Supporting Information

Crystal Structures of Cisplatin Bound to a Human Copper Chaperone

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Supporting Methods

Protein Purification

Atox1 was expressed from a pET21b vector in BL21(DE3) *E. coli* as described previously.[1] Protein used for monomer structural studies (Pt-Atox1) was eluted from the final Superdex 75 size exclusion column in 20 mM MES pH 6.5 containing 200 mM NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol. Fractions containing pure protein were pooled and concentrated to > 1 mM (concentration determined by UV-visible absorbance at 280 nm, ε = 3884 M^{-1} cm^{-1}). Tris(2-carboxyethyl)phosphine (TCEP) was added to purified Atox1 to a final concentration of 10 mM and the sample was incubated overnight on ice. The reduced protein (1-2 mL) was then transferred to a Coy anaerobic chamber and dialyzed twice (30 min, room temperature) in a Pierce 3.5 kDa MWCO dialysis cartridge versus 200 mL of 100 mM MES, pH 6.5. This anaerobic dialysis step was intended to remove TCEP and EDTA while keeping the Atox1 cysteine residues reduced prior to cisplatin loading. However, the presence of TCEP in the crystal structure and ESI-MS data indicate that the dialysis protocol was not sufficient to remove all the TCEP.

For dimer structure determination (Pt-$(Atox1)_2$), Atox1 was eluted from the Superdex 75 column in 50 mM HEPES pH 7.5 containing 200 mM NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol. Fractions containing pure protein were pooled and concentrated to > 1 mM (concentration determined by UV-visible absorbance at 280 nm, ε = 3884 M^{-1} cm^{-1}). Dithiothreitol (DTT) was added to the purified protein at 10 mM concentration and the sample was incubated overnight on ice. The reduced protein was then transferred to a Coy anaerobic chamber and dialyzed four times (room temperature) versus 400 mL of 20 mM MES, pH 6.0, 50 mM NaCl with buffer changes every two hours and then overnight. This dialysis step was

included to remove the DTT. The length of time and the number of buffer changes were optimized to eliminate reducing agent-PtAtox1 adducts as detected by ESI-MS. The minor pH changes between this preparation and that described above were only introduced to ensure maximum reducing capacity of the DTT and to improve protein stability, and probably have little bearing on the oligomerization state of the cisplatin-Atox1 adduct.

Cisplatin Loading

Cisplatin was dissolved in 20 mM MES pH 6.0 containing 50 mM NaCl at concentrations ranging from 1 to 4 mM, aliquoted, and stored at -80 ºC. All cisplatin stocks were stored and manipulated shielded from ambient light. The exact platinum concentrations of stock solutions were determined by inductively-coupled plasma atomic emission spectroscopic (ICP-AES) analysis. Prior to use, cisplatin aliquots were thawed at 37 °C and vortexed extensively. Solutions were bubbled with argon gas for 5 min, and then sealed and transferred to the anaerobic chamber. Equimolar amounts of cisplatin stock solutions were added to diluted protein samples in the dialysis buffers described above. Addition of excess cisplatin (> 1 mM overall concentration) resulted in protein and/or cisplatin precipitation. All solutions were vortexed and incubated at room temperature in the dark in the anaerobic chamber. Once the reactions were complete as determined by optical spectroscopy, any excess cisplatin was removed by two cycles of dialysis against 400 mL (30 min, room temperature) of 20 mM MES pH 6, 50 mM NaCl in the anaerobic chamber followed by dialysis into buffer appropriate for subsequent experiments.

UV-visible Spectroscopy

Cisplatin-Atox1 reactions at 1 mM concentration (1:1) were evaluated by UV-visible spectroscopy using a Hewlett-Packard 8452A diode array spectrophotometer in the Keck Biophysics facility at Northwestern University. Spectra were recorded from 190-700 nm on samples diluted 21-fold (50 μL reaction + 1 mL buffer) and measured under ambient conditions in a quartz cuvette. No evidence of protein precipitation was observed in these experiments.

