | Cu\(_{\text{II}}\)-\(\mu_4\)S | 209 (0)            | Cu\(_{\text{II}}\)-S |

Table S8: Vibrations predicted for the 1-hole SH\(^{-}\) bridged model of \( \text{Cu}_Z \) and the 1-hole OH bridged model of \( \text{Cu}_Z^* \). Stretching vibrations of the \( \mu_2 \) edge ligand are highlighted in grey.
Figure S8: Pictorial representations of the vibrations of the 1-hole SH⁻ model listed in Table S4.

Figure S9: TD DFT predicted absorption spectra of the 1-hole SH⁻ bridged model of Cu₂ (top) and the OH bridged model of Cu₂⁻ (bottom) with two functionals: A) B3LYP, and B) B98.
| Bond Lengths (Å) | 1-hole SH | 2-hole SH⁺ | 2-hole S⁻ |
|-----------------|-----------|-----------|-----------|
| Cu₁-S₁          | 2.437     | 2.385     | 2.383     |
| Cu₂-S₁          | 2.264     | 2.270     | 2.288     |
| Cu₃-S₁          | 2.229     | 2.232     | 2.229     |
| Cu₄-S₁          | 2.255     | 2.217     | 2.202     |
| Cu₁-S₂          | 2.351     | 2.340     | 2.229     |
| Cu₄-S₂          | 2.478     | 2.448     | 2.278     |
| Cu₁-S₁-Cu₄      | 91.0°     | 97.5°     | 95.8°     |
| Cu₁-S₂-Cu₄      | 87.8°     | 92.6°     | 98.0°     |
| S₁-S₂           | 3.379     | 3.164     | 3.010     |
| Cu₁-Cu₄        | 3.350     | 3.463     | 3.403     |
| Cu₁-Cu₃        | 3.320     | 3.177     | 3.341     |
| Cu₄-Cu₄        | 2.694     | 2.660     | 2.682     |
| Cu₃-Cu₃        | 3.162     | 3.208     | 3.164     |
| Cu₃-Cu₄        | 3.343     | 3.317     | 3.383     |

Table S9: Comparison of important bond lengths and angles of the broken symmetry singlet 2-hole 4CuS(SH) and 4Cu₂S models with the computational model of 1-hole Cu₂ (B3LYP, TZVP on Cu, S, and ligating N atoms, and SV on all remaining atoms, PCM of 4.0).

Figure S10: Structural models of 2-hole SH⁻ with second sphere Asp residues and 2-hole S⁻ model after internal proton transfer.
Figure S11: Dependence of the $\Delta\Delta E$ for deprotonation of the 2-hole 4CuS(SH) model (+1 charge) relative to the 1-hole 4CuS(SH) model (neutral) on the dielectric used for the PCM.

Figure S12: Comparison of the experimental absorption spectrum of 2-hole Cu$_2$ (black) and the TD DFT predicted absorption spectrum of the broken symmetry singlet 2-hole 4Cu2S model (green, B3LYP; blue, B98; TZVP on Cu$_4$S$_2$N$_7$, SV on remainder, PCM of 4.0).
Table S10: Predicted vibrations of the 2-hole 4Cu2S model of 2-hole Cu$^2_z$ and the 1-hole SH$^-$ model of 1-hole Cu$^2_z$, vibrations with edge ligand character highlighted in grey.

| Energy (cm$^{-1}$) | Vibration | Energy (cm$^{-1}$) | Vibration |
|-------------------|-----------|-------------------|-----------|
| 461               | S-H bend // to Cu$_{I}-$Cu$_{IV}$ | 320       | Cu$_{II}$-$\mu_4$S-Cu$_{IV}$ sym |
| 426               | S-H bend $\perp$ to Cu$_{I}-$Cu$_{IV}$ | 310       | Cu$_{III}$-$\mu_4$S |
| 344               | Cu$_{II}$-$\mu_4$S-Cu$_{IV}$ sym | 312       | $\mu_2$S-Cu$_{II}$-$\mu_4$S sym, Cu$_{III}$-$\mu_2$S |
| 309               | $\mu_2$S-Cu$_{II}$-$\mu_4$S antisym, Cu$_{III}$-$\mu_2$S | 297       | Cu$_{II}$-$\mu_4$S |
| 299               | Cu$_{III}$-$\mu_4$S | 256       | Cu$_{IV}$-$\mu_2$S |
| 242               | Cu$_{IV}$-$\mu_2$S | 202       | Cu$_{I}$-$\mu_4$S |

