Supporting Information

for

Copper binding promotes the interaction of cisplatin with human copper chaperone Atox1

Zhaoyong Xi, Wei Guo, Changlin Tian, Fuyi Wang, Yangzhong Liu

Experimental Details

Synthesis of ^{15}N-labeled cisplatin

The ^{15}N-labeled cisplatin, *cis*-[$PtCl_2(^{15}NH_3)_2$] was synthesized according to the literature method.[1]

Protein expression and purification

The genes encoding Atox1 was amplified via PCR from a human cDNA library and inserted into pST-SG1 vector using the ligation-independent cloning method. The resulting plasmid was transformed into *E. coli* BL21(DE3) Gold. Cells were grown at 37 °C in LB medium until the OD_{600} of 0.8 was reached, followed by incubation with 0.8 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h. Cells were harvested by centrifugation at 4000 rpm at 18 °C for 20 min. The protein was purified using Ni^{2+} affinity chromatography. The $(His)_6$ tag was removed by the TEV protease digestion. Protein was further purified through gel filtration on Superdex 75 16/60 column (GE Healthcare).

NMR spectroscopy

^{15}N-labeled cisplatin were dissolved in Milli-Q water to 3 mM and then lyophilized. Protein samples were prepared in 50 mM sodium phosphate (pH 7.0) containing 10% (v/v) D_2O and 5-fold dithiothreitol (DTT). For the preparation of Cu^I-Atox1 samples, equimolar amounts of Cu(I) complex $[Cu(CH_3CN)_4]^+$ was added to apo-Atox1 in the buffer containing DTT. The NMR samples were prepared prior to NMR experiments by mixing protein solutions with the lyophilized cisplatin to give a final concentration of 0.8 mM for both the protein and cisplatin. ^{1}H-^{15}N HSQC spectra were recorded immediately to monitor the reaction. NMR spectra were recorded on a 700 MHz Varian Inova spectrometer at 25 °C. The spectral widths were set as 8 ppm (^{1}H) and 60 ppm (^{15}N, centered at -60 ppm). The sequence was optimized with a delay $1/(4J_{NH})$ of 3.42 ms. ^{15}N chemical shifts of cisplatin were referred to $^{15}NH_4Cl$.

HPLC-ESI-MS

The Atox1 (0.2 mM) samples were incubated with equimolar cisplatin at 25 °C for different time. The resulting mixtures were directly analyzed by HPLC-ESI-MS. The reaction mixtures of Atox1 with equimolar cisplatin at 25 °C for 4 h were separated by HPLC with a Zorbax eclipse XDB-C8 column (4.6×250 mm, 100 Å, 5 μm, Agilent) to remove unbound cisplatin. Then, trypsin (from Promega) was added in a molar ratio of 1:40 (trypsin:Atox1) to the solution of purified protein adduct. The resulting mixture (pH 7.8) was incubated for 6 h at 37 °C for digestion prior to HPLC-ESI-MS analysis.

Positive-ion electrospray ionization mass spectra were obtained on a Micromass Q-TOF mass spectrometer (Waters) coupled to a Waters CapLC HPLC system. Atox1 and Pt-Atox1 adducts were separated on a Symmetry-C8 column (4.6×50 mm, 100 Å, 5 μm, waters). The tryptic digests of Atox1 and Pt-Atox1 adducts were separated on a Symmetry-C18 column (1.0×50 mm, 100 Å, 3.5 μm, Waters). Mobile phases were A: 95% H_2O containing 4.9% acetonitrile and 0.1% formic acid, and B: 95% acetonitrile containing 4.9% H_2O and 0.1% formic acid. The separation of Pt-Atox1 adducts was achieved with a 20 min linear gradient from 1% to 80% of B at a rate of 30 μL min^{-1}, and the tryptic peptides were eluted with a 50 min linear gradient from 1% to 45% of B at a rate of 30 μL min^{-1}. The eluents were directly infused into the mass spectrometer through the ESI probe. The spray voltage of the mass spectrometer was 3.30 kV and the cone voltage was 35 V. The desolvation temperature was 140 °C and source temperature 80 °C. Nitrogen was used as both cone gas and desolvation gas with a flow rate of 40 L h^{-1} and 400 L h^{-1}, respectively. The collision energy was set to 10 V. The MS spectra were acquired in the range of 500-1800 *m/z*. Collision-induced dissociation MS/MS spectra were obtained in the range of 100–2000 *m/z*, and the collision energy was set to 15-20 eV with argon as a collision gas. All *m/z* values corresponding to the mass-to-charge ratios of the most abundant isotopomer of the observed ions were calibrated first versus a NaI calibration file and then the respective tryptic peptides of native Atox1. Mass Lynx(ver. 4.0) software was used for analysis and post processing.

