Supplementary Information

An Unexpected All-Metal Aromatic Tetranuclear Silver Cluster in Human Copper Chaperone Atox1

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**Experimental**

**Protein Sample preparation for Single-molecule AFM.** Glasses were first immersed into chromic acid for 2h to remove impurities. After rinsing with Mili-Q water, glasses was covered by silane-PEG-NHS solution in DMSO (Nanocs inc, 5kDa, 1mg ml$^{-1}$) for 2h. Glasses were rinsed with large amount of DMSO to remove the unreacted silane-PEG-NHS. Then glasses was covered by NH$_2$-BG solution in DMSO (10μg ml$^{-1}$) for 2h, so protein snap can directly bind to glasses. Finally, rinsing glasses with Mili-Q water to remove unreacted NH$_2$-BG. Glasses was used immediately after finishing the modification process. For cantilever was coated by Au, protein cys-Xmod-doc can directly bind on it.

**Crystallization.** Crystal screening was performed at 293 K by the sitting-drop vapour-diffusion method. A 200 nanolitre protein solution (27 mg/mL) was mixed with 200 nanolitre reservoir solution and equilibrated against 30 microlitre reservoir solution. Commercial crystallization kits from Hampton Research and Qiagen were used for crystal screening. Initial crystals of Atox1 were observed under the following condition: 0.2 M tri-sodium citrate, 20 % (w/v) PEG 3350. Single crystals were obtained by further optimization of salt concentration and pH values. For heavy atom derivative crystals preparation, we added 5 mM AgNO$_3$ to a cryo-protection solution (0.2 M tri-sodium citrate, 20 % (w/v) PEG 3350, 25% glycerol), soaked the crystals for about 4 hours and then the data were collected at home source diffraction system. For Crystal optimizing, protein was incubated with 5mM TCEP and 5mM AgNO3 on ice for 3 hours. A 200 nanolitre protein solution (29 mg/mL) was mixed with 180 nanolitre reservoir solution and 20 nanolitre lysozyme seed, and equilibrated against 15 microlitre reservoir solution. Final crystals of Atox1 were observed under the following condition: 0.2M LiCl, 0.1M Tris pH 8, 20% PEG 6,000. For heavy atom derivative crystals preparation, we added 5 mM AgNO$_3$ and 5 mM TCEP to a reservoir solution (0.2M LiCl, 0.1M Tris pH 8, 20% PEG 6,000), soaked the crystals for about 4 hours, and then added 5 mM AgNO3 to a cryo-protection solution. The data were collected at SSRF BL18U1.

The contents of the unit cell were analysed using the Matthews coefficient (Matthews, 1968). Molecular replacements were performed using MOLREP (Vagin & Teplyakov, 2010) and Phaser (McCoy, 2007). The models were refined by iterative cycles of manual building using Coot (Emsley et al., 2010) followed by simulated annealing. Subsequent stages of refinement were carried out with REFMAC5 (Murshudov et al., 2011) within the CCP4 suite (Winn et al., 2011; Collaborative Computational Project, Number 4, 1994) and manual improvement in Coot. All structural representations were generated using PyMOL (DeLano, 2002) with subsequent ray tracing.

**Protein expression.** The DNA fragment encoding the Homo sapiens protein Atox1 protein was synthesis by Nanjing GenScript Biotechnology Corporation. The fusion protein 6×His-TEV-Atox1 was sub-cloned into a pET28a vector by standard polymerase chain reaction (PCR) methods, and the resulting construct was subsequently transformed into BL21 (DE3) cells. The fusion protein was expressed in LB medium containing 50 μg/mL kanamycin after induction with 1 mM IPTG at 15°C. To obtain purified Atox1, *E.coli* BL21 (DE3) cells containing the recombinant plasmid that had been cultured overnight were collected by centrifugation. The pellet was re-suspended in buffer (50 mM Tris-HCl pH 7.0, 500 mM NaCl and 5% v/v glycerol) and dissociated by microfluid. The supernatant was obtained by centrifuging the cell lysate at 20,000 rpm and 277 K for 1 h. Standard Ni-affinity chromatography (His-Trap FF) was performed for preliminary purification of the His-tagged fusion protein from the supernatant. The enrichment fusion protein was digested by TEV
protease at 277 K overnight. Ni-affinity chromatography (His Trap HP) was used again to obtain Atox1, which was separated from the 6×His -TEV fusion protein. High purity Atox1 was obtained after further purified by size-exclusion chromatography (Superdex 30) and was concentrated.

For the loop bypass mutant, the sequence of Coh-(GB1)4-Atox1-linker-Atox1-(GB1)4-Snap is as follows:

**Coh:**
MGTALTDGRMTYDLPKGSSAATKPVEVTKVFDTAADAAGQTVEFKVSAGAEKGYATTGYHIVYDER LEVVATKGTAYAKGAALEDSLAKAENNGNGVFSAGADDDFGADGVMWTVLKVPAADAKAGDVYPIIDV AYQWDPSKGDLDFTDNKDAQGKLMQAYFFTQGIKSSSNpstDEYLVKA-NATYADGVIAYIAIKAGEP

**GB1:**
MDTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVGEWYTDDATKTFTVTE ATOX1:
MPKHEFSVMTCGGCAGAEAVSRVLKLGGVKVIDLPPNKKVCIIEHSMDDLALTLKKTGKTIVSYLGE
**Linker:**
RSGGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSGSGGSRS
**Snap:**
GGGMDKDCEMKRTTLPANTYPANLHYYNNATLKDAGFYEGGLAVKEWLLAHEHRLGKPGLGA

**Cys-Xmod-Doc:**
CGGNTVTSAVTQVYEIESVDFGYFNFETFDFTAQIKKAVLHTVYMETYGTDGDGVAVVLRWEYSEPVDITAEL TFGDATPANTYKAVNKFYEPVQYNATLKDAGFYEGGLAVKEWLLAHEHRLGKPGLGA

**Mass Spectrometry.** Proteins were injected into a reverse phase HPLC (Agilent 1200 series HPLC, Agilent Technologies) with a ProSwift™ RP-3U LC Column (4.6×50 mm, SS, Thermo Scientific™). Positive ion Electrospray Ionization (ESI) mass spectra for intact protein were obtained with an Agilent 6224 mass spectrometer equipped with an ESI interface and a time-of-flight (TOF) mass detector (Agilent Technologies). Mass spectra were analyzed and deconvoluted using an Agilent software MassHunter version B.04.00 (Agilent Technologies).