Figure S13: Pictorial representations of the vibrations of the 2-hole $\mu_2$S$^-$ model listed in Table S6.
## Calculated Structures

1-hole SH° small model (B3LYP; tzvp Cu₄S₂N₇/sv; PCM=4.0)

| Atom | X    | Y    | Z    |
|------|------|------|------|
| Cu   | 15.86139 | 27.55597 | 1.79755 |
| Cu   | 12.74928 | 26.73854 | 0.98044 |
| Cu   | 13.70871 | 25.35849 | -1.6976 |
| Cu   | 15.23553 | 24.5845  | 0.38261 |
| S    | 16.63083 | 25.38456 | 2.26727 |
| S    | 14.67052 | 26.70118 | -0.14984 |
| N    | 10.8479  | 26.8082  | 0.28351 |
| N    | 9.04306  | 26.5699  | -0.97603 |
| N    | 12.66796 | 22.11305 | -4.17502 |
| N    | 12.93728 | 23.70057 | -2.64538 |
| N    | 14.65004 | 20.62702 | -0.93098 |
| N    | 14.93705 | 22.59769 | 0.04673 |
| N    | 12.44895 | 26.55091 | 3.02619 |
| N    | 12.20806 | 27.04802 | 5.17805 |
| N    | 14.94305 | 28.69998 | 3.4348 |
| N    | 13.757   | 30.195   | 4.58299 |
| N    | 16.54144 | 29.32417 | 0.89527 |
| N    | 16.71898 | 31.01201 | -0.53001 |
| N    | 13.24926 | 26.94095 | -3.02532 |
| N    | 12.54999 | 28.77303 | -4.08699 |
| C    | 10.34988 | 26.98969 | -0.93461 |
| C    | 9.82961  | 26.26193 | 1.05574 |
| C    | 8.69711  | 26.10499 | 0.28674 |
| C    | 7.36497  | 25.56102 | 0.61601 |
| C    | 13.47622 | 23.11861 | -3.71994 |
| C    | 11.56026 | 22.04478 | -3.34597 |
| C    | 11.72884 | 23.03508 | -2.39655 |
| C    | 10.79505 | 23.40098 | -1.29493 |
| C    | 15.32373 | 21.81892 | -0.96343 |
| C    | 13.79875 | 20.64682 | 0.16634 |
| C    | 13.98108 | 21.87646 | 0.76882 |
| C    | 13.32991 | 22.439   | 1.98394 |
| C    | 11.82032 | 27.34213 | 3.89135 |
| C    | 13.26121 | 25.71403 | 3.78026 |
| C    | 13.12532 | 26.00638 | 5.11981 |
| C    | 13.79395 | 25.449   | 6.31296 |
| C    | 13.96169 | 29.59036 | 3.36096 |
| C    | 15.40285 | 28.7359  | 4.74617 |
| C    | 14.68279 | 29.66452 | 5.4767 |
| C    | 14.78501 | 30.121   | 6.88399 |
| C    | 16.40314 | 29.6851  | -0.37487 |
| C    | 16.97783 | 30.45348 | 1.58238 |
| C    | 17.0973  | 31.51697 | 0.70766 |
| C    | 17.51601 | 32.92799 | 0.886 |
| C    | 13.30511 | 28.27144 | -3.0407 |
| C    | 12.44548 | 26.56758 | -4.08474 |
1-hole OH small model (B3LYP; tzvp Cu₄S₂N₇/sv; PCM=4.0)

2.2

C 21.76000010000 43.46800050000 13.8479930000
C 22.45598100000 42.85002300000 15.0341560000
N 22.20486900000 41.54007300000 15.4668000000
2-hole SH small model, S=0 (B3LYP; tzvp Cu_4S_2N_7/sv; PCM=4.0)