Mass Spectrometry

Electrospray ionization mass spectrometry (ESI-MS) was performed in the Integrated Molecular Structure Education and Research Center (IMSERC) at Northwestern University. Cisplatin-Atox1 adducts (1 mM) were submitted following overnight dialysis versus 400 mL of 5 mM ammonium acetate (two changes, 4 °C). Samples were analyzed on an Agilent (Wilmington, DE) 6210 ToF-LC/MS mass spectrometer. Analyses were performed in the positive ion ESI mode using the reflectron configuration. The instrument was calibrated with the Agilent ESI low concentration tune solution. Operational parameters are as follows: mass range 100-3000 m/z, vcap 3515 V, drying gas 5.0 L/min, gas temperature 300 °C, skimmer 65 V, fragmentor 205 V, ion focus -108 V. Samples were injected using flow injection on an Agilent 1200 HPLC system with 90% MeOH, 10% H_2O with 0.1% TFA as the carrier stream. All solvents used were HPLC grade or better.

ICP-AES Analysis

All protein solutions were diluted 100-fold in 5% trace metal grade nitric acid and analyzed using a Varian Vista-MPX CCD simultaneous ICP-AES instrument in the Northwestern IMSERC. Platinum standards ranging from 0.5 to 20 ppm were prepared in the

same fashion using platinum standard stock solution (1000 ppm, Sigma). Protein concentrations were determined by UV-visible absorbance at 280 nm. Values are reported as the average ± standard deviation (s.d.) for three independent experiments.

Crystallization and Structure Determination

Cisplatin-Atox1 adducts were dialyzed versus 200 mL of 20 mM MES, pH 6.0 containing 20 mM NaCl and 5% glycerol in the anaerobic chamber (1 hr, room temperature). Hexagonal rod-shaped crystals were obtained for the TCEP-reduced sample (14 mg/mL Atox1) using the hanging drop vapor diffusion method with 2.0 M Li_2SO_4, 0.1 M MES pH 6.0 as a precipitant. For DTT-reduced samples, hexagonal spear-shaped crystals were obtained using 14 mg/mL Atox1 with 1.5 M Li_2SO_4, 0.1 M MES, pH 6.0, 50 mM NaCl as a precipitant. Trays were incubated at 20 ºC in the dark under aerobic conditions. Crystals were soaked in cryoprotectant solution (1.1-1.5 M Li_2SO_4, 30% glycerol) for less than 5 min, mounted on loops, and flash cooled in liquid nitrogen. The TCEP reduced crystals belong to the $P3_221$ space group with unit cell dimensions $a = b = 54.07$ Å, $c = 55.58$ Å and one molecule per asymmetric unit. The DTT reduced crystals belong to the $P6_5$ space group with unit cell dimensions $a = b = 78.29$ Å, $c = 54.34$ Å and two molecules per asymmetric unit.

Data were collected at the Life Sciences Collaborative Access Team (LS-CAT) beamlines at the Advanced Photon Source (APS). Use of the Advanced Photon Source was supported by the U. S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357. Use of the LS-CAT Sector 21 was supported by the Michigan Economic Development Corporation and the Michigan Technology Tri-Corridor for the support of this research program (Grant 085P1000817). Data for the monomer structure (Pt-Atox1) were