**Inductively coupled plasma mass spectrometry (ICP-MS) experiments.** To confirm the binding condition of Atox1 fusion protein and Ag, we conducted ICP-MS detection in a 220 μl complex solution containing Atox1 fusion protein and Ag. As a result, we found that the molar ratio of Ag (0.278 × 10⁻⁴ mol) and Atox1 (0.064 × 10⁻⁶ mol) was about 4.34 in Ag-Atox1 complex.

**Single-molecule AFM experiments.** Single-molecule AFM experiments were carried out on a commercial AFM (Force Robot 300, JPK, Berlin, Germany). All the force-extension experiments were carried out in Tris-HCl buffer (25mM Tris, 72mM NaCl). Protein sample (0.1mg ml⁻¹,150μl) was directly deposited on a freshly cleaved glass surface for 2h and was washed with buffer to remove unreacted protein. We modified the cantilever tip with cys-Xmod-doc. The gold-coated
cantilever was immersed in a protein solution (0.1 mg ml\(^{-1}\)) for 1 hour at room temperature to allow the formation of gold-thiol linkage. The physically adsorbed proteins were removed by rinsing the cantilever tip with deionized water for at least 5 times in an incubator. Then, the sample chamber was filled with 1ml buffer before the measurement. The spring constant of the AFM cantilevers (Biolever-RC-150VB-70 from Olympus) was calibrated using the equipartition theorem before each experiment, with a typical value of 6pN nm\(^{-1}\). The pulling speed was 400 nm s\(^{-1}\) for all traces.

**EPR measurement.** 0.2 mM Atox1 was incubated with 10 molar equivalents of AgNO\(_3\) in 20 mM Tris buffer containing 200 mM NaCl. Then the spin-trapping agent 5,5-dimethylpyrroline-N-oxide (DMPO) was added to the reaction mixture. After shaking for 1 minute, the sample was transferred to a quartz capillary tube. For MTSSL labeling, 0.2 mM Atox1 was incubated with 10 molar equivalents of MTSSL overnight at 4°C. Then the reaction mixture was added to Ni-NTA to remove free spin label. The continuous-wave electron paramagnetic resonance (CW-EPR) spectra were recorded on a Bruker A300 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) at X-band (9.5 GHz).

**Crystallization.** Crystal screening was performed at 293 K by the sitting-drop vapour-diffusion method. A 200 nanolitre protein solution (27 mg/mL) was mixed with 200 nanolitre reservoir solution and equilibrated against 30 microlitre reservoir solution. Commercial crystallization kits from Hampton Research and Qiagen were used for crystal screening. Initial crystals of Atox1 were observed under the following condition: 0.2 M tri-sodium citrate, 20 % (w/v) PEG 3350. Single crystals were obtained by further optimization of salt concentration and pH values. For heavy atom derivative crystals preparation, we added 5 mM AgNO\(_3\) to a cryo-protection solution (0.2 M tri-sodium citrate, 20 % (w/v) PEG 3350, 25% glycerol), soaked the crystals for about 4 hours and then the data were collected at home source diffraction system.

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**Theoretical Analyses and Computational Modeling.**

In Figure 3, the electronic structure of Ag\(_{42}^{2+}\) cluster based on the experimentally measured geometry (Figure 2) have been investigated using density functional theory (DFT) with Amsterdam Density Functional 2019 program. The calculations were done using PBE exchange-correlation functional and the TZP Slater basis sets. Frozen core
approximation was applied to the [1s^2…4p^6] core of Ag, and Zeroth-Order Regular Approximation to the Dirac Equation (ZORA) was used to account for the scalar relativistic (SR) effect.

The constrained DFT geometry optimization was done at the level of SR-ZORA PBE/DZP with Grimme D3-BJ Dispersion Correction. The model of constrained geometry optimization adopted [Ag_4]^q+ cluster inside the frozen experimental cavity, with size about 5 Å around the [Ag_4]^q+ core. The calculated molecular orbital (MO) energy levels and wavefunctions of Ag_4^{2+} cluster are listed in Table S3-S5, which were analyzed using Hückel method based on three kinds of geometry structures with T_d, D_{4h} and D_{2h} symmetry. The MO energies and frontier MOs of optimized geometry structures with T_d, D_{4h} and D_{2h} symmetries and experimentally measured structure with C_2 symmetry were studied by ADF 2019 program with level of PBE/TZP, frozen core approximation and ZORA scalar relativistic method.

The ELF color-filled map and multicenter bond index were calculated using Gaussian-16B and Multiwfn-3.8 at the level of PBE0/def2-TZVP. The nucleus-independent chemical shift (NICS) of Ag_4^{2+} cluster based on the experimentally measured geometry was studied using Multiwfn and Gaussian with level of B3LYP/def2-SVP.