3 1

Cu 15.65823 27.6691 1.76304
Cu 12.71994 26.73202 1.00224
Cu 13.67199 25.38976 -1.7519
Cu 15.13829 24.56938 0.31009
S 16.42529 25.49663 2.17437
S 14.63163 26.67881 -0.14801
N 10.86237 26.74981 0.26729
N 9.04303 26.56999 -0.97598
N 12.66798 22.1131 -4.1748
N 12.92966 23.71812 -2.66591
|   | 14.64999 | 20.62699 | -0.931 |
|---|----------|----------|--------|
|   | 14.88984 | 22.61465 | 0.00911|
|   | 12.45192 | 26.55184 | 3.02778|
|   | 12.20805 | 27.048   | 5.178  |
|   | 14.91333 | 28.68303 | 3.44487|
|   | 13.75701 | 30.19498 | 4.58298|
|   | 16.26094 | 29.4106  | 0.91155|
|   | 16.71891 | 31.01203 | -0.53007|
|   | 13.18331 | 26.95353 | -2.973  |
|   | 12.55002 | 28.77302 | -4.08693|
|   | 10.35241 | 26.97255 | -0.94179|
|   | 9.84675  | 26.19506 | 1.03856 |
|   | 8.70442  | 26.0773  | 0.27821 |
|   | 7.36497  | 25.561   | 0.616  |
|   | 13.46777 | 23.13181 | -3.74207|
|   | 11.56972 | 22.0385  | -3.33535|
|   | 11.73015 | 23.03996 | -2.39734|
|   | 10.79497 | 23.4009  | -1.29518|
|   | 15.28617 | 21.83291 | -0.99861|
|   | 13.81914 | 20.63496 | 0.18141 |
|   | 13.97005 | 21.87766 | 0.763  |
|   | 13.33002 | 22.43901 | 1.984  |
|   | 11.82306 | 27.34716 | 3.89329 |
|   | 13.2546  | 25.70624 | 3.78464 |
|   | 13.11952 | 26.0025  | 5.12274 |
|   | 13.79396 | 25.44901 | 6.31297 |
|   | 13.94218 | 29.59062 | 3.3666  |
|   | 15.38147 | 28.71118 | 4.75373 |
|   | 14.67593 | 29.65485 | 5.47957 |
|   | 14.78501 | 30.12099 | 6.88399 |
|   | 16.28589 | 29.72726 | -0.37961|
|   | 16.70913 | 30.53016 | 1.61332 |
|   | 17.00374 | 31.54335 | 0.72083 |
|   | 17.51601 | 32.92798 | 0.886  |
|   | 13.28317 | 28.28487 | -3.02466|
|   | 12.37101 | 26.57334 | -4.02729|
|   | 11.96963 | 27.6871  | -4.71963|
|   | 11.08807 | 27.77501 | -5.87496|
|   | 10.87409 | 27.40063 | -1.79015|
|   | 9.99723  | 25.91193 | 2.07512 |
|   | 8.41702  | 26.6477  | -1.77291|
|   | 7.08371  | 24.69058 | -0.00801|
|   | 6.57709  | 26.32741 | 0.47908 |
|   | 7.33239  | 25.23752 | 1.6693  |
|   | 12.85021 | 21.51652 | -4.97735|
|   | 14.3999  | 23.40961 | -4.22592|
|   | 10.77958 | 21.30489 | -3.46479|
|   | 10.24189 | 24.3311  | -1.5157 |
|   | 10.0522  | 22.60013 | -1.14234|
|   | 11.32988 | 23.55388 | -0.34016|
|   | 14.79657 | 19.84305 | -1.56071|
|   |   |   |
|---|---|---|
| H | 16.00453 | 22.09087 -1.77067 |
| H | 13.21065 | 19.78219 0.46689 |
| H | 12.793 | 23.38338 1.76934 |
| H | 14.07684 | 22.6436 2.77402 |
| H | 12.59578 | 21.72944 2.4015 |
| H | 11.10413 | 28.12369 3.6494 |
| H | 11.85958 | 27.49391 6.02222 |
| H | 14.40875 | 24.57601 6.0382 |
| H | 14.46563 | 26.19402 6.78366 |
| H | 13.0734 | 25.12166 7.08631 |
| H | 13.6838 | 29.83922 2.47931 |
| H | 16.18711 | 28.06534 5.09139 |
| H | 13.092 | 30.9355 4.78751 |
| H | 15.0989 | 31.18162 6.94018 |
| H | 13.82454 | 30.03576 7.42673 |
| H | 15.5341 | 29.52566 7.43179 |
| H | 16.02564 | 29.07325 -1.20544 |
| H | 16.7967 | 30.53756 2.69452 |
| H | 16.84709 | 31.49349 -1.41634 |
| H | 18.48593 | 33.06843 0.37169 |
| H | 16.8118 | 33.6779 0.47704 |
| H | 17.66894 | 33.1585 1.95304 |
| H | 13.83849 | 28.91408 -2.33661 |
| H | 12.11695 | 25.53968 -4.2302 |
| H | 12.46179 | 29.74958 -4.35484 |
| H | 11.58618 | 28.22798 -6.75503 |
| H | 10.74471 | 26.77154 -6.17578 |
| H | 10.18697 | 28.38732 -5.67346 |
| H | 17.52601 | 25.61015 1.39473 |