collected at a wavelength of 0.90511 Å; data for the dimer structure (Pt-$(Atox1)_2$) were collected at a wavelength of 0.97872 Å. Data were processed using the HKL2000 package.[2] Both structures were solved with PHASER[3] using the coordinates of Cd(II)-Atox1 (PDB accession code 1FE0) as a starting model. Both structures were also solved independently by Pt single wavelength anomalous dispersion (SAD) phasing using SHARP.[4] Model building and refinement were performed with Coot[5] and Refmac5[6], respectively. The final model for Pt-Atox1 consists of residues 2-67, one platinum ion, one sulfate molecule, one TCEP molecule, and 92 water molecules. The final model for Pt-$(Atox1)_2$ consists of residues 2-68 for each monomer, one platinum ion, two ammine ligands, three sulfate ions, and 104 water molecules. Ramachandran plots calculated with PROCHECK[7] indicate that 100% of the residues for both structures are in the allowed and additionally allowed regions. The diffraction-component precision index (DPI) errors calculated by SFCHECK[8] are 0.074 for Pt-Atox1 and 0.160 for Pt-$(Atox1)_2$. The occupancies of the Pt(II) ions were modeled such that minimal negative density around the metal center was observed in the difference Fourier map. The Pt(II) occupancy in the Pt-Atox1 structure was best modeled as 0.75 as compared to 0.40 for the Pt-$(Atox1)_2$ structure. The ammine ligands in the Pt-$(Atox1)_2$ structure were also modeled at an occupancy of 0.40. Figures were generated with PyMOL.[9]

Supporting Information References

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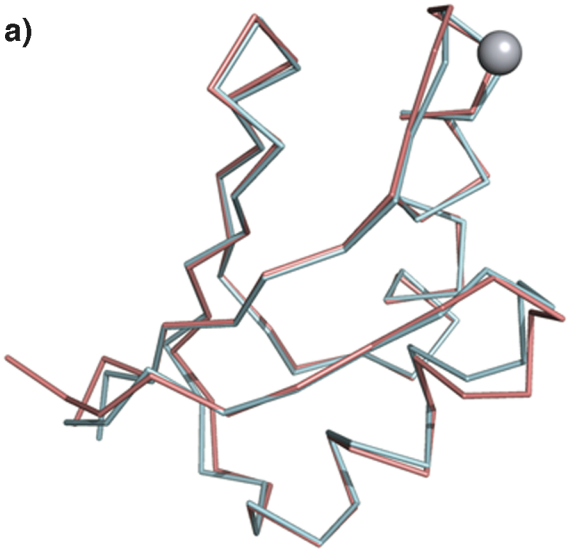
Table S1. Crystallographic data collection and refinement statistics.

	Pt-Atox1	Pt-(Atox1)$_2$
Data collection		
Space group	$P3_221$	$P6_5$
Cell dimensions		
a, *b*, *c* (Å)	54.07, 54.07, 55.58	78.29, 78.29, 54.34
α, β, γ (°)	90.00, 90.00, 120.00	90.00, 90.00, 120.00
Resolution (Å)	50.00-1.60 (1.63-1.60)	50.00-2.14 (2.18-2.14)
R_{sym} or R_{merge}	0.118 (0.503)	0.078 (0.401)
I / σI	24.9 (3.3)	23.4 (3.5)
Completeness (%)	99.7 (99.7)	99.9 (100)
Redundancy	19.5 (9.5)	5.6 (5.5)
Refinement		
Resolution (Å)	46.83-1.60	28.76-2.14
No. reflections	12060	10032
R_{work} / R_{free}	0.179/0.210	0.186/0.228
No. atoms	609	1132
Protein	496	1010
Ligand/ion	21	18
Water	92	104
B-factors		
Protein	17.0	22.1
Ligand/ion	33.9	68.1
Water	39.6	38.4
R.m.s. deviations		
Bond lengths (Å)	1.252	1.092
Bond angles (°)	0.010	0.009

a)



b)