**Cartesian Coordinates of Geometry Structures**

Experimental structure of Ag_4-(Atox1)_2 7DC1:

| Element | X    | Y    | Z    |
|---------|------|------|------|
| Ag      | -27.203000 | 46.371000 | 7.945000 |
| Ag      | -29.133000 | 45.802000 | 9.934000 |
| Ag      | -26.557000 | 46.744000 | 11.514000 |
| Ag      | -25.099000 | 48.131000 | 9.525000 |
| N       | -28.361000 | 48.860000 | 4.792000 |
| C       | -27.081000 | 49.589000 | 4.574000 |
| C       | -25.891000 | 48.627000 | 4.676000 |
| C       | -26.946000 | 50.740000 | 5.578000 |
| O       | -27.127000 | 50.312000 | 6.930000 |
| H       | -26.820000 | 51.001000 | 7.524000 |
| H       | -28.777896 | 49.166964 | 5.647553 |
| H       | -25.999884 | 51.192138 | 5.473347 |
| N       | -26.071000 | 47.402000 | 5.159000 |
| C       | -24.953000 | 46.440000 | 5.296000 |
| C       | -25.498000 | 45.017000 | 5.233000 |
| Atom | x     | y     | z     |
|------|-------|-------|-------|
| H    | -30.047000 | 49.522000 | 12.131000 |
| H    | -27.715817  | 48.692270  | 13.809163 |
| H    | -31.334040  | 48.113718  | 13.985628 |
| N    | -28.016000  | 46.279000  | 14.300000 |
| C    | -27.742000  | 44.830000  | 14.163000 |
| C    | -26.237000  | 44.590000  | 14.226000 |
| O    | -25.502000  | 45.571000  | 14.221000 |
| C    | -28.370000  | 44.300000  | 12.863000 |
| S    | -27.427000  | 44.616000  | 11.334000 |
| H    | -29.351000  | 44.763000  | 12.753000 |
| H    | -27.307000  | 46.940000  | 14.018000 |
| H    | -28.197000  | 44.279000  | 14.986000 |
| H    | -28.399000  | 43.216000  | 12.971000 |
| N    | -25.839000  | 43.341000  | 14.289000 |
| H    | -26.507184  | 42.599581  | 14.227122 |
| N    | -24.223000  | 43.428000  | 12.051000 |
| C    | -23.426000  | 43.710000  | 10.846000 |
| C    | -22.787000  | 45.095000  | 10.920000 |
| O    | -21.827000  | 45.385000  | 10.095000 |
| H    | -24.061230  | 43.662325  | 9.986285  |
| H    | -25.219626  | 43.373298  | 11.989804 |
| N    | -23.287000  | 45.955000  | 11.803000 |
| C    | -22.813000  | 47.356000  | 11.926000 |
| C    | -21.364000  | 47.398000  | 12.437000 |
| O    | -20.645759  | 48.406067  | 12.210060 |
| C    | -23.761000  | 48.198000  | 12.775000 |
S       -25.290000   48.687000   11.927000
H       -23.235000   49.103000   13.080000
H       -24.023000   45.640000   12.418000
H       -22.816000   47.806000   10.933000
H       -24.058000   47.576000   13.620000
N       -19.574000   47.352000    9.242000
C       -19.899000   48.589000    8.487000
C       -21.367000   48.923000    8.616000
H       -21.697000   49.462000    7.728000
H       -20.357313   46.730866    9.217233
H       -19.660005   48.444394    7.454106
H       -21.523000   49.544000    9.498000
H       -21.941000   48.002000    8.715000
C       -25.232000   53.048000   10.431000
C       -25.384000   51.557000   10.600000
N       -26.443000   51.013000    9.724000
H       -26.739000   50.112000   10.072000
H       -24.315127   53.366371   10.881427
H       -25.638000   51.342000   11.638000
H       -26.085000   50.911000    8.785000
H       -25.219998   53.289418    9.388660
H       -24.440000   51.086000   10.325000
O       -28.102000   48.675000    9.730000
O       -24.500000   50.069000    7.917000
H       -24.854000   49.476000    8.584000
H       -24.476000   50.964000    8.264000
| Atom | X-Coordinate | Y-Coordinate | Z-Coordinate |
|------|--------------|--------------|--------------|
| O    | -28.468000   | 51.143000    | 11.618000    |
| H    | -28.631000   | 51.743000    | 10.887000    |
| H    | -28.219000   | 50.281000    | 11.276000    |
| H    | -19.317839   | 49.400287    | 8.872961     |
| H    | -18.781616   | 46.905970    | 8.825845     |
| H    | -31.410458   | 39.645987    | 10.175530    |
| H    | -33.123137   | 41.430587    | 10.585878    |
| H    | -24.918352   | 43.101477    | 5.060124     |
| H    | -25.276182   | 41.946486    | 6.779506     |
| H    | -25.887694   | 41.107471    | 8.704739     |
| H    | -33.345573   | 49.336101    | 8.556107     |
| H    | -28.983998   | 49.045776    | 4.032157     |
| H    | -27.093978   | 50.003599    | 3.587674     |
| H    | -27.713832   | 51.469906    | 5.354642     |
| H    | -20.965998   | 46.570093    | 12.985693    |
| H    | -23.767818   | 43.291125    | 12.930815    |
| H    | -22.655830   | 42.972869    | 10.754481    |
| H    | -24.867553   | 43.130722    | 14.398882    |
| H    | -29.848057   | 41.442364    | 6.473268     |
| H    | -25.482274   | 48.523065    | 3.692618     |
| H    | -25.150599   | 49.102190    | 5.285017     |
| H    | -29.281473   | 46.330082    | 15.766375    |
| H    | -29.948755   | 46.332473    | 14.173964    |
| H    | -29.757537   | 48.465496    | 15.871345    |
| H    | -27.507097   | 48.809032    | 15.424549    |
| H    | -30.716503   | 49.736480    | 14.104463    |
Optimized structure of \([\text{Ag}_4]^+\) in constrained 7DC1 cavity:

- Ag: -27.174248, 46.886293, 7.647061
- Ag: -28.912106, 45.580500, 9.744174
- Ag: -26.811643, 46.856738, 11.823799
- Ag: -24.848644, 47.709082, 9.780124
- N: -28.361000, 48.860000, 4.792000
- C: -27.081000, 49.589000, 4.574000
- C: -25.891000, 48.627000, 4.676000
- C: -26.964000, 50.740000, 5.578000
- O: -27.127000, 50.312000, 6.930000
- H: -26.820000, 51.001000, 7.524000
- H: -28.77896, 49.166964, 5.647553
- H: -25.999884, 51.192138, 5.473347
- N: -26.071000, 47.402000, 5.159000
- C: -24.953000, 46.440000, 5.296000
- C: -25.498000, 45.017000, 5.233000
- O: -26.714000, 44.871000, 5.238000
- C: -24.180000, 46.719000, 6.596000
- S: -24.925000, 46.060000, 8.125000
- H: -24.091000, 47.800000, 6.706000
- H: -26.998000, 47.118000, 5.441000
- H: -24.248000, 46.558000, 4.473000
- H: -23.226000, 46.203000, 6.487000
- N: -24.615000, 44.048000, 5.170000
- H: -23.638879, 44.256229, 5.231875
N  -25.498000  42.692000  7.408000
C  -26.141000  42.143000  8.613000
C  -27.660000  42.282000  8.539000
O  -28.391000  41.595000  9.364000
H  -25.782175  42.669416  9.472669
H  -24.658993  43.170734  7.666614
N  -28.155000  43.145000  7.656000
C  -29.605000  43.435000  7.533000
C  -30.366000  42.201000  7.022000
O  -31.598116  42.083025  7.249031
C  -29.860000  44.677000  6.684000
S  -29.519000  46.245000  7.532000
H  -30.906000  44.674000  6.379000
H  -27.514000  43.625000  7.041000
H  -29.993000  43.662000  8.526000
H  -29.173000  44.624000  5.839000
N  -31.221000  40.627000  10.217000
C  -32.130000  41.528000  10.972000
C  -31.685000  42.966000  10.843000
H  -31.594000  43.409000  11.835000
H  -30.419820  41.001675  9.750384
H  -32.124594  41.248882  12.004939
H  -32.419000  43.523000  10.261000
H  -30.719000  43.003000  10.339000
C  -33.325000  48.376000  9.028000
C  -31.958000  47.762000  8.859000
N    -30.957000  48.407000   9.735000
H    -30.670000  47.758000  10.454000
H    -34.059238  47.741331   8.577457
H    -31.645000  47.875000   7.821000
H    -33.540104  48.486214  10.070345
H    -32.022000  46.709000   9.134000
H    -30.154000  48.677000   9.186000
H    -27.840000  49.445000   9.220000
H    -28.104000  48.893000  10.664000
O    -31.111000  46.252000  11.542000
H    -30.238000  45.932000  11.782000
H    -31.435000  45.761000  10.784000
N    -28.134000  48.991000  14.667000
C    -29.405000  48.247000  14.885000
C    -29.167000  46.736000  14.783000
C    -30.460000  48.722000  13.881000
O    -30.008000  48.649000  12.529000
H    -30.047000  49.522000  12.131000
H    -27.715817  48.692270  13.809163
H    -31.334040  48.113718  13.985628
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H  -25.219626  43.373298  11.989804
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C  -21.367000  48.923000  8.616000
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| Element | X       | Y       | Z       |
|---------|---------|---------|---------|
| H       | -20.357313 | 46.730866 | 9.217233 |
| H       | -19.660005  | 48.444394  | 7.454106 |
| H       | -21.523000  | 49.544000  | 9.498000 |
| H       | -21.941000  | 48.002000  | 8.715000 |
| C       | -25.232000  | 53.048000  | 10.431000 |
| C       | -25.384000  | 51.557000  | 10.600000 |
| N       | -26.443000  | 51.013000  | 9.724000 |
| H       | -26.739000  | 50.112000  | 10.072000 |
| H       | -24.315127  | 53.366371  | 11.638000 |
| H       | -25.638000  | 51.342000  | 11.638000 |
| H       | -26.085000  | 50.911000  | 8.785000 |
| H       | -25.219998  | 53.289418  | 9.388660 |
| H       | -24.440000  | 51.086000  | 10.325000 |
| O       | -28.102000  | 48.675000  | 9.730000 |
| O       | -24.500000  | 50.069000  | 7.917000 |
| H       | -24.854000  | 49.476000  | 8.584000 |
| H       | -24.476000  | 50.964000  | 8.264000 |
| O       | -28.468000  | 51.143000  | 11.618000 |
| H       | -28.631000  | 51.743000  | 10.887000 |
| H       | -28.219000  | 50.281000  | 11.276000 |
| H       | -19.317839  | 49.400287  | 8.872961 |
| H       | -18.781616  | 46.905970  | 8.825845 |
| H       | -31.410458  | 39.645987  | 10.175530 |
| H       | -33.123137  | 41.430587  | 10.585878 |
| H       | -24.918352  | 43.101477  | 5.060124 |
| H       | -25.276182  | 41.946486  | 6.779506 |
| Atom | X (Å) | Y (Å) | Z (Å) |
|------|-------|-------|-------|
| H    | -25.887694 | 41.107471 | 8.704739 |
| H    | -33.345573  | 49.336101  | 8.556107 |
| H    | -28.983998  | 49.045776  | 4.032157 |
| H    | -27.093978  | 50.003599  | 3.587674 |
| H    | -27.713832  | 51.469906  | 5.354642 |
| H    | -20.965998  | 46.570093  | 12.985693 |
| H    | -23.767818  | 43.291125  | 12.930815 |
| H    | -22.655830  | 42.972869  | 10.754481 |
| H    | -24.867553  | 43.130722  | 14.398882 |
| H    | -29.848057  | 41.442364  | 6.473268 |
| H    | -25.482274  | 48.523065  | 3.692618 |
| H    | -25.150599  | 49.102190  | 5.285017 |
| H    | -29.281473  | 46.330082  | 15.766375 |
| H    | -29.948755  | 46.332473  | 14.173964 |
| H    | -29.757537  | 48.465496  | 15.871345 |
| H    | -27.507097  | 48.809032  | 15.424549 |
| H    | -30.716503  | 49.736480  | 14.104463 |
| H    | -26.053039  | 53.545990  | 10.903018 |