2-hole S² small model, S=0 (B3LYP; tzvp Cu₄S₂N₇/sv; PCM=4.0)

|   |   |   |
|---|---|---|
| Cu | 15.23096 | 24.54935 0.33875 |
| Cu | 13.69156 | 25.36458 -1.69996 |
| Cu | 12.72744 | 26.7311 0.9859 |
| Cu | 15.86465 | 27.51747 1.76888 |
| S  | 14.68315 | 26.4056 -0.07963 |
| S  | 16.51355 | 25.4504 1.99241 |
| N  | 12.54991 | 28.77318 -4.08708 |
| N  | 13.19784 | 26.95621 -2.97113 |
| N  | 16.71899 | 31.01205 -0.53007 |
| N  | 16.43966 | 29.3618 0.9126 |
| N  | 13.75697 | 30.19496 4.58298 |
| N  | 14.94252 | 28.69924 3.43732 |
| N  | 12.20789 | 27.04799 5.17804 |
| N  | 12.45695 | 26.56105 3.02715 |
| N  | 14.91995 | 22.59702 0.0319 |
| N  | 14.65012 | 20.62699 -0.93089 |
| N  | 12.93552 | 23.70174 -2.64707 |
| N  | 12.66792 | 22.11308 -4.17514 |
N  9.04337  26.56951 -0.97597
N  10.84714  26.80972  0.28202
C  11.08806  27.77497 -5.875
C  12.38747  26.57393 -4.02609
C  13.28722  28.28486 -3.02417
C  17.51602  32.92796  0.88603
C  17.05995  31.5301  0.71226
C  16.87644  30.48831 1.60001
C  16.36265  29.69905 -0.36809
C  14.78504  30.121  6.88397
C  14.68257  29.66443  5.47722
C  15.40228  28.73477  4.74763
C  13.96085  29.58894  3.36234
C  13.79403  25.449  6.31295
C  13.13251  26.01241  5.11786
C  13.27666  25.72799  3.77759
C  11.81775  27.34344  3.89363
C  13.32961  22.43902  1.9837
C  13.97423  21.87485  0.76551
C  13.80258  20.64127  0.16947
C  15.31348  21.81949 -0.97485
C  10.79524  23.4011 -1.29457
C  11.72825  23.03453 -2.39658
C  11.56107  22.04323 -3.34506
C  13.4748  23.1203 -3.72212
C  7.36485  25.56119  0.61606
C  8.69775  26.10366  0.28668
C  9.83006  26.26128  1.05555
C  10.34807  26.99185 -0.93595
H  11.58121  28.23556 -6.75421
H  10.18324  28.38033 -5.66729
H  10.74994  26.77085 -6.18066
H  12.45435  29.74916 -4.35258
H  12.14106  25.5374 -4.22699
H  13.83722  28.91795 -2.33419
H  17.70765  33.14153  1.95094
H  16.7643  33.65893  0.52895
H  18.45583  33.12808  0.33492
H  16.76636  31.51246 -1.41272
H  17.03037  30.4828  2.67438
H  16.07333  29.04137 -1.18197
H  15.55258  29.5401  7.42247
H  13.83073  30.00422  7.4337
H  15.07093  31.18939  6.95249
H  13.081  30.92512  4.78527
H  16.21413  28.09681  5.08683
H  13.38432  29.82837  2.47361
H  13.0651  25.07745  7.05924
H  14.4328  26.19931  6.82032
H  14.44271  24.60381  6.02913
| X  | Y  | Z   |
|----|----|-----|
| H  | 11.8598 | 27.49027 | 6.02333 |
| H  | 13.96091 | 25.02466 | 3.1543 |
| H  | 11.09018 | 28.11267 | 3.65152 |
| H  | 12.59394 | 21.73068 | 2.40185 |
| H  | 14.07736 | 22.65344 | 2.76973 |
| H  | 12.80116 | 23.8644 | 1.76515 |
| H  | 13.1721 | 19.79849 | 0.43624 |
| H  | 16.04945 | 22.07264 | -1.73186 |
| H  | 14.78856 | 19.84731 | -1.56672 |
| H  | 11.3341 | 23.55731 | -0.34285 |
| H  | 10.04849 | 22.6042 | -1.13714 |
| H  | 10.24713 | 24.33448 | -1.51556 |
| H  | 10.76411 | 21.31941 | -3.48745 |
| H  | 14.41537 | 23.39153 | -4.19308 |
| H  | 12.85262 | 21.52133 | -4.98 |
| H  | 7.31662 | 25.28373 | -0.9207 |
| H  | 6.55674 | 26.29475 | 0.76945 |
| H  | 7.12596 | 24.65425 | 0.02654 |
| H  | 8.42793 | 26.61311 | -1.78301 |
| H  | 9.97651 | 26.00687 | 2.10035 |
| H  | 10.87719 | 27.39855 | -1.79088 |