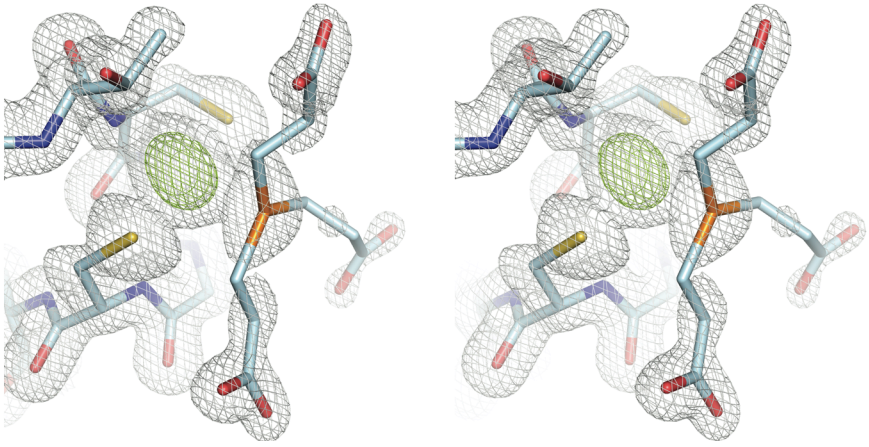


Figure S1 (a) Overlay of Pt-Atox1 with one monomer of Cu(I)-Atox1 (PDB accession code 1FEE). (b) Stereo view of the $2F_o$-F_c electron density map (light grey, contoured at 1.35σ) at the metal binding site in the Pt-Atox1 X-ray structure. The anomalous difference Fourier map showing the Pt(II) ion is superimposed in green (contoured at 9σ).

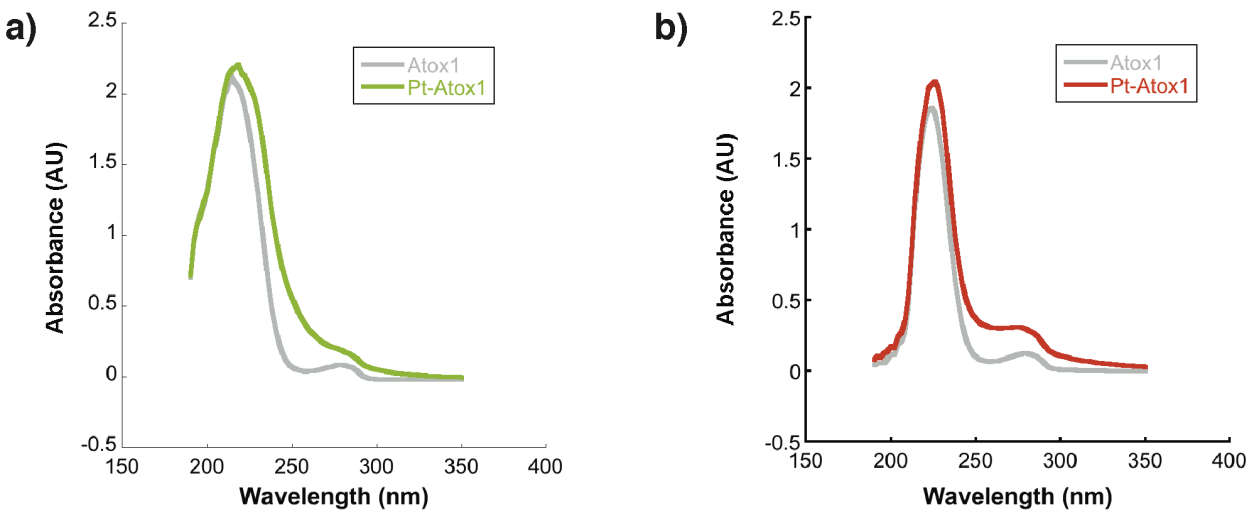


Figure S2 Optical spectra of cisplatin-Atox1 adducts. Reduced Atox1 (1 mM) was incubated with equimolar cisplatin for 4 hrs at ambient temperature in an anaerobic chamber in the dark. (a) Atox1 reduced with TCEP and then loaded with cisplatin. (b) Atox1 reduced with DTT prior to cisplatin loading.