Protein aggregation assay

The protein aggregation was analyzed using Tricine-SDS-PAGE. 0.3 mM Protein samples (apo-Atox1 or Cu^{I}-Atox1) were incubated with equimolar platinum complexes at 25 °C for different time (10 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h) before loading to the gel.

[1] S. J. S. Kerrison, P. J. Sadler, *J. Chem. Soc., Chem. Commun.* **1977**, 861.

Table S1. Species observed in the ESI-MS spectra

Common species	Formula	Observed *m/z*	Calculated *m/z*
a1: $[Atox1+Pt(NH_3)_2Cl+7H]^{8+}$	$C_{332}H_{560}N_{91}O_{106}S_6ClPt$	993.11	993.11
a2: $[Atox1+Pt(NH_3)_2(H_2O)+6H]^{8+}$	$C_{332}H_{561}N_{91}O_{107}S_6Pt$	990.90	990.87
a3: $[Atox1+Pt(NH_3)_2+6H]^{8+}$	$C_{332}H_{559}N_{91}O_{106}S_6Pt$	988.63	988.62
a4: $[Atox1+Pt(NH_3)+6H]^{8+}$	$C_{332}H_{556}N_{90}O_{106}S_6Pt$	986.50	986.49
a5: $[Atox1+Pt+6H]^{8+}$	$C_{332}H_{553}N_{89}O_{106}S_6Pt$	984.37	984.36
a6: $[Atox1+Pt(NH_3)(DTT)+6H]^{8+}$	$C_{336}H_{566}N_{90}O_{108}S_8Pt$	1005.72	1005.74
a7: $[Atox1+Pt(DTT)+6H]^{8+}$	$C_{336}H_{563}N_{89}O_{108}S_8Pt$	1003.60	1003.61
a8: $[Atox1+Pt(NH_3)+Pt(DTT)+4H]^{8+}$	$C_{336}H_{564}N_{90}O_{108}S_8\ Pt_2$	1029.87	1029.86
a9: $[Atox1+Pt+Pt(DTT)+4H]^{8+}$	$C_{336}H_{561}N_{89}O_{108}S_8\ Pt_2$	1027.73	1027.73
a10: $[Atox1+Pt(NH_3)_2+Pt+4H]^{8+}$	$C_{332}H_{557}N_{91}O_{106}S_6\ Pt_2$	1012.74	1012.73
a11: $[Atox1+Pt(NH_3)+Pt+4H]^{8+}$	$C_{332}H_{554}N_{90}O_{106}S_6Pt_2$	1010.61	1010.61
a12: $[Atox1+2Pt+4H]^{8+}$	$C_{332}H_{551}N_{89}O_{106}S_6Pt_2$	1008.49	1008.48

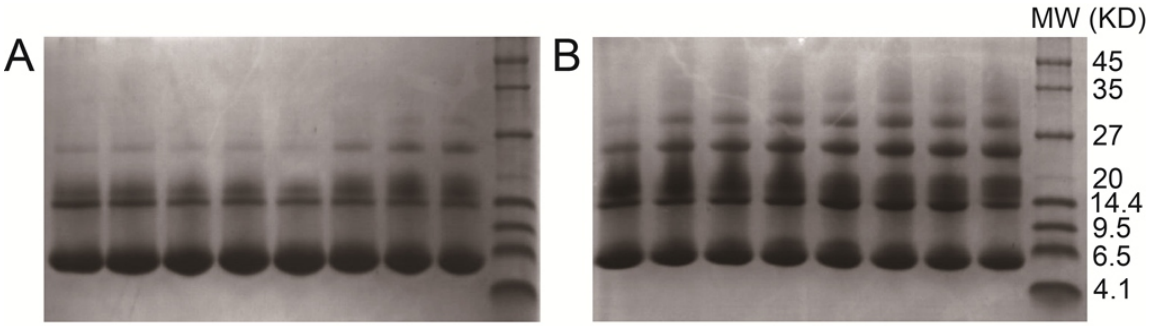

Fig. S1. Platinum complexes induced apo-Atox1 and Cu-Atox1 aggregation monitored by tricine-SDS-PAGE. Incubation time of the samples is 10 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h from left to right. (A) cisplatin+apo-Atox1. (B) cisplatin+Cu^I-Atox1. Results clearly show that severer protein aggregations are present in the reaction of Cu^I-Atox1.