1-hole SH\(^+\) model with 2Asp (B3LYP; tzvp Cu\(_5\)S\(_2\)N\(_7\)/sv; PCM=4.0)

| X  | Y  | Z   |
|----|----|-----|
| Cu | 15.9695 | 27.55711 | 1.76019 |
| Cu | 12.75043 | 26.7413 | 0.96994 |
| Cu | 13.6932 | 25.37288 | -1.70069 |
| Cu | 15.22099 | 24.58413 | 0.37046 |
| S  | 16.61098 | 25.36324 | 2.30761 |
| S  | 14.67326 | 26.69678 | -0.15078 |
| N  | 10.84102 | 26.82347 | 0.29123 |
| N  | 9.04306 | 26.5699 | -0.97603 |
| N  | 12.66792 | 22.11308 | -0.17497 |
| N  | 12.92804 | 23.72008 | -2.64333 |
| N  | 14.65004 | 20.62703 | -0.3098 |
| N  | 14.93876 | 22.59562 | 0.04962 |
| N  | 12.44279 | 26.54683 | 3.02602 |
| N  | 12.20805 | 27.04802 | 5.17804 |
| N  | 14.95042 | 28.69976 | 3.43587 |
| N  | 13.75699 | 30.195 | 4.58299 |
| N  | 16.64422 | 29.29802 | 0.89741 |
| N  | 16.71898 | 31.012 | -0.53 |
| N  | 13.14751 | 26.94168 | -2.96127 |
| N  | 12.55001 | 28.77301 | -4.08699 |
| C  | 10.34778 | 26.99764 | -0.9294 |
| C  | 9.82238 | 26.275 | 1.06069 |
| C  | 8.69473 | 26.10925 | 0.28759 |
| C  | 7.36496 | 25.56102 | 0.61601 |
| C  | 13.46194 | 23.12044 | -3.72314 |
| C  | 11.57259 | 22.04894 | -3.34071 |
| C  | 11.72994 | 23.04761 | -2.39382 |
|  |  |  |  |
|---|---|---|---|
| C | 10.79509 | 23.40095 | -1.29496 |
| C | 15.31596 | 21.82223 | -0.96848 |
| C | 13.80569 | 20.64189 | 0.17123 |
| C | 13.98919 | 21.87066 | 0.77545 |
| C | 13.32991 | 22.439 | 1.98394 |
| C | 11.82056 | 27.34211 | 3.8904 |
| C | 13.25285 | 25.70808 | 4.74856 |
| C | 14.68122 | 29.6637 | 5.47788 |
| C | 14.78501 | 30.121 | 6.884 |
| C | 16.43354 | 29.68924 | -0.36477 |
| C | 17.095 | 30.42796 | 1.57303 |
| C | 17.14007 | 31.5014 | 0.6959 |
| C | 17.51601 | 32.92799 | 0.886 |
| C | 13.29111 | 28.26567 | -3.03413 |
| C | 12.30579 | 26.57747 | -3.99555 |
| C | 11.93459 | 27.69617 | -4.69784 |
| C | 11.08801 | 27.77498 | -5.875 |
| H | 10.88485 | 27.39689 | -1.78358 |
| H | 9.96493 | 26.02313 | 2.10674 |
| H | 8.43726 | 26.59815 | -1.79054 |
| H | 7.12854 | 24.65413 | 0.0247 |
| H | 6.55238 | 26.29087 | 0.42904 |
| H | 7.31882 | 25.28051 | 1.68169 |