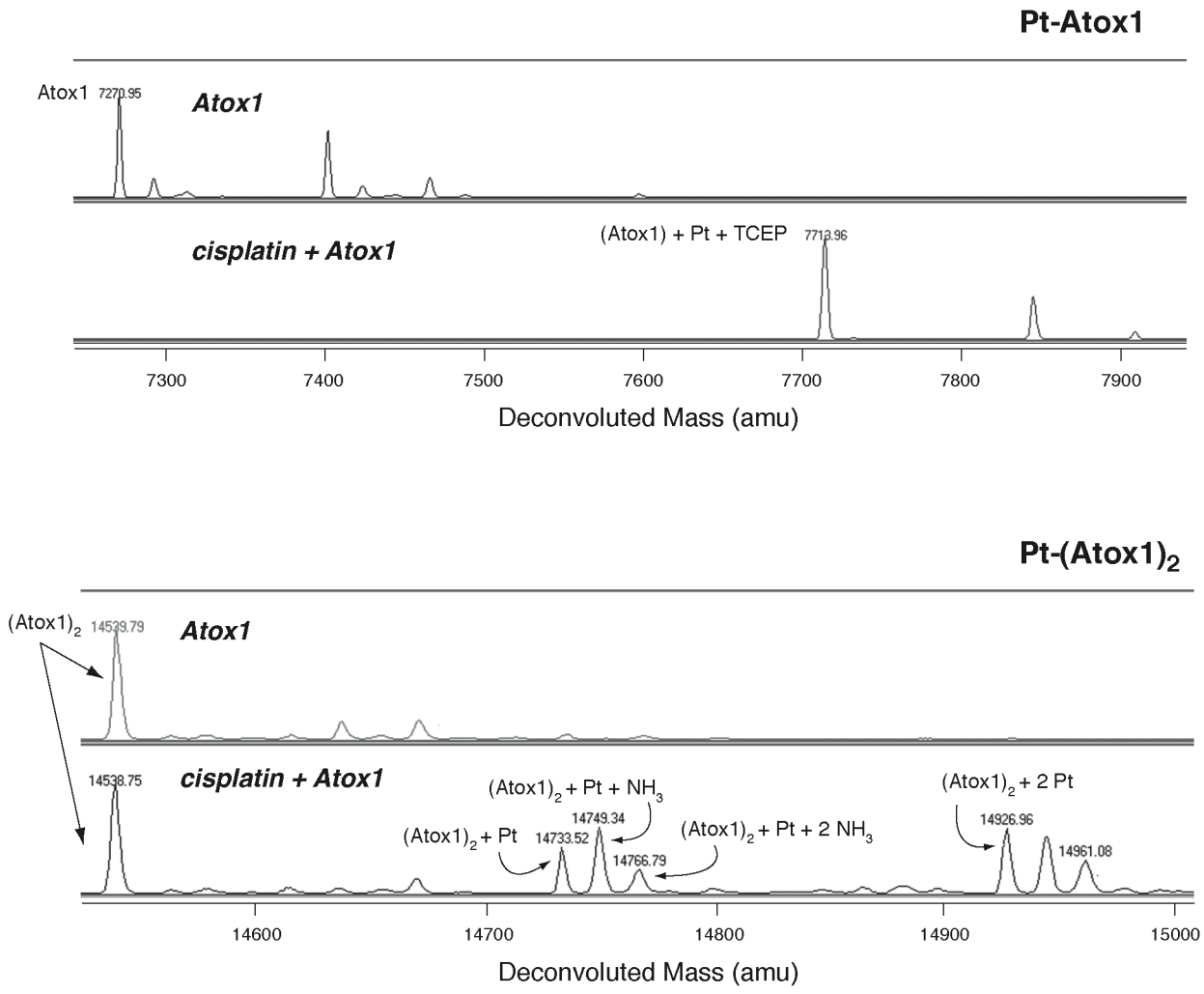


Figure S3 Electrospray ionization mass spectrometry (ESI-MS) deconvoluted spectra of cisplatin-Atox1 adducts. The apo and platinated samples are compared for Pt-Atox1 (top) and Pt-$(Atox)_2$ (bottom). Note that peak intensity does not necessarily correlate with the quantity of individual products in the platination reaction. Ligand dissociation may occur as result of the conditions required to achieve ionization.