Optimized structure of [Ag₄]^{2+} in constrained 7DC1 cavity:

| Atom | X (Å) | Y (Å) | Z (Å) |
|------|-------|-------|-------|
| Ag   | -27.189764 | 47.238952 | 7.715822 |
| Ag   | -28.860772 | 45.762323 | 9.769021 |
| Ag   | -27.301700 | 47.134770 | 11.812722 |
| Ag   | -25.130535 | 47.709555 | 9.787557 |
| N    | -28.361000 | 48.860000 | 4.792000 |
| C    | -27.081000 | 49.589000 | 4.574000 |
| Element | X   | Y   | Z   |
|---------|-----|-----|-----|
| C       | -25.891 | 48.627 | 4.676 |
| C       | -26.964 | 50.740 | 5.578 |
| O       | -27.127 | 50.312 | 6.930 |
| H       | -26.820 | 51.001 | 7.524 |
| H       | -28.778 | 49.167 | 5.648 |
| H       | -25.999 | 51.192 | 5.473 |
| N       | -26.071 | 47.402 | 5.159 |
| C       | -24.953 | 46.440 | 5.296 |
| C       | -25.498 | 45.017 | 5.233 |
| O       | -26.714 | 44.871 | 5.238 |
| C       | -24.180 | 46.719 | 6.596 |
| S       | -24.925 | 46.060 | 8.125 |
| H       | -24.091 | 47.800 | 6.706 |
| H       | -26.998 | 47.118 | 5.441 |
| H       | -24.248 | 46.558 | 4.473 |
| H       | -23.226 | 46.203 | 6.487 |
| N       | -24.615 | 44.048 | 5.170 |
| H       | -23.639 | 44.256 | 5.232 |
| N       | -25.498 | 42.692 | 7.408 |
| C       | -26.141 | 42.143 | 8.613 |
| C       | -27.660 | 42.282 | 8.539 |
| O       | -28.391 | 41.595 | 9.364 |
| H       | -25.782 | 42.669 | 9.473 |
| H       | -24.658 | 43.170 | 7.666 |
| N       | -28.155 | 43.145 | 7.656 |
| C       | -29.605 | 43.435 | 7.533 |
|   | X-Coordinate     | Y-Coordinate     | Z-Coordinate     |
|---|------------------|------------------|------------------|
| C | -30.366000       | 42.201000        | 7.022000         |
| O | -31.598116       | 42.083025        | 7.249031         |
| C | -29.860000       | 44.677000        | 6.684000         |
| S | -29.519000       | 46.245000        | 7.532000         |
| H | -30.906000       | 44.674000        | 6.379000         |
| H | -27.514000       | 43.625000        | 7.041000         |
| H | -29.993000       | 43.662000        | 8.526000         |
| H | -29.173000       | 44.624000        | 5.839000         |
| N | -31.221000       | 40.627000        | 10.217000        |
| C | -32.130000       | 41.528000        | 10.972000        |
| C | -31.685000       | 42.966000        | 10.843000        |
| H | -31.594000       | 43.409000        | 11.835000        |
| H | -30.419820       | 41.001675        | 9.750384         |
| H | -32.124594       | 41.248882        | 12.004940        |
| H | -32.419000       | 43.523000        | 10.261000        |
| H | -30.719000       | 43.003000        | 10.339000        |
| C | -33.325000       | 48.376000        | 9.028000         |
| C | -31.958000       | 47.762000        | 8.859000         |
| N | -30.957000       | 48.407000        | 9.735000         |
| H | -30.670000       | 47.758000        | 10.454000        |
| H | -34.059238       | 47.741331        | 8.577458         |
| H | -31.645000       | 47.875000        | 7.821000         |
| H | -33.540104       | 48.486214        | 10.070346        |
| H | -32.022000       | 46.709000        | 9.134000         |
| H | -30.154000       | 48.677000        | 9.186000         |
| H | -27.840000       | 49.445000        | 9.220000         |
| Atom | X   | Y   | Z   |
|------|-----|-----|-----|
| H    | -28.104000 | 48.893000 | 10.664000 |
| O    | -31.111000 | 46.252000 | 11.542000 |
| H    | -30.238000 | 45.932000 | 11.782000 |
| H    | -31.435000 | 45.761000 | 10.784000 |
| N    | -28.134000 | 48.991000 | 14.667000 |
| C    | -29.405000 | 48.247000 | 14.885000 |
| C    | -29.167000 | 46.736000 | 14.783000 |
| C    | -30.460000 | 48.722000 | 13.881000 |
| O    | -30.008000 | 48.649000 | 12.529000 |
| H    | -30.047000 | 49.522000 | 12.131000 |
| H    | -27.715817 | 48.692270 | 13.809163 |
| H    | -31.334040 | 48.113718 | 13.985628 |
| N    | -28.016000 | 46.279000 | 14.300000 |
| C    | -27.742000 | 44.830000 | 14.163000 |
| C    | -26.237000 | 44.590000 | 14.226000 |
| O    | -25.502000 | 45.571000 | 14.221000 |
| C    | -28.370000 | 44.300000 | 12.863000 |
| S    | -27.427000 | 44.616000 | 11.334000 |
| H    | -29.351000 | 44.763000 | 12.753000 |
| H    | -27.307000 | 46.940000 | 14.018000 |
| H    | -28.197000 | 44.279000 | 14.986000 |
| H    | -28.399000 | 43.216000 | 12.971000 |
| N    | -25.839000 | 43.341000 | 14.289000 |
| H    | -26.507184 | 42.599581 | 14.227122 |
| N    | -24.223000 | 43.428000 | 12.051000 |
| C    | -23.426000 | 43.710000 | 10.846000 |
|    |   X    |   Y    |    Z  |
|----|--------|--------|-------|
| H  | -24.867553 | 43.130722 | 14.398882 |
| H  | -29.848057  | 41.442364 |  6.473268 |
| H  | -25.482274  | 48.523065 |  3.692618 |
| H  | -25.150599  | 49.102190 |  5.285017 |
| H  | -29.281473  | 46.330082 | 15.766375 |
| H  | -29.948755  | 46.332473 | 14.173964 |
| H  | -29.757537  | 48.465496 | 15.871345 |
| H  | -27.507097  | 48.809032 | 15.424549 |
| H  | -30.716503  | 49.736480 | 14.104463 |
| H  | -26.053039  | 53.545990 | 10.903018 |