| H | 12.97541 | 21.40807 | -4.95937 |
| H | 14.407 | 23.3848 | -4.18949 |
| H | 10.77962 | 21.31657 | -3.46765 |
| H | 10.2158 | 24.31627 | -1.5172 |
| H | 10.07076 | 22.58483 | -1.12365 |
| H | 11.33028 | 23.58531 | -0.34526 |
| H | 14.75134 | 19.87286 | -1.60379 |
| H | 16.03426 | 22.08321 | -1.73991 |
| H | 13.16049 | 19.80707 | 0.42785 |
| H | 12.81148 | 23.38867 | 1.75231 |
| H | 14.06482 | 22.6485 | 2.784 |
| H | 12.58164 | 21.7365 | 2.39047 |
| H | 11.10683 | 28.12317 | 3.64461 |
| H | 13.89941 | 24.96786 | 3.32146 |
| H | 11.87978 | 27.5096 | 6.02037 |
| H | 14.42511 | 24.58913 | 6.03312 |
| H | 14.45336 | 26.19795 | 6.79559 |
| H | 13.07267 | 25.10164 | 7.07841 |
| H | 13.4034 | 29.83417 | 2.46537 |
| H | 16.2171 | 28.10108 | 5.09014 |
| H | 13.09005 | 30.93537 | 4.77659 |
| H | 15.06598 | 31.191 | 6.95267 |
| H | 13.8333 | 29.9995 | 7.43858 |
| H | 15.55749 | 29.54385 | 7.42013 |
| Atom | x  | y  | z    |
|------|----|----|------|
| H    | 16.05601 | 29.05647 | -1.16197 |
| H    | 17.34278 | 30.40378 | 2.63102 |
| H    | 16.51978 | 31.56279 | -1.44881 |
| H    | 18.44899 | 33.18824 | 0.34679 |
| H    | 16.72902 | 33.60716 | 0.50466 |
| H    | 17.67571 | 33.15415 | 1.95496 |
| H    | 13.90266 | 28.91058 | -2.41183 |
| H    | 12.0234  | 25.54705 | -4.18398 |
| H    | 12.58505 | 29.75659 | -4.34739 |
| H    | 10.70606 | 26.77554 | -6.14415 |
| H    | 10.20945 | 28.43689 | -5.73133 |
| H    | 11.62853 | 28.16492 | -6.76183 |
| H    | 17.75833 | 25.30369 | 1.59519 |
| C    | 12.16299 | 18.93701 | -6.787 |
| C    | 13.59632 | 19.43191 | -6.54551 |
| O    | 14.56594 | 18.76222 | -7.0102 |
| O    | 13.722   | 20.53727 | -5.86164 |
| C    | 14.196   | 33.28501 | -3.744 |
| C    | 14.88901 | 32.08996 | -3.11738 |
| O    | 14.28993 | 30.96104 | -3.09233 |
| O    | 16.05912 | 32.33457 | -2.61277 |
| H    | 11.65481 | 18.74735 | -5.8205 |
| H    | 11.5754  | 19.71319 | -7.31524 |
| H    | 13.91077 | 34.00392 | -2.95039 |
| H    | 14.88664 | 33.81767 | -4.42342 |
| H    | 13.28879 | 32.98596 | -4.29565 |
| H    | 12.16479 | 18.01024 | -7.38428 |