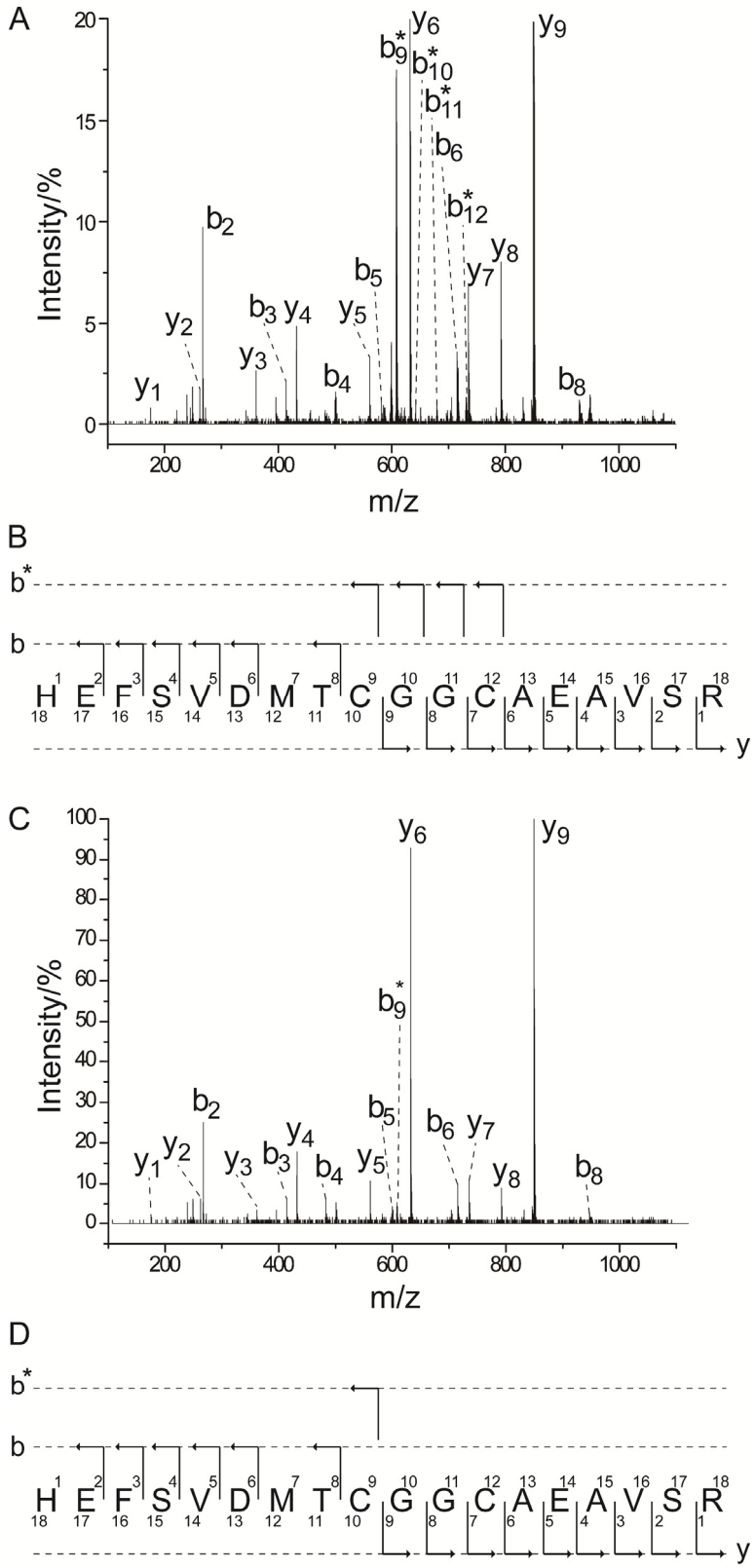

Fig. S2 ESI-MS/MS spectra of the triply-charged ion d2 at *m/z* 703.92 from trypsin digestion of the platinated Atox1 by cisplatin. (A) apo-Atox1; (C) Cu^{I}-Atox1. Fragmentation schemes based on the spectra (A) and (C) are shown in (B) and (D), respectively.

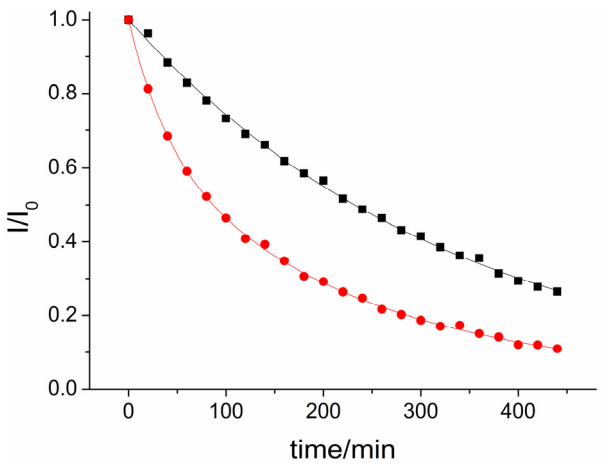

Fig. S3 Plots of the ratio of unreacted cisplatin (I) to initial cisplatin (I_0) *versus* time for the reaction of cisplatin with apo-Atox1 (black square and line) or Cu^{I}-Atox1 (red circle and line) in the presence of excess GSH. Similar to the reactions in the presence of DTT, the $t_{1/2}$ is much shorter in the reaction of Cu^{I}-Atox1 (86 min) than that of apo-Atox1 (232 min).