Experimental structure (C₂) of Ag₄ Cluster:

|    |   X    |   Y    |    Z  |
|----|--------|--------|-------|
| Ag | -0.514904 | -1.748836 |  0.236102 |
| Ag | -2.305118  |  0.390645 | -0.236102 |
| Ag |  0.514904  |  1.748836 |  0.236102 |
| Ag |  2.305118  | -0.390645 | -0.236102 |

Optimized T₆-[Ag₄]²⁺ Cluster:

|    |   X    |   Y    |    Z  |
|----|--------|--------|-------|
| Ag |  1.060049  | -1.060049 |  1.060049 |
| Ag | -1.060049  |  1.060049 |  1.060049 |
| Ag | -1.060049  | -1.060049 | -1.060049 |
| Ag |  1.060049  |  1.060049 | -1.060049 |

Optimized D₄h-[Ag₄]²⁺ Cluster:

|    |   X    |   Y    |    Z  |
|----|--------|--------|-------|
| Ag | -1.469085  | -1.469085 |  0.000000 |
| Ag | -1.469085  |  1.469085 |  0.000000 |
| Ag |  1.469085  | -1.469085 |  0.000000 |
| Ag |  1.469085  |  1.469085 |  0.000000 |
Optimized $D_{2h}$-$[\text{Ag}_4]^{2+}$ Cluster:

| Element | X        | Y        | Z        |
|---------|----------|----------|----------|
| Ag      | 2.692548 | 0.000000 | 0.000000 |
| Ag      | 0.000000 | 1.448782 | 0.000000 |
| Ag      | 0.000000 | -1.448782| 0.000000 |
| Ag      | -2.692548| 0.000000 | 0.000000 |

Optimized $D_{4h}$-$[\text{C}_4\text{H}_4]^{2+}$ Cluster:

| Element | X        | Y        | Z        |
|---------|----------|----------|----------|
| C       | 0.000000 | 0.952783 | 0.000000 |
| C       | 0.000000 | 0.000000 | 1.061328 |
| C       | 0.000000 | -0.952783| 0.000000 |
| C       | 0.000000 | 0.000000 | -1.061328|
| H       | 0.000000 | 2.040396 | 0.000000 |
| H       | 0.000000 | -2.040396| 0.000000 |
| H       | 0.000000 | 0.000000 | 2.129351 |
| H       | 0.000000 | 0.000000 | -2.129351|
| Single thiol-metal bond | Protein | ref |
|------------------------|---------|----|
| Au-S                   | ~165 pN | 1  |
| Cu-S                   | ~171 pN | 1  |
| Zn-S                   | ~170 pN | 2  |
| Zn-S                   | ~90 pN  | 3  |
| Fe-S                   | ~211 pN | 4  |
| Fe-N                   | ~160 pN | 5  |
| Fe-O                   | ~127 pN | 6  |
| Ag-S                   | ~64 pN Atox1 | This work |
| Protein            | Atox1 for 5F0W                      | Atox1 for 7DC1                      |
|--------------------|------------------------------------|------------------------------------|
| Method             | Sitting-drop vapour-diffusion       | Sitting-drop vapour-diffusion       |
| Plate type         | Corning 3552                       | Corning 3552                       |
| Temperature (K)    | 293                                | 293                                |
| Protein concentration | 27 mg/mL                         | 29 mg/mL                          |
| Buffer composition of protein solution | 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM TCEP | 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM TCEP |
| Composition of reservoir solution | 0.2 M tri-sodium citrate, 20 % (w/v) PEG 3350 | 0.2M LiCl, 0.1M Tris pH 8, 20% (w/v) PEG 6,000 |
| Volume and ratio of drop | 200 nL protein/200 nL reservoir | 200 nL protein/200 nL reservoir |
| Volume of reservoir | microlitre.                       | microlitre.                       |
Table S3. X-ray data collection and refinement statistics

We finished all of the data collection works by use F-RE++ and R-AXIS IV of RIGAKU.

| Data collection | 5F0W | 7DC1 |
|-----------------|------|------|
| Space group     | P6_2 | P3_21 |
| Cell dimensions |      |      |
| a,b,c(Å)       | 112.49, 112.49, 56.63 | 104.49, 104.493, 29.188 |
| α,β,γ(°)       | 90.00, 90.00, 120.00 | 90.000, 90.000, 120.000 |
| Resolution     | 50.00-2.70 | 19.47-1.75 |
| Rmerge         | 12.9(43.7) | 5.6 (61.6) |
| I/σI           | 17.78(4.68) | 17.0 (2.3) |
| Completeness(%) | 90.2(87.4) | 98.5 (97.1) |
| Redundancy     | 6.2(6.3) | 4.6 (4.3) |