1-hole S² model with 2Asp (B3LYP; tzvp Cu₄S₃N₇/sv; PCM=4.0)
|     |         |         |         |         |
|-----|---------|---------|---------|---------|
| H   | 13.43236| 29.88732| 2.45265 |
| H   | 16.47555| 28.42552| 4.97273 |
| H   | 12.99251| 30.81908| 4.81911 |
| H   | 14.87287| 29.79291| 7.43003 |
| H   | 15.653   | 29.65293| 7.37992 |
| H   | 16.1888  | 30.27557| 2.46049 |
| H   | 16.45652| 31.56612| -1.423  |
| H   | 18.25635| 33.33484| 0.16869 |
| H   | 16.58946| 33.52293| 0.76424 |
| H   | 17.9025  | 33.10585| 1.90519 |
| H   | 13.90351| 28.89371| -2.40472|
| H   | 12.03578| 25.54348| -4.19275|
| H   | 12.59168| 29.75814| -4.34077|
| H   | 10.71237| 26.77391| -6.14831|
| H   | 10.20415| 28.42996| -5.72737|
| H   | 11.62276| 28.17252| -6.76254|
| C   | 12.163   | 18.93703| -6.787  |
| C   | 13.42821| 19.78692| -6.98195|
| O   | 14.26187| 19.44778| -7.87501|
| O   | 13.55497| 20.82272| -6.20122|
| C   | 14.19601| 33.285   | -3.744  |
| C   | 14.86178| 32.06629| -3.14697|
| O   | 14.2432 | 30.94958 | -3.15968|
| O   | 16.02686| 32.28712| -2.62439|
| H   | 12.09768| 18.58065| -5.73994|
| H   | 11.2597 | 19.54972| -6.97801|
| H   | 13.88638| 33.97103| -2.93017|
| H   | 14.90925| 33.84405| -4.37769|
| H   | 13.30456| 33.01609| -4.33636|
| H   | 12.16522| 18.07078| -7.46931|

2-hole S\textsuperscript{2} model with 2Asp (B3LYP; tzvp Cu\textsubscript{4}S\textsubscript{2}N\textsubscript{7}/sv; PCM=4.0)
2-hole SH⁺ model with 2Asp (B3LYP; tzvp Cu₄S₂N₇/sv; PCM=4.0)

Cu  15.76265  27.66164  1.74807
Cu  12.72241  26.75078  0.99493
Cu  13.66954  25.40454 -1.76279
Cu  15.11444  24.58004  0.29396
S   16.40051  25.46064  2.21048
S   14.62762  26.69306 -0.15971
N   10.85238  26.77985  0.27818
N   9.04299  26.56986 -0.976
N   12.66792  22.11295 -4.17504
N   12.92263  23.74294 -2.66624
N   14.65006  20.62707 -0.93097
| Atom | X         | Y         | Z       |
|------|-----------|-----------|---------|
| N    | 14.87461  | 22.62157  | -0.00051|
| N    | 12.4478   | 26.5512   | 3.02729 |
| N    | 12.208    | 27.04799  | 5.17798 |
| N    | 14.9326   | 28.69181  | 3.44185 |
| N    | 13.757    | 30.195    | 4.583   |
| N    | 16.41388  | 29.35958  | 0.92171 |
| N    | 13.13618  | 26.9533   | -2.94886|
| N    | 12.54998  | 28.77305  | -4.08703|
| C    | 10.34925  | 26.98457  | -0.93603|
| C    | 9.83584   | 26.22594  | 1.04828 |
| C    | 8.70048   | 26.08911  | 0.2816  |
| C    | 7.36501   | 25.56107  | 0.61601 |
| C    | 13.45241  | 23.13555  | -3.74992|
| C    | 11.58562  | 22.0451   | -3.32609|
| C    | 11.7353   | 23.05564  | -2.39175|
| C    | 10.79525  | 23.40109  | -1.29489|
| C    | 15.26272  | 21.84388  | -1.01444|
| C    | 13.83416  | 20.62718  | 0.19208 |
| C    | 13.97577  | 21.87412  | 0.76729 |
| C    | 13.3298   | 22.43893  | 1.98395 |
| C    | 11.82532  | 27.34954  | 3.89253 |
| C    | 13.24617  | 25.70137  | 3.78294 |
| C    | 13.11533  | 25.99828  | 5.12171 |
| C    | 13.79401  | 25.449    | 6.31299 |
| C    | 13.96022  | 29.59599  | 3.36183 |
| C    | 15.38854  | 28.71751  | 4.75482 |
| C    | 14.6751   | 29.65502  | 5.4804  |
| C    | 14.785    | 30.121    | 6.884   |
| C    | 16.33101  | 29.72423  | -0.36525|
| C    | 16.88271  | 30.47782  | 1.61299 |
| C    | 17.06896  | 31.51606  | 0.71355 |
| C    | 17.516    | 32.928    | 0.886   |
| C    | 13.28781  | 28.28087  | -3.03136|
| C    | 12.29147  | 26.58049  | -3.98254|
| C    | 11.92854  | 27.69627  | -4.69218|
| C    | 11.08799  | 27.775    | -5.87501|
| H    | 10.87686  | 27.40356  | -1.78577|
| H    | 9.98214   | 25.95449  | 2.08864 |
| H    | 8.42664   | 26.62681  | -1.78172|
| H    | 7.09877   | 24.67909  | 0.00108 |
| H    | 6.56801   | 26.3154   | 0.46392 |
| H    | 7.32897   | 25.24983  | 1.67316 |
| H    | 12.97174  | 21.40339  | -4.9854 |
| H    | 14.38528  | 23.41024  | -4.2346 |
| H    | 10.80014  | 21.3019   | -3.43452|
| H    | 10.20818  | 24.31088  | -1.51851|
| H    | 10.07743  | 22.57872  | -1.13057|
| H    | 11.32101  | 23.58354  | -0.33908|
| H    | 14.77138  | 19.85998  | -1.58661|
| H    | 15.95073  | 22.11618  | -1.8088 |
Electronic Supporting Information References