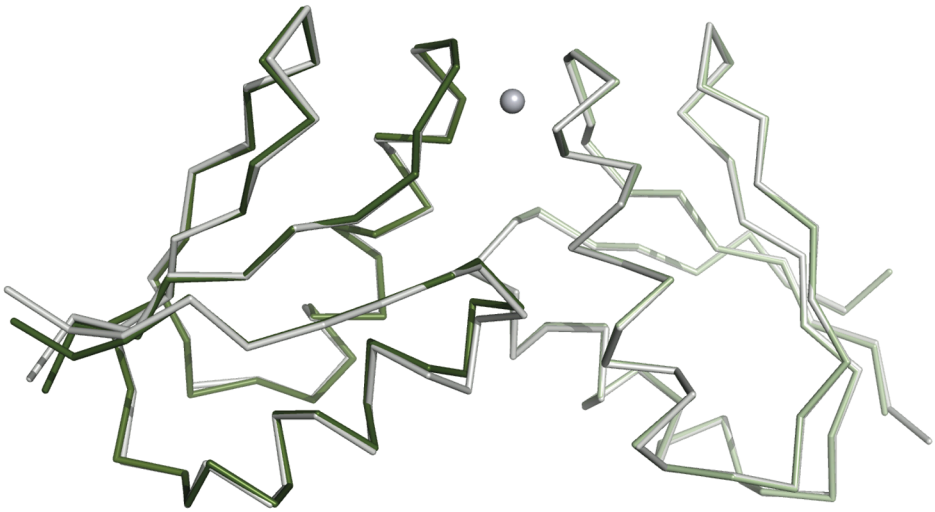


Figure S4 Overlay of Pt-$(Atox1)_2$ (green) with Cu(I)-Atox1 (grey) (PDB accession code 1FEE). The Pt(II) in Pt-$(Atox1)_2$ is shown as a gray sphere.

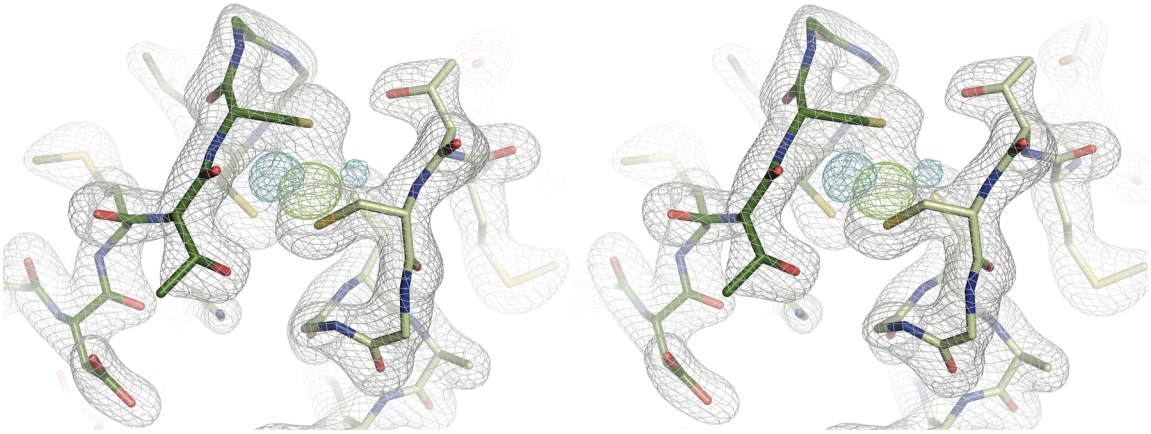


Figure S5 Stereo view of the $2F_o$-F_c electron density map (light gray, contoured at 1.35σ) at the metal center in the Pt-$(Atox1)_2$ structure. The anomalous difference Fourier map showing the Pt(II) ion is superimposed in green (contoured at 12σ). The F_o-F_c difference density (blue, 4σ) shows two positive peaks in the vicinity of the Pt(II) site that were best modeled as ammine ligands.