**Refinement**

|                         | 5F0W | 7DC1 |
|-------------------------|------|------|
| Resolution(Å)           | 24.48-2.70 | 19.47-1.75 |
| No. unique reflections  | 10336 | 18311 (1002) |
| R_work / R_free         | 0.26/0.29 | 0.18/0.20 |
| No. atoms               |      |      |
| Protein                 | 2056 | 1019 |
| Ligand/ion              | 8    | 4    |
| Water                   | 12   | 133  |
| B-factors               |      |      |
| Protein                 | 31.55 | 28.44 |
| Ligand/ion              | 38.73 | 27.41 |
| Water                   | 23.05 | 39.01 |
| R.m.s.deviations        |      |      |
| Bond lengths            | 0.014 | 0.005 |
| Bond angles             | 1.62  | 1.259 |

*Values in parentheses are for highest-resolution shell.
### Table S4. The Hückel MO energies and MO wavefunctions of planar D$_{2h}$-Ag$_4$ cluster.

| MO        | Eigenvalue | Energy    | Eigenfunctions                              |
|-----------|------------|-----------|---------------------------------------------|
| LUMO+2(a$_g$) | $\chi_4 = 1.5616$ | $\alpha - 1.5616 \beta$ | $\Psi_4 = (0.4352 \phi_1 + 0.4352 \phi_2 - 0.5573 \phi_3 - 0.5573 \phi_4)$ |
| LUMO+1(a$_u$) | $\chi_3 = 1.0000$ | $\alpha - \beta$ | $\Psi_3 = (0.7071 \phi_1 - 0.7071 \phi_2)$ |
| LUMO(b$_u$) | $\chi_2 = 0.0000$ | $\alpha$ | $\Psi_2 = (-0.7071 \phi_3 + 0.7071 \phi_4)$ |
| HOMO(a$_g$) | $\chi_1 = -2.5616$ | $\alpha + 2.5616 \beta$ | $\Psi_1 = (0.5573 \phi_1 + 0.5573 \phi_2 + 0.4352 \phi_3 + 0.4352 \phi_4)$ |

### Table S5. The Hückel MO energies and wavefunctions of square planar D$_{4h}$-Ag$_4$ cluster.

| MO        | Eigenvalue | Energy    | Eigenfunctions                              |
|-----------|------------|-----------|---------------------------------------------|
| LUMO+1(a$_g$) | $\chi_4 = 2.0000$ | $\alpha - 2.0000 \beta$ | $\Psi_4 = (0.5000 \phi_1 - 0.5000 \phi_2 + 0.5000 \phi_3 - 0.5000 \phi_4)$ |
| LUMO(b$_{2u}$) | $\chi_3 = 0.0000$ | $\alpha$ | $\Psi_3 = (0.7071 \phi_1 - 0.7071 \phi_3)$ |
| LUMO(b$_{1u}$) | $\chi_2 = 0.0000$ | $\alpha$ | $\Psi_2 = (0.7071 \phi_1 - 0.7071 \phi_4)$ |
| HOMO(a$_g$) | $\chi_1 = -2.0000$ | $\alpha + 2.0000 \beta$ | $\Psi_1 = (0.5000 \phi_1 + 0.5000 \phi_2 + 0.5000 \phi_3 + 0.5000 \phi_4)$ |

### Table S6. The Hückel MO energies and wavefunctions of the tetrahedron T$_d$-Ag$_4$ cluster.

| MO        | Eigenvalue | Energy    | Eigenfunctions                              |
|-----------|------------|-----------|---------------------------------------------|
| LUMO(t$_2$) | $\chi_4 = 1.0000$ | $\alpha - 1.0000 \beta$ | $\Psi_4 = (0.5000 \phi_1 - 0.5000 \phi_2 + 0.5000 \phi_3 - 0.5000 \phi_4)$ |
| LUMO(t$_2$) | $\chi_3 = 1.0000$ | $\alpha - 1.0000 \beta$ | $\Psi_3 = (0.5000 \phi_1 + 0.5000 \phi_2 - 0.5000 \phi_3 - 0.5000 \phi_4)$ |
| LUMO(t$_2$) | $\chi_2 = 1.0000$ | $\alpha - 1.0000 \beta$ | $\Psi_2 = (0.5000 \phi_1 - 0.5000 \phi_2 - 0.5000 \phi_3 + 0.5000 \phi_4)$ |
| HOMO(a$_1$) | $\chi_1 = -3.0000$ | $\alpha + 3.0000 \beta$ | $\Psi_1 = (0.5000 \phi_1 + 0.5000 \phi_2 + 0.5000 \phi_3 + 0.5000 \phi_4)$ |
Table S7. The MO contours and energy of the frontier MOs of $T_d$, $D_{4h}$, $D_{2h}$ and the $C_2$ experimental measured structures $[\text{Ag}_4]^{2+}$ cluster (isosurface = 0.03 a.u.) .