1. S. Dell’Acqua, S. R. Pauleta, J. J. G. Moura and I. Moura, *Philosophical Transactions of the Royal Society B-Biological Sciences*, 2012, 367, 1204-1212.
2. E. M. Johnston, S. Dell’Acqua, S. Ramos, S. R. Pauleta, I. Moura and E. I. Solomon, *Journal of the American Chemical Society*, 2014, 136, 614-617.
3. T. Rasmussen, B. C. Berks, J. N. Butt and A. J. Thomson, *Biochemical Journal*, 2002, 364, 807-815.
4. M. Prudencio, A. S. Pereira, P. Tavares, B. Besson, I. Cabrito, K. Brown, B. Samyn, B. Devreese, J. Van Beeumen, F. Rusnak, G. Fauque, J. J. G. Moura, M. Tegoni, C. Cambillau and I. Moura, *Biochemistry*, 2000, 39, 3899-3907.
5. A. Pomowski, W. G. Zumft, P. M. H. Krones and O. Einsle, *Nature*, 2011, 477, 234-U143.
6. K. Brown, K. Djunic-Carugo, T. Haltia, I. Cabrito, M. Saraste, J. J. G. Moura, I. Moura, M. Tegoni and C. Cambillau, *Journal of Biological Chemistry*, 2000, 275, 41133-41136.
7. S. Ghosh, S. I. Gerelsky, S. D. George, J. M. Chan, I. Cabrito, D. M. Dooley, J. J. G. Moura, I. Moura and E. I. Solomon, *Journal of the American Chemical Society*, 2007, 129, 3899-3907.
8. M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery Jr., J. E. Peralta, F. Ogliaro, M. J. Bearpark, J. Heyd, E. N. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. P. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, N. J. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, Ö. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski and D. J. Fox, Gaussian, Inc., Wallingford, CT, USA, 2009.
9. M. D. Hanwell, D. E. Curtis, D. C. Lonie, T. Vandermeersch, E. Zurek and G. R. Hutchison, *Journal of Cheminformatics*, 2012, 4.
10. W. Humphrey, A. Dalke and K. Schulten, *Journal of Molecular Graphics*, 1996, 14, 33-38.
11. A. L. Tenderholt, Version 2.3.2 edn.
12. I. Bar-Nahum, A. K. Gupta, S. M. Huber, M. Z. Ertem, C. J. Cramer and W. B. Tolman, *Journal of the American Chemical Society*, 2009, 131, 2812-+.
13. F. Neese, *Wiley Interdisciplinary Reviews-Computational Molecular Science*, 2012, 2, 73-78.