| MO diagram | $T_d$ | $D_{4h}$ | $C_2$, Exp | $D_{2h}$ |
|------------|-------|--------|-----------|--------|
| E / Hartree | ![MO diagram](image) | ![MO diagram](image) | ![MO diagram](image) | ![MO diagram](image) |
| LUMO+2     | ![MO diagram](image) | ![MO diagram](image) | ![MO diagram](image) | ![MO diagram](image) |
|            | $t_2$, $E = -0.4406$ | $b_{2g}$, $E = -0.3784$ | $a$, $E = -0.3859$ | $a_g$, $E = -0.3961$ |
| LUMO+1     | ![MO diagram](image) | ![MO diagram](image) | ![MO diagram](image) | ![MO diagram](image) |
|            | $t_2$, $E = -0.4406$ | $e_{1u}$, $E = -0.4611$ | $b$, $E = -0.4450$ | $b_u$, $E = -0.4247$ |
| LUMO       | ![MO diagram](image) | ![MO diagram](image) | ![MO diagram](image) | ![MO diagram](image) |
|            | $t_2$, $E = -0.4406$ | $e_{1u}$, $E = -0.4611$ | $b$, $E = -0.4717$ | $b_u$, $E = -0.4760$ |
| HOMO       | ![MO diagram](image) | ![MO diagram](image) | ![MO diagram](image) | ![MO diagram](image) |
|            | $a_1$, $E = -0.5699$ | $a_{1g}$, $E = -0.5468$ | $a$, $E = -0.5450$ | $a_g$, $E = -0.5459$ |
Table S8. The calculated NICS values (ppm) of the Ag$_4^{2+}$ cluster

| Structure | Number of Bq$_{x}^{a}$ | NICS (ppm)$^{b}$ |
|-----------|------------------------|------------------|
|           | 1                      | -15.9            |
|           | 2                      | -16.4            |
|           | 3                      | -16.4            |
|           | 4                      | -8.3             |
|           | 5                      | -9.7             |

$^{a}$ Bq$_{x}$ represents the ghost atom with numbering x. $^{b}$ For benzene: -8.0 ppm at the center and -10.2 ppm at 1 Å above the ring plane.

Table S9. Constrained DFT optimization results of the [Ag$_4$]$^{q+}$ core in the experimental cavity of 7DC1.

| Specie                | Total Charge | q | Bond Length (Å) | Dihedral Angle (°) |
|-----------------------|--------------|---|-----------------|--------------------|
| Ag$_4$-(Atox1)$_2$ 7DC1 (Experiment) |              |    | Ag1-Ag2 | Ag1-Ag4 | Ag2-Ag3 | Ag3-Ag4 | Ag1-Ag3 | Ag2-Ag4 | Ag1-Ag2-Ag4-Ag3 | Ag2-Ag1-Ag3-Ag4 |
|                       |              |    | 2.83   | 3.17   | 3.17   | 2.83   | 3.65   | 4.68   | 150.8  | 157.0  |
| [Ag$_4$] in cavity of 7DC1 | 0            | +4 | 3.02   | 3.26   | 3.22   | 2.96   | 4.19   | 4.59   | 166.3  | 167.4  |
|                         | -2           | +2 | 3.03   | 2.96   | 2.91   | 3.02   | 4.10   | 4.21   | 151.8  | 152.5  |
|                         | -4           | 0  | null (not converged) |


Figure S1. The liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS) measurement of Atox1 and silver ions. (A) LC-ESI-MS of Atox1 protein (Found: 7458.55Da, expected: 7458.94Da) and Atox1 in complex with different equivalents of Ag ion. (B) (C) (D) ESI-MS of Atox1 protein and Atox1 in complex with different equivalents of Ag ion in the different buffer conditions including 0.01 to 1 mM DTT.
**Figure S2.** NMR analysis of Ag⁺ binding to Atox1. (A) Overlay of $^1$H,$^{15}$N-HSQC NMR spectra of Atox1 apo-Atox1 before (red) and after (blue) adding equimolar Ag⁺. (B) Chemical shift perturbation $\Delta\delta$ against the residue number ($\Delta\delta = \sqrt{(\Delta\delta_n)^2 + (\Delta\delta_\alpha / 5)^2}$).

**Figure S3.** Atox1 crystal structures in the presence of metals (Cu-Atox12 PDB accession code 1FEE, Hg-Atox12 PDB accession code 1FE4, Cd-Atox12 PDB accession code 1FE0, CisPt-Atox12 PDB accession code 3IWX).
Figure S4. The rare cases we observed in the single-molecule force spectroscopy experiments. The SMFS measurements on the engineered chimeric polyprotein in the presence of Ag, and the rupture of Ag4-(Atox1)2 complex proceeded in two steps.
Figure S5. Reported Atox1 crystal structures in the presence of metals and Ag₄-(Atox1)₂ in this work. Crystal structure of Ag bound to an Atox1 dimer with 2.70 Å (PDB accession code 5F0W).
Figure S6. Close-up view showing details of the tretrasilver cluster in Atox1 dimer(5F0W). The distance between Ag1 and Ag4 is 3.01 Å. The distance between Ag1 and Ag3 is 2.95 Å. The distance between Ag2 and Ag3 is 2.95 Å. The distance between Ag2 and Ag4 is 2.89 Å. The Angle Ag4-Ag1-Ag3 is 65.01°, and the angle Ag4-Ag2-Ag3 is 66.56°. The four silver ions are situated nearly on the same plane, forming four Ag-Ag bonds with an average dihedral angle of 171°.
Figure S7. (A) Crystal structure of Ag bound to an Atox1 dimer with 1.75 Å (PDB accession code 7DC1). (B) The superimposed 2Fo-Fc electron density map of Ag₄-(Atox1)₂. 2Fo-Fc electron density map (gray, 1.00σ) of Ag₄-(Atox1)₂ metal center with anomalous difference Fourier density showing the Ag ions superimposed (green, 6σ).
Figure S8. The ELF color-filled map of Ag₄²⁺ generated by Multiwfn. Significant electron-pair density in the center of the cluster supported delocalized 4-center weak bonding.
**Figure S9.** The CW-EPR spectra measurement of Atox1, Atox1-Ag, Ag-Atox1 with DMPO and MTSSL-Atox1